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Extracellular CIRP (eCIRP) and Inflammation

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Abstract

Cold-inducible RNA-binding protein (CIRP) was discovered two decades ago while studying the mechanism of cold stress adaptation in mammals. Since then, the role of intracellular CIRP (iCIRP) as a stress-response protein has been extensively studied. Recently, extracellular CIRP (eCIRP) was discovered to also have an important role, acting as a damage-associated molecular pattern (DAMP), raising critical implications for pathobiology of inflammatory diseases. During hemorrhagic shock and sepsis, inflammation triggers the translocation of CIRP from the nucleus to the cytosol and its released to the extracellular space. eCIRP then induces inflammatory responses in macrophages, neutrophils, lymphocytes and dendritic cells. eCIRP also induces endoplasmic reticulum stress and pyroptosis in endothelial cells by activating the NF- κ B and inflammasome pathways, and necroptosis in macrophages via mitochondrial DNA damage. eCIRP works through the TLR4-MD2 receptors. Studies with CIRP^{-/-} mice reveal protection against inflammation, implicating eCIRP to be a novel drug target. Anti-CIRP Ab or CIRP-derived small peptide may have effective therapeutic potentials in sepsis, acute lung injury and organ ischemia/reperfusion injuries. Current review focuses on the pathobiology of eCIRP by emphasizing on signal transduction machineries, leading to discovering novel therapeutic interventions targeting eCIRP in various inflammatory diseases.

Graphical Abstract

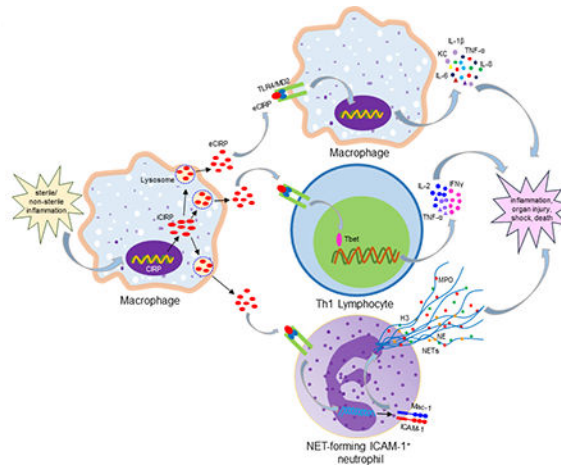
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Authorship

PW discovered eCIRP and conceived the idea of the project. MA compiled literature review. MA, MB, PW outlined the concept of the review. MA, MB wrote the manuscript and prepared the figures and table. PW reviewed, provided constructive criticism and edited the manuscript. PW supervised the study.

Disclosures

MA has no financial conflicts of interests. One of the authors (PW) is an inventor of patent applications covering the fundamental concept of targeting CIRP for the treatment of inflammatory diseases, licensed by TheraSource LLC. PW is a co-founder of and MB is a director at TheraSource LLC.



Keywords

CIRP; eCIRP; DAMP; neutrophils; macrophage; sepsis; hemorrhage; inflammation; ALI; ischemia/reperfusion

Introduction

Cold-inducible RNA-binding protein (CIRP) was first described in late 90's as an RNA chaperone controlling the expression of cell cycle proteins in hibernating animals [1]. Since then, intense research on intracellular CIRP (iCIRP) has revealed its role in the regulation of a variety of cellular stress responses, including mRNA stability [2], cell proliferation [3], cell survival [4, 5], circadian clock gene modulation [6], telomerase maintenance [7], stress adaptation [8], and tumor formation and progression [9, 10]. These iCIRP activities, which allow cells to cope with various cellular stress conditions, have been reviewed in detail [8]. In contrast to iCIRP, extracellular CIRP (eCIRP) was recently discovered to act as a damage-associated molecular pattern (DAMP) promoting inflammation and injury [11]. The identification of eCIRP as a new DAMP has originated a new era of investigation in inflammation pathobiology. Serum levels of eCIRP not only were found to be elevated in individuals admitted to the intensive care unit (ICU) with hemorrhagic shock (HS), but also correlated with organ injury [11]. Furthermore, it was demonstrated that eCIRP was able to promote inflammation independently [11]. In cells subjected to hypoxia, CIRP translocates from its usual location in the nucleus to the cytosol [1, 12], and is then released to the extracellular space [11]. eCIRP then acts as a DAMP increasing macrophage production of proinflammatory cytokines [11]. Since its original discovery as a DAMP, eCIRP's emerging proinflammatory role has been progressively extended to include additional target cells, such as neutrophils, lymphocytes, epithelial cells, and endothelial cells [13–21]. eCIRP promotes inflammation by binding to the TLR4-MD2 receptor complex, leading to the activation and nuclear translocation of the transcription factor nuclear factor- κ B (NF- κ B) [11, 13]. Accordingly, CIRP^{-/-} mice are protected and exhibit attenuated inflammation and organ injury in preclinical models of inflammatory diseases [11, 13]. These observations suggested that targeting eCIRP could be an effective therapeutic strategy for the treatment of

inflammatory diseases. Indeed, we have now shown that treatment with anti-CIRP polyclonal antibodies or with the CIRP-derived small peptide C23 dramatically rescues animals from inflammation [11, 22–26]. In this article, we will review i) eCIRP's role in pathobiology focusing on CIRP's induction and release, ii) eCIRP's mechanism of action, and iii) the development of eCIRP-targeting therapeutic interventions to ameliorate inflammatory diseases.

CIRP

CIRP is an evolutionarily conserved RNA chaperone [8]. It consists of an N-terminal RNA recognition motif (RRM) and a C-terminal arginine-rich (RGG) region structurally similar to stress-induced RNA-binding proteins in various species [1, 8]. CIRP has three protein-coding splice variants which are transcribed during normo- and hypothermic conditions [27]. The precise effects of these variants in the protein structure and function remain to be adequately studied. The shortest transcript is mostly expressed at 37°C, while two other longer transcripts harboring an internal ribosome entry site (IRES) are expressed at 32°C [28]. During hypothermia the core and alternate promoters and an alternative splicing tool become active for generating CIRP's transcripts [29]. Non-hibernating animals such as hamsters express a long CIRP transcript in their hearts. This transcript contains an additional insert with an internal stop codon at the open reading frame (ORF), leading to a truncated protein and aberrant function. In contrast, hibernating animals predominantly express the short isoform with a complete ORF [9, 29, 30]. Overall, CIRP expression levels are altered in response to stress by versatile mechanisms, suggesting a wide range of adaptation to various external and internal challenges.

CIRP is predominantly found in the nucleus, where it regulates RNA transcription and processing, whereas in the cytoplasm iCIRP regulates mRNA translation and turnover [1, 8]. The cDNA sequence of CIRP has been cloned and characterized in numerous species, with >210 homologues currently identified in fish, amphibians, reptiles, birds and mammals [1, 31–33]. CIRP from human, mouse and rats, in particular, have elevated sequence homology. The human CIRP cDNA encodes an 18-kD protein whose amino acid sequence is 95.3% similar to that of mouse CIRP [31]. Mouse and rat CIRP have a sequence similarity of 100% [32]. In addition to homologues and orthologues, CIRP has at least two paralogues: RNA-binding motif protein 3 (RBM3) – an RNA-binding protein upregulated in response to low temperature [9], and aggrecan promoter-binding protein-1 (APBP-1) – a 19-kDa DNA/RNA binding protein [34]. CIRP is constitutively and ubiquitously expressed at low levels, with studies showing its presence in the brain, lungs, heart, liver, kidneys, and endometrium, as well as in macrophages and neutrophils [11, 13, 35]. Recently, both CIRP expression and release have been shown to be induced in cellular stress and inflammatory conditions [11]. Since CIRP exerts fundamentally different functions depending on its localization, it is critical to clearly establish the unique characteristics and activities of iCIRP and eCIRP.

Intracellular CIRP, an RNA chaperone

iCIRP is synthesized in the cytoplasm and gets back into the nucleus to modulate homeostatic RNA transcription and processing [1, 8]. In cardiomyocytes, for example, iCIRP regulates cardiac repolarization by binding to and inhibits the translation of mRNAs

encoding Ito channel proteins [36]. However a fraction of iCIRP can also be found in the cytoplasm. iCIRP's cytoplasmic fraction is particularly increased by cellular stressors such as hypothermia and ischemia [1, 8, 12]. iCIRP's nucleocytoplasmic distribution is regulated by methylation of its C-terminal domain [12]. The shift in iCIRP's subcellular localization, such as that elicited by ischemic and inflammatory states, results in a change in iCIRP's role. Once in the cytoplasm, iCIRP coordinates translational reprogramming by promoting the synthesis of specific proteins. iCIRP selectively binds to the 3'-untranslated region (UTR) of ribosomal fraction RNA transcripts harboring CIRP's RNA signature motif [8]. By binding to its target mRNAs, iCIRP inhibits their deadenylation, preserving their poly-A tail length and thus increasing their stability and preventing their degradation [2, 37, 38]. iCIRP also enhances the expression and activity of human antigen R (HuR) protein, which further positively regulates mRNA stability and translation by interacting with AU-rich element located in the 3'-UTR of many mRNAs [39]. Furthermore, iCIRP regulates the translation of its target mRNAs at the translation initiation phase by interacting with eIF4G [40]. All of these mechanisms lead to enhanced translational activity [2, 37, 38]. Indeed, iCIRP has been shown to promote the translation of genes involved in DNA repair [37, 41], cellular redox metabolism [40], cellular adhesion [42], circadian homeostasis [6], reproduction [2], telomerase maintenance [7], and genes associated with the translational machinery [8].

Physiological hypothermia within a scrotum is needed for normal spermatogenesis in mammals. Elevated levels of iCIRP are found in the nucleus of primary spermatocytes which play a pivotal role in spermatogenesis [43]. A recent protein-transcriptome screening study reveals that iCIRP binds to several transcripts encoding circadian oscillator proteins, like CLOCK, implicating iCIRP's role in maintenance of circadian mRNAs [6]. iCIRP promotes keratinocyte cell survival and proliferation following ultraviolet (UV) exposure via phosphorylated signal transducer and activator of transcription 3 (STAT3)-dependent induction of cyclin D1 and Bag-1/S expression [44]. iCIRP has also been shown to accelerate the cell cycle progression of cultured mouse embryonic fibroblasts by binding to dual specificity tyrosine-phosphorylation regulated kinase 1B (Dyrk1b) [3].

In contrast with its role in keratinocyte proliferation during UV irradiation, iCIRP can inhibit cell proliferation by upregulating the expression of a cell cycle inhibitor protein p27Kip1 during mild hypothermia [4]. In addition to the positive post-transcriptional regulation, upon stress conditions nuclear iCIRP migrates to cytoplasmic stress granules (SGs) where it serves as a translational repressor, thus negatively controlling protein synthesis in general [12]. The genes induced by iCIRP is thought to be a function of the intensity (mild, moderate or severe) and timing (early or late exposure) of the stressors. In a recent study, for example, the neuronal expression of iCIRP was increased in rats subjected to chronic hypoxia, which in turn inhibited HIF- α and, thus, protected neuronal cells from apoptosis [45]. Besides, its role in regulation of mRNA stability and translation in the cytoplasm, nuclear iCIRP has also been shown to play a role in post-transcriptional regulation of its targets by controlling alternative polyadenylation that leads to generating alternative mRNA transcripts [46]. Thus, iCIRP coordinates translational reprogramming involving several post-transcriptional mechanisms to regulate physiological process such as spermatogenesis, circadian rhythm, cell proliferation, differentiation, and survival.

Extracellular CIRP: A novel mediator in the inflammation field

Under physiologic conditions, CIRP is found predominantly in the nucleus [1]. During hypoxia and inflammation, however, CIRP is translocated from the nucleus to the cytoplasm and gradually released to the extracellular space [11]. The recent discovery of eCIRP has led to the investigation of its role as a DAMP in inflammatory diseases.

Extracellular CIRP in ischemic and inflammatory diseases

Increased tissue and serum levels of CIRP have been reported in several ischemic and inflammatory diseases. While serum CIRP was undetectable in the healthy volunteers, its levels were elevated in the serum of the patients admitted to the ICU with hemorrhagic shock [11], which is characterized by a global deficiency in oxygen delivery and low grade inflammation [47]. In the rodent model of hemorrhage, serum CIRP was also elevated, and correlated with increased mRNA and protein expression levels in the heart and liver [11]. Similarly, serum and tissue levels of CIRP were also elevated in a number of organ-targeted ischemia and reperfusion models characterized by sterile inflammation, including rodent models of hepatic ischemia [26], mesenteric ischemia [24], ischemic acute kidney injury (AKI) [48] and stroke [21]. Furthermore, serum and tissue levels of CIRP are also elevated in sepsis [11, 13, 22, 49]. In an ICU-based study, circulating levels of CIRP in septic patients were significantly higher in the non-survivors than in the survivors [49], and correlated with sepsis severity as measured by APACHE II and SOFA scores, serum creatinine level, procalcitonin level, and mortality rate [49]. These observations were corroborated by increased serum and lung levels of CIRP in a murine model of polymicrobial sepsis [11, 13]. Furthermore, increased serum and tissue levels of CIRP have also been determined in chronic inflammatory diseases. Increased expression of CIRP has been shown in the airways and the alveolar epithelium of the lungs from chronic obstructive pulmonary disease (COPD) patients [50]. Similarly, the CIRP expression in alveolar epithelial cells was increased in mice treated with cold air [16]. Increased serum and synovial fluid levels of CIRP were also found in the patients with rheumatoid arthritis (RA) and osteoarthritis (OA), and the increased synovial levels of CIRP correlated with disease activity in two independent studies [51, 52]. In the colonic mucosa of patients with ulcerative colitis, expression of CIRP was correlated with the expression of proinflammatory cytokines, anti-apoptotic proteins and stem cell markers [53, 54]. Using an animal model of colitis, CIRP^{-/-} mice had decreased susceptibility to colonic inflammation through decreased expression of proinflammatory cytokines in the colonic lamina propria cells compared with WT mice [53]. In the murine colitis associated cancer (CAC) model, CIRP deficiency led to decreased expression of proinflammatory, apoptotic and stem cells, leading to attenuated tumorigenic potential [53, 54]. Accordingly, the expression of CIRP was higher in tissue samples from patients with oral squamous cell carcinoma (OSCC) than in matched normal tissues [55]. CIRP's role in cancer has been recently reviewed [8, 10]. CIRP has also been implicated in binge drinking. Binge drinking is associated with cerebral dysfunction [56]. Mice exposed to binge drinking levels of alcohol have increased brain CIRP mRNA and protein levels [19]. Furthermore, microglial cells exposed to alcohol *in vitro* increased their expression and release of CIRP [19], suggesting that microglial cells could be the source of the increased levels of CIRP in the brain during binge drinking. Taken collectively, these observations

demonstrate that eCIRP levels are elevated and correlate with severity and injury in a wide range of ischemic and inflammatory states (Table 1).

Mechanism: How does CIRP's expression, translocation and secretion occur?

As indicated by its name, iCIRP's transcription and translation are induced by mild hypothermia such as the testicular physiologic temperature and suppressed by elevated temperature [1, 31, 43, 57]. iCIRP is also upregulated by UV radiation [44] and acute hypoxia [11, 21]. In macrophages, hypoxia, endotoxin and alcohol can each upregulate the expression of iCIRP via pathways involving HIF- α , TLR4, NF- κ B, Egr-1, AP-1, and STAT1, and cause eCIRP release [11, 19, 58]. The activation of glycogen synthase kinase 3 β (GSK3 β) and insulin-like growth factor 1 (IGF1) can also increase the expression of iCIRP [59, 60]. To dissect the mechanism of iCIRP expression during mild hypothermia, a recent study revealed the involvement of transient receptor potential vanilloid (TRPV) 3 and transient receptor potential cation channel subfamily M (TRPM) 8 ion channel proteins, but independently of their ion channel activities [61]. The transcription factor specificity protein 1 (sp1) is induced by hypoxia and has been found to bind to the CIRP's cold responsive element, suggesting that it may mediate hypoxia-induced CIRP transcription [62, 63]. Recently, the overexpression of miRNA-23a has also been shown to induce the expression of CIRP during prolonged hypoxia condition, possibly through its binding to CIRP's transcriptional enhancer [45]. A recent study also revealed that hypothermia increases intracellular calcium levels via TRPV4 which regulates CIRP's expression in response to cold stress [64]. CIRP has various transcripts, which possibly differ between species and may have respond differently to inducers, enhancers and repressors [8]. Further studies evaluating each CIRP transcript in homeostasis inflammation will provide valuable insights into the mechanisms of expression, localization and secretion of CIRP with potentially significant implications to CIRP's effective therapeutic targeting.

In addition to being regulated at the transcriptional and translational levels, iCIRP's function is also regulated by its subcellular translocation. Specifically, stressors such as oxidative stress, endoplasmic reticulum (ER) stress, osmotic shock and heat shock may cause iCIRP methylation and subsequent migration from the nucleus to the SGs in the cytoplasm, where untranslated mRNA becomes sequestered [8, 12]. However, while hypothermia markedly induces CIRP synthesis in various cell types, it does not lead to the accumulation of CIRP in SGs [12]. Likewise, UV irradiation, which induces SGs, promotes CIRP's nuclear export without further assembly into SGs [12]. These observations suggest that distinct stressors may affect iCIRP translational reprogramming differently via their unique modulation of iCIRP transcript expression and subcellular localization.

CIRP's nuclear cytoplasmic translocation and release has been investigated using a green fluorescent protein (GFP)-tagged CIRP expression system in macrophages exposed to hypoxia [11]. Under physiologic normoxia, the GFP-tagged CIRP protein was concentrated in the nucleus, while hypoxia caused its enrichment in the cytoplasm, followed by its release to the extracellular space [11]. CIRP's release was not attributable to necrosis, as other intracellular proteins were not detected in the culture supernatants after hypoxia [11]. Since CIRP does not contain a signal peptide, its secretion cannot be mediated through the

conventional ER-Golgi dependent classical pathway. Biochemical fractionation revealed CIRP's enrichment at the lysosomal compartment of macrophages undergoing hypoxia, suggesting that CIRP is released by lysosomal secretion [11] (Figure 1). However, it is still unclear exactly how CIRP gets into the lysosomal compartment and is released without disrupting the cell membrane. An *in vitro* study using macrophages showed that cellular necrosis does not cause CIRP release, thus suggesting that passive release is not a major source of eCIRP during hypoxia or endotoxemia [11]. However, under *in vivo* condition like other DAMPs, passive release due to cell necrosis might play a role for eCIRP in injury such as I/R and sepsis [13, 25, 65]. Future studies on the involvement of the inflammasome-induced pore forming protein gasdermin D, various carrier/transport proteins and the involvement of ER stress molecules for active release of CIRP during inflammation help understand how eCIRP gets secreted to the extracellular space.

Post-translational modifications regulate CIRP's subcellular localization. CIRP's methylation, for example, is required for its cytoplasmic translocation [12, 66]. Similarly, CIRP's phosphorylation by GSK3 β and CK2 also affects its cellular localization, as demonstrated by the reduced CIRP cytoplasmic translocation in cells exposed to UV and treated with CK2 inhibitors or GSK3 β inhibitors [37]. Further studies of CIRP's post-translational phenotypes after hypoxia, endotoxemia or alcohol exposure are expected to reveal insights into the pathophysiology of inflammatory diseases and data potentially useful for the development of treatments targeting CIRP cytoplasmic translocation and secretion.

Facts and findings: Temperature-dependent and -independent expression of CIRP

In mammalian cells, relative to the body's steady-state temperature (36.5–37.5°C) CIRP's transcription levels reach its peak at mild to moderate hypothermia (28–34°C) and drop significantly at extreme hypothermia (15–25°C) [1, 67, 68]. In contrast, hyperthermia (39–42°C) causes substantial decreases in CIRP in cultured cells [43, 69]. These temperature-dependent CIRP expression studies have been conducted mostly in neurons, fibroblasts and male germ cells [43, 67, 68, 70]. CIRP can also be induced by a wide range of stimuli such as endotoxemia, hypoxia, radiation, toxins and drugs in a temperature-independent manner [9, 11, 71–73]. The temperature-independent CIRP's expression studies such as in hypoxia and endotoxemia were performed mainly on macrophages [11]. During mild hypothermia, the transcription factor Sp1 is recruited to the mild-cold responsive element located in the close proximity of the CIRP gene, leading to increased CIRP expression [62]. Whereas under hypoxia or inflammatory conditions CIRP is upregulated through HIF1 α - and NF- κ B-dependent pathways [11, 74]. Therefore, temperature-dependent and -independent induction of CIRP's transcription involve distinct intracellular signaling pathways, transcription factors, and possibly also cell-types.

CIRP's expression in cold response changes over time. When cells are exposed to hypothermia, CIRP transcription raises within 3 h and reaches maximal expression at 12 h, but drops precipitously within 8 h of rewarming [67, 75]. In normothermia, time-dependent changes in the quantity of CIRP within the cells (iCIRP) and in the extracellular space (eCIRP) have been studied in macrophages following hypoxia and reoxygenation. iCIRP's expression was dramatically increased at 7 h of reoxygenation, while decreased to its

original levels at 24 h of reoxygenation [11]. On the other hand, the highest level of eCIRP was found at 24 h after reoxygenation [11]. In a murine model of hemorrhagic shock, eCIRP serum levels increased after hemorrhage in a time-dependent manner [11]. Although in terms of the kinetics of CIRP's expression were done in *in vitro* and *in vivo* conditions, its stability and degradation statuses were not reported yet. Furthermore, eCIRP's circulating half-life has not yet been determined, although a single exogenous administration of eCIRP is sufficient to cause persistent inflammation and organ injury [17].

Mechanism: How does extracellular CIRP exaggerate inflammation?

eCIRP's role as a key inflammatory mediator has been scrutinized in various inflammatory disease conditions. CIRP^{-/-} mice subjected to models of hemorrhagic shock, organ-targeted ischemic/reperfusion injury and sepsis exhibit protective effects, such as the attenuated organ dysfunction and improved survival, suggesting eCIRP plays a critical role as in the development of inflammation and injury in these disease models [11, 13, 24, 26, 65]. Indeed, administration of recombinant CIRP to healthy rats is sufficient to increase serum levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6 and high mobility group box-1 (HMGB1), and induce liver injury, as assessed by increased levels of the organ injury markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [11]. Additionally, healthy mice injected with recombinant CIRP develop lung injury, as indicated by lung histology [17]. eCIRP-induced lung injury is characterized by increased infiltration of neutrophils into the lungs, increased vascular endothelial permeability, and activation of vascular endothelial cells [17, 20]. The effects of eCIRP and its mechanism of action have already been studied in a number of immune cells, including macrophages, lymphocytes, neutrophils and dendritic cells [11, 13–15, 76]. eCIRP's effects have also been studied in endothelial and epithelial cells [17, 20, 50] (Figure 2).

Macrophages

Macrophages treated with recombinant CIRP release TNF- α and HMGB-1 [11]. eCIRP-mediated expression of proinflammatory cytokines and chemokines has been determined in murine primary macrophages, RAW264.7 mouse macrophage M1-like cells, THP-1 human monocyte-like cells and BV-2 murine microglial cells [11, 21, 25]. Since the pattern recognition receptors (PRRs) TLR4, TLR2 and RAGE are known to regulate macrophage release of TNF- α , the response to recombinant CIRP was evaluated in macrophages deficient in each of these receptors [11]. While TLR4-deficient macrophages are unable to release TNF- α in response to recombinant CIRP, RAGE- and TLR2-deficient macrophages respond to eCIRP as well as WT macrophages, indicating that the TLR4 pathway is key for macrophage responsiveness to eCIRP [11]. eCIRP's dependence on TLR4 was further confirmed *in vivo* [11]. While WT mice injected with recombinant CIRP exhibited a dose-dependent increase in the serum levels of the proinflammatory mediators TNF- α , IL-6 and HMGB1 and the organ injury markers AST and ALT, these effects were not recapitulated in TLR4^{-/-} mice [11]. Surface plasmon resonance (SPR) analysis showed that CIRP binds to the TLR4-MD2 complex, as well as to TLR4 and MD2 individually, with very high affinity, strongly suggesting that eCIRP activates macrophages via its direct binding to the TLR4-MD2 complex [11]. eCIRP's binding to TLR4 in macrophage causes NF- κ B activation and nuclear translocation, ultimately leading to the induction and release of proinflammatory

cytokines and chemokines [11] (Figure 2). Recombinant CIRP also showed high affinity binding to RAGE and TLR2, although the precise biological relevance of these two receptors for CIRP-mediated macrophage activation remains to be determined [11].

eCIRP has also been identified to augment trauma-induced sterile inflammation through macrophage necroptosis [18]. Following tissue damage in trauma, eCIRP activates macrophage production of reactive oxygen species (ROS) via the TLR4-myeloid differentiation factor 88 (MyD88) signaling pathway [18]. This ultimately activates endonuclease G, which serves as an executor for mtDNA fragmentation in macrophages, ultimately triggering necroptosis and thus furthering inflammation in trauma [18].

Neutrophils

Neutrophils are the most abundant circulating leukocytes and are critical effector cells of the innate immune system [77]. Neutrophils express a wide range of PRRs, including TLRs, Fc receptors, and complement components [77, 78]. Furthermore, neutrophils have the capacity to kill microorganisms through phagocytosis, degranulation of cytotoxic proteins and peptides, and release of neutrophil extracellular traps (NETs) [79, 80]. Following infection or trauma, neutrophils rapidly migrate to inflamed tissues where they release a large number of microbicidal components such as lactoferrin, lysozyme, proteases, myeloperoxidase (MPO) and ROS, including hydrogen peroxide and superoxide, for elimination of invaded pathogens [81, 82]. However, excessive accumulation of activated neutrophils can lead to deleterious effects by disrupting the endothelial cell barrier, damaging cells and the extracellular matrix and causing unrestrained inflammation [83, 84]. Excessive lung neutrophil accumulation in the interstitial and alveolar spaces, for example, causes lung injury in mouse models of hemorrhagic shock, mesenteric ischemia, and sepsis [20, 84]. CIRP^{-/-} septic mice, however, only develop a mild form of lung injury with mild neutrophil infiltration of the lungs and mostly preserved lung architecture compared with septic WT mice [20], suggesting that eCIRP plays a key role in the development of acute lung injury (ALI) in septic mice. Indeed, injection of healthy mice with recombinant CIRP is sufficient to induce lung injury characterized by increased lung levels of TNF- α and IL-1 β and accumulation of neutrophils in the lungs [17]. Similarly, hepatic ischemia results in overproduction of the chemokine macrophage inflammatory protein-2 (MIP-2) in the affected liver tissue, which promotes neutrophil infiltration of the liver ultimately contributing to liver injury [26]. Conversely, treatment of mice subjected to hepatic ischemia and reperfusion with eCIRP neutralizing antibodies significantly reduced levels of proinflammatory chemokines, and decreased associated neutrophil recruitment to the liver [26].

Although neutrophils have traditionally been considered to be a relatively homogeneous population, emerging evidence indicates the existence of phenotypic and functional heterogeneity among neutrophils [85, 86]. eCIRP induces deleterious subsets of neutrophils during sepsis [13, 14]. The effect of eCIRP on the phenotype and function of neutrophils is described below.

NET-forming ICAM-1⁺ neutrophils

ICAM-1, also known as CD54, is a member of the Ig-like gene family [87] that plays a pivotal role in cell adhesion, migration, and aggregation via the interactions with its ligands, the integrins such as leukocyte functional antigen-1 (LFA-1) and macrophage receptor-1 (Mac-1) [88]. ICAM-1 is predominantly expressed on the endothelial and epithelial cells and to a lesser extent on certain subsets of leukocytes [89, 90]. Its expression is up-regulated by stimulation with endotoxin and proinflammatory cytokines [89–91]. Neutrophil ICAM-1 expression increases after stimulation with LPS and TNF- α , and results in increased neutrophil-neutrophil adherence and aggregation [92]. The ICAM-1 expressing neutrophils have enhanced effector functions through the increase of phagocytosis and ROS generation [93]. A recent study demonstrated increased expression of C1RP and higher frequencies and numbers of ICAM-1⁺ neutrophils in the lungs of WT mice with sepsis but few ICAM-1⁺ neutrophils in the lungs of C1RP^{-/-} mice with sepsis, suggesting that eC1RP participates in the induction of ICAM-1⁺ neutrophils [13]. Indeed, *in vitro* treatment of primary murine neutrophils with recombinant C1RP significantly induced their surface ICAM-1 expression [13] (Figure 2). The induction of ICAM-1⁺ neutrophils by eC1RP was significantly attenuated in neutrophils treated with TLR4 neutralizing antibodies or an NF- κ B inhibitor, indicating that the induction of ICAM-1⁺ neutrophils by eC1RP is mediated through the TLR4 and NF- κ B pathway [13]. Neutrophils exposed to certain stimulants extrude NETs, which consist of chromatin filaments coated with citrullinated histones and neutrophil enzymes such as MPO and elastase [94]. NETs contribute to pathogen clearance [95] but, in sepsis, excessive NET formation has a deleterious effect by promoting tissue damage and thereby perpetuating inflammation [96]. In the lungs of septic mice, ICAM-1⁺ neutrophils showed significantly higher levels of iNOS and NETs compared to ICAM-1⁻ neutrophils [13]. Furthermore, eC1RP-induced ICAM-1⁺ neutrophils had significantly higher frequencies of iNOS and NETs than ICAM-1⁻ neutrophils [13]. Taken together, these observations indicate that ICAM-1⁺ neutrophils not only are phenotypically distinct, but also functionally relevant. eC1RP upregulation of NET-producing neutrophils is mediated through the induction of peptidylarginine deiminase 4 (PAD4), a rate limiting step in activation of NETs [13, 97]. Thus, eC1RP induces NET-forming ICAM-1⁺ neutrophils, a novel neutrophil subset participating in the pathogenesis of sepsis-associated lung injury.

Reverse migrated neutrophils

The conventional paradigm consists of neutrophil irreversible and unidirectional migration from the vasculature into the tissue [98]. However, recent studies have reported the ability of neutrophils to return to the bloodstream after migrating to the extravascular space through a process known as reverse transendothelial migration (rTEM) [85, 99–101]. These reverse migrated (RM) neutrophils have prolonged lifespan and are associated with pulmonary inflammation following cremaster muscle ischemia and reperfusion injury in mice, suggesting that RM neutrophils may contribute to dissemination of inflammation [100, 101]. Furthermore, RM neutrophils exhibit a proinflammatory phenotype, with increased levels of superoxide and high surface ICAM-1 expression [101]. In fact, the surface expression of ICAM-1 and CXCR1 have been used to differentiate RM neutrophils (ICAM-1^{hi} CXCR1^{lo}) from circulating (ICAM-1^{lo} CXCR1^{hi}) and tissue resident (ICAM-1^{lo} CXCR1^{lo}) neutrophils [99]. As discussed in the previous section, eC1RP induces iNOS and NET producing

ICAM-1⁺ neutrophils [13]. A recent study revealed that eCIRP also induces neutrophil rTEM. Septic WT mice had significantly more RM neutrophils in the circulation than septic CIRP^{-/-} mice [14]. Additionally, circulating RM neutrophils increased after intratracheal administration of recombinant CIRP to healthy mice, demonstrating that eCIRP is sufficient to induce neutrophil rTEM [14].

Neutrophil rTEM requires disruption of junctional adhesion molecule (JAM)-C forming endothelial cell (EC) junctions [101]. In the murine cremaster muscle ischemia and reperfusion injury model, lipid chemoattractant leukotriene B4 (LTB4) upregulated in the inflamed tissues induced neutrophil production of the proteolytic enzyme neutrophil elastase (NE), which disrupted JAM-C at EC junction, thus facilitating neutrophil rTEM [100]. This observation is in line with a recent study that showed increased NE and decreased JAM-C in the lungs of mice with sepsis or injected with recombinant CIRP [14]. *In vitro* neutrophil stimulation with recombinant CIRP significantly induced NE in cell culture supernatants, and the supernatant of eCIRP-stimulated neutrophils dramatically decreased EC surface expression of JAM-C, but not when NE blocking antibodies were added to the conditioned media [14]. These results indicate that eCIRP causes neutrophils to produce NE, which decreases EC surface expression of JAM-C, and provide the mechanism by which eCIRP promotes neutrophil rTEM [14] (Figure 3).

Lymphocytes and dendritic cells

During sepsis, T cells are activated, as evidenced by increased expression of CD69 and CD25 in CD4⁺ splenic T cells and of CD69 in CD8⁺ splenic T cells [15]. Excessive T cell activation, however, leads to T cell exhaustion via activation-induced cell death (AICD) [102]. eCIRP has been found to promote T lymphocyte activation in sepsis (Figure 2). Mice injected recombinant CIRP showed significantly increased the expression of activation markers on CD4⁺ and CD8⁺ splenic T cells [15]. This effect, however, was not seen in TLR4-deficient mice [15]. Additionally, treatment with recombinant CIRP promoted CD4⁺ T cell polarization towards a Th1 hyperinflammatory profile [15]. Thus, eCIRP plays an important role in T-cell dysregulation during sepsis in a TLR4-dependent manner. eCIRP has also been shown to induce dendritic cell activation and migration, as well as to enhance presentation of eCIRP-bound antigens to T-cells, revealing a novel role for eCIRP in the activation of professional antigen presenting cells [76].

Lung endothelial and epithelial cells

eCIRP has been shown to bind with high affinity to TLR4 to increase the release of pro-inflammatory cytokines *in vitro* and *in vivo* [11]. It is not yet known whether eCIRP can be internalized by itself or after binding to TLR4. Another unexplored possibility is that eCIRP or iCIRP could interact with intracellular PRRs such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the RIG-like helicases (RLHs), which would give a potential new direction on its effect on innate immune system.

The inflammasome is a multimolecular platform which assembles as a complex of NLR proteins, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1 (or caspase-5) to release proinflammatory

Neutralizing antibody against eCIRP

The first study of therapeutic approach based on targeting eCIRP was performed using polyclonal CIRP neutralizing antibodies raised in rabbit [11]. The antibodies effectively inhibited recombinant CIRP-induced TNF- α production in macrophages [11]. Administration of eCIRP neutralizing antibodies during fluid resuscitation to rats with hemorrhagic shock significantly reduced inflammation, measured by serum and hepatic levels of TNF- α and IL-6, neutrophil infiltration of the liver, and organ injury measured by serum AST and ALT [11]. Additionally, the survival rate of hemorrhaged rats administered CIRP neutralizing antibodies was significantly higher than that of hemorrhaged rats administered control IgG [11]. eCIRP neutralizing antibodies were also administered to mice with polymicrobial sepsis. Similarly to the hemorrhaged rats, septic mice treated with CIRP neutralizing antibodies had attenuated inflammation, less organ injury, and improved survival [11].

The beneficial effects of treatment with CIRP neutralizing antibodies have also been documented in a model of hepatic ischemia. Mice with hepatic ischemia treated with CIRP neutralizing antibodies exhibited markedly reduced inflammation, neutrophil infiltration of the liver, hepatocellular damage, and liver histological damage [26]. CIRP neutralizing antibodies also dramatically decreased the amount of apoptosis and nitrosative stress [26]. Ultimately, treatment with CIRP neutralizing antibodies lead to significant improvement in the survival after hepatic ischemia [26].

CIRP-derived small peptide

Recombinant CIRP binds to the TLR4-MD2 complex as well as to TLR4 and MD2 independently [11]. In order to identify small molecular inhibitors for eCIRP, overlapping 15-mer peptides covering the entire length of the human CIRP protein sequence were screened for their binding affinity for MD2 using SPR technology. Compound #23 (C23) peptide, covering human CIRP amino acid residues 111–125 was identified to have the highest affinity for MD2 [11]. Computational modeling predicted that C23 fits into MD2's pocket. Initial studies showed that C23 was able to reduce ICAM-1 expression and IL-1 β release in ECs stimulated with recombinant CIRP [11]. Moreover, administration of C23 to rats with hemorrhagic shock significantly reduced EC activation in the lungs [11]. These observations revealed the potential for developing C23 as a novel adjunctive therapeutic for hemorrhage resuscitation.

Further studies showed that C23 dose-dependently inhibited the production of TNF- α in rat peritoneal macrophages and in THP-1 cells exposed to recombinant CIRP, by protecting the degradation of I κ B α , and inhibiting NF- κ B nuclear translocation [22, 25]. When administered to septic mice, C23 was protective and attenuated inflammation (IL-6, TNF- α , and IL-1 β) and liver injury (LDH, ALT) and kidney injury (creatinine, BUN, NGAL, KIM-1) [22]. C23-treated mice also had improved lung histology, less TUNEL-positive cells, and decreased pulmonary EC activation (E-selectin, ICAM). Importantly, C23 improved the survival rate after sepsis [22]. Similar studies have shown that treatment with C23 also protects mice from hemorrhagic shock [11] and ischemic AKI [25] and mesenteric

ischemia [24], underscoring the eCIRP's critical role in inflammation and suggesting that C23 be developed as an effective treatment for sepsis and other inflammatory diseases.

CIRP-based vaccination strategy

Immunotherapy is an active area of cancer research [105], and many approaches are being developed to target tumor-induced immune suppression. A recent study suggests that eCIRP indirectly exerts anti-tumor effect by serving as an adjuvant enhancing T-cell reaction to tumor antigens [76]. To prove this concept, the investigators created a fusion protein combining amino acids of CD8 T cell's TCR specific epitope from OVA and three amino-acid flanking residues linked to the N-terminus of murine CIRP and named it SIIN-CIRP [76]. SIIN-CIRP protein induces DC maturation, cytokine production and migration in a TLR4-dependent manner [76] (Figure 2). SIIN-CIRP protein also enhances antigen presentation. When DC were pre-incubated with SIIN-CIRP and co-cultured with OT-I CD8 T-cells, strong interactions were observed in SIIN-CIRP-treated DC with CD8 T cells, which further led to T cell proliferation and differentiation [76]. Thus, eCIRP improves tumor antigen presentation by DC and activate effector T cells to promote tumor rejection. Additionally, CIRP-enhanced tumor immunogenicity was further improved by combination with conventional adjuvants as well as with antibodies neutralizing PD-1/PD-L1, CTLA-4 and IL-10 [76]. Therefore, CIRP-based vaccination strategies are able to circumvent tumor-induced immune suppression and may further improve the anti-tumor effects of checkpoint inhibitors.

Future perspectives and conclusions

iCIRP and eCIRP are now known to have very different functions. iCIRP acts as an RNA chaperone to coordinate stressor-related translational reprogramming [1, 8], while eCIRP functions as a DAMP to promote and amplify the inflammatory response [11, 17]. The marked improvement of inflammation and tissue injury in animal models treated with eCIRP targeting strategies underscores eCIRP's critical role in the pathogenesis of inflammation, and provides a rational framework for further developing these therapeutic agents as treatments for patients with inflammatory diseases [11, 22, 24]. Although studies have considerably expanded our understanding of eCIRP's role in acute inflammatory diseases, its role in chronic inflammatory diseases, Alzheimer's disease, fibrosis, asthma and autoimmunity remains to be explored. Likewise, studies have focused on eCIRP's role in macrophage, neutrophil and T cell biology, but it remains to be investigated in other cell types such as B and NK cells. eCIRP induction of NETs [13, 97] suggests that it may also influence extracellular traps in other cells, *e.g.* macrophages (METosis) and B cells (BETosis). Other knowledge gaps in eCIRP pathobiology include the biological significance of its binding to RAGE and TLR2, as well as the possibility of activation of additional receptors, such as MARCO, FcRs and P₂X₇ [106–108]. eCIRP has been shown to activate NF- κ B and the Nlrp3 inflammasome [17, 22], raising questions about eCIRP's connection to autophagy, which is linked to innate immune responses [109]. In addition, since miRNAs play an important role in regulating gene expression and function [110], it is worthwhile to investigate iCIRP's regulation by miRNA in both health and diseased states. Various miRNAs are transported by RNA-binding proteins towards multivesicular bodies (MVBs) for exosome loading and to the plasma membrane for secretion [111]. As such, iCIRP's role

in miRNA processing, exosomal loading or the secretion of miRNA by MVBs may also be worth investigating. A better understanding of eCIRP's secretion through exosomes, microvesicles or exocytosis is likely to generate additional targets to treat inflammation. A better understanding of each post-translational form of iCIRP is also needed. Identification of various forms of extracellular CIRP and their putative receptors will help reveal the pathophysiology of diseases and provide the rationale for the design of novel therapeutics. The recent studies focused on therapeutic approaches by targeting eCIRP using its neutralizing Abs or by developing small peptide that can mask its receptor [11, 22, 23]. Since there is still a lack of adequate information about how this molecule is released during inflammation, therapeutic targeting by interfering with its release would be promising. However, since lysosomal mediated CIRP release was discovered [11], therefore the use of molecules/drugs that inhibit lysosome release and ER stress inhibitors could be of importance for better clinical use in various diseases.

In conclusion, the discovery of eCIRP's role as a new DAMP has led to a new era in inflammation research, and numerous diseases and cells have been found to be profoundly affected by this molecule. eCIRP targeting strategies underline how critical eCIRP's proinflammatory activities are. Many knowledge gaps still exist and will need to be addressed. Many more disease conditions are likely to benefit from research targeting eCIRP. Thus, further discovering eCIRP's black box will help change CIRP-oriented research from bench to bedside.

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List of abbreviations

iCIRP	intracellular cold-inducible RNA-binding protein
eCIRP	extracellular cold-inducible RNA-binding protein
DAMP	damage-associated molecular pattern
TLR	Toll-like receptor
MD2	myeloid differentiation factor 2
NF-κB	nuclear factor- κ B
C23	compound#23
STAT3	signal transducer and activator of transcription 3
COPD	chronic obstructive pulmonary disease
EC	endothelial cells
ALT	alanine aminotransferase

LDH	lactate dehydrogenase
TNF	tumor necrosis factor
IL	interleukin
HMGB1	high mobility group box 1
TRPV	transient receptor potential vanilloid
ICAM-1	intercellular adhesion molecule-1
NETs	neutrophil extracellular traps
iNOS	inducible nitric oxide synthase
rTEM	reverse transendothelial migration
RM	reverse migrated
JAM-C	junctional adhesion molecule-C
NE	neutrophil elastase
BUN	blood urea nitrogen
NGAL	neutrophil gelatinase-associated lipocalin
KIM-1	kidney injury molecule-1
MPO	myeloperoxidase
MIP	macrophage inflammatory protein
Cox	cyclogenase
casp-3	caspase-3
PMN	polymorphonuclear neutrophils
mt	mitochondrial
NLRP3	NACHT, LRR and PYD domains-containing protein 3
ESR	erythrocyte sedimentation rate
CRP	c-reactive protein
MAPK	mitogen activated protein kinase
ALI	acute lung injury
I/R	ischemia/reperfusion
RA	rheumatoid arthritis
UC	ulcerative colitis

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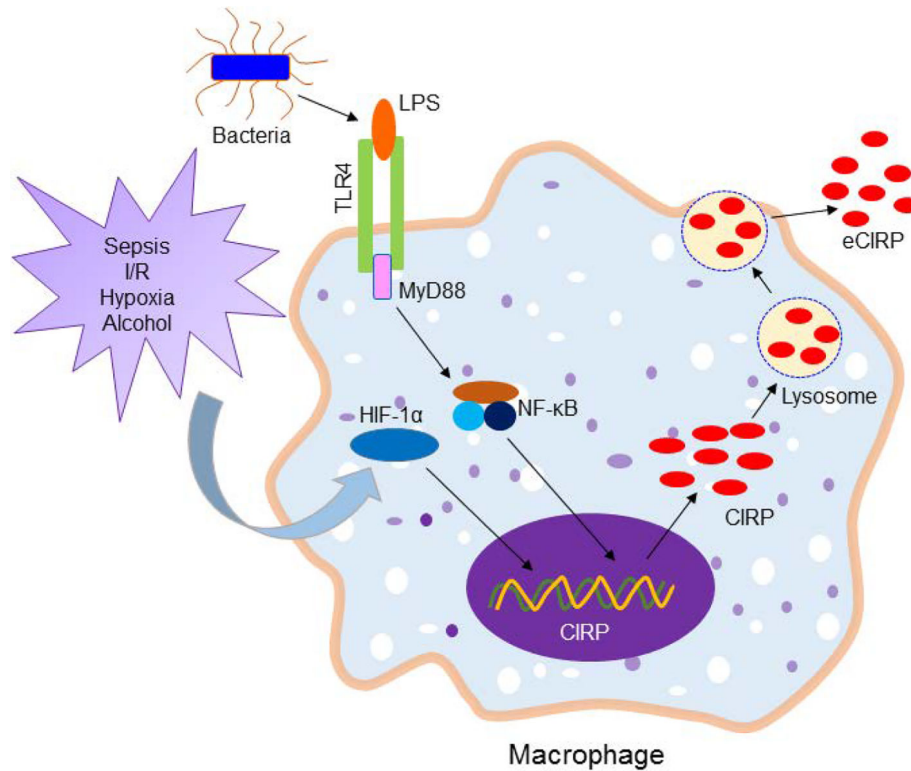


Figure 1: Release of eCIRP.

Sepsis and sterile injury promote global or localized infectious and hypoxic environment which activate TLR4 and HIF-1 α . Bacterial LPS binds to TLR4 and recruits MyD88 to transduce downstream signal for activating NF- κ B. HIF-1 α and NF- κ B serve as potent transcription factors to increase the expression of CIRP at mRNA levels. During inflammation or hypoxia cytoplasmic CIRP protein is enriched in the lysosomes. The lysosomes containing CIRP are then excreted outside the cells through exocytosis. eCIRP, extracellular cold-inducible RNA-binding protein; I/R, ischemia/reperfusion; TLR4, Toll-like receptor 4; HIF-1 α , hypoxia inducible factor-1 α ; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; MyD88, myeloid differentiation factor 88.

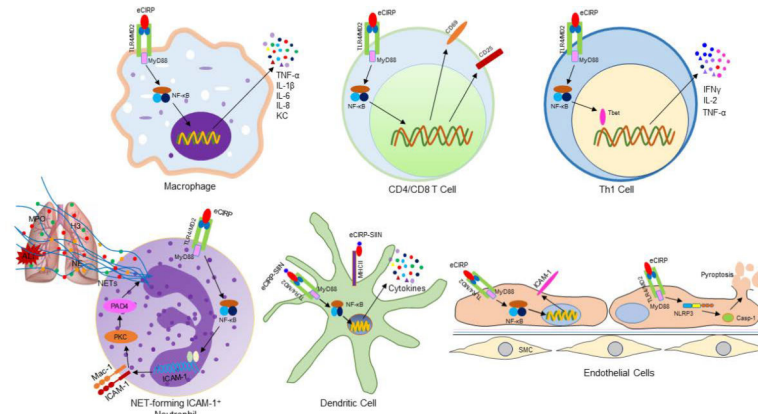


Figure 2: Proinflammatory effects of eCIRP.

eCIRP binds to its receptor TLR4/MD2 complex expressed in various cell types. In macrophages and lymphocytes, eCIRP activates NF- κ B through TLR4/MD2 complex and increases the expression of proinflammatory cytokines and chemokines, and T cell activation markers such as CD69 and CD25. eCIRP skews proinflammatory Th1 type T cells by activating the master transcription factor T-bet. In neutrophils, through the classical TLR4/MD2 and NF- κ B pathways eCIRP upregulates the expression of ICAM-1 on their surface. ICAM-1 transduces downstream signaling to activate PAD4-dependent NET formation. Increased NETosis by ICAM-1⁺ neutrophils in turn causes acute lung injury. In the DCs, a CIRP fusion protein prepared by combining amino acids of CD8 T cell's TCR specific epitope from OVA and three amino-acid flanking residues linked to the N-terminus of murine CIRP named SIIN-CIRP induces DC maturation, cytokine production and migration in a TLR4-dependent manner. SIIN-CIRP protein also enhances antigen presentation. In the ECs, eCIRP increases the expression of ICAM-1 through TLR4/MD2 and NF- κ B pathways. In addition, eCIRP induces NLRP3 inflammasome through TLR4/MD2 and NF- κ B-dependent pathways. Increased activation of inflammasome leads to caspase-1 activation and subsequently IL-1 β and IL-18 production and pyroptosis. eCIRP, extracellular cold-inducible RNA-binding protein; TLR4, Toll-like receptor 4; NF- κ B, nuclear factor- κ B; MyD88, myeloid differentiation factor 88; ICAM-1, intercellular cell adhesion molecule-1; DCs, dendritic cells; ECs, endothelial cells; SMC, smooth muscle cells; PAD4, peptidyl arginase deaminase 4; NLRP3, NACHT, LRR and PYD domains-containing protein 3.

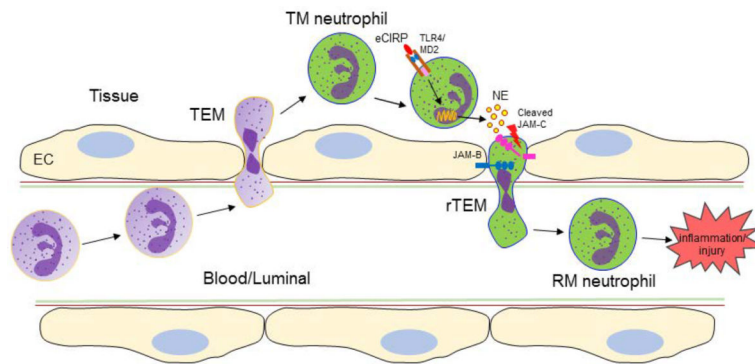


Figure 3: Neutrophil reverse transendothelial migration: role of eCIRP.

During inflammation neutrophils migrate from the blood to the tissues across the EC barrier through transendothelial migration. During inflammation eCIRP is increased in the blood and tissues. The TM neutrophils in the tissues are activated by eCIRP-TLR4 axis to release NE. The EC barrier is maintained by the interaction between JAM-C with its counterpart JAM-B. NE cleaves the JAM-C expressed on the surface of EC and enables TM neutrophils to pass through the disrupted EC barrier towards luminal direction. This process of neutrophil migration from the tissues to the blood during inflammation is called rTEM. The neutrophils that undergo rTEM is called RM neutrophils which further fuel inflammation. eCIRP, extracellular cold-inducible RNA-binding protein; TLR4, Toll-like receptor 4; EC, endothelial cells; TEM, transendothelial migration; TM, transmigrated; NE, neutrophil elastase; JAM-B/C, junctional adhesion molecule-B/C; rTEM, reverse transendothelial migration; RM, reverse migrated.

Table 1:

The role of eCIRP in inflammatory diseases.

Inflammatory diseases	eCIRP's source	eCIRP's effects on cells/tissues	Inflammatory/injury markers assessed	Therapeutic outcomes of targeting eCIRP	References
sepsis	blood, monocyte, macrophage, liver, heart, lungs	macrophage, lymphocyte, neutrophil, EC, hepatocyte, lung, spleen, kidney	ALT, LDH, TNF α , IL-6, HMGB1, Th1 cell, CD69 ⁺ and CD25 ⁺ T cell, ICAM-1 ⁺ neutrophil, NETs, iNOS, RM neutrophil, NE, JAM-C, histology, apoptosis, E-selectin, ICAM-1, creatinine, BUN, NGAL, KIM-1	CIRP ^{-/-} mice, anti-CIRP Ab and C23 reduce inflammatory cytokines, chemokines, activated immune cells, NETs, and iNOS and improve survival. TLR4 ^{-/-} mice attenuate eCIRP-induced lymphocyte activation	11, 13, 14, 15, 22
hemorrhage	blood, macrophage, liver, heart	blood, macrophage, EC, liver, lung	ALT, AST, LDH, TNF α , IL-6, IL-1 β , HMGB1, MPO, histology, EC barrier, ICAM-1	CIRP ^{-/-} mice, anti-CIRP Ab and C23 reduce inflammatory markers, MPO, histology, ICAM-1, EC barrier and improve survival	11, 23
alcohol-induced brain inflammation	microglial cell, brain	blood, brain	ALT, AST, LDH, TNF α , IL-1 β	CIRP ^{-/-} mice have reduced levels of ALT, AST, LDH, TNF α , and IL-1 β	19
cerebral ischemia and reperfusion	microglial cell, brain	microglial cell, neuroblastoma cell, brain	TNF α , caspase, Bcl2, NF- κ B	CIRP ^{-/-} mice have reduced infarct size and volume, less AIF1 expression in microglia	21
hepatic ischemia and reperfusion	blood	blood, liver	ALT, AST, LDH, histology, IL-6, MIP-2, neutrophils (Gr-1), apoptosis, iNOS, Cox-2	anti-CIRP Ab reduces ALT, AST, LDH, IL-6, MIP-2, iNOS, Cox-2, neutrophils, apoptosis, and improves survival	26
ALI	recombinant CIRP injection	EC, lung	TNF α , IL-1 β , ICAM-1, L-selectin, NAD(P)H oxidase, ROS, Nlrp3, ASC, casp-1, EC leakage, pyroptosis, BiP, pIRE1 α , sXBP1	CIRP ^{-/-} mice have reduced ER stress in lung	17, 20

Inflammatory diseases	eCIRP's source	eCIRP's effects on cells/tissues	Inflammatory/injury markers assessed	Therapeutic outcomes of targeting eCIRP	References
sepsis-induced ALI	lung	blood, arterial EC, lung	AST, IL-6, IL-1 β , MIP-2, KC, MPO, histology, apoptosis, BiP, pIRE1 α , sXBP1, CHOP, c-Casp-12, CD31, NF- κ B	CIRP ^{-/-} mice have reduced ER stress, NF- κ B, IL-6, IL-1 β , MIP-2, KC, MPO, histology, and apoptosis	13, 14, 20
renal I/R	blood, kidney	macrophage, blood, kidney	TNF α , IL-6, IL-1 β , KC, NGAL, KIM-1, BUN, creatinine, histology, apoptosis, neutrophils, nitrotyrosine, Cox-2	CIRP ^{-/-} mice, anti-CIRP Ab and C23 attenuate BUN, creatinine, inflammatory and injury markers and improve survival	25, 48
fracture trauma and hemorrhage (two hits)	blood	macrophage, lung	mtDNA fragmentation, apoptosis, ROS, autophagy, necroptosis	TLR4 ^{-/-} and MyD88 ^{-/-} mice protect against CIRP-induced mtDNA fragmentation	18
intestinal I/R	blood	blood, small intestine, lung	AST, LDH, TNF α , IL-6, histology, apoptosis, casp-3, MIP-2, MPO, Ly6G ⁺ PMN	CIRP ^{-/-} mice, C23 attenuate IL-6, LDH, histology, apoptosis, casp-3, and MPO	24, 65
RA	blood, synovial fluid, monocyte	blood, synovial fluid	ESR, CRP, IL6, TNF α	not studied	51, 52
COPD	airway epithelial cell	epithelial cells	IL-1 β , TNF α , MUC5AC, MAPK	CIRP siRNA attenuates cold-induced expression of IL-1 β , TNF α , MUC5AC, and MAPK	16
UC	colon mucosa	colon	histology, F4/80 cell, apoptosis, TNF, IL-1, IL-17, IL-23, BCL2, BCLXL, Sox2, tumor size	CIRP ^{-/-} mice show less colonic inflammation, small tumor size and apoptosis	53, 54

Abbreviations: EC, endothelial cells; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; HMGB1, high mobility group box 1; ICAM-1, intercellular adhesion molecule-1; NETs, neutrophil extracellular traps; eCIRP, extracellular cold-inducible RNA-binding protein; iNOS, inducible nitric oxide synthase; TLR, Toll-like receptor; RM, reverse migrated; JAM-C, junctional adhesion molecule-C; NE, neutrophil elastase; BUN, blood urea nitrogen; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; MPO, myeloperoxidase; MIP, macrophage inflammatory protein; NF- κ B, nuclear factor- κ B; Cox, cyclogenase; casp-3, caspase-3; PMN, polymorphonuclear neutrophils; mt, mitochondrial; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ESR, erythrocyte sedimentation rate; CRP, c-reactive protein; MAPK, mitogen activated protein kinase; ALI, acute lung injury; I/R, ischemia/reperfusion; RA, rheumatoid arthritis; COPD, chronic obstructive pulmonary disease; UC, ulcerative colitis.