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Role of antimicrobial peptide cathelicidin in thrombosis and thromboinflammation

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Thrombosis is a frequent cause of cardiovascular mortality and hospitalization. Current antithrombotic strategies, however, target both thrombosis and physiological hemostasis and thereby increase bleeding risk. In recent years the pathophysiological understanding of thrombus formation has significantly advanced and inflammation has become a crucial element. Neutrophils as most frequent immune cells in the blood and their released mediators play a key role herein. Neutrophil-derived cathelicidin next to its strong antimicrobial properties has also shown to modulates thrombosis and thus presents a potential therapeutic target. In this article we review direct and indirect (immune- and endothelial cell-mediated) effects of cathelicidin on platelets and the coagulation system. Further we discuss its implications for large vessel thrombosis and consecutive thromboinflammation as well as immunothrombosis in sepsis and COVID-19 and give an outlook for potential therapeutic prospects.

KEYWORDS

thrombosis, immunothrombosis, cathelicidin (LL-37), platelets, thromboinflammation, LL-37

Introduction

Thrombosis and thrombo-embolism are common causes of death and major health issues worldwide (1). Thrombosis can occur in arteries, veins and the microcirculation, and thus can affect all parts of the cardiovascular system. While arterial thrombosis (commonly associated with atherosclerosis) can result in myocardial infarction, peripheral artery disease and stroke, deep vein thrombosis and pulmonary embolism are major complications of thrombus formation in the venous system. Microvascular thrombosis is triggered mainly in the setting of septic or sterile inflammation, as recently demonstrated impressively in COVID-19 infection, where thrombo-embolic complications turned out as main drivers of mortality (2, 3). For a long time, platelets and the coagulation system have been considered the main players in thrombosis and eventually developed successful targets of therapeutic approaches. However, current antithrombotic strategies (i.e. platelet inhibition and anticoagulation) also affect physiological hemostasis and thereby increase

bleeding risk (4). Over the last decade inflammation has been established as a hallmark in the pathophysiology of thrombosis (5). Today, thrombotic and inflammatory processes are considered as inseparably linked and regulated by a complex interaction between immune cells, platelets, and soluble factors (5). While inflammatory diseases constitute a risk factor for both arterial (6–8) and venous thrombosis (9, 10), primary thromboembolic events can directly induce local tissue inflammation and a systemic inflammatory response (11–14). Crosstalk between thrombosis and inflammation in some settings can also be beneficial, as formation of thrombi inside blood vessels can support the immune system facilitating pathogen recognition and destruction, a process termed immunothrombosis (15).

Cathelicidins are antimicrobial peptides that form an effective component of the innate immune system (16–18). Various cells can release these peptides with neutrophils constituting the major source in the blood stream (16, 19–21). Neutrophil-activation is a key feature of thromboinflammation and cathelicidin is abundantly released and able to influence both inflammation and thrombosis (5, 22, 23). This article summarizes recent findings of how cathelicidin affects different aspects of thrombotic processes and highlight its consequences for thrombosis and thromboinflammatory disorders. Eventually, we also discuss these neutrophil-derived antimicrobial peptides as possible therapeutic targets.

General features of cathelicidin

Structure and function

Cathelicidins are antimicrobial peptides usually 10–50 residues in length and constitute a crucial component of the innate immune response (16). Though the mature form of the peptides is diverse in length, composition, net charge, and structure, the organization of the coding sequence is well conserved across species even beyond mammals including several vertebrates (24–26). Overall, the biological structure of cathelicidin peptides is diverse and complex, reflecting the many different roles that these peptides play in the immune system. The molecules are generally characterized by a net positive charge and a high proportion of hydrophobic amino acids and share some common structural features (16, 17, 27). Cathelicidins often contain a conserved region called the cathelin domain (28), which is thought to play a role in regulating the activity of the peptide (29). Further, many cathelicidins are alpha-helical peptides, that allows interaction with the membranes of bacterial cells and disrupture of their integrity (30, 31).

Humans express only one cathelicidin, hCAP18 gene is transcribed to a precursor peptide, which is extracellularly cleaved into the active form, that is called LL-37 because it consists of 37 amino acids. The mouse analogue is referred to as cathelicidin-related antimicrobial protein (CRAMP) (16). LL-37 is mainly expressed by immune cells (mainly neutrophils, macrophages, dendritic cells, and natural killer cells), but also by epithelial cells of the skin, eyes, gastrointestinal, genital and respiratory tract (32–34). It is released upon pathogen-mediated (bacteria, viruses, fungi, parasites) endoplasmatic reticulum stress (35), and also by NF-kB-induced inflammatory signals (16, 36), while active vitamin D and several other factors, such as short-chain fatty acids and some cytokines induce cathelicidin transcription (33, 37, 38).

Cathelicidin next to physical interaction with negatively charged membranes can directly or indirectly activate a variety of surface receptors or intracellular targets that are structurally unrelated (16, 39). The most studied receptor interacting with human cathelicidin is formyl peptide receptor like-1 (FPR2), a G-protein-coupled receptor with downstream effects on chemotaxis and angiogenesis (40). Further chemokine (C-X-C motif) receptor 2 (CXCR2), MrgX2, EGFR, IGF1R, or purinergic receptors P2X7 ionotropic receptor and P2Y11 have been associated to cathelicidin (16, 41, 42). After binding to nucleic acids, cathelicidin can enhance cell responses to self-nucleic acids released from damaged and dying cells, by permitting recognition by intracellular recognition systems such as Toll-like receptor (TLR) mitochondrial antiviral-signaling protein (MAVS) and stimulator of interferon genes (STING) (24, 43). Downstream pathways of cathelicidin signaling result in transcription and translation, including the modulation of NF-κB inhibitor-α (IκBα) and several kinase pathways (e.g. mitogen-activated protein kinases (MAPKs) p38, extracellular signal-regulated kinase 1 and 2, JUN N-terminal kinase (JNK) and phosphoinositide 3-kinase) (24, 44–46).

Antimicrobial and immune-modulatory effects

The first and most extensive investigated function of cathelicidin is its direct antimicrobial activity (44, 47, 48). The cationic and amphophilic character allows direct binding to negatively charged pathogen membranes or nucleic acids (24, 49, 50). These physical properties are prerequisites to induce membrane permeability, pore formation and eventually disruption leading to effective killing of pathogens, which makes cathelicidin a crucial player in the first line of immune defense. Cathelicidin is also effective against viral infection by direct interaction with viral particles and consecutive destabilizing their envelopes (51–54). Recent studies reported a strong interaction with the SARS-CoV2 receptor binding domain (RBD) of the spike protein, and cathelicidin thereby reduced the binding capacity of the cellular SARS-CoV2 receptor ACE2 (55).

Next to their antimicrobial activity, cathelicidin exerts pleiotropic effects on different cell types including both pro- and anti-inflammatory effects (16, 24, 44, 56). These could be explained by their strong affinity to bind other molecules and thereby modulate their function, resulting in enormous variations of the net effects, that can either be beneficial or detrimental in different pathophysiological context and tissue environments. Immune-modulatory effects of cathelicidin most importantly contain enhanced cellular killing capacities e.g. in neutrophils or T-cells (57–60), degranulation of mast cells (61–65), differentiation and polarization of immune cells such as pro-inflammatory macrophage differentiation (66–68), leukocyte recruitment (56, 69), neutralization of bacterial molecules that normally induce

proinflammatory immune responses such as LPS (36, 41, 56, 70–73) and induction of type I interferon response (24, 74).

Thrombo-modulatory mechanisms of cathelicidin

Direct effects on platelets

Platelets play a central role in thrombus formation in arteries, veins and microvessels. They interact frequently with immune cells to propagate thromboinflammation (5). Platelets are exposed to released cathelicidin locally at sites of thrombus formation but also systemically in the blood stream. Interestingly, data on direct effects of cathelicidin on platelets are scarce.

In a first study from 2015 investigating possible side effects of cationic antimicrobial peptide-based therapeutic strategies, P-selectin exposure on platelets was not observed after *in vitro* treatment of human platelet rich plasma (PRP) with LL-37 in concentrations from 0.025 mg/mL to 0.1 mg/mL (approximately 5-20 μM) (75). Another study reported inhibitory effects of LL37 on agonist-induced (ADP, U46619, collagen and thrombin at medium dose concentrations) expression of P-Selectin, platelet aggregation and fibrinogen-triggered platelet spreading of isolated human platelets. Mechanistically these observations were linked to reduced phosphorylation of Akt- and Src-kinases, however, cytotoxicity should be taken into consideration since *in vitro* concentrations of LL-37 peptide were in the range of 0.1 mM to 1.2 mM (76). Importantly, cytotoxic effects on eukaryotic cells have been described under culture conditions in the presence of 0.1 mM of LL-37 (77). Two studies published in 2018 more extensively focused on cathelicidin effects exerted on platelets at lower concentrations (20, 78), which are more likely to be achieved locally in inflammatory settings *in vivo* (47, 79–82). Pircher et al. reported that LL-37 in isolated washed human platelets dose-dependently induced alpha degranulation (P-selectin and CD40L surface expression and release) as well as release of IL-1β and HMGB1 (high mobility group box1) starting at 5 μM, which promoted platelet-neutrophil-aggregate formation and neutrophil activation. The suggested molecular mechanism involves glycoprotein VI receptor and downstream signaling through protein tyrosine kinases Src/Syk and phospholipase C. Blockade of other possible receptors for cathelicidin (as described in other cell types) such as FPR1, FPR2, or purinergic P2X7 did not alter LL-37-dependent platelet activation. Interestingly, LL-37 at the same concentrations did not induce GPIIb/IIIa-activation, and did not affect fibrinogen-dependent platelet spreading or platelet aggregation in PRP (both spontaneous and agonist-induced). In line with these findings, Salamah et al. reported comparable effects on LL-37-induced alpha degranulation in isolated platelets (78). Additionally, they observed increased fibrinogen binding, platelet aggregation and spreading. Interestingly, LL-37 hereby induced intracellular calcium mobilization comparable to that induced by cross-linked collagen-related peptide (CRP-XL 1 mg/mL), albeit with faster kinetics. Moreover, this study found low levels of LL-37 stored in platelet granules which were, in analogy to neutrophil responses, released upon activation indicating auto- and paracrine mechanisms of activation. Mechanistically, in mice CRAMP bound to formyl peptide receptor 2 (FPR2/ALX)/Fpr2/3, which is an orthologue to human FPR2/ALX, a Gi-coupled receptor for LL-37/CRAMP on immune cells (83, 84), whose expression has also been reported in megakaryocytes and human and mouse platelets (85, 86). Activation of FPR2/ALX/Fpr2/3 on platelets can increase P-selectin secretion and fibrinogen binding by reducing cAMP-dependent signaling that is known as an inhibitor of platelet functions (78). Accordingly, plasma of mice with psoriasis (that show significantly increased levels of CRAMP) considerably activated wildtype platelets but failed to do so in Fpr2/3-deficient mice (87). In addition to classical platelet activation, both LL-37-stimulated human platelets but also platelet intrinsic cathelicidin showed considerable antibacterial activity *in vitro* characterized by increased binding and killing of bacteria (88). In summary, cathelicidin is a potent activator of platelets that contributes to thromboinflammation (Figure 1).

Direct effects on coagulation and bleeding

The plasmatic coagulation system is an essential factor for thrombus stabilization as well as its resolution. Few studies have investigated possible interactions of cathelicidin and coagulation pathways. In an *in vitro* study that analyzed interactions of cathelicidin and related components with blood cells, LL-37 at concentrations of 50 μg/mL did not influence PT (prothrombin time) and aPPT (activated partial thromboplastin time) in poor platelet plasma, while at higher concentrations (0.2 mg/mL) both PT and aPTT were significantly prolonged (75). However, latter concentrations are unlikely to be reached locally *in vivo* (47, 80, 82). At lower concentrations LL37-dependent alterations in whole blood coagulation were not observed, neither in clotting time nor in clot thickness (75). Mice deficient in hematopoietic CRAMP did not show differences in both extrinsic or intrinsic coagulation ex vivo as assessed by ROTEM thrombelastometry (20). In mouse tail bleeding assays, injection of human LL-37 reduced bleeding time (78), however, no changes were observed in mice deficient for hematopoietic CRAMP (20). In summary, while cathelicidin exerts various effects on platelet functions, the peptides have minimal (if any) effects on blood coagulation at concentrations expected *in vivo* in the absence of inflammatory conditions or infections. New light has been shed on the effects of cathelicidin on the coagulation system in the context of Covid-19 (89), which is associated thromboembolic complications such as pulmonary embolism (2, 3, 90). In this condition levels of LL-37 were negatively correlated with thrombin time and positively related to plasma fibrinogen level suggesting pro-coagulatory effects. Indeed, LL-37 (0-50 ug/mL) increased activity of thrombin and FXa (Factor Xa), fibrinogen, and prothrombin, respectively (91). Further, LL-37 might decrease heparin effects by binding to heparin due to its cationic and amphipathic properties (92), which might influence coagulation. In summary, effects of cathelicidin on the coagulation system are not clear yet with dose-dependency possibly playing a role (Figure 1).

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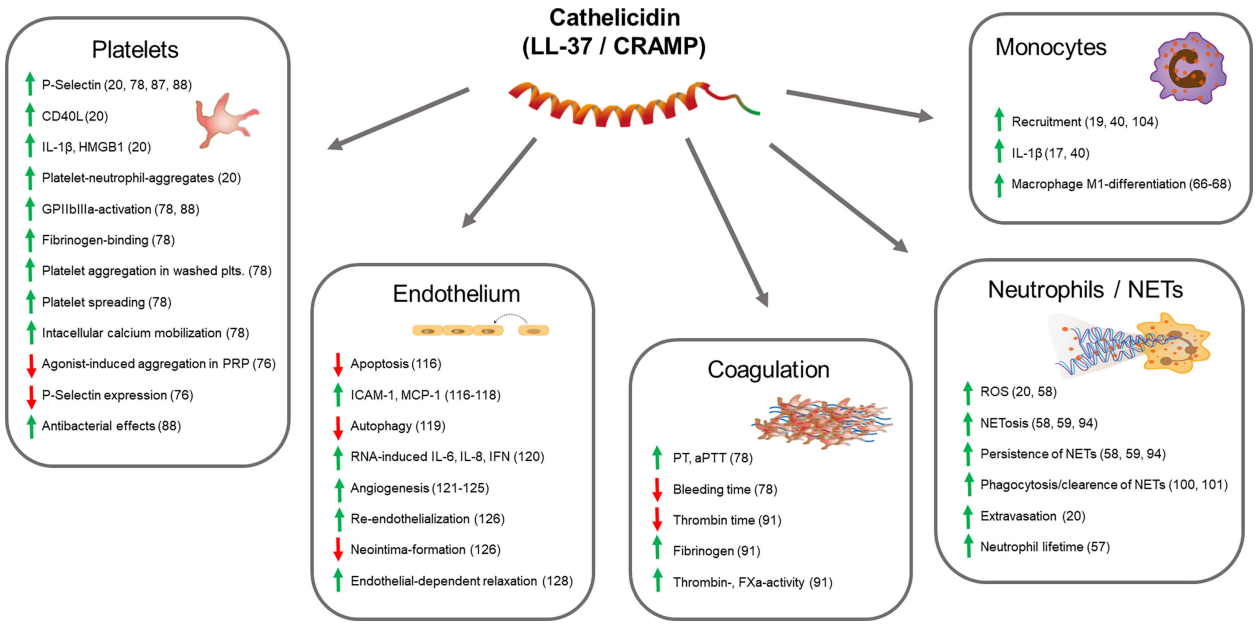

FIGURE 1
Thrombo-modulatory mechanisms of cathelicidin. The scheme displays effects of cathelicidin on cell types (platelets, endothelial cells, neutrophils, monocytes) and systems (coagulation) that are directly involved in thrombosis and thromboinflammation. Arrows indicate up-/down-regulation or increase/decrease of molecules or functions, respectively. References are indicated in brackets.

Effects on neutrophil activation and NETs (Neutrophil extracellular traps)

Next to direct effects on platelets or plasmatic coagulation, cathelicidin might modulate thrombosis *via* immune-cell-mediated mechanisms. Neutrophils play a crucial role in venous and arterial thrombosis as well as in immunothrombosis (15, 22, 93). Beside representing the main source of blood cell-derived cathelicidin neutrophil themselves can be activated by human LL-37 in an auto- and paracrine fashion (24). LL-37 potentiates neutrophil respiratory burst and induces NETosis partially through FPR2 (58, 59, 94). Hereby LL-37 translocates towards the nucleus and can disrupt the nuclear membrane (58). Neutrophil extracellular traps (NETs) are released by neutrophils upon activation and a potent mechanism to capture invading pathogens (95). Besides that, NET formation can stimulate coagulation through multifaceted mechanisms including NET-mediated platelet activation, disintegration of tissue factor pathway inhibitor (TFPI), and direct binding of vWF and activating factor XII (15, 96). Cathelicidin is a major component of neutrophil secondary granules and can be released and adhere to NETs (97, 98). In arterial thrombi, extracellular cathelicidin was abundantly associated with NETs (20). While NETs could simply serve as scaffold for cathelicidin to approach pathogens, the specific role of cathelicidin within NETs has been controversially discussed. LL-37 has been reported to be essential for NET survival and persistence by protecting neutrophil DNA from cleavage by bacterial nucleases (58, 59, 94), however, other studies suggested that LL-37 could also help clean NETs by binding to DNA and condensing it to denser assemblies for more effective phagocytosis by macrophages (99, 100). Further, cathelicidin-induced platelet activation led to increased platelet-neutrophil-interaction and thereby enhanced neutrophil ROS production and NETosis. Eventually, CRAMP-activated platelets enhanced injury-induced neutrophil extravasation in mice cremaster muscle venules (20). Thus, cathelicidin associate with extracellular nucleosomes that affect NET functions and provide a platform for interactions with adjacent cells or pathogens (Figure 1).

Effects on monocytes and other immune cells

Monocytes are important cells of the host defense system, but also play a role in thrombotic processes (15). Mechanistically, they express plenty of tissue factor once they are activated, which initiates the coagulation system (101, 102). Cathelicidin not only serves as chemoattractant for neutrophils and monocytes (17, 40) but can upregulate the expression and release inflammatory factors from monocytes, such as IL-1β (17). *In vivo*, cathelicidin recruits classical monocytes to the arteriolar endothelium in the mouse cremaster muscle through formyl-peptide receptor 2 (FPR2), a chemotactic receptor (19, 40, 103). Overall, might contribute to immunothrombosis and thromboinflammation in a monocyte-dependent manner.

In addition to effects on myeloid cells, cathelicidin can potentiate T-helper cell differentiation into a Th17 phenotype (104), thereby linking innate and adaptive immune responses. Th17/IL-17A-mediated inflammatory response in turn has been associated to a pro-atherosclerotic phenotype (105) and prothrombotic effects in autoimmune diseases (106). Further, stimulation of PBMCs (peripheral blood mononuclear cells) with

LL-37 was associated with reduced programmed cell death protein 1 (PDCD1) mRNA expression in patients with acute coronary syndrome (107) (Figure 1).

Effects on endothelial cells

Next to blood and immune cells the vascular endothelium is an essential regulator of hemostasis and functional endothelial cells continuously release antithrombotic mediators such as nitric oxide and prostaglandin to prevent blood clotting (108–111). While disruption of the endothelium leads to exposure of subendothelial collagen to the blood stream with consecutive rapid thrombus formation, also endothelial dysfunction is associated with a prothrombotic state and additionally leads to progressive vascular inflammation (22, 112–114). Activated endothelial cells express adhesion molecules including P-selectin, E-selectin, vWF, ICAM, and VCAM that recruit neutrophils, platelets, and monocytes (114). Effects of cathelicidin on endothelial cells are not understood in detail and are probably context-related. While LL-37 was shown to inhibit apoptosis of endothelial cells by neutralizing LPS (115), endothelial cells showed LL-37-dependent NF-kappaB-activation, expression of ICAM-1 and monocyte chemoattractant protein 1 (115–117). Further, LL-37 induces autophagy in endothelial cells but enhances cell death in autophagy-dysfunctional conditions, that plays a role in the pathogenesis of atherosclerosis (118). *In vitro*, LL-37 rapidly activated endothelial IL-6 gene expression, but this effect was temporary and not observed after several hours. However, LL-37 strongly boosted upregulation of IL-6, IL-8 and interferon beta expression induced by double-stranded RNA-analogon polyinosinic:polycytidylic acid (polyI:C). In contrast, while endothelial uptake of viral DNA was facilitated by LL-37, DNA-induced inflammatory response was abrogated (119). While abovementioned rather pro-inflammatory effects are clearly related to prothrombotic consequences, other studies describe cathelicidin-dependent effects that would rather decrease thromboinflammation. So, LL-37 has been shown to induce endothelium-dependent angiogenesis *via* FPR2-receptor and to stimulate proliferation and formation of vessel-like structures in cultivated endothelial cells (120). Similarly, the murine homologue CRAMP induced prostaglandin-dependent angiogenesis *in vivo* (121). Immobilized forms of cathelicidin *in vitro* showed mitogenic effects on endothelial cells that were comparable to those of vascular endothelial growth factor (VEGF) (122). Further, LL-37 induced tumor stromal cells to express strong pro-angiogenic factors that eventually facilitated tumor progression (123). Consequently therapeutic approaches of topical application of recombinant LL37 promoted wound healing through vascularization (124). In line with this, neutrophil-born LL-37/CRAMP promoted re-endothelization potentially through the same receptor (125). This limited neointima formation at sites of endothelial injury, mediated by recruitment and increased survival of endothelial outgrowth cells (125, 126). Further, LL-37 promoted endothelium-dependent relaxation in human omental veins mediated by FPR2-dependent release of nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) but not prostanoids (127) (Figure 1).

Cathelicidin in thrombotic and thromboinflammatory diseases

Release in thrombosis and thromboinflammation

Several cell types involved in thromboinflammation such as neutrophils, endothelial cells and macrophages express cathelicidin (16), with neutrophil-born likely to be the main source particularly in acute thrombotic processes (20). Neutrophil-activation is found in both pathogen-triggered immunothrombosis as well as sterile thrombosis with consecutive thromboinflammation. In microbial infection as well as sterile inflammation pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) as well as many inflammatory cytokines (e.g. IL-8, IL-6, IFN-γ, TNF α) and chemokines activate neutrophils and trigger cathelicidin release (24, 31, 128–133). In thrombosis activated platelets interact with neutrophils *via* P-selectin and P-selectin glycoprotein ligand 1 as well as glycoprotein Ib and macrophage-1 antigen, which mediate reciprocal activation directly *via* physical cell interactions as well as release of soluble mediators (23, 102, 134–136). Inflammatory signals lead to cathelicidin gene expression in response to endoplasmic reticulum (ER) stress and NF-κB activation (35). Additionally, cathelicidin stored as inactive precursor in azurophilic granules can be rapidly released by degranulation during immune responses (132, 137). Cathelicidin can be released, but also be exposed and bound to surface membranes and extracellular nucleosomes such as NETs as described above, which is a key feature in thrombosis (20, 97, 100, 138).

Although the effects of cathelicidin on different cell types have been extensively studied *in vitro*, the dynamics of release and concentration *in vivo* remain speculative. This is in part due to limitations in rapid local sample acquisition as well as technical *in vitro* handling, since the positively charged peptides readily stick to negatively charged surfaces such as cell membranes and also laboratory tubes. The concentrations of cathelicidin shown to have effects on platelets and other blood cells *in vitro* are not reached by those measured in the systemic circulation (79), although higher local concentrations have been found in bronchoalveolar lavage (BAL) fluid and at different mucosal sites (47, 80, 82). Although cathelicidin is considered to have a short half-life, it is very likely that relevant amounts of neutrophil-derived cathelicidin are exposed to blood components and other cells at sites of thrombosis. Though, neutrophils are considered the main source of cathelicidin in thromboinflammation that have capacity of immediate release of high amounts with influence on acute processes (20), release by other cells such as monocytes/macrophages, endothelial, smooth muscle cells or even platelets (16, 78, 88, 139) might contribute to ongoing inflammation with effects on development of chronic vascular diseases such as atherosclerosis (19, 116) (Table 1).

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TABLE 1 Cathelicidin expression and proposed function in thromboinflammatory diseases.

Disease	Main findings and mechanism	Ref.
Atherosclerosis	LL-37 detected in human atherosclerotic plaques	(116, 139)
	Decreased lesion size in CRAMP-/- ApoE-/- mice due to reduced recruitment of classical inflammatory monocytes and neutrophils	(19)
	CRAMP-mtDNA complexes aggravate atherosclerosis in ApoE-/- mice	(140)
	T cells reactive to mouse cathelicidin modulate plaque calcification in ApoE-/- mice	(107)
Thrombosis and haemostasis	Neutrophil-derived cathelicidin found in human coronary artery thrombi and FeCl3 induced mouse carotid thrombi	(20)
	Deficiency of (hematopoietic) CRAMP reduced FeCl3-induced carotid artery thrombosis and ligation-induced platelet adhesion	(20, 91)
	IV injection of LL-37 shortened bleeding time in mice	(78)
	IV injection of LL-37 or CRAMP accelerated arterial thrombosis in mice	(91)
	Injection of LL-37 decreased thrombus weight in arterio-venous shunt thrombosis in rats	(76)
Myocardial infarction and ischemia	LL-37-reactive CD8+ effector T-cells are associated with acute coronary events in human	(107)
	Systemic plasma levels of LL-37 transiently decreased in patients with STEMI, but are higher levels in culprit lesion	(141)
	CRAMP protects against cardiomyocyte apoptosis and cardiac I/R injury via activation of Akt and ERK and nuclear export of FoxO3a	(142)
	CRAMP aggravates ischemia-reperfusion injury via TLR4 and NLRP3-inflammasome activation	(143)
	Basal plasma levels of LL-37 associated with lower risk of ischemic events within the first 3 years after STEMI	(144)
	Cathelicidin recruits and retain bone marrow-derived stem/progenitor cells after myocardial infarction	(145–147)
	Cathelicidin-biofunctionalized stent reduced the incidence of in-stent-restenosis due to reduced neointima formation	(125)
	LL-37/CRAMP increases angiogenesis in rabbit hindlimb ischemia and wound revascularizatoin in mice	(120)
Immunothrombosis and sterile thromboinflammatory microvascular diseases	Protective role of cathelicidin in mouse models of sepsis by neutralization of LPS and induction of NETosis	(31, 58, 72, 94, 148, 149)
	Hematopoietic CRAMP-deficient mice protected from acid-induced lung injury and reduced pulmonary NETosis	(20)
	IV injection of high doses of human LL-37 or murine CRAMP induces pulmonary microvascular thrombosis in mice	(91)
	LL-37 strongly induces NETs in systemic lupus erythematosus	(150)
	Strong cathelicidin-dependent platelet activation in psoriasis	(87)
Covid-19	Neutrophil-derived LL-37 inversely correlated with disease severity in Covid19-infection	(151)
	Epithelial LL-37 was upregulated by SarsCov-2 spike protein and elevated in plasma of Covid19-patients	(91)
	Intranasal administration of LL-37 decreased lung infection in mice infected with human ACE2 expressing adenovirus	(55)
	LL-37 levels negatively correlated with thrombin time but positively correlated with fibrinogen level	(91)

Cathelicidin in atherothrombotic and large vessel disease

Cardiovascular diseases remain the leading cause of mortality worldwide despite current therapeutic interventions. The majority of cardiovascular deaths are caused by myocardial infarction and stroke following rupture of an atherosclerotic plaque and subsequent thrombotic arterial occlusion. Atherosclerosis is nowadays considered an inflammatory disease and immune cells, including neutrophils, play a role in all steps of atherothrombosis (152–156).

In this context, LL-37 has been detected in human atherosclerotic plaques, where it interacts with macrophages and endothelial cells (116, 139). A functional role in the progression of atherosclerosis was identified in ApoE-deficient mice (a commonly used mouse model for atherosclerosis), where cathelicidin deficiency significantly decreased atherosclerotic lesion size (19). Mechanistically neutrophil-derived cathelicidin enhanced recruitment of classical inflammatory monocytes and neutrophils (19). Another study indicated that CRAMP-mtDNA complexes aggravate atherosclerotic lesion formation in ApoE-deficient mice and suggests that LL-37-mtDNA complex acts as a key mediator of atherosclerosis formation (140). Furthermore, T cells reactive to mouse cathelicidin may be involved in modulating plaque calcification in ApoE-deficient mice (107).

Rupture of atherosclerotic plaques is the primary cause for arterial thrombosis and associated mortality in myocardial infarction and stroke. LL-37 was abundant in coronary artery thrombi of patients with acute myocardial infarction as well as mouse arterial thrombi induced by ferric chloride injury (20). In this context cathelicidin derived almost exclusively from neutrophils and was abundantly associated to neutrophil-derived nucleosomes (NETs). Deficiency in hematopoietic CRAMP delayed ferric chloride-induced carotid artery occlusion and decreased thrombus size and stability; similarly, carotid artery ligation-induced platelet adhesion was reduced (20, 91), while bleeding time was unaffected (20). An independent study showed that intravenous injection of LL-37 (20 μM) into mice shortened bleeding time (78). Accordingly, addition of LL-37 (10-50 μM) to human whole blood concentration-dependently increased thrombus formation ex vivo on collagen-coated slides under arterial shear rates (78). Recently, similar effects with accelerated arterial thrombosis in mice were described upon injection of both human LL-37 or murine CRAMP (91). Interestingly in a slightly higher dose (10-15 mg/kg corresponding approximately 50 μM in the blood) LL-37 decreased thrombus weight in a model of arterio-venous shunt thrombosis in rats (76). Future studies should address the role of cathelicidin in venous thrombosis and thromboembolism.

Cathelicidin in myocardial infarction and ischemia

Though one could assume that cathelicidin could promote acute myocardial infarction, the best example for atherothrombosis, its role in this setting remains unclear. A recent study suggested that the persistence of LL-37-reactive CD8+ effector T-cells may be involved in acute coronary events (107). Observations from clinical studies showed that systemic plasma levels of LL-37 transiently decreased in patients with ST-segment myocardial infarction (STEMI) as compared to patients without or stable coronary artery disease, but were restored within 24 hours (141). However, local plasma levels in the culprit lesion were higher than in the systemic circulation (141). Explanations for these observations are speculative, as excessive trapping of the positively charged peptides at injury sites but also binding to heparin (which is routinely administered early even after suspicion of acute myocardial infarction) are possible. As cathelicidin release is likely to promote acute events, its role for consecutive myocardial injury is controversially discussed, as discrepant impact on ischemia-reperfusion injury have been described, including both protective effects on cardiomyocytes as well as harmful response *via* activation of Akt/ERK and nuclear export of FoxO3a or *via* TLR4- and NLRP3-inflammasome activation, respectively (142, 143). Interestingly, higher basal plasma levels of LL-37 were associated with lower risk of ischemic cardiovascular events within the first 3 years after STEMI (144).

Further, cathelicidin may recruit and retain bone marrow-derived stem/progenitor cells (BMSPC) after myocardial infarction, which potentially could help post-AMI remodeling and recovery (145–147). On the other hand neutrophils after myocardial infarction induce excessive release of platelets and thereby boost the risk of recurrent ischemia (157). Another study showed that LL37/CRAMP increases angiogenesis in rabbit hindlimb ischemia model as well neovascularization of wounds in mice (120). Noteworthy, as neutrophil-born cathelicidin promoted angiogenesis and re-endothelialization, in an experimental model of stent-thrombosis (stenting of occluded arteries is the first-line intervention in acute coronary syndrome) a cathelicidin-biofunctionalized stent reduced the incidence of in-stent-restenosis (125).

Cathelicidin in immunothrombosis

Immunothrombosis is a critical process in which pathogen-induced inflammation uses microvascular thrombosis to combat infections and defend pathogens (5, 15, 96). Immunothrombosis can facilitate pathogen recognition, compartmentalization, trapping and killing, but also prevent spreading. Mechanistically the process is based on complex and reciprocal interplay between platelets, the coagulation system and innate immune cells. Herein the role of neutrophils is to highlight and their property to undergo NETosis is a key feature to induce thrombosis (96, 158–161). Considering cathelicidin an essential component of neutrophil granules as well as NETs, it might play a central role in this context. Surprisingly its precise role has not been investigated in a specific model of immunothrombosis so far. However, several studies showed that cathelicidin has a protective role in mouse models of sepsis which is mediated by several mechanisms (31, 72, 148). Hereby, next to direct antibacterial properties and neutralization of LPS, LL-37 strongly induced of NETosis in sepsis (58, 94, 149, 162), which in term is a main driver of immunothrombosis. While the term

"immunothrombosis" has developed with respect to thrombosis in response to pathogens, similar mechanisms are observed in response to sterile inflammatory conditions such as ARDS (acute respiratory distress syndrome) or autoimmune diseases (5, 163, 164). In a mouse model of sterile lung injury deficiency of hematopoietic cathelicidin reduced pulmonary NETosis and systemic markers of thromboinflammation (20). Interestingly, a recent study described spontaneous microvascular thrombus formation in the lung of mice 10 minutes after intravenous injection of high doses (30 mg/kg) of human LL37 or murine CRAMP (91). However, it remains unclear whether local thrombus formation or thromboembolism was responsible for these observations. Further, LL-37 has shown to be a strong inducer of NETs in systemic lupus erythematosus (150) that correlates with both micro- and macrovascular thrombosis (98, 129, 165). In psoriasis, a systemic autoimmune disease mainly affecting skin and joints, cathelicidin levels are locally but also systemically elevated (60, 87). Strong cathelicidin-dependent platelet activation has been described (87) and may contribute to the prothrombotic state in these patients (166).

Cathelicidin in Covid-19

The importance of the crosstalk between inflammation and thrombosis got notable attention in the context of the Covid-19-pandemic. Soon after the beginning of the global spread of SARS-CoV-2-virus it became evident that next to acute respiratory distress syndrome, cardiovascular events such as venous thromboembolism, MI and stroke were major causes of fatality in infected patients (2, 3, 90). Numerous studies have highlighted the role of NETosis and thromboinflammation in this context (5, 167–169). Given the outstanding position of cathelicidin at the crossroads of infection, inflammation and thrombosis several studies have focused on a pathophysiological role and speculated with therapeutic prospects (170, 171). Human cathelicidin LL-37 exerted antiviral properties by reducing SARS-CoV-2 binding capacity to its cellular receptor ACE2 (55) and neutrophil-derived LL-37 was inversely correlated with disease severity supporting protective effects in Covid19-infection (151). A study particularly focusing on possible effects of cathelicidin on thrombotic processes, observed that epithelial LL-37 was upregulated by the spike protein and consequently elevated in plasma of Covid-19 patients. LL-37 levels negatively correlated with thrombin time but positively correlated with fibrinogen level. Cathelicidin enhanced platelet activation and activity of coagulation factors Xa (FXa) and thrombin, suggesting that it significantly contributes to prothrombotic state in Covid-19-infection (91).

Cathelicidin as possible therapeutic target

Ever since cathelicidin has been discovered more than 20 years ago, the promise to take therapeutic advantage of the peptide has shined on the horizon. Particularly its antimicrobial effects have been considered as useful synergism to conventional antibiotics in bacterial infections (31, 48, 172–174). Nevertheless, most of the pre-clinical studies have failed to advance the therapeutic implications for the clinic. One reason for this might be a relatively narrow window of bioavailability, because peptides are quickly broken down by proteases, which limits routes of administration. This problem has not been reliably managed so far even with modern technologies, such as nanoparticle-carriers, which modulate its functions (175, 176). Local applications such as the cornea of the eye or skin lesions may be accessible for therapeutic approaches of cathelicidin-modifying therapies (124, 177, 178). However, systemic interventions are hampered by context-related pleiotropy and complex immunomodulatory effects, and by consecutive unwanted side effects. For example in immunothrombotic settings it is challenging to find a therapeutic range in which cathelicidin successfully combats microbes and on the other hand does not drive excessive thromboinflammation (5, 31). With respect to sterile thrombotic disorders, where antimicrobial properties might be dispensable, a straightforward approach for pharmacologic inhibition of cathelicidin remains still challenging as beneficial effects on ischemic tissues might escape (142, 143, 179). Therefore, to take therapeutic advantage of cathelicidin particularly in thrombosis and thromboinflammation, it is essential to know its precise pathophysiological spatial and temporal role in several tissues. Though, this still will not automatically overcome above-listed problems and make cathelicidin a grateful target in thrombosis, however it could propose interesting new or more easily accessible downstream targets, such as platelet GPVI- and FPR2-receptors or even the P-selectin-PSGL1-axis as master regulator of platelet-neutrophil-interaction (20, 23, 78, 180).

Conclusion

Cathelicidin – in particular of neutrophil origin – plays an important role in both inflammation and thrombosis, and thereby in principle represents an excellent therapeutic target candidate for thrombosis and thromboinflammation. So far clinical application has been limited by in part poorly understood and highly variable effects on tissues. Further research extending our knowledge on the precise function of cathelicidin in health and disease might overcome limitations and bring advantages for the treatment of inflammatory diseases.

Author contributions

QZ, QU, and JP did literature research and wrote the review. CS and JP supervised and revised the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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