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CROSS-TALK BETWEEN THE PLASMINOGEN/PLASMIN SYSTEM AND INFLAMMATION RESOLUTION:

Pro-resolving properties of the Plg/Pla system

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Abstract

The Plasminogen/Plasmin (Plg/Pla) system, best known for its classical role in thrombolysis, has been recently highlighted as a regulator of other biological processes in mammals, including key steps of the resolution of inflammation. Inflammation resolution is a complex process coordinated by different cellular effectors, notably leukocytes, and active mediators, and is initiated shortly after the inflammatory response begins. Once the inflammatory insult is eliminated, an effective and timely engagement of pro-resolution programs prevent persistent inflammation, thereby avoiding excessive tissue damage, fibrosis, and the development of autoimmunity. Interestingly, recent studies demonstrate that Plg/Pla and their receptor, Plg-R_{KT}, regulate key steps in inflammation resolution. The number of studies investigating the involvement of the Plg/Pla system in these and other aspects of inflammation, including degradation of extracellular matrices, immune cell migration, wound healing, and skeletal growth and maintenance, highlights key roles of the Plg/Pla system during physiological and pathological conditions. Here, we discuss robust evidence in the literature for the emerging roles of the Plg/Pla system in key steps of inflammation resolution. These findings suggest that dysregulation in Plg production and its activation plays a role in the pathogenesis of inflammatory diseases. Elucidating central mechanisms underlying the role of Plg/Pla in key steps of inflammation resolution either in pre-clinical models of inflammation or in human inflammatory conditions, can provide a rationale for the development of new pharmacological interventions to promote resolution of inflammation, and open new pathways for the treatment of thromboinflammatory conditions.

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Declaration of Interest
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Fibrinolysis; inflammation resolution; plasminogen; plasmin; Plg-R_{KT}

1. Introduction

Coagulation and inflammation are highly integrated processes triggered after tissue injury that prevent excessive bleeding, constrain, or eliminate invading pathogens and dead cells, among other functions, in order to reestablish vascular and tissue integrities [1]. It is widely known that most inflammatory signals induce pro-coagulant responses and that several components of the coagulation pathway have potent pro-inflammatory actions [2]. This crosstalk amplifies and maintains activation of both systems until they are counter-regulated by endogenous mechanisms (as discussed below) to avoid further tissue damage due to excessive inflammation and hypercoagulation [3, 4].

Fibrin is the final product of the coagulation cascade and the major component of the blood clot. The fibrinolytic or plasminogen/plasmin (Plg/Pla) system is activated to break down fibrin polymers within blood clots, thereby preventing thrombosis [5]. In addition, anticoagulant mechanisms counterbalance the synthesis or action of pro-coagulant factors [6]. Concomitantly, endogenous resolution programs operate to switch the production of pro-inflammatory toward pro-resolving molecules, by regulating leukocyte migration and clearing apoptotic cells to actively terminate inflammation and ensure the resumption of homeostasis [3, 7].

Dysregulation of any component of the coagulation/fibrinolytic and inflammation/resolution systems can affect the entire homeostatic balance, contributing to the pathophysiology of cardiovascular, respiratory, metabolic, and autoimmune diseases [8]. Notably, exacerbated inflammation and coagulopathy are hallmarks of deadly infectious diseases such as severe Coronavirus Disease-19 (COVID-19) [9, 10].

The molecular and cellular events that occur during coagulation, fibrinolysis and the onset of inflammation have been extensively investigated. However, the study of mechanisms underpinning the resolution phase of inflammation and, particularly the interplay between inflammation resolution and fibrinolysis, is an emerging area. Furthermore, accumulating evidence from recent studies, which are discussed in this review, expands the initially attributed function of Plg/Pla during acute inflammation (as reviewed elsewhere [11]) to highlight Plg/Pla as agonists of inflammation resolution.

2. Overview of the plasminogen/plasmin system

Plasminogen (Plg) is a glycoprotein of ~90 kDa comprised of 791 amino acids that is synthesized predominantly in the liver with lower expression in other tissues including the brain, adrenal gland, and kidney. Plg is found at relatively high concentrations (1.5-2μM) in human plasma and it is also present in interstitial tissues following plasma exudation during inflammation and tissue injury [12–14]. Plg is the zymogen of plasmin (Pla), a broad-

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spectrum enzyme composed of an N-terminal heavy chain (~65 kDa) and a C-terminal light chain (25 kDa) that contains the proteolytic active site [12].

Plg is converted into Pla through cleavage of the Arg561-Val562 peptide bond by either tissue-type (tPA) or urokinase-type (uPA) Plg activators [15]. Intravascular fibrinolysis, the breakdown of fibrin polymers in blood clots into smaller fragments (e.g., D-dimer), occurs primarily through tPA-triggered Pla generation, whereas uPA is more associated with tissue repair or remodeling [5]. Fibrinolysis is dependent on interactions between lysine residues in fibrin and lysine-binding sites (LBS) present in the Plg N-terminal chain [16]. Plg activation occurs more efficiently on cell surfaces than in solution due to the concomitant binding of Plg and its activators to their respective cellular receptors [17–19]. Of note, at least twelve Plg receptors have been identified so far, including α-enolase, Plg-R_{KT}, $\alpha M\beta 2$, and the annexin A2/S100A10 complex [18, 19].

Pla activity is low under physiological conditions, which means its activation into Pla occurs on a “need-only” basis, for example, once fibrin clots are formed [20]. The proteolytic activity of soluble Pla, i.e. not fibrin-bound Pla, is primarily regulated by α2-antiplasmin and also by α2-macroglobulin [21]. In addition, Plg activator inhibitor types-1 and −2 (PAI-1 and PAI-2, respectively) control tPA and uPA activities to prevent further Pla generation [22]. Thrombin-activatable fibrinolysis inhibitor (TAFI) is also an important regulator of the Plg/Pla system [23]. TAFI removes lysine and arginine residues from fibrin, impairing Plg binding to fibrin and, consequently, attenuating fibrinolysis [23].

Pla has several other substrates in addition to fibrin, including coagulation factors, pro-metalloproteinases, matrix proteins, and complement factors, and thus activates other proteolytic systems, suggesting that the Plg/Pla system has several other physiological functions apart from fibrinolysis, such as wound healing, and skeletal growth and maintenance [24, 25]. This concept is reinforced by the broad expression of Plg receptors by different cell types including myeloid, endothelial, neuronal cells, and fibroblasts in distinct body tissues [18].

The interaction of plasmin(ogen) with immune cells points to an important role of the fibrinolytic system in regulating immune responses [19, 20]. In addition, several lines of evidence suggest that the hemostatic and immune systems have been highly interconnected during evolution [1]. In primitive organisms, such as the horseshoe crab, the coagulation and innate immune systems are integrated [1]. These systems became much more complex and specialized in phylogenetically higher species, notably in *Homo sapiens;* however, the two-way crosstalk between coagulation and inflammation has persisted during evolution [1]. For instance, fibrin formation is known to trigger inflammation, and the interaction between activated platelets and neutrophils enhances leukocyte recruitment into inflammatory tissues [1, 26]. In addition, components of the immune system, such as pro-inflammatory cytokines can activate the coagulation system by inducing, for example, the expression of tissue factor (TF) and PAI-1 [2]. Interestingly, pro-inflammatory cytokines can also induce the expression of Plg [27] and its activators [2]. However, the study of the interplay between fibrinolysis, or thrombus dissolution, and the resolution phase of inflammation is more recent and remains to be better understood.

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3. Inflammation

Inflammation is an essential immune response that has enabled survival in relatively hostile environments under the potential threat of multiple infectious agents [28]. The vascular events that occur during an inflammatory response (vasodilatation, increased blood flow and vascular permeability) are an intrinsic part of the early stage or the “onset phase” of acute inflammation [29]. These events allow the accumulation of blood-derived leukocytes in affected tissues [notably polymorphonuclear (PMN) cells] to patrol and phagocytose invading microorganisms, particles, or dead cells [30]. On a histological level, maximal PMN cell infiltration is deemed to be the peak of acute inflammation [31]. Acute inflammation is usually resolved within a few hours or days [3, 7, 32].

The aim of the inflammatory process is to restore or adapt to a new state of tissue homeostasis, regardless of the causative agent (infectious or non-infectious) [30]. However, inflammation can be harmful to the host if excessive or persistent, leading to further tissue damage and hence complications, such as fibrosis, autoimmunity, and loss of function [30]. These are hallmarks of chronic inflammatory conditions that are prevalent in modern society, including cancer, obesity, cardiovascular, neurodegenerative, and some respiratory diseases [33]. Notably, elements of the coagulation pathway may induce or exacerbate inflammation and vice-versa, reinforcing the crosstalk between these two systems. For instance, the major trigger of blood coagulation, TF, can induce inflammation [34]; whereas C-reactive protein, an acute-phase reactant, induces TF expression in monocytes [35].

Inducers of inflammation are detected by sensors (receptors) expressed in leukocytes and other resident cells, such as endothelial cells, that produce soluble mediators as a response to stimuli [29]. These soluble mediators from diverse characteristics act on various target tissues and cells, which respond by altering their functional states and by producing further mediators to build an effective inflammatory response against damaging stimuli [30]. Another classic event triggered during inflammation mediated by microbial infections is the formation of neutrophil extracellular traps (NETs) [36]. During infections, neutrophils recruited to the affected tissue can release extracellular DNA, granule proteases, and histones, to prevent proliferation and dissemination of microorganisms [36]. NETs` release is an important mechanism for host infection control but their sustained activation is associated with uncontrolled inflammatory responses and the pathophysiology of several diseases [37].

The characteristics and consequences of an inflammatory response depend on several factors, including the type of inducer, sensors, activated cells, balance of pro- and anti-inflammatory mediators, affected tissues, and intensity/duration of inflammation [29]. Moreover, recent studies have revealed that the successful termination of inflammation depends on the timely production of pro-resolving mediators, that directly stimulate the resolution of inflammation [3, 7, 32].

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4. Resolution of inflammation

In the past, inflammation was believed to passively fade away once the inflammatory stimulus was eliminated, with a gradual dilution of pro-inflammatory mediators and leukocytes within the affected tissue over time. However, robust evidence from the last decades indicates that inflammation resolution starts shortly after the acute inflammatory response begins [38], and is an active rather than a passive process that involves complex and tightly coordinated actions of various cells, as well as spatial and temporal production of mediators to reestablish tissue homeostasis [39–41]. The resolution phase of inflammation can be defined as the interval between maximum neutrophilic infiltration to the time point at which neutrophils are depleted from the affected tissue, which parallels an increase in mononuclear cell infiltration [42].

Akin to the five cardinal signs of inflammation, resolution of inflammation is also associated with five basic pillars, which are: i) tissue clearance of pathogens, dead cells, and debris; ii) re-establishment of endothelial barrier integrity and vascular perfusion; iii) tissue regeneration; iv) fever remission; and v) alleviation of inflammatory pain [43]. To achieve these pillars of resolution, key molecular and cellular events occur, such as inhibition of pro-inflammatory mediators and induction of pro-resolving ones, decrease of neutrophil numbers through cessation of cell recruitment and induction of apoptosis, skew of macrophages toward anti-inflammatory/pro-resolving phenotypes, with further removal of apoptotic neutrophils from the affected tissue [3, 7, 32, 38, 39, 41]. Figure 1 illustrates the sequential events during self-resolving inflammation as outlined above, and the role of Plg/Pla in key steps of this process, which will be discussed in detail below.

Pro-resolving mediators comprise a large and growing family of mediators, including proteins/peptides [e.g., annexin A1 (AnxA1), glucocorticoid-induced leucine zipper (GILZ), Angiotensin-(1–7) and melanocortins], lipids [named specialized pro-resolving mediators (SPMs), including lipoxins (LXA4), resolvins (RvD), maresins (MaR), and protectins], gases (e.g., hydrogen sulfide, and carbon monoxide), and neuromodulators under control of the vagus nerve (e.g., netrin-1 and acetylcholine) [39, 40]. The cellular and molecular processes activated by pro-resolving mediators can both reduce the magnitude of the inflammatory response and stimulate its resolution, which means that they possess both anti-inflammatory and pro-resolving effects, but without causing immunosuppression [39, 40]. Mounting evidence suggests that pro-resolving mediators also enhance the clearance of microorganisms [40], stimulate tissue regeneration, attenuate fibrosis, alleviate pain [39], and help to prime adaptive immunity in the post-resolution phase of acute inflammation [3, 44].

Regarding the role of pro-resolving mediators during hemostasis, a temporal cluster of endogenously produced prothrombotic and proinflammatory eicosanoids mediators as well as SPMs has been identified during coagulation [45]. It was further demonstrated that pro-resolving mediators derived from omega-6 and omega-3 fatty acids (SPMs) are naturally produced in a time-dependent manner during venous thrombosis progression *in vivo*, notably during the onset of thrombus resolution [46]. Exogenous administration of the RvD4 significantly reduces thrombus size in mice with deep vein thrombosis [46].

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Additionally, RvD4 promotes the resolution of thrombus inflammation by triggering the synthesis of other docosahexaenoic acid-derived SPMs, inhibiting neutrophil migration and NETs release, increasing the percentage of cells in early apoptosis, and shifting macrophage phenotypes from M1 to M2 phenotype within the thrombus [46]. Notably, both lipid and protein pro-resolving mediators, including RvD1 and AnxA1 have been shown to be protective in thromboinflammatory sickle cell disease (SCD) [47, 48]. Indeed, by using pre-clinical models of SCD or human samples, it was demonstrated that these mediators act by inducing several pro-resolving mechanisms, including increasing efferocytosis of SCD erythrocytes and neutrophils, reducing inflammatory cytokine and vascular inflammation and inhibiting the thrombotic NET phenotype associated with neutrophil activation during thromboinflammatory conditions [47]. Given the temporal and mechanistic relationship between clot dissolution and resolution of inflammation during thrombus resolution, and the role of pro-resolving mediators in thromboinflammatory diseases, targeting fibrinolytic pathways could represent a novel therapeutic approach to resolve inflammation.

5. Emerging roles of the Plg/Pla system on inflammation resolution

5.1. The role of Plg/Pla in neutrophil apoptosis and efferocytosis

The shutdown of neutrophil function by apoptosis is essential for their removal from inflamed tissues, thereby avoiding further tissue injury [49]. Neutrophils undergoing apoptosis display engulfment molecules that promptly attract macrophages to phagocytize them, in a process termed efferocytosis [50].

Previous reports have documented enhanced plasminogen binding to apoptotic cells surface [51], and that the proteolytic activation of Plg to Pla is required for the phagocytic removal of apoptotic cells [52]. Moreover, necrotic cells also generate surface-bound Pla, and Pla enhance the efferocytosis of necrotic cells by dendritic cells via its protease activity, while producing an immunosuppressive state in dendritic cells [53]. In an elegant study, Das *et al.* showed that Plg enhances phagocytosis of apoptotic thymocytes *in vitro* by modulating the expression of genes involved in several of the main steps of the phagocytosis pathway, including recognition and engulfment, and phagosome maturation/ processing [54]. Moreover, Plg deficiency hampers the clearance of apoptotic cells *in vivo* [54]. Conversely, intrapleural injection of Plg or Pla at the peak of LPS-induced pleurisy promoted the resolution of neutrophilic inflammation by increasing neutrophil apoptosis and their removal by efferocytosis [55]. In addition, *in vitro* experiments showed that neutrophil treatment with Plg/Pla decreases neutrophil survival induced by LPS, overriding the pro-survival effect of LPS [55]. Moreover, Plg/Pla improved the efferocytic functions of mouse peritoneal macrophage when apoptotic neutrophils were provided as prey in the peritoneal cavity [55]. Mechanistically, neutrophil apoptosis and efferocytosis were dependent on Pla-protease activity and up-regulation of AnxA1 [55], a glucocorticoid-induced protein with anti-inflammatory and pro-resolving functions [39, 40], including in thromboinflammatory conditions [47]. Notably, deletion of either Plg or Plg-R_{KT} decreases the ability of macrophages to phagocytose apoptotic neutrophils both *in vivo* and *in vitro* associated with lower expression of AnxA1 and the scavenger receptor CD206, as compared to wild-type (WT) mice [56]. Indeed, $Plg^{-/-}$ mice also has higher neutrophils numbers and defective

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neutrophil apoptosis/efferocytosis during sepsis compared to WT mice, features that are rescued after Pla-treatment of septic WT mice [57]. The role of Plg/Pla in modulating neutrophil apoptosis and efferocytosis is shown in Figure 2.

5.2 Effects of Plg/Pla on mononuclear cell migration and macrophage polarization

Monocytes migrate from the bloodstream to peripheral tissues, where they differentiate into macrophages or dendritic cells, during both homeostasis and inflammation [58]. Recruitment of mononuclear cells to inflamed tissues is essential for the clearance of pathogens, apoptotic cells, and cellular debris [58]. In pre-clinical models of self-resolving inflammation, recruitment of mononuclear cells reaches a peak during the resolving phase of inflammation [42, 56].

Macrophages are able to adopt different phenotypes, depending on the inflammatory context or phase of inflammation. M1 (or classically activated) macrophages play major roles during the early stages of infection or injury. Microbial constituents (e.g., LPS) and pro-inflammatory cytokines [e.g., interferon (IFN)-γ] can induce the polarization of macrophages into the M1-like phenotype that produces high levels of pro-inflammatory mediators, such as IL-1β and TNF, and microbicidal molecules, such as reactive oxygen species and nitric oxide [59]. The macrophage phenotype progresses from M1 toward M2 (or alternatively activated type), as the resolution of inflammation evolves [56]. M2 macrophages secrete anti-inflammatory cytokines (e.g., IL-10 and TGF-β) and have higher efferocytic ability compared with M1 macrophages [59]. In vivo, M2 macrophages ($CD11b^{high}$) can further differentiate into Mres macrophages ($CD11b^{low}$), also known as “satiated” macrophages, as they have lost their ability to engulf apoptotic cells [60]. In addition to producing anti-inflammatory cytokines, Mres macrophages exhibit antioxidant and anti-fibrotic properties [61]. Once the resolution of inflammation is completed, resolutive macrophages migrate to the lymph nodes, where they convey signals to lymphocytes and other immune cells to build an adaptive immune response [44].

Studies revealed that plasmin(ogen) is a key factor for monocyte recruitment [56, 62–64]. In fact, Plg expression and Pla activity are high during the resolving phase of acute inflammation, when mononuclear cells predominate in inflammatory exudates [55]. In addition, Plg deficiency interferes with lymphocyte and dendritic cell recruitment, without affecting PMN migration [56, 62, 64].

The fibrinogen-binding motif for integrin $\alpha_M\beta_2$ is required for Plg-mediated mononuclear cell migration through fibrin matrices and Pla-mediated fibrinolysis loosens the migratory constraints on macrophages imposed by the $\alpha_M\beta_2$-dependent interaction with fibrin(ogen) [64]. It is noteworthy that Plg-dependent mononuclear cell recruitment also depends on Plg binding to cellular receptors, including Plg-R_{KT}, which is highly expressed in monocytes [65]. Indeed, monocyte recruitment is lower in Plg-$R^{-/-}$ mice compared to WT during thioglycolate-induced peritonitis [65]. In addition, genetic deletion of either Plg or Plg-R_{KT} results in decreased mononuclear cell migration during the resolving phase of LPS-induced pleurisy [56]. Since Plg-R_{KT} does not have an intracellular domain to transduce signaling by itself [66], its effects appear to be mediated by activation of Plg to Pla. One proposed explanation is that after Plg binds to its cell surface receptors (e.g.,

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Plg-R_{KT}), it adopts a conformation that favors its cleavage into Pla to exert its biological functions [67, 68], including the production of CCL2 [63], an important chemokine for the recruitment of mononuclear cells [69]. Indeed, there were lower CCL2 levels alongside lower recruitment of mononuclear cells into the pleural cavity of either $Plg^{-/-}$ or Plg-$R_{KT}^{-/-}$ mice during LPS-induced pleurisy [56]. Moreover, Plg/Pla from local macrophages are required for extravascular fibrin clearance by promoting endocytic uptake of fibrin by CCR2-macrophages [70].

Pla formation is also required for Plg-dependent mononuclear cell recruitment, as mutation of the Arg-Val site for Plg activators (resulting in a non-activatable Plg) or treatment with Pla inhibitors, including aprotinin, suppresses macrophage cell recruitment to inflammatory sites [63, 71, 72]. Once Pla is formed, it degrades extracellular matrices and activates other proteolytic enzymes, such as MMP-9 that facilitate cell migration [72]. Activation of pro-MMP-9 is reduced in the peritoneal fluid of thioglycolate injected-WT mice treated with anti-Plg-R_{KT} mAb and in $Plg^{-/-}$ mice in an abdominal aortic aneurysm model [65, 72]. The role of Plg/Pla in modulating mononuclear cell recruitment is shown in Figure 3.

Our group have shown that pleural injection of Pla or Plg increases the number of M2 ($F4/80^{high}/Gr1^{-}/CD11b^{high}$) and Mres ($F4/80^{med}/Cd11b^{low}$) macrophages, without affecting the number of M1 macrophages ($F4/80^{low}/Gr1^{+}/Cd11b^{med}$) 48h after injection [55]. In addition, Pla induces the expression of the M2 markers CD206 and arginase-1, while reducing the basal levels of inducible nitric oxide, an M1 marker [55]. The levels of TGF-β and IL-10, which are known inducers of M2-like macrophages, are also increased in the pleural fluid after Pla or Plg injection [55]. Corroborating these findings, *in vitro* experiments using murine or human macrophages, demonstrated that treatment with Plg/Pla stimulates the release/expression of the M2 markers (IL-10, TGF-β, CD206, and arginase-1) while inhibiting the release/expression of M1 markers (TNF-α, HLA, and CD86) in LPS+IFN-γ-stimulated macrophages [56]. Indeed, Borg and coworkers found increased TGF-β levels and decreased expression of CD86 and HLA-DR by monocyte-derived dendritic cells after Pla treatment [53]. Of note, pro-inflammatory $CD14^{++}CD16^{+}$ human and $Ly6C^{high}$ mouse monocytes express significantly higher levels of Plg-R_{KT} on their surfaces and present higher Plg binding capacity than other monocytes subsets [73].

It is known that macrophage polarization stimuli, such as IL-4 and IL-10 induce activation of the signal transduction and activators of transcription (STATs) to induce gene expression associated with the M2-like phenotype [59]. Regarding the signaling pathway elicited by Plg/Pla during macrophage polarization, our group and another showed that Pla induces STAT-3 phosphorylation in monocytes and macrophages [56, 74]. Interestingly, total and phosphorylated levels of STAT3 were lower in $Plg^{-/-}$ mice during sepsis, compared to $Plg^{+/+}$ mice [75]. Moreover, IL-10- and IL-4-induced polarization to the M2-like phenotype is impaired in murine bone marrow-derived macrophages (BMDMs) from $Plg^{-/-}$ and Plg-$R_{KT}^{-/-}$ mice, which is associated with reduced STAT3 phosphorylation and lower expression of Arginase-1 and CD206 M2-markers [56]. Notably, while both $Plg^{-/-}$ or Plg-$R_{KT}^{-/-}$ mice exhibit higher M1 numbers during LPS-induced pleurisy [56] and sepsis [57], the levels of P-STAT-1 were similar in LPS+IFN-γ-stimulated BMDMs compared to BMDMs from WT mice, suggesting that Plg/Pla binding to their receptors, which are

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increased in inflammatory monocytes [73], is important to skew inflammatory macrophages toward regulatory phenotypes, and that pro-inflammatory profiles are favored in the absence of Plg/Pla signaling. This hypothesis is in line with our recent findings using a pre-clinical model of severe sepsis, in which Pla-treatment of septic mice decreased M1 macrophages numbers, alongside several other inflammatory/damage parameters, improving mice survival [57]. In line with this, there were higher numbers of neutrophils and M1 macrophages accompanied of increased lethality rates in uPA receptor (uPAR)$^{-/-}$ mice subject to sepsis [57]. Indeed, it was reported in a mice model of inflammatory bowel disease (IBD) that uPAR absence leads to an increased production of pro-inflammatory cytokines, M1 macrophage polarization, and defective phagocytosis [76]. Interestingly, uPAR expression is downregulated in macrophages derived from the inflamed mucosa of patients with IBD [76]. The roles of Plg/Pla in modulating macrophage reprogramming are depicted in Figure 3.

5.3. The role of Plg/Pla in modulating the production of mediators of inflammation

The genetic deletion of Plg hampers the production of the anti-inflammatory cytokine IL-10 by macrophages stimulated with LPS+IFN-γ [56]. Conversely, pre-treatment of human macrophages with Pla increases the release of IL-10 and reduces the secretion of the pro-inflammatory cytokine TNF induced by LPS+IFN-γ [56]. Pla also increases the levels of IL-10 and TGF-β in mouse macrophages [56], and increases TGF-β levels without affecting IL-10 release by monocyte-derived dendritic cells [53]. In addition, pre-treatment of murine macrophages with Pla decreases release of TNF and IL-1β in response to IFN-γ [77]. Aiding to these findings, septic-Plg$^{-/-}$ mice show higher levels of IL-6, while treatment of septic-WT mice with Pla reduces the levels of IL-6 and CXCL1 (neutrophil chemoattractive chemokine), associated with decreased neutrophil recruitment into the peritoneal cavity [57]. Noteworthy, it has been demonstrated that Plg can be induced by IL-6 [27] and vice-versa, i.e. injection of Plg/Pla into the pleural cavity of mice induces IL-6 release [55, 63], which is associated with Plg/Pla-induced macrophage polarization to regulatory phenotypes in nonphlogistic conditions [55]. Although the mechanism underlying IL-6 modulation by Plg/Pla remains to be unraveled, given our recent findings of Plg/Pla-mediated IL-6 regulation in septic condition [57] and the known pleiotropic inflammatory actions of IL-6 [78], one can hypothesize that the role of Plg/Pla in modulating IL-6 levels depends on the inflammatory milieu.

It was reported Pla effect in reducing LTB4, a pro-inflammatory lipid mediator, while inhibiting neutrophil chemotaxis and the release of superoxide radicals [79]. However, Pla has been shown to possess pro-inflammatory actions in other scenarios. When used in high concentrations *in vitro*, Pla induces the production of pro-inflammatory mediators such as the cytokines TNF-α and IL-1 [11], and the lipids LTB4 and PGE2 [80, 81]. Moreover, Plg is activated during some inflammatory conditions, such as acute graft-versus-host disease and macrophage activation syndrome, and Pla inhibition prevents mortality in these inflammatory conditions [82, 83]. In contrast, Sugimoto *et al.* showed that Plg expression and Pla activity are higher also at the resolution phase of inflammation of LPS-induced pleurisy, and Pla given at the peak of inflammation promotes inflammation resolution [55]. Yet, Pla given to septic mice decreases the exacerbated inflammation and sepsis-induced lethality rates [57]. Notably, activation of the Plg/Pla system also up-regulates the

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expression and release of AnxA1 [55] and activates the AMPK pathway [77], which is engaged by several pro-resolving mediators, including AnxA1 and protectin DX [84, 85]. Another important point of interest is to know if pro-resolving mediators would affect the Plg/Pla system and vice-versa. Of note, RvD3 inhibit PAI-1 secretion by monocytes [86], which would ultimately decrease the inactivation of Plg activators. Therefore, the complex dynamics of Plg/Pla in inflammation seem to be dependent on the concentration used *in vitro* and on clinical condition or time of inhibition attempted in *in vivo* studies.

The interesting concept that "the beginning programs the end" [38] explored by Dr. Serhan Lab to describe the activation and actions of pro-resolving molecules, aligns nicely with the Plg/Pla pathways that can activate important pathways during the onset of inflammation [11] but, in a timely and temporal manner, may induce signaling leading to inflammation resolution. In this regard, our hypothesis is that there is a proper therapeutic window for Pla administration in the course of inflammatory diseases that harnesses key steps of resolution of inflammation while preventing unwanted effects. The role of Plg/Pla in modulating the production of inflammatory mediators is shown in Table 1.

5.4. The role of Plg/Pla in modulating neutrophil infiltration and NET release

Emerging studies have demonstrated the importance of Plg/Pla on neutrophil function, notably in the context of its deficiency or supplementation by using pre-clinical models of diseases [57, 87]. An elegant study has shown increased neutrophilic infiltration and NETs formation during periodontal immunopathology in $Plg^{-/-}$ mice. Mechanistically, the authors showed that fibrin accumulation due to the lack of fibrinolysis in $Plg^{-/-}$ mice leads to persistent neutrophil activation and NETs release [87]. These data corroborate with our recent study using a murine model of sepsis, in which $Plg^{-/-}$ mice displayed higher lethality rates associated with increased neutrophils numbers, defective apoptosis, liver fibrin deposition, IL-6 levels, tissue damage and systemic release of NETs, as compared to $Plg^{+/+}$ mice [57]. Noteworthy, administration of Plg/Pla to septic-WT mice decreases neutrophil numbers, while promotes their apoptosis with further removal by efferocytosis, decreases cytokines, tissue damage, fibrin deposition and NETs release, events that render mice less susceptible to sepsis [57]. Correspondingly, t-PA inhibits pro-inflammatory pathways induced by LPS in macrophages and administration of t-PA to septic mice decreases lethality rates by blocking the toxicity of LPS [88]. Noteworthy, Pla administration to septic mice also decreased neutrophilic infiltration into the tissue [57], a characteristic that is seen associated with fibrin deposition in some pre-clinical models using $Plg^{-/-}$ mice [25, 87]. It remains to be uncovered whether Pla-treatment decreases neutrophil migration or has a more relevant role in neutrophil apoptosis and removal. Nevertheless, these data indicate an inability of the inflammatory phase to resolve in the absence of Plg and open a window of opportunity for fibrinolytic therapy in some inflammatory diseases.

Mechanistically, pre-treatment of human neutrophils with Plg/Pla decreased NETs release in a process dependent on Pla protease activity and lysine binding sites. Interestingly, it has been reported that pro-resolving mediators, including AnxA1 and RvT-series, reduce NETs by enhancing neutrophil apoptosis or NETs uptake by macrophages, favoring NETs clearance at inflammatory sites [89]. Although the mechanisms by which Pla modulates

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NETs release have not been established, given the known role of Plg/Pla on neutrophil apoptosis and macrophage efferocytosis [53, 55], one can hypothesize a similar pathway would also apply to Plg/Pla. Of interest, the DNA-bound elastase of NETs degrades plasminogen, reduces plasmin formation, and decreases fibrinolysis [90], highlighting the intricate mechanism of regulation between the Plg/Pla systems and neutrophil activation. The role of Plg/Pla in modulating neutrophil infiltration and NET release is shown in Figure 4.

6. Defective production or activation of Plg during inflammatory conditions

The role of Plg/Pla in modulating the inflammatory response has been reinforced by studies showing lower levels of Plg or decreased Pla generation during exacerbated or non-resolving inflammatory conditions [91–94]. Recently, low plasminogen and high PAI-1 levels were shown to be associated with increased inflammation and sepsis severity *in vivo* [57]. Indeed, an acute fibrinolysis shutdown occurs early in septic shock and associates with increased morbidity and lethality [91]. In COVID-19 patients, low circulating plasminogen levels strongly correlate with mortality [92]. Interestingly, plasma Plg levels significantly decrease post-burn and remain low during the peak of inflammation and coagulopathy [93]. Moreover, low Plg levels positively correlate with organ failure in patients with severe burn [93]. It has also been demonstrated that Pla generation is delayed in mice on a high-fat diet, a model of chronic inflammatory metabolic disease [94]. However, it is noteworthy that Plg/Pla deficiency or inhibition of Pla activity may have protective actions in other inflammatory contexts, such as macrophage activation syndrome [82]. The putative dual role of Plg/Pla in inflammation has been elegantly reviewed elsewhere [20, 95].

7. Conclusion and perspectives

It has become increasingly evident that the biological functions of the Plg/Pla system extend beyond the dissolution of the fibrin clot. Studies *in vitro* and using pre-clinical models of acute inflammation have revealed that Plg, and particularly Pla, regulate several key steps of inflammation resolution (Figure 1), at least in part through AnxA1, warranting reestablishment or adaptation of injured tissue to a new state of homeostasis. However, one cannot disregard the putative dual role of Plg/Pla on inflammation and that actions of Plg/Pla are likely to be context dependent, which should be better explored in future studies focused on other inflammatory contexts, such as infectious and chronic inflammatory conditions.

Targeting fibrinolytic pathways emerges as a promising therapeutic approach for clinical conditions associated with defective resolution of inflammation that greatly affect human health, including cardiovascular, metabolic, and respiratory diseases. Indeed, clinically moderate COVID-19 patients quickly improved lung function after Plg inhalation by increasing oxygen saturation [96]. In addition, granulocyte microvesicles with a high Pla generation capacity [97] or local Pla-injection into peritoneum [57] improves the outcome of septic mice, pointing out for a beneficial effect of Pla-based therapy in infectious disease with thromboinflammatory characteristics. Moreover, subcutaneous injections of Plg accelerate healing of radiation-induced wounds, by reducing neutrophil accumulation

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and decreasing the expression of pro-inflammatory genes (e.g., and TNF, IL-1β, IL-6 and CXCL1) [98]. Notably, many of these Plg-downregulated genes are pro-fibrotic, suggesting that Plg can redirect the healing process to resemble the physiological state with reduced fibrosis [98]. Since Pla, when administered intravenously, can be rapidly inhibited by endogenous inhibitors present at very high concentrations in the blood [21], there is a need for the development of formulations that increase Pla half-life. Indeed, nanoformulations using heparin/polyarginine based polymers or liposomes loaded with Pla increase Pla stability, showing promising effects in a mouse model of stroke [99, 100]. Future clinical trials will be necessary to establish the effectiveness and safety of Plg/Pla-based therapeutics.

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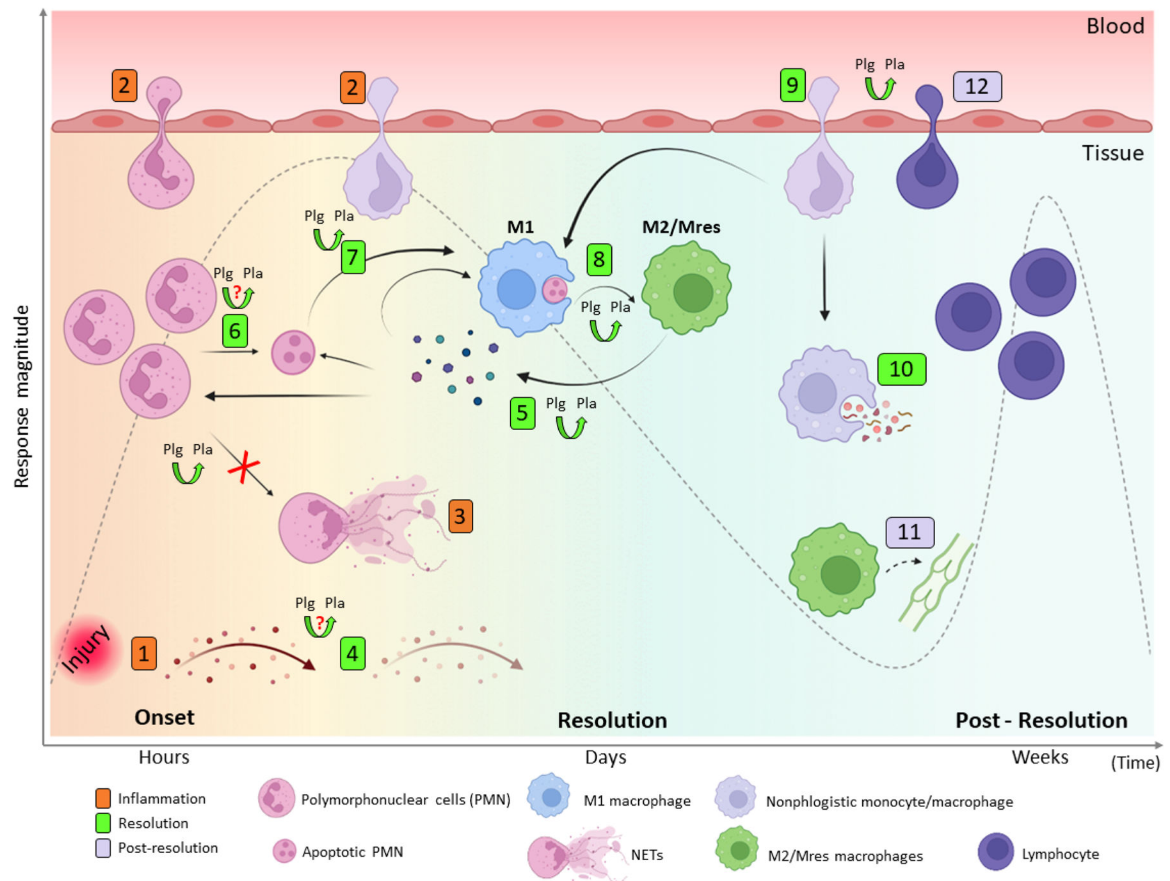

Figure 1. Sequential events during self-resolving inflammation and the role of Plg/Pla in key events.

At the onset of the inflammatory response resident cells sense the inciting stimulus, become activated and, in turn, produce and release soluble pro-inflammatory mediators (1). Changes in the microvasculature and increased levels of chemoattractants in the tissue favor the influx of PMNs from circulation to the inflammatory site, followed by monocytes/macrophages (2). Infiltrating leukocytes perform their effector functions in infected/injured tissue, including phagocytosis of microorganisms and NETs release (3) in order to pathogen elimination. The resolution of inflammation is characterized by the catabolism of pro-inflammatory mediators and shutdown of pro-inflammatory signaling pathways (4) alongside production of pro-resolving mediators (e.g. Annexin A1 and SPMs) (5). This milieu favors neutrophil apoptosis (6) and their further removal by efferocytosis (7). Efferocytosis reprograms pro-inflammatory M1 macrophages toward regulatory/resolving phenotypes (e.g. M2/Mres) (8), which amplify the resolution process by additional production of pro-resolving molecules (5). Additional recruitment of monocytes in a nonphlogistic manner (9) amplifies the clearance of apoptotic cells (7) and debris (e.g. fibrin, necrotic cells remains) (10). The migrated leukocytes leave the tissue drained by lymphatic vessels (11). The tissue is repopulated by adaptive immune cells, which command the magnitude of a subsequent immune response, e.g. caused by a secondary inciting agent (12). Curved green arrows indicate events induced by Plg/Pla. Red question markers indicate specific process that Pla-protease activity needs to be covered. Created with BioRender®.

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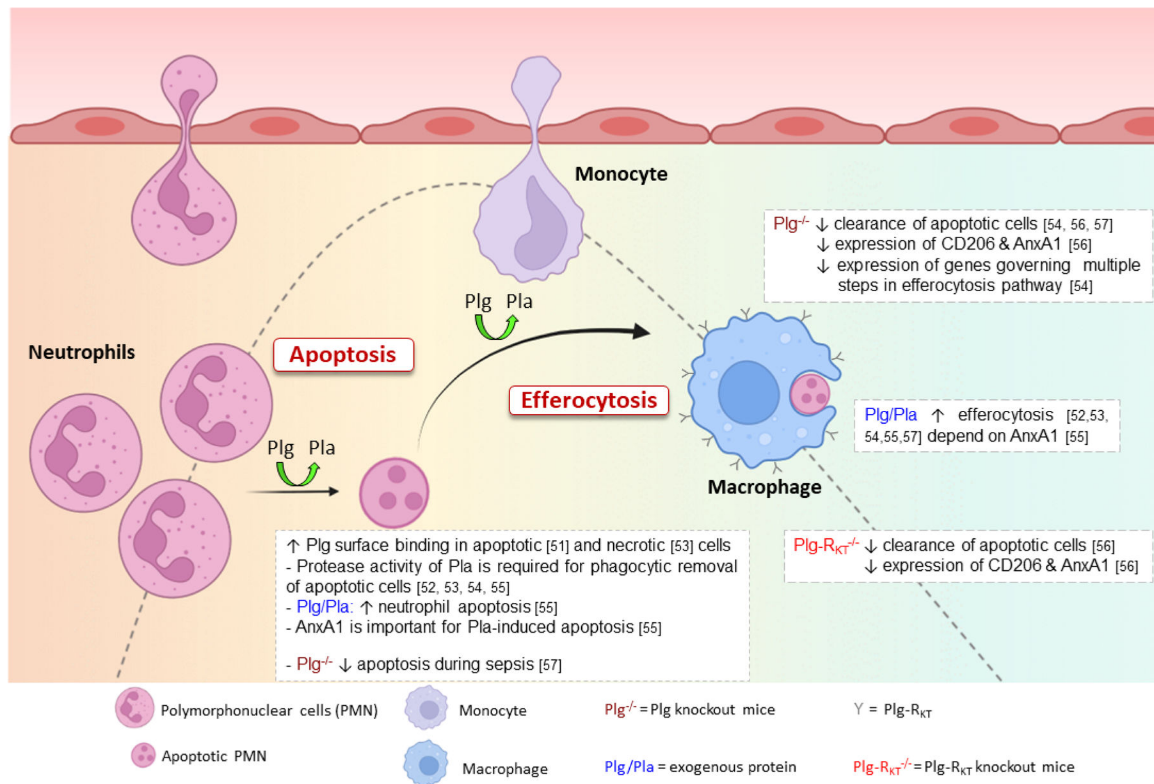

Figure 2. Key findings of the effects of plasminogen/plasmin and Plg-R_{KT} in apoptosis and efferocytosis.

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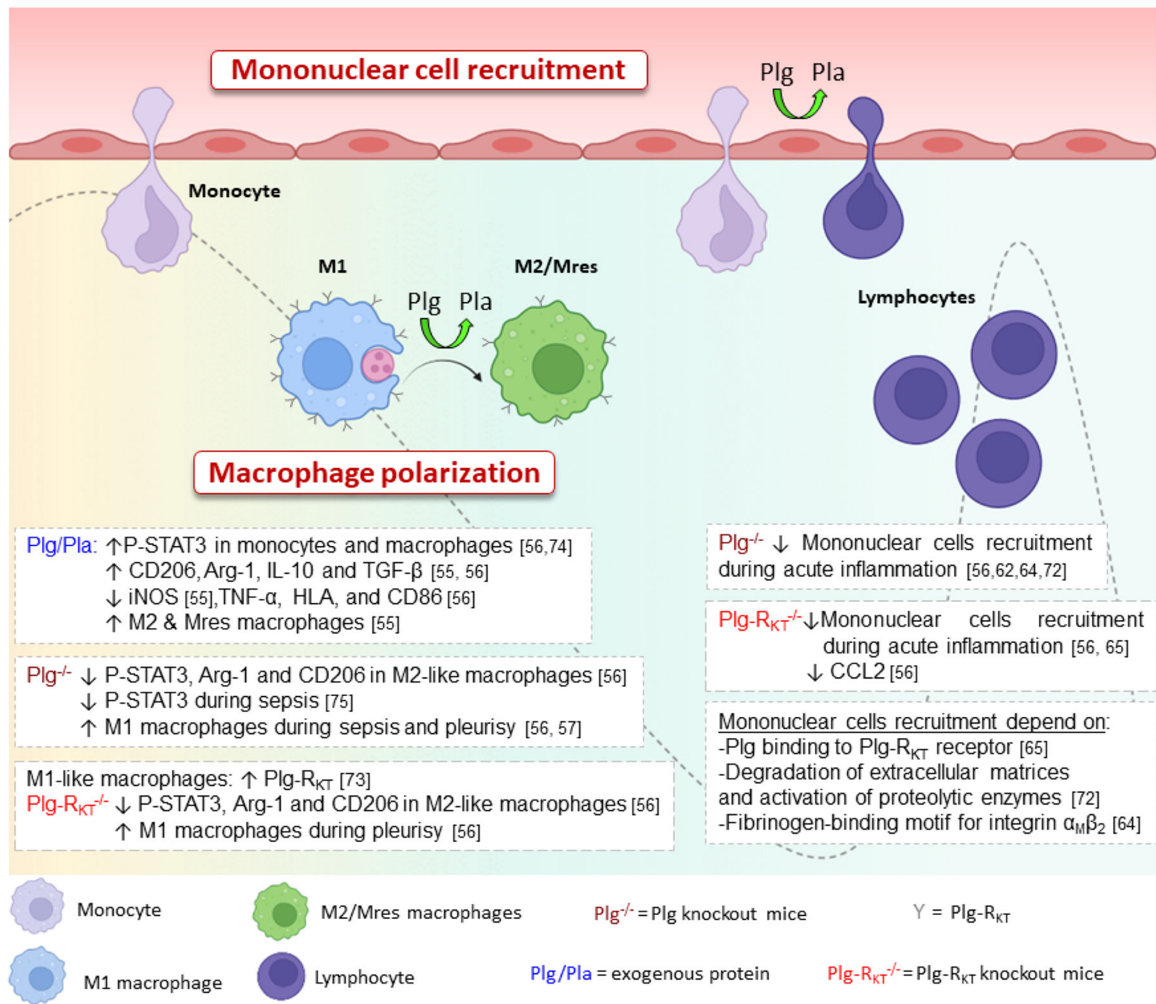

Figure 3. The role of plasminogen/plasmin and Plg-R_{KT} on mononuclear cell recruitment and macrophage polarization.

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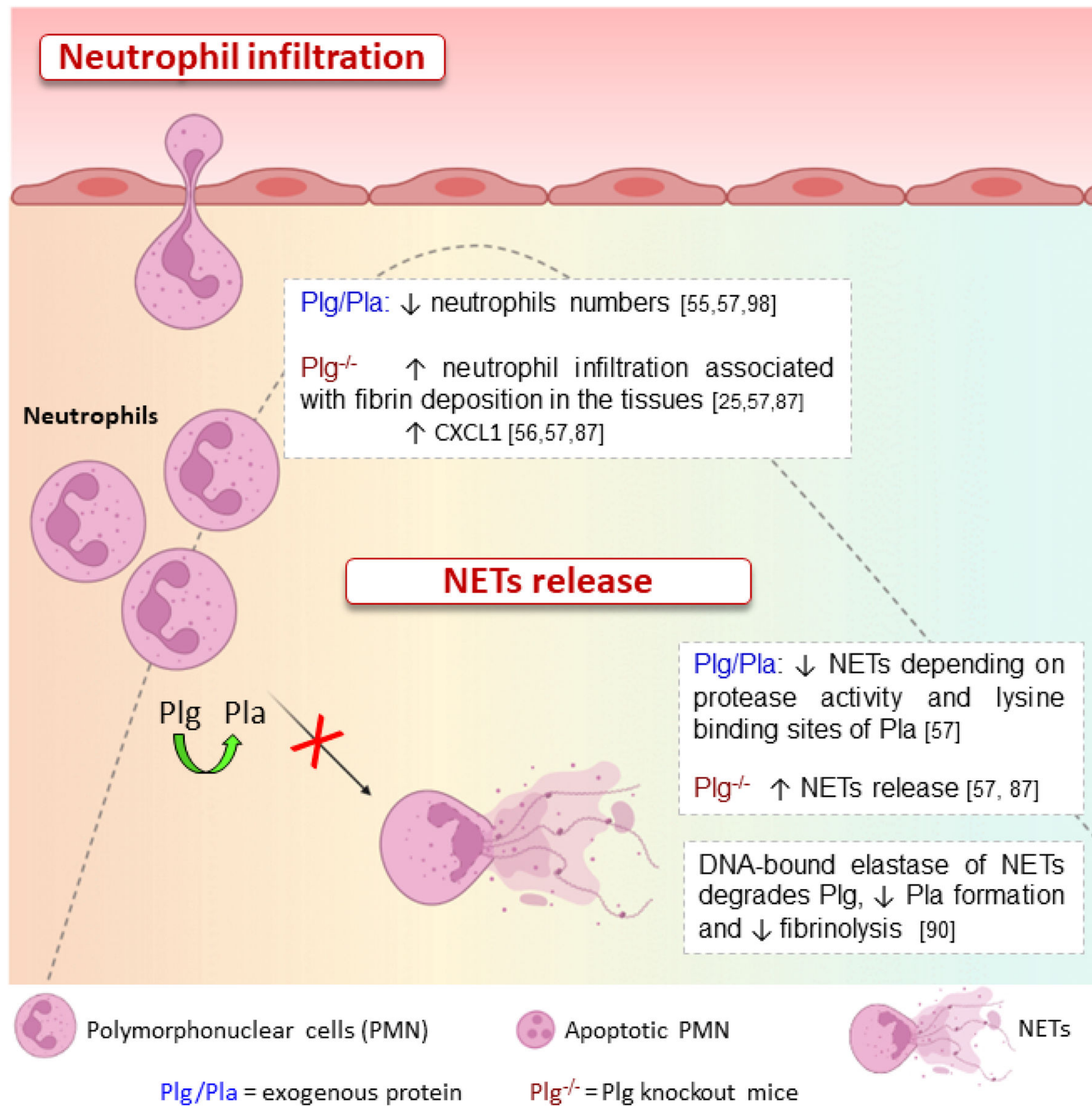

Figure 4. Effect of plasminogen/plasmin in features of neutrophils: recruitment and NETs release.

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Table 1:

Effects of plasminogen/plasmin on the production of mediators of inflammation/resolution. ↓ = decreased protein levels or RNA expression; ↑ = increased protein levels or RNA expression; AnxA1 = Annexin A1; LTB4 = leukotriene B4; $Plg^{-/-}$ = Plg knockout mice; Plg/Pla = Plasminogen or Plasmin exogenously administered *in vivo* or *in vitro* stimulation.

	Pro-inflammatory mediators	Anti-inflammatory / pro-resolving mediators
Plg/Pla	↓ TNF [56, 77, 98] ↓ CXCL1 [57, 98] ↓ IL1-β [77, 98] ↓ IL-6 [57, 98] ↓ LTB4 and superoxide radical release [79]	↑ IL-10 [55, 56] ↑ TGF-β [53, 55, 56] ↑ AnxA1 [55]
$Plg^{-/-}$	↑ IL-6 [57] ↑ CXCL1 [57, 87]	↓ IL-10 [56] ↓ AnxA1 [56]