DAMPS ACROSS THE TREE OF LIFE, VOLUME 2: REGULATED CELL DEATH AND IMMUNE RESPONSES

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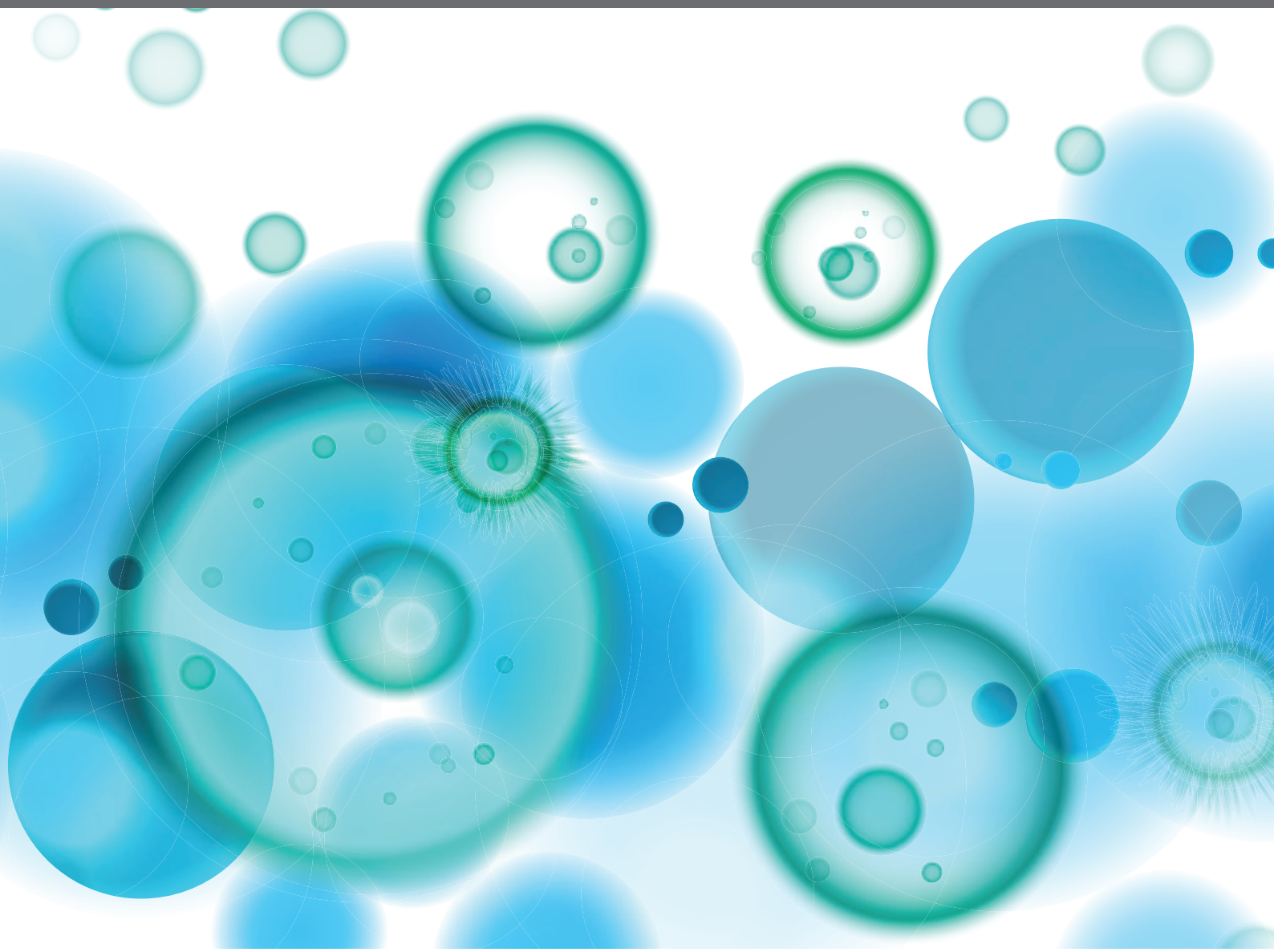
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88974-354-4
DOI 10.3389/978-2-88974-354-4

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Citation: Seong, S.-Y., Land, W. G., Anders, H.-J., Heil, M., Maffei, M. E., eds. (2022). DAMPs Across the Tree of Life, Volume 2: Regulated Cell Death and Immune Responses. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-354-4

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frontiers in Immunology

MINI REVIEW
published: 25 August 2020
doi: 10.3389/fimmu.2020.01918

The Critical Roles and Mechanisms of Immune Cell Death in Sepsis

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Sepsis was first described by the ancient Greek physicians over 2000 years ago. The pathophysiology of the disease, however, is still not fully understood and hence the mortality rate is still unacceptably high due to lack of specific therapies. In the last decade, great progress has been made by shifting the focus of research from systemic inflammatory response syndrome (SIRS) to multiple organ dysfunction syndrome (MODS). Sepsis has been re-defined as infection-induced MODS in 2016. How infection leads to MODS is not clear, but what mediates MODS becomes the major topic in understanding the molecular mechanisms and developing specific therapies. Recently, the mechanism of infection-induced extensive immune cell death which releases a large quantity of damage-associated molecular patterns (DAMPs) and their roles in the development of MODS as well as immunosuppression during sepsis have attracted much attention. Growing evidence supports the hypothesis that DAMPs, including high-mobility group box 1 protein (HMGB1), cell-free DNA (cfDNA) and histones as well as neutrophil extracellular traps (NETs), may directly or indirectly contribute significantly to the development of MODS. Here, we provide an overview of the mechanisms and consequences of infection-induced extensive immune cell death during the development of sepsis. We also propose a pivotal pathway from a local infection to eventual sepsis and a potential combined therapeutic strategy for targeting sepsis.

Keywords: sepsis, extensive immune cell death, damage-associated molecular patterns (DAMPs), multiple organ dysfunction syndrome (MODS), extracellular histones, immunosuppression

OPEN ACCESS

Edited by:
Walter Gottlieb Land, Université de Strasbourg, France

Reviewed by:
Jaewoo Lee, Duke University, United States
Julien Pottecher, Hôpitaux Universitaires de Strasbourg, France

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *14 February 2020*
Accepted: *16 July 2020*
Published: *25 August 2020*

Citation:
Cheng Z, Abrams ST, Toh J, Wang SS, Wang Z, Yu Q, Yu W, Toh C-H and Wang G (2020) The Critical Roles and Mechanisms of Immune Cell Death in Sepsis. Front. Immunol. 11:1918. doi: 10.3389/fimmu.2020.01918

SEPSIS

Sepsis is still the leading cause of death in most intensive care units (ICU) with an unacceptably high mortality rate (10–20%), although there has been a significant decrease in mortality rates in recent decades (from 1994 to 2014) (1, 2). Center for Disease Control in the United States estimated that over half a million people developed sepsis there per year with about a 1.5% increase per annum (3). A recent investigation of a cohort of 568 patients who died in six hospitals in the United States showed that sepsis presented in 300 patients (52.8%) and was the most common immediate cause of death in 198 patients (34.9%), indicating that sepsis is still the major cause of death in hospitals (4). For years, it was believed that high morbidity and mortality were due to systemic inflammatory response syndrome (SIRS), but many clinical trials to inhibit inflammation failed to improve survival (5–7). In 2016, sepsis has been redefined as multiple organ dysfunction syndrome (MODS) caused by a dysregulated host response to infection (8) and is now termed Sepsis-3.

This has changed the focus from SIRS (9, 10) to MODS. Thus, finding what mediates MODS is now the major challenge in understanding the pathophysiology of sepsis (11).

DISCOVERY OF IMMUNE CELL DEATH IN SEPSIS

Cheadle et al. (12) reported that a significant lymphopenia occurred in a group of trauma patients with sepsis. Years later, lymphopenia in sepsis began to attract increased attention (13–16). In human, depletion of both B cells and CD4+ T lymphocytes caused by sepsis-induced apoptosis were reported (16). In baboon and murine sepsis models, extensive apoptosis of lymphoid tissue was also found (17–19). Rapidly progressing lymphocyte exhaustion after severe sepsis has been widely recognized (20) and early circulating lymphocyte apoptosis was associated with poor outcome in patients with sepsis (21, 22). Thus, a number of research groups have focused on the role of altered cell death in contributing to MODS in sepsis and clinical trials for a new type of therapy has emerged (23–26).

TYPES OF IMMUNE CELL DEATH AND CLINICAL RELEVANCE

Lymphocyte death occurs in the spleen, thymus, and lymphoid tissues (27). The peripheral lymphocyte count is also dramatically reduced in both sepsis models and patients (16, 22, 24). Changes in the subsets of lymphocyte involved varies depending on the bacterial origin of sepsis (28), but there is no doubt that both T and B lymphocyte subsets are significantly changed by sepsis. CD3+, CD3+CD4+, and CD3+CD8+ lymphocyte counts drop significantly in septic patients, while CD3+CD4+ lymphocytes return to normal after 14 days in most patient survivors, but this is not true of the CD3+CD8+ counts (29). The ratio of Th1/Th2 helper cells has been found to be significantly lower in sepsis (30). Circulatory Th1, Th2, Th17, and Treg as well as Th1/CD4 $^{+}$ ratios are significantly lower in non-survivors compared to survivors (31). The αβ and γδ T cell subsets are all reduced in sepsis, but the $CD3^{+}$ $CD56^{+}$ γδ T cells show the largest decrease, and their loss is strongly associated with septic severity and mortality (32, 33). Sepsis causes progressive and profound depletion of B lymphocytes in patients (16). Thus, the percentage of CD19+CD23+ was significantly lower in patients who died of septic shock than in survivors (34). In a mouse polymicrobial sepsis model, substantial apoptosis of lamina propria B cells mediated by FasL has been reported (35).

Not only are B and T lymphocytes susceptible to programmed cell death, many other types of immune cells including neutrophils, macrophages and dendritic cells are also vulnerable to cell death in sepsis (22, 36, 37). Neutrophils are the first line of defense against invading bacteria. Neutrophils phagocytose bacteria or form neutrophil extracellular traps (NETs), and both these mechanisms are critical for clearance of invading bacteria (38). After taking up bacteria, neutrophils undergo a respiratory burst and die (39). NETs formation is also a novel program for cell death (40–42). Therefore, large numbers of neutrophils die during sepsis. In mouse models, apoptosis of mouse peritoneal macrophages may be due to the release of HMGB1 in sepsis (43). Dendritic cells have unique capabilities to regulate the activity and survival of T and B cells. Thus splenic interdigitating dendritic cells (IDCs) and follicular dendritic cells (FDCs) initially expand in sepsis. The FDCs expand to fill the entire lymphoid zone of spleen, which is otherwise occupied by B cells (44). Twelve hours after the onset, these dendritic cells undergo apoptosis (44). In contrast, natural killer (NK) cell counts increase in early sepsis and higher levels predict mortality in severe sepsis (45). Thus, the ratio of NK cells to CD4+ lymphocytes was used to predict the mortality of patients with sepsis (46). NK cells also contribute to the lethality of a murine model of sepsis, and NK cell-depleted and NK cell-deficient mice showed much high survival rates than wild type controls (47).

MECHANISMS OF IMMUNE CELL DEATH

Apoptosis is the major mechanism of lymphocyte death in sepsis (35, 48). Both the death receptor and mitochondrial pathways activated by multiple triggers are involved in apoptosis of a broad range of subsets of lymphocyte (49). Apoptosis could occur via p53-dependent and -independent pathways (50). The increase in apoptosis in the thymus, spleen, lungs, and gut during polymicrobial sepsis of mice is mediated by FasL via death receptors, but not by endotoxins nor TNF-α (14, 35). Monocytes can induce Fas-mediated apoptosis of T lymphocytes (51). Caspase-1 is involved in apoptosis of splenic B lymphocytes (52). Activation of caspase-3 and externalization of phosphatidylserine in CD4+, CD8+, and CD19+ lymphocytes were reported in patients with sepsis (53). Activation of programmed cell death ligand 1 (PD-L1) pathway is involved in T cell exhaustion in patients with sepsis (54). In addition, endoplasmic reticulum (ER) stress can mediate lymphocyte apoptosis in sepsis (55). Bcl-2 is an anti-apoptosis protein and is found to be reduced in sepsis (56). Overexpression of Bcl-2 in septic mice provides protection by decreasing lymphocyte apoptosis (57, 58). In $CD4^{+}$ T and B lymphocytes isolated from septic patients, the Bcl-2 protein was decreased but the expression of pro-apoptotic proteins Bim, Bid, and Bak were massively upregulated (23, 53). It has also been reported that overexpression of histamine H4 receptors counteracts the effect of NF-kB in contributing to splenic cell apoptosis in sepsis (59).

There is no doubt that multiple factors are involved in lymphocyte apoptosis, but the detailed molecular mechanisms are still not fully understood. In addition, apoptosis has been the major focus of cell death in last two decades, but recently other processes have emerged, e.g., pyroptosis, necroptosis, ferroptosis, parthanatos, entotic cell death, NETotic cell death, immunogenic cell death, and mitotic catastrophe, to explain the complexity of cell death (60). Pyroptosis is caused by rapid plasma-membrane rupture by non-selective gasdermin-D pore and releases of DAMPs (61). Neutrophil and endothelial cell pyroptosis has been considered as a major pathological factor in sepsis (62, 63). Increased membrane permeabilization in necroptosis releases specific DAMPs, and lipid peroxidation in ferroptosis may be

involved in renal failure (64–66). These regulated cell deaths may turn to necrosis if their resolution is delayed (67). The roles and mechanisms of different types of cell death in sepsis is far from clear and more work need to be done to understand how the immune cells die so extensively in sepsis.

Neutrophil respiratory burst and NETosis all involve generation of reactive oxygen species (ROS) and NADPH oxidase pays a critical role (40). Endotoxin reduced CD95-mediated neutrophil apoptosis occurs via cIAP-2 activation and the degradation of caspase-3 (68). The detailed molecular mechanisms of neutrophil respiratory burst, NETosis, and homeostasis will not be discussed in this review.

In summary, the types of cell death and underlying molecular mechanisms are still not fully understood, although the subpopulations of immune cells that die during sepsis is almost clarified.

ROLES AND CONSEQUENCE OF IMMUNE CELL DEATH

It is known that the extent of immune cell death is strongly associated with severity and mortality of sepsis. However, the biological roles are still not clear. The direct cause-effect relationship of extensive immune cell death with sepsis has not yet been proven. When splenectomy to remove the largest lymph organ in mice prior to septic modeling was undertaken, it is found that this procedure protects mice against secondary sepsis (69, 70). This observation suggests that extensive splenocyte death is potentially pathogenic in sepsis. Neutrophil death, particularly NETosis, has been reported to be involved in the development of multiple organ failure in sepsis (71–73). Abrams et al. (74) recently showed that strong NETs formation mainly occurs in severe sepsis and is associated with disseminated intravascular coagulation (DIC) and ultimately poor outcomes. Patel et al. (75) recently showed that a reduction in *ex vivo* PMA-induced NETosis of neutrophils isolated from patients with severe sepsis is associated with poorer outcomes. This observation demonstrates the pathological role of *in vivo* NETs formation, a mechanism that eliminates the majority of pro-NETosis neutrophils. This result is also consistent with the current general consensus (72, 74). However, the pathological role of immune cell death in sepsis is still not fully understood, but the following mechanisms are widely considered to be very important.

DAMPs and Histone Release

The "danger" theory was proposed by Matzinger in 1994 (76) that damaged cells initiate immune responses by releasing substances were termed damage-associated molecular patterns (DAMPs) by Walter Land in 2003 (77). DAMPs represent danger-associated or damage-associated molecular patterns, which are released from the cell through activation of inflammasome or passively following cell death (78–80) and recognized by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) NOD-like receptors, DNA sensors, C-type lectin receptor, and non-PRR DAMP receptors, including RAGE receptor (81). Many DAMPs that origin from extracellular matrix and different components or organelles of cells have been identified, such as histones, DNAs, HMGB1, heat shock protein, and ATP. More information can be found in a recent review (82). In sepsis, a large number of immune cell death releases a large quantity of DAMPs (83, 84). Similarly, NETs are released from neutrophils during inflammation (41). These NETs are brokendown into free DNA and histones and become a source of DAMPs (72, 85). DAMPs trigger the host's immune response, activate coagulation and mediate MODS (86–88). Therefore, they play a central role in development of sepsis and its progression (84, 89). DAMPs include a large group of molecules and are involved in different pathological processes during sepsis.

Release of chromatin protein HMGB1 triggers inflammation and mediates endotoxin lethality in mice (90, 91). HMGB1 facilitates LPS entering cells to trigger pyroptosis, which plays an important role in sepsis (63, 92, 93). In 2009, extracellular histones were shown to be major mediators of death in sepsis (94) and have attracted more and more attention. Extracellular histones bind to the cell membrane and form pores to allow calcium influx which leads to calcium overload, which directly damages cells that contacted (87, 88). Histones also induce rapid thrombocytopenia, increase thrombin generation and contribute to DIC (95–99). Anti-histone antibodies and non-anticoagulant heparin neutralize extracellular histones and improves survival in sepsis (87, 88, 99–101). Recently, the role of extracellular histones in the development of MODS in critical illnesses and animal models, including sepsis, pancreatitis, and trauma, has been demonstrated (86). Mitochondrial DNA released into the cytosol or outside cells also serves as DAMPs and play important roles in sepsis (11, 102). In addition, circulating cell-free DNA is associated with poor outcomes in patients with severe sepsis (103–106). The pathological roles of these cell-free DNAs are not clear but strengthening blood clots resistant to fibrin lysis may facilitate DIC development (107). A recent report shows increased S100 proteins, including A8/A9 and A12, which are types of DAMPs, are associated with a higher risk of death in patients with sepsis (108).

NETs Formation

Although NETs are an important source of DAMPs, NETs formation has specific roles in thrombosis, DIC and microcirculatory impairment. NETs formation induces organ injury and exacerbates the severity of sepsis (42, 73, 74, 109–112). Suppression of NETosis using PAD4 inhibitors or cleavage of NETs using DNase 1 improves survival in a murine sepsis model (113), but other reports showed the opposite effect (114, 115). Recently it has been reported that delayed, not early treatment with DNase 1 reduces organ injury and improves outcome in sepsis model (116). These observations strongly indicate the complex roles of NETs formation during sepsis.

Coagulopathy and DIC

Sepsis-induced coagulopathy and DIC play a major role in microcirculatory impairment and MODS development (117). DAMPs play important roles in septic coagulopathy (118). Extracellular histones are the most important DAMPs

that promote coagulation activation by inducing rapid thrombocytopenia, enhancing thrombin generation, impairing thrombomodulin-dependent protein C activation, damaging endothelial cells and increasing tissue factor activity (95–99). cfDNA exert both pro- and anti-fibrinolytic effects and NETs serve as scaffolds for immunothrombosis and promote intracellular coagulation together with platelets (107, 119, 120). The overall consequence is the development of coagulopathy and DIC, which significantly enhance disease severity and worsen the outcomes (74, 86, 99, 104, 105).

Immune Suppression

As our understanding of the pathophysiology in sepsis has improved, we now know that the role of immunosuppression is more important than previously thought. IL-7, as an immune-adjuvant therapy that increased absolute lymphocyte counts and in circulating CD4+ and CD8+ T cells (3–4 fold), and T cell proliferation and activation (121), supports this contention. However, why IL-7 protected mice with sepsis but showed no effects on 28-days survival of patients with sepsis is not clear and further investigation is required (122). IL-15 is also reported to prevent apoptosis, reverse innate and adaptive immune dysfunction, and improve survival in murine models of sepsis (123). Changes associated with immunosuppression is more obvious in patients who died of sepsis than those who survived (31, 124). Immune cell death, particularly T and B lymphocytic apoptosis, is a major contributor to the development of immunosuppression (15, 32, 125), besides the usual anti-inflammatory cytokine release, such as that of IL-10 (126). Myeloid-derived suppressor cells (MDSCs) are closely related to neutrophils and monocytes. They are immature myeloid cells that have immunosuppressive functions and play important roles in the development of immunosuppression in sepsis (2, 127–129). DAMPs activate TLR-4 to enhance MDSCs accumulation (130). Many DAMPs possess both pro- and anti-inflammatory properties to induce both immune response and immunosuppression, which has been well-studied in trauma (131). Recently, the roles of PD-1 and PD-L1 in sepsis as key mediators of T-cell exhaustion in infections have been investigated (132, 133). Blocking PD-1 or PD-L1 inhibits lymphocytic apoptosis, reverses monocyte and immune dysfunction, and improves survival during sepsis (54, 134–136). Monneret et al. (137) demonstrated that after septic shock anti-inflammatory response became dominate with high IL-10 and low HLA-DR on monocytes, a surrogate marker of monocyte non-responsiveness (138). IL-7 and anti-PD-1 or blocking IL-10 reverse sepsis-induced immunosuppression, including increasing HLA-DR expression and IFN-γ production, and improve survival in mouse models (126, 139). Monitoring HLA-DR, PD-1, or PD-L1 may guide clinical immunotherapies (140). All available evidences showed no doubt that immunosuppression is the major pathological feature and immunotherapies will become a critical management in severe sepsis with poor outcomes.

In summary, the major consequence of immune cell death is the DAMPs release and NETs formation, both of which contribute to the development of coagulopathy and MODS. Another major consequence is immunosuppression. All these consequences are the major pathological changes during severe sepsis, strongly indicating that DAMPs and NETs are critical in the development of severe sepsis.

INHIBITION OF IMMUNE CELL DEATH IN SEPSIS AND POTENTIAL DOWNSTREAM THERAPY

Caspase inhibitors, which inhibit apoptosis and improve the survival of immune cells, have been demonstrated to improve survival in sepsis. Thus, caspase-3$^{-/-}$ mice have decreased levels of apoptosis (141, 142). Increasing anti-apoptotic proteins, such as Bcl-2, or decreasing pro-apoptotic proteins, such as Bim, reduces immune cell apoptosis and improves survival in septic animal models (57, 58, 142–145). The Protease Inhibitor (PI) class of antiretroviral agents also significantly improved survival of mouse septic models by reducing lymphocyte apoptosis (146). These anti-apoptosis therapies have been demonstrated in animal models (147), but there have been no successful clinical trials in humans as yet.

Therapies with immune modulators have attracted more attention in recent years. The success of the IL-7 clinical trial shed some light on the management of sepsis (121). Immunotherapy is potentially a major strategy (145, 148, 149), but the focus of research has shifted from simply suppressing the immune response to immune modulation and precision medicine based on immune status (148, 150–153). Targeting immune cell checkpoints during sepsis is also a potential therapeutic strategy (154).

Another promising strategy is to neutralize DAMPs, including histones, DNAs and HMGB1. Anti-histone therapy has been proposed by Xu et al. from 2009 (94). Anti-histone antibodies or heparin can neutralize extracellular histones and reduce their toxicity so as to increase survival rates in septic animal models, but no clinical trial has been reported yet (86–88, 94, 100). Normal heparin has anticoagulant activity which may cause side effects if it is used at a wrong time with high doses. Non-anticoagulant heparin has been developed and hold the promise for future clinical application (100, 155). DNase 1, used to digest free DNA or NETs, has also been shown to increase the survival rate of septic animal models (116, 156). Many reagents targeting HMGB1, its release or downstream pathways have been reported, but no drug has yet been fully developed for clinical management of sepsis (157, 158).

Correction of downstream events, such as coagulopathy, have been trialed. Activated protein C, an anti-coagulant enzyme, was used clinically for a few years, but was withdrawn from the market due to failure in randomized controlled trials (159). It is very difficult to justify the correct time to use anti-coagulants and fibrinolysis reagents, such as low-molecular-weight heparin, antithrombin, thrombomodulin, and tissue factor inhibitors (117). Therefore, anti-coagulant therapy for sepsis is difficult to

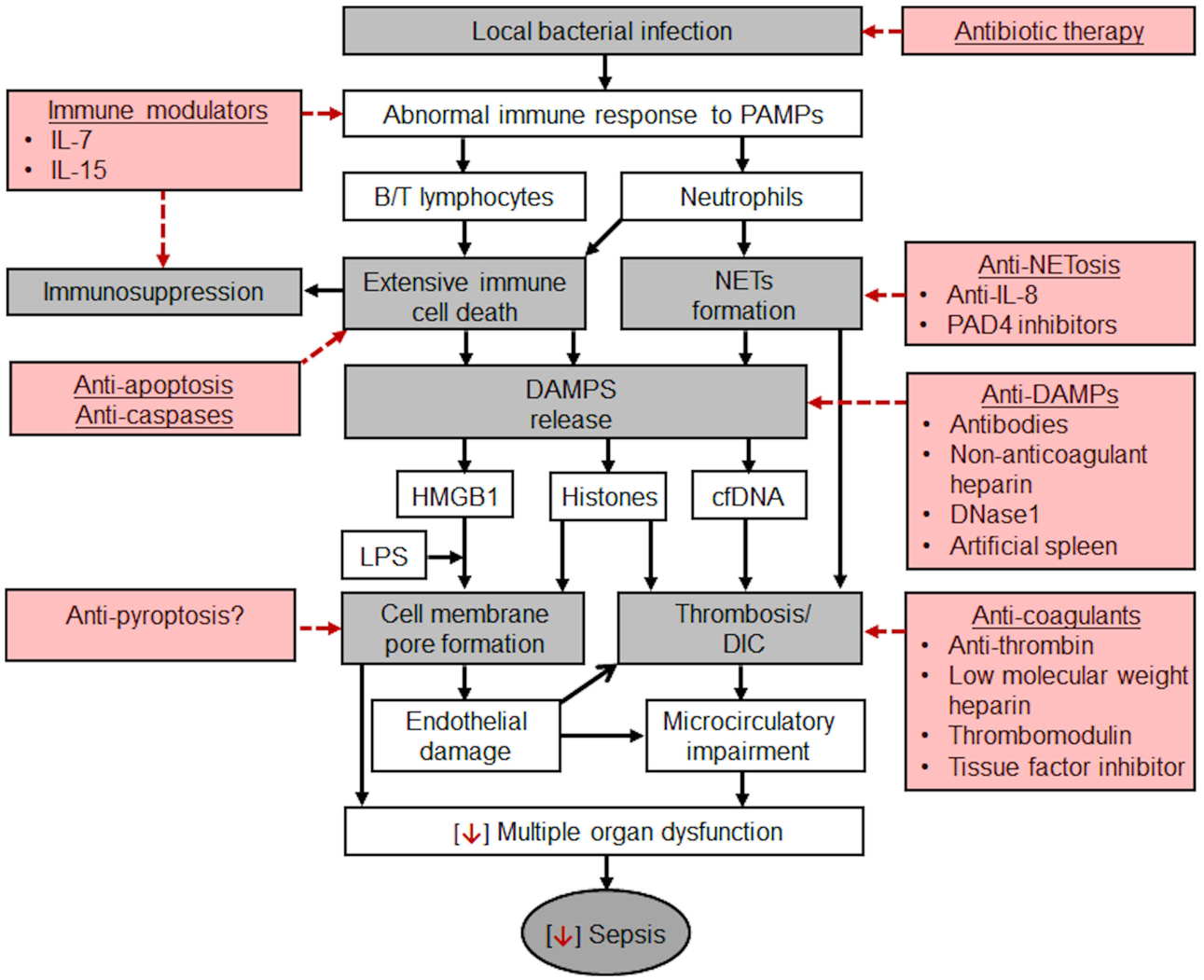

FIGURE 1 | Potential pathological mechanisms of sepsis which develops from a local bacterial infection and potential therapeutic strategies. Gray boxes: pathways from a local infection to sepsis. Once a local bacterial infection causes host abnormal immune responses to pathogen-associated molecular pattern (PAMPs), extensive immune cell death, including B/T lymphocytes (spleen, thymus, lymphoid tissues, and peripheral blood), and neutrophils could occur and result in immunosuppression. Neutrophils could also form NETs. NETs and immune cell death could release a large quantity of DAMPs, mainly HMGB1, cfDNA, and histones. HMGB1 can delivers LPS into cells to trigger pyroptosis by forming pores in the cell membrane. Extracellular histones could also bind to cell membrane to form pores which may cause calcium overload and subsequently endothelial damage and organ injury. Indirectly, extracellular histones activate coagulation to form thrombi in the microvascular lumen to impair microcirculation. cfDNA could serve as scaffolds for thrombosis or stabilize thrombi by increasing their resistance to fibrinolysis. Microcirculatory impairment is the major feature of sepsis and a major contributor to MODS. Red boxes: Potential therapeutic strategies. Besides early diagnosis, prompt use of effective antibiotics, and supportive therapies, such as maintaining blood pressure and circulation, improving microcirculation, and protecting individual organs, the potential specific therapies include the combination of modulating immune status, preventing immune cell death and NETosis, neutralizing or clearing DAMPs. These new approaches could become the leading research directions in reducing the mortality rate of sepsis.

use clinically. Developing therapies to target upstream events appears a better strategy.

CLINICAL PERSPECTIVE

Sepsis was first described by the ancient Greek physicians. Despite millennia of experience with this illness, we are still investigating the nature of sepsis. In the last decade, great progress has been made by shifting the focus of research from SIRS to MODS. However, the pathophysiology of sepsis is still not fully understood, particularly the roles of extensive immune cell death and DAMPs. Many types of DAMPs could directly or indirectly mediate MODS by their cytotoxicity or by triggering inflammation and activating coagulation, respectively. Therefore, the axis of infection, immune response, immune cell death, DAMPs release and MODS could be the central pathological pathway in the transition of a local infection to sepsis (**Figure 1**).

Targeting this central pathological pathway is already underway. However, fully understand the pathophysiology of sepsis is still the first task toward the success in clinical management.

DISCUSSION

There is no doubt that extensive immune cell death is a major driver of sepsis. This mainly involves T and B lymphocyte apoptosis in the spleen, thymus, lymphoid tissues, and circulation. Neutrophil apoptosis, respiratory burst, and NETosis are also involved in this event. Macrophages and dendritic cells may also be involved, but their contributions may be negligible. However, the mechanism of how bacterial infection leads to extensive immune cell death is still not fully understood. Moreover, significant gaps still exist in our understanding of how extensive immune cell death proceeds to the development of sepsis. The obvious consequence of immune cell death would be immunosuppression but no direct link has been demonstrated. It is clear that the release of large quantities of DAMPs can enhance inflammation, directly damage endothelial cells, impair microcirculation and cause multiple organ injury, but to what extent these DAMPs contribute to the development of sepsis is still unclear. Some DAMPs, such as histones and NETs, strongly activate coagulation and eventually lead to DIC. Therefore, the importance of DAMPs in sepsis development and progression cannot be underestimated. In the future, targeting the axis of immune cell death-DAMPs release-and microcirculatory impairment, will become the most comprehensive strategy to reduce the unacceptably high mortality rate of sepsis.

AUTHOR CONTRIBUTIONS

ZC, ZW, and QY wrote the first draft. JT and SW edited the reference. SA drew the diagram. WY, C-HT, and GW supervised the work and edited final version of paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by British Heart Foundation (PG/14/19/30751 and PG/16/65/32313), and Scholarships from China Scholarship Council (CSC:20160609156) and Southeast University (YBJJ1740) to ZC.

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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.

frontiers in Immunology

MINI REVIEW
published: 16 September 2020
doi: 10.3389/fimmu.2020.575890

Thrombomodulin as a Physiological Modulator of Intravascular Injury

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Thrombomodulin (TM), which is predominantly expressed on the endothelium, plays an important role in maintaining vascular homeostasis by regulating the coagulation system. Intravascular injury and inflammation are complicated physiological processes that are induced by injured endothelium-mediated pro-coagulant signaling, necrotic endothelial- and blood cell-derived damage-associated molecular patterns (DAMPs), and DAMP-mediated inflammation. During the hypercoagulable state after endothelial injury, TM is released into the intravascular space by proteolytic cleavage of the endothelium component. Recombinant TM (rTM) is clinically applied to patients with disseminated intravascular coagulation, resulting in protection from tissue injury. Recent studies have revealed that rTM functions as an inflammatory regulator beyond hemostasis through various molecular mechanisms. More specifically, rTM neutralizes DAMPs, including histones and high mobility group box 1 (HMGB1), suppresses excessive activation of the complement system, physiologically protects the endothelium, and influences both innate and acquired immunity. Neutrophil extracellular traps (NETs) promote immunothrombosis by orchestrating platelets to enclose infectious invaders as part of the innate immune system, but excessive immunothrombosis can cause intravascular injury. However, rTM can directly and indirectly regulate NET formation. Furthermore, rTM interacts with mediators of acquired immunity to resolve vascular inflammation. So far, rTM has shown good efficacy in suppressing inflammation in various experimental models, including thrombotic microangiopathy, sterile inflammatory disorders, autoimmune diseases, and sepsis. Thus, rTM has the potential to become a novel tool to regulate intravascular injury via pleiotropic effects.

Keywords: thrombomodulin, damage-associated molecular patterns, disseminated Intravascular coagulation, neutrophil extracellular traps, high mobility group box 1, immunothrombosis

OPEN ACCESS

Edited by:
S. Y. Seong,
Seoul National University, South Korea

Reviewed by:
Péter Gál,
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Kagoshima University, Japan

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *24 June 2020*
Accepted: *19 August 2020*
Published: *16 September 2020*

Citation:
Watanabe-Kusunoki K, Nakazawa D, Ishizu A and Atsumi T (2020) Thrombomodulin as a Physiological Modulator of Intravascular Injury. Front. Immunol. 11:575890. doi: 10.3389/fimmu.2020.575890

INTRODUCTION

Endothelial cells coordinate vascular homeostasis, including vessel permeability, provision of a lining surface, and coagulation system regulation. To prevent unnecessary clotting, the endothelium expresses anti-coagulant factors, such as tissue factor pathway inhibitor and thrombomodulin (TM), and regulators of platelet activation, such as nitric oxide, prostacyclin, and ADPase, at steady state. When traumatic vascular injury occurs, platelet aggregation and the activated blood coagulation system invoke a thrombus to prevent blood loss. Moreover, damaged endothelium reduces the expression of anti-coagulant and platelet molecules, and releases pro-coagulant factors via the activation of nuclear factor-kappa B (NF-κB) signaling, consequently

enhancing thrombus formation. Meanwhile, during non-traumatic intravascular injury, including disseminated intravascular coagulation (DIC), atherosclerosis, and thrombotic microangiopathy (1), the endothelium collaborates with the blood coagulation system and platelets to cope with the traumatic situation, possibly forming an unwanted thrombus. In addition, cross-talk between the activated coagulation system and inflammatory signaling leads to mutual amplification (2). Accordingly, damage-associated molecular patterns (DAMPs) released from injured tissues and blood cells activate the innate immune system and elicit vascular inflammation (3, 4). DAMPs directly activate platelets and indirectly induce platelet aggregation via interaction with neutrophils, leading to an enhancement of the pre-existing pro-coagulant state. This series of events of coagulation and blood cell activation, collectively referred to as immunothrombosis, is supposed to physiologically enclose and effectively kill invading microbes as part of an innate immune response (5). The structural basis of the immunothrombotic clot is formed by fibrin, consisting of coagulant factors, platelets, and leukocytes. The immunothrombus can also be involved in the development of non-infectious diseases, including ischemia-reperfusion, drug-induced tissue damage, autoimmune diseases, and cancer as an executor of intravascular injury. In the pro-coagulant state, TM derived from altered endothelium serves to maintain vascular homeostasis by participating in the coagulation system. Furthermore, TM possesses multiple regulatory properties against inflammation beyond its anti-coagulant effect, which could possibly contribute to the termination of intravascular injury (6, 7).

ANTI-COAGULANT EFFECTS OF TM IN VASCULAR BIOLOGY

TM is a transmembrane glycoprotein encoded by the *THBD* gene, and it is expressed on endothelium, immune cells (including neutrophils, macrophages, monocytes, and dendritic cells), vascular smooth muscle cells, keratinocytes, and lung alveolar epithelial cells (8–10). The structure of TM comprises five domains; each domain possesses a different function. Surface domains are a lectin-like domain (TMD1), a domain with six epidermal growth factor-like structures (TMD2), and a serine- and threonine-rich domain (TMD3). Certain stimuli, including tissue factor, orchestrate the coagulation cascade and produce thrombin as a coagulant executor. In response to thrombin production, thrombomodulin on the endothelium acts as a thrombin receptor to reduce the ability of thrombin that converts fibrinogen to fibrin and activates platelet. The thrombin-thrombomodulin complexes activate protein C and the activated protein C (APC) inactivates Va and VIIIa, resulting in the suppression of thrombin generation (11, 12). As such, TM naturally serves to terminate excessive intravascular coagulation.

ANTI-INFLAMMATORY EFFECTS OF TM

The surface TMD1 domain has no anti-coagulant effects, but has various anti-inflammatory properties. TM directly acts as a natural regulator of inflammation via its lectin-like domain TMD1 by (1) inhibiting leukocyte-mediated intravascular injury, (2) neutralizing DAMPs, including high mobility group box 1 (HMGB1) protein and histones, (3) binding to bacteria-derived components, and (4) suppressing the complement system. (1) Transgenic mice with a genetically deleted TMD1 domain showed increased mortality in endotoxin-induced sepsis, together with the finding that adhesion molecule expression and neutrophil infiltration were increased in TMD1-deficient endothelium (13). *Ex vivo* studies have shown that additional TMD1 binds to endothelial antigen during inflammation, competitively inhibiting leukocyte migration and adhesion (14). Furthermore, we (15) showed that recombinant TM (rTM), containing TMD123, directly binds to neutrophils via the macrophage-1 antigen (Mac-1) receptor, and thus inhibits neutrophil activation. In addition, rTM affects lymphocytes to inhibit pro-inflammatory cytokine/chemokine production during an inflammatory response. (2) Necrotic parenchymal cells and neutrophil extracellular traps (NETs) release HMGB1 and histones into the extracellular space. The former is a nuclear chromatin-binding protein that transduces intracellular pro-inflammatory signals via toll-like receptor 4 (TLR4) and the receptor for advanced glycation endproducts (RAGE) (16). The latter exerts distinct biological effects, including direct cell toxicity, exacerbation of immune responses via TLR stimulation, and the activation of platelets, consequently exacerbating DIC, thrombosis, post-ischemic organ damage, and sepsis (17, 18). TM potentially neutralizes these DAMPs, attenuating intravascular injury and organ damage (19, 20). (3) The TMD1 domain potentially binds to the Lewis Y antigen of lipopolysaccharide (LPS) that has pro-inflammatory properties, as it can interact with CD14 and TLRs, thus inhibiting excessive inflammatory responses (21). (4) TM and its TMD1 domain regulate the complement system by eliciting complement-inhibitory signals (22). Abnormal complement activation leads to endothelial dysfunction, including thrombotic microangiopathy. TM may negatively regulate the alternative complement pathway by enhancing complement factor I-mediated inactivation of C3b. In addition, TM interferes with thrombin-mediated complement factor C5 activation, which involves the production of anaphylatoxin, and the formation of a membrane attack complex. TMD2

Abbreviations: AE-IPF, Acute exacerbation of idiopathic pulmonary fibrosis; ANCA, Anti-neutrophil cytoplasmic antibody; APC, Activated protein C; DAMPs, Damage-associated molecular patterns; DIC, Disseminated intravascular coagulation; EGPA, Eosinophilic granulomatosis with polyangiitis; GPA, Granulomatosis with polyangiitis; GVHD, Graft-vs.-host disease; HMGB1, High mobility group box 1; HUS, Hemolytic uremic syndrome; IRI, Ischemia-reperfusion injury; LPS, Lipopolysaccharide; Mac-1, Macrophage-1 antigen; MPA, Microscopic polyangiitis; MPO, Myeloperoxidase; NETs, Neutrophil extracellular traps; NF-κB, Nuclear factor-kappa B; RAGE, Receptor for advanced glycation endproducts; rTM, Recombinant thrombomodulin; STEC, Shiga toxin-producing *Escherichia coli*; sTM, Serum thrombomodulin; TAFI, Thrombin activatable fibrinolysis inhibitor; TA-TMA, transplant-associated thrombotic microangiopathy; TLR, Toll-like receptor; TM, Thrombomodulin; TMA, Thrombotic microangiopathy; TTP, Thrombotic thrombocytopenic purpura; VEGF, Vascular endothelial growth factor.

and TMD3 also exert indirect anti-inflammatory effects via APC production, which activates protease-activated receptor-1 on the endothelium to induce cell protection by inhibiting NF-κB signaling (23). Furthermore, TM-thrombin binding enhances the activation of thrombin activation of fibrinolysis inhibitor (TAFI) that degrades bradykinin and complement factors (24), contributing to the regulation of inflammation. Collectively, TM regulates inflammation, the complement system, and endothelial protection in addition to anti-coagulation during intravascular injury, consequently preserving intravascular homeostasis.

NETS AND TM

Various stimuli induce NETs through their own NETs-signaling mechanisms. However, regardless of the type of trigger, the NETs resulting from it could become major sources of DAMPs, and act as initiators of immunothrombosis in the face of intravascular injury (25, 26). Thus, NETs have the potential to become a therapeutic target for treatment of immunothrombosis-related diseases. Previously, rTM has been reported to downregulate several types of NET formation. Shimomura et al. showed that rTM inhibited NET formation following treatment with LPS-primed platelets by suppressing TLR4 signaling (27, 28). Studies by Shrestha et al. (29) indicated that rTM treatment ameliorated histone-induced sepsis by neutralizing extracellular histones and suppressing the formation of NETs (20). These previous reports implied indirect effects against neutrophils. Recently, we (15) could show the direct effect of rTM binding to neutrophils, which inhibited auto-antibody-mediated NET formation. In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, pathogenic myeloperoxidase (MPO)-ANCA binds to MPO expressed on tumor necrosis factor α-primed neutrophils, and the Fc region of ANCA crosslinks with the Fcγ receptor coupled with Mac-1 on neutrophils to activate spleen tyrosine kinase signaling and ROS production, which results in peptidylarginine deiminase 4 activation and NET formation (30–32). In this scenario, rTM binds to Mac-1 to competitively interfere with ANCA binding on neutrophils, and inhibits downstream signaling, which suppresses ANCA-induced NET formation. Thus, TM potentially has direct and indirect inhibitory effects on NET formation, which contributes to the resolution of intravascular inflammation and immunothrombosis (**Figure 1A**).

EXPERIMENTAL EVIDENCE OF RTM-MEDIATED RESOLUTION OF INFLAMMATORY INTRAVASCULAR INJURY [SEPSIS, ISCHEMIC REPERFUSION INJURY, THROMBOTIC MICROANGIOPATHY (TMA), AND MACROANGIOPATHY]

Of note, rTM containing all the extracellular domains acts not only as an anti-coagulant, but also displays anti-inflammatory properties, hence contributing to the resolution of various diseases (**Figure 1B** and **Table 1A**).

Sepsis

Sepsis involves multi-organ dysfunction with systemic inflammatory processes, immune dysregulation, coagulopathy, and other physiological responses. Among these processes, NETs and necrotic cell-derived DAMPs directly injure the endothelium and contribute to the development of immunothrombosis through the activation of platelets, coagulation systems, and recruitment of neutrophils (17, 25, 89, 90). In a mouse histone-induced septic model, pretreatment with rTM reduced mortality rates by neutralizing histones (20). In a rat sepsis/peritonitis model (33) and a murine LPS-induced septic model (34), rTM controlled sepsis-related immunothrombosis by limiting abnormal hemostasis and NET formation.

Ischemia-Reperfusion Injury (IRI)

IRI occurs in response to the physiological processes that accompany tissue ischemia with inadequate oxygen supply. This is followed by reperfusion that drives regulated necrosis and subsequent inflammatory responses, leading not only to local organ damage, but also to remote organ injury in the form of necroinflammation (91, 92). In the animal brain, heart, lung, and liver, rTM (the entire ectodomain with lectin-like domain TMD1) ameliorated IRI tissue damage via anti-inflammatory effects, including neutralization of HMGB1 and histones, subsequently triggering the TLR4 signaling pathway (13, 38–40, 93). In a mouse model of renal IRI, ischemia-initiated tubular epithelial cell necrosis released extracellular histones and induced NET formation, which further contributed to remote lung injury (94). Interestingly, rTM (35) and a histone-neutralizing antibody (94) ameliorated remote organ damage, but did not have sufficient effects on local kidney injury. Conversely, inhibition of regulated necrosis, including necroptosis, mitochondrial necrosis, and ferroptosis, rescued local kidney injury at primary lesions, but had less effect on remote organ injury compared with histone neutralization (94). The discrepancy between local and remote injury was compatible with the phenomenon observed in an rTM-treated intestinal IRI mouse model, in which rTM improved remote liver injury, but not local intestinal damage (37). These findings imply that primary necrotic organ injury might develop based on the intracellular signaling cascades arising in response to IRI, but remote organ injury might mainly be caused by DAMPs and inflammatory responses, which could provide a better understanding of DAMP-related IRI pathogenicity.

TMA

TMA is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and organ injury. The underlying pathogenesis of TMA is understood to be endothelial dysfunction, which is caused by bacterial toxins, deficiency or dysfunction of the complement system, deficiency or

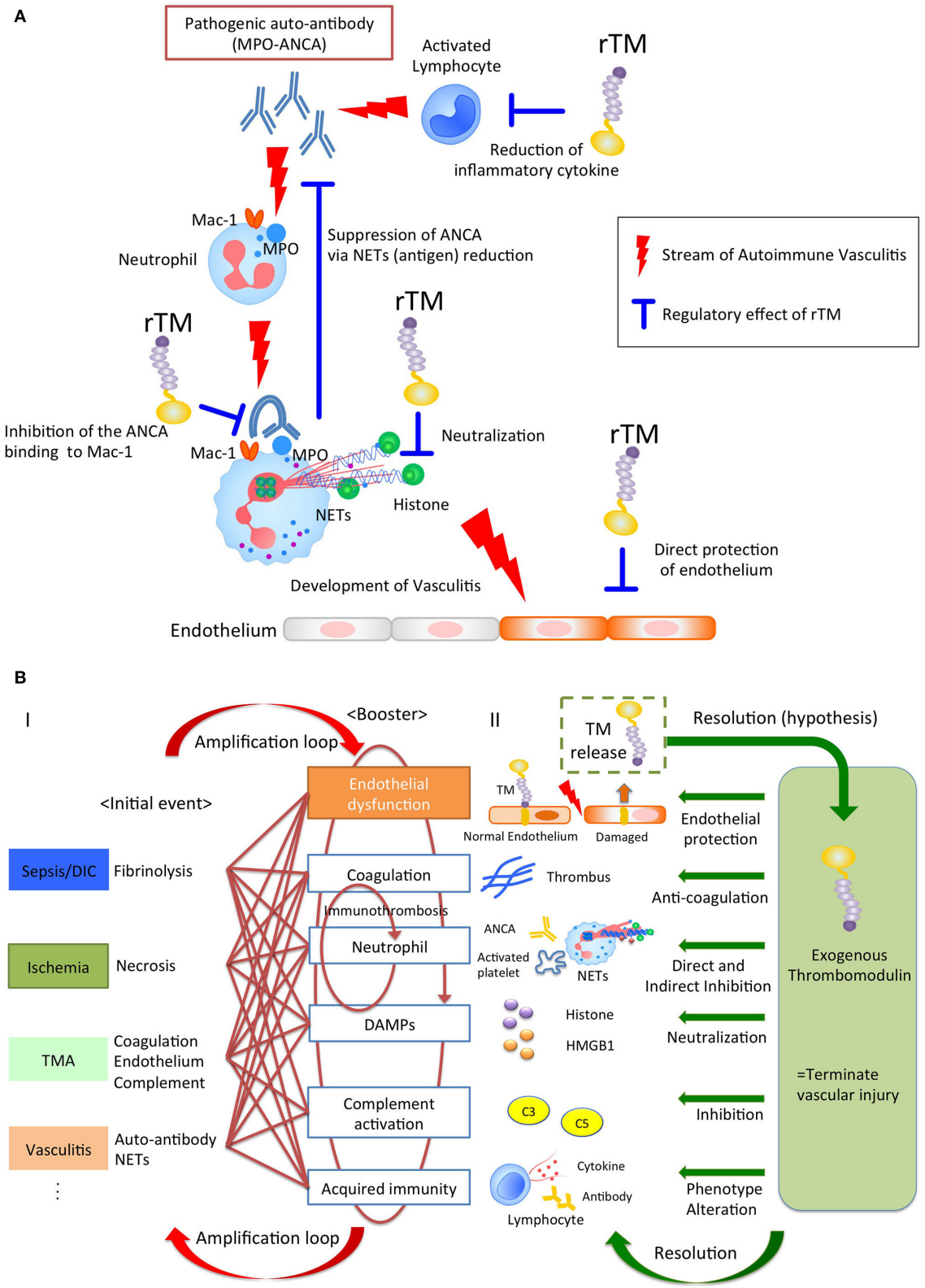

FIGURE 1 | (A) The pleiotropic effects of rTM in autoimmune vasculitis. Pathogenic anti-neutrophil cytoplasmic antibody (ANCA) produced by lymphocytes binds to neutrophil antigen, inducing neutrophil extracellular traps (NETs). The NETs components cause vasculitis and could become auto-antigens, resulting in the further

(Continued)

FIGURE 1 | ANCA production. rTM suppresses the pro-inflammatory lymphocytes and inhibits the ANCA binding to Mac-1 on neutrophil, resulting in the suppression of NETs, which leads to the reduction of auto-antigens and ANCA production. Furthermore, rTM neutralizes cytotoxic extracellular histones in NETs and directly protects endothelium. Collectively, rTM could regulate the multiple points in pathogenesis of autoimmune vasculitis. **(B)** Thrombomodulin terminates auto-amplification of intravascular injury. (I) Intravascular injury in sepsis, ischemia-reperfusion injury, thrombotic microangiopathy, and vasculitis develops due to fibrinolysis, necrosis, coagulation/endothelial dysfunction, and neutrophil activation, respectively, as an initial event. In the next step, these events appear jointly with endothelial dysfunction, coagulation, neutrophil activation, damage-associated molecular patterns, complement activation, and acquired immunity to exacerbate the disease. In particular, immunity and coagulant systems collaborate to generate robust immune-thrombi, which accelerate intravascular injury, leading to an amplification loop.
(II) Thrombomodulin is released into the intravascular space after endothelial injury and serves to counteract excessive coagulation and inflammation via its pleiotropic effects.

TABLE 1A | Experimental evidence on recombinant thrombomodulin (rTM, including TMD1, TMD23, and TMD123 domains) in animal disease models.

Animal model	Outcomes	Mechanisms	References
Histone-induced thrombosis (mouse)	Improved mortality and thrombosis	Neutralization of histones	(20)
Cecal ligation and puncture-induced peritonitis (rat)	Improved coagulopathy	Regulation of NETs	(33)
LPS-induced sepsis (mouse)	Improved mortality	Neutralization of HMGB1	(34)
Renal ischemia-reperfusion injury (mouse)	Improved lung injury (remote organ)	Regulation of NETs	(35)
Renal ischemia-reperfusion injury (rat)	Improved renal function and histology	Reduction of leukocyte infiltration	(36)
Intestinal ischemia-reperfusion (mouse)	Increased survival and liver damage (remote organ)	Regulation of NETs	(37)
Myocardial ischemia (mouse)	Reduced myocardial damage	Suppression of leukocyte-endothelial interaction and TLR signaling	(13, 38)
Lung ischemia-reperfusion injury (mouse)	Suppressed protein leakage	Reduction of leukocyte infiltration	(39)
Cerebral ischemic injury (mouse)	Reduced infarct volume	Neutralization of HMGB1	(40)
Anti-glomerular basement membrane glomerulonephritis (rat)	Improved histology	Neutralization of HMGB1	(41)
Experimental autoimmune encephalomyelitis (mouse)	Improved clinical and pathological severity	Neutralization of HMGB1	(42)
ANCA-associated vasculitis (rat and mouse)	Improved renal and lung vasculitis	Suppression of NETs, acquired immunity	(15)
Hemolytic uremic syndrome (mouse)	Improved mortality and renal histology	Regulation of the complement system	(43)
Diabetic glomerulopathy (mouse)	Improved nephrosis	Inhibition of the complement system and inflammasome	(44, 45)
Arthritis (mouse)	Improved arthritis	Complement inhibition	(46)
Acute respiratory distress syndrome (mouse)	Increased survival rate	Neutralization of HMGB1 and increase in regulatory T cells	(47)
Bleomycin-induced pulmonary fibrosis (mouse)	Improved lung damage	Inhibition of transforming growth factor-β1 and HMGB1	(48, 49)
Bronchial asthma (rat)	Improved lung function	Modulation of dendritic cells	(9)
Pre-eclampsia (rat)	Improved maternal and fetal conditions	Improvement of hypo-perfusion	(50)
Recurrent spontaneous miscarriage (mouse)	Improved fetal resorption	Increase of VEGF expression	(51)
Lung metastasis (mouse)	Inhibited invasion and metastasis of cancer cells	Thrombin-independent mechanism	(52)
Pancreatic cancer (mouse)	Suppressed tumor growth	Inhibition of NF-κB activation	(53)
Atherosclerosis (mouse)	Improved atherosclerotic change	Anti-autophagic action and inhibition of thrombin-induced endothelial activation	(54, 55)
Aortic aneurysm (mouse)	Suppressed aneurysm	Inhibition of HMGB1-RAGE signaling	(56, 57)

ANCA, anti-neutrophil cytoplasmic antibody; HMGB1, high mobility group box 1; LPS, lipopolysaccharide; NETs, neutrophil extracellular traps; NF-κB, nuclear factor-kappa B; RAGE, receptor for advanced glycation end product; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

TABLE 1B | The levels of serum thrombomodulin (TM) in diseases with intravascular injury.

Disease	References	Levels of sTM	Correlation	With
Sepsis/DIC	(58)	–	Positive	DIC, multiorgan dysfunction, mortality
	(59)	Increase	Positive	Disease severity, mortality
Cerebral infarction	(60)	Increase	–	–
	(61)	No change	Inverse	Disease severity
	(62)	Increase	No	Disease severity
	(63)	Increase	Positive	Disease progression
<Autoimmune disease>				
Systemic lupus erythematosus	(64–66)	Increase	Positive	Disease activity
ANCA-associated vasculitis (GPA)	(67, 68)	Increase	Positive	Disease activity
ANCA-associated vasculitis (GPA or MPA)	(69)	–	Positive	Disease activity
ANCA-associated vasculitis (EGPA)	(70)	–	Positive	Disease activity
Diabetes	(71)	Increase	Positive	Nephropathy and/or Retinopathy
	(72–74)	Increase	Positive	Nephropathy
	(75)	–	Inverse	Risk of type 2 Diabetes
<Cardiovascular disease>				
Coronary heart disease	(76)	No change	–	–
	(77, 78)	–	Inverse	Risk of coronary heart disease
	(79)	Increase	–	–
	(80)	–	Positive	Risk of coronary heart disease
	(81)	–	None	Risk of coronary heart disease
Atherosclerosis	(82, 83)	Increase	–	–
	(77)	–	Positive	Risk of carotid atherosclerosis
	(84)	Increase	Positive	Sclerotic changes in hypertensive retinopathy
	(85)	Increase	Positive	Intima-media thickness
Aortic aneurysm	(86)	Increase	Positive	Risk factors for atherosclerosis
Pre-eclampsia	(87, 88)	Increase	–	–

ANCA, anti-neutrophil cytoplasmic antibody; DIC, disseminated intravascular coagulation; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; sTM, serum thrombomodulin.

inhibition of ADAM-TS13, drug-induced reactions, and transplant complications (95). The major disorders are hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). *Escherichia coli* (O157:H7) induces HUS by producing Shiga toxins, which bind to endothelial cells in the kidney and brain, triggering them to undergo cell death by inhibiting protein synthesis and inducing the secretion of Von Willebrand factor multimers, which leads to endothelial injury and microthrombi (96, 97). In mice, TM deficiency (more specifically, lectin-like domain TMD1) exacerbated Shiga toxin-producing *E. coli* (STEC)-HUS (98). Furthermore, in STEC-HUS-induced mice, rTM treatment protected them from kidney injury by regulating intravascular inflammation, complement dysfunction, and the coagulation system (43).

Macroangiopathy, Including Aortic Aneurysm

Aortic aneurysm develops in association with certain risk factors, including age, genetic predisposition, atherosclerosis, and smoking. The underlying pathogenesis is characterized by chronic vascular inflammation and degradation of collagen-producing structural matrix proteins, which weaken the aortic wall (99). In a $CaCl_2$-induced abdominal aortic aneurysm model, rTM [entire ectodomain (56) and lectin-like domain TMD1 (57)] treatment ameliorated abdominal aortic aneurysm by suppressing inflammatory mediators, macrophage recruitment, and HMGB1-RAGE signaling. In an apolipoprotein E-deficient atherosclerosis model, rTM (TMD23) inhibited autophagy-related cell death of aortic endothelial cells, preventing the progression of atherosclerosis (54). *In vitro* studies have shown

that rTM directly binds to fibroblast growth factor receptor 1 on the endothelium, which activates the phosphatidylinositol 3-kinase-AKT/mammalian target of rapamycin complex 1 signaling pathway, and inhibits autophagy (54, 100). These findings indicate that TM could potentially mediate large vessel homeostasis by controlling immunological responses and endothelium protection.

EXPERIMENTAL EVIDENCE OF RTM AS AN IMMUNE MODULATOR BEYOND AN INFLAMMATORY REGULATOR

In previous sections, the anti-inflammatory effects of rTM against intravascular injury were mainly described. In autoimmune diseases, including Goodpasture's syndrome (41) and autoimmune encephalomyelitis (42), rTM ameliorated the disease by suppressing inflammation and neutralizing DAMPs. Interestingly, recent reports have indicated that rTM acts as an immune modulator in addition to serving as an inflammatory regulator. In our study, rTM affected acquired immunity as well as neutrophil activation to resolve autoimmune vasculitis (15). Pathogenic ANCA auto-antibodies play a pivotal role in the development of ANCA-associated vasculitis. In this regard, rTM binds to antibody-producing lymphocytes to alter their activities from pro-inflammatory to anti-inflammatory, which contributes to the reduction of ANCA production and the resolution of the disease. Furthermore, Takagi et al. (9) reported that rTM ameliorated the ovalbumin-induced asthma model by regulating pathogenic dendritic cells. In a graft-vs.-host disease (GVHD) model, rTM increased regulatory T cells via the induction of anti-apoptotic Mcl-1 expression, resulting in the improvement of GVHD (101, 102). Similarly, rTM ameliorated acute respiratory distress syndrome in mice with an increase in regulatory T cells (47). Van De Wouwer et al. (46) showed that rTM (lectin-like domain TMD1) improved mouse arthritis by suppressing excessive inflammatory responses by macrophages and complement activation. As such, rTM could potentially modulate systemic acquired immunity in response to intravascular injury separately from maintaining local vessel homeostasis.

CLINICAL EVIDENCE FOR RTM-BASED STRATEGIES

Several studies have reported the serum TM level to examine its role in various diseases. Sepsis (58), ischemic disease (63), and autoimmune diseases (64) showed high levels of soluble TM in serum and plasma that reflected prevailing endothelial injury, indicating that soluble TM levels might be useful for disease diagnosis (**Table 1B**). Does endogenous soluble TM protect from intravascular injury in human disease? In coronary heart disease, the level of soluble TM is inversely correlated with disease severity (77), implying that endogenous TM might contribute to the resolution of this disease. However, because soluble TM is released from damaged endothelium to counteract the disease, soluble TM levels are often found to increase with disease severity (**Table 1B**) (77). Meanwhile, genetic polymorphisms of TM could influence the disease beyond the quantity of TM, which might explain the discrepancy between the titer and disease (103). It might be difficult to determine the role of endogenous TM based on soluble TM levels. However, the efficacy of additional TM has been clinically revealed with regard to several diseases during the past two decades.

DIC

In randomized, double-blind clinical trials, in which patients with DIC associated with hematologic malignancy or infection were treated with rTM or heparin, rTM improved DIC, and alleviated hemorrhagic complications compared with heparin (104). Although rTM therapy did not reduce all-cause mortality in a large clinical trial, *post-hoc* subgroup analysis stratified by the persistence of abnormal coagulation showed a tendency to decrease mortality (105). Meanwhile, a one-arm prospective trial revealed the effectiveness of rTM in solid tumor-associated DIC (106). Moreover, rTM administration could potentially be useful for treatment of obstetric DIC. During pregnancy, placental abruption, bleeding, and hypoxia could drive DIC underlying obstetric disorders, which is associated with maternal and fetal morbidity and mortality (107). A retrospective comparative study revealed that rTM significantly improved clinical and laboratory findings compared with controls in patients with obstetric DIC (108).

TMA

TMA is associated with high mortality regardless of the underlying disease, including HUS, TTP, transplant complications, and drug side effects. In a case series of three patients with HUS, rTM ameliorated clinical outcomes with improvements reflected in reduced platelet counts and excessive complement activation (109). Furthermore, rTM could be beneficial for patients with transplant-associated (TA)-TMA. The latter is a severe complication after hematopoietic stem cell transplantation. The putative etiology is endothelial injury, which is caused by cytotoxic agents, infections, and GVHD (110). A case report (111) and retrospective cohort study (112, 113) showed the effectiveness of rTM with favorable clinical features and overall survival. Likewise, hepatic sinusoidal obstructive syndrome shows clinical manifestations characterized by hepatomegaly, jaundice, ascites, fluid retention, and thrombocytopenia following hematopoietic stem cell transplantation, with pathogenesis mechanisms similar to those of TA-TMA (114). Moreover, patients treated with rTM showed remission and survival rates equivalent to that of patients receiving defibrotide, which is the only recommended therapy for sinusoidal obstructive syndrome (115).

Acute Exacerbation of Idiopathic Pulmonary Fibrosis (AE-IPF)

AE-IPF is a lethal condition associated with endothelial damage and abnormalities of the coagulation system (116, 117). HMGB1 is involved in the pathophysiology of pulmonary fibrosis (48). Furthermore, NETs are identified in the bronchi of patients

diagnosed with AE-IPF, and are believed to contribute to disease progression (118). Kataoka et al. (119) reported that rTM therapy resulted in improved mortality rates compared with the control group (rTM vs. control: 30 vs. 65%). However, similar to the sepsis clinical trial, a large randomized phase III study in patients with AE-IPF did not show the superiority of rTM using the state of the control as primary endpoint (120). The cause is thought to be the heterogeneous pathology in the comparison group. Therefore, an appropriate study protocol with stratified risk factors is required.

Clinical Perspectives of rTM Therapy via the Anti-inflammatory and Immune-Regulatory Effects

Although the efficacy of rTM has not been clinically shown in autoimmune disease and inflammatory disorder, several experimental data represent the potential to overcome these diseases. *In vitro* and animal studies indicate that rTM possesses the direct immunomodulatory effects in innate and acquired immunity independently of anti-coagulant effect (9, 15). Based on animal studies (**Table 1A**), rTM is being clinically expected to contribute to resolving diseases with inflammation including diabetes mellitus, arthritis, bronchial asthma, and ischemic-reperfusion injury. In particular, autoimmune ANCA vasculitis, which is characterized by immune dysregulation and intravascular injury, might be a candidate for rTM treatment. However, the dosage of rTM in many experimental situations (15, 33, 41) is 15–50 times of therapeutic dosage in patients with DIC and the effective concentration as an anti-inflammatory and immune-regulatory property remains unclear. Thus, in the future the indications of rTM therapy and the suitable dosage with no serious complications such as bleeding tendency should be carefully addressed.

CONCLUSIONS

Immunothrombosis during intravascular injury leads to organ damage and further intravascular injury via cellular and molecular signaling, including excessive inflammation, coagulation, and cell activation. rTM regulates the immunothrombosis to terminate inflammation/coagulation, neutralize DAMPs, and affect immunity. The administration of rTM has the potential to become a novel therapeutic strategy for various diseases associated with immunothrombosis-mediated intravascular injury.

AUTHOR CONTRIBUTIONS

KW-K and DN conducted the literature research and reviewed all articles. AI and TA edited the article. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant number JP 18K15968).

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing.

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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.

frontiers in Immunology

REVIEW
published: 07 October 2020
doi: 10.3389/fimmu.2020.581300

DAMPs in Unilateral Ureteral Obstruction

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Damage-associated molecular patterns (DAMPs) are released from tubular and interstitial cells in the kidney after unilateral ureteral obstruction (UUO). DAMPs are recognized by pattern recognition receptors (PRRs), which mediate the initiation of an immune response and the release of inflammatory cytokines. The animal model of UUO is used for various purposes. UUO in adult mice serves as a model for accelerated renal fibrosis, which is a hallmark of progressive renal disease. UUO in adult mice enables to study cell death, inflammation, and extracellular matrix deposition in the kidney. Neonatal UUO is a model for congenital obstructive nephropathies. It studies inflammation, apoptosis, and interstitial fibrosis in the neonatal kidney, when nephrogenesis is still ongoing. Following UUO, several DAMPs as well as DAMP receptors are upregulated. In adult UUO, soluble uric acid is upregulated and activates the NOD-like receptor family, pyrin domain containing-3 (NLRP3) inflammasome, which promotes fibrosis, apoptosis, and reactive oxygen species (ROS) injury. Further DAMPs associated with UUO are uromodulin, members of the IL-1 family, and necrotic cell DNA, all of which promote sterile inflammation. In neonatal UUO, the receptor for advanced glycation endproducts (RAGE) is highly upregulated. RAGE is a ligand for several DAMPs, including high mobility group box 1 (HMGB1) and S100 proteins, which play an important role in renal fibrosis. Additionally, necroptosis is an important mechanism of cell death, besides apoptosis, in neonatal UUO. It is highly inflammatory due to release of cytokines and specific DAMPs. The release and recognition of DAMPs initiate sterile inflammation, which makes them good candidates to develop and improve diagnostic and therapeutic strategies in renal fibrosis and congenital obstructive nephropathies.

Keywords: damage-associated molecular patterns (DAMPs), unilateral ureteral obstruction (UUO), inflammation, innate immunity, kidney

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *08 July 2020*
Accepted: *21 September 2020*
Published: *07 October 2020*

Citation:
Wyczanska M and Lange-Sperandio B (2020) DAMPs in Unilateral Ureteral Obstruction. Front. Immunol. 11:581300. doi: 10.3389/fimmu.2020.581300

INTRODUCTION

Sterile inflammation is a response to acute or chronic tissue injury without pathogens being involved. However, how does the body recognize damage? The activation of the immune system as a response to pathogens is possible by detection of molecular motifs conserved in so-called pathogen-associated molecular patterns (PAMPs). In the case of sterile injury, the immune system reacts in a similar way. Damage-associated molecular patterns (DAMPs) are intracellular molecules that are released as a

response to sterile injury and are able to activate innate immunity just like PAMPs. DAMPs and PAMPs are recognized by pattern recognition receptors (PRRs), which then mediate the initiation of an immune response (1, 2). PRRs can be of several types, like Toll-like receptors (TLRs), NOD-like receptors (NLRs), AIM2-like receptors (ALRs), RIG-I-like RNA helicases, C-type lectin receptors (CLRs), and more (3, 4). DAMPs are molecules that have specific functions inside the cell; they operate as signals of cell damage only when they are released into the cytosol or the extracellular space (5). Released DAMPs expose hydrophobic portions of molecules that are naturally hidden within living cells and can thus be recognized as danger signals (6, 7). One way for DAMPs to leave the cell is to be passively released from dying cells. It is important to differentiate between different cell death pathways here. Apoptosis, being a non-inflammatory programmed way of cell death, does not lead to the release of DAMPs. By contrast, necroptosis, necrosis, and pyroptosis, induce inflammatory responses through the release of cytokines and DAMPs (8). DAMPs can also be secreted by living cells, which are exposed to life-threatening stress. High mobility group box 1 (HMGB1), for instance, is a DAMP that can be secreted by stressed cells without involving the endoplasmic reticulum (9). HMGB1 release is induced in monocytes by lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α or interleukin (IL)-1. Upon activation, HMGB1 exits the nucleus into the cytoplasm, translocates into the cytoplasmic organelles and is released through lysosome exocytosis.

DAMPs play a role in a variety of kidney diseases and could be used as biomarkers, or reveal novel drug targets for inhibiting the inflammatory response. In this review, we focus on DAMPs in unilateral ureteral obstruction (UUO) in mice (**Table 1**), which is used as a model for various purposes. UUO in adult mice is an experimental model of renal injury, leading to tubulointerstitial fibrosis (26). Renal fibrosis is the final common pathway of numerous kidney diseases leading to end-stage renal disease with dialysis or renal transplantation, as no effective treatments exits yet (23). UUO enables to study different stages of fibrosis development in an accelerated manner, like inflammatory cell infiltration, tubular cell death, extracellular matrix (ECM) deposition, and tubular atrophy (**Figure 1**) (28, 29). Urinary tract obstruction in the newborn mouse kidney also permanently impairs renal development (29). Neonatal UUO at the second day of life investigates the pathological mechanisms of congenital obstructive nephropathy (30, 31), which is the most common identifiable cause of chronic kidney disease in children and infants (32, 33). Neonatal UUO studies inflammation, apoptosis, and interstitial fibrosis in the neonatal kidney, while nephrogenesis is still ongoing. In humans, nephrogenesis is completed before birth by the 34-36 gestational week. By contrast, nephrogenesis in mice ceases 1-2 weeks postnatally. Therefore, performing UUO in newborn mice allows studying the effect of ureteral obstruction on kidney development. This experimental urinary tract obstruction in neonatal mice is analogous to the obstruction arising in the midtrimester human fetus with congenital obstructive nephropathy (28). UUO in neonatal and adult mice leads to sterile inflammation and thus to upregulation and release of DAMPs (**Figure 2**). DAMPs released during tissue injury, together with the immune receptors that recognize these, most likely contribute to the development of renal fibrosis (23). This review focuses on danger signals associated with obstruction in adult and neonatal kidneys.

TABLE 1 | List of known DAMPs in UUO.

DAMPs	Putative Receptors	Pro-fibrotic	References
Biglycan	TLR2, TLR4, NLRP3	N/A	(10)
Decorin	TLR2, TLR4	○	(11, 12)
HMGB1	TLR2, TLR4, TLR9, RAGE	●	(13–15)
IL-1α	IL-1R	N/A	(16, 17)
IL-33	ST2	●	(18, 19)
Necrotic DNA	TLR9, ALR	●	(20, 21)
sUa	NLRP3	●	(22)
S100A8/A9	TLR2, TLR4, RAGE	●	(23, 24)
Uromodulin	TLR4	○	(25)

Black dot indicates that the criterion is fulfilled; white dot indicates that the criterion is not fulfilled; N/A, no data available for the UUO model. TLR, toll-like receptor; RAGE, receptor for advanced glycation end-products; NLRP3, NOD-, LRR-, and pyrin domains-containing protein 3; HMGB1, high mobility group box 1; ALR, absent in melanoma 2-like receptors; sUa, soluble uric acid.

FIBROSIS

Renal fibrosis is the hallmark of progressive renal disease and involves glomerular sclerosis and interstitial fibrosis (26). Fibrogenesis is considered a failed wound healing process after an injury. Processes leading to fibrosis are: proliferation of interstitial fibroblasts with myofibroblast transformation and deposition of a large amount of ECM components (34). There are several markers that are used to characterize fibrosis, like α-smooth muscle actin (αSMA) (34) or collagen I, III, and IV (35). Epithelial-mesenchymal transition (EMT) is also a process associated with UUO and renal fibrosis. It is a mechanism by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells (36). These multipotent stromal cells can differentiate into a variety of cell types. Epithelial cells dedifferentiate to mesenchymal cells as a repair mechanism, however, in the case of chronic or repetitive injury they can differentiate into myofibroblasts, thus building fibrotic scar tissue (37, 38). Following UUO monocytes infiltrate the renal interstitium and release cytokines such as transforming growth factor β1 (TGF-β1) (26). TGF-β1 promotes either apoptosis of tubular epithelial cells, leading to tubular atrophy, or EMT, leading to fibrosis. Several DAMPs, like IL-33, HMGB1, and biglycan also play a role in EMT (36, 39–41). Chronic inflammation is a critical process in fibrogenesis. Following kidney injury various pro-inflammatory stimuli activate fibroblasts (42). Fibroblasts can be activated by DAMPs through ligation with TLRs (43) (**Figure 2**).

CELL DEATH AND INFLAMMASOMES

Necrosis and apoptosis are cell death mechanisms and both occur during UUO (28, 44). During apoptosis the plasma membrane integrity is maintained, whereas during necrosis it raptures (45). Additional difference between apoptosis and

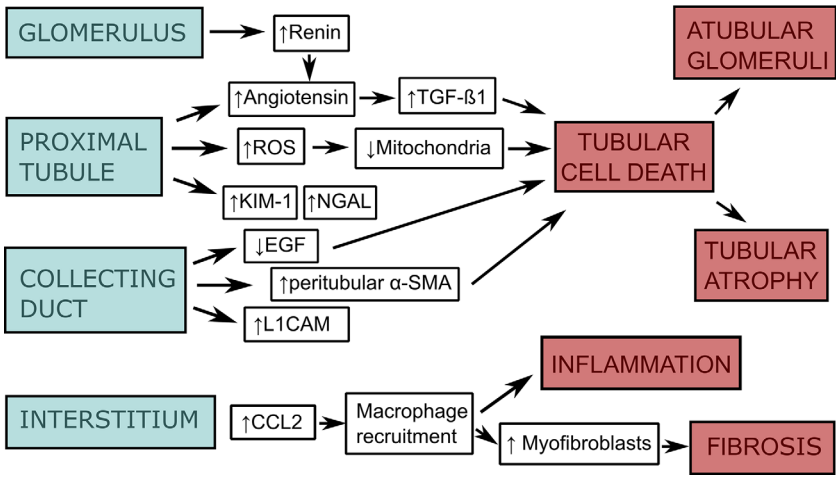

FIGURE 1 | Scheme of the pathophysiology of unilateral ureteral obstruction (UUO). As a response to unilateral ureteral obstruction the glomerulus increases vascular renin production along with activation of the renin-angiotensin system, which leads to stimulation of transforming growth factor-β1 (TGF-1β). The proximal tubular epithelium activates the renin-angiotensin system as well. Additionally, it increases production of reactive oxygen species (ROS), which impair mitochondrial function, as well as kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). Collecting duct injury leads to downregulation of epidermal growth factor (EGF) and upregulation of peritubular mesenchymal collars that express α-smooth muscle actin (α-SMA) and L1 cell adhesion molecule (L1CAM). Injury of glomeruli, proximal tubule, and collecting duct lead to tubular cell death (apoptosis, necrosis, and necroptosis), which itself leads to atubular glomeruli and tubular atrophy. In the interstitium, there is an upregulation of chemokines (CCL-2, CCL-5) and adhesion molecules as an response to obstruction. This leads to macrophage recruitment, interstitial inflammation, and stimulation of myofibroblast proliferation, which causes fibrosis. The figure is adapted from (27).

necrosis is the release of DAMPs, which is absent in apoptosis. Necrosis was seen as an unregulated form of cell death for a long time, but there are several cell death modalities of regulated necrosis, like necroptosis and pyroptosis. Engagement of receptors for Fas, TNF, or TNF-related apoptosis-inducing ligand (TRAIL) can lead to programmed cell death, apoptosis, through recruitment and activation of caspase-8 (46). However, in presence of caspase-inhibitors the cell death mechanism switches to a more rapid and necrotic mode of cell death, so-called necroptosis (47). Necroptosis is a well-characterized form of regulated necrosis, mediated by receptor interacting protein kinase-3 (RIPK3) and its substrate mixed lineage kinase like (MLKL) (48). Necroptosis is seen as a trigger for inflammation through release of DAMPs due to rapid cell rupture (49). DAMPs released through cell death can themselves trigger other endothelial and epithelial cells to undergo further cell death (**Figure 2**). It is unlikely, that both necrosis and necroptosis release the exact same cluster of DAMPs. DAMPs released by accidental cell death and secondary necrotic cells are well studied, however, studies about specific DAMPs associated with necroptosis were rarely conducted until recently (49). A crucial step of the necroptotic cell death pathway is phosphorylation of MLKL, thus pMLKL is seen as a marker for necroptosis (50).

Pyroptosis is a programmed necrosis that involves the activation of inflammasomes (45). Inflammasomes are intracellular sensors, they can be activated by extracellular DAMPs through the ligation of DAMPs and TLRs on cell surface. This process activates the nuclear factor (NF)-κB signaling pathway (51) or mitochondrial ROS production (22) and triggers the inflammasome. NOD-like receptor family, pyrin domain containing-3 (NLRP3) is one of the best-studied inflammasomes. Its activation results in inflammation. NLRP3 canonical activation in macrophages and other immune cells requires two steps: priming and activation. Priming is stimulated by binding of DAMPs or PAMPs to TLRs or cytokine receptors. It generally involves NF-κB signaling and expression of NLRP3, pro-caspase-1, pro-IL-1β, and pro-IL-18 (52, 53). Activation induces inflammasome assembly and caspase activation. NLRP3 proteins bind to apoptosis-associated speck-like proteins, which recruit pro-caspase-1 proteins that are cleaved into mature caspase-1. Active caspase-1 then processes pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18. It also cuts gasdermin-D into N-terminal and C-terminal fragments, of which gasdermin-D-N creates extensive membrane pores, causing leakage of IL-1β, IL-18, and other cell compartments, which sets up the sterile inflammation (53). This process is called pyroptosis. Known DAMPs released due to pyroptosis after NLRP3 activation are HMGB1, IL-1α, and apoptosis associated speck-like protein containing a CARD (caspase activation and recruitment domain) (8). These are also known to be upregulated after UUO (13, 54, 55). The NLRP3 inflammasome itself can be activated by a variety of DAMPs such as uric acid crystal, silica crystals, ATP, asbestos, alum, and X-ray (53).

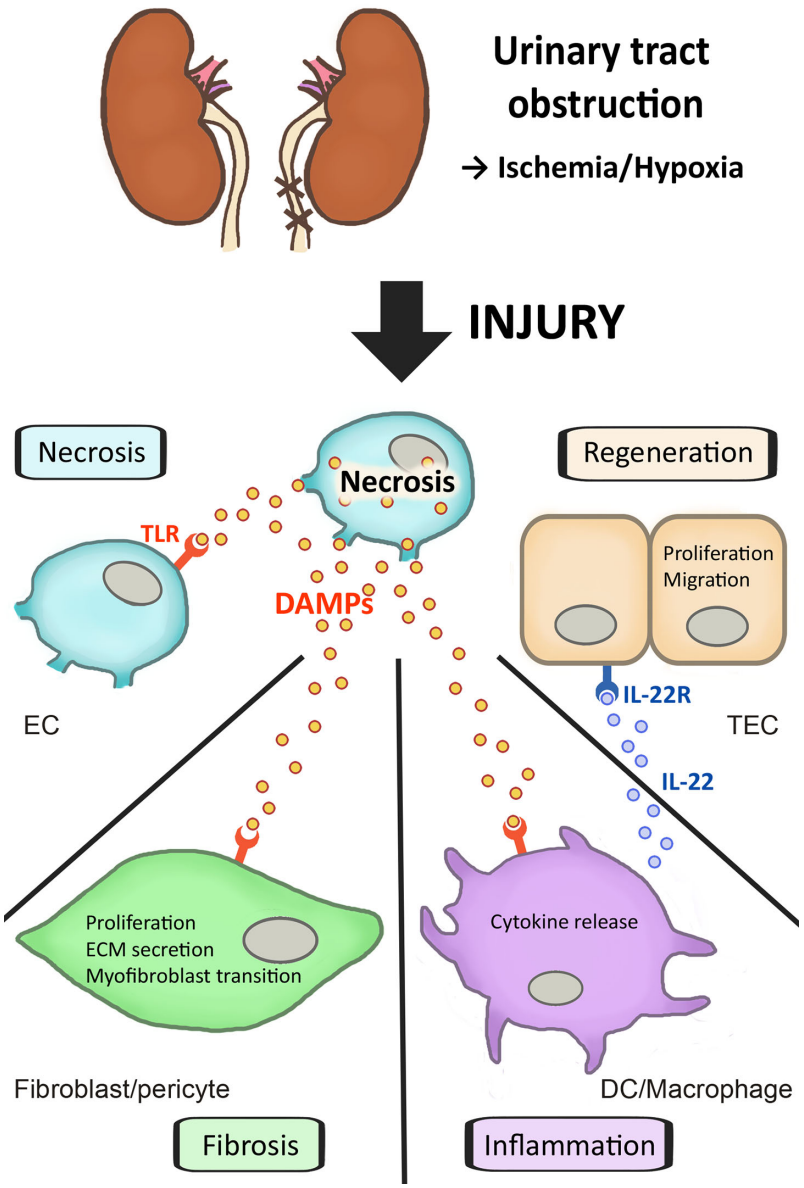

FIGURE 2 | Different outcomes of cell death and DAMP release due to urinary tract obstruction. Unilateral ureteral obstruction causes cell injury and necrosis, as well as the regulated forms necroptosis and pyroptosis. Due to cell stress and cell death DAMPs are released by injured endothelial (EC) and tubular epithelial (TEC) cells. These DAMPs activate PRRs such as TLRs on other cells. This can lead to further renal cell necrosis, with amplification of DAMPs. Fibroblasts and pericytes activated by DAMPs trigger fibrosis through proliferation, ECM secretion and myofibroblast transition. Activated dendritic cells (DC) and macrophages release cytokines and chemokines, which initiate an inflammatory response. IL-22 secreted by renal DCs, on the other hand, is able to activate the IL-22 receptor on TECs, which accelerates tubular re-epithelialization, thus promoting regeneration of TECs.

IL-1α AND IL-33

IL-1α and IL-33, which count as cytokines as well as DAMPs, are actively released during necroptosis (16, 45). IL-1α can also be actively secreted by epidermal epithelial cells (56). IL-33 was detected in the plasma of RIPK1-deficient mice and is dependent on the presence of RIPK3 and MLKL (16). Taken together with the observations of increased IL-33 expression in necroptotic epidermal keratinocytes (57), IL-33 was categorized as a necroptotic DAMP. IL-33 is able to activate basophils and eosinophils, as well as to induce type 2 immune responses (58). Recently it was shown that necroptosis and necroinflammation are accompanied phenomena in neonatal kidneys with ureteral obstruction (17). Biochemical analyses showed a decrease in caspase-8 expression and an increase in RIPK3 and pMLKL expression, indicating a role of necroptosis in UUO. IL-1α

and IL-33 were measured in this study. IL-1α was significantly upregulated in the kidneys following obstruction. In the neonatal UUO model, in contrast to the adult one (18), the expression of IL-33 was downregulated. It is unknown, why IL-33 expression decreased in this particular disease model despite clear indications of necroptosis. A possible explanation is that interferon gamma (IFNγ), an immunomodulatory cytokine that was also upregulated following UUO, is able to downregulate pro-fibrotic IL-33 under certain conditions (59). IL-33 has been linked to fibrosis (18, 19) and can induce EMT *in vitro* (19). In HK-2 cells IL-33 was able to promote the cellular motility and migration capabilities of these cells. IL-33 activates the p38 mitogen-activated protein kinase (MAPK) signalling pathway, which induces the EMT process (60). The more pro-inflammatory and less pro-fibrotic state after neonatal UUO could be linked to the IFNγ activity and IL-33 downregulation. Even though the expression of only two possible necroptotic DAMPs were measured in this study, it can be assumed that due to necroptosis, other DAMPs are released as well and contribute to further inflammation and/or fibrosis after ureteral obstruction in the kidney.

IL-1–EXTENDED FAMILY

Two members of the IL-1 extended family, IL-1α and IL-33 are referred to as cytokines that also act as DAMPs (61). The IL-1 extended family consists of IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ. All members play different biological activities involved in innate immunity (62). A recently published hypothesis argues that all members of the extended IL-1 family function as DAMPs (63). IL-1α and IL-1β are released from dying cells due to necroptosis and pyroptosis. Additionally, IL-18 is released from pyroptotic cells and IL-33 from necroptotic cells. Three of these cytokines (IL-1α, IL-1β, IL-18) are known to be upregulated during UUO (64, 65), IL-33 is upregulated in adult UUO (18), but downregulated in neonatal UUO (17). However, do all these cytokines qualify as canonical DAMPs? The definition of DAMPs is not completely clear. They are often portrayed as molecules that would only be released upon cell death and through certain pathways would initiate an inflammatory response (66). Most candidate DAMPs are structurally divers molecules that do not share common mechanisms of action. The author argues that the IL-1 family cytokines are good DAMP candidates due to their ability to drive inflammation in sterile injury. They are activated (IL-1β, IL-18, IL-36α, IL-36β, IL-36γ) and released mostly in cell death processes (67). One important argument is that the IL-1 family cytokines signal via receptors that contain intracellular Toll/IL-1 receptor signaling motifs, just like a subset of PAMPs. Additionally both receptor classes share signaling intermediates, like MyD88, and IRAK kinases. The main difference is stated in a part of the definition of DAMPs. DAMPs have a non-inflammatory "day job" within the cytosol or nucleus of cells, which cytokines generally do not have (68). Most members of the IL-1 family (IL-1β, IL-18, IL-36α, IL-36β, IL-36γ) require proteolytic processing to activate their biological activity, putting them in an inactive state, without function, until activation of the inflammasome or necrosome. An exception here would be cellular IL-1α, which has been associated with cellular senescence and other functions, as well as IL-33, which is expressed by a variety of cells and has a possible role in regulating gene expression (69). Given this, only IL-1α and IL-33 can be seen as both cytokines and DAMPs. However, if the cellular non-inflammatory active function of DAMPs would be neglected, then the other cytokines could also be counted as DAMPs. The quiescent state of the IL-1 family cytokines inside the cell seems to be the only argument against their categorization as DAMPs.

SOLUBLE URIC ACID

Uric acid, a purine catabolism product, is a DAMP released from injured and dying cells (70). Upon crystallization, it activates the immune system. It triggers the NLRP3 inflammasome activation through phagocytosis (71). Recently it has been reported that beside uric acid crystals, soluble uric acid (sUA) is also able to act as a DAMP and activate the NLRP3 inflammasome (22). Elevated serum uric acid induces inflammation dependent on mitochondrial ROS production and changes in the redox state. It is released in a hypoxic environment (72) and able to trigger NLRP3 through production of mitochondrial ROS, which leads to caspase-1 activation and IL-1β production. To study this, a murine UUO model was used, as it leads to increased levels of sUA. Additionally, correlation between tissue damage and the degree of sUA formation was observed (22). This also confirms the findings that NLRP3 plays a role in the acute phase following UUO (51, 73). Accordingly, $NLRP3^{-/-}$ mice demonstrate reduced inflammation, tubular injury and fibrosis after UUO, which is associated with reduced caspase-1 activation and IL-1β and IL-18 maturation (64, 65). Inhibition of NLRP3 in UUO would have beneficial effects, as it plays a key role in sterile inflammation and fibrosis. However, NLRP3 is also involved in antiviral responses (74), so it may be more beneficial to inhibit DAMPs, like crystalized or soluble uric acid that trigger this inflammasome, without losing the protection from viruses. Inhibitors of uric acid like the xanthine oxidase inhibitors allopurinol and febuxostat are used to treat chronic kidney disease patients with hyperuricemia (75). Allopurinol treatment in UUO mice reduces type 1 collagen mRNA levels and hydroxyproline, the main amino acid that forms collagen (22). It also reduces the mRNA expression of *Il-33* and *Nlrp3*. The administration of febuxostat to mice after UUO inhibits the induction of proinflammatory and fibrogenic cytokines (76). It suppresses TGF-β, type I collagen and α-SMA expression and thus fibrosis. Treatment of patients with obstructive nephropathies with allopurinol or febuxostat may be therefore promising in the suppression of uric acid induced fibrosis.

NECROTIC CELL DNA

DNA has the ability to impact immunity itself or by forming complexes with other molecules and create unique danger signals (20). For the stimulation of immunity DNA has to have access to

internal cell sensors. Extracellular DNA, either released from pathogens or by necrosis of host cells, can reenter another cells when bound to antibodies or nucleic acid-binding proteins. Necrotic cell DNA triggers dendritic cells and macrophages to mature phenotypically and functionally (77). As UUO in mice induces tubular necrosis (44), necrotic DNA is released and functions as DAMP in this model. Absent in melanoma 2 (AIM2) inflammasome is typically activated by pathogen DNA and triggers innate immunity, but it can also be activated by DNA released from dying cells (78). AIM2 is a cytosolic PRR that assembles an inflammasome in response to double-stranded DNA. Its activation drives proteolytic maturation of the proinflammatory cytokines IL-1β and IL-18, and pyroptosis (78). AIM2 has a protective role in microbial infection but a pathological one in sterile inflammation. Recently it has been shown, that *Aim2* deficiency reduces renal injury, fibrosis, and inflammation in adult mice after UUO (21). AIM2 is upregulated in the tubular epithelium and in inflammatory infiltrates in the kidney. In UUO-induced renal inflammation and injury, AIM2 is activated in recruited macrophages by uptake of necrotic cell DNA and aggravates the pathological state. *Nlrp3*$^{-/-}$*Aim2*$^{-/-}$ mice were used to examine the relative contribution of the inflammasomes NLRP3 and AIM2 to renal injury. There were no significant phenotypic differences between *Nlrp3*$^{-/-}$*Aim2*$^{-/-}$ mice compared with *Aim2*$^{-/-}$ mice, suggesting a partially redundant role for the inflammasomes during renal injury. *Nlrp3*$^{-/-}$*Aim2*$^{-/-}$ mice had less injury, inflammation, and fibrosis compared with WT mice. However, still ongoing injury and inflammation in the injured kidney indicates an important role of other inflammasomes after ureteral obstruction. It also shows that inhibition of just one type of inflammasome might be able to reduce sterile inflammation and fibrosis, but not prevent it entirely.

MITOCHONDRIAL DAMPS

Mitochondrial dysfunction plays an important part in various chronic inflammatory diseases, including UUO (64, 79). Mitochondrial damage causes production of mitochondrial reactive oxygen species, aberrant calcium mobilization, potassium efflux, reduction in cytoplasmic levels of NAD^+, and upregulation of extracellular ATP (80). These changes have been shown to be involved in NLRP3 activation. In case of mitochondria injury or dysfunction production of mitochondrial DAMPs is possible (5, 80). Cytochrome C is a small soluble electron carrier hemeprotein that transfers electrons from complex III to complex IV to facilitate cell energy production (81). It is released in apoptotic cell death to trigger non-inflammatory cell death processes. However, when translocated into the extracellular space, cytochrome C functions as a DAMP. Cardiolipin is a phospholipid of mitochondria and confined to it (82). Due to mitochondrial stress or dysfunction it can undergo oxidation and be released into the extracellular milieu as a DAMP (83). Cardiolipin can directly bind and activate NLRP3 (84). Mitochondrial N-formyl peptides, which are released upon injury, can bind to formyl peptides receptors on neutrophils, monocytes, and dendritic cells and activate these (80). Mitochondrial DNA also seems to act as a DAMP (85). Upon opening of the mitochondrial permeability transition pore fragments of mitochondrial DNA are released from mitochondria (86). If this mitochondrial DNA enters thy cytoplasm, extracellular space or circulation, it can engage multiple pattern-recognition receptors and trigger pro-inflammatory responses (85). There is an ongoing debate, whether mitochondrial DNA is a bona fide DAMP following necroptotic killing (8). Recent findings however suggest that extracellular intact mitochondria are released during necroptosis and indeed act as danger signals (87). The released mitochondria were determined to be intact, as they did not emit detectible amounts of mitochondrial DNA. These extracellular mitochondria activate cytokine production in macrophages and cell maturation of dendritic cells, which classifies them as DAMPs (87). There has been no research on blocking these DAMPs in UUO, nonetheless UUO causes mitochondrial stress and dysfunction (64, 79), as well as necroptosis (17). It is probable that mitochondrial DAMPs play an important role in sterile inflammation and renal fibrosis following UUO. UUO decreases nuclear factor erythroid 2-related factor 2 (Nrf-2) translocation and activity, which is accompanied with an increase of mitochondrial BCL2 associated X protein translocation and an increase of cytosolic cytochrome c release (88). Overexpression of Nrf-2 attenuates mitochondrial dysfunction and has anti-fibrotic effects in UUO (88, 89). It is unknown whether the anti-fibrotic effect results from Nrf-2 induced reduction of TGF-β expression and hydroxyproline level alone or whether the reduction of cytochrome c release might also play a role. Research on mitochondrial DAMPs during UUO might be important in the future.

S100A8/A9

The calcium binding protein S100A8/A9 is a DAMP that activates the receptor for advanced glycation end-products (RAGE) (24). RAGE is a multiligand pattern recognition receptor linked to chronic inflammation (90, 91). RAGE binds and mediates the cellular response to a variety of DAMPs. It is expressed at low level under normal physiological conditions, but is highly upregulated in chronic inflammation due to the accumulation of various ligands. RAGE has been identified as a receptor directly mediating leukocyte recruitment *in vivo*. S100A8/A9 heterodimer is expressed and released by phagocytes and has been shown to induce chemotaxis, cytoskeleton reorganization, and cytokine expression through activation of macrophages and neutrophils (92). It can be either passively released via necrosis, cellular damage, or neutrophil extracellular traps formation of myeloid cells, or actively from myeloid cells during acute or chronic local inflammation. S100A8/A9 exerts a critical role in initiating an inflammatory response by stimulation leukocyte recruitment and inducing cytokine secretion (93). Adult S100A9$^{-/-}$ mice lacking the S100A8/A9 heterodimer that were subjected to UUO were protected from renal fibrosis (23). S100A8/A9 mediates renal fibrosis, tubular apoptosis, and crucial events for epithelial-mesenchymal transition in the kidney after UUO. High concentrations of

S100A8/A9 induce a caspase-independent cell death, possibly necrosis, in tubular epithelial cells, thus leading to further release of DAMPs. Blocking S100A8/A9 activity has been shown to be beneficial in a variety of diseases (92, 93) and it could be a crucial factor for the reduction of fibrosis. Furthermore, RAGE, the receptor of S100A8/A9 is upregulated early in neonatal mice after UUO (94). This upregulation induces activation of the transcription factor NF-κB and its target genes, including proinflammatory cytokines. $RAGE^{-/-}$ mice showed less tubular apoptosis and less interstitial fibrosis after neonatal UUO (95). Besides inhibition of inflammasomes and specific DAMPs, blocking DAMP receptors may be a promising target to treat sterile inflammation and fibrosis.

HMGB1

High-mobility group box-1 (HMGB1) is also shown to be a DAMP released by necroptotic cells (96). HMGB1 is the best characterized DAMP and it is also a ligand of RAGE. (97). It has been identified as an important extracellular mediator in local and systematic inflammation (98). In the nucleus, HMGB1 organizes nucleosomes and DNA and regulates gene transcription (99). Due to cell injury or activation, nuclear HMGB1 is released into the cytoplasm, where it is involved in inflammasome activation as well as regulation of the autophagy/apoptosis balance through activation of immune and endothelial cells. Translocated HMGB1 has chemokine, cytokine, neuroimmune, and metabolic activities (99). HMGB1 can be actively secreted by macrophages/monocytes in response to inflammatory stimuli or passively released by necrotic cells (49, 96, 100). The release mode of HMGB1 can be divided into two groups: burst-mode and sustained-mode. Different durations of the release, being either 7.1 or 109 min on average, were observed (96). In the burst-mode HMGB1 is rapidly released from the cytoplasm, probably due to existing cytoplasmic membrane damage, in the sustained-mode the release is slowed down. The sustained-mode release of HMGB1 shows a balance between the extent of pore forming activity and membrane repair capacity of associated proteins. The biological significance of these two different modes remains unclear. However, it demonstrates a possible plasticity of cell death pathways and release of DAMPs. Thus, HMGB1 could play a role in prognosis and therapy. Furthermore, an acidic environment is able to trigger HMGB1 release *in vitro* (13). Thus, besides cell death it is hypothesized that acidosis, due to UUO or other pathologies, may cause release of HMGB1 or other DAMPs leading to inflammation. HMGB1 is upregulated after UUO in adult mice (13–15). It can induce the classically activated macrophages (M1) phenotype at the early stage of UUO (13). M1 activation is associated with injury, inflammation, and production of reactive nitrogen and oxygen species. Inhibition of HMGB1 diminished the presence of M1 macrophages (13). The treatment also resulted in an upregulation of M2 macrophages in the early stage of injury. As no previous M2 macrophage activation was observed in this stage of UUO, the M2 macrophages after inhibition of HMGB1 were likely to be converted from M1 macrophages. Additionally, inhibition of HMGB1 attenuated UUO-induced interstitial inflammation and blocked the injury-induced collagen deposition in the kidney. This indicates an important role of HMGB1 in sterile inflammation and fibrosis after UUO. Another link to fibrosis is that HMGB1 expression is highly elevated in diabetic nephropathy, which results in apoptosis and EMT progression of podocytes due to inhibition of autophagy (39). Downregulation of HMGB1 inhibits EMT progression.

DECORIN AND BIGLYCAN

Decorin and biglycan are small leucine rich proteoglycans. They are important components of the ECM. Recent studies however also show their involvement in different signaling pathways, indicating a role in autophagy, host immune responses and fibrosis (11). Decorin is the best studied proteoglycan; it regulates collagen fibrillogenesis and is a key factor for the mechanical integrity of such tissues as skin, tendon and ligaments (101). Additionally, it interacts with a variety of growth factors and thus has tumor suppressive, anti-inflammatory and anti-fibrotic properties. Decorin can be cleaved by proteases and cytokines and the cleavage fragments act as DAMPs. Decorin and biglycan activate as DAMPs the production of TNF α, IL-12, and macrophage inflammatory protein 2 in macrophages by binding to TLR4/2 (11). Decorin has anti-fibrotic activities through inhibition of TGF-β activities (101). Furthermore, decorin inhibits connective tissue growth factor signaling in fibroblasts, inhibits apoptosis of renal tubular epithelial cells and down-regulates microRNA miR-21 (43). The inhibition of these processes further alleviates interstitial fibrosis. In UUO decorin is highly upregulated (12, 102). Furthermore, decorin deficient mice show aggravation of renal fibrosis, highlighting the anti-fibrotic properties of this proteoglycan (12). Inhibition of decorin in renal sterile inflammation would have negative effects on fibrosis. However, inhibition of cleaved decorin, which functions as a DAMP, or the factors necessary for the cleavage could have beneficial effects. This should be elaborated in future research.

Biglycan can be found in most tissues as a stationary component of the ECM (10). However, upon release from injured cells or secretion by activated macrophages, biglycan becomes available in its soluble form and acts as a DAMP. Biglycan is involved in the activation of the NLRP3 inflammasome in sterile inflammation, leading to secretion of mature IL-1β. Similar to decorin, the expression of biglycan is upregulated after UUO (12). Biglycan deficient mice after UUO display lower levels of active caspase-1 and mature IL-1β, leading to reduction of infiltrating macrophages and less renal injury (10). Inhibition of biglycan attenuates inflammation, but its role in renal fibrosis is not yet clear (11). However, an upregulation of biglycan induces EMT by TGFβ activation (36). Biglycan binds extracellular TGFβ1 and modulates its access to the TGFβ

receptors. TGFβ induces EMT, via a group of specific transcription factors (36, 103). Hence, an upregulation of biglycan seems to have pro-fibrotic properties.

UROMODULIN

Uromodulin (UMOD), also known as Tamm-Horsfall protein, is the most abundant protein in normal human urine (104). UMOD has been assigned a role in a variety of functions: modulating renal ion channel activity, intertubular communication, salt/water balance, inflammatory response, mineral crystallization, and bacterial adhesion (105). UMOD is synthesized in thick ascending limb tubular epithelial cells (106). It reaches the plasma membrane in a monomeric form. Its luminal release into the urine and subsequent polymerization is dependent on its cleavage mediated by the serine protease hepsin (107). Additionally, small amounts of UMOD are also released basolaterally into the interstitium and blood and show a positive association with kidney function (108). However, this positive effect results from monomeric UMOD. Polymeric UMOD in serum stimulates an inflammatory response (109). Many studies do not distinguish between these two possible states of UMOD. Polymerized UMOD is not immunostimulatory inside the tubular lumen, but once leaked into the interstitial compartment, it functions as a DAMP (56). UMOD can activate TLR4 on myeloid dendritic cells, leading to maturation of these cells (110). It has also the ability to activate the NLRP3 inflammasome leading to IL-1β release (111). Recently, UUO studies with UMOD deficient adult mice were conducted (25). In $UMOD^{+/+}$ mice UMOD protein expression increased 9-13x above sham levels following obstruction. In $UMOD^{-/-}$ mice apoptosis and cellular debris were reduced. The intensity of the interstitial inflammatory response was evaluated by F4/80 monocyte/macrophage protein levels. These were significantly lower (50%) in the $UMOD^{-/-}$ mice, showing a proinflammatory function of UMOD after UUO. However, there were no significant difference in renal fibrosis between $UMOD^{+/+}$ and $UMOD^{-/-}$ mice. This suggests that in the absence of UMOD interstitial macrophages are recruited that are distinct and functionally polarized to a more robust fibrogenic phenotype. Blocking extratubular polymerized UMOD may be an interesting target to treat patients with obstructive nephropathies.

DISCUSSION

Extensive progress has been made in the field of DAMPs in recent years. New DAMPs and the corresponding pathways have been identified, as well as different release modes. DAMPs play an important role in UUO, as they drive inflammation and can have pro-fibrotic functions. They present possibilities for new biomarkers and anti-inflammatory therapies. At present, there is a lack of precise and reliable markers of urinary tract obstruction (31). Prenatal diagnosis of obstructive nephropathies is important as it allows for the planning of appropriate prenatal and postnatal care. It is key to distinguish between kidneys that do not need surgery and kidneys that would deteriorate and lose function or growth potential without. The perfect biomarker for renal fibrosis should be specific, non-invasive, directly involved in the mechanisms of fibrosis, with the ability to reflect treatment effects, and have low or no background in healthy individuals (112). DAMPs secreted in the urine may be future biomarkers in patients with congenital obstructive nephropathies and renal fibrosis, respectively. In a variety of diseases DAMPs are already used as biomarkers (92, 93, 113, 114). They can differentiate between diseases (114) and recognize inflammation as well as the site of infection or sterile injury (92). DAMPs assign valid outcome prognoses (115, 116) and help to differentiate between beneficial and harmful immune responses (115). Recently it has been shown that different isoforms of HMGB1 provide information on the type of injury (113). HMGB1 can be either slowly excreted from stressed or inflammatory cells, or rapidly released from dying cells. Non-acetylated HMGB1 is released from dying cells, whereas acetylated HMGB1 is associated with active secretion. This finding improves diagnostics, as it helps to estimate the severity of the inflammatory response. As for diagnostic purposes it is advised to use a mix of biomarkers, as under specific circumstances one biomarker could be inhibited and thus deliver false results. This can be seen in the case of IL-33, which is used as a marker for necroptotic cell death. However, IL-33 wasn't upregulated after obstruction in the neonatal kidney despite evidence for necroptosis (17). A variety of factors, especially organ development or different diseases can alter the expression of certain biomarkers. There is a risk of false negative or false positive results if not taken into account.

Besides being used as biomarkers, DAMPs and DAMP associated pathways may also play a role in therapy. Inhibition of cell death pathways, like necroptosis can be helpful in inflammatory diseases, but only if the necroptotic cell death plays a major role (117). Inhibition of inflammasomes and receptors can be beneficial in reducing inflammation and fibrosis in the kidney. Knock-out of *Nlrp3* and *Aim2* resulted in less injury, inflammation, and renal fibrosis after obstruction (21). Neonatal $RAGE^{-/-}$ mice showed less tubular apoptosis and interstitial fibrosis after UUO (95). There are several inhibitors of NLRP3 or RAGE (55, 91), however inhibition of inflammasomes and their receptors can also be harmful, as this would hinder pathogen detection. PAMPs and DAMPs are both recognized by PRRs like TLRs (4, 118) Inhibition of receptors would also inhibit their ability to detect PAMPs and initiate an inflammatory response to fight the infection (118–120). If a sterile inflammation would be accompanied or followed by a bacterial infection, receptor inhibition would worsen rather than improve the state of the patient. Another important issue is that most inhibitors block only one inflammasome (55) and therefore reduce inflammation only to a certain extent. An alternative would be to block several DAMPs associated with sterile inflammation during UUO. Inhibition of HMGB1 attenuated UUO-induced interstitial inflammation and renal fibrosis (13). Adult $S100A9^{-/-}$ mice that were subjected to UUO were protected

TABLE 2 | Open questions.

Do mitochondrial DAMPs contribute to renal fibrosis?
Do other inflammasomes, beside NLRP3 and AIM2, play a role in sterile inflammation and fibrosis after UUO?
Can DAMPs be used as suitable markers for renal fibrosis and the severity of UUO?
Are the results of DAMP inhibition seen in mice reproducible in patients?

from renal fibrosis (23). HMGB1 and S100A8/A9 are well studied DAMPs and a variety of inhibitors have been designed that are used to reduce inflammation in diseases and injury (92, 93, 113). These inhibitors are widely used against harmful inflammation; their use against fibrosis needs to be studied in future research.

It should be considered that DAMPs are not always harmful and can have beneficial effects on repair. TLR2 on renal progenitor cells is activated by certain DAMPs and accelerates tubular repair (43). Additionally, TLR4 on dendritic cells, when activated by DAMPs, triggers IL-22 release. IL-22 activates the IL22-receptor on tubular epithelial cells and accelerates tubular re-epithelialization (**Figure 2**) (43, 121). HMGB1 recruits bone marrow derived mesenchymal stem cells and thus promotes repair (6). It also plays a role in proliferation and differentiation of tissue-associated resident stem cells. Moreover, HMGB1 promotes angiogenesis, which is required for tissue repair. These differences between beneficial and harmful functions of HMGB1 may be due to its redox state (113, 122) and should be further investigated. Directly inhibiting DAMPs could be helpful to fight sterile inflammation, however fine tuning might be a better option. Overexpression of DAMPs is harmful; however, they also contribute to tissue repair and healing. Research into DAMPs as biomarkers and their use in therapeutic application, especially regarding inflammation and fibrosis in the kidney, is a promising field for future research. There are still many open research questions that need to be answered (**Table 2**).

AUTHOR CONTRIBUTIONS

MW and BL-S wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

BL-S is supported by a grant from the German Research Foundation (DFG La 1257/5-1).

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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.

The handling editor declared a shared affiliation, though no other collaboration, with the authors MW and BL-S.

frontiers in Immunology

REVIEW
published: 07 October 2020
doi: 10.3389/fimmu.2020.568513

Extracellular DNA—A Danger Signal Triggering Immunothrombosis

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Clotting and inflammation are effective danger response patterns positively selected by evolution to limit fatal bleeding and pathogen invasion upon traumatic injuries. As a trade-off, thrombotic, and thromboembolic events complicate severe forms of infectious and non-infectious states of acute and chronic inflammation, i.e., immunothrombosis. Factors linked to thrombosis and inflammation include mediators released by platelet granules, complement, and lipid mediators and certain integrins. Extracellular deoxyribonucleic acid (DNA) was a previously unrecognized cellular component in the blood, which elicits profound proinflammatory and prothrombotic effects. Pathogens trigger the release of extracellular DNA together with other pathogen-associated molecular patterns. Dying cells in the inflamed or infected tissue release extracellular DNA together with other danger associated molecular pattern (DAMPs). Neutrophils release DNA by forming neutrophil extracellular traps (NETs) during infection, trauma or other forms of vascular injury. Fluorescence tissue imaging localized extracellular DNA to sites of injury and to intravascular thrombi. Functional studies using deoxyribonuclease (DNase)-deficient mouse strains or recombinant DNase show that extracellular DNA contributes to the process of immunothrombosis. Here, we review rodent models of immunothrombosis and the evolving evidence for extracellular DNA as a driver of immunothrombosis and discuss challenges and prospects for extracellular DNA as a potential therapeutic target.

Keywords: thrombosis, leukocytes, platelets, vasculitis, mouse model, stroke, sepsis

OPEN ACCESS

Edited by:
Janos G. Filep,
Université de Montréal, Canada

Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *01 June 2020*
Accepted: *09 September 2020*
Published: *07 October 2020*

Citation:
Shi C, Yang L, Braun A and Anders H-J (2020) Extracellular DNA—A Danger Signal Triggering Immunothrombosis. Front. Immunol. 11:568513. doi: 10.3389/fimmu.2020.568513

INTRODUCTION

Evolution positively selected four major danger response programs, i.e., inflammation, clotting, epithelial healing, and mesenchymal healing because they assure survival upon traumatic injury (1). Blood clotting and inflammation are early responses that immediately create barriers. Clotting creates an inside-out barrier for blood loss and clotting and inflammation both create an outside-in barrier for pathogen entry. Balanced clotting can prevent fatal bleeding and balanced inflammation can prevent fatal sepsis. However, trade-offs exist and largely contribute to prevalent disease pathomechanisms in clinical medicine (2). Thrombotic and thromboembolic events are important complications in severe forms of infectious and non-infectious states of acute and chronic inflammation, i.e., immunothrombosis (3). Proinflammatory mediators released from platelets, complement, and lipid mediators link clotting and inflammation as do certain integrins, and neutrophil extracellular traps (NETs).

As a novel entry, extracellular deoxyribonucleic acid (DNA) can elicit profound proinflammatory and pro-thrombotic effects in the extracellular space (4). Pathogens release

DNA together with other pathogen-associated molecular patterns (PAMPs). Dying parenchymal cells release extracellular DNA together with other danger- or damage-associated molecular patterns (DAMPs) and neutrophils release DNA by forming neutrophil extracellular traps (NETs) during infection, trauma or other forms of vascular injury (5–7). Extracellular DNA localizes to the sites of injury and experimental studies employing deoxyribonuclease (DNase)-deficient mouse strains or recombinant DNase demonstrate a functional contribution of this extracellular DNA to the development of immunothrombosis.

In this review, we summarize basic knowledge about the process of immunothrombosis, and discuss the role of extracellular DNA as a modulator of thrombosis in the arterial and venous segments of the vasculature. Furthermore, we describe several mouse models to study the process of immunothrombosis in different disease settings.

Experimental Models of Venous Thrombosis

Venous thrombosis is a common clinical health care problem and causes congestion and pain when affecting the deep veins of the limbs or acute thoracic pain, dyspnea, and shock when affecting the pulmonary arteries. Pulmonary embolism is a severe life-threatening complication of deep vein thrombosis. Venous thrombosis frequently develops in the perianal venous plexus region and usually presents as painful swelling at the site of the blood clot. The most popular rodent model of venous thrombosis is obstruction of the inferior vena cava via a surgical intervention, which generates clots of sufficient size for measuring clot weight and for histopathological characterization of the clot material (8). Here, we introduce several types of venous thrombosis models which are studied in mice (**Table 1**).

Inferior Vena Cava Stasis Occlusion Model

The stasis occlusion variant is a model of permanent inferior vena cava (IVC) ligation, mimicking the clinical condition of complete vascular occlusion. Technically, the IVC and all collateral side branches distal to the left renal vein are ligated. Thrombus formation in this model involves venous stasis and local release of tissue factor (TF) with augmented coagulation inside the IVC (9, 80). The advantages of this model are its low mortality, high frequency of thrombus generation, and highly consistent thrombus sizes (10, 11). Ultrasound can sequentially monitor thrombus progression and to select an optimal time point for harvesting the thrombus. This model has proven valuable to study the interactions between the venous wall and thrombus progression from acute (first 2–3 days) to chronic inflammation as well as to study the subsequent remodeling of the venous wall (12, 13). As a relevant discrepancy to most venous thromboses in human, blood flow does not establish. Peternel et al. used the stasis occlusion model in rats and found it well-suited for evaluating antithrombotic therapies (14).

Inferior Vena Cava Stenosis Model

Partially reducing rather than completely blocking venous blood flow is more similar to the process of venous thrombus in humans. Technically, this implies only partial ligation of side branches of the IVC and using a wire as a spacer during IVC ligation that, once removed, maintains a small lumen with a residual flow avoiding endothelial cell damage (15–21). These subtle modifications mimic a residual flow that is typical for human venous thrombosis and critical for its pathophysiology. As a disadvantage, the thrombus formed in the IVC is generally smaller and the size is more variable (22). This model allows us to better study early thrombotic events (16).

Electrolytic Inferior Vena Cava Thrombosis Model

Cooley et al. first described thrombus induction by electrical injury to the common femoral vein of mice (23, 24). Diaz et al. modified the protocol by applying a constant current to a copper wire inserted into the IVC. The electrical current induces free radicals inside the wire, which subsequently activate endothelial cells with minor cell damage (25–27). A thrombus develops quickly in the direction of the blood flow and thrombus sizes are highly consistent. This venous thrombosis model is used to study pro-thrombotic, anti-thrombotic, and thrombolytic therapies (28–30). Moreover, this model can mimic the early and late stages of venous thrombosis. Disadvantages include long procedure time and potential venous wall injury.

Ferric Chloride ($FeCl_3$) Inferior Vena Cava Thrombosis Model

Local application of $FeCl_3$ solution causes oxidative damage to the surface of the exposed venous wall followed by thrombus formation (31, 32). To achieve this, a small piece of filter paper soaked with a defined concentration of $FeCl_3$ solution is applied to the IVC for 3 min (33). As toxin exposure allows only a short observation period, thrombus size is usually small with little thrombus material for evaluation. Gustafsson et al. combined $FeCl_3$-induced vessel injury with IVC stenosis in rats to obtain a larger thrombus size (34).

Recurrent Inferior Vena Cava Thrombosis Model

Patients with deep vein thrombosis face a high risk of post-thrombotic syndrome and 30% experience recurrent thrombosis with 45% occurring in the ipsilateral leg within the following 10 years. Attempts to model recurrent thrombosis employ first the electrolytic method and 21 days later a secondary thrombus is induced using either a second electric insult or a ligature-based method. At the time of the second intervention, the primary thrombus has been incorporated into the venous wall, and the lumen has recovered. This clinically more relevant model has proven valuable to study the biology of recurrent deep vein thrombosis (35).

Experimental Models of Arterial Thrombosis

Arterial thrombosis is followed by territorial ischemia and infarcts during spontaneous rupture of atherosclerotic plaques, or in patients with an anti-phospholipid syndrome or with trauma. Arterial thrombosis is the central pathologic mechanism contributing to myocardial infarction and ischemic stroke (81). It is a major health concern in terms of cardiovascular morbidity

TABLE 1 | Animal models of immunothrombosis.

Model	Strengths	Weaknesses	References
Venous thrombosis			
IVC ligation model (stasis model)	Thrombus size is highly consistent.	It completely blocks blood flow.	(9–15)
IVC stenosis model	Thrombus reduces blood flow, endothelial cell damage.	Thrombus formation is strain-dependent, clamp relevant injury is unclear.	(16–22)
Modified IVC stenosis model	Thrombus reduces blood flow, no endothelial cells damage.	Variable in thrombus incidence and size.	(16–22)
Electrolytic IVC model (EIM)	Thrombus size is highly consistent, with no endothelial cells damage.	Long operation time.	(23–30)
$FeCl_3$ IVC model	Produces thrombus within minutes, thrombus size is time-dependent.	Transmural vein wall injury, the thrombus is small, only be used to study early time points.	(31–34)
Recurrent IVC model	Most clinically relevant.	Twice surgeries on the same mouse.	(35)
Arterial thrombosis			
Photothrombotic model	Localize the ischemic lesion, minimal variation in infarction, low mortality and invasiveness, highly reproducible.	The translational impact is poor.	(36–47)
Thromboembolic clot models	Any kind of embolus-like material can be used, perfectly matches human embolic stroke.	High variability in infarct size, embolic material not lysisable, high price.	(48–52)
Microsphere/macrosphere model	Infarcts with penumbras, induce ischemic cell death and inflammation. Occlusion can be postponed.	Permanent ischemia, multiple vessels occluded, blood flow redistribution, immediate disruption of the blood-brain barrier and vasogenic edema.	(53–55)
Cholesterol clot model	Cholesterol crystal triggers clots formation, appropriate for thrombolytic agent study, low mortality, low invasiveness, highly reproducible.	Requires a high degree of surgical skill, the high variability of infarct size, localized ischemic region.	(56)
Thrombotic microangiopathy			
Acquired TTP model	A simple approach leads to salient features of TTP.	It requires rabbit or mouse antibodies.	(57–61)
Hereditary TTP model	Spontaneous thrombocytopenia	High mortality.	(61)
HIT/T model	Severe thrombocytopenia, allowing pre-clinical studies.	Needs high doses of heparin, Four factors (Heparin, hPF4, FcγRIIA, and anti-heparin/hPF4 antibodies) are present simultaneously.	(62–65)
Disseminated intravascular coagulation (DIC)			
Sepsis-related DIC model	Inducible DIC with multiple organ failure, suitable for candidate drugs testing.	Mice are relatively resistant to endotoxin. Needs more than bolus injection.	(66–74)
CLP-related DIC model	Inducible DIC with multiple organ failure. Technically easy, reproducible and similar to human disease.	High mortality and variability.	(75–79)

IVC, inferior vena cava; TTP, thrombotic thrombocytopenic purpura; HIT/T, heparin-induced thrombocytopenia/thrombosis; CLP, cecal ligation and puncture.

and mortality and has become an attractive drug target for the treatment of these diseases. A variety of reproducible animal models have been developed to investigate the pathomechanisms of arterial thrombosis (**Table 1**).

Photothrombosis Model of Cerebral Stroke

The photothrombotic model uses a photosensitive dye (e.g., Rose Bengal) that after injection responds to illumination across the intact or thinned skull with laser light of a specific wavelength (36, 37). Light exposure induces the formation of oxygen and superoxide radicals damaging surrounding endothelial cell membranes. Endothelial damage promotes local activation and aggregation of platelets. As a consequence, platelet-rich thrombi occluding cerebral microvessels, and causing cortical ischemic infarcts. The photothrombotic stroke model involves the neuroplasticity of perilesional and contralesional brain tissues (38, 39). Modifications of the classical photothrombotic stroke model mimic also a perilesional penumbra. A ring filter model produces a central area of brain injury surrounded by tissue without thrombosis (40), but whether this model sufficiently reflects the penumbra in a human disease context is still under discussion (41). Other modifications include the targeting of individual brain arterioles or implantable optical fibers to produce small subcortical infarcts (42, 43), which surround areas of hypoperfusion with characteristics resembling an inverted penumbra (40). The photothrombotic stroke model allows real-time analysis of many parameters in freely moving rats and mice with acute stroke without the need for anesthesia (44, 45). The activating light can be placed into the specific cortical region of the desired brain area. Using this *in vivo* model, highly reproducible infarct size and low mortality are suitable to study repair mechanisms of the brain and related long-term functional outcomes (46, 47). Since microvascular clots are unusually platelet-rich recombinant tissue plasminogen activator (t-PA) can resolve such clots to a limited extent (42).

Thromboembolic Stroke Model

This model is frequently referred to study human thromboembolic stroke, injecting thrombus-like materials into the cerebral vessels, and the internal carotid artery (48, 49). Depending on the size and amount of the injected material, this model is characterized by leading to one or multiple arterial occlusions followed by ischemic infarcts in the respective territory. Compared to models of middle cerebral artery occlusion (MCAO), cerebral thromboembolism models-induced brain infarcts are surrounded by a well-defined penumbra but infarct sizes are more variable. This model is suitable to study the pathomechanisms of arterial immunothrombosis and the effects of thrombolytic drugs in this process (50, 51). However, the emitting source of the embolus is still not part of this model (52).

Microsphere/Macrosphere Embolic Stroke Model

Embolic stroke can also be induced by injection of synthetic large-sized macrospheres (diameter between 300 and 400 μm) or small-sized microspheres (diameter between 20 and 50 μm) into the cerebral artery. Many different materials, such as silicone, collagen, and titanium dioxide have been used to induce embolic stroke *in vivo* (53). This model has been characterized by permanent ischemia as the fibrinolytic system cannot dissolve such spheres. Microspheres cause multifocal and heterogeneous small infarcts due to microembolization into multiple arteries (54). Unlike thrombus formation, microspheres block blood vessels suddenly, leading to a rapidly developing edema and redistribution of blood flow (55). Although the macrosphere model induces similar infarct development to the ligation models, it does not allow to study the effect of thrombolytic drugs.

Cholesterol Embolism Model

We recently developed a model of cholesterol embolism by injecting cholesterol crystals into the left renal artery of mice (56). According to the size and number of cholesterol crystals, intra-arterial injection leads to multiple microvascular thrombotic occlusions followed by ischemic territorial infarcts. Interestingly, these occlusions are sensitive to thrombolytic therapy preventing tissue infarction and kidney failure. However, it does not appear to be the crystals themselves but rather the blood clots surrounding the crystals that cause vascular obstruction, tissue ischemia, and organ failure (56). As a disadvantage, infarct size is highly variable in this model but the degree of organ failure tightly correlates with the injected crystal dose.

Experimental Models of Thrombotic Microangiopathy

Thrombotic microangiopathies (TMAs) are heterogeneous disorders characterized by thrombocytopenia, microangiopathic hemolytic anemia, renal failure, and neurological symptoms (82). Complex histopathological features have been detected in TMAs, including thrombosis in arterioles and capillaries with abnormalities in the endothelium and vessel wall [**Table 1**; (83)]. TMAs can result from having numerous different pathophysiological mechanisms resulting in a spectrum of distinct but frequently overlapping clinical presentations, as discussed in detail elsewhere (84). An important element is genetic and acquired complement system alterations that either alone or in combination with other triggers cause TMA. Such triggers of uncontrolled complement activation at the endothelial interface include infections, bacterial toxins, certain drugs, and malignancies. Placental as well as maternal factors can trigger TMA during pregnancy that can present with different clinical features referred to by a traditional nomenclature, i.e., (pre-) eclampsia or hemolysis-elevated liver enzymes and low platelet count (HELLP) syndrome. Another entity relates to the von Willebrand factor (vWF) cleaving protease disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13)-induced damages. Given this complexity of disease pathomechanisms, animal models of TMA can mimic only selective scenarios of the broad clinical spectrum of TMA. Some are presented here.

Thrombotic Thrombocytopenic Purpura (TTP) Models

TTP develops from absence or inactivation of the ADAMTS13, leading to the accumulation of vWF multimers and the formation of microvascular thrombi with ischemic end-organ damage (57, 82). Two important mouse models have been developed to study the ADAMTS13 function *in vivo*. The TTP-ADAMTS13 proteolytic activity inhibition model is based on the administration of human anti-ADAMTS13 recombinant single-chain variable region antibody fragments (scFv's), which inhibits the enzymatic activity of ADAMTS13 in mice (58, 59). This *in vivo* treatment leads to persisted ADAMTS13 deficiency for over 2 weeks and the formation of microvascular thrombi (58, 60). Administration of Shiga toxin-2 to these mice results in lethal TMA affecting the brain, heart, and kidney (61). In another mouse model, ADAMTS13-deficient mice are challenged with a second hit to develop TTP, e.g., the infusion of Shiga toxin causes a syndrome closely resembling human TTP with widespread vWF-rich and fibrin-poor hyaline thrombi in the microvasculature of multiple organs (62).

Heparin-Induced Thrombocytopenia (HIT) and Thrombosis

Heparin can trigger an immune-mediated thrombocytopenic disorder characterized by venous and arterial thrombus formation via antibodies against complexes of human platelet factor 4 (PF4) and heparin (63, 64). Heparin-induced thrombocytopenia in mice requires transgenic expression of human PF4 and a lack of the genetic equivalent of human Fc gamma receptor IIA (FcRIIA). As a third requirement, mice are injected with anti-heparin-PF4 immunoglobulin (IgG) and heparin (64, 65, 85). Although this combination of causal factors is not identical to the clinical scenario in patients, the mouse model is suitable to study HIT. Also, in some cases, lethal TTP with disseminated arterial and venous thrombi have been described in mouse models of HIT.

Disseminated Intravascular Coagulation (DIC) Model

Thrombocytopenia is frequently observed in septic patients who have a systemic activation of immunothrombotic mechanisms (66, 86, 87). Several important models have been developed to study the pathology of DIC in mice. In the endotoxemia model,

injection of lipopolysaccharide (LPS), zymosan or *E. coli* bacteria in mice initiates an overwhelming activation of innate immunity and procoagulant pathways that can lead to DIC with multiple organ dysfunction (67–70). Pathophysiological characteristics of this treatment are reduced platelet count, prolonged bleeding time, decreased plasma fibrinogen levels, and increased plasma D-dimer levels (71–73). This model is often used for the testing of drug candidates (74, 75).

Caecal Ligation and Puncture Model

This represents the gold standard for research on polymicrobial sepsis (76, 77). It consists of DIC-like microvascular thrombosis and multiple organ failure representing an irreversible stage of sepsis (78, 79).

Cellular Components and Molecular Mechanisms of Immunothrombosis

Platelets

Studies using mouse models of sepsis revealed the accumulation of platelets in the microvasculature. Indeed, LPS injection resulted in thrombocytopenia in mice, the accumulation of platelets was found in the lung and liver. Several Toll-like receptors (TLRs) were identified in human and mouse platelets to bind a major component of the wall of gram-negative microorganisms (LPS), transmitting signals between platelets and the innate immune system, thereby inducing inflammatory responses. TLR-2 and−4 on human and mouse platelets bind LPS and increase nitric oxide and cyclic guanosine monophosphate (GMP) levels, and activate protein kinase G (88). TLR4 activates the nuclear factor-κB (NF-κB) and the mitogen-activated protein (MAP) kinases increasing interleukin6 (IL-6), cyclooxygenase (COX-2), and prostaglandin E2 (PGE2) production (89). Platelet TLR-1- and−4 are involved in the development of microvascular thrombosis and sepsis-induced intravascular coagulation by triggering platelet degranulation, which releases proinflammatory cytokines from alpha (α)-granules and promotes platelet-neutrophil interaction (**Figure 1**). Several other isoforms of TLRs have been studied in human and mouse platelets, connecting TLR signaling to pathogenesis of virus-induced thrombocytopenia, and intravascular coagulation. Platelet glycoprotein (GP) Ib and αIIbβ3 integrins are involved in this process, together with an extracellular matrix bridge formed by vWF and fibrinogen. It has been shown that collagen-mediated-activation of GPVI signaling in platelets plays an important role in platelet adhesion onto the inflamed endothelium (90). Altogether, these results suggest that platelets can distinguish between cellular immunity and hemostasis using a combination of different platelet TLRs and, depending on the ligand binding of the pathogens, platelet TLRs can transduce effector signals to immune cells.

Endothelial Injury, TF, and Thrombus Formation

In the inflamed vessel wall, endothelial cells start to expose TF and extracellular matrix molecules at the luminal surface, which induces functional crosstalk between platelets, immune cells and activates the coagulation cascade. Platelets and neutrophils are the first blood cells adhering to the luminal endothelial surface of the inflamed vessel wall. Platelet GPIb binds vWF, GPVI binds collagen, laminin, and fibrin (91, 92). Besides these interactions, α2β1 integrin and GPV bind collagen, and α6β1 interacts with laminin during thrombus formation (93, 94). Thrombus growth involves additional platelet recruitment, thereby accelerating the coagulation cascade and the immune response, which stabilizes the growing thrombus on the endothelium surface (**Figure 1**). Thrombin generation amplifies platelet granule secretion priming the innate immune system. Granule-resident factors released by platelets have diverse effects on the innate immune system, including monocyte cell differentiation (95), neutrophil cell migration (96), phagocytosis, and cytokine responses. For example, platelet granules contain second wave mediators [adenosine triphosphate (ATP) and serotonin], plasma factors, TFs, fibrinogen, and (pro)-inflammatory cytokines, and chemokines. Platelet serotonin released from delta (δ)-granules could significantly increase neutrophil accumulation and extravasation during inflammation (97). Platelet inflammatory cytokines interleukin-1 (IL-1), regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES), platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), and epidermal growth factor (EGF) enhance local inflammatory responses and supported by platelet chemokines, such as chemokine (C-C motif) ligand 5 (CCL5), chemokine (C-X-C motif) ligand 4 (CXCL4), and 7 (CXCL7) that activate monocytes and neutrophils. Interestingly, monocytes express TF in certain pathological conditions (98, 99), connecting the innate immune system to the coagulation cascade. In addition, microparticles released by monocytes bind the platelet surface to accumulate TFs and to promote coagulation (100).

Platelet-Immune Cell Interactions

Platelet attachment to the inflamed vessel walls is supported by the interaction between platelets and extracellular matrix components, thereby promoting the interaction of platelets with immune cells and endothelial cells. Indeed, the interaction of surface receptors of activated immune cells and platelets strongly influences innate immune responses. It has been shown that liver-resident macrophages (Kupffer cells) can interact with platelets via platelet GPIb receptor and the exposed vWF on the Kupffer cell surface, e.g., during bacterial infection-induced immunothrombosis (101). Such platelet-immune cell conjugates correlate to the severity of sepsis (102). Although the detected lifetime of this conjugates in the peripheral blood is very short, this interaction activates integrins and induces granule secretion. Platelet-neutrophil adhesion to the endothelium involves the interaction between neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) and αMβ2 integrin to platelet P-selectin and GPIb, respectively. Neutrophil macrophage antigen 1 (Mac-1) also binds platelet GPIb and αIIbβ3 integrin in the presence of fibrinogen, inducing exposure of proinflammatory chemokines CXCL4 and CCL5 (103). P-selectin and PSGL-1 interactions also contribute to the formation of platelet-monocyte conjugates resulting in monocyte activation. Also, monocyte-resident Mac-1 receptor and platelet adhesion receptors GPIb, junction

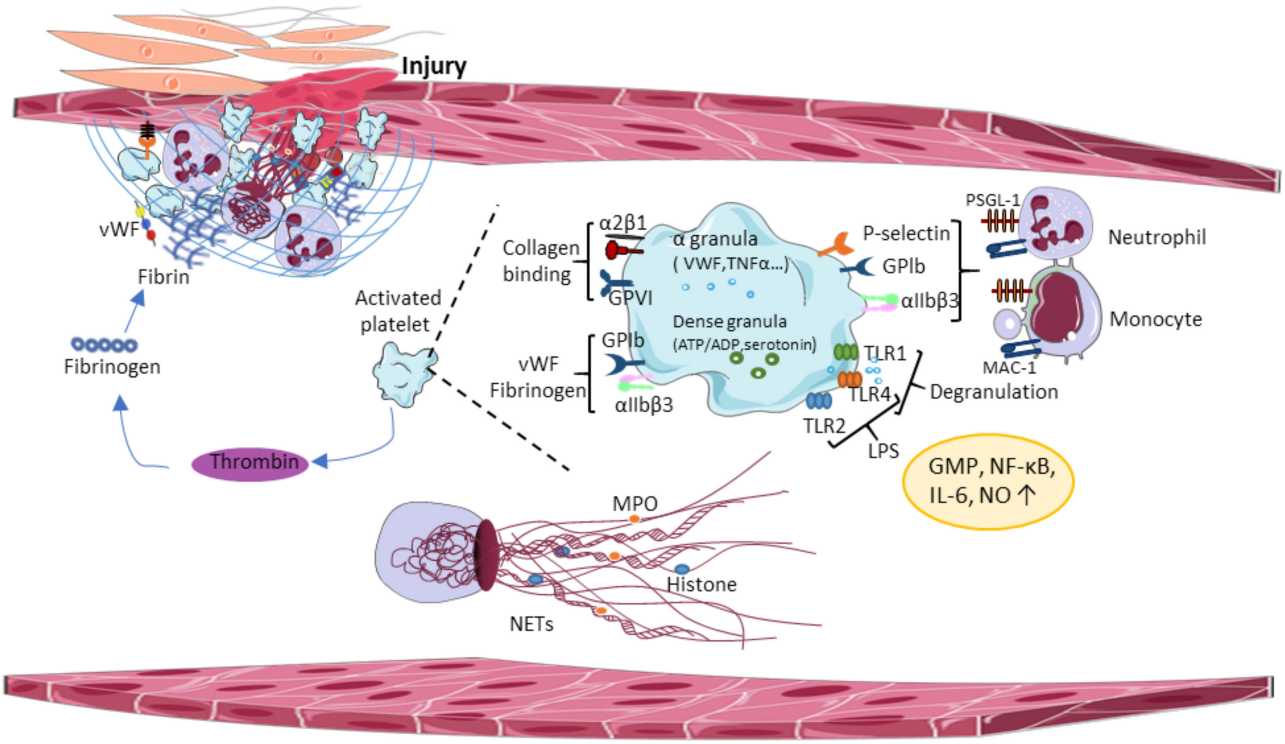

FIGURE 1 | Central paradigms of immunothrombosis. Damaged vessel walls and injured endothelial cells release tissue factor (TF) and extracellular matrix molecules, inducing functional crosstalk between platelets and leading to platelet aggregation. Activated platelets promote thrombin formation thereby enhancing platelet degranulation and fibrin formation. Activated platelets release proinflammatory cytokines from α-granules, which promotes platelet-neutrophil interaction and triggers the release of the NETs. vWF, von Willebrand Factor; GPIb, glycoprotein Ib; GPVI, glycoprotein VI; TLR, toll-like receptor; LPS, lipopolysaccharides; NO, nitric oxide; GMP, guanosine monophosphate; NF-κβ, nuclear factor-κ beta; IL-6, interleukin 6; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; MAC-1, macrophage antigen 1.

adhesion molecule 3 (JAM3), or αIIbβ3 integrin form transient interactions (104).

Platelets and NET Formation

Electron micrographs showed adhesion and aggregation of activated platelet within a fibrous meshwork of NETs (105). In addition, platelet aggregation accurred in a time-dependent manner, and DNase treatment simultaneously cleared NETs and platelets (105). The release of NET into the circulation is followed by platelet adhesion and aggregation, which together with histones released from NETs promote thrombus formation and growth (4). The NET release was observed also in non-infectious inflammatory conditions, such as venous-, microvascular-, and cancer-related thrombosis, acute lung injury, endothelial damage with trauma, autoimmune diseases, preeclampsia, and systemic lupus erythematosus (106). Interestingly, the structure of thrombi in the presence of NETs is more rigid and less permeable. In a mouse model of sepsis, TLR2, and TLR4 on the surface of platelets in liver sinusoids and lung capillaries contribute to platelet-neutrophil interaction and NET formation (107). In addition, synchronized activation of surface integrins and chemokine receptors induce NET formation (108). Thromboxane A2 (TxA2)-released from activated platelets can also amplify NET formation and this process is inhibited by aspirin (109).

Coagulation

The blood coagulation cascade operates in three steps: (i) formation of prothrombin activator, (ii) conversion of prothrombin to thrombin, and (iii) conversion of fibrinogen to fibrin (**Figure 1**). The first step involves the intrinsic coagulation pathways. The intrinsic pathway is activated by exposure of endothelial collagen and the extrinsic pathway is activated through TF released by injured endothelial cells. The following two steps encase platelet aggregates and red blood cells into a fibrin network and attach it to the damaged endothelium. At sites of the damaged vessel wall, platelet activation and degranulation convert inactive IL-1 to the active form by thrombin cleavage, thereby connecting the coagulation system to immunothrombosis. NET release influences the coagulation cascade by activating coagulation factor XII (FXII), inactivating anticoagulant tissue factor pathway inhibitor (TFPI), and by providing an active surface for platelet adhesion and aggregation. All of these mechanisms lead to the inhibition of fibrinolytic activity, thereby promoting thrombus formation and growth.

TABLE 2 | Experimental evidence for the role of extracellular DNA in immunothrombosis.

Model	Type of evidence	References
Venous thrombosis	IVC model, ecDNA were present in thrombosis, DNase degrades ecDNA, breaks down NETs, reduces thrombus size.	(16, 114, 115)
	Acute limb IRI model, DNase I reduced DNA traps, inflammation, Thrombin-Anti-Thrombin-III expression, and enhanced post-ischemic hind limb perfusion.	(116)
	Ex vivo, DNA-histones complexes improved stability and rigidity of thrombus, and DNase promotes clot lysis.	(117)
Arterial thrombosis	Murine models of atherosclerosis, DNase I reduced atherosclerosis burden.	(118)
	Ischemic stroke model, circulating nucleosomes and DNA was increased after ischemic stroke. DNase I reduced infarct size and improved stroke outcome.	(119)
	Cholesterol clot model, ecDNA were presented in crystal clots, DNase prevented clots formation, reduced organ infarction.	(56)
	Thrombi collected from stroke patients, neutrophils were abundant in all thrombi, and NETs contributed to the composition of all thrombi especially in their outer layers.	(120, 121)
Thrombotic microangiopathies syndromes	HIT/T model, thrombi including neutrophils, extracellular DNA. While neutrophil depletion abolishes thrombus formation, DNase treatment limited venous thrombus size.	(122, 123)
	Sepsis-induced DIC in the murine model, ecDNA were presented in thrombus, the blood vessel of lung occluded in DNase deficient mice, DNase treatment prevented NETs clot. Time-dependent increase of cfDNA, later administration of DNase reduced cfDNA, inflammation, and suppressed organ damage.	(124, 125)
	In a murine CLP model, later administration of DNase 4 or 6 h after CLP resulted in reduced cell-free DNA, inflammation, prevented organ damage, and improved survival.	(126)
	In acute TMA patients, levels of DNase activity of plasma showed a significant reduction in compared with healthy controls, plasma-mediated degradation of NETs is reduced in patients with acute TMA.	(127)

IVC, inferior vena cava; ecDNA, extracellular DNA; cfDNA, cell-free DNA; NET, neutrophil extracellular trap; HIT/T, heparin-induced thrombocytopenia/thrombosis; CLP, cecal ligation and puncture.

PRO-THROMBOTIC DANGER-ASSOCIATED MOLECULAR PATTERNS (DAMPs): THE ROLE OF EXTRACELLULAR DNA IN IMMUNOTHROMBOSIS

Among the mediators released from injured cells, extracellular DNA acts as pro-thrombotic DAMP (110–112). Released chromatin forms similar functional structures as the fibrin network to trap red blood cells, platelets, and coagulation factors including TF and fibrin (113). Here, we discuss some experimental pieces of evidence derived from studies on venous, arterial and microvascular thrombosis, and ischemic stroke (**Table 2**).

Contribution of Extracellular DNA to Venous Thrombosis

Several animal models established the role of extracellular DNA in venous thrombosis (**Table 2**). Ligation of the IVC in mice can increase plasma levels of extracellular DNA during several days (114). Immunofluorescence studies revealed colocalization of extracellular DNA with histones and vWF in the thrombus. DNase I administration protected mice from thrombosis at 6 h and 48 h in this model, indicating that the extracellular DNA itself is a critical component of fibrin-rich thrombi. Several experimental studies confirmed the presence of extracellular DNA in venous thrombi induced by the restriction of blood flow (16, 114, 115). In a mouse model of acute limb ischemia-reperfusion injury, DNase I treatment significantly reduced the presence of extracellular DNA traps, immune cell infiltration, thrombin-anti-thrombin-III generation, and enhanced post-ischemic hind limb perfusion. Interestingly, neutrophil depletion resulted only in a small reduction in DNA traps without inducing any skeletal muscle injury, or hind limb perfusion (116). Indeed, *ex vivo* experiments showed that DNA-histone complexes stabilized the fibrin network resulting in a higher rigidity of an artificial thrombus that was resistant to t-PA. In contrast, adding DNase I promoted clot lysis in combination with t-PA (117). Thus, evolving data in a set of different models of venous thrombosis consistently demonstrated a role of extracellular DNA in venous thrombosis.

Contribution of Extracellular DNA to Arterial Thrombosis

Numerous studies on animal models suggested the role of extracellular DNA in arterial thrombosis. In murine models of atherosclerosis, DNase I treatment resulted in a reduced burden of atherosclerosis (118). Recently, we showed that in a murine model of cholesterol embolism, extracellular DNA can be a non-redundant component of crystal clots forming within a few hours upon embolization and vascular occlusion. Similar to the platelet purinergic receptor P2Y12 antagonism with clopidogrel, DNase I treatment significantly reduced the numbers of obstructed arteries, decreased ischemic-related organ failure, and tissue infarction (56). In addition, preincubation of washed platelets with DNAse I inhibited platelet activation, P-selectin exposure,

and aggregation response to a collagen-related peptide (CRP) and thrombin. Furthermore, treatment with DNAse I inhibits ATP release and the formation of a fibrin network.

Contribution of Extracellular DNA in Ischemic Stroke

In stroke patients, extracellular DNA components have been also observed in ischemic brain tissues, possibly contributing to stroke development. In support of this, histological analysis of thrombi collected from stroke patients revealed that a large number of nucleated leukocytes presented in all thrombosis specimens, neutrophils were abundant in all observed thrombi, and NETs were found in all thrombi, in particular in their outer layers (119, 120). In a murine model of transient middle cerebral artery occlusion (tMCAO), increased levels of circulating nucleosomes and DNA were found after ischemic stroke. Under hypoxic conditions, an increased level of extracellular chromatin was detected. Moreover, targeting extracellular chromatin components with DNase I improved stroke outcome (121). Strikingly, adding DNase I to t-PA significantly accelerated the *ex vivo* lysis of thrombi compared to t-PA alone (119, 120).

Contribution of Extracellular DNA to Thrombus Formation in the Microvasculature

Recent experimental evidence suggests that extracellular DNA plays an important role in DIC-related organ dysfunction, probably caused by elevated levels of circulating thrombin, high platelet aggregation, vascular leakage, the release of proinflammatory cytokines, and NET formation (128). In sepsis-induced DIC, large numbers of NETs are accumulated mainly in the microvasculature of the lung and liver (124). Studies using DNase-deficient mice reported that the formation of NET clots associates with TMA and DIC, including schistocytes, hemolytic anemia, and organ failure due to vascular occlusions. Similar observations have been detected in patients with severe bacterial infections (124). Studies using multicolor confocal intravital microscopy studies showed the presence of aggregated platelets and fibrin clots together with extracellular DNA in septic tissues (125). NETosis is regulated by the citrullinating enzyme peptidyl arginine deiminase 4 (PAD4) which induces decondensation of the chromatin through arginine modification of histones. Accordingly, in mouse models of sepsis, deficiency of PAD4, or DNase I treatment significantly inhibited systemic intravascular thrombin activity, reduced platelet aggregation, and improved microvascular perfusion (125). Patients with acute TMA show lower plasma levels of DNase I when compared with healthy controls (127). In a murine caecal ligation and puncture model, a time-dependent increase in cell-free DNA was accompanied by systemic inflammation (126). Interestingly, early administration of DNase I at 2 h after caecal ligation and puncture resulted in a drop in circulating cell-free DNA levels, increased inflammation, and organ damage in the lungs and kidneys. In contrast, later administration of DNase I, 4 or 6 h after caecal ligation and puncture, resulted in less cell-free DNA and inflammation, preventing organ damage and improving survival (126).

In a mouse model of HIT, thrombi are composed of neutrophils, extracellular DNA, citrullinated histone H3, and platelets. Interestingly, neutrophil depletion or Pad4-deficiency abrogates thrombus formation and DNase I treatment reduced the size of venous thrombi (122, 123).

As the studies on animal models supported a therapeutic potential of recombinant DNase I against thrombus formation in different types of vessels, this concept deserves further investigation at the clinical level. Recently, clinical studies suggested that endogenous DNase I activity could represent a therapeutic biomarker during acute myocardial infarction (129). Accordingly, coronary NET burden and endogenous DNase activity are shown as predictors of myocardial infarct size and stenosis resolution (130). Indeed, recombinant DNase I can accelerate t-PA-mediated lysis of human coronary and cerebral thrombi *ex vivo* (119, 120). Patients with acute microvascular thrombosis displayed reduced DNase I activity (127). Timely and efficient removal of extracellular DNA is required to prevent excessive thrombus formation. The restoration of plasma DNase I activity possibly represents a new therapy for thrombotic complications.

Cellular Sources of Extracellular DNA in Immunothrombosis

Extracellular DNA could be released by activated immune cells such as neutrophils and monocytes, by apoptotic platelets or by the damaged vasculature (131–134). Therefore, it is difficult to identify precisely the sources of extracellular DNA that contribute to thrombus formation *in vivo* context. Neutrophils are considered as a major source of extracellular DNA when they release their chromatin as NETs (105, 135). As indicated above, NETs are critical for the development of sepsis-induced intravascular coagulation regardless of the inciting bacterial stimulus (gram-negative, gram-positive, or bacterial products). Indeed, many clinical and experimental studies use extracellular DNA as a marker for NETs in the circulation. NETs and extracellular DNA are present in patients with HIT. In patients with myocardial infarction, blood samples contain DNA, nucleosomes, myeloperoxidase, and neutrophil elastase, and their plasma levels correlated with the burden of NETs, detected within coronary thrombi, as well as with the infarct size (130). In ischemic stroke, thrombi in cerebral arteries stain positive for neutrophils, extracellular DNA, and neutrophil elastase, suggesting NET formation (119).

Extracellular traps are also released from monocytes, referred to as METs. METs have a similar web-like structure comprising DNA, granular enzymes, and citrullinated histones, and procoagulant activity, similar to NETs (132). Besides neutrophils and monocytes, it has been reported that eosinophils also form extracellular traps (134).

Another source of extracellular DNA can be released by necrotic vascular or parenchymal cells. During thrombosis-induced tissue ischemia, the majority of cells die primarily via a process of necrosis, this process releases nuclear DNA into the extracellular space and bloodstream. Injured cardiomyocytes are probably a major source of extracellular DNA in patients

with myocardial infarction (136, 137). Another source is injured endothelial cells at the site of vascular obstruction (56, 138). Finally, activated platelets release DNA from their mitochondria. Although the total amount of mitochondrial DNA per platelet is low, the large numbers of platelets involved in blood clotting also render platelets as a potentially significant source of extracellular DNA (139, 140). Taken together, numerous sources contribute to the pool of extracellular DNA in immunothrombosis.

Therapeutic Potential of Recombinant DNAse I in Immunothrombosis

In a mouse model of sepsis-induced intravascular coagulation, NET release coincided with increased platelet aggregation, thrombin generation, and fibrin clot formation (125). DNase I treatment reduced NET formation and degraded extracellular DNA, which was associated with inhibited platelet aggregation and microvascular obstructions (**Figure 2**). In the LPS-induced sepsis mouse model, NET release and fibrin clot formation were inhibited by the combined treatment of DNase I with the thrombin inhibitor argatroban. However, these treatments did not influence bacterial dissemination (141). In line with this, in septic patients, NETs also significantly increased the generation of thrombin and fibrin clot formation, and this effect was reduced by DNase I treatment (142). Of note, DNase I treatment leads to the release of NET components into the bloodstream, which may elicit procoagulant activity and intravascular thrombosis in septic patients. Free extracellular DNA fragments enhance the intrinsic coagulation pathway (143), which is also observed in patients with deep vein thrombosis (144), leading to tissue hypoxia and endothelial damage.

It is known that DNA intercalates with fibrin to form a scaffold that stabilizes clot structure in the bloodstream (4), therefore DNA-fibrin complexes have a fundamental effect on clots lysis. In plasma of septic patients, extracellular DNA significantly delayed t-PA-mediated clot lysis times by forming DNA-plasmin-fibrin ternary complex which results in a densely packed clot structure (145). Elevated levels of extracellular DNA in plasma from septic patients promoted thrombin generation (146). DNA alone or NETs inhibited plasminogen activation and t-PA-induced resolution of plasma clots (147). In a murine model of HIT, PF4 combines with NET-forming complexes that selectively bind HIT-induced antibodies, DNase I treatment limited venous thrombus size (148). Extracellular DNA markedly affects the hemostatic system by activating factor XI (FXI) and factor XII (FXII) (149). Extracellular DNA present in the fibrin clot inhibits the antithrombotic activities of anticoagulants, such as unfractionated heparin and enoxaparin (150). In contrast, RE31 DNA aptamers inhibit thrombin formation, accelerates fibrinolysis *in vitro*, and suppress thrombosis *in vivo* (151, 152).

In cystic fibrosis the lung is frequently affected by recurrent bacterial infections and chronic inflammation causing progressive lung destruction; the development of thick mucus in small bronchioles and peribronchial regions of the lung thereby triggering permanent bacterial infection. Infiltrated neutrophils release granular content to eliminate the pathogens, and also release high concentrations of extracellular DNA, forming NETs in the inflamed bronchioles, which contribute to airway damage, aggravating mucus viscosity, and its mucociliary clearance from the bronchioles. Blood samples from patients with cystic fibrosis showed an increased number of activated platelets (153), which form cell conjugates with monocytes and neutrophils (153, 154). Increased platelet aggregation responses to adenosine diphosphate (ADP) and thrombin receptor-activating peptide (TRAP), and second-wave mediators (TxA2, ATP, serotonin), and α-granule-resident proteins [tumor necrosis factor alpha (TNFα), CD40 ligand (CD40L), leukotriene B4 (LTB4), and interleukins] were also detected (155, 156). Plasma levels of platelet granule-resident proteins are correlated with a decreased lung function of these patients (157, 158). DNase I treatment showed significant improvement in rheological parameters in cystic fibrosis, reducing the thick mucus layer by cleaving the extracellular DNA of NETs. Therefore, patients can release more easily the accumulated mucus up from the inflamed lung tissue.

The literature also describes that following bacteremia, neutrophils recruited to the liver sinusoids enhance the clearance of pathogens from the circulation (107, 159). Similar to the phenomenon observed in septic lung tissues, and in liver sinusoids, neutrophils also release intravascular NETs (69). Blocking NET formation by DNase I reduced the capture of circulating bacteria in the liver, resulting in increased dissemination of bacteria to distant organs.

NET formation was also detected in acute ischemic stroke, located in the outer layer of developing thrombi, and consequently, the increase of extracellular DNA content in the blood plasma correlates with stroke severity. Although thrombolysis with t-PA administration promotes fibrin degradation in the occluded vessel of acute ischemic stroke, t-PA-resistant clot formation has been frequently observed in platelet-rich arterial thrombi. Hence, fibrin accumulation in the growing thrombi is limited at the early phase of acute ischemic stroke (160, 161). Interestingly, the co-administration of DNase I with t-PA accelerated thrombolysis *ex vivo*. However, DNase I treatment alone had no thrombolytic effect *ex vivo*. These results suggest that both fibrin and NET formations can be targeted simultaneously to induce successful thrombolysis and recanalization of the artery in acute ischemic stroke (120). In line with these results, combined treatment of DNase I with t-PA also attenuated infarct size in a murine model of myocardial ischemia-reperfusion injury. Again, DNase I or t-PA treatment alone had no beneficial effects in this mouse model (162). Altogether, these results suggest that DNase I and t-PA treatment together improve both myocardial and cerebral post-ischemic infarction. However, a clinically implemented and safe pharmacological strategy of DNase I treatment is currently established in patients with cystic fibrosis (163) and limited clinical trials investigated the thrombolytic effects of NET degradation in other disease conditions. Altogether, these data suggest that in some cases, DNA-targeted therapies by DNase I may improve thrombolysis and inhibit coagulation. Therefore, further investigation is necessary to establish the role of DNase I treatment in immunothrombosis.

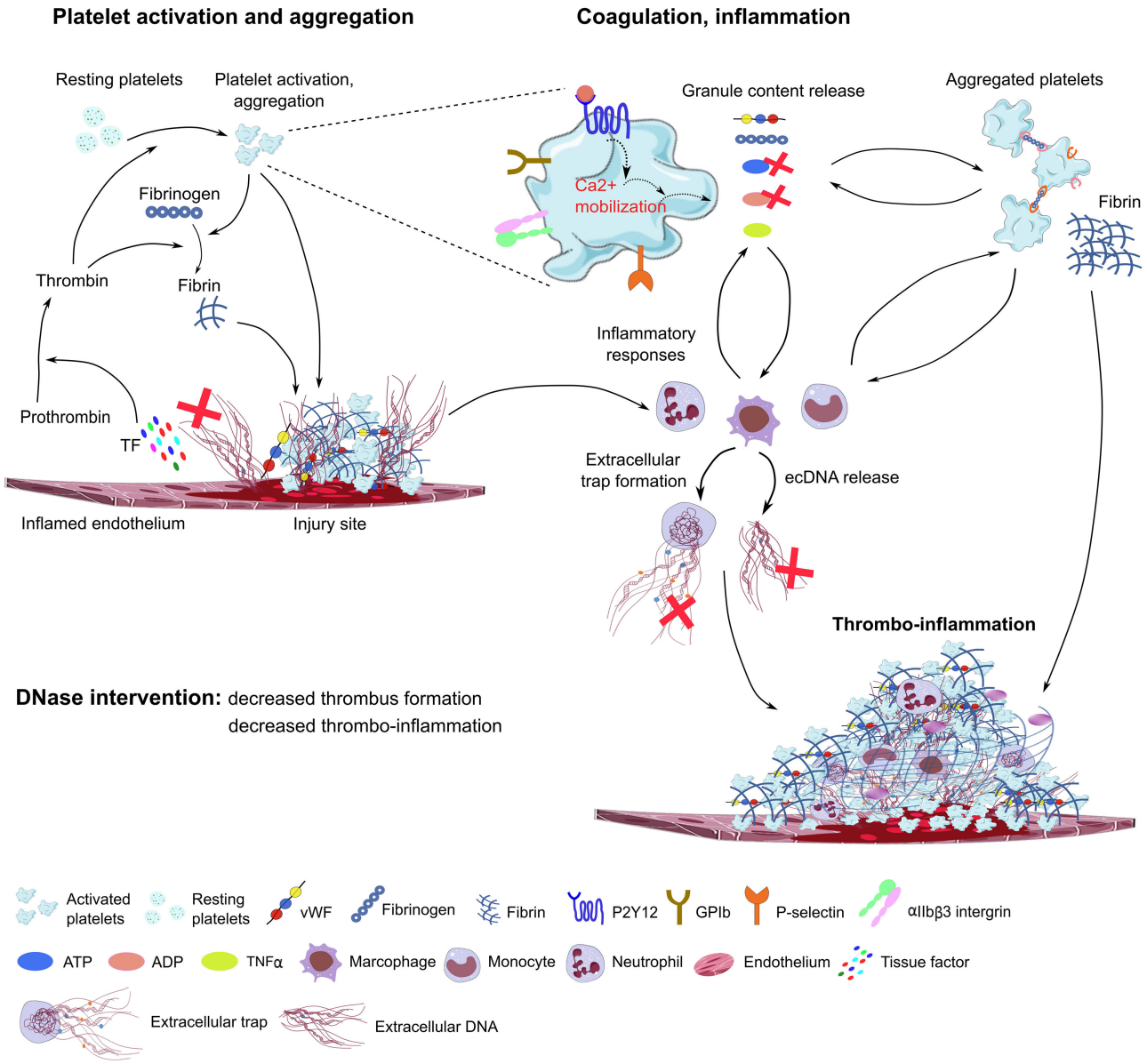

FIGURE 2 | Proposed model of DNase function in immunothrombosis. Damaged endothelial cells release tissue factor (TF) and ecDNA. TF activates the coagulation cascade, converting prothrombin to thrombin, which further activates platelets through PAR receptors. The ecDNA acts as DAMP and directly activates platelets and triggers inflammatory responses. The damaged endothelial layer exposes extracellular matrix proteins (collagen, laminin), and accumulates vWF, fibrinogen and other blood plasma proteins on the endothelial surface, further supporting platelet adhesion and activation through platelet specific glycoprotein receptors (GPIb, GPVI) and integrins (αIIbβ3, α2β1). During degranulation, second wave mediators (ATP, ADP, serotonin), extracellular matrix components (vWF, fibrinogen), and inflammatory cytokines are released by activated platelets, triggering thrombus formation and enhancing immune cell responses and NET formation. Platelet purinergic receptors (P2Y1, P2Y12) are activated by ADP, further promoting platelet aggregation and thrombus growth. P-selectin exposure on the plasma membrane of activated platelets increases procoagulant activity and supports platelet-immune cell interaction and NET formation. In the process of immunothrombosis, DNase could inhibit NETosis by fragmenting DNA within the NETs, thereby dissociating platelet-rich components from the endothelial surface, and inhibiting thrombus growth. DNAse may also inhibit purinergic signals in platelets and immune cells. TF, tissue factor; ec-DNA, extracellular deoxyribonucleic acid; vWF, von Willebrand Factor; ADP, adenosine diphosphate; ATP, adenosine triphosphate; TNFα, tumor necrosis factor alpha (TNFα); GPIb, glycoprotein Ib; GPVI, glycoprotein VI, DNase, deoxyribonuclease; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

CONCLUSIONS AND FUTURE PERSPECTIVES

Immunothrombosis is a complex process involving numerous elements of the cascades of coagulation and inflammation. *In vivo* preemptive administration of recombinant DNase I not only cleaves deposits of extracellular DNA but also inhibits ATP release from platelet δ-granules and prevents the formation of fibrin network. Extracellular DNA may directly induce fibrin formation, thereby enhancing thrombus growth. Studies

analyzing the role of extracellular DNA in immunothrombosis related to either the use of DNase-deficient mice or the recombinant DNase I. It worth to postulate that DNAse I treatment may limit thrombus formation by inhibiting the function of platelet-derived second wave mediators, such ATP.

Several questions remain unanswered: What is the main source of extracellular DNA during the early phase of blood clotting *in vivo*? How does the extracellular DNA released from infarcted tissues may contribute to the clot formation and the resistance to the fibrinolysis? Is extracellular DNA a suitable therapeutic target in humans beyond the anticoagulants or fibrinolytic drugs? Does recombinant DNase I have a better safety profile compared to the anticoagulants? A better understanding of the role of extracellular DNA in a immunothrombosis context is required to clarify these issues.

AUTHOR CONTRIBUTIONS

CS, H-JA, and AB wrote the manuscript and prepared tables. LY and CS prepared the figures. H-JA and AB revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (AN372/16-2, 20-2, 24-1) to H-JA and 374031971-TRR 240/A09 to AB. CS and LY were recipients of a stipendium from the Chinese Scholarship Council.

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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.

frontiers in Immunology

REVIEW
published: 16 November 2020
doi: 10.3389/fimmu.2020.601740

Extracellular Vesicles and Damage-Associated Molecular Patterns: A Pandora's Box in Health and Disease

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *01 September 2020*
Accepted: *21 October 2020*
Published: *16 November 2020*

Citation:
Picca A, Guerra F, Calvani R, Coelho-Júnior HJ, Landi F, Bernabei R, Romano R, Bucci C and Marzetti E (2020) Extracellular Vesicles and Damage-Associated Molecular Patterns: A Pandora's Box in Health and Disease. Front. Immunol. 11:601740. doi: 10.3389/fimmu.2020.601740

Sterile inflammation develops as part of an innate immunity response to molecules released upon tissue injury and collectively indicated as damage-associated molecular patterns (DAMPs). While coordinating the clearance of potential harmful stimuli, promotion of tissue repair, and restoration of tissue homeostasis, a hyper-activation of such an inflammatory response may be detrimental. The complex regulatory pathways modulating DAMPs generation and trafficking are actively investigated for their potential to provide relevant insights into physiological and pathological conditions. Abnormal circulating extracellular vesicles (EVs) stemming from altered endosomal-lysosomal system have also been reported in several age-related conditions, including cancer and neurodegeneration, and indicated as a promising route for therapeutic purposes. Along this pathway, mitochondria may dispose altered components to preserve organelle homeostasis. However, whether a common thread exists between DAMPs and EVs generation is yet to be clarified. A deeper understanding of the highly complex, dynamic, and variable intracellular and extracellular trafficking of DAMPs and EVs, including those of mitochondrial origin, is needed to unveil relevant pathogenic pathways and novel targets for drug development. Herein, we describe the mechanisms of generation of EVs and mitochondrial-derived vesicles along the endocytic pathway and discuss the involvement of the endosomal-lysosomal in cancer and neurodegeneration (i.e., Alzheimer's and Parkinson's disease).

Keywords: Alzheimer's disease, damage-associated molecular patterns, endo-lysosomal system, inflammation, innate immunity, mitochondrial-derived vesicles, Parkinson's disease, quality control system

INTRODUCTION

Inflammation is part of the innate immunity response to pathogens or molecules released upon tissue injury, collectively indicated as damage-associated molecular patterns (DAMPs) (1). This non-specific first line of organismal defense is mounted upon binding of DAMPs to a set of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and inflammasomes that sense

DAMPs and elaborate an immune response (2, 3). Albeit DAMPs-triggered inflammation is protective towards harmful stimuli *via* the coordination of their clearance, promotion of tissue repair, and restoration of tissue homeostasis, an excessive inflammatory response in the setting of persistent stimuli may be detrimental. Indeed, if dysregulated or not timely resolved, inflammation contributes to the development of several disease conditions (e.g., autoimmune diseases, cardiovascular disease, neurodegeneration, and cancer) (4, 5). Hence, a hyper-resolution response aimed at limiting hyper-inflammation and triggered by DAMPs-activated/initialized innate immune cells is in place (6). This pro-resolving pathway is possibly mediated by suppressing/inhibiting inducible DAMPs (SAMPs) (6).

A large deal of research has been devoted to understanding the complex regulatory pathways involved in DAMPs production and trafficking. The endo-lysosomal system that includes a set of dynamic and inter-convertible intracellular compartments such as early-, recycling-, and late endosomes, and lysosomes is a major component of such response. Along with this, autophagosomes are autophagy executors that deliver intracellular contents to lysosomes (7). The fusion of endosomes and/or autophagosomes with lysosomes installs an acidic environment and enables cargo degradation for recycling unnecessary components into re-usable biological building blocks (e.g., carbohydrates, proteins, lipids, and nucleotides) within the cell (7). These events are accomplished *via* vesicle trafficking, protein sorting, and selective cargo degradation. In particular, two opposite sorting systems are in place: the endosomal sorting complex required for transport (ESCRT) that supports cargoes degradation and the retromer complex that allows specific retrograde cargo retrieval (7).

Mitochondria are highly interconnected organelles that form a dynamic network by contacting the endoplasmic reticulum (ER), lysosomes, and the actin cytoskeleton (8, 9). While inter-mitochondrial junctions allow mitochondrial membrane cristae remodeling between adjacent mitochondria (10), mitochondrial fusion enables the mixing of matrix and intermembrane space contents (11). Recently, an additional mechanism of mitochondrial interconnection based on tube-like protrusions (mitochondrial nanotunnels) has been described (9). Mitochondrial nanotunnels may be especially relevant in establishing connections between organelles immobilized within post-mitotic tissues (e.g., skeletal muscle, myocardium), in which fusion events are limited (9). Finally, Golgi-derived vesicles contribute to the maintenance of mitochondrial homeostasis through participating in mitochondrial dynamics (12).

Altered regulation of the endosomal-lysosomal system has been implicated in several age-related conditions, including cancer and neurodegeneration, and might therefore be targeted for therapeutic purposes (13). Remarkably, small extracellular vesicles (sEVs) isolated from primary fibroblasts of young humans have shown to ameliorate senescence biomarkers in cells obtained from old donors (14). A major task of the endosomal-lysosomal system is the disposal of dysfunctional, but not severely damaged mitochondria *via* a housekeeping process of mitochondrial quality control (MQC) (15). Herein, we provide an overview on vesicle trafficking along the endocytic pathway, the generation of exosome and mitochondrial-derived vesicles (MDVs), and discuss the involvement of the endosomal-lysosomal system in physiological and pathological conditions, including cancer and neurodegeneration [i.e., Alzheimer's (AD) and Parkinson's disease (PD)].

GENESIS OF ENDO-LYSOSOMAL VESICLES

Exosomes are EVs of endosomal origin with a diameter of 50-150 nm. The biogenesis of exosomes is associated with the generation and fate of multivesicular bodies (MVBs) (16). These organelles owe their name to the accumulation of intraluminal vesicles (ILVs) after inward budding of plasma membrane microdomains, fission, and release (16). ILVs have a small diameter (50-150 nm) and are identified as exosome precursors. As part of the endocytic trafficking, endosomal organelles undergo maturation and MVBs, moving from cell's periphery to the center along microtubules, mature in late endosomes. For this reason, MVBs are considered to be newborn late endosomes derived from the maturation of early endosomes. However, according to an alternative model, MVBs are identified as intermediate transporters between early and late endosomes (17). Realistically, MVBs can follow two alternative directions: 1) toward fusion with other MVBs or late endosomes to undergo maturation and acidification, thus becoming lysosomes for cargo degradation or 2) toward the plasma membrane to fuse and release into the extracellular space ILVs, such as exosomes (16) (**Figure 1**).

Hence, the biogenesis of MVBs and exosomes is closely related. There are two different mechanisms that guide the origin of MVBs. In fact, they can originate *via* the sequential action of ESCRT or from endosomes containing lipid rafts (16, 18). The ESCRT system consists of five cytosolic complexes [i.e., ESCRT 0, I, II, III, and vacuolar protein sorting (VPS) 24] (19) and its role in exosome biogenesis has been proven by the identification of several ESCRT proteins in exosomes purified from different cell culture types or biological fluids. For this reason, ESCRT proteins are now used as exosomal markers (20).

ESCRT 0 recognizes a specific group of ubiquinated proteins on early endosomes referred to as phosphatidyl inositol monophosphate (PI3P) enriched domains. The recognition of ubiquitin and PI3P areas occurs through the interaction with the two subunits of the ESCRT 0 complex: HRS (hepatocyte growth factor regulated tyrosine kinase substrate) and STAM1/2 (signal transducing adaptor molecule 1/2). This is the first transition step from early endosomes to MVBs followed by the HRS-mediated recruitment of ESCTR I to endosomes (17, 21–23). ESCRT I in mammalian cells is a heterodimeric complex composed by tumor susceptibility gene 101 (TSG101), VPS28, VPS37A-D, and the ortholog of the yeast Mvb12 (22). In this step, the formation of stable vacuolar domains starts through TSG101 action carrying on the maturation of early endosome into MVBs (24). ESCRT I takes the place of ESCRT 0 and recruits

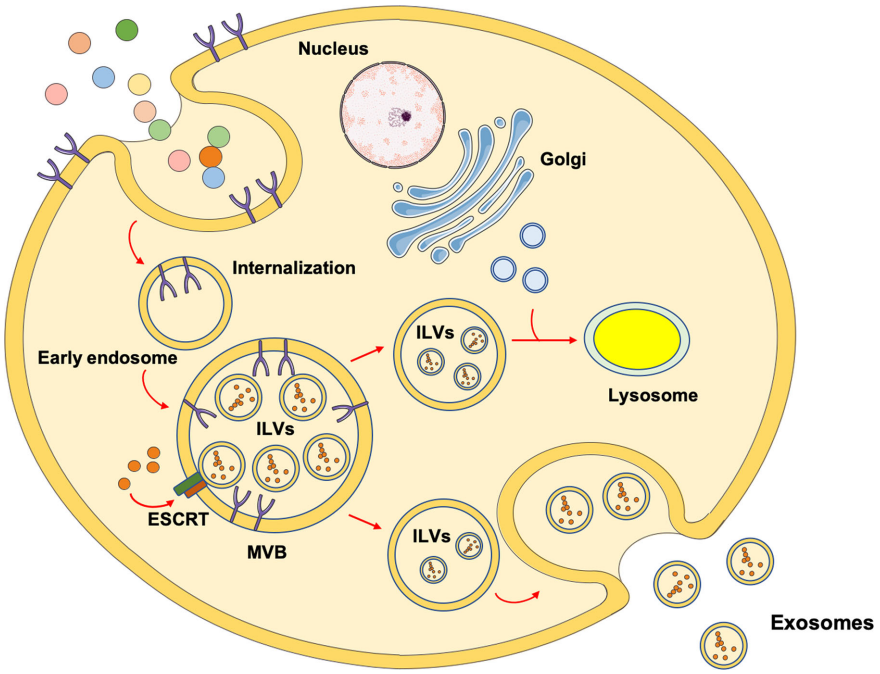

FIGURE 1 | Schematic representation of the mechanisms involved in exosomes biogenesis. The most investigated mechanism through which exosomes are generated involves endocytosis after receptor/ligand binding at the cell's membrane. After the ligand dissociates from its receptor, it is located into an early endosome. The receptor can either be recycled and relocated on the membrane surface or degraded into lysosomes. Through the activity of the endosomal sorting complex request for transport (ESCRT), the early endosome maturates into multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). Eventually, MVBs migrate toward the plasma membrane and fuse to release ILVs as exosomes. As an alternative route, MVBs can fuse with other MVBs or late endosomes and receive vesicles containing lysosomal enzymes from trans Golgi, evolving into lysosomes for degradative purposes.

ESCRT II that, in mammalian cells, is composed of ELL-associated protein of 30 kDa (EAP) 30, EAP20, and EAP45 (22). ESCRT III is a heterotetrameric complex composed by VPS20-CHromatin-Modifying Protein (CHMP) 6, Sucrose Non-Fermenting protein (SNF) 7-CHMP4, VPS24-CHMP3, and VPS2-CHMP2 subunits. ESCRT II recruits ESCRT III through the interaction between EAP20 and CHMP6, while CHMP-6 has been shown to regulate cargo sorting (25). ESCRT III has the role of recruiting deubiquitinating enzymes to remove ubiquitin residues from the protein with consequent complete invagination of the membrane and generation of ILVs. This is the last crucial step for the entry of cargoes into ILVs (22, 26). ESCRT III recruits accessory subunits, such as BRO1/ALIX (BCK1-like resistance to osmotic shock protein-1/apoptosis linked gene 2 interacting protein X) for cargo deubiquitination (27), and could also play a role in the fusion of MVBs with late endosomes (26, 28, 29). Finally, the interaction between ESCRT III and VPS4 allows the VPS4 ATPase activity to determine the final membrane budding, scission, and detachment of ESCRT subunit for recycling and cargo delivery (22). Thus, the whole process of ILV budding, cargo selection, membrane remodeling, and the incorporation of ILVs into MVBs is regulated by the ESCRT complex. However, only a few ESCRT components are necessary in this process, including HRS, TSG101, and STAM1 (ESCRT 0/I) (30). Indeed, the silencing of these proteins induces a decrease in exosome secretion, while an increase of exosome release is observed by inhibiting CHMP4C, VPS4B, VTA1 and ALIX (ESCRT III complex) (30). ALIX also interacts with several ESCRT proteins (e.g., TSG101 and CHMP4) and is involved in regulating protein composition/cargo loading, budding of ILVs, and MVB incorporation (31). Recent studies have also indicated that ALIX is crucial for the connection between syndecans and the ESCRT machinery through the binding of syntenins. Syntenins are soluble proteins acting as intracellular adaptors, *via* their PDZ domains that recruit syndecans. These latter are membrane proteins carrying heparan sulfate chains (HS) that are necessary to bind adhesion molecules and growth factors allowing them to interact with their receptors and assist in the endocytic process. This heterotrimeric complex is involved in endosomal budding and exosomes biogenesis (31, 32).

The exosome biogenesis can also follow an ESCRT-independent pathway. Indeed, even in the setting of simultaneous depletion of core ESCRT proteins, MVB and exosome biogenesis can still ensue *via* specific membrane lipid composition. Endosomes, which have domains enriched in cholesterol and sphingolipids, named lipid rafts, are able to curve inward and determine the formation of MVBs with the support of the pH gradient across the membrane (33). In this case, phospholipases mediate the synthesis of ceramides from sphingolipids and assure endosome membrane invaginations without ESCRT assistance. In fact, cone-shaped structures of ceramides, alone or associated with cholesterol, generate areas

suitable for membrane deformation and ILV budding (34). The conversion of sphingomyelin in ceramide is catalyzed by neutral sphingomyelinases (SMases) which are enzymes located in the Golgi but also in the plasma membrane favoring exosomal biogenesis. Indeed, the inhibition of SMases reduces exosome secretion in specific cell types (35).

Originally identified in B lymphocytes and implicated in several cellular processes like cell fusion, cell migration and cell adhesion, the three tetraspanins CD9, CD81, and C63 are acknowledged as exosomal markers for their abundance in exosomes (36). These proteins generate the TEM domain (tetraspanin-enriched domain) and are composed by four transmembrane domains that interact with several other proteins, cholesterol, and gangliosides. Cargo sorting and formation of ILVs are mediated by the tetraspanins. Indeed, CD9 cooperates in the fusion of plasma membrane, while CD63 interacts with the PDZ syntenin domain (37).

The mechanisms through which MVBs move towards the plasma membrane for the release of exosomes instead of their fusing with lysosomes are presently unclear. Nevertheless, during the fusion of MVBs with the plasma membrane, the interaction between specific proteins and lipids determines exosome secretion, a process involving SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) proteins and small GTPases (38). Indeed, exosomes secretion is inhibited by overexpression of R-SNARE VAMP7 (vesicle-associated membrane protein 7), which induces enlargement of MVBs and their clustering at the cell's periphery (39). The transport of MVBs towards the plasma membrane is regulated by microtubules and microfilaments such that the modulation of the expression of cortactin induces changes in the release of exosomes (40). Moreover, members of the Ras-related in brain (RAB) protein family, known for their role in endosomal trafficking, are also involved in exosome biogenesis and release. In this regard, several studies have shown a pivotal role for RAB27 and RAB35 in the docking of MVBs at the plasma membrane (41–43), while the silencing of RAB7A, the master regulator of late endocytic pathway, decreases syntenin-mediated exosome secretion (31, 44) or increases the release of CD9- and CD81-positive exosomes in cisplatin resistant cancer cells (45, 46).

DAMPs of different nature can be shuttled *via* EVs. Of note, mitochondria can also exploit this pathway for preserving organelle homeostasis. The mechanisms assisting in the generation of EVs from mitochondria are discussed in the next paragraph.

Mitochondrial-Derived Vesicles

MDVs are generated by the selective incorporation of protein cargoes, including outer and inner membrane constituents, and matrix content. These vesicles have a uniform size (from 70 to 150 nm) and can follow two distinct fates: 1) they can fuse with MVBs and/or late endosomes for degradation (47) or extracellular secretion (13, 48); 2) they can be delivered to a subpopulation of peroxisomes (49).

Upon mitochondrial stress and isolation of mitochondria *in vitro*, it is possible to observe the formation of MDVs enriched in oxidized protein (50), revealing a mitochondrial stress-dependent selective cargo incorporation. An elegant work by Soubannier et al. (50) showed that MDVs carrying the outer membrane pore protein voltage-dependent anion channel (VDAC) are generated after the production of xanthine oxidase/xanthine-induced reactive oxygen species (ROS), while generation of ROS upon treatment with the complex III inhibitor antimycin A determines MDV formation without enrichment in VDAC, thus suggesting that MDVs can transport any oxidized cargo.

The protein kinase phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and the cytosolic ubiquitin E3 ligase Parkin are required for the generation of MDVs targeted to the endocytic pathway and, finally, to the lysosomes (51). Both mutated in familial forms of Parkinson's disease (52, 53), PINK1 and Parkin are known relevant factors in MQC and inducers of the mitophagic pathway. PINK1 is targeted to mitochondria but is normally degraded very rapidly (54–56). Indeed, during the import process at the site of mitochondria, a set of matrix processing peptidases and presenilins-associated rhomboid-like protein (PARL) cleave PINK1, thereby allowing its release from the mitochondrial import channel and subsequent cytosolic proteolytic degradation (56). However, in the setting of damaged mitochondria, the import machinery is inactivated thus determining the trapping of PINK1 within or near the import channel at the mitochondrial outer membrane (55). Here, PINK1, by exposing its kinase domain to the cytosol, induces Parkin phosphorylation. As a consequence, a stable recruitment of Parkin at the mitochondria and a Parkin-dependent ubiquitination of several proteins at the mitochondrial surface occur (57). Finally, a set of autophagic adaptor proteins recognize mitochondrial Parkin-ubiquitinated proteins and deliver damaged organelles to the autophagosome for subsequent disposal (57).

Sugiura et al. (58) proposed a model in which they predicted a similar mechanism in PINK1- and Parkin-mediated MDV transport. The authors hypothesized that a local mitochondrial oxidative damage or complex assembly defects may induce protein aggregation at the mitochondrial import site that may clog the import process into the organelle. Along with this, the oxidation of phosphatidic acid and cardiolipin alters the membrane curvature which may support an early outward bending of the mitochondrial membrane, thus forming MDVs (59). Hence, a dual role for MDVs generation can be envisioned. On the one hand, MDVs can be considered as the first step of MQC, accomplished through the extrusion of damaged proteins as an attempt to avoid complete mitochondrial dysfunction. This would occur in the setting of mildly damaged organelles in which the autophagic pathway is not triggered (47, 51). On the other hand, severe mitochondrial dysfunction and uncoupling could induce a switch from local displacement of mitochondrial content to a complete arrest of PINK1 in all import channels, followed by the recruitment of autophagic mediators and degradation of the whole organelle (**Figure 2**).

Such a view supports the hypothesis of including the delivery of MDVs to lysosomes among MQC mechanisms. Indeed, cells perform MQCs *via* four different mechanisms: 1) degradation of unfolded and oxidized proteins within the mitochondrial matrix or

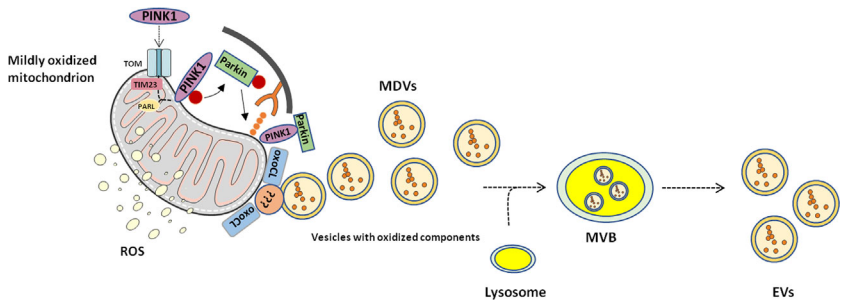

FIGURE 2 | Proposed mechanism of mitochondrial-derived vesicle generation and release. Mitochondrial-derived vesicles (MDVs) may represent an additional level of mitochondrial quality control through which mildly damaged mitochondria are targeted and displaced. Phosphatase and tensin homolog-induced kinase 1 (PINK1) and Parkin prime damaged mitochondria for disposal. Membrane curvatures generated by oxidized cardiolipin (oxoCL) and other unknown proteins allow generation of MDVs that form multivesicular bodies (MVBs) within the endolysosomal system. Eventually, MVBs are extruded from the cell as extracellular vesicles (EVs). PARL, presenilin-associated rhomboid-like; ROS, reactive oxygen species; TIM23, translocase of inner mitochondrial membrane 23; TOM, translocase of the outer mitochondrial membrane.

intermembrane space by mitochondrial protease (60); 2) ubiquitination and delivery of mitochondrial outer membrane proteins to the cytosolic proteasome (61, 62); 3) activation of mitophagy to remove severely damaged mitochondria, whether linked to global protein misfolding or depolarization (63), and 4) generation and delivery of MDVs to lysosomes to protect the cell from premature mitophagy by removal of PINK1 and Parkin from each failing import channel.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS AND STERILE INFLAMMATION

Chronic sterile inflammation ensues in several pathological conditions for which a common thread may reside into dysregulated EV trafficking. Therefore, a deeper understanding of the pathways generating EVs and triggering innate immunity may help clarify the events linking cellular dyshomeostasis with peripheral changes. The generation of MDVs orchestrated by mitochondrial-lysosomal crosstalk (64) is a strong candidate mechanism linking the two processes. Indeed, while operating as an housekeeping system in healthy mitochondria (16), in the setting of failing mitochondrial fidelity pathways, the clearance of dysfunctional organelles *via* MDVs may release noxious material with the potential of triggering inflammation (64). This response, mediated by the release of interferons (IFNs), pro-inflammatory cytokines, and chemokines, is part of innate immunity and starts with the recognition of an infectious agent (either viral or bacterial) that binds and activates membrane or cytoplasmic immune sentinel molecules termed PRRs [reviewed in (65)]. Of these, membrane-bound TLRs and the cytosolic retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs, RIG-I, and MDA5) are the best characterized in the setting of viral infections (66). Upon detection of double-stranded RNA produced during viral genome replication (67), TLR3 located in the endolysosomal compartment signals the binding *via* Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF) and activates the IκB kinase (IKK) complex and the IKK-related kinases TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε. As a result of this activation, the translocation of nuclear factor-kappa B (NF-κB) and IFN-regulatory factors (IRFs) to the nucleus and their activation occur, thus inducing the production of type I and III IFNs together with a set of inflammatory chemokines including the regulated on activation normal T cell expressed and secreted (RANTES), and IFN-γ-inducible protein 10 (IP-10) (68–70). Viral RNAs can also be sensed in the cytoplasm by the RLRs, which signal *via* the mitochondrial antiviral signaling protein (MAVS) adaptor located at the mitochondrial outer membrane. Following the RLR-MAVS pathway, the activation of IKK and IKK-related kinases and, subsequently, NF-κB and IRFs occurs (71–73). Once induced, IFNs upregulate the expression of hundreds of IFN-stimulated genes (ISGs), ultimately installing an antiviral response that halts viral replication and spread (74). Along shared pathways, mitochondrial DAMPs can also trigger inflammation. In particular, mitochondrial DNA (mtDNA), due to its bacterial ancestry and its hypomethylated CpG motifs, is a potent trigger of innate immunity response involving the release of pro-inflammatory mediators installing an inflammatory milieu (75, 76). Indeed, mtDNA can interact with PRRs including TLRs, but also NOD-like receptors (NLRPs), and the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS–STING) systems (77, 78). The TLR pathway is engaged by mtDNA *via* its binding to TLR9 at the endolysosomal level, followed by the recruitment of the innate immune signal transduction adaptor myeloid differentiation primary response 88 (MyD88). The latter, by activating the mitogen-activated protein kinase, triggers inflammation *via* NF-κB signaling (79–81). Alternatively, mtDNA can ignite inflammation as part of the innate immunity response either *via* inflammasome or cGAS–STING system activation at the cytosolic level (82–86). The cGAS–STING DNA-sensing pathway operates *via* the TBK1/IRFs/IFNs pathways described above as part of inflammation mounted in the presence of viral infections (84–86). The activation of the STING pathway is also triggered as part of

neutrophil activation and neutrophil extracellular trap (NET) formation, a specific cell death route characterized by the extrusion of chromatin-bound cytosolic content (87). NETs have been implicated in the pathogenesis of autoimmune disorders. In particular, NETs enriched in oxidized mtDNA stimulate a type I IFN response and have been implicated in lupus-like diseases (88). In systemic lupus erythematosus, mtDNA binding to the histone-like protein mitochondrial transcription factor A (TFAM) has shown to assist in rerouting oxidized mtDNA of neutrophils to lysosomes for degradation (89). Once extruded, TFAM-oxidized mtDNA complexes are powerful immune system activators (89). Similarly, the release of activated platelet-derived microparticles enriched with high-mobility group box 1 (HMGB1) protein has been described in systemic sclerosis (90). This DAMP molecule might contribute to vasculopathy and tissue fibrosis possibly *via* the presentation of HMGB1 to neutrophils to induce their activation and consequent endothelial damage (90).

Finally, the engagement of NLRP3, the best studied multi-subunit inflammasome system, elicits caspase-1 signaling and promotes caspase-1-dependent cleavage and activation of interleukin (IL) 1 and 18 *via* binding to adaptor molecules (91). This route of inflammation is particularly relevant to mitochondrial dysfunction since the synergistic activation of redox-sensitive inflammation and inflammasome reinforce inflammation (92). The molecular triggers of the inflammatory response *via* inflammasome are unclear. However, bacterial-like motifs of mtDNA are sensed by NLRs (93). Furthermore, NLRP3 is involved in facilitating the organization of the mitochondrial transition pore and assist in mtDNA release (94). A self-sustaining circle involving mitochondrial damage, ROS production, and consequent mtDNA damage/DAMPs release triggered by NLRP3 activators has been hypothesized (83). In particular, damaged/oxidized mtDNA/DAMPs are preferentially sensed and bound by NLRP3 (83).

Following the view of MQC failure as a source of MDVs/DAMPs, we will discuss in the next section the main literature supporting the involvement of mitophagy impairment and DAMPs release in the setting of cancer and two common neurodegenerative diseases (AD and PD).

IMPLICATION OF EXTRACELLULAR VESICLES AND DAMAGE-ASSOCIATED MOLECULAR PATTERNS IN DISEASE

Cancer

Although the involvement of DAMPs in cancer pathogenesis is debated, the installment of an inflammatory milieu is recognized as a factor favoring tumor progression (95, 96). In particular, increasing levels of pro-inflammatory mediators, including IFN-γ, IL1, IL6, lymphotoxin (LT)-β, tumor necrosis factor alpha (TNF-α), and transforming growth factor β, have been implicated in the promotion of carcinogenesis (95–97), for their potential role in modulating DAMPs expression and release (95, 98). Intracellular and extracellular DAMPs are, indeed, hallmarks of cancer that have been implicated in the early stages of carcinogenesis (95). While oxidative stress triggers the release of DAMPs in the extracellular space thus stimulating hyper-inflammation and immune injury, the loss of intracellular DAMPs, [i.e., HMGB1, histones, ATP, and DNA] induces genomic instability, epigenetic alterations, telomere attrition, reprogrammed metabolism, and impaired degradation (98). In the setting of such DAMPs-mediated pathogenic changes, cancer initiation and development are favored. Along with this, the release of ATP, IL1α, adenosine, and uric acid have also been implicated in carcinogenesis *via* induction of inflammation, immunosuppression, angiogenesis, and tumor cell proliferation (95) (**Figure 3**).

Strikingly, inflammatory pathways may also be activated by damaged mitochondrial constituents displaced within MDVs (65) that may trigger caspase-1 activation and secretion of pro-inflammatory cytokines (99). Interestingly, adaptive immunity responses are suppressed by PINK1 and Parkin that redirect MDVs toward lysosomal degradation to prevent endosomal loading with mitochondrial cargoes on major histocompatibility complex (MHC) class I molecules for antigen presentation purposes (48). Furthermore, the possibility that MDVs are used by cells as a homeostatic mechanism by horizontal mitochondrial transfer cannot be disregarded (100). Bone marrow mesenchymal stromal cells (BM-MSCs) eliminate damaged depolarized mitochondria through EVs and export them to neighbouring macrophages (101). Macrophages, in turn, recycle these MDVs to secrete exosomes which contain microRNAs (miRNAs) that inhibit TLR stimulation and induce macrophage tolerance to transferred damaged mitochondria (101). Moreover, cells with impaired mitochondria are able to transfer and take up fully-functional mitochondria displaced within MVDs to rescue aerobic respiration (102–105). A mitochondrial transfer was also shown between A549 mtDNA depleted (p^0) lung cancer cell and BM-MSCs to rescue respiration in lung cancer cells lacking mtDNA-encoded subunits of the electron transport chain (ETC) (104). However, vesicles enriched in whole mitochondria or mitochondria void of envelops can also be released and serve as DAMPs in pathological conditions, including tissue injury and cancer (106). In particular, the release of mitochondria by damaged mesenchymal stem cells has been found to function as a danger signal to activate their rescue properties (107). The uptake of whole mitochondria by epidermal growth factor-activated human osteosarcoma cells *via* macropinocytosis has also been described (108).

Recent findings indicate that cancer cells can reprogram their energy metabolism to adapt and survive in unfavorable microenvironments *via* EVs (109). Indeed, an efficient mitochondrial respiration is required by cancer cells to maintain their tumorigenicity (110). Upon acquisition of mtDNA through EVs, estrogen receptor (ER)-positive breast cancer can evolve from hormonal therapy sensitive (HTS) to dormant (HTD) or resistant (HTR) with poorer outcome. EVs from patients with HTR disease contain full mitochondrial

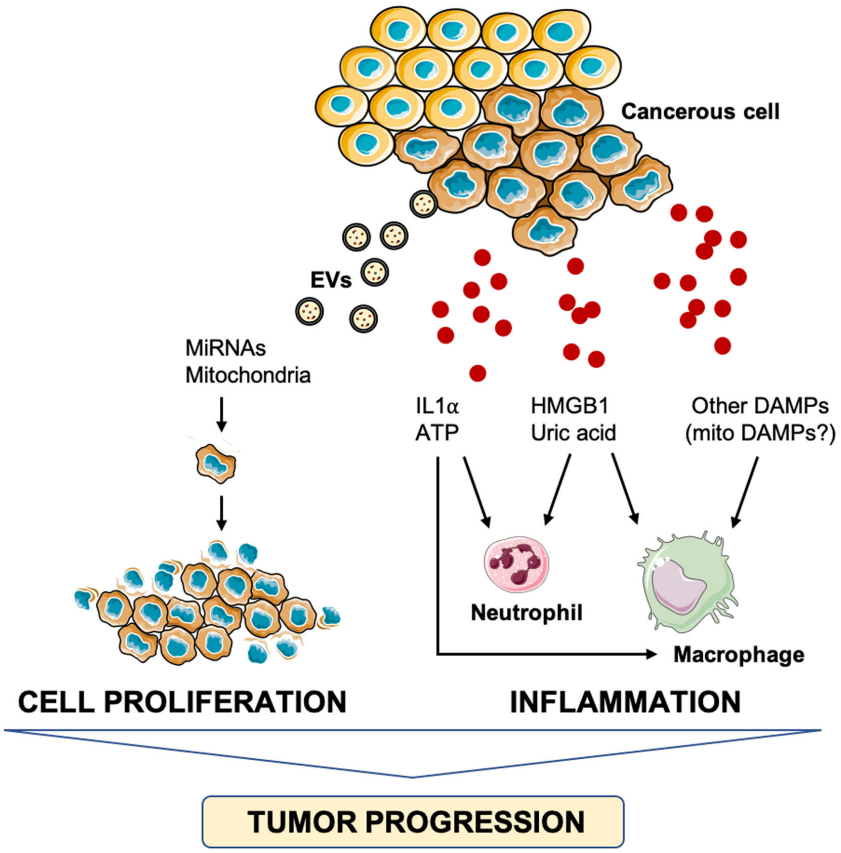

FIGURE 3 | Schematic representation of the main pathways triggered by damage-associated molecular patterns and involved in tumor progression. ATP, adenosine triphosphate; DAMPs, damage-associated molecular patterns; HMGB1, high-mobility group box 1, IL1α, interleukin 1α; miRNA, micro RNA.

genome that might have been transferred to HTS/HTD cells to sustain oxidative phosphorylation, an exit from dormancy and the development of HTR disease (111). Additional findings show that EVs from melanoma, ovarian and breast cancer tissues contain mitochondrial membrane proteins and active mitochondrial enzymes that are not detected in healthy controls (112), thus corroborating the hypothesis that energy metabolism reprogramming in cancer cells may occur also *via* EVs.

Similar to HMGB1 and histones, miRNAs can also be releases in the extracellular space as DAMPs in cancer (113, 114). Recent work has shown an exosomes-dependent pathway to secrete miRNA in cancer cells (115). For instance, in pancreatic cancer cells, exosomes containing miR-212-3p are secreted and lead to decreased expression of MHC II in dendritic cells (DCs), thereby inducing immune tolerance (116). Another system used by cancer cells to escape their recognition by the immune system is based on PD-L1. This factor binds to the PD1 receptor on immune cells thereby inhibiting proliferation and survival of CD8+ cytotoxic T lymphocyte (117). A recent study has shown that exosomes derived from lung cancer express PD-L1 and this is implicated in immune escape and promotion of cancer growth (118). These mechanisms enable cancer cell survival, proliferation, and undisturbed dissemination into other bodily districts, even located at long distance from the primary neoplastic mass. Thus, exosomes are useful shuttles for cancer cells to elude the immune system's response and achieve undisturbed survival and proliferation.

Moreover, cancer cells can also transfer miRNAs *via* exosomes to favor angiogenesis. These miRNAs of exosomal origin are ultimately DAMPs promoting cancer proliferation. Indeed, their secretion is induced under oxidative stress (119). An elegant work by Deng et al. (120) showed that gastric cancer cells released exosomes containing miR-155 to increase the expression of vascular endothelial growth factor (VEGF) and promote proliferation and tube formation of vascular cells. In further support to the role of exosomal miRNAs in promoting angiogenesis are findings showing a strong enhancement of angiogenesis and tumor growth in mice under the infusion of exosomes containing miR-155 (120).

Finally, DAMPs may also act as a suppressor of tumor progression by promoting immunogenic cell death. Under physiologic conditions, cell death linked to normal turnover is not immunogenic and does not activate PRRs, such as TLRs and NLRPs (121). In contrast, immunogenic cell death is essential for tumor suppression after chemotherapeutic treatments (122).

Immunogenic and non-immunogenic cell death are characterized by different biochemical and metabolic events. In particular, during immunogenic cell death, antigens from dying cells are incorporated by DCs and presented bound to MHC to mount a T cell immune response. In this context, co-stimulatory signals and cytokines are required for differentiation of specific T cells (123). The preapoptotic exposure of calreticulin on the plasma membrane of dying cells promotes their uptake by DCs (124). Interestingly, the release of HMGB1 in the surroundings of dying cells (125) induces an increase in tumor antigen presentation and regulates the TLR4-dependent immune response (126). The role of the NLRP3 inflammasome is crucial for the immune response against dying tumor cells as it interacts with the adaptor molecule apoptosis-associated speck-like protein to induce caspase-1 activation (127). The caspase-1 pathway is involved in the production of proinflammatory cytokines (i.e., IL1β and IL18) which are essential to induce an immunogenic response (127). Notably, ATP released from dying tumor cells mediates immunogenic cell death *via* the activation of the NLRP3 inflammasome (128). Therefore, understanding the fine-tuning of DAMPs release may be crucial for unveiling new pathways that modulate tumor cell's death vs. survival.

Neurodegeneration

As a first line of defense against microbes, microglial cells of the central nervous system (CNS) preserve tissue homeostasis by clearing out damaged neurons and limiting the spread of infections. This macrophage population accomplishes these housekeeping activities by triggering inflammation *via* the release of cytokines and by instigating ROS production (129). However, upon prolonged stressors, a persistent microglia activation installs a pro-inflammatory and pro-oxidant environment that impinges on tissue homeostasis. A state of chronic, low-grade inflammation is observed during aging (i.e., inflamm-aging) which has been associated also with metabolic changes in microglia (130, 131). The age-related microglial and metabolic reshaping plays relevant roles in the context of AD and PD (132, 133). Indeed, neuroinflammation may represent a common thread in a large set of neurological disorders for which DAMPs of different origins, including mitochondrial, may support disease progression (134) (**Figure 4**).

Alzheimer's Disease

AD is the most common age-associated dementia and is characterized by neuronal degeneration mainly in the

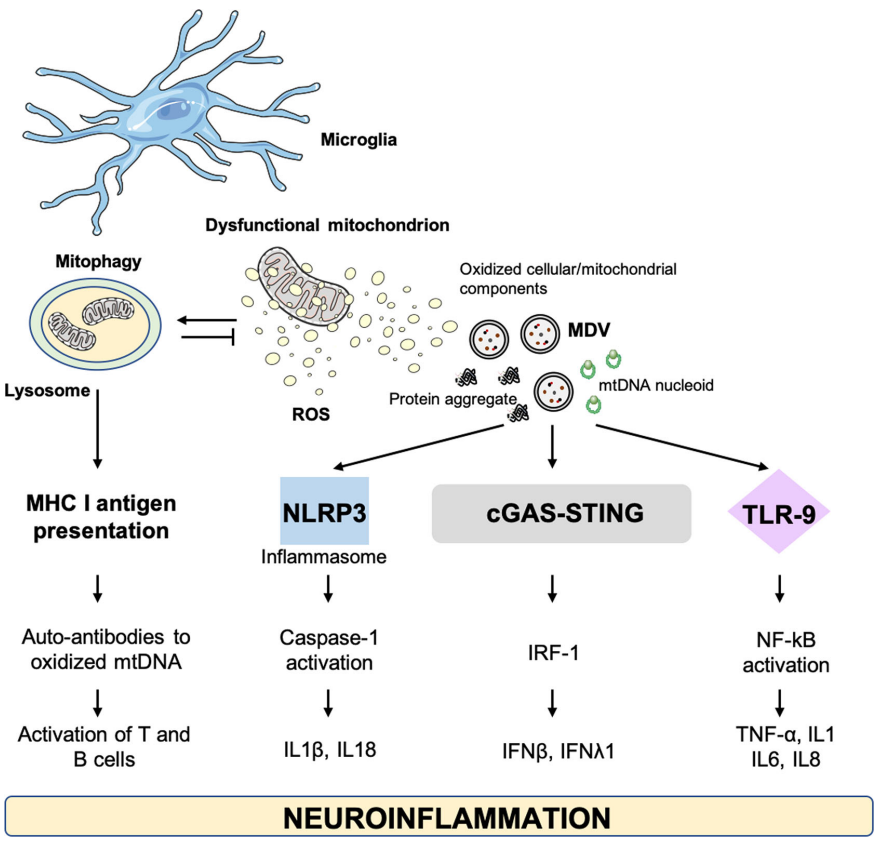

FIGURE 4 | Cellular alterations and damage-associated molecular patterns involved in neuroinflammation. cGAS–STING, GMP-AMP synthase–stimulator of interferon genes; IFN, interferon; IL, interleukin; IRF-1, IFN-regulatory factor 1; MDV, mitochondrial derived vesicle; MHC, major histocompatibility complex; mtDNA, mitochondrial DNA; NLRP3, NOD-like receptor 3; NF-kB, nuclear factor kappa B; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.

neocortex and the *hippocampus* (12). The extracellular deposition of amyloid beta (Aβ) aggregates and intracellular neurofibrillary tangles are distinctive histopathological traits of AD (12). Amyloid plaque deposition instigates microglia activation which, in turn, promotes the development of a pro-inflammatory environment through the release of inflammatory cytokines, including IL1β, IL6 and TNF-α (135). This neuroinflammatory response may represent an inter- and intracellular signaling system between microglia and astrocytes aimed at clearing damaged neuronal components (136). Indeed, in the setting of inefficient intracellular quality control (137), the persistence of damaged components and hyper-inflammation may favor the generation and spread of Aβ peptides, thereby triggering neurotoxicity (135).

Dysregulation of the endo-lysosomal system contributes to the generation of amyloid plaques and AD pathogenesis. Indeed, Aβ42 aggregates, the most pathogenic Aβ peptides, have been detected in the soma of neurons at the level of lysosomes or lysosome-derived components (138). Furthermore, neurons from AD transgenic mice show enlarged and dysfunctional MVBs in the presence of Aβ42 accrual (139). As a consequence of MVB dysfunction, higher levels of the amyloid precursor protein (APP) are secreted extracellularly in this murine model (139). In the endosomal compartment is also located the activity of the β-site APP-cleaving-enzyme (BACE1), a hub for the intracellular trafficking of APP and a relevant contributor to amyloid plaque generation (140). Conversely, a retrograde transport of APP from endosomes to the trans Golgi network is in place to reduce Aβ production (141). Notably, an impairment in the retromer complex activity has been involved in AD pathogenesis (141).

Circulating levels of HMGB1 and the soluble form of the receptor for advanced glycation end products (RAGE) have been detected in the serum of AD patients. The concentration of these DAMPs correlate with the extent of Aβ deposition (142). Moreover, HMGB1 and thrombin proteins have been identified as pro-inflammatory mediators contributing to dysfunction of the blood-brain barrier (BBB) (142). Similarly, serum levels of the brain-derived protein S100B have been associated with the severity of the disease (143). The administration of the S100B inhibitor pentamidine was able to reduce the levels of S100B and RAGE and blunt Aβ-induced gliosis and neuroinflammation in a mouse model of AD (144).

Mitochondrial dysfunction and the ensuing oxidative stress have also been involved in the pathogenesis of AD. Indeed, a lower copy number and a higher levels of mtDNA heteroplasmy have been found post-mortem in brains of people with AD (145–147). In addition, oxidative damage to mitochondrial components has been described as an early event in AD, which suggests a role for oxidative stress in disease pathogenesis (148, 149). Interestingly, Aβ peptide aggregates and neurofibrillary tangles can impact mitochondrial function by binding to proteins of the mitochondrial import machinery (150). As a result, increased ROS production occurs (151). The mitochondrial localization of fragments of the E4 variant of apolipoprotein E, the main susceptibility gene for sporadic AD, has also been reported and associated with mitochondrial dysfunction and oxidative stress in hippocampal neurons (152, 153).

While primary mitochondrial deficits have been observed in AD, aberrant mitochondria can also result from defective quality control mechanisms, especially mitophagy. In particular, a vicious circle between defective mitophagy and mitochondrial dysfunction may be triggered Aβ and phosphorylated Tau (p-Tau), ultimately leading to neuronal disruption (154–156). Altered expression of the mitophagy receptor disrupted-in-schizophrenia 1 (DISC1) has been reported in AD patients, transgenic AD mice, and cultured cells treated with Aβ (157). DISC1 is a promoter of mitophagy that binds to microtubule-associated proteins 1A/1B light chain 3 (LC3) and protects synaptic plasticity from the toxicity of Aβ accrual (157). The positive effect exerted by the pharmacological restoration of mitophagy on cognitive dysfunction and Aβ proteinopathy in APP/PS1 mice highlights the central role of defective mitophagy in AD pathogenesis (158). Following pro-mitophagy pharmacological treatments, reduced levels of Tau phosphorylation and mitigation of inflammation induced by microglia activation have also been observed (154). As such, a link between neuronal bioenergetic failure resulting from defective MQC, inflammation, and neuronal loss can also be hypothesized in AD (92). Following mitophagy impairment, cGAS–STING-DNA-mediated inflammation has been described in neurodegeneration (159) and NLRP3-induced inflammation has been observed in AD [reviewed in (160)].

A defective mitophagy and the resulting accrual of dysfunctional mitochondria in AD may instigate the extrusion of damaged organellar components with consequent stimulation of innate immunity (77, 78). Mitochondrial DAMPs have been retrieved within circulating EVs in several age-related conditions, including neurodegeneration (161, 162). Whether this mechanism is relevant to AD is worth being explored.

Parkinson's Disease

PD is the second most common age-related neurodegenerative disorder (163) and is characterized by a progressive degeneration of dopaminergic neurons of the *substantia nigra pars compacta* and dopamine depletion in the *striatum* (164). These histopathological and biochemical abnormalities underlie a set of motor (i.e., bradykinesia, postural inability, rigidity, and tremor) and non-motor signs and symptoms (e.g., constipation, depression, sleep disorders, cognitive dysfunction) (164).

Neuroinflammation is a noticeable feature of PD (165). In particular, the HMGB1-TLR4 axis seems to plays an important role. Higher serum levels of HMGB1 and TLR4 protein have been detected in PD patients and correlated with disease stage (166). Moreover, the administration of anti-HMGB1 monoclonal antibody in a rat model of PD was able to reduce inflammation by preserving the BBB and lowering IL1β and IL6 secretion (167). The chemokine fractalkine (CX3CL1), which is mainly expressed by neurons and serves as a modulator of microglial-neuronal communication, has been indicated as a possible biomarker for PD (168). Increased levels of the S100B

protein were also detected in the *substantia nigra* and cerebrospinal fluid of persons with PD and in the ventral midbrain of a murine PD model treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (169). Notably, the ablation of S100B in the murine model was neuroprotective by reducing microgliosis and the expression of both RAGE and TNF-α (169). Noticeably, a systemic inflammatory signature, involving IL8, IL9, and macrophage inflammatory protein 1α and 1β, has been identified in older adults with PD (170).

A defective cellular quality control, manifested by deposition of aberrant α-synuclein in dopaminergic neurons, is acknowledged as an important mechanism underlying neurodegeneration in PD (171). The accumulation of α-synuclein at the mitochondrial complex I has been shown to impair its activity (171). Such an inhibitory function, together with mutations in genes encoding for the mitochondrial regulators Parkin, PINK1, and protein deglycase DJ-1 have been linked with enhanced ROS generation in PD (172, 173) and α-synuclein aggregation (174–177). Derangements in mtDNA homeostasis, including large deletions, have been also detected in neuronal cells of the *substantia nigra* of persons with PD (178–180). These observations indicate that mitochondrial dysfunction plays a major role in the pathogenesis of familial PD (181). On the other hand, PD in its sporadic form recapitulates all major hallmarks of aging (182). Indeed, MQC derangements and the generation of DAMPs have been indicated as a major contributors to the co-occurrence of mitochondrial dysfunction and neuroinflammation in PD (159, 183, 184). An innate immune response triggered by defective autophagy and impaired disposal of damaged mitochondria has been described in mice lacking PINK1 or parkin gene (PARK2) (159). Moreover, the activity of the mitophagy mediator Parkin mediates a mitophagic control over inflammation (48). In particular, Parkin regulates adaptive immunity *via* the presentation of mitochondrial antigens to endosomes for loading onto MHC class I molecules (48). Similarly, the intracellular trafficking regulator RAB7A exerts also a mitochondrial antigen presentation role by controlling the fusion of MDVs with late endosome for their subsequent degradation (48). The function of RAB7A as a mitochondrial antigen-presenting system in immune cells *via* MDV trafficking ensures that the process can be finalized in the absence of PINK1 or Parkin (48). Indeed, alterations in PINK1/Parkin expression and activity in PD result in MQC dysregulation and possibly neuroinflammation *via* mitochondrial antigen presentation by MDVs (48). Recent work by our group described the presence of mitochondrial DAMPs among circulating EVs in older adults with PD along with a specific inflammatory signature (162). In particular, higher serum concentrations of small EVs including exosomes of endosomal origin were identified in older adults with PD (162). However, lower levels of MDVs were retrieved in people with PD relative to non-PD controls (162). A lower secretion of MDVs in older adults with PD is in keeping with the hypothesis of intracellular accrual of dysfunctional mitochondrial secondary to engulfed MQC system (162). According to this view, MDV generation may serve as a housekeeping mechanism that complements MQC to preserve cell homeostasis (15). A link between mitochondrial damage and inflammatory and metabolic disarrangements in PD has also been proposed (184, 185); however, the molecular mechanisms linking these processes are missing. An involvement of the cGAS–STING-DNA driven inflammation in neurodegeneration following mitophagy impairment has been reported (159). Indeed, higher circulating levels of the pro-inflammatory cytokines IL6 and IFNβ have been detected in Pink and Parkin knockout mice challenged with exhaustive exercise (159). Notably, the deletion of STING or the administration of IFNα/β receptor-blocking antibody was able to blunt this response, thus suggesting that the accrual of dysfunctional mitochondria may trigger inflammation in people with PD (159). Among the ever-growing list of molecules linking mitochondrial dysfunction to systemic inflammation in PD, the fibroblast growth factor 21 (FGF21) has emerged as a relevant mediator (162). Indeed, FGF21 has been indicated as a "mitokine" for its association with impaired MQC in neurons of murine models of tauopathy and prion disease (186). Taken as a whole, these findings suggest that a deeper understanding on the intracellular and extracellular trafficking of DAMPs and vesicles, including those of mitochondrial origin, may be key to unveiling relevant pathogenetic pathways of PD and, hence, novel targets for drug development.

CONCLUSION

Cells bearing DAMPs receptors sense and bind extracellular DAMPs as triggers of inflammation and fibrotic responses. Higher levels of circulating DAMPs have been identified during aging and related to inflamm-aging (3, 98, 187). A multicomponent senescence-associated secretory phenotype consisting of cytokines, chemokines (CXCLs), growth factors, and proteases has also been reported (188–191). While these secreted molecules contribute to preserving cell homeostasis in healthy tissues (192), the installment of an age-associated chronic secretory phenotype is a candidate pathway for the deployment of pathological hallmarks of aging, (e.g., inflamm-aging, tumorigenesis, loss of cell stemness). A core of circulating factors has been identified among plasma biomarkers of aging; however, their relationship with DAMPs is still unclear. The identification of circulating EVs stemming from altered regulation of the endosomal-lysosomal system in several age-related conditions, including cancer and neurodegeneration, holds hope for targeting this route for therapeutic purposes (13). Therefore, a deeper understanding of the complex, dynamic, intracellular and extracellular trafficking of DAMPs and vesicles, including those of mitochondrial origin, may be key to unveiling relevant pathogenic pathways and novel targets for drug development.

AUTHOR CONTRIBUTIONS

Conceptualization: AP, CB, EM, and FG. Writing (original draft preparation): AP, CB, EM, and FG. Writing (review and editing): RC, HC-J, and RR. Supervision: FL and RB. Funding acquisition:

CB and RB. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Innovative Medicine Initiative-Joint Undertaking (IMI-JU #115621), AIRC (Associazione Italiana per la Ricerca sul Cancro) Investigator grant 2016 #19068 to CB, Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) to Consorzio Interuniversitario Biotecnologie (DM 1049, 29/12/2018; CIB N. 112/19 to CB), intramural research grants from the Università Cattolica del Sacro Cuore (D3.2 2020), and the nonprofit research foundation "Centro Studi Achille e Linda Lorenzon".

ACKNOWLEDGMENTS

The figures were drawn using the freely available Servier Medical Art resource (http://www.servier.com/Powerpoint-image-bank).

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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.

frontiers in Immunology

REVIEW
published: 07 December 2020
doi: 10.3389/fimmu.2020.609441

Distinct Molecular Mechanisms Underlying Potassium Efflux for NLRP3 Inflammasome Activation

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The NLRP3 inflammasome is a core component of innate immunity, and dysregulation of NLRP3 inflammasome involves developing autoimmune, metabolic, and neurodegenerative diseases. Potassium efflux has been reported to be essential for NLRP3 inflammasome activation by structurally diverse pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Thus, the molecular mechanisms underlying potassium efflux to activate NLRP3 inflammasome are under extensive investigation. Here, we review current knowledge about the distinction channels or pore-forming proteins underlying potassium efflux for NLRP3 inflammasome activation with canonical/non-canonical signaling or following caspase-8 induced pyroptosis. Ion channels and pore-forming proteins, including P2X7 receptor, Gasdermin D, pannexin-1, and K2P channels involved present viable therapeutic targets for NLRP3 inflammasome related diseases.

Keywords: P2X7 receptor, pannexin-1, Gasdermin D, K2P channels, TWIK protein-related acid-sensitive potassium channel 2, THIK-1, inflammasome

OPEN ACCESS

Edited by:
Walter Gottlieb Land, Université de Strasbourg, France

Reviewed by:
Marco Di Gioia, Boston Children's Hospital and Harvard Medical School, United States
Chunfu Zheng, Fujian Medical University, China

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *23 September 2020*
Accepted: *09 November 2020*
Published: *07 December 2020*

Citation:
Xu Z, Chen Z-m, Wu X, Zhang L, Cao Y and Zhou P (2020) Distinct Molecular Mechanisms Underlying Potassium Efflux for NLRP3 Inflammasome Activation. Front. Immunol. 11:609441. doi: 10.3389/fimmu.2020.609441

NLRP3 INFLAMMASOME

Inflammasomes are intracellular multiprotein complexes and core components of innate immunity (1–3). To date, the NOD-like receptor (NLR) family and the PYHIN family have been reported to form inflammasomes (4). These are composed of six NLR family proteins, including NLRP1, NLRP2, NLRP3, NLRP6, NLRC4, NLRP12, and two members of the PYHIN family, including AIM2 and IFI16 (5, 6).

Among various inflammasomes, NLRP3 inflammasome has been widely under investigation because of its most significant clinical relevance (7, 8). NLRP3 inflammasome consists of sensory protein NLRP3, adaptor protein ASC (the adaptor molecule apoptosis-associated speck-like protein containing a CARD), and effector protein caspase-1 (7, 8). Canonical NLRP3 inflammasome activation requires two steps: priming and activation. The priming process leads to the expression of NLRP3, pro-IL-1β, and pro-IL-18, which could be initiated by Toll-like receptors (TLR) ligands (9). The activation process promotes the assembly of inflammasome complexes, cleaving pro-caspase-1 to form active caspase-1, thereby cleaving pro-IL-1β and pro-IL-18 to release mature IL-1β and IL-18 (**Figure 1**).

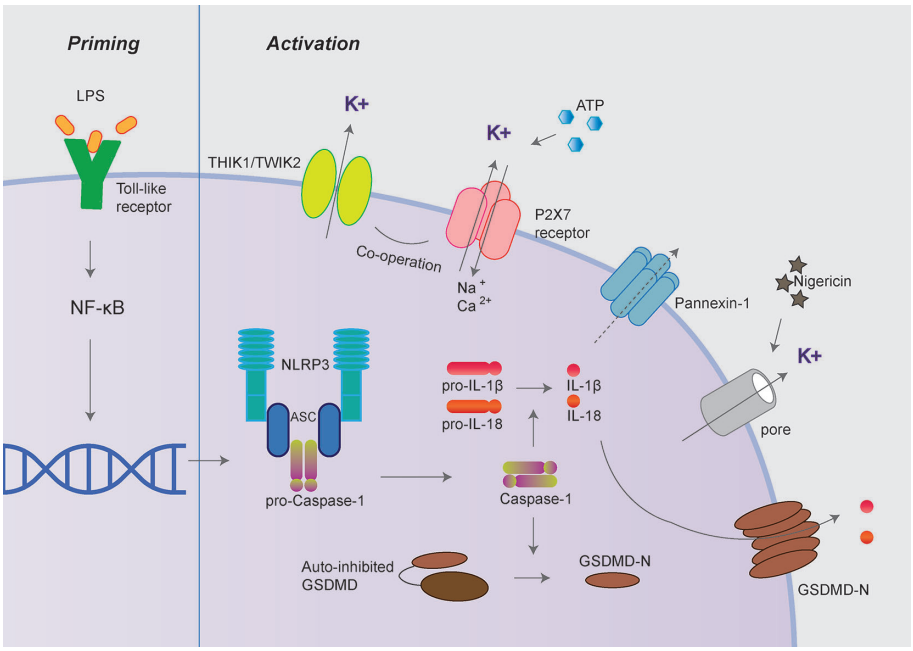

FIGURE 1 | Canonical NLRP3 inflammasome activation. Canonical NLRP3 inflammasome activation includes two signals: priming and activation. The priming process leads to the expression of NLRP3, pro-IL-1β and pro-IL-18 could be provided by Toll-like receptors activation. The activation process promotes the assembly of inflammasome complexes through various PAMPs or DAMPs, including extracellular ATP, nigericin, and particulate matters. The activation of NLRP3 inflammasome cleaves pro-caspase-1 to active caspase-1, thereby cleaves pro-IL-1β and pro-IL-18 to produce mature IL-1β and IL-18. Besides, activated caspase-1 also cleaves GSDMD to release its N-terminal domain, which forms pores at the plasma membrane and mediates the release of mature IL-1β and IL-18. The P2X7 receptor, Pannexin-1, TWIK2, and THIK1 have been proposed to mediate potassium efflux during NLRP3 inflammasome activation under different circumstances.

Besides, activated caspase-1 also cleaves Gasdermin D (GSDMD) to release its N-terminal domain, which forms pores at the plasma membrane and induces a rapid, pro-inflammatory form of cell death termed "pyroptosis" (10–12). Intriguingly, the activation process of NLRP3 inflammasome could be provided by surprisingly various types of PAMPs (pathogen-associated molecular pattern) or DAMPs (danger-associated molecular pattern). These include extracellular ATP, pore-forming toxins (nigericin and maitotoxin, etc.), particulate matter (urate crystalline MSU, aluminum adjuvant, silica, and asbestos), and misfolded proteins related to neurodegenerative diseases (fibrillar Aβ protein; α-synuclein) (10–12). The dysregulated activation of NLRP3 inflammasome is closely related to various auto-inflammatory or chronic inflammations, such as gout, atherosclerosis, obesity, Alzheimer's disease, Parkinson's disease, and type 2 diabetes (13–15). Besides the canonical activation process, the non-canonical inflammasome pathway is mediated by caspase-11 in mouse cell or caspase-4/caspase-5 in a human cell in response to cytoplasmic bacterial lipopolysaccharide (LPS) (**Figure 2**) (16, 17).

Cytoplasmic LPS directly binds the caspase recruitment domain (CARD) of caspase-4/5/11, triggering caspase-4/5/11 cleaves GSDMD to initiate pyroptosis (18, 19). Caspase-11 mediated pyroptosis in response to cytosolic LPS is critical for antibacterial defense and septic shock in mice as demonstrated that GSDMD$^{-/-}$ and caspase11$^{-/-}$ mice could be protected against LPS-induced lethality (20, 21). Besides directly causing pyroptosis, the non-canonical inflammasome also promotes the canonical NLRP3 inflammasome to cause the maturation and release of IL-1β and IL-18 (19).

ION CHANNELS AND PORE-FORMING PROTEINS MEDIATING POTASSIUM EFFLUX DURING NLRP3 INFLAMMASOME ACTIVATION

It has been well accepted that potassium (K^+) efflux is both necessary and sufficient for NLRP3 inflammasome activation in most cases (22–25). First, a large reduction of intracellular potassium concentration was observed to activate the NLRP3 inflammasome by ATP, nigericin, and crystal molecules (23). Furthermore, incubation of primed macrophages in a K^+-free medium was sufficient to trigger NLRP3 inflammasome activation (26). In contrast, NLRP3 inflammasome activation could be blocked by high concentrations of extracellular potassium (30-45 mM) (23, 26). Besides, AIM2 and NLRC4 inflammasomes activation was not affected by high concentrations of extracellular K^+, indicating potassium's specific role in modulating NLRP3 inflammasome (23–25).

Structurally diverse DAMPs/PAMPs employ distinct mechanisms to cause potassium efflux to activate the NLRP3 inflammasome. Firstly, the existing research mainly focuses on the molecular mechanism of potassium efflux during ATP-induced NLRP3 inflammasome

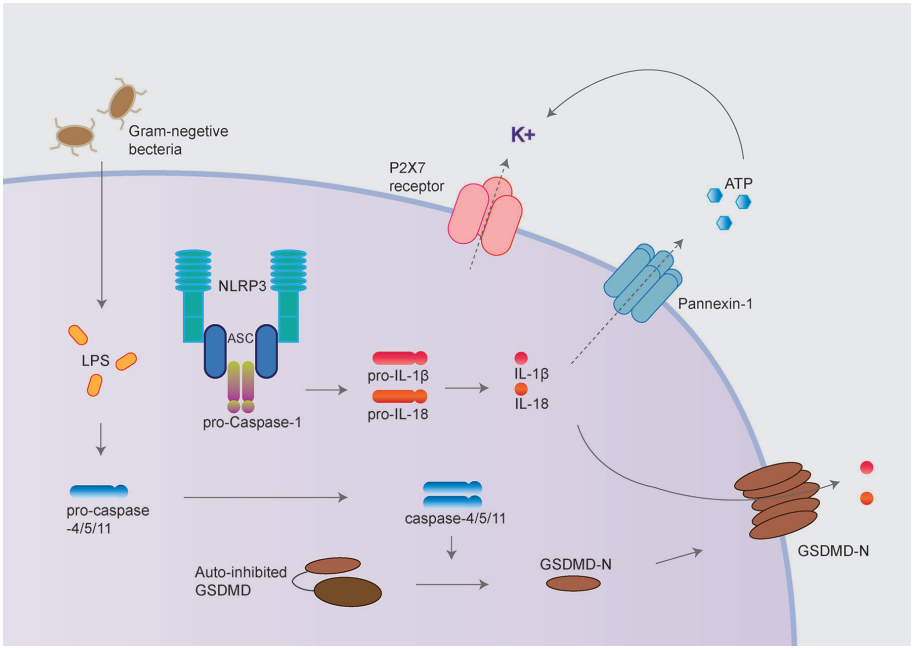

FIGURE 2 | NLRP3 inflammasome activation following non-canonical inflammasome activation. The non-canonical inflammasome pathway is mediated by caspase-11 in mouse cell and caspase-4/caspase-5 in a human cell in response to cytoplasmic bacterial lipopolysaccharide (LPS). Caspase-4/5/11 cleaves GSDMD to initiate pyroptosis, thus leads to NLRP3 inflammasome activation. GSDMD and P2X7 receptor/Pannexin-1 have been proposed to mediate potassium efflux, which underlies the mechanism of NLRP3 inflammasome activation following non-canonical inflammasome.

activation. The P2X7 receptor, pannexin-1, and K2P channels have been reported to participate in the process above (24, 25, 27). Secondly, toxins such as nigericin directly promote potassium efflux by forming pores on the plasma membrane. NLRP3 inflammasome could also be activated following the non-canonical inflammasome or caspase-8 mediated pyroptosis, which also depends on potassium efflux (28–31). Controversially, GSDMD, and pannexin-1 have been proposed to mediate potassium efflux in the above process to activate NLRP3 inflammasome (19, 29, 30, 32, 33). This section will review the current knowledge about ion channels' roles and pore-forming proteins mediating potassium efflux during NLRP3 inflammasome activation under different circumstances.

P2X7 Receptor

The P2X7 receptor is an ATP-gated cation-selective channel widely expressed in various immune cells (34). At resting conditions, extracellular ATP concentration is at low levels (<10 nM/L), which will be massively increased to several tens or hundreds of μmoles/l within stressed or dying cells (35). The elevated extracellular ATP activates the P2X7 receptor, which then mediates potassium efflux and thus leads to NLRP3 inflammasome activation (34, 36–38).

Besides, the canonical NLRP3 inflammasome, P2X7 receptor, and pannexin-1 (see *Pannexin-1*) also have been reported to participate in non-canonical inflammasome (39) coordinately. It was reported that the activated caspase-11 cleaves pannexin-1 followed up by ATP release, which in turn activates the P2X7 receptor to mediate potassium efflux and NLRP3 inflammasome activation (39). Correspondingly, the P2X7 receptor ablation significantly reduced the mortality of mice and IL-1β secretion in peritoneal fluid in a sepsis mice model (39). However, this study is contradicted with studies by several other groups that we will discuss in the next section.

Gasdermin D

Gasdermin D (GSDMD) has been identified as the executor of pyroptosis activated by caspase-1/4/5/11 in 2015 (19, 31, 40). Full-length GSDMD includes the N-terminal (GSDMD-N) and C-terminal repressor domain (GSDMD-C) interacting with each other in the absence of stimulation. This auto-inhibitory conformation is released upon efficient cleavage at a conserved glutamic acid residue (D276 in mouse and D275 in human GSDMD) caspase-1/4/5/11, dividing GSDMD into GSDMD-N and GSDMD-C. The generation of GSDMD-N allows it to insert into the plasma membrane and form large oligomeric pores, leading to IL-1β and IL-18 secretion and pyroptosis. Kayagaki et al. and Shi et al. reported that potassium pass through the pore-forming GSDMD, which further leads to NLRP3 inflammasome activation during non-canonical inflammasome activation (18, 19). Besides mediating pyroptosis and NLRP3 inflammasome activation, GSDMD was recently reported to restrain type I interferon response to cytosolic DNA by driving potassium efflux (41).

Caspase-8 has long been considered to play key roles in extrinsic apoptosis and suppress necroptosis by inhibiting RIPK1/RIPK3 and MLKL. More recently, three independent studies have demonstrated the "apoptotic" caspase-8 also could cleave GSDMD leading to

pyroptosis-like cell death, further triggering NLRP3 inflammasome activation in murine macrophages (**Figure 3**) (29, 30, 32). It has been proposed that potassium efflux underlies NLRP3 inflammasome activation, followed by caspase-8 mediated pyroptosis. However, three groups disagree with the molecular mechanism underlying potassium efflux in the process above. Orning et al. and Sarhan et al. suggest that NLRP3 inflammasome activation is dependent on GSDMD-mediated potassium efflux based on delays in ASC oligomerization in GSDMD$^{-/-}$ cells (29, 30). However, Chen et al. observed normal caspase-1 processing in GSDMD$^{-/-}$ and/or GSDME$^{-/-}$ (Gasdermin E; another member of Gasdermin protein) cells, which suggests NLRP3 inflammasome activation is not dependent on GSDMD or GSDME (32).

Pannexin-1

The pannexin-1 is a non-selective, large-pore channel that releases potassium and nucleotides, including ATP (42, 43). Pannexin-1 is expressed in most cell types and functionally auto-inhibited by its cytoplasmic C-terminal domain. In response to apoptosis, the pannexin-1 channel can be functionally activated by caspase-3 mediated cleavage of the inhibitory C-terminal domain (44, 45).

The relationship between pannexin-1 and NLRP3 inflammasome is still controversial. By using pannexin-1 inhibitors or siRNA, Pelegrin et al. reported that pannexin-1 is responsible for IL-1β release upon NLRP3 inflammasome agonists ATP or nigericin (46–48). However, this channel was lately reported to be dispensable for canonical NLRP3 inflammasome activation using pannexin-1 knockout mice (49).

Together with the P2X7 receptor, pannexin-1 was also implicated in promoting pyroptosis and NLRP3 activation during non-canonical inflammasome activation (discussed in *P2X7 Receptor*) (39). In LPS-induced sepsis mouse models, the ablation of pannexin-1 significantly reduced mice mortality, which indicates the role of pannexin-1 in non-canonical inflammasome activation (39). This finding is at odds with the observation that caspase-11 drives NLRP3 inflammasome activation through GSDMD pores (18, 19, 40). A recent study further pointed out that pannexin-1 is dispensable for canonical or non-canonical inflammasome activation within pharmacological inhibition and two other macrophages strain with pannexin-1 ablation (33).

Interestingly, during the NLRP3 inflammasome activation following caspase-8 activated pyroptosis, Chen et al. observed that potassium efflux mediated by pannexin-1 but not GSDMD is critical for NLRP3 inflammasome activation following caspase-8 mediated pyroptosis (32, 33).

K2P Channels

Two-pore domain potassium (K2P) channels comprise a major and structurally distinct subset of mammalian K+ channel superfamily, including fifteen K2P subtypes that form six subfamilies (TWIK, TASK, TRESK, TALK, THIK, and TRESK) (50, 51). K2P channels contribute to the background leak currents, responsible for maintaining the resting membrane potential in nearly all cells. They are regulated by various physical, chemical, and biological stimuli and implicated in multiple physiological processes. In recent years, significant roles of K2P channels for the

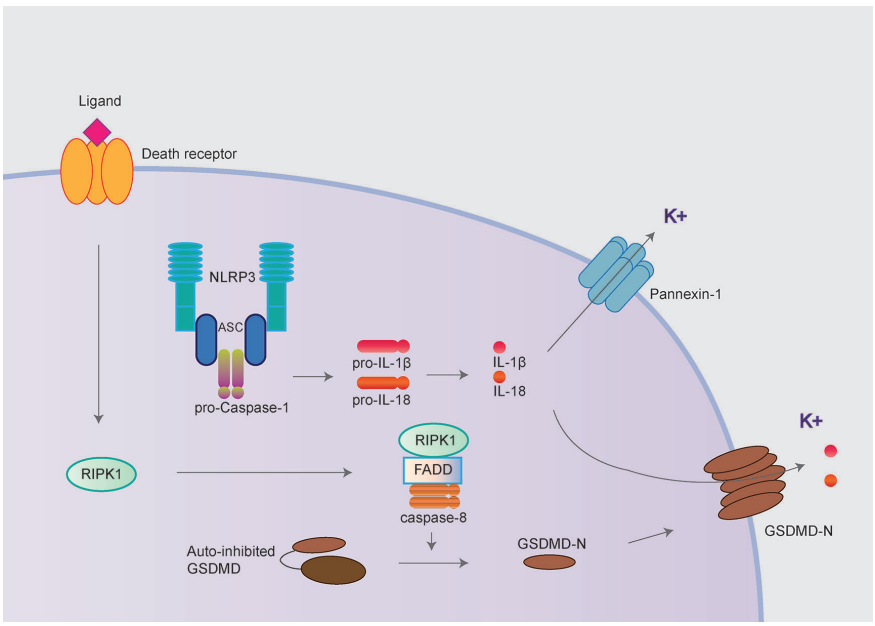

FIGURE 3 | NLRP3 inflammasome activation following caspase-8 mediated pyroptosis. The NLRP3 inflammasome could also be activated following by caspase-8 mediated pyroptosis. The "apoptotic" caspase-8 cleaves GSDMD and further mediated potassium efflux leading to NLRP3 inflammasome activation in murine macrophages. In contrast, Chen et al. demonstrated the pannexin-1 but not GSDMD mediating potassium efflux contributed to NLRP3 inflammasome activation following caspase-8 mediated pyroptosis.

activation of NLRP3 inflammasome and innate immunity have been gradually revealed (27, 52).

TWIK2 is a member of K2P channels, highly expressed in the gastrointestinal tract, blood vessels, and immune system (53). Given that TWIK2 showed no or little conductance in heterologous expression systems, the physiological functions of TWIK2 is poorly understood (54). Interestingly, a recent study demonstrated that pharmacological inhibition or genetic deletion of the TWIK2 channel blocked the activation of NLRP3 inflammasome induced by ATP and thus reduced the release of caspase-1 and IL-1β (27). In contrast, the TWIK2 channel had no effect on the activation of NLRP3 inflammasome activated by imiquimod or nigericin. The TWIK2 channel was mechanistically suggested to cooperate with the P2X7 receptor activated by extracellular ATP, thus mediated potassium efflux required for NLRP3 inflammasome activation.

Furthermore, TWIK2 deletion prevents inflammatory lung injury in sepsis mice (27). Besides TWIK2, THIK1 channel, another member of K2P channels, was recently discovered to play key roles in microglia (52). THIK1 channel was reported to be the main potassium channel expressed in microglia. Pharmacological inhibition or gene knockout of THIK1 depolarizes microglia, decreasing microglial ramification, reducing surveillance function, and IL-1β secretion. This study indicates that THIK1 is necessary for NLRP3 inflammasome activation and immune surveillance in microglia.

THE MECHANISMS OF POTASSIUM EFFLUX DURING NLRP3 INFLAMMASOME ACTIVATION

K^+ efflux is proposed as an important event upstream of NLRP3 inflammasome activation, and the decrease in intracellular K+ can activate the NLRP3 inflammasome; however, the mechanisms of potassium efflux during NLRP3 inflammasome activation is not understood. Macrophages expressing a constitutively active mutant NLRP3 R258W, which could not be suppressed by high extracellular concentrations of potassium, suggests that potassium efflux may be related to NLRP3 protein conformational change (23). Two individual studies show that potassium efflux is essential for NLRP3 and NEK7 interaction, which is an important part of the assembly of the NLRP3 inflammasome, given that the interaction disappears with high extracellular concentrations of potassium (55, 56). These studies suggest that potassium efflux may be closely related to the conformational change of NLRP3 protein and NLRP3-NEK7 interaction during NLRP3 activation, and the underlying mechanism ought to be further investigated. Moreover, K+ efflux might promote NLRP3 activation by mitochondrial dysfunction and mtROS production (57).

SUMMARY AND OUTLOOK

Given the critical role of NLRP3 inflammasome in autoimmune, metabolic, and neurodegenerative diseases and the essential role of potassium efflux in NLRP3 inflammasome activation, it is of great significance to explore the molecular mechanisms underlying potassium efflux during NLRP3 inflammasome activation under different circumstances.

The important role of the P2X7 receptor and GSDMD in immune responses has gained a lot of attention, both academically and industrially (31, 34). An inhibitor JNJ-55308942 targeting the P2X7 receptor is now in phase I clinical study to treat neuroinflammation (58). The role of pannexin-1 in NLRP3 inflammasome activation following caspase-11 or caspase-8 induced pyroptosis is still under debate. Furthermore, although crystalline substances also depend on potassium efflux to activate NLRP3 inflammasome, this process's mechanism is not clear and needed to be resolved in the future.

Last but not least, the lately identified TWIK2 and THIK1 channels were the only "specific" potassium channels involved in NLRP3 inflammasome activation (27, 52). Both TWIK2 and THIK1 channels could be attractive therapeutic targets for the treatment of NLRP3 inflammasome related autoimmune diseases in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by research grants from the National Natural Science Foundation of China (81973333), the Young Scholars of Pearl River in Guangdong Province, and the Natural Science Foundation of Guangdong Province (2019A1515010964) to PZ.

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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.

frontiers in Immunology

REVIEW
published: 21 January 2021
doi: 10.3389/fimmu.2020.611347

STING, the Endoplasmic Reticulum, and Mitochondria: Is Three a Crowd or a Conversation?

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The anti-viral pattern recognition receptor STING and its partnering cytosolic DNA sensor cGAS have been increasingly recognized to respond to self DNA in multiple pathologic settings including cancer and autoimmune disease. Endogenous DNA sources that trigger STING include damaged nuclear DNA in micronuclei and mitochondrial DNA (mtDNA). STING resides in the endoplasmic reticulum (ER), and particularly in the ER-mitochondria associated membranes. This unique location renders STING well poised to respond to intracellular organelle stress. Whereas the pathways linking mtDNA and STING have been addressed recently, the mechanisms governing ER stress and STING interaction remain more opaque. The ER and mitochondria share a close anatomic and functional relationship, with mutual production of, and inter-organelle communication *via* calcium and reactive oxygen species (ROS). This interdependent relationship has potential to both generate the essential ligands for STING activation and to regulate its activity. Herein, we review the interactions between STING and mitochondria, STING and ER, ER and mitochondria (vis-à-vis calcium and ROS), and the evidence for 3-way communication.

Keywords: STING, cGAS, mitochondria, endoplasmic reticulum, unfolded protein response, reactive oxygen species

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *28 September 2020*
Accepted: *04 December 2020*
Published: *21 January 2021*

Citation:
Smith JA (2021) STING, the Endoplasmic Reticulum, and Mitochondria: Is Three a Crowd or a Conversation? Front. Immunol. 11:611347. doi: 10.3389/fimmu.2020.611347

INTRODUCTION

Nature has a dramatic capacity for repurposing. The same pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) on invaders such as bacteria and viruses also respond to endogenous products, particularly those generated during tissue damage (damage associated molecular patterns or DAMPs (1)). For example, Toll Like Receptor 4 (TLR4) not only recognizes bacterial cell wall lipopolysaccharide, but also responds to components of the extracellular matrix such as fibrinogen and fibronectin that are released during infectious and immune damage (2, 3). Not all endogenous PRR stimuli are from infection-mediated tissue damage, as PRRs are also involved in normal physiologic function. For instance, TLRs direct development and cell fate in *Drosophila*, *C. elegans* and mice (4). In the brain, TLRs modulate neuronal connectivity and function (5). "Sterile" PRR engagement also drives pathology: In autoimmune disease (e.g. lupus), nucleotide-activated receptors such as TLR7 (RNA) and TLR9 (DNA) respond to material released from apoptotic cells, regulating inflammation in a cell-specific manner (6, 7).

Most PRRs, such as TLRs, C-lectin type receptors, Retinoic acid-inducible gene I (RIG-I) like receptors, inflammasomes and Nod-like Receptors (NLRs), reside on the plasma membrane, within endosomes or within the cytosol. These locations prime PRRs to respond to both pathogen and endogenous products in the extracellular space or cytosol. In contrast, Stimulator of Interferon Gene (STING) resides in the endoplasmic reticulum (ER), particularly in ER-mitochondrial appositions, with its triggering face to the cytosol (8). This unique location is not only useful for detecting cytosolic invaders; the organelle associations position STING to respond to alarm signals generated by the mitochondria and ER. Interestingly, the multi-molecular inflammation generating machinery triggered by RIG-I family PRRs and inflammasomes, including the lynchpin mitochondrial anti-viral signaling protein (MAVS) aggregates at the mitochondria (9); The MAVS C-terminal transmembrane domain inserts in the outer mitochondrial membrane where it nucleates the formation of filamentous signaling platforms (10, 11). Involvement of, and crosstalk between these organelles may critically contribute to PRR signaling by increasing signal amplitude and providing further context (intracellular stress). In this review, we will focus on the crosstalk between STING, ER and mitochondria. Although many previous lines of inquiry have focused on dyads in this triangle (**Figure 1**, conceptual framework for this review), we posit that DAMP-stimulated STING signaling may reflect three-way communication between these organelles and STING.

THE KEY PLAYERS: CGAS AND STING

Viruses depend exclusively upon host building blocks and machinery to produce the RNA and DNA strands that encode their genomes. During some portion of their lifecycle (e.g. uncoating, creating progeny), viral genomic nucleic acid will be present in the host cytosol. Thus, sensors of cytosolic nucleic acids such as STING constitute a vital defense that has been in place across 600 million years of evolution (12, 13). STING directly binds cyclic-di-nucleotides (CDNs). STING also "senses" cytosolic dsDNA indirectly *via* its "partner" in detection, cyclic-GMP-AMP (cGAMP) synthase (cGAS); upon binding dsDNA, cGAS generates endogenous cyclic-di-nucleotides that serve as the actual STING ligands. Although multiple molecules may detect cytosolic dsDNA in addition to cGAS (e.g. Gamma interferon inducible protein 16 (IFI16), Dead box helicase 41 (DDX41)), cGAS is the primary dsDNA-sensor required for STING activation by dsDNA (14, 15). The role of these other sensors remains unclear, though IFI16 promotes cGAS activation and enhances STING phosphorylation, translocation, and Tank binding kinase 1 (TBK1) recruitment (16, 17). DDX41 may promote IFN-induced cGAS expression (18). cGAS senses cytosolic DNA, but in the resting state in macrophages and other cell types, the vast majority of cGAS resides inside the nucleus, sequestered by chromatin (19–21). One study from Barnett et al. also placed cGAS at the plasma membrane *via* an N-terminal phosphoinositide interaction; the basis for this discrepancy with the other studies is not clear (22).

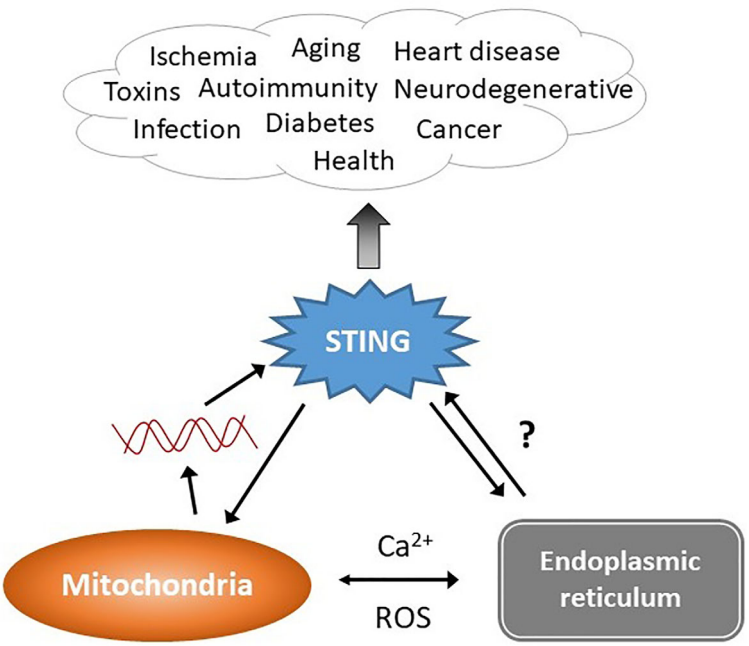

FIGURE 1 | STING stimulation by stressed organelles: an interactive triad. STING plays a critical role in preserving health but also mediates disease, even in the absence of infectious triggers. Mitochondrial DNA (red lines) has recently emerged as a trigger of STING activation. The endogenous ligand mediating the ER-STING reciprocal relationship is not clear. The endoplasmic reticulum (ER) and mitochondria share a very close anatomic and functional relationship, and together modulate homeostatic and pathologic levels of intracellular calcium (Ca^{2+}) and reactive oxygen species (ROS). This relationship may generate the "missing ligand" for ER stress-mediated STING activation *via* mitochondrial DNA release.

Recent cryo-electron microscopy structural data has elucidated the inhibitory relationship between chromatin and cGAS that prevents self-recognition: an acidic patch on the nucleosome histone H2A-H2B heterodimer occupies the dsDNA binding site, preventing cGAS activation and dimerization (23–27). This new information begs the question of how nuclear cGAS responds to pathogenic challenges, as described in the setting of HIV2 recognition (28). Perhaps nuclear proteins such as non-POU domain-containing octamer-binding protein (NONO) extract cGAS from the nucleosomes during infection to enable response to nucleus-located viruses. The mechanism by which nuclear cGAS access cytosolic dsDNA is equally mysterious. Clarification of this process awaits further study.

cGAS recognizes dsDNA at least 45nt in length, retrovirus-transcribed cDNA and Y-form DNA with an overhanging stretch of guanines (29–31). Retroviral triggers of cGAS include both pathogen-derived nucleic acid and potentially endogenous retroviruses (30, 32). cGAS directly binds the DNA deoxyribose sugar phosphate backbone, explaining the sequence-independence of recognition (33). DsDNA recognition is both length and concentration dependent, requiring a size ~1 kb at more physiologic levels (31). DsDNA-bound cGAS forms liquid-phase like droplets, potentially increasing local DNA concentration and valency (34). The formation of two by two structures (2 strands of DNA, 2 cGAS molecules) induces a conformational change in cGAS, which activates its nucleotidyl-transferase enzymatic activity (35). Using ATP and GTP as initial substrates, cGAS catalyzes the production of an asymmetric cyclic-di-nucleotide product with a 2'-5' phosphodiester bond between the 2' hydroxyl of GMP and 5' phosphate of AMP, and a 3'-5' phosphodiester bond linking the 3- hydroxyl of AMP back to the 5' phosphate of GMP, referred to as 2'3'-cGAMP (36, 37). STING also binds the bacterial second messenger cyclic-di-GMP, which was the first identified ligand for STING, cyclic-di-AMP produced by Gram-positive bacteria such as *Staphylococcus* and *Listeria*, and bacterial origin 3'3'-cGAMP (38–40). Interestingly, the affinity of STING for bacterial products is much lower (>1–2 logs) than the endogenously generated 2'3'-cGAMP, suggesting an anti-viral evolutionary priority (37, 41, 42). Another possibility is that bacteria serendipitously coopted STING's CDN-binding capacity to enhance type I interferon (IFN) production by the host cells, which benefits multiple bacterial species (13, 43, 44).

In its inactive state, the STING molecule, which has 4 membrane-spanning helices, resides in the ER plasma membrane as a dimer, with its v-like CDN binding domain facing the cytosol (45). Upon binding cGAMP, STING undergoes a conformational change that enables a lid-like 4-pass beta sheet to flop down over the CDN binding site in a "closed" position, and rotates the cytosolic portion 180 degrees. This rotation allows for higher order STING oligomerization and lateral stacking (46–48). CDNs such as cyclic-di-GMP stabilize STING in a more open position vs. cGAMP, perhaps explaining their lower affinity and activity (41, 49). In most vertebrates, with few exceptions, this lid also contains a flexible extended random coil C-terminal tail (CTT, amino acids 341–379 in humans) that has binding sites for TBK1 family kinases (TBK1 and Inhibitor of nuclear factor kappa B kinase (IKK)ε) (50). Some TBK1 associates constitutively with STING dimers, but the TBK1 dimer's kinase domains face away from each other, preventing cis-phosphorylation (51). The higher order structures promote TBK1 trans-phosphorylation and activation. STING phosphorylation on Thr376 enhances TBK1 association ~20-fold (47). TBK1 also phosphorylates STING on Ser365/366 (mouse/human), forming a binding site for the interferon-regulatory transcription factor IRF3 (52). TBK1 phosphorylates IRF3, enabling the dimerization of IRF3 required for nuclear entry (53). The kinase domain of STING-attached TBK1 cannot access the cis-IRF3 molecule and thus relies on the close proximity of other TBK1 molecules to accomplish IRF3 activation (51). Changes in STING localization appear to be very important for specific activation steps in the TBK1-IRF3 signaling pathway. Following CDN binding, STING transits *via* the ER Golgi intermediate compartment (ERGIC) to the Golgi in a Coat protein complex II (COPII), Sar1 GTPase, ADP ribosylation factor (Arf1)-dependent manner (54, 55). Blocking this transition with agents like Brefeldin A, or the *Shigella flexneri* IpaJ protein prevents TBK1 association and phosphorylation (55, 56). Activated TBK1-STING then clusters together in peri-nuclear punctae where IRF3 is phosphorylated in multi-molecular "signalosome" complexes. These multi-molecular complexes also result in the activation of nuclear factor kappa-B (NF-κB), which then cooperates with IRF3 to induce the prototypic IFN gene *IFNB1* and promotes pro-inflammatory cytokine transcription. For a summary of cGAS-STING activation, see **Figure 2**.

STING is most widely known for IFN stimulation, and secondarily pro-inflammatory cytokine stimulation *via* NF-κB. However, STING triggers multiple signaling cascades: STING activates MAP kinase signaling, STAT6, inflammasomes (e.g. NLR family pyrin domain containing 3 (NLRP3)), autophagy and apoptosis (57–62). STING also suppresses translation, inhibiting viral infection, independently of eukaryotic initiation factor 2α (63). The detailed mechanisms by which STING initiates these different functions remain to be elucidated. Consider NF-κB activation for example: multiple reports document the necessity of the CTT and TBK1 for NF-κB activation (50, 58, 64). TBK1 does appear to be critical for IRF3 activation, an observation that has borne up over time. However, in myeloid cells, either TBK1 or IKKε can mediate NF-κB activation (50). TBK1 or IKKε activates Mitogen-activated protein kinase kinase kinase 7 (TAK1) and thus inhibitor of nuclear factor kappa-B kinase subunit (IKKβ/IKKα), resulting in inhibitor of κB (IκB) phosphorylation, IκB proteasomal degradation and nuclear factor kappa B (NF-κB) nuclear translocation (58). In myeloid cells, STING-dependent NF-kB activation did not require Tumor necrosis factor associated factor 6 (TRAF6). An alternatively spliced form of STING lacking the CTT, designated as MITA-related protein (MRP), functions as a dominant negative of IFN production yet activates NF-κB signaling independently of TBK1 (65). In the setting of genotoxic DNA damage, STING activates NF-κB independently of cGAS (and cGAMP) *via* association with p53, TRAF6, and IFIT16 (66). The zebrafish STING CTT contains an extra tail-end module that enhances NF-κB activation through increased recruitment of TRAF6 (67). In zebrafish, TRAF6 was essential for both NF-κB and IRF3 activity. Interestingly, TBK1 deletion in

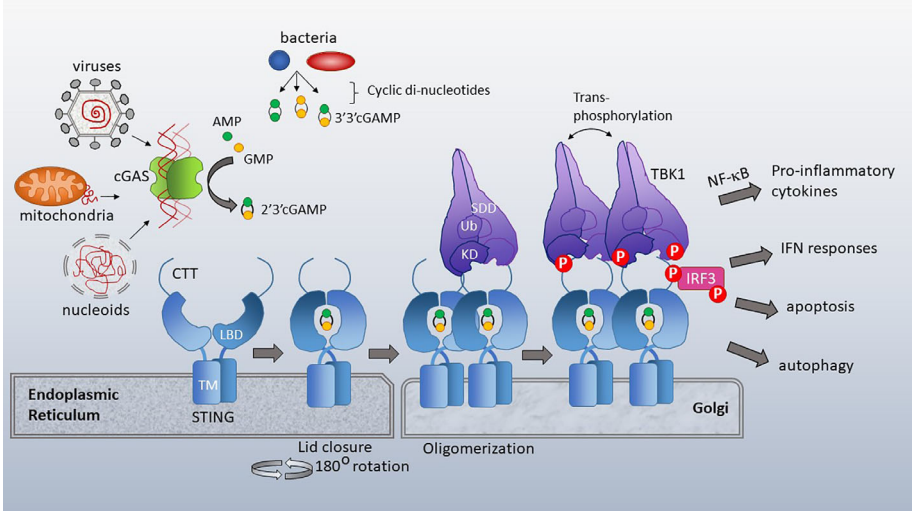

FIGURE 2 | cGAS and STING activation. Cytosolic dsDNA from viruses, mitochondria or nucleoids formed during nuclear breakdown bind cGAS, triggering its catalytic formation of 2'3' cGAMP. 2'3' cGAMP serves as a ligand for STING, which resides in the ER with its ligand binding domain (LBD) facing the cytosol. TM=transmembrane domain. Bacterial cyclic-di-nucleotides, such as cyclic-di-AMP, cyclic-di-GMP and 3'3' cGAMP also bind STING. Upon ligand binding, the cytosolic domains of STING close over the di-nucleotide ligand and rotate 180 degrees, enabling lateral stacking. STING translocates to the Golgi where it oligomerizes. This oligomerization enhances trans-phosphorylation of the STING CTT (C terminal tail)-associated TBK1 family kinases. TBK1 has a scaffold and dimerization domain (SDD), ubiquitin like domain (Ub) and Kinase domain (KD). Activated TBK1 phosphorylates the STING CTT, enabling recruitment and subsequent phosphorylation of IRF3. TBK1 family kinases also activate signaling pathways leading to NF-κB nuclear translocation. STING activation has diverse immune stimulatory outputs including pro-inflammatory cytokine responses (via NF-κB), interferon responses (via IRF3), apoptosis and autophagy. STING/TBK1 structural cartoon adapted from (51).

zebrafish only decreased IFN production by ~60% and NF-κB not at all, suggesting some flexibility and substitution capacity in STING modular functional domains. Together, these studies support context-dependent requirements for TBK1-family kinases and specific NF-κB activating pathways.

Evolution poses other questions regarding primordial STING function and NF-κB activation. Recognizable STING and cGAS orthologs are present in unicellular choanoflagellates, pre-dating NF-κB (68). *Nematostella vectensis*, a sea anemone that diverged from human ancestors >500 million years ago, possesses a STING molecule with only ~29% aa identity to human STING, but virtually an identical crystal structure to the STING core (12). Interestingly, the Mab-21 domain containing nucleotidyl-transferases such as cGAS date back as far as STING, but the *Nematostella* homologue makes 3'3'-cGAMP, not 3'2'-cGAMP (12). *Nematostella* cGAS also lacks the zinc-binding region present in vertebrates that is required for dsDNA binding. TBK1 and NF-κB also date back to *Nematostella*, but the CTT only developed in vertebrates, so it is not clear if primordial (pre-CTT) STING stimulates NF-κB in a TBK1-dependent manner (13). Thus, even though all the components were present early in evolution, their interactions and scope of activity remain a mystery.

The mechanisms by which STING induces autophagy are also not entirely clear. It has been proposed that the STING core moiety (lacking the CTT) contains a primordial autophagy function: In reconstitution experiments in HEK293 cells, the STING core was sufficient for initiating autophagy upon stimulation with exogenous cGAMP. Further, this core autophagy function exerted anti-viral activity – particularly against DNA virus such as Herpes Simplex Virus 1 (HSV1), but not the RNA virus Sendai virus (SeV). In this report, upon transit to the Golgi, the CTT-deleted STING core initiated Microtubule-associated protein 1A/1B-light chain 3 (LC3) lipidation through a non-canonical mechanism involving WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) and Autophagy related 5 (Atg5), but not Unc-51 Like Autophagy Activating Kinase 1 (ULK1) and the Vacuolar protein sorting 34 (VPS34) complex. Although Mammalian target of rapamycin (mTOR) regulates autophagy, STING did not dephosphorylate or inhibit mTOR (54). However, in a recent report examining S365A and CTT deletion mutants in mice, HSV-1 viral resistance was IFN independent, but required the CTT and TBK1 for both autophagy and viral resistance. S365 phosphorylation was also important for enhancing NF-κB activation in macrophages (64). Multiple components of the cGAS-STING activation cascade interact with autophagy related molecules and pathways: TBK1 has been noted to activate mitophagy *via* phosphorylation of Calcium Binding And Coiled-Coil Domain 2 (NDP52), p62, TAX1BP1 and optineurin (69). Following translocation to the Golgi, STING co-localizes with p62, LC3 and Atg9a (59). cGAS may also participate in autophagy induction independently of STING: cGAS binds Beclin1, releasing Run domain Beclin-1 interacting and cysteine-rich containing (RUBICON, a potent negative regulator of autophagy) thus stimulating autophagy (70). In summary, multiple signaling routes link STING-cGAS signaling with autophagy, and activation of any particular pathway(s), or dependence upon specific STING moieties, may be context-dependent.

STING also stimulates apoptosis and cell death *via* multiple mechanisms (71). During "intrinsic" apoptosis, mitochondria

form Bcl2 Associated X (BAX)/Bcl2 antagonist killer 1 (BAK)-dependent micropores, resulting in mitochondrial outer membrane permeabilization, release of cytochrome c, and caspase activation (72). BAX/BAK macropores subsequently enable herniation of inner mitochondrial membranes and extrusion of mitochondrial DNA (mtDNA) (73). STING promotes phosphorylation of receptor-interacting protein kinase 3 (RIP3), which activates p53 upregulated modulator of apoptosis (PUMA, another pro-apoptotic Bcl2 family member), leading to mitochondrial outer membrane permeabilization (74). IRF3 and p53 also coordinately upregulate PUMA and Noxa (60). Moreover, activated IRF3 binds BAX directly *via* its BH3 domain, stimulating apoptosis (75). STING activation also leads to mitochondria-induced apoptosis indirectly *via* ER stress and its multiple pro-apoptotic programs (more on this connection below and in (71)). When apoptosis is inhibited by infection or genetically, STING-dependent type I IFN and TNF promote regulated necrosis or "necroptosis" (76, 77). Depending upon STING signaling strength and cell type, STING trafficking to lysosomes following autophagy induction results in lysosomal permeabilization and so called "lysosomal cell death" (61). Lysosomal rupture induces potassium efflux, and secondary NLRP3 activation, stimulating pyroptosis (61, 78). It should be noted however, that STING-inflammasome cooperation does not invariably increase pyroptosis (62).

Both cGAS and STING are subject to multiple types of transcriptional and post-translational modifications and regulation, reviewed extensively elsewhere (79–81), with a few examples presented here. IFN increases cGAS and STING expression, driving a positive feedback loop (18, 82). Both cGAS and STING expression are suppressed by DNA methylation in many tumors (83). Palmitoylation of STING in the Golgi is essential for its oligomerization and activity (84). cGAS can be inhibited by Protein Kinase B (Akt) phosphorylation and glutamylation (TTLL4 and TTLL6) (85, 86). Complex ubiquitination can activate or inhibit cGAS and STING by targeting them for degradation (79). The autophagic flux stimulated by STING may facilitate its lysosomal destruction post-stimulation in an ULK1-dependent manner (87). The same pro-apoptotic caspases stimulated by cGAS-STING operate to inhibit their activation: During intrinsic apoptosis, initiator and effector caspases (Caspase 9 and Caspase 3) result in cleavage of cGAS, STING and IRF3, thus limiting further STING signaling (88, 89). Inflammasome processed caspase1 also cleaves cGAS and inhibits its enzymatic activity following Gasdermin-D dependent K+ influx (90, 91). Regulation of STING/cGAS activity by ROS and calcium will be described below.

STING IN THE "STERILE" PATHOLOGY OF DISEASE STATES

Although cGAS and STING are poised to respond to pathogens, increasing evidence supports their critical role in a number of "sterile" physiologic and pathologic conditions, including cancer, heart disease, diabetes, neurodegenerative disease, lupus as well as normal aging/cellular senescence (**Figure 3**) (92–98). For example, gain of function mutations in STING and diminished nuclease activity lead to distinctive IFN-driven autoinflammatory conditions such as STING Associated Vasculopathy presenting in Infancy (SAVI) and Aicardi Goutieres syndrome, respectively (93). Increased STING trafficking to the Golgi (and sustained activation) results in autoimmunity in COPA syndrome (99). Lupus patients show a strong type I IFN signature in their peripheral blood, and a sub-group of lupus patients (~15%) has elevated circulating cGAMP (100, 101). However some autoimmune conditions dependent upon other PRRs may be regulated by STING and worsen with STING deficiency (102, 103). In regards to cancer, STING essentially mediates the anti-tumor effect of radiation (104). IFN promotes maturation and antigen presentation by CD8α+ dendritic cells (DC) and thus priming and activation of tumor infiltrating CD8 T cells and Natural Killer (NK) cells (49, 105). Related to these consequences of STING activation, STING agonists have shown promise as anti-cancer therapeutic agents (106). On the other hand, STING can promote toleragenic responses *via* Indoleamine-pyrrole 2,3-dioxygenase (IDO), especially with low antigenicity tumors, and induce the T cell exhaustion stimulus Programmed death ligand 1 (PDL1) (49, 107, 108). Inter-tumor cell cGAMP transfer and subsequent STING activation can also facilitate metastasis (109). Regarding cardiovascular disease, cGAS critically mediates inflammation post-myocardial infarction (MI), and induces CXCL10, iNOS expression and M1 differentiation. In this setting, STING or cGAS deficiency improved post-MI survival (110). STING activity may attenuate type I diabetes, but exacerbate type II diabetes and the associated metabolic syndrome (97, 111, 112). The dual positive and negative effects of STING on health mandate caution in modulating STING activity therapeutically.

One of the major questions posed by these observations relates to the source of "endogenous" STING/cGAS ligands in sterile pathology. In health, self DNA might be expected to be sequestered in the nucleus and within mitochondria. However, it has become clear that nuclei and mitochondria are not as "air tight" as previously thought. To maintain the status quo in health, nucleases patrol the cytosol and extracellular milieu, degrading rogue cytosolic nucleic acids. Even in the face of this nucleotide cleanup crew, increasing evidence suggests mtDNA and under certain conditions, nuclear and extracellular DNA serve as stimuli for STING. Extracellular dsDNA from apoptotic cells can be taken up by endocytosis, or in the forms of microvesicles and exosomes (113, 114). Internalized dsDNA translocates from lysosomes (especially if deficient in DNAse) into the cytosol (115). Extracellular cGAMP that evades Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) degradation can also be taken up by and stimulate cells (116). Studies in senescence, infection, neurodegenerative diseases, cancer and lupus have greatly elucidated the generation (and recognition) of mitochondrial and genomic DNA ligands. Genomic DNA will be discussed briefly, but the remaining focus will be on mtDNA.

Without killing the involved cell, genotoxic stress can result in partial breakdown of the laminin nuclear envelope structure and extrusion of so-called "micronuclei" into the cytoplasm (117). In senescence, telomere dysfunction and breakdowns in DNA repair lead to genotoxic stress. In cancer, genotoxic stress may

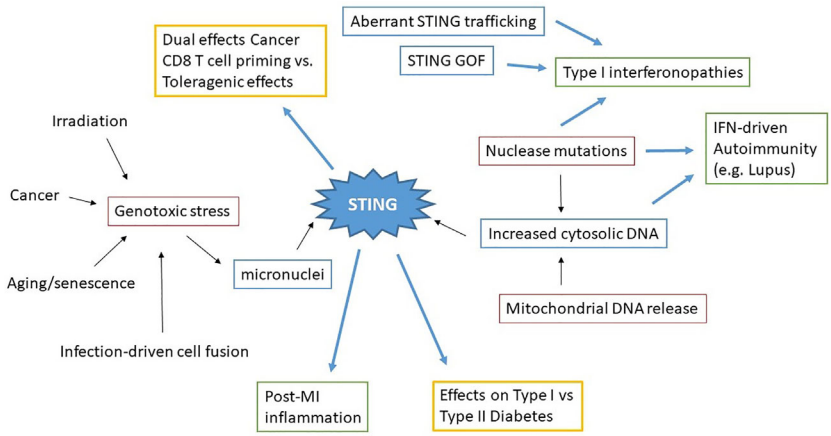

FIGURE 3 | Concept map of STING in sterile pathology. Irradiation, cancer, aging/senescence and infection can drive genotoxic stress, resulting in the generation of micronuclei. Nuclease mutations, deficiencies, and mitochondrial DNA release lead to increased cytosolic dsDNA. These immediate drivers of cytosolic dsDNA are in red boxes. STING aberrantly activated through these processes, as well as STING mutations and altered STING regulation (blue boxes) all result in pathologic disease states. Excess STING-dependent IFN and inflammatory cytokines contribute to pathology (green boxes) in Type I interferonopathies, autoimmunity and post-MI (Myocardial Infarction). However the effects of STING on other types of pathologies (Cancer, Diabetes) can vary (yellow boxes) depending upon the specific situation.

result from aberrant mitosis, mutation in DNA-repair enzymes as well as exogenously applied radiation. Micronuclei only form during cell division, and breakdown of the micronuclei envelope during mitosis is required for recognition (117, 118). This damaged genomic DNA in micronuclei then becomes an available substrate for cGAS (95). It is unclear how the extra-nuclear DNA avoids nucleosomal inhibition of cGAS, unless the DNA dissociates from histones. The chromatin state in micronuclei is not well described. Once cytosolic micronuclei dsDNA stimulates cGAS, the resulting cGAMP can also transfer across cellular boundaries *via* gap junctions (109, 119). For instance in cancer, cGAMP from tumor cells transfers to STING-expressing myeloid and B-cells that produce natural killer cell-stimulating IFN (120). One bacterium, *Burkholderia pseudomallei*, induces cell fusion, genomic instability and aberrant mitosis resulting in micronuclei formation. Interestingly, in this case, the micronuclei triggers STING activation and IFN transcription but not IFN protein, and STING/autophagy-dependent cell death. Polyethylene glycol induced cell fusion also triggers IFN gene expression and autophagy, suggesting a critical role for the cell fusion process (121). Micronuclei detected in human Huntington's disease embryonic stem cell-derived neurons have been linked to inflammation and autophagy (122).

FURTHER CUES ON ENDOGENOUS LIGANDS FROM LUPUS AND RELATED CONDITIONS: IMPORTANT ROLES FOR NUCLEASES AND MITOCHONDRIAL DNA

Discovery of the linkage between nuclease deficiencies and type I interferonopathies has thrown the requirement for nucleotide "clean-up" into sharp relief. The cytosolic nuclease TREX1/DNAseIII was one of the first described molecular associations with type I IFN-driven diseases (123, 124). TREX1 deficiency and mutations have been implicated in Aicardi-Goutieres syndrome (AGS), Familial chilblain lupus, systemic lupus erythematosus (SLE) and retinal vasculopathy with cerebral leukodystrophy (RVCL) (123, 125–127). Full deletions are more likely to be associated with the severe early-onset manifestations, as in AGS, whereas heterozygous deficiencies are more common in complex milder or later onset conditions, such as familial chilblain lupus (126). AGS also results from defects in RNAse H2, which cleaves RNA from DNA to decrease DNA damage, and SAM and HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1 (SAMHD1), a dNTPase that acts at stalled replication forks and regulates reverse transcription to cDNA (128–130). Mice deficient in extracellular DNAseI develop high titer anti-nuclear antibodies and glomerulonephritis (131). Lysosomal DNAseII deficiency is embryonic lethal in mice, but completely rescued by cGAS or STING absence. Interestingly *DNaseII-/-Ifnar* (type I IFN receptor)-/- mice survive to adulthood, but develop rheumatoid arthritis-like disease, most likely reflecting the NF-κB stimulating activity of STING (132). TREX1, which is located in the cytosol and localizes to the ER, is active against ssDNA, nicked end dsDNA, and retroviral cDNA (produced for instance during HIV-1 infection) (133). TREX1 may also degrade endogenously transcribed retroviral elements (124, 134). Mutations in the DNAse region are mostly associated with AGS, whereas DNAse intact C-terminal truncations have been identified in RVCL and SLE (93). The non-DNAse TREX1 functions may be mediated through its association with and regulation of oligosaccharyltransferase, as C-terminal TREX1 mutations result in production of large amounts of immunostimulatory free glycans (135).

The association of SLE-like type I IFN driven diseases with these rare nuclease mutations serves as proof of principle

regarding the importance of endogenous DNA in driving autoimmune disease. Work over the last decade has shed light on pathogenic mechanisms that may be more prevalent. Cells from lupus patients, most prominently neutrophils tend to extrude their nuclear contents in stringy structures rich in histones and dsDNA known as neutrophil extracellular traps (NETs). These NETS also contain inflammatory proteins such as Cathelecidin LL37 antimicrobial peptides and High Mobility Group Box 1 (HMGB1) (136). Wang et al. reported the presence of mitochondrial (mt)DNA in ex-vivo stimulated NETs and increased anti-mtDNA antibodies in lupus patients. These antibodies correlated with IFN scores and disease activity scores, including lupus nephritis scores, better than the standard anti-dsDNA. This group also found mtDNA in NETs in SLE subject kidney biopsies. MtDNA-anti-mtDNA complexes were strong stimulators of plasmacytoid DC IFN, even more so than anti-dsDNA. This group then performed a proof of concept trial with metformin, which decreases mitochondrial respiratory chain complex I and NADPH oxidase activity, suggesting that oxidation played a key role in pathogenesis (137). MtDNA lacks protective histones and DNA repair enzymes present in the nucleus and thus, is more susceptible to oxidation. Oxidized DNA is also resistant to TREX1 degradation, making this a plausible scenario (138). This study concluded the IFN was TLR9-dependent, but the evidence was very indirect.

Two further studies from 2016 further explored the relationship between oxidized mtDNA and lupus. Caielli et al. reported that neutrophils from healthy subjects that sustain mitochondrial damage extrude DNA. This damaged mtDNA dissociates from the mitochondrial transcription factor A mitochondrial (TFAM) molecule that packages it into nucleoids en route to lysosomes for degradation. Dissociation requires protein kinase A (PKA)-mediated TFAM phosphorylation. Exposure of either type I IFN-treated neutrophils or neutrophils from lupus patients to anti-RNP immune complexes decreased the cAMP required for PKA activation. The TFAM-associated nucleoids remained in the cytosol, became oxidized and were released from the cell through unclear means. TLR9 and RAGE participated in uptake of the TFAM-associated oxidized mtDNA nucleoids by DC, thereby stimulating IFN production. In support of this mechanism, oxidized mtDNA autoantibodies were present in a fraction of patients and oxidized mtDNA nucleoids visualized in SLE patient neutrophils (139). In the report by Lood et al, they tied oxidized mtDNA to STING-dependent IFN as follows: anti-RNP immune complex stimulation increased mitochondrial ROS. Mitochondrial ROS resulted in hypopolarization, translocation of mitochondria to the plasma membrane and release of oxidized mtDNA into the extracellular milieu. This oxidized mtDNA was a potent stimulus of IFN production by peripheral blood mononuclear cells (PBMC) and monocytic THP1 cells. In mice, injection of oxidized mtDNA induced IFN in a STING-dependent manner. Furthermore, lupus patient low-density granulocytes spontaneously released NETS enriched in mtDNA in a mitochondrial ROS-dependent manner. As proof of principle, they administered a mitochondrial ROS antagonist (mitoTEMPO) to mice continuously *via* a pump, decreasing disease severity in lupus prone MRL/lpr mice. MtDNA oxidation occurred independently of NADPH-oxidase, in *Nox* knockout mouse cells and chronic granulomatous disease patients (140). Together these reports firmly establish a link between mitochondrial ROS, oxidized mtDNA and IFN generation in lupus (summarized in **Figure 4**). Some of the differences, for instance TLR9 vs. STING dependence, might reflect species in some experiments (mouse vs. human) as well as mtDNA stimulated target cell (DC vs. PBMC and monocytes).

Beyond lupus, mtDNA appears to play a role in STING activation in other sterile diseases, such as cancer, and toxin-stimulated injury. Cisplatin-induced acute kidney injury depended upon mitochondrial damage and stimulation of STING. Interestingly, in this report, STING stimulated NF-κB but not type I IFN production, yet more evidence that different STING outputs can be uncoupled (141). In regards to cancer, oxidized mtDNA sensing by STING promoted the antitumor effect of irradiated immunogenic cancer cells (142). DC appropriated oxidized mtDNA released from dying irradiated tumors and then cross-presented antigen to cytotoxic CD8 T cells.

Mitochondria also mediate STING stimulation in a variety of infectious settings, in effect, functioning as both DAMP and PAMP. STING plays an unanticipated role in responding to RNA viruses, with mitochondria mediating the interaction. Interestingly, many of these viruses express mitochondria targeting proteins. Dengue virus M protein targets the mitochondrial membrane, forming pores that result in swelling and loss of membrane potential (143). NS2B3 cleaves mitofusins 1 (Mfn1) and Mfn2, influencing the structure and function of mitochondria (144). Dengue-induced mitochondrial stress and damage results in release of mtDNA into the cytosol (145). NS2B3 also directly cleaves STING, limiting production of type I IFN (146). Encephalomyocarditis and influenza induce mtDNA release into the cytoplasm *via* viroporins, stimulating cGAS and DDX41-dependent immune responses (147). Although Herpesviruses are DNA viruses, the HSV1 gene product UL12, that depletes TFAM and results in enlarged mitochondrial nucleoids and mtDNA release, is essential for full IFN production and anti-viral activity (145). Different strains of *Mycobacterium tuberculosis* induce varying amounts of mitochondrial stress and mtDNA release stimulating cGAS/STING-dependent IFN (148, 149). *M. abscessus* induces IFN and NLRP3 activity *via* mitochondrial oxidative stress. In this setting, IFN and IL-1β exhibited a mutually inhibitory reciprocal relationship (150).

STING AND THE ER: CROSS TALK AND CROSS REGULATION

The studies highlighted above describe a connection between mitochondria and STING activation *via* the release of mtDNA, which is oxidized in many cases. Increasing evidence also supports communication and cross-regulation between the ER and STING, in which an ER stress response known as the "Unfolded Protein Response" (UPR) takes center stage (UPR recently reviewed in (151) and in (152), **Figure 5**). The ER serves as the protein-producing factory of the cell. Different types of physiologic

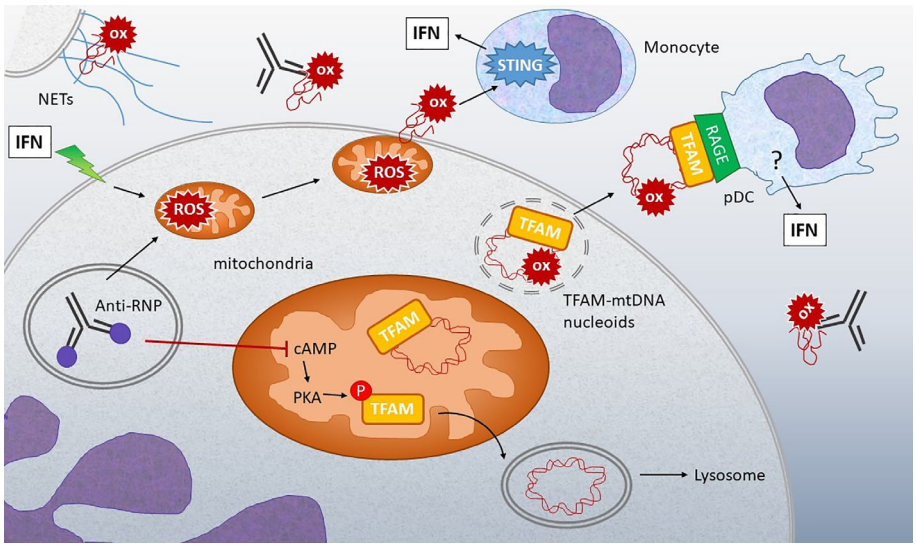

FIGURE 4 | Connection between oxidized mtDNA and Lupus. Stimulation of IFN-treated neutrophils or neutrophils from lupus patients with anti-RNP immune complexes can lead to release of oxidized (ox) mtDNA by multiple mechanisms: 1) Stimulation of NETosis, with extrusion of DNA containing oxidized mtDNA. 2) Increased mitochondrial ROS leads to membrane translocation and extrusion of oxidized mtDNA into the extracellular milieu. 3) Anti-RNP and type I IFN decrease the levels of cyclic AMP (cAMP), a second messenger required for activation of protein kinase A (PKA), which normally phosphorylates TFAM, enabling its release from mtDNA. When TFAM is released, the mtDNA can then go to the lysosome for degradation. If PKA is inhibited, TFAM remains associated with mtDNA in nucleoids that accumulate in mitochondria and then are released from the neutrophils through unclear mechanisms. Extracellular oxidized mtDNA is sensed by monocytes in a STING-dependent manner and internalized by pDC *via* RAGE receptors. Downstream of RAGE, the IFN-generating sensor in pDC is unclear, although both oxidized and non-oxidized mtDNA stimulation of pDC is TLR9-dependent (139). The abundance of anti-mtDNA antibodies in lupus and correlation with disease support the critical involvement of these mechanisms in disease pathogenesis (137, 139, and 140).

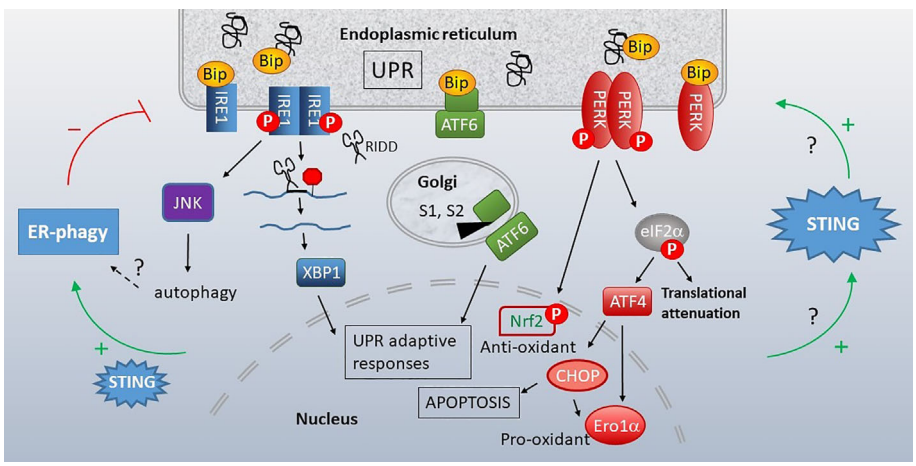

FIGURE 5 | Unfolded Protein Response (UPR), STING and autophagy. When cellular insults or protein production demands compromise ER function, the ER initiates the UPR. Misfolding proteins bind the chaperone BiP, releasing it from three stress sensors, IRE1 (blue), ATF6 (green) and PERK (red). IRE1 is a bifunctional kinase/endonuclease that initiates JNK-dependent signaling and excises a 26bp stretch from the XBP1 mRNA, removing a premature stop codon *via* frameshift mutation. IRE1 also decreases ER load through more promiscuous endonuclease activity (RIDD). Upon release of BiP, ATF6 translocates to the Golgi, where S1 and S2 proteases generate an active transcription factor. PERK kinase phosphorylates eIF2α, resulting in global translational attenuation apart from select mRNAs such as ATF4. ATF4 promotes transcription of the pro-apoptotic transcription factor CHOP and Ero1α oxidoreductase. PERK also leads to nuclear factor erythroid 2–related factor 2 (Nrf2) nuclear translocation and resulting anti-oxidant responses. The UPR promotes STING activity and STING increases the UPR (right green arrows). This STING-dependent increase in UPR also enhances autophagy of ER components ("ER-phagy", left side), which can limit ER stress responses. Many questions remain regarding the mechanistic details connecting STING, UPR and ERphagy.

demands and insults that impact protein folding, including increased protein production, misfolding proteins, nutrient deprivation, hypoxia, calcium and oxidative dysregulation, all lead to induction of the UPR. The UPR encompasses three primary signaling arms set in motion by the activation of ER-membrane associated stress sensors, serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α (IRE1), Protein Kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Very briefly, in the absence of stress, these sensors associate with an ER-protein folding chaperone GRP78 (Immunoglobulin heavy chain binding protein (BiP)). An overabundance of unfolded protein results in the release of these sensors from BiP, thus activating UPR signaling. IRE1 is both a kinase and endonuclease which processes the X-box binding protein 1 (XBP1) transcription factor mRNA, yielding the active transcription factor. XBP1 promotes production of ER chaperones, ER associated degradation (ERAD) proteins, and ER expansion. In certain settings, IRE1 also displays non-specific endonuclease function decreasing protein load, a process termed Regulated IRE1-dependent decay (RIDD). IRE1 kinase signals *via* NF-κB and JNK pathways, stimulating inflammation, autophagy and apoptosis. PERK is a kinase whose activity results in the phosphorylation of eukaryotic initiation factor 2 α (eIF2α). Phosphorylation inhibits global mRNA translation apart from select transcripts with 5'Cap-independent upstream open reading frames such as ATF4. PERK regulates amino acid acquisition, redox status and apoptosis *via* induction of the C/EBP Homologous Protein (CHOP) transcription factor. ATF6 is a proto-transcription factor that traffics to the Golgi following BiP dissociation. There, Site1 and Site2 proteases cleave ATF6 to an active transcription factor that induces ER chaperones and with XBP1, increases ER capacity. As the UPR accomplishes much of its adaptive program through gene transcription, it is often monitored experimentally by quantitating UPR target gene expression. Together these pathways re-establish proteostasis (proteome homeostasis) by decreasing protein load, at least temporarily, and enhancing ER function. If ER stress is profound or fails to resolve, these pathways trigger apoptosis.

The UPR intersects with, and activates pro-inflammatory signaling [extensively reviewed elsewhere (153–155)]. Moreover, cells undergoing ER stress respond to PRRs with synergistic cytokine production; thus, the UPR acts as an amplifier for pathogen recognition (153, 156, 157). In TLR4-stimulated macrophages, IFN-β was one of the most dramatically enhanced cytokines by ER stress (157). Further, using chemical UPR inducers and oxygen-glucose deprivation, we found that ER stress was sufficient for phosphorylation and nuclear translocation of IRF3 (158). Interestingly, IRF3 activation was only STING-dependent for certain types of ER stress induction, such as oxygen glucose deprivation and treatment with Thapsigargin, which induces ER stress *via* inhibition of the calcium-regulating sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump (159). Oxygen glucose deprivation can also increase cytosolic calcium *via* modulation of SERCA, Ryanodine receptors (RyR) or other receptors (160, 161). Tunicamycin, an UPR-inducing N-linked glycosylation inhibitor resulted in IRF3 phosphorylation through a STING-independent, but ATF6-dependent mechanism (158). Calcium may be key for STING activation by the UPR, but increased cytosolic calcium, introduced with a calcium ionophore, was not sufficient in the absence of ER stress.

The linkage between ER stress and STING activation detected with *in vitro* pharmacologic manipulation was observed in several disease models: Patrasek et al. reported that in early alcoholic liver disease, alcohol induced ER stress, which resulted in STING activated IRF3 and IRF3-dependent apoptosis (162). In traumatic brain injury, a PERK inhibitor abrogated STING activation and ameliorated damage (163). In a report from Cui et al, *Mycobacterium bovis* STING activation of TBK1 and IRF3 was dependent upon ER stress (164). In this study, IRF3-BAX-initiated apoptosis required TBK1 activity. We found that during *Brucella abortus* infection, induction of the UPR was critical for STING activation and induction of the STING-dependent type I IFN program genes. However, this study also brought up an interesting, somewhat thorny observation: Induction of the UPR was STING dependent (lower in STING-/- cells), so there is reciprocal regulation. Further, type I IFN enhanced UPR induction (165). This type of reciprocal crosstalk was also described in a report on heart inflammation and fibrosis. Angiotensin II induced STING expression and increased IFN in cardiac myocytes in an ER stress dependent manner. In this study, STING-/- mice exhibited decreased ER stress following aortic banding (166). Moretti et al. described STING activated ER stress and autophagy induction in the setting of *Listeria innocua* infection. *Listeria* c-di-AMP stimulated STING, resulting in upregulation of ER stress markers, inhibition of mTOR and autophagy. In this particular type of autophagy, "ER phagy", ER membranes that were autophagocytosed included ER markers, ER stress proteins and STING. They observed a yin-yang relationship between ER stress and autophagy: ERphagy reduced ER stress (especially the PERK pathway). Inhibition of autophagy (and increased ER stress) resulted in apoptosis. PERK deletion decreased IFN production and autophagy, suggesting an ER-stress feed forward mechanism (167). Putting these studies together, ER stress can induce STING activity and STING increases ER stress and ER stress-dependent autophagy.

A report by Wu et al. examined the molecular basis for the UPR-STING connection by focusing on a STING gain of function (GOF) mutant associated with SAVI (168). Patients afflicted with SAVI develop early onset vasculitis, rash and interstitial lung disease (169). SAVI is largely IRF3-independent in mice, suggesting non-IFN STING activities are important drivers of the inflammatory disease (170). Wu et al. found increased expression of UPR markers, BiP and CHOP in GOF human T cells, less so in B cells and not in macrophages, MEFs or fibroblasts (UPR was cell type-dependent). In the Jurkat cell line, the STING GOF mutant was not sufficient for UPR induction but synergized with T cell receptor (TCR) stimulated ER stress. In wild type murine T cells, TCR engagement typically induces ER stress, but not apoptosis. In the wild type T cells, the strong STING agonist DMXAA and TCR stimulation, but not DMXAA alone, significantly increased ER stress. Similarly, the GOF STING mutant synergized with TCR signaling to increase

ER stress and IRF3-independent apoptosis. Furthermore, Wu et al. defined a requisite "UPR motif" within STING, in aa322–343, a highly conserved sequence encoding a helix on the exterior of the STING dimer, next to the CDN ligand-binding domain. Residues R331 and R334 were particularly important for UPR function. A deletion around the IRF3 binding site (343–354) abolished IFN, but not UPR or NF-κB outcomes (168).

STING AND CALCIUM

In addition to the UPR, a second related aspect of ER function, calcium regulation, has been implicated in STING activation and function. The ER serves as the primary calcium storage within the cell, maintaining a huge gradient across the ER membrane. ER calcium concentrations are estimated at 2mM with free calcium at 500μM, whereas cytoplasmic calcium is in the 100–100 nM range (171, 172). These high concentrations of calcium are critical for optimum function of the ER protein-folding machinery. ER-resident calcium binding proteins with low affinity but high capacity include the chaperones calreticulin, calnexin, BiP, grp94 and Protein disulfide isomerase (PDI) (173). Calreticulin and calnexin work with Erp57, the thiol disulfide isomerase, to form disulfide bonds and promote protein folding (174). Calreticulin and calnexin also direct protein trafficking through the ER and ERAD (175). The BiP ATPase prevents protein aggregation (176). Calsequestrins and Chromogranins further buffer ER calcium. Three families of proteins, SERCA, Inositol triphosphate receptors (IP3R) and RyRs, mediate the tremendous ER-cytosol gradient and regulate calcium release. Expression and relative roles of these proteins is cell-dependent. For instance, there are 3 SERCA genes with multiple splice variants, but SERCA2b is most widely expressed, has the highest calcium affinity of the SERCAs and is primarily responsible for maintaining high ER calcium (177). Type 1 IP3R is located throughout the ER but type 3 IP3R localize to the mitochondrial associated membranes (MAMs) and primarily transmit calcium to mitochondria (178). RyRs are expressed most prominently in muscle, but even at lower concentrations, they may exert strong effects, as RyRs release ~20x more calcium into the cytosol than IP3Rs (179). Stromal interaction protein 1 (STIM1) is a transmembrane calcium sensor that senses ER calcium levels through its EF hand and other calcium binding sites (180). When ER calcium is depleted, STIM1 translocates to the plasma membrane where it binds the Calcium release-activated calcium channel protein 1 (Orai1) resulting in capacitative calcium entry, also known as Store operated calcium channel (SOC) entry (181).

STING monomers share 2 Ca2+ binding sites when they form dimers, and a certain amount of cytosolic calcium appears necessary for activation (182). For instance, during dsDNA-stimulated STING activation in macrophages, calcium chelators such as BAPTA and mitochondrial calcium export inhibitors (CGP37157 sodium pump inhibitor) both reduce IRF3 and NF-κB activation (183). W-7, a potent calmodulin inhibitor also reduced STING activation by a pharmacologic STING-stimulating adjuvant (184). Early sensing of HCMV (human cytomegalovirus) and Sendai virus membrane perturbations and ensuing STING activation is calcium dependent (185). Cyclosporin A (the calcineurin inhibitor) decreases mitochondrial calcium release and STING-dependent IFN in macrophages (183). Short-term elevations in cytosolic calcium increase STING activity through the following mechanism: calcium binds and activates Calcium/calmodulin-dependent protein kinase II (CAMKII), which then phosphorylates 5' AMP-activated protein kinase (AMPK) (186). AMPK represses ULK1, which phosphorylates (and negatively regulates) STING (87). However, saturating levels of cytosolic Ca^{2+} (as following ionomycin treatment) can also inhibit STING activation, so a happy medium is required by STING for optimal function (187). The importance of the calcium-STING connection has borne out in lupus: dipyridamole (a Ca channel blocker and cGMP phosphodiesterase inhibitor) reduces cytokine production in SLE T cells (188). CAMKIV is overexpressed in lupus nephritis and CAMK deficiency or inhibitors (e.g., KN-93) decrease disease in murine lupus models (189, 190).

Calcium homeostasis is also important for controlling STING location in its basal state and during activation. The ER calcium sensor, STIM1 physically interacts with and inhibits STING activation and translocation to Golgi (191). Exogenously increased STIM1 greatly decreases STING activation and UPR induction. The STIM1-STING inhibition is mutual, in that STING inhibits STIM1 translocation. When STING is absent, STIM1 enriches at the plasma membrane, and mediates increased calcium entry *via* SOC.

Not only does calcium regulate STING activity and location, but STING, in a reciprocal fashion, may regulate calcium levels. STING associates closely with ER SERCA pumps and mitochondrial calcium transporters VDAC1 and VDAC3 in the MAMs (192). Direct association between STING and IP3Rs increases cytosolic calcium release and drives lethal coagulation during sepsis (193). The STING GOF mutant (chronic STING activity) exhibits decreased ER Ca^{2+} release and lower influx across the plasma membrane. However, acute T cell receptor signaling and activation of the GOF mutant resulted in increased calcium-dependent ER stress. Exacerbating this effect with the SERCA pump inhibitor Thapsigargin (but not Tunicamycin) synergized with the STING GOF mutation in inducing apoptosis (168). Thus, STING may regulate calcium homeostasis and set thresholds for calcium-mediated signaling and apoptosis. For a summary of STING and calcium reciprocal regulation, see **Figure 6**.

THE ER MITOCHONDRIAL CONNECTION: COMMUNICATION *VIA* ROS AND CALCIUM

It is evident how mtDNA could stimulate STING *via* cGAS. However, it is much less apparent how ER stress or calcium mechanistically stimulates STING without an activating ligand. This conundrum brings us to the base of our conceptual tripod (**Figure 1**): the ER-mitochondria connection. The ER and mitochondria share a close relationship, both anatomically in the MAMs and functionally. Mitochondria host metabolic pathways,

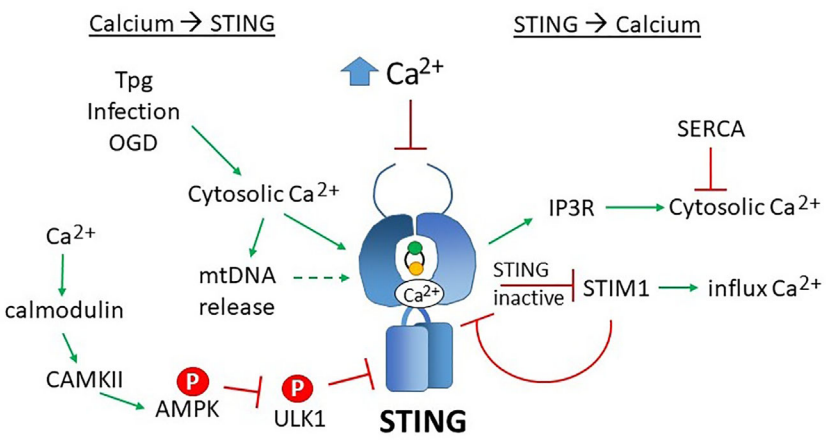

FIGURE 6 | Reciprocal effects of calcium on STING activity and STING on calcium homeostasis. Increases in cytosolic calcium (Ca^{2+}) enhances STING activity through multiple mechanisms: 1) calcium directly binds STING dimers, promoting cyclic-di-nucleotide signaling, 2) increased cytosolic calcium enhances mitochondrial DNA extrusion (thus triggering cGAS), and 3) calcium stimulated calmodulin activates CAMKII, which phosphorylated AMPK, which then inhibits ULK1, a STING inhibitor. SERCA pump inhibitors Thapsigargin (Tpg), infection and oxygen glucose deprivation (OGD) increase cytosolic calcium, thereby stimulating STING. The mechanisms underlying these observations are not yet established. Too much calcium (as in ionomycin treatment) inhibits STING activity. On the right, STING stimulates IP3R-dependent calcium release, a process that may be counteracted by SERCA activity. In its inactive state, STING sequesters STIM1 in the ER, preventing extracellular calcium entry. STIM1 reciprocally "tethers" STING to the ER, inhibiting its activity.

biosynthetic activities, ATP generation, and buffer calcium, generate most of the cellular reactive oxygen species (ROS), and regulate cell death by apoptosis. The ER synthesizes lipids and steroids, regulates calcium, and through oxidative protein folding, generates ROS. We hypothesize that the close connection and functional feedback between these organelles may generate the "missing ligand" in the form of released mitochondrial DNA. Below we will review their interconnections (**Figure 7**) focusing on two "coins of the realm", calcium (touched on above) and reactive oxygen species (ROS).

Close apposition between ER membranes and mitochondria at the MAMs or MERCs (mitochondria ER contacts) enables phospholipid transfer, calcium movement, and redox control and regulates mitochondrial fusion and fission, inflammasome activation, autophagy, and apoptosis (96, 194). Consider phospholipid synthesis as a prime example of the ER mitochondrial partnership: ER synthesized phosphatidylserine goes to the mitochondria where it is decarboxylated to phosphatidylethanolamine, which then returns to the ER to be methylated to phosphatidylcholine, the most common lipid in cell membranes (195). During mitochondrial fission, the ER first wraps around mitochondria (196). Constriction of the mitochondria *via* ER-bound inverted formin 2 (INF2) requires actin polymerization and increased ER-mitochondria calcium transfer (197). Protein folding requires abundant ATP generated by mitochondrial respiration.

Multiple molecular interactions anatomically bridge the two organelles, facilitating the exchange of small molecules and calcium. These interacting partners include mitofusin2 (Mfn2) on the ER and mitofusin1 (Mfn1) on mitochondria, Vesicle associated membrane protein B (VAPB) and Protein tyrosine phosphatase interacting protein 51 (PTPIP51), IP3R3 and VDAC1 (voltage dependent anion channel 1) ((198–200), **Figure 7**). Mitofusins not only controls ER structure, but also regulate mitophagy and facilitate calcium transfer. VABP and PTPIP51 also facilitate calcium transfer between ER and mitochondria and regulate autophagy (199). On the mitochondrial side, the outer mitochondrial membrane is permeable to calcium through the VDAC channels, but the inner mitochondrial membrane is much less so. However, the steep negative membrane potential generated by respiration can drive the mitochondrial calcium uniporter (MCU) (201). On the external surface, VDACs form pores that allow release of small molecules such as ATP, metabolites, superoxide anions, and cytochrome c. GRP75 stabilizes the bridge between VDACs and IP3Rs (200). Therefore, when the ER releases calcium, particularly through IP3R, the mitochondria are well situated to act as a calcium buffer, maintaining optimally low cytoplasmic calcium. Too much ER calcium release, though and the mitochondria initiate apoptosis. ER stress sensors (more on this below) and protein-folding chaperones also cluster at the MAMs, including BiP, calnexin, calreticulin, ERp44, ERp57, and Sigma 1 receptor (202, 203).

Both ER and mitochondria generate ROS during normal physiologic function and pathologic intracellular stress. ROS also play a critical role in immunity, for instance in NF-κB induction, macrophage phagocytic function and inflammasome activation (204–206). The ER accounts for about 25% of total cellular ROS, primarily produced during protein folding (207). Formation of the inter- and intra-molecular disulfide bonds required for protein structure requires an oxidizing milieu. The Protein disulfide isomerase (PDI) oxidoreductase catalyzes the formation, reduction and isomerization of disulfide bonds. PDI family members ERp57 and ERp72 also form disulfide bonds (208). In order to re-oxidize PDI, electrons are transferred to Endoplasmic Reticulum Oxidoreductase 1 Alpha (ERO1α) *via* a flavin adenine cofactor, and from there to molecular oxygen, ultimately generating H2O2 (209, 210). During ER stress, ERO1α is one of

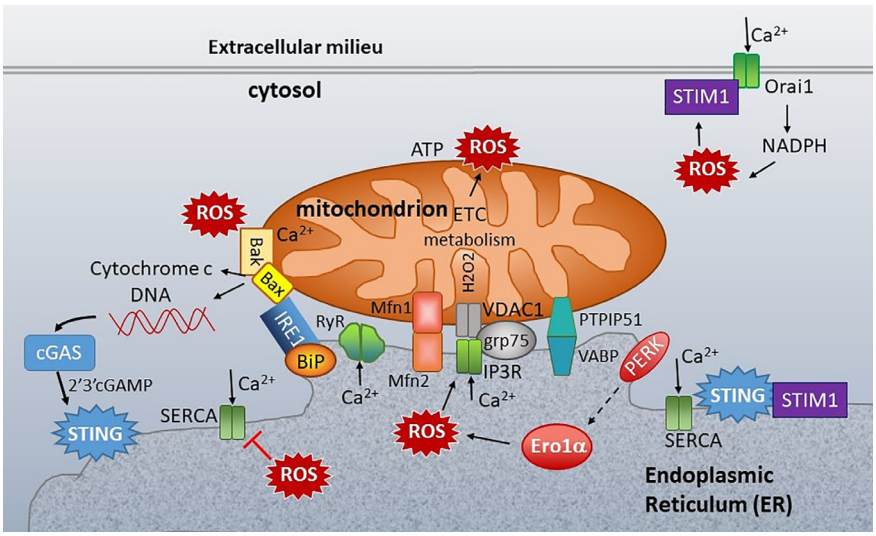

FIGURE 7 | ER-mitochondria connections at the ER mitochondria-associated membranes (MAMs). Mitochondria are closely associated with ER membranes through multiple sets of molecular bridges, including the mitofusins (Mfsn) that regulate mitochondria fission/fusion, the inositol triphosphate receptor (IP3R) calcium channel and non-selective voltage-dependent anion channel (VDAC) stabilized by GRP75, and Vesicle APC-Binding Protein (VAPB) and protein tyrosine phosphatase-interacting protein 51 (PTIP51), which also regulate calcium flux. ER stress sensors inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and folding chaperones (e.g. GRP78/BiP) congregate at the ER mitochondria- associated membranes (MAMs). STING is also enriched at the MAMs. In the resting state, STING associates with STIM1. ER calcium is primarily regulated by three types of calcium channel: Sarcoplasma/ER calcium ATPase (SERCA), which pumps calcium (Ca^{2+}) into the ER, and the IP3Rs and ryanodine receptors (RyR), which release ER calcium. Mitochondrial respiration and the action of the electron transport chain (ETC) generate ROS. Protein folding is the primary source of ROS generation in the ER. PERK indirectly induces (dashed arrow) Endoplasmic Reticulum Oxidoreductase 1 Alpha (Ero1α) expression, which is one of the primary sources of ER ROS. ROS decrease ER calcium by inhibiting SERCA and activating IP3R and RyR. ROS also stimulate the translocation of Stromal interaction molecule 1 (STIM1) from ER to plasma membrane, where it interacts with Calcium release-activated calcium channel protein 1 (Orai1) to enable store operated calcium entry (SOC). SOC stimulates NADPH oxidase, generating a positive feedback loop. At the mitochondria, too much calcium and ROS stimulate Bak/Bax mediated release of cytochrome c and extrusion of mitochondrial DNA (mtDNA). The mtDNA stimulates cGAS production of 2'3'-cGAMP, an activating ligand for STING. Calcium regulating molecules are in green, apoptosis in solid yellow, and UPR-associated molecules as in **Figure 5**.

the key downstream targets induced by PERK (via CHOP) to increase folding capacity (211). Too much ERO1α however causes a hyperoxidizing environment, excessive ROS production and thus induces ER stress (212). NADPH family oxidoreductases NOX2, NOX4 and NOX5 also localize to the ER (213, 214). Other ER oxidoreductases filling a similar role to ERO1 include vitamin K epoxide reductase, quiescin sulfydryl oxidase and peroxiredoxin IV (215). Glutathione peroxidases and GSH help scavenge excess ROS (216). Binding of oxidized glutathione peroxidase to BiP enhances its chaperone activity (217). GSH also reduces disulfide bonds in improperly folded proteins. However, there are relatively low levels of GSH in the ER, predisposing to the oxidizing environment. Ratios of GSH:GSSG are 1:1–3:1 vs. 30–100:1 in the cytosol (218).

Mitochondria generate the lion's share of ROS in the cell *via* fatty acid beta-oxidation, respiration (electron transport chain (ETC) Complex I and III, cytochrome b5 reductase) and other metabolic enzymes including monoamine oxidase, a-ketoglutarate dehydrogenase, pyruvate dehydrogenase, flavoprotein ubiquinone oxidoreductase (219). A subset of NOX4 localizes to the mitochondria where it regulates mitochondrial bioenergetics (220). Superoxide anions are the primary ROS produced by mitochondria. Mitochondrial ROS are scavenged by super oxide dismutases (SODs), glutaredoxin, glutathione, thioredoxin, glutathione reductases, peroxidase and peroxiredoxins (215, 216). Negative feedback loops also keep ROS generation in check. For instance, mitochondrial ROS stabilize Hypoxia inducible factor 1 alpha (HIF1α), which decreases the Krebs cycle and electron transport chain (ETC) activity and stimulates mitophagy (221–224). Mitochondrial fission and fusion also exhibit cross-regulation with ROS; for instance, reactive oxygen species modulator 1 (ROMO1) decreases ROS production and maintains structural integrity by enhancing OPA1 Mitochondrial Dynamin Like GTPase (OPA1) oligomerization (increasing metabolic function and ROS production). However, excessive ROS inactivates ROMO1, leading to cleavage of OPA1, loss of mitochondrial cristae, mitochondrial fragmentation and decreased respiration (225). As an example of positive feedback, excessive ROS induces translocation of the fission-requiring dynamin protein Drp1, and fission promotes mitochondrial ROS over-production (226, 227).

ROS production and calcium fluxes intercommunicate within organelles and between the ER, cytosol and mitochondria on many different levels (**Figure 7**) [reviewed in (171)]. At steady state, constitutive ER calcium release *via* IP3R supports mitochondrial oxidative phosphorylation (228). However, boosting the Krebs cycle dehydrogenases and activating NO synthase increases ROS. ER calcium release that causes cytoplasmic Ca^{2+} spikes generates a

"nanodomain" of mitochondria-generated H2O2, which in turn induces a positive feedback increase in calcium release (229). ROS directly regulate the activity of ER calcium channels. For instance, oxidation of RyRs causes calcium leak, further ROS generation and muscle weakness (230). ROS inhibit SERCA by preventing ATP binding, thus depleting ER calcium and increasing cytosolic calcium. ERO1α highly enriches at the MAMs in normal oxidizing conditions (231). ERO1α activity generates H2O2, which oxidizes IP3Rs and results in increased activity and calcium flux out of the ER (232, 233). In the cytosol, increased calcium efflux *via* IP3R stimulates CAMKII, which then exacerbates the situation by stimulating NOX2-dependent ROS production. NOX2 can also stimulate mitochondrial ROS and mitochondrial superoxide activates NOX2 (234, 235). A stressed ER in need of more ATP for folding could thus communicate *via* ROS and calcium to mitochondria to increase ATP production. However too much cytosolic calcium or excess ROS results in opening of the mitochondrial membrane permeability transition pore, resulting in cytochrome c loss, compromise of ECT function (generating more ROS) and initiation of apoptosis (232). Besides activating ER calcium channels, ROS (hydrogen peroxide) also stimulate translocation of STIM1 and possibly STIM2 to the plasma membrane, increasing cytosolic calcium through SOC entry (236, 237). Here also, there is feed-forward reciprocal regulation: STIM1 and Orai1 calcium channels contribute to ROS generation by NADPH oxidase, and NOX2 drives STIM1-mediated SOC entry (238, 239). Putting these observations together, optimum calcium concentrations and limited release enhances communication between organelles and increases their function (i.e. protein folding and metabolic respiration), but excess ROS production and calcium movement out of the ER into mitochondria or cytosol initiates problematic positive feedback loops that can drive apoptosis.

The UPR further impacts ROS and calcium signaling and is in turn regulated by them. The pharmacologic agent Thapsigargin, a SERCA pump inhibitor, rapidly and potently induces the UPR by depleting ER calcium. Oxidized cholesterol causes inflammatory ER stress in macrophages (240). In skeletal muscle, free fatty acids increase oxidative stress and mitochondrial dysfunction, thus leading to ER stress and autophagy. The Sigma1R, which modulates IP3R activity and calcium flux, decreases ER stress and stabilizes IRE1 oligomerization and generation of pro-survival responses (202, 241). TLR signaling induces IRE1 activation and XBP1 production *via* NOX2 by an unclear mechanism (156). In the direction of ER stress to calcium/ROS, ER stress increases cytosolic calcium to the point of calcium-dependent mitochondrial outer membrane permeabilization and apoptosis. PERK, which is abundant at the MAMs, contributes to ROS-driven mitochondrial stress and apoptosis (242). PERK both increases ROS *via* ERO1α induction and conversely induces anti-oxidant responses *via* nuclear factor erythroid 2–related factor 2 (Nrf2). PERK directly phosphorylates Nrf2, leading to its dissociation from Kelch-like ECH-associated protein 1 (KEAP1) which prevents Nrf2 nuclear translocation (243). With ATF4, Nrf2 induces SODs, Heme oxygenase-1, glutathione transferase and uncoupling mitochondrial protein 2 (UCP2) (215). PERK regulation of proteostasis (and oxidative ROS-generating protein folding) can also have a large impact on cell capacity to survive ER stress (244).

EVIDENCE FOR A TRIAD OR GUILT BY ASSOCIATION?

To this point, we have addressed the various dyads between STING and mitochondria, STING and ER, and mitochondria and ER, but what evidence is there for a three-way interaction? UPR activation has been previously implicated in some of the same settings where mitochondrial damage is now taking the spotlight. Consider the case of STING and the RNA virus Dengue virus. The elaboration of viral mitochondria-targeting proteins and resulting mitochondrial stress and damage was described above. Dengue replicates in ER-derived vesicles and also induces the UPR. Viral induction of PERK and IRE1 signaling pathways increase viral autophagy and replication (245). Similarly, in the case of Cisplatin induced acute kidney injury, Cisplatin has long been known to cause ER stress and UPR activation (246). In these two scenarios, mtDNA-induced STING activation and UPR coexist in the same pathologic setting, but the whether these manifestations are interconnected or occur independently is not yet clear.

More work on the relationship between UPR and mitochondria had been done in cancer (**Figure 8**). One report suggested ER stress contributes to mitochondrial exhaustion of CD8 T cells (247). In a murine sarcoma model, tumor-infiltrating PD1+ cells had greater levels of mitochondrial ROS. Mitochondrial ROS correlated with mitochondrial dysfunction as evident by lower oxygen consumption rates. PERK inhibition decreased mitochondrial ROS in PD1+ cells. PERK inhibitor and ERO1 inhibitor treated T cells exhibited both higher O2 consumption rates and improved IFN-γ production. IFN-γ, produced by tumor-infiltrating CD8 T cells and NK cells, enhances cytotoxicity and antigen presentation, and exerts direct anti-tumor effects – although in some settings, the cytokine may be pro-tumorigenic (248). Further, PERK deficiency and PERK and ERO1α inhibitor treatment of T cells resulted in higher energy reserve and enhanced anti-tumor activity *in vivo*. Others have shown constitutive XBP1 activation by ROS (lipid peroxidation byproducts more specifically) drives tumor progression by limiting antigen presentation and T cell activation (249). Thus, through IRE1 or PERK, the UPR can have a deleterious effect on cancer containment. However, STING agonists (which can increase ER stress) improve CD8 T cell anti-tumor activity, despite increasing PDL1 expression (49). When it comes to T cell regulation, STING may exert competing effects on IFN production vs. ER stress and exhaustion. Thus, in developing therapeutics, the various effects of STING agonists on mitochondria and UPR signaling in CD8 T cells or their interacting DCs will require clarification in specific contexts.

The effects of UPR/PERK-mitochondria signaling may also be cell-specific. Myeloid derived suppressor cells (MDSC) have been implicated in tumor progression. These cells show signs of UPR activation correlating with chemoresistance (250). Thapsigargin treatment expanded splenic MDSC and enhanced tumor growth whereas the UPR inhibitor TUDCA had the opposite effect.

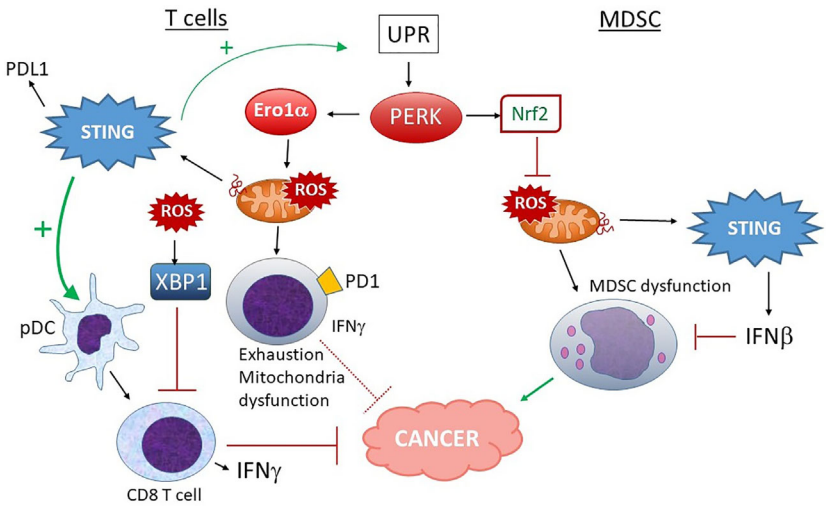

FIGURE 8 | Different outcomes of PERK activation in T cells vs. MDSC and STING input. In T cells, PERK and ERO1α increase ROS production, leading to mitochondrial dysfunction, increased exhaustion and lower IFNγ production, rendering these PD1+ T cells less adept at fighting tumors. ROS-stimulated XBP1 also decreases T cell activation. Although STING activation could make matters worse by increasing UPR induction and PDL1 expression, many studies indicate a positive role for STING and type I IFN in pDC-dependent CD8 T cell activation and anti-tumor activities, suggesting a balance of effects. On the other hand, in MDSC, PERK-stimulated Nrf2 activity predominates. Nrf2 prevents mitochondrial ROS and dysfunction. Mitochondrial ROS also leads to dsDNA extrusion and STING activation, which further inhibit MDSC *via* Type I IFN signaling. MDSC promote tumor progression.

Mohamed et al. reported that the PERK pathway was highly activated in MDSC in tumors (251). PERK enhanced MDSC-mediated immunosuppression *via* Nrf2, preventing oxidative damage, mitochondrial DNA release and DNA sensing *via* cGAS/STING. Ablating PERK in the myeloid branch delayed tumor growth. In a separate report, CHOP contributed to MDSC activity. However, the PERK effect noted by Mohamed et al. required Nrf2, not CHOP. PERK deficient MDSC exhibited increased cellular ROS, altered mitochondrial morphology, membrane potential, reduced oxygen consumption and release of mtDNA. The mtDNA activated STING and induced type I IFN. Blocking STING or IFNAR restored the immunosuppressive effect of MDSC in the absence of PERK. Thus, in the case of MDSC, PERK promoted suppressor cell "well-being" and inhibited STING activation through its anti-oxidant activities (251). Interestingly, Nrf2 also antagonizes STING expression by mRNA destabilization (252).

Thus, in some settings STING agonists hold dramatic promise as anti-tumor agents. However, other reports suggest they may increase metastases and tumor progression. We are just beginning to scratch the surface of how STING regulates different types of tumors and the different cells in tumor environments. Understanding the potential mechanisms by which ER stress and mitochondrial dysfunction interact and regulate STING is lagging further behind but a ripe area for further study.

SUMMARY AND PERSPECTIVE

An underlying hypothesis in this review is that ER stress may activate STING in the absence of an obvious ligand *via* calcium/ROS mediated mitochondrial damage and mtDNA release. To illustrate how this might work based on the previous discussion consider cancer once more: In tumor microenvironments, unregulated cellular proliferation may outstrip the neo-vascular supply of nutrients including oxygen, glucose and amino acids. This lack of nutrients negatively impacts ER function, triggering the UPR. Hypoxia may directly uncouple electron transport and damage mitochondria. However, it is also likely that the disruptions in ER calcium homeostasis, ROS production and stress will lead to mitochondrial damage and release of mtDNA into the cytosol. cGAS would then sense the mtDNA and generate cGAMP, which stimulates STING to produce type I IFN. This scenario raises multiple questions: It may be a logical fallacy to invoke crosstalk between all three corners of the triad; just because A goes to B and B goes to C, doesn't mean A requires B to get to C. The effect of hypoxia on mitochondria may be sufficient in the absence of ER stress to cause mtDNA release. ER stress may activate STING in some unknown way without the mitochondrial intermediary, for instance by stabilizing STING oligomerization or altering STING trafficking.

The data presented above raise other questions regarding ER stress-mitochondria-STING interactions: It is still unclear why calcium mobilization during ER stress was necessary for Thapsigargin and oxygen-glucose induced STING activation—was it because of a unique calcium-dependent effect on mitochondria and subsequent mitochondrial DNA release or another mechanism? Was the role of the ER stress simply to generate ROS? Another issue is how the UPR could trigger mtDNA release without initiating apoptosis. The UPR triggers multiple pathways converging on mitochondria-dependent intrinsic apoptosis including suppression of anti-apoptotic molecules, induction of pro-apoptotic molecules and JNK

signaling, in addition to the calcium and ROS dysregulation described above (253). The decision between mitochondrial DNA release and apoptosis may simply be a matter of degree of ER stress and relative amount of mitochondrial destruction, but such a threshold model would require further experimental support. There is certainly evidence for a yin-yang balance between apoptosis and mtDNA stimulation of STING in that caspase deficiency increases STING induced IFN (254). Alternatively, in addition to Bax-Bak pores, VDAC pores in oxidatively stressed mitochondria enable mtDNA extrusion, perhaps promoting STING activation short of apoptosis (255).

Let us come back full circle. What is the physiologic need for repurposing PRRs such as STING? One possibility is the context added by DAMPs; inside the cells, sufficient damage from pathogens can trigger PRRs to amplify immune responses. However, endogenous PRR stimulation represents a double-edged sword with its own perils, as manifest by the involvement of STING in heart disease, cancer and autoimmunity. In cancer, STING stimulation by endogenous stressors not only can bolster innate and adaptive anti-tumor immunity but can also undermine anti-tumor defenses. Similarly, STING may drive type I interferonapathies, but STING deficiency exacerbates autoimmunity triggered by other PRRs. STING is particularly well situated to respond to organelle-generated alarm signals resulting from disruptions in calcium homeostasis and critically increased reactive oxygen species. The close apposition of ER and mitochondria and calcium-ROS cross talk between these organelles offers the tantalizing possibility that stress initiated in either organelle could ultimately generate the required ligand for STING and regulate STING activity. It will be interesting to see how elucidation of the underlying mechanisms leading from intracellular stress and damage to STING activation unfolds. Linear sequential pathways are much easier to assess *via* common tools such as expression modulation or inhibitors, but reciprocal regulation and mutually augmenting feedback loops present much more of a challenge. Despite these issues, it remains important to determine the key intermediaries and interactions within these pathways under different scenarios, because this knowledge will be critical for guiding therapeutic interventions.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

All funding for this work comes from the University of Wisconsin-Madison (institutional support).

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 08 March 2021
doi: 10.3389/fimmu.2020.612452

Cross Kingdom Immunity: The Role of Immune Receptors and Downstream Signaling in Animal and Plant Cell Death

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *29 December 2020*
Published: *08 March 2021*

Citation:
Roudaire T, Héloir M-C, Wendehenne D, Zadoroznyj A, Dubrez L and Poinssot B (2021) Cross Kingdom Immunity: The Role of Immune Receptors and Downstream Signaling in Animal and Plant Cell Death. Front. Immunol. 11:612452. doi: 10.3389/fimmu.2020.612452

Both plants and animals are endowed with sophisticated innate immune systems to combat microbial attack. In these multicellular eukaryotes, innate immunity implies the presence of cell surface receptors and intracellular receptors able to detect danger signal referred as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Membrane-associated pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), receptor-like kinases (RLKs), and receptor-like proteins (RLPs) are employed by these organisms for sensing different invasion patterns before triggering antimicrobial defenses that can be associated with a form of regulated cell death. Intracellularly, animals nucleotide-binding and oligomerization domain (NOD)-like receptors or plants nucleotide-binding domain (NBD)-containing leucine rich repeats (NLRs) immune receptors likely detect effectors injected into the host cell by the pathogen to hijack the immune signaling cascade. Interestingly, during the co-evolution between the hosts and their invaders, key cross-kingdom cell death-signaling macromolecular NLR-complexes have been selected, such as the inflammasome in mammals and the recently discovered resistosome in plants. In both cases, a regulated cell death located at the site of infection constitutes a very effective mean for blocking the pathogen spread and protecting the whole organism from invasion. This review aims to describe the immune mechanisms in animals and plants, mainly focusing on cell death signaling pathways, in order to highlight recent advances that could be used on one side or the other to identify the missing signaling elements between the perception of the invasion pattern by immune receptors, the induction of defenses or the transmission of danger signals to other cells. Although knowledge of plant immunity is less advanced, these organisms have certain advantages allowing easier identification of signaling events, regulators and executors of cell death, which could then be exploited directly for crop protection purposes or by analogy for medical research.

Keywords: pattern recognition receptors, Toll-like receptors, NOD-like receptors, pathogen-associated molecular patterns, damage-associated molecular patterns, hypersensitive response, regulated cell death

INTRODUCTION

Eukaryotic cells have evolved complex defense mechanisms in order to combat microbial challenges and preserve organism integrity. Both plants and animals are endowed with a conserved innate immune system able to neutralize pathogens and to contain the infection. It uses specialized receptors to detect microbial patterns, pathogen-derived compounds, and danger signals and elicits an adapted response. The immune response includes a transcriptional reprogramming, the production of antimicrobial molecules, the activation of a regulated cell death program in infected cells and the release of soluble factors such as cytokines and phytohormones able to signal away from the original infection site and alert the host organism of danger. In plants, a systemic resistance is transiently established to prevent forthcoming microbial assault (1). In addition, vertebrates have evolved an adaptive immune system involving specialized cells able to produce a stronger, specific immune response and ensure a long-term protection.

The activation of cell death processes at the site of pathogen attack constitutes an efficient strategy shared by plants and animals to protect the organism from pathogen invasion by directly destroying the pathogen niche. Cell death can also produce alert signals for neighboring cells through the release of intracellular components that can elicit or amplify the anti-microbial response. During a microbial infection, three types of regulated cell death are classically described in animals for confining pathogen progression: apoptosis, pyroptosis, and necroptosis (2, 3). Apoptosis is defined by specific morphological criteria that include cell shrinkage, condensation of the chromatin and fragmentation of the nucleus, plasma membrane blebbing, and the formation and release of apoptotic bodies that are engulfed through a phagocytosis-like process named efferocytosis. At the molecular level, apoptosis involves a cascade of events that culminates in the activation of specific proteases belonging to the caspase family responsible for cell dismantling (2). Of note, apoptosis is a "silent form" of cell death that does not directly cause inflammatory response because of conserved plasma membrane integrity and efferocytosis. Efferocytosis has even been linked to the resolution of inflammation, required for the clearance of dead cells after infection, reducing the production of inflammatory factors by phagocytes and progressively allowing the restoration of homeostasis (4). On the opposite, pyroptosis and necroptosis are associated with the release of pro-inflammatory molecules allowing the establishment of the adaptive immunity. They both involve pore-forming proteins [gasdermin-D (GSDMD) in pyroptosis and mixed lineage kinase domain-like (MLKL) in necroptosis] that trigger membrane permeabilization and osmotic imbalance leading to cell swelling. Many connections exist between signaling pathways leading to apoptosis, pyroptosis, and necroptosis, and these cell death processes can occur simultaneously.

Recognition of invading microbes by plants can also trigger a specific cell death referred to as the hypersensitive response (HR) which efficiently blocks the spreading of biotrophic pathogens in healthy tissues by limiting their access to plant metabolites (5). However, the molecular events involved in HR have not yet been completely deciphered. HR-associated cell death is characterized by an early rupture of the plasma membrane associated with some apoptosis-like features such as cytoplasm shrinkage, chromatin condensation, and nucleus disruption (6). These events are associated with plant-specific cell death features including the dismantling of tonoplast and the vacuolar collapse (7). Consequently, the release of active hydrolases and proteases from collapsed vacuoles can trigger autophagy-like processes (8). Thus, HR appeared as a plant regulated necrosis displaying some feature of necroptosis or pyroptosis in animals (5, 9). As observed during necroptosis and pyroptosis, leakage of the cellular content can constitute alert signals for neighboring cells and prepare them to cope with infections. All of these concerted events insure a global and effective defense response.

The theory that innate immune response is elicited by specialized receptors which recognize conserved microbial components referred to as pathogen-associated molecular patterns (PAMPs) was laid out by Medzhitov and Janeway (10), rewarded by the Nobel Prize in 2011. The first pattern recognition receptor (PRR) was described in plants as a cell surface receptor encoded by the rice gene *Xa21*, which confers resistance to the bacteria *Xanthomonas oryzae* pv. *Oryzae* (11). For many years, two major strategies to study the plant innate immunity have existed. The first was based on a genetic approach: certain varieties of a plant species express *R* genes leading to the perception of microbial effectors encoded by avirulence genes (*Avr*) and then generally to the establishment of the HR. This is the basis of the gene-for-gene concept (*R-Avr*) described by Flor on flax and then widely used for genetic breeding of crop plants (12). The second used a biochemical approach coupled with pharmacological studies. Several teams have purified microbial-derived compounds, commonly referred to as elicitors (chitin, flagellin, elicitin, β-glucans, …) able to trigger plant immune responses. They also characterized high-affinity plasma membrane binding sites interacting with elicitors and showing biochemical features of receptors. To combine the results of these two approaches and to get closer to the concept of PAMPs existing in mammals came the concept of PAMP-Triggered Immunity (PTI), that takes into account the recognition of PAMPs by surface receptors, and Effector-Triggered Immunity (ETI), related to the recognition of effectors (pathogen *Avr* gene products) by intracellular receptors (encoded by *R* genes). However, this PTI-ETI concept proposed by Jones and Dangl (13) was a bit controversial because it was too binary (ETI or PTI) and did not reflect all the existing shade of gray levels of plant immune responses, notably during symbiotic interactions. Recently, new models have emerged in the plant innate immunity with the concept of invasion patterns (14) and signs of danger perceived by a complex plant surveillance system that can produce secondary host-derived immunogenic factors termed phytocytokines (15).

This review aims at highlighting similarities and specificities of the immune responses existing in mammals and plants, mainly focusing on recent advances in the discovery of immune receptors and the involvement of signaling pathways leading to cell death.

MEMBRANE-ASSOCIATED PRRs

Cells employ a large number of cell surface or endosomal receptors to sense PAMPs and endogenous danger signals referred to as damage-associated molecular patterns (DAMPs) to engage defense responses. In vertebrates, membrane-bound immune receptors belong to the Toll-like receptors (TLRs) family, which are located either at the cell surface or within the endosomal compartment, and C-type lectin receptors (CLRs) located at the cell surface. Plant PRRs are mainly plasma membrane-localized and are divided into two categories: the receptor-like kinases (RLKs) and the receptor-like proteins (RLPs) (**Figure 1**). In both plants and vertebrates, recognition of PAMPs/DAMPs by membrane PRRs primarily activates transcriptional programs that culminate in the production of antimicrobial molecules and in the implementation of an adaptive response of the host to counteract the pathogen attack.

Membrane-bound PRRs are composed of an N-terminal extracellular domain that functions as a ligand recognition and binding domain, a transmembrane intermediate and a C-terminal cytoplasmic signal transducing domain (**Figure 1**). In animals, PAMPs are recognized by tandem copies (16–22) of LRR (leucine-rich repeat) in TLRs and by C-type lectin-like domains in CLRs (23). Thirteen TLR paralogs were described in vertebrates. Cell surface TLRs (TLR1, 2, 4–6, and 10) recognize components of microbial membranes such as lipids, lipoproteins, membrane anchored proteins, or extracellular proteins bound to pathogens such as heat shock proteins (HSP) 60 and 70 while TLRs found within endosomal compartment (TLR3, 7–9, and 11–13) likely sense virus and bacteria-derived nucleic acids or endogenous nucleic acids in some pathological conditions. On the other hand, CLRs are dedicated to the defense against fungal infections (23). CLRs can also sense commensal fungi and thus contribute to maintain homeostasis (24, 25). Molecular mechanisms of signal transduction in animal cells are well characterized. They involve the presence of conserved modular domains found in receptors, adaptor proteins or signaling effectors and mediate, through homotypic interaction, the assembly of oligomeric signaling platforms favoring the activation by proximity of signaling effectors. In TLRs, the intracytoplasmic signal transduction domain is the conserved Toll/IL-1 receptor (TIR) domain, also found in the adaptor proteins myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), TIR-domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM). Ligand binding triggers TLR homo- or hetero-dimerization, conformational change in their intracellular TIR domain and the consecutive recruitment *via* TIR-TIR homotypic interaction of adaptor proteins to form a signaling platform and initiate downstream signaling pathway. TLR stimulation engages MAPKs and/or NF-κB and ultimately promotes inflammatory response that constitutes the first line of defense in mammals and appears critical to maintain homeostasis and tissue integrity. This includes the production of antimicrobial molecules, pro-inflammatory cytokines, and immune cell differentiating mediators responsible for the recruitment and activation of specialized immune cells to the site of infection (**Figure 2**) (16). However, in some situations such as a sustained infection, the presence of pathogens that resist to inflammatory defense or in some pathological conditions [e.g., some cancers, X-linked lymphoproliferative syndrome type 2 (XLP-2)] (17, 18), several TLRs such as TLR3 that recognizes virus-derived double stranded RNAs and RNAs released from damaged cells, and TLR4 that senses LPS from Gram-negative bacteria, can also trigger cell death (19–21). For CLRs, the signal transduction motif is the immunoreceptor tyrosine-based activation motif (ITAM) or ITAM-like motif that mediates the binding and the activation *via* phosphorylation of the spleen tyrosine kinase (Syk). Generally,

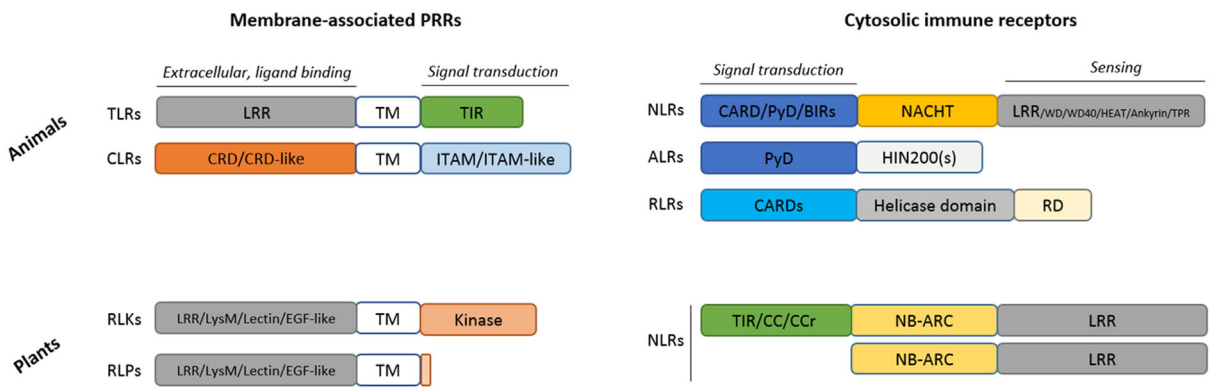

FIGURE 1 | Structural comparison of the main immune receptors found in animals and plants. ALR, AIM2 (absent in melanoma 2)-like receptors; BIR, Baculovirus Inhibitor of apoptosis protein Repeat; CARD, caspase recruitment domain; CC, coiled coil domain; CCr, CC-RPW8; CLR; C-type lectin receptors; CRD, Carbohydrate-Recognition Domain; EGF-like, Epidermal Growth Factor like; HIN200(s), Hematopoietic Interferon-inducible Nuclear protein with a 200 amino acid repeat; ITAM, Immunoreceptor Tyrosine-based Activation Motif; LRR, leucine-rich repeat; LysM, Lysin Motif; NACHT, NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) or TP1 (telomerase-associated protein); NB-ARC, Nucleotide-Binding domain Apaf1, Resistance, CED4; NLR, Nucleotide-binding and oligomerization domain (NOD)-Like Receptor (animals) or Nucleotide-Binding Domain (NBD)-containing LRRs (plants); PYD, Pyrin effector Domain; RD, Regulator Domain; RLK, Receptor-Like Kinase; RLP, Receptor-Like Protein (contain a short cytoplasmic domain devoid of kinase activity); RLR, RIG-I–like receptors; TIR, Toll/Interleukin-1 receptor; TM; Transmembrane; TLRs, Toll-like receptors.

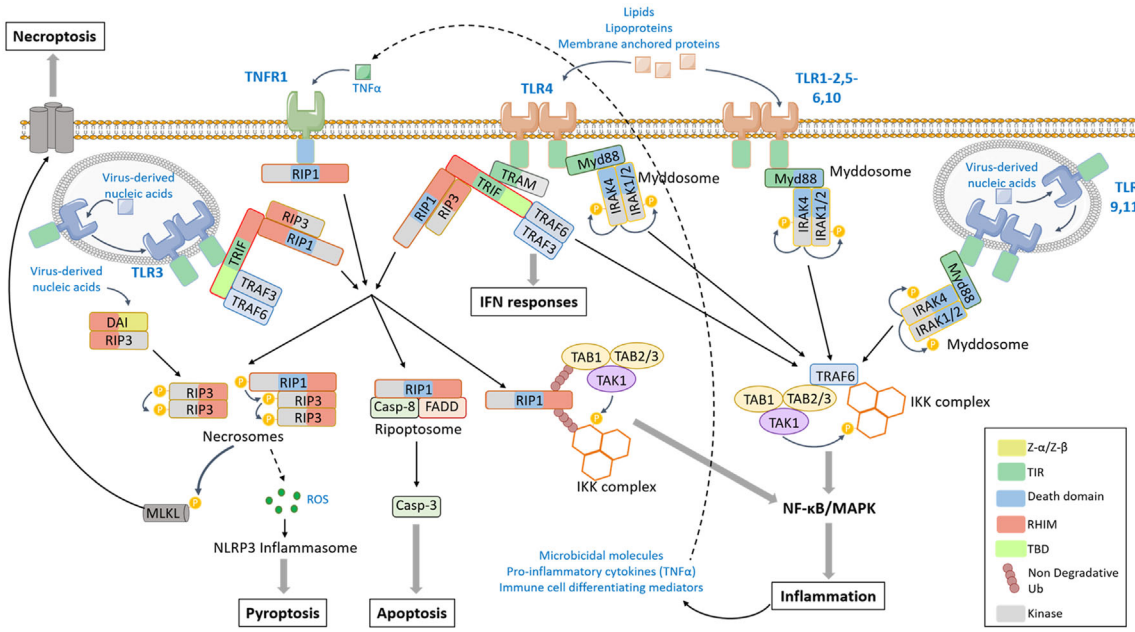

FIGURE 2 | TLR-mediated signaling pathway. The activation of TLR (toll like receptor) 1–2, 4–6, or 10 by lipids, lipoproteins or membrane-anchored proteins and the activation of TLR7-9, 11–13 by virus-derived nucleic acids induce the dimerization of TLRs and the recruitment of Myd88 (myeloid differentiation factor 88) and IRAKs *via* homotypic interaction domains, forming the Myddosome. IRAKs catalyze phosphorylation cascade leading to the recruitment of TRAF6 [tumor necrosis factor (TNF) receptor associated factor 6]. In turn, TRAF6 promotes the activation by proximity of TAK1 (tumor growth factor-β-activated kinase 1)/TAB1-3 (transforming growth factor-activated kinase1-binding protein 1, 2, and 3) and the IKK (Inhibitor of κB kinase) complexes that result in the activation of MAPK (Mitogen-activated protein kinases), NF-κB (nuclear factor-kappa B)-signaling pathways, and pro-inflammatory response. TLR3 and TLR4 stimulation induces the recruitment of TRIF (TIR-domain-containing adaptor-inducing IFN-β) through a TIR-TIR homotypic interaction. The adaptor TRAM (Trif-related adaptor molecule) serves as a bridge between TLR4 and TRIF. In turn TRIF can recruit TRAF3 that engages IFN (Interferon) response, TRAF6 that leads to MAPK and NF-κB activation, or the kinases RIP (Receptor Interacting Protein) 1 and/or RIP3. When poly-ubiquitinated, RIP1 can recruit TAK1/TAB1-3 and IKK complexes and activates the pro-inflammatory response. In a non-ubiquitinated form, RIP1 can assemble with FADD and caspase-8 to form the ripoptosome that leads to caspase cascade activation and apoptotic cell death or can activate RIP3 in the necrosome. RIP3 catalyzes the activating phosphorylation of MLKL, which oligomerizes and translocates into the plasma membrane to form pores and induces necroptosis. The DAI (DNA-dependent activator of IRFs) can also directly recruit RIP3 *via* RHIM homotypic interaction to induce MLKL phosphorylation and necroptosis in response to virus-derived nucleic acids. The necrosome has also the ability to induce ROS (Reactive oxygen species) production resulting in NLRP3 (NOD (Nucleotide-binding oligomerization domain)-like receptor protein) inflammasome and pyroptosis (details of pyroptosis available in **Figure 4**). The TLR-mediated production of TNF-α can induce an autocrine stimulation of TNFR1 that can lead to RIP1 engagement and an amplification of the signal.

CLR stimulation does not trigger cell death but results in MAPKs, NF-κB, or interferon-regulatory factors (IRFs) activation (23).

By comparison, plants have evolved hundreds of membrane PRRs able to recognize a wide variety of PAMPs such as flagellin, peptidoglycan, chitin, elicitin, polygalacturonase, and xylanase (22, 26) or DAMPs such as fragments of the plant cell wall extracellular matrix (oligogalacturonides from pectins, cellobiose, and cellodextrines from cellulose and xyloglucans from hemicellulose) (27, 28), extracellular ATP, NAD^+, or secondary endogenous phytocytokines such as systemin, rapid alkalinization factors (RALFs), pathogen-associated molecular pattern-induced peptides (PIPs), and elicitor peptides (PEPs) (15). The diversity of sensed PAMPs is made possible thanks to the presence of different extracellular domains (**Figure 1**). For example, LRRs found in flagellin sensing 2 (FLS2) sense flagellin or its 22-amino acid epitope flg22 (29, 30), whereas Lysin motif (LysM) specifically recognizes chitin oligosaccharides in chitin elicitor receptor kinase 1 (CERK1) associated with LYK4/5 (31–34) or peptidoglycan with lysin-motif proteins LYM1/LYM3 (35). Epidermal growth factor (EGF)-like domain present in the wall-associated kinase 1 (WAK1) senses oligogalacturonides released during the degradation of the plant cell wall by fungal hydrolases (36, 37), whereas the lectin domains present in AtLecRK1.9 (DORN1) and AtLecRK1.8 perceive extracellular ATP and NAD^+, respectively (38, 39).

Unlike animal PRRs that require the recruitment of adaptor proteins to transduce signal, plant membrane-associated RLKs are endowed with kinase activity thanks to the presence of a cytoplasmic kinase domain (KD) (**Figure 1**). PAMP-sensing induces oligomerization of RLKs and kinase activation that directly triggers numerous cellular responses (**Figure 3**) such as ion fluxes across the plasma membrane, a burst of reactive oxygen species (ROS), a phosphorylation cascade activating MAPKs, and transcription factors leading to the expression of defense genes which encode enzymes involved in the production of defensive secondary metabolites (e.g., phytoalexins, callose, and lignin that reinforce plant cell wall) (26). Because of their very short cytoplasmic domain (about 24 amino acids) devoid of

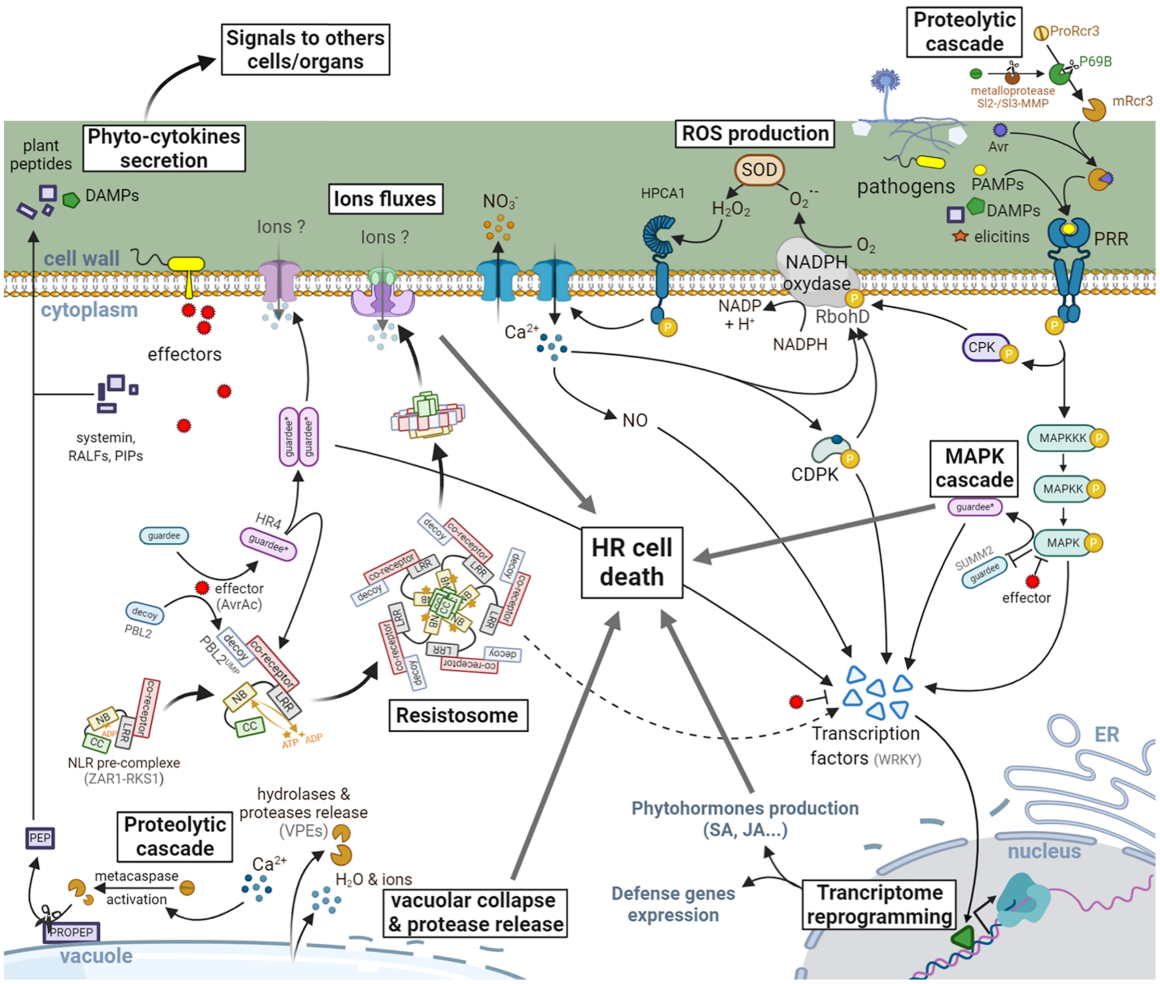

FIGURE 3 | Signaling events leading to HR in plants. PRRs are activated by the recognition of eliciting molecules resulting from the degradation of plant cells (DAMPs) or released by the pathogens (PAMPs, elicitins, apoplastic avirulence factors: Avr). The signal is then transduced by a cascade of phosphorylation events involving MAPKs, cytoplasmic protein kinases (CPKs), and transcription factors, mainly from the WRKY family. This phosphorylation can also activate the NADPH oxidase RbohD, leading to the production of ROS [O_2^- transformed into hydrogen peroxide (H_2O_2) by a superoxide dismutase (SOD)]. An influx of intracellular Ca^{2+}, initiated quickly after perception of H_2O_2 by HPCA1 leads to the production of nitric oxide (NO), as well as the activation of transcription factors *via* the calcium dependent protein kinases (CDPKs). This is followed by a reprogramming of the transcriptional activity leading to the expression of defense genes involved in the synthesis of phytohormones (SA, JA, …), the antimicrobial phytoalexins or even the release of hydrolytic enzymes (glucanases, chitinases, …) from the pathogenesis-related proteins family. In the meantime, effectors secreted by pathogens to counter the plant's defenses can also be directly or indirectly (*via* the recognition of a modified host-protein) recognized by NLRs. This recognition generally induces a conformational change in the protein (noted here by an asterisk and a color change), allowing the exchange of ADP by an ATP and therefore the activation of the NLR leading in some cases to macromolecular complexes such as the resistosome or to the activation of transcription factors. These larger-scale molecular complexes have been proposed to act *via* the recruitment of other signaling actors leading to a potentiation of the defenses already in place or by the formation of pores in the plasma membrane. A HR cell death is then observed locally to block the spreading of the pathogen. This will also be associated with the release of DAMPs, phytohormones and phyto-cytokines which will transmit information to neighboring cells and organs to prevent future infections in healthy tissues. Some plant peptides (e.g., PEPs) can be matured by metacaspase-mediated cleavage and released in the apoplast to prime immune responses in neighboring cells, thus enabling the establishment of a local resistance.

kinase activity, RLPs are not able to transduce signal but function as co-receptors heterodimerized with RLKs that finally elicit a similar cascade of immune signaling events. Most of the PAMPs and DAMPs recognized by plant cell surface PRRs are unable to elicit specific plant cell death. Only some proteinaceous elicitors secreted by pathogens in the apoplast can trigger HR in a plant species dependent manner.

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD)-LIKE RECEPTORS (NLRs)

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), also generalized as nucleotide-binding domain (NBD)-containing LRRs (NB-LRR in plants), are a

class of immune proteins found across both plant and animal kingdoms, with some exceptions in taxa such as Algae, Nematoda, and Drosophila. They were previously classified in plants as R-proteins in the gene-for-gene model. In the animal kingdom, NLRs are the most represented family of cytosolic immune receptors, along with ALRs [AIM2 (absent in melanoma 2)-like receptors] and RLRs (RIG-I–like receptors) (**Figure 1**). ALRs recognize cytosolic DNAs and RLRs sense cytosolic RNAs that include virus nucleic acids as well as endogenous microRNAs (miRNAs) whereas NLRs sense cytosolic PAMPs (e.g., bacteria-secreted toxins, components of bacteria with pore forming activity, internalized LPS, viral proteins) but also markers of intracellular stresses acting as DAMPs such as ATP, uric acids, ROS, metabolic products, or cell-derived peptides that are released by cells in response to endogenous (e.g., ER stress, disruption of ion gradients) or environmental stresses. Stimulation of cytosolic immune receptors can trigger inflammatory response. However, for most of them, the main response is the release of the pro-inflammatory molecules IL-1β and IL-18 and pyroptosis (**Figure 4**). NLR stimulation can also indirectly lead to apoptosis, necroptosis, or activate autophagy process to clear pathogen (40). In plants, NLRs directly or indirectly detect pathogen-secreted effectors (i.e., virulence proteins) to generally promote HR cell death.

Although NLRs are found in both animals and plants, they seem to result from an independent evolutionary process (41). They are characterized by the presence of a central NBD (also named NOD) that may be originated from a prokaryotic class of AAA^+ ATPases. Both mammals and plants would have selected the NBD for the flexibility of its architecture. It catalyzes the ADP/ATP exchange which promotes the NLR oligomerization and activation. ATP-bound active forms are generally unstable and rapidly recycled into inactive forms or degraded. This mode of activation allows the NLR to quickly react after detection of pathogens and enable to preserve the organism from a costly and useless mobilization of defenses in their absence. Animal NLRs are characterized by a "NACHT" [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) or TP1 (telomerase-associated protein)] NBD subtype while plants NLRs possess an "ARC" (Apaf1, Resistance, CED4) NBD subtype, also found in some animal adaptor proteins involved in apoptosis such as Apaf-1 (42).

In addition to NBD, LRR is common to both sides. As in animal TLRs, this domain is involved in sensing microbe

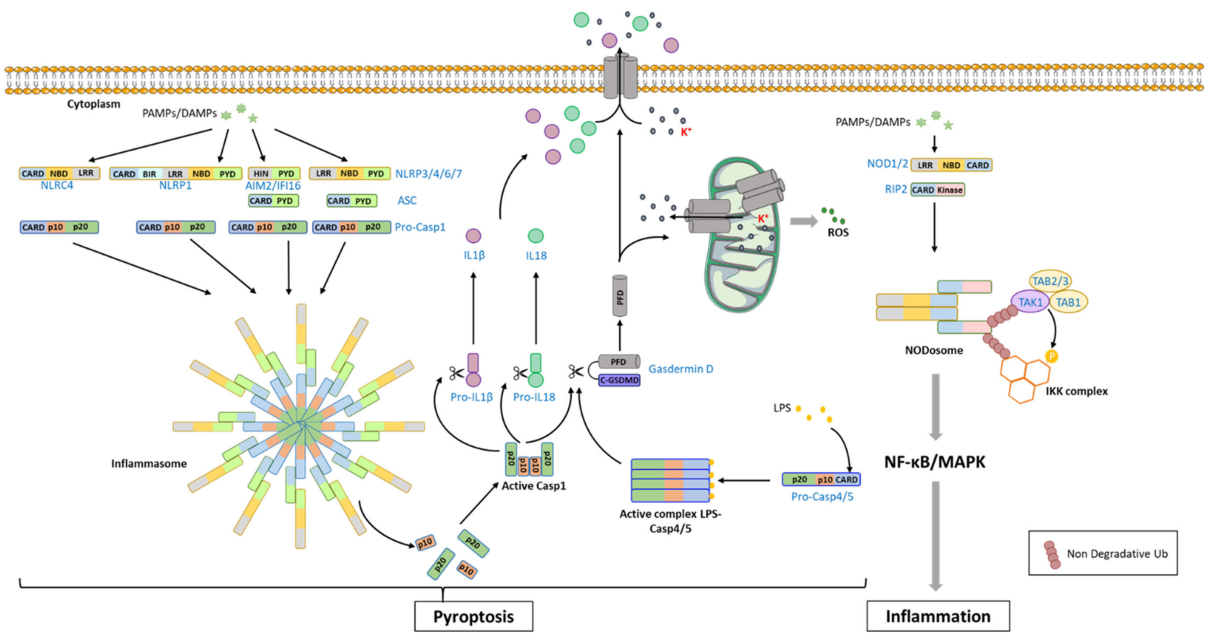

FIGURE 4 | NLR-mediated signaling pathway in mammals. The recognition of PAMPs/DAMPs [Pathogen-Associated Molecular Patterns/Damage-Associated Molecular Patterns) by NLRs (NOD (Nucleotide-binding oligomerization domain)-like receptor proteins: NLRCs, NLRPs] promotes the recruitment of procaspase-1 (Pro-Casp1) through homotypic domain interaction, forming a large cytoplasmic complex named inflammasome. In some situation, the adaptor ASC (apoptosis-associated speck-like protein containing a CARD domain) can take part to the complex, bridging the receptor to procaspase-1. The procaspase-1 undergoes auto-activation and processing of its 2 active sub-units (p10 and p20) which assemble into a tetrameric active form. Active caspase-1 catalyzes the cleavage of proIL-18 (proInterleukine-18), proIL-1β (proInterleukine-1β) into mature cytokines, and gasdermin-D releasing the PFD domain that can oligomerizes and anchors into the membrane to form pores. Processing of gasdermin-D can also be ensured by caspase-4 and caspase-5, which are activated after LPS binding. The gasdermin PFD can also form pores into the mitochondrial membrane that induce K^+ release and ROS production. In addition, pores created in the plasma membrane by PFD also lead to IL1β and IL18 release and to an ion imbalance that finally results in pyroptosis. The recognition of PAMPs/DAMPs by NOD1/2 promotes the formation of the NODosome composed of the sensor and the protein RIP2 (Receptor Interacting Protein 2) through CARD-dependent homotypic interaction. RIP2 can then recruit TAB1/TAB2/3/TAK1 and IKK complexes that engage MAPK and NF-kB signaling pathways leading to the production of pro-inflammatory cytokines.

effectors or modified host-proteins. As an alternative, some animal NLRs own other motif domains with sensing activity such as WD/WD40, HEAT, Ankyrin, or TPR (tetratricopeptide) motifs. LRR domain is widespread among immune receptors of any kinds, probably because of its pre-disposition for forming mismatches thus easily creating diversity and therefore new potential recognition sites, as suggested by Baggs et al. (43). After invasion pattern recognition, LRRs undergo a conformational change allowing NLR switch from an inactive NBD-ADP-bound closed conformation to an active NBD-ATP-bound open conformation and the recruitment of downstream signaling effectors (44, 45). LRR has also the ability to negatively regulate the NBD through intramolecular interactions that prevent its oligomerization (46).

The domains found in the N-term part of the protein are involved in the transduction of downstream signaling (45, 47). In animals, these domains belong to the death-fold superfamily which are homotypic interacting domains also found in adaptor and signaling molecules. The pyrin effector domain (PYD) is the most represented and characterizes the NLRP (NOD-like receptor protein) sub-family of NLRs (also named NALP). The NLRC sub-family contains one or two caspase-activation and recruitment domains (CARD), NLRA the acidic transactivation domain and NLRB the baculoviral inhibitory repeat-like domain (BIR) (**Figure 1**) (40).

In plants, if present, the N-term accessory domain could be of three main types: TIR defining the TIR-NB-LRR (TNLs) group, CC (Coiled-Coil) which defines the CNLs subgroup of NLRs, or CCr (the CC domain subtype with high similarity with the non-NBD-LRR resistance gene RPW8) that characterizes RNLs (48, 49). In addition, different structure variants lacking one or two of the previously described NLR domains can be found (42, 50, 51). Some of these truncated forms, also termed "adapters" or "helpers", amplify the immune response in plants or could serve as effector baits.

Because of the wide diversity and shorter lifespan of pathogens, different modes of NLR-mediated detection of pathogens have emerged, allowing them to bypass the recognition issue due to their obvious slowly evolution (43, 52). In addition to the classic direct detection of one effector by its related NLR, different ways to activate NLRs have been observed such as the "guard and decoy concept" (42). In this strategy, a decoy protein is in close relation with and constitutively represses the activity of a guard NLR. Effectors or endogenous danger signals mediate decoy modification such as direct cleavage, phosphorylation, acetylation, or uridylation (53–56). These post-translational modifications (PTMs) finally disrupt the inhibition signal allowing the NLR to be fully active (42). In this way, many pathogen effectors can be only detected by monitoring a small number of targets, whether directly involved in defenses (guardee model) or not (decoy model). Moreover, as NLRs are often working by pairs or oligomers, it is not rare to observe an "autoimmune" phenotype, i.e., a constitutive activation of NLRs in the absence of pathogen. This phenotype can result from mutations within the molecule (57, 58) or might also be the consequence of the absence of its related inhibitor, as well as an inappropriate negative interaction between closely related NLRs (59, 60). This is particularly common in the case of crossed plant populations, where inappropriate allelic combinations generate underdeveloped or self-deteriorating seedlings due to the fitness imbalance between growth and defense (61, 62). This phenomenon, also known as hybrid incompatibility, thus facilitates the natural selection of related units. In connection with this, integration of decoy domains inside the NLR protein may have been selected more easily, as the probability of recombination being more important in genetically distant partners than in linked proteins. This then gives rise to the last NLR activation strategy described by Jones et al. (42), the "integrated decoy" model, exemplified by the RRS1 TLR that contains an integrated WRKY transcription factor domain, target of numerous effectors, or the NLR RGA5 that holds an integrated heavy metal-associated copper binding domain.

DOWNSTREAM SIGNALING EVENTS LEADING TO CELL DEATH IN ANIMALS

In Animals, although many interplays between cell death signaling pathways exist, pyroptosis is primarily the result of the stimulation of intracellular immune receptors (NLRs and cytosolic DNA sensors) while apoptosis and necroptosis are mainly triggered by membrane-associated TLRs.

NLR-Induced Pyroptosis

The signaling pathway induced by NLR stimulation has been extensively studied in animals and is about to be well characterized [for review, see (63)]. Sensing of PAMPs or DAMPs by the C-term domain triggers NLRs oligomerization and the recruitment of downstream adaptors or signaling proteins to the N-terminal death fold domain *via* homotypic domain interaction, forming large, cytoplasmic, ring-like multi-protein complexes. The best characterized are known as inflammasomes and result in pyropotosis. However, some NLRs such as NOD1 and NOD2 from the NLRC subfamily are not able to induce the assembly of an inflammasome but of a NODosome that ultimately results in the activation of a transcriptional program through NF-κB and/or MAPK signaling pathways (**Figure 4**) (64–66).

Inflammasome is built by the association of a cytoplasmic immune receptor and the effector pro-caspase 1, a cysteine protease from the caspase family (**Figure 4**). Pro-caspase 1 is composed by a CARD-containing prodomain and two active subunits. Its recruitment into the inflammasome *via* CARD-dependent homotypic interactions induces an activating dimerization and subsequent auto-cleavage of the two active subunits that assemble into the tetramer active form. Once activated, caspase-1 induces the processing of the pro–IL-1β and pro–IL-18 into mature cytokines. Caspase-1 can also catalyze the cleavage and activation of the pore-forming protein gasdermin D. Gasdermin D belongs to the pore-forming protein family gasdermin (GSDM) characterized by the presence of an N-terminal pore-forming domain (PFD).

It is synthetized as a precursor composed of two domains linked by a loop. Caspase-1 cleaves Gasdermin D within the loop, releasing the PFD that oligomerizes and anchors into the inner plasma membrane to form pores. Inflammasome-mediated Gasdermin D activation results in IL-1β and IL-18 release, and ionic imbalance that culminates in cell swelling (67, 68). The cleavage of gasdermin D can also be performed by the human CARD-containing caspase-4 and -5 (mouse caspase-11) that is activated in the so-called non-canonical inflammasome thanks to their ability to directly sense LPS by their CARD domain (69). Interestingly, Gasdermin D is also able to form pores into bacterial and organelle membrane such as mitochondria or endoplasmic reticulum that can result in potassium efflux, calcium mobilization and ROS generation (70).

Among NLRs, NLRP1, -3, -4, -6, -7, and NLRC4 can form inflammasome. Moreover, the cytosolic DNA sensors AIM2 and IFI16 also have the ability to form it (71–73). For NLRP3 and AIM2 that do not contain a CARD domain, the molecular adaptor ASC (apoptosis-associated speck-like protein containing a CARD domain), that owns both a pyrin and a CARD domain, is recruited as an intermediate bridge to link the sensor to the pro–caspase-1 (**Figure 4**). ASC adaptor is also observed in the NLRC4 and NLRP1 inflammasomes, stabilizing the interaction between the NLR and the pro–caspase-1 (74). The activation process of NLRP3-inflammasome is the most documented (75). It involves a priming step that can be provided by sensing LPS by TLRs which induces NF-kB-dependent expression of NLRP3 and ASC (76). As the intracellular amount of these proteins have reached a sufficient level, NLPR3-inflammasome activation can be completed by sensing DAMPs (such as ATP) or some PAMPs (viral RNA or proteins, bacterial-derived compounds). Some PTMs such as phosphorylation (77) and de-ubiquitination (78) could also be of importance for the priming step.

TLR-Mediated Apoptosis and Necroptosis

As described above, TLR-mediated signal transduction involves the assembly of multiprotein signaling platforms thanks to the presence of homotypic interacting domains in the receptors, adaptors and effector proteins (16). Ligand binding triggers TLR homo- or heretodimerization, conformational change in their intracellular TIR domain and the consecutive recruitment *via* TIR-TIR homotypic interaction of adaptor proteins. Schematically, TLR signaling pathways are subdivided into MyD88-dependent and TRIF-dependent signaling. Briefly, MyD88 can bind most of endosomal and cell-surface-TLRs. In turn, it promotes the recruitment of serine/threonine IL-1R–associated kinase (IRAK) family members, *via* homotypic interaction with its C-term death domain (DD), to form a multiprotein complex named Myddosome. IRAK4 catalyzes a phosphorylation reaction that leads to the recruitment of the E3-ubiquitine ligase TRAF6 [tumor necrosis factor receptor (TNFR)-associated factor 6], the subsequent recruitment and activation of TAK1/TAB1/3 and IKK complexes. This signaling platform engages MAPKs and NF-κBs, and that ultimately leads to the expression of pro-inflammatory cytokines that include TNFα (tumor necrosis factor-α) (16) (**Figure 2**).

The endosomal TLR3 and the cell surface TLR4 recruit the adaptor TRIF. While TLR3-TRIF binding is direct *via* TIM-mediated homotypic interaction, TLR4 requires the adaptor TRAM that bridges TLR4 to TRIF. TRIF can recruit TRAF3 and/or TRAF6 thanks to the presence of TRAF-binding motifs in the N-terminal part of the protein. TRAF6 promotes subsequent MAPK or NF-κB-dependent transcriptional program while TRAF3 engages the IRF3-activating signaling pathway and the expression of type I IFN genes (16) (**Figure 2**). In addition to the TIR and TRAF-binding domains, TRIF owns a C-terminal RIP homotypic interaction motif (RHIM) also found in the receptor-interacting kinases (RIPs) RIP1 and RIP3 (79).

The serine/threonine kinase RIP1 has been identified in 1995 because of its capacity of binding death receptors from TNFR superfamily (80). Thus, RIP1-dependent signaling pathway can be activated by TLR3 and TLR4 as described above and also indirectly by the other TLRs *via* the NF-κB-dependent production of TNFα and autocrine stimulation of TNFR1. RIP1 appears as the core component of signaling platforms, at the crossroad between inflammatory response, apoptotic and necroptotic cell death signaling pathways [for review see (81)] (**Figure 2**). When modified by non-degradative ubiquitin chains, RIP1 constitutes a scaffold for the recruitment of the kinase complexes TAK1/TAB1/TAB2 and IKK that activate MAPK and NF-κB-dependent transcriptional response. In a non-ubiquitinated form, RIP1 promotes the assembly of a secondary cytoplasmic cell death signaling platforms thanks to its kinase activity. Associated with the adaptor FADD (Fas-associated protein with DD) and caspase-8, it promotes caspase-dependent apoptotic cell death *via* the activation of the proteolytic cascade involving the caspase-3 (82). RIP1 can recruit its closely related protein RIP3 *via* their respective RHIM domain and forms a necrosome (**Figure 2**). Independently of RIP1, RIP3 can also be directly engaged by TLR3 and TLR4 *via* a RHIM-dependent binding to TRIF, and also by the cytosolic DNA sensor DAI from RLR family that owns a RHIM domain (83).

RIP3 is the main effector of necroptosis (84). It is activated by homodimerization and autophosphorylation. RIP3 promotes the activating phosphorylation of MLKL (85). Once activated, MLKL oligomerizes and translocates to the plasma membrane where it interacts with phosphatidylinositols and forms pores or cation channels responsible for membrane permeabilization and disruption and cell death (84, 86). Necroptosis has been associated with the production of ROS. Deletion of RIP3 completely blocked ROS production suggesting that this event occurs downstream of necrosome activation (87).

The concept of necroptosis has emerged over the two last decades but its contribution to the control of pathogen infections is still not very well understood. Experimental necroptosis model usually requires an inhibition of apoptotic signaling pathway, suggesting that necroptosis occurred as a secondary event (88). The role of RIPK3-mediated cell death in antiviral response was highlighted by the analysis of mice deficient in RIPK3 that appeared highly susceptible to Influenza A virus or vaccinia virus (87, 89). In the same manner, macrophages or lung epithelial cell death induced by some bacteria (that include *Salmonella typhimurium*, *Staphylococcus aureus*, *Staphylococcus*

marcescens, or *Streptococcus pneumoniae*) are inhibited by RIPK3 deficiency or necroptosis inhibitors (90–92). Necroptosis could constitute an important defense mechanism against infection with pathogens able to bypass apoptosis induction by expressing anti-apoptotic proteins.

DOWNSTREAM SIGNALING EVENTS LEADING TO CELL DEATH IN PLANTS

NLR-Mediated HR

ETI-related HR cell death is frequently associated with early signaling events such as NLR oligomerization, activation of a MAPK cascade, ROS generation, transcriptional reprogramming, and a later accumulation of phytohormones associated to plant immune responses (93, 94). Once the pathogen is detected through the direct recognition of an effector or *via* a modification of a host molecular target, larger immune receptor complexes are built. Recently, an NLR supramolecular structure termed resistosome and showing similarities with the mammalian inflammasomes has been discovered in plants. In this case, the bacterial pathogen *Xanthomonas campestris* injects into the host plant cells the AvrAC effector which uridylates the PBS1-like protein 2 (PBL2) decoy receptor-like cytoplasmic kinase (RLCK). This modification ($PBL2^{UMP}$) triggers its association with the second RLCK resistance-related kinase 1 (RKS1) that interacts with the NLR Hop-Z–activated resistance 1 (ZAR1). This allows the NBD to become active by exchanging its ADP to ATP (95, 96). Subsequent ATP binding triggers the pentamerization of the ZAR1-RKS1-PBL2 resistosome complex (**Figure 3**). The assembled supramolecular complex forms a funnel-shaped structure with a pore diameter ranging from ~10 to 30 Å. Because of its structural resemblance with the hemolytic pore forming protein fragaceatoxin C and the partial requirement of its association with the plasma membrane for the induction of cell death, Wang and collaborators hypothesized this complex to be pore-forming. The ZAR1 resistosome would thus disrupt the plasma membrane integrity and/or alter the ions homeostasis by acting in a similar manner to the MLKL and gasdermins in mammals (9). Nonetheless, we cannot rule out that the ZAR1 resistosome could also serve as a docking site for others immune actors. Furthermore, some NLRs complexes appear to directly activate the expression of transcription factors, rather than associating with the membrane, to finely tune cell death and immune responses in plants. In that way, the *Arabidopsis thaliana* (Arabidopsis) helper NLR AtNRG1 (N-requirement gene 1) forms a cell death signaling hub when it is associated with the heterodimer formed by the lipase-like protein enhanced disease susceptibility1 (AtEDS1) interacting with the senescence-associated gene101 (AtSAG101) (97–99). However, if AtEDS1 interacts with phytoalexin-deficient 4 (AtPAD4), this molecular complex associates with another helper NLR AtADR1 (accelerated disease resistance 1) to promote the expression of immune genes *via* transcriptional reprogramming (99).

In Arabidopsis, HR cell death pathway is regulated by a MAPK cascade, involving the MAPK kinase kinase MEKK1, the MAPK kinases MKK1/2, and the MAPK MPK4. Interestingly, the CC-NLR protein SUMM2 (suppressor of *mkk1/2*, 2) has been shown to monitor this MAPK cascade (100). Indeed, the Arabidopsis *mekk1*, *mkk1/2*, and *mpk4* mutants or plants in which *MEKK1* has been silenced exhibited spontaneous cell death and constitutive immune responses such as defense gene activation and ROS production (101–106), whereas mutations in SUMM2 suppressed the cell death of *mekk1*, *mkk1/2*, and *mpk4* mutants (100). A screening of Arabidopsis T-DNA insertion lines identified SUMM2, MEKK2, and calmodulin-binding receptor-like cytoplasmic kinase 3 (CRCK3) as key regulators of MEKK1 depletion-induced cell death (107). At the opposite, overexpression of CRCK3 induced a SUMM2- and MEKK2-dependent cell death (106). Altogether, these results suggest that a dedicated plant MAPK cascade plays an important role in the HR signaling, which is under the tight control of different regulator proteins.

The onset of HR is also often associated with the production of ROS. Most of the apoplastic ROS generated during plant-pathogen interactions are produced *via* plasma membrane-localized enzymes with homology to the mammalian phagocytes NADPH oxidases (NOXs) and called respiratory burst oxidase homologs (RBOHs) in plants (108). These enzymes generate apoplastic superoxide ions ($O_2^{\cdot-}$) that rapidly dismutate to hydrogen peroxide (H_2O_2), a well-known microbicide. Following infection by a bacterial pathogen possessing the avirulence gene *AvrRpm1*, HR is reduced in Arabidopsis *AtrbohD* mutant and *AtrbohD/F* double mutant plants, indicating that RBOHs and ROS promote and/or mediate cell death (109). This process requires the concomitant production of nitric oxide (NO; see below). Nevertheless, infection with an avirulent oomycete pathogen in Arabidopsis *AtrbohD/F* double mutant plants caused more HR and resistant phenotype even though ROS production was suppressed (109). Similarly, Arabidopsis *nca1* (no catalase activity 1) and *cat2* (catalase 2) mutants, supposed to have an increased H_2O_2 level, surprisingly showed reduced cell death when infected by a bacterial pathogen expressing *AvrRpm1* (110). At the molecular level, the discovery that RBOHD activity is positively and negatively regulated by many PTMs *via* different phosphorylation or ubiquitination events suggests a finely tuned control of the spatio-temporal ROS production during plant-pathogen interaction (111–114). Thus, the connection between ROS and HR still needs clarifications. Interestingly the Arabidopsis LRR-RK HCPA1 has been recently shown to perceive extracellular H_2O_2 *via* cysteine oxidation to trigger Ca^{2+} influx, which then leads to immune responses such as stomatal closure to delay the pathogen penetration through this natural opening in plants (115).

PRR-Triggered Signaling Events Leading to HR

Apoplastic elicitors which trigger HR are more the exception than the rule. Nevertheless, Avr2/4/5/9 proteins secreted by the fungus *Cladosporium fulvum* induce HR in tomato expressing the corresponding surface RLP Cf-2, Cf-4, Cf-5, or Cf-9, respectively

(116–118). Similarly, fungal endopolygalacturonases secreted by *Botrytis cinerea* trigger plant cell death in a specific Arabidopsis ecotype (Columbia-0) co-expressing the RLPs RBPG1 (responsiveness to *Botrytis cinerea* polygalacturonases1) associated with SOBIR1 (suppressor of BIR1-1) (119). Elicitin proteins secreted by different species of *Phytophthora* can also trigger HR in different solanaceous plants after recognition by the RLP ELR (elicitin response) which forms a molecular receptor complex with the RLK BAK1 (brassinosteroid insensitive 1-associated receptor kinase 1) (120).

Cryptogein produced by *Phytophthora cryptogea* proved to be an efficient biological tool to study the mechanism underlying HR. More precisely, *Phytophthora cryptogea* induces an immune response in tobacco plants characterized by a HR and a transient systemic resistance conferring protection against numerous micro-organisms, including virulent ones (121). Cryptogein secreted by this oomycete is the major inducer of plant immunity and mimics the effects of the oomycete once applied to tobacco plants and cell suspensions (122, 123). Cryptogein is an elicitin protein of 10 kDa that acts as sterol carriers and could supply *Phytophthora* species with sterols, these latter being sterol auxotrophs (124). *In vitro* binding assays provided first evidences that cryptogein is recognized by a putative plasma membrane receptor (125). Even if its molecular identity has not been reported so far, it is plausible to assume that the RLP ELR fulfils this role (120).

The cellular and molecular processes leading to the cryptogein-induced HR have been widely studied, mostly using tobacco cell suspensions. Cells undergoing cell death show a vacuole shrinkage within few hours (126). This vacuole volume loss has been functionally linked to fast and ample nitrate effluxes across the plasma membrane resulting from the activity of anion-permeable channels (126, 127). Accordingly, inhibition of these effluxes, as well as Ca^{2+} influxes from the extracellular space, suppressed or delayed cell death (128). A similar result was observed in cryptogein-treated tobacco cells in which the activity of protein kinases, including MAPK, has been suppressed (129). All together, these data highlighted that the machinery leading to cell death is a dynamic process requiring early signaling events. These latter also include the production of NO and ROS. More precisely, cryptogein triggers within minutes the activation of a plasma membrane NADPH oxidase (NtRBOHD) which produces $O_2^{\cdot-}$ simultaneously dismutated into H_2O_2 through the activity of superoxyde dismutases (130). Interestingly, a coordinated action of ROS and NO has been highlighted (131). Indeed, the production of H_2O_2 is a prerequisite for NO synthesis and functions as impairment of NtRBOHD expression compromises NO production as well as its involvement in cell death. In turn, NO negatively regulates the level of H_2O_2 through the formation of peroxynitrite ($ONOO^-$) resulting from the chemical combination between NO and $O_2^{\cdot-}$. The formation of $ONOO^-$ might mitigate the effects of H_2O_2 and provide a mean to control the intensity of cell death. The possibility that NO also mitigates H_2O_2 production and the amplitude of HR through the inhibition of NtRBOHD by S-nitrosation, a NO-dependent post-translational protein modification, has been reported in other plant-pathogens models (132). However, the occurrence of this mechanism in cryptogein-treated cells has not been confirmed (131).

Caspase-like activities have also been detected in plants during HR. If genes encoding true orthologs of caspases (cysteine dependent aspartate-directed proteases) are absent in the plant genomes, many proteases involved in HR have been identified such as metacaspases in the cytosol, vacuolar processing enzymes (VPEs) in the vacuole or saspase, cathepsin B or papain-like cysteine protease (PLCP-like Rcr3 or Pip1) in the apoplast (9). Interestingly, plant metacaspases are lysine- and arginine-specific, whereas caspases found in mammals are aspartate-specific, indicating a different substrate specificity of these plant enzymes. Based on their protein structure, the phylogenetic analysis of plant metacaspases indicated three major clades that can be divided into type I with, or without, a zinc finger motif in the N-terminus region, and type II harboring a linker region between the two subunits of 10 and 20 kDa of the caspase-like regulatory and catalytic domains (133). In Arabidopsis, the type I metacaspase AtMC1 promotes HR during biotic stresses whereas AtMC2 acts antagonistically by inhibiting plant cell death without any different pathogen dissemination in both *atmc1* and *atmc2* mutants (134). The type II metacaspase AtMC4 is a calcium-dependent cysteine protease which cleaves the PROPEP1 phyto-cytokine in order to release PEP1 in the apoplast, itself detected by the PEPR1 PRR in neighboring cells to amplify plant immune responses during the damage-triggered immunity (135). The recent resolution of the AtMC4 crystal structure highlights the inhibitory role of the large linker domain which blocks activation and substrate access to the catalytic domain (136). Concerning VPEs, they have been shown to exhibit similar enzymatic properties as the animal caspase 1 except that they are active in the vacuole (7). They possess an autocatalytic conversion of the inactive proprotein (pVPE) into the mature active mVPE (137). In tobacco plants infected by the tobacco mosaic virus (TMV), caspase-1 inhibitors or *VPE* gene silencing reduces the caspase-1–like activity associated to the rupture of the vacuolar membrane normally leading to the virus-induced HR (138). During plant immunity, bacterial harpin-induced cell death was compromised in *Nicotiana benthamiana VPE*-silenced plants (139) and inhibitors of caspase-1 delayed the HR cell death normally triggered in tobacco cells by the oomycete elicitor cryptogein (126). In Arabidopsis, a *vpe* null mutant lacking the four *VPE* genes (α–, β–, γ–, and δ–*vpe*) was unable to show any VPE or caspase-like 1 activity (140) and γ-*vpe* mutant was more susceptible to viral, bacterial or fungal infection (141). The extracellular proteases Pip1 and Rcr3 also actively participate to the perception of the fungal pathogen *C. fulvum* in tomato. *C. fulvum* secretes Avr2 into the apoplast which associates with Rcr3 and Pip1. The mentioned complexes perceived by Cf-2, a RLP, trigger HR and induce resistance to *C. fulvum* (142). Recently, the first proteolytic cascade has been discovered in plants where the extracellular immune protease proRcr3 is cleaved by the subtilase P69B in mature Rcr3 that interacts with Avr2 before that the molecular complex binds to the Cf-2

RLP (143). Interestingly, this P69B subtilase has been previously shown to be itself the target of the matrix metalloproteinases Sl2-MMP and Sl3-MMP (144, 145), suggesting that these MMP are initiator proteases whereas Rcr3 plays an effector role in this cascade, as shown for intracellular caspases in mammals.

CELL DEATH REGULATIONS IN MAMMALS AND PLANTS

In host-pathogen interactions, the control of the regulated cell death is crucial for both partners. Host have to adapt their defense responses to contain pathogen development while avoiding their own lethal outcome. At the opposite, pathogens need to modulate the regulated cell death to ensure their infection cycle.

Cross Regulation Mechanisms Between Immune Signaling Pathways

Organisms need to adapt their response as the infection evolves. Thus, many interplays and cross-regulation mechanisms exist between the different immune cell signaling pathways. The stimulation of one receptor can cause different responses including the activation of transcriptional programs or the engagement of cell death pathways, depending on the nature, the duration and the intensity of the stimuli. These responses can occur simultaneously or successively and cells have the ability to change the response very quickly. In animals, the interplay between TLR and NLR signaling pathways is illustrated by the activation of NLRP3-inflammasome in a two-step process. The first priming step involves the TLR-mediated, NF-κB-dependent expression of *NLRP3*. Then DAMPs or PAMPs can stimulate the NLRP3-inflammasome assembly and pyroptosis (76). Thus, NLRP3-inflammasome-mediated pyroptosis is only activated when the TLR-mediated immune response is not sufficient to neutralize pathogens. As illustrated **Figure 2**, TLR4 is able to trigger NF-kB-dependent pro-inflammatory response, IFN-response, necroptosis, or apoptosis. This implies the presence of accurate regulation mechanisms. In addition to promote the production of microbicidal molecules and pro-inflammatory mediators, TLR-mediated NF-κB activation induces the expression of several survival proteins such as cellular inhibitors of apoptosis (cIAPs) (146). cIAPs act as E3-ubiquitin ligases promoting poly-ubiquitination of RIP1. Therefore, they are required for the TLR-dependent activation of transcriptional programs while they inhibit RIP-dependent apoptotic and necroptotic pathways (18, 147). The presence of a second signal that neutralizes cIAP activity could convert the transcriptional signal to a cell death signal. It is interesting to note that some viruses such as baculovirus express proteins from IAP family (148) that block cell death of infected cells and allow viral propagation. The different cell death signaling pathways are also interconnected and have the ability to regulate each other [for review, see (75)]. Blocking apoptosis signaling pathway is generally a prerequisite for detecting necroptosis because the apoptotic caspase-8 can inhibit the activity of key components of the necroptosis signaling pathway including RIP1 and RIP3 (84, 88). On the contrary, caspase-8 can activate the NLRP3-inflammasome (149) or directly cleave the caspase-1 leading to pyroptosis (150). Conversely, inflammasomes can connect caspase-8 to induce apoptosis (151). The necroptotic effector RIP3 is also able to cause NLRP3-inflammasome activation and pyroptosis *via* ROS production (152, 153).

Although less documented, such cross-regulations between immune receptor-mediated signaling pathways probably exist in plants. Mutations or overexpression of some plant immune receptors such as the RLK CERK1, the co-receptor BAK1, its closest homolog BKK1 (BAK1-like 1) or the RLP BIR2 (BAK1-interacting RLK 2) can trigger an enhanced cell death (154–157). This discovery suggests that a plant surveying system probably guards these immune receptors from inhibition by pathogen effectors to trigger HR (9, 158, 159).

Modulation of Immune Signaling Pathways by Post-Translational Modifications

Molecular mechanisms that dictate the response to immune receptor stimulation are not completely understood and are subject of intense research. Because of their flexibility and speed of implementation, PTMs constitute a remarkable and effective process to modulate the intracellular signaling and to regulate the communication networks between cell transduction pathways.

In mammals, as reported above, the priming step required for the full activation of NLRP3-inflammasome has also been shown to involve some PTMs of NLRP3 such as phosphorylation (77) and de-ubiquitination (78). A nice example is given by RIP proteins (RIP1, RIP2, and RIP3) for which the recruitment into various multiprotein signaling platforms, the kinase activity and the ability to engage downstream signaling pathways is orchestrated by PTMs, mainly ubiquitination and phosphorylation (160). The serine/threonine kinase RIP1 is recruited to the TLR3 and TLR4-associated signaling complex (**Figure 2**) and RIP2 is associated with the cytosolic NLRs NOD1 and NOD2 (161) (**Figure 4**). When polyubiquitinated RIP1 and -2 function as a molecular scaffold. It promotes the recruitment and the activation of the kinase complexes IKK and TAB1/TAB2/TAK1 that promote a pro-survival and pro-inflammatory response (**Figure 2**) (146, 160, 161). On the other hand the non-degradative ubiquitination also completely inhibits RIP1 kinase activity that is essential for the assembly of secondary cytoplasmic cell death signaling platforms leading to apoptosis or necroptosis (18, 160). Of note, some viruses have developed strategies to counteract the death of infected cells by modulating RIP ubiquitination (162).

The plant immune responses are also finely regulated by PTMs of signaling proteins. The ubiquitin-proteasome system (UPS) plays an essential role in plant immunity. Among UPS components, E3 ubiquitin ligases have been particularly studied. Wang et al. (163) showed that the E3 ligase OsPUB15 interacts with the homodimerized PID2K, a transmembrane RLK, which confers rice resistance against *Magnaporthe oryzae*. Interestingly, the authors demonstrated that the overexpression of *OsPUB15* led to an enhanced resistance against the pathogen, correlated with up-regulation of some defense genes, excessive accumulation of

ROS and plant cell death, suggesting that ubiquitination of PID2K could favor its activity. However, in this case, the observed lesions were so important that they conducted to the plant death. Another example of the importance of UPS in plant immunity is provided by studies investigating the function of the ATPase cell division cycle 48 (CDC48). CDC48 is a highly conserved chaperone-like protein from yeast to plants and animals [also named VCP (vasolin-containing protein) or p97 in mammals]. This protein catalyzes the disassembly of protein complexes and/or the extraction of ubiquitinated proteins from membranes or chromatin in order to deliver them to the proteasome. Therefore, it plays an important function in UPS and, more generally, proteostasis (164). Investigations of CDC48 function in plant immunity demonstrated that the tobacco CDC48 isoform rapidly accumulates in its hexameric active structure in tobacco cells exposed to cryptogein (165). A screening for its binding partners allowed to the identification of key regulators of the redox status, including cytosolic ascorbate peroxidase (cAPX) a pivotal enzyme for ROS removal (165, 166). In CDC48-overexpressing tobacco cells, the activity of cAPX was impaired, leading to severe decrease in the cell capacity to respond to oxidative stress (167). Accordingly, a faster and pronounced cell death was observed in those cells. Although speculative, the involvement of CDC48 in cell death could also be explained by its ability to promote the degradation of cell death repressors. In animals, several studies have reported a role for its ortholog p97 in viral spreading (poliovirus, herpes simplex virus, cytomegalovirus, or influenza virus) by regulating the cycle of viral replication in infected cells (168). However, the mechanisms and the relationship with immune receptor-induced cell death have not been clearly demonstrated.

Histone deacetylases (HDACs) of type 2 (HD2s) have also been identified as important regulators of the cryptogein-induced HR in tobacco. HD2s design a plant specific family of nuclear histone deacetylases (169). Two HD2s tobacco isoforms, NtHD2a and NtHD2b, were shown to undergo a fast phosphorylation in tobacco cells treated with cryptogein (170). This process was followed by a decrease both at the transcript and protein levels. Interestingly, silencing of *HD2* in cell suspensions or *in planta* led to a faster and amplified cell death manifested by exacerbated HR symptoms. Based on these data, it has been proposed that NtHD2a and NtHD2b act as constitutive negative regulators of HR by modulating the expression or activity of HR regulators or effectors which identities remain to be discovered. In a similar manner, a role for histone deacetylases in the acquisition of cell resistance phenotype has also been observed in mammal macrophages. An upregulation of HDAC8 has been correlated with the acquisition of a resistance phenotype to anthrax lethal toxin (LeTx). HDAC inhibitors sensitized cells to LeTx-induced pyroptosis while inversely upregulation of HDAC8 prevents LeTx-induced cell death (171).

Regulation of Immune Signaling Pathways by miRNAs

As NLRs are often tightly linked to strong immune process including cell death, these proteins need to be finely tuned to avoid any deleterious impact on the plant fitness in the absence of pathogen. In accordance, animals and plants possess several regulation processes among which are miRNAs. These regulatory elements function similarly in animals and plants and are in the same way excised from long primary miRNA transcripts by Dicer or Dicer-like enzymes (such as DCL1) before being loaded into an RNA-induced silencing complex (RISC) and to repress the gene by DNA methylation or by cleavage, destabilization or translational inhibition of its messenger RNA (mRNA) (172). These miRNAs are involved in the regulation of different biological processes, and particularly studied in plants in development and defense contexts. In addition to interact with exogenous nucleic acid and defend plant cells against viral pathogens (173), some host miRNAs also target their own transcripts encoding immune receptors such as the NLR proteins (174–177). This is believed to allow them to control their immune reactions in the absence of pathogen and therefore to avoid any unnecessary waste of energy. In addition, since some bacterial and viral pathogens infect their host by blocking its miRNA interference process, the decrease in these miRNAs would also, at the same time, lead to an accumulation of the previously repressed immune receptors, ultimately leading to a potentiation of defenses (174, 176, 178).

Regulation of Plant Immunity by Phytohormones

Plant immunity is also regulated by a complex network of phytohormones, which integrate signals from biotic and abiotic stresses in order to finely tune the spatio-temporal expression of the different immune responses. Among them, salicylic acid (SA) and jasmonic acid (JA) play major roles and their antagonism is believed to specifically adapt the plant immunity to biotrophic or necrotrophic pathogens, respectively (179). SA has been shown to positively regulate the HR cell death during interaction with biotrophic pathogens whereas JA seems to be more important for the plant resistance against invading necrotrophs or insects. Actually, low level of SA downregulates HR cell death whereas high level of SA triggers plant cell death (180). Moreover, this hormonal balance between SA and JA seems to finely regulate plant cell death locally as SA accumulates into the HR-related cell death zone whereas JA level increases in the surrounding area to act antagonistically with the SA-pathway (181).

Pathogens Interfere With Cell Death Signaling Pathways to Their Own Benefits

According to their infection cycle, some pathogens also interfere with regulated cell death signaling pathways to their own benefits (182). So, it is generally accepted that viruses and biotrophic pathogens whose survival is fully dependent on the intracellular machinery of host cells can delay or inhibit cell death contrary to necrotrophic ones which take nutrients from dead cells. However, the classification does not always reflect the complexity of the pathogen cycle infection. The strategies used by pathogens to evade host defenses in order to favor their multiplication and spread have been widely studied in animal cells. Many viruses or bacteria deliver anti-apoptotic proteins

TABLE 1 | Main signaling pathways driving cell death or transcriptional reprogramming in response to activation of membrane-associated or intracellular immune receptors in animals and plants.

		Animals	Plants
Membrane-associated receptors	**Receptors**	**TLRs, CLRs**	**RLKs, RLPs**
	Signal transduction domain	homotypic interacting domains	kinase domains
	Mechanisms of signal transduction	assembly of multiprotein complexes in which executors are activated by proximity	PRR oligomerization, kinase activation & trans-phosphorylation
	Signaling pathways leading to transcriptional reprogramming	MAPK, NF-κB or IRFs (MyD88, TRIF or RIP1-dependent)	MAPKs and phosphorylation-dependent kinases ROS/NO production Ca^{2+} influx transcription factors activation Phyto-cytokines secretion
	Cell Death	**Necroptosis** **Apoptosis**	**Hypersensitive Response (HR)***
	Cell death signaling, executors, associated features, regulation	Signaling platforms: Necrosome Ripoptosome Key executors: RIP3, pore-forming MLKL (RIP1 dependent or independent) RIP1, Caspases cascade (caspase-8, -3, -7) associated features: ROS, membrane permeabilisation Silent form of cell death	Ions fluxes across the PM Production of NO and ROS Inhibition/degradation of cell death repressors (HD2s…) Proteases activation (metacaspases in the cytosol, VPEs in the vacuole) Phyto-cytokines secretion Chromatine condensation, nucleus disruption, vacuolar collapse
Intracellular receptors	**Receptors**	**NLRs, RLRs, ALRs**	**NLRs**
	Signal transduction	Homotypic interacting domains	Proteins interaction
	Mechanisms of signaling activation	Assembly of multiprotein complexes in which executors are activiated by proximity	Effectors detection (direct) or proteins modification (indirect)
	Signaling pathways leading to transcriptional reprogramming	NODosome assembly leading to MAPK & NF-κB	Unknown (direct activation of transcription factors by NLRs or linked to pore-forming structures in the PM?)
	Cell Death	**Main: Pyroptose** **Alternative: Necroptose**	**Hypersensitive response (HR)**
	Signaling pathways/platforms leading to cell death	Inflammasome	Resistosome, other NLR complexes
	Key executors of cell death	Caspase 1, pore-forming Gasdermin D	Unknown (pore-forming Ca^{2+}-dependent activation of proteolytic cascade?)
	Associated-features, regulations	IL-1β, IL-18 release, membrane permeabilisation, ionic unbalance, ROS production	ROS & NO production, phytohormones accumulation, membranes permeabilization, release of active proteases and phyto-cytokines

ALR, AIM2 (absent in melanoma 2)-like receptors; AtEDS1, Arabidopsis thaliana enhanced disease susceptibility 1; AtNRG1, Arabidopsis thaliana N-requirement gene 1; AtSAG101, Arabidopsis thaliana senescence-associated gene101; CLR, C-type lectin receptors; HD2, Histone deacetylase; HR, Hypersensitive response; IL, Interleukin; IRF, Interferon-regulatory factors; MAPK, Mitogen-activated protein kinase; MLKL, Mixed lineage kinase domain-like; MyD88, myeloid differentiation factor 88; NLR, Nucleotide-binding and oligomerization domain (NOD)-Like Receptor [animals] or Nucleotide-Binding Domain (NBD)-containing LRRs [plants]; NO, Nitric oxide; PLCP, papain-like cysteine proteases; PM, plasma membrane; RLK, Receptor-Like Kinase; RIP, receptor-interacting kinase; RLP, Receptor-Like Protein (contains a short cytoplasmic domain devoid of kinase activity); RLR, RIG-I-like receptors; ROS, Reactive oxygen species; TLRs, Toll-like receptors; TRIF, TIR-domain-containing adaptor-inducing IFN-b; VPE, Vacuolar processing enzyme.
**HR cell death induced by membrane-associated receptors is an exceptional outcome.*

that directly block apoptotic, necroptotic and/or pyropotic machineries. For example, caspase-1 and/or caspase-8 involved in pyroptosis and apoptosis (**Figures 2** and **4**), respectively, can be directly inhibited by serpin (serine proteinase inhibitor) homologs encoded by poxvirus, the influenza virus protein NS1, the vaccinia virus protein B15N or effectors molecules secreted by *Pseudomonas aeruginosa* or *Yersinia* spp. (183). The necroptosis executor RIP3 can be sequestered by MLKL

homologs produced by poxviruses (184). RIP1 involved in TLR3 and 4 signaling pathways (**Figure 4**) is also frequently found as a target. Virus or bacteria effectors can modulate the PTM of RIP1 such as phosphorylation or ubiquitination, thereby affecting its kinase activity and its cell death-promoting ability (apoptosis or necroptosis). Thus, the latent membrane protein 1 (LMP1) from Epstein-Barr virus (EBV) and op-IAP produced by baculovirus can promote the poly-ubiquitination of RIP1 (162, 185) and the *Yersinia pestis* effector YopJ/P modulates the phosphorylation status of RIP1 by targeting the kinases TAK1 and IKK or MK2 (186). Pathogens can bypass host defense mechanisms by blocking signaling pathways just downstream of the pathogen recognition-receptor. This is illustrated by the enterohemorrhagic bacteria type 3 that produces a protease that cleaves the RHIM domain owned by RIP1, RIP3, TRIF, the adaptor proteins involved in TLR3 and TRL4-mediated signaling pathways and the cytosolic DNA sensor DAI (187). On the other hand, RHIM-homotypic interaction that mediates the assembly of the necrosome, as well as the recruitment of RIPs to sensors (**Figure 4**) can be affected by the presence of viral RHIM-containing proteins such as the proteins ICP6 and ICP10 produced by Herpes simplex virus (188), vaccinia virus innate immune evasion protein E3 (189), or vIRA encoded by the murine cytomegalovirus (190).

What is the situation in plants? Biotrophic or hemibiotrophic phytopathogens have to keep plant cells alive to ensure their infection cycle. In this way, they secrete many effectors which target receptors or key signaling components to suppress host immunity triggered by their own invading patterns (191, 192). As examples, RipAY produced by *Ralstonia solanacearum* inhibits SA-dependent defense responses and HR induced by the effector RipE1 in *Nicotiana benthamiana* (193), and RipAK suppresses catalase activity and HR of *Nicotiana tabacum* (194). *Phytophtora infestans* AVR3a targets the E3 ligase CMPG1 and suppresses HR induced by the elicitin INF1 in *Nicotiana benthamiana* (195, 196). In addition, it has been shown that *P. infestans* PexRD2 interacts with the KD of MAPKKKε, a positive regulator of cell death, increasing the susceptibility of *Nicotiana benthamiana* to this pathogen (197).

Inversely, necrotrophic phytopathogens favor plant cell death to ensure the infection spreading. For example, the broad host range necrotrophic plant pathogen *Sclerotinia sclerotiorum* secretes oxalic acid (OA) which is considered as a key molecule for its pathogenesis. It has been shown that OA has opposite roles: i) to suppress host oxidative burst and then HR at early state of infection allowing the establishment of the pathogen and ii) to activate plant cell death, *via* ROS production, facilitating disease development (198, 199). Besides OA secretion, *Sclerotinia sclerotiorum* produces several effectors or toxins inducing plant cell death (200). For some of them, secreted in apoplast such as SsNE1-SsNE5, the death-inducing signal is mediated by the BAK1/SOBIR1 receptor complex (200). The involvement of these RLK was already highlighted for the necrotizing activity of the xylanase BcXYG1 secreted by *Botrytis cinerea* (201). Others would be internalized in host cells as the "effector-like" protein SsSSVP1 which interacts with QCR8, a subunit of plant cytochrome complex in mitochondrial respiratory chain (202). This leads to the loss of function of QCR8 and to plant cell death induction. Actually, numerous phytopathogens secrete several toxins or effectors to induce cell death. Around 180 apoplastic cell death-inducing proteins (CDIPs) have been identified and for some of them, the associated receptors are known (203, 204). Other toxins are internalized in host cells and interact in some case with NB-LRR (182). Thus, the Arabidopsis susceptibility to *Cochliobolus victoriae* is due to the interaction between the secreted toxin victorin with the NB-LRR LOV1. In this case, the pathogen co-opts HR to facilitate its development (205).

A beneficial effect of cell death for pathogens to ensure infection cycle has also been described in mammals. It is well illustrated by the human immunodeficiency virus (HIV) that hijacks the immune surveillance by promoting the pyroptosis of immune cells ($CD4^+$ lymphocytes) (206).

CONCLUDING REMARKS

Although number of questions still remain to address, the intense research of the scientific communities on innate immunity in mammals during the three last decades and recent technological advances gave rise to a relative clear scheme of the cell death-signaling pathways activated in response to immune receptors (**Table 1**). By comparison, the understanding of the immune receptor-induced cell death signaling pathways remains incipient in plants although the use of Arabidopsis mutants allowed the identification of signaling molecules and regulators of HR (207–209). Up to date, more than 600 RLKs and 100 NLRs have been inventoried but only few have been characterized and many have not even been identified yet. The abundance of immune receptors, the different processes used for their activation as well as the diversity of cellular models make the decoding of cell death signaling pathways very difficult. The generic name HR does not reflect the complexity of signaling pathways and, as in mammals, recognition of PAMPs, DAMPs or effectors does probably not lead to the engagement of one unique cell death response but likely activates different cell death-signaling pathways with specific features and outcomes. The cell death signal also likely depends on the cell type and plant species.

The plant immune response is associated with different biochemical modifications and cellular signals that include MAPK activation, oxidative and nitrosative bursts, calcium fluxes, phytohormones production, protease activation, and transcriptional reprogramming (5). Studies analyzing their involvement in immune receptor-induced cell death reported controversial results and their direct role in transducing cell death signal is still debated. A closer characterization of the spatial and temporal aspects of these cellular events could probably provide a better view of their involvement. By putting animal models into perspective, we can hypothesize that the stimulation of plant membrane PRRs or NLRs have the ability to engage (i) signaling pathways leading to transcriptional reprogramming responsible for phytohormones production

and expression of defense genes, and (ii) a cell death signaling pathway that can culminate into ions imbalance and in the rupture of plasma membrane, both regulated by interplays and cross-regulation mechanisms.

The analysis of plant NLRs structure showed that the signal transduction domains belong to conserved homotypic interacting domain family. This suggests that mechanisms of activation involve the assembly of multiprotein platforms. A range of evidences indeed suggests that plant NLRs form signaling platforms to promote cell death (39, 96–99, 204, 210), as observed for mammals NLRs. The recent works of Wang and colleagues (96, 210) highlighting the presence of a resistosome that can translocate into plasma membrane to probably form pore-like structures provided very important elements into understanding the plant NLR-mediated cell death signaling. It is interesting to note that the lumen of the funnel-shape structure found in the resistosome has negative electrostatic potentials given by two negatively charged glutamic acid residues (96). Such negatively charged glutamate residue is thought to be critical for anion selectivity in several human Ca^{2+} voltage-dependent channels (211, 212). It is thus tempting to speculate that this selectivity for cations could be associated with a death process. The similarities between the ZAR1-resistosome and NLR inflammasomes as discussed in the recent review of Xiong et al. (213) suggest that the NLR-mediated cell death signaling pathway could be a conserved process.

While the role and mechanisms of activation of proteases (i.e., caspases-1, -8, and -3) in PRR-induced signaling pathways start to be well characterized in mammals, the activation mechanisms of plant proteases and their importance in the immune-receptor-induced HR remain important questions to address. Indeed, our knowledge about the proteolytic cascades involved in HR cell death is really scarce and fragmentary. Of interest, AtMC4 has been recently shown to be activated by Ca^{2+} (136), suggesting a link between calcium influx, metacaspase activation and release of mature phytocytokines. However, many things remain to discover such as the different proteolytic cascades involved, their initiation and regulation during plant HR.

FUTURE APPLICATIONS FOR PLANT PROTECTION

In Arabidopsis, the screening of vast mutant collections and naturally occurring ecotypes, as well as forward genetic approaches, has led to the successful identification of novel immune receptors involved in HR cell death. In crops, analyzing genomic variations within different cultivars but also the "wild" relative species and their introgression lines allowed to map the Quantitative Trait Loci (QTLs) related to disease resistance. Although QTLs will mostly carry *R*-genes, they may also contain PRR genes (encoding RLKs or RLPs). In a scientific point of view, it is interesting to note that PRRs can be successfully transferred from one plant species to another to provide a novel source of resistance. A very effective demonstration was achieved in tomato (from the Solanaceae family), where the transfer of the EFR RLK receptor (from the Brassicaceae family) led to a great resistance of plants against a wide range of different bacterial pathogens (214). Different studies also showed that the ectodomain and KDs from distinct PRRs can be combined in order to form chimera receptors with preserved signal transduction. Such a chimeric receptor was built from the chitin-binding ectodomain of OsCEBiP and the KD of Xa21. This chimera receptor was able to initiate HR in rice thus conferring to the plant a highly improved resistance to the fungus *Magnaporthe oryzae*. This strategy thus represents an interest for practical use in disease resistance engineering (215).

Analysis of the polymorphism occurring in plant immune receptors or cell death regulators in different cultivars or species could lead to the identification of more efficient variants. As an alternative to the transgenic approach, conventional breeding can be assisted by the use of molecular markers that help to deliver the desired gene into the crop, pyramiding it with other genes important for the plant resistance such as *R*-genes.

All over, the immune receptor-based breeding, the transfer and creation of novel chimeric PRRs might be applicable as an alternative in agriculture disease and pest management, as a "tailored immune-receptor therapy" that might provide more durable and broader resistance when associated with *R*-genes.

AUTHOR CONTRIBUTIONS

TR, M-CH, DW, AZ, LD, and BP contributed equally to the writing of this review. All authors contributed to the article and approved the submitted version.

FUNDING

TR, M-CH, and BP are financially supported by Agence Nationale de la Recherche (ANR) and Agence Française pour la Biodiversité (AFB) ("ChitoProtect" project, grant # ANR-19-ECOM-0008) and Institut Carnot Plant2Pro ("VitiLYKs" project, grant # C4520). TR, M-CH, DW, AZ, LD, and BP have been supported by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (MESRI). DW is supported by the MESRI, Investissements d'Avenir program, project "Initiatives Science Innovation Territoire Economie en Bourgogne-Franche-Comté" ("Structure, Function and Roles of Nitric Oxide Synthases in Algal Responses to Environemental Stresses (NOISELESS)" project, grant # RA18041.AEC.IS) and the ANR ("Algae-Nitric Oxide Synthases" project, grant # ANR-18-CE20-0022-02). LD is supported by grants from "La Ligue contre le cancer" Comité de la Côte d'Or, the "Conseil Regional de Bourgogne-Franche-Comté", the ANR, ("Investissements d'Avenir" program, grant # ANR-11-LABX-0021) and the European Union program FEDER.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 May 2021
doi: 10.3389/fimmu.2021.640315

Prednisolone Suppresses the Extracellular Release of HMGB-1 and Associated Inflammatory Pathways in Kawasaki Disease

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 11 December 2020
Accepted: 04 May 2021
Published: 17 May 2021

Citation:
Ueno K, Nomura Y, Morita Y and Kawano Y (2021) Prednisolone Suppresses the Extracellular Release of HMGB-1 and Associated Inflammatory Pathways in Kawasaki Disease. Front. Immunol. 12:640315. doi: 10.3389/fimmu.2021.640315

Innate immune activity plays an essential role in the development of Kawasaki disease (KD) vasculitis. Extracellular release of high mobility group box-1 (HMGB-1), an endogenous damage-associated molecular pattern protein that can activate the innate immune system and drive host inflammatory responses, may contribute to the development of coronary artery abnormalities in KD. Prednisolone (PSL) added to intravenous immunoglobulin treatment for acute KD may reduce such abnormalities. Here, we evaluate the dynamics of HMGB-1 and therapeutic effects of PSL on HMGB-1-mediated inflammatory pathways on KD vasculitis *in vitro*. Serum samples were collected prior to initial treatment from patients with KD, systemic juvenile idiopathic arthritis (sJIA), and from healthy controls (VH), then incubated with human coronary artery endothelial cells (HCAECs). Following treatment of KD serum-activated HCAECs with PSL or PBS as a control, effects on the HMGB-1 signaling pathway were evaluated. Compared to that from VH and sJIA, KD serum activation induced HCAEC cytotoxicity and triggered extracellular release of HMGB-1. KD serum-activated HCAECs up-regulated extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and, p38 phosphorylation in the cytoplasm and nuclear factor kappa B (NF-κB) phosphorylation in the nucleus and increased interleukin (IL)-1β and tumor necrosis factor (TNF)-α production. PSL treatment of KD serum-activated HCAECs inhibited extracellular release of HMGB-1, down-regulated ERK1/2, JNK, p38, and NF-κB signaling pathways, and decreased IL-1β and TNF-α production. Our findings suggest that extracellular HMGB-1 plays an important role in mediating KD pathogenesis and that PSL treatment during the acute phase of KD may ameliorate HMGB-1-mediated inflammatory responses in KD vasculitis.

Keywords: pediatrics, Kawasaki disease (KD), DAMPs (damage-associated molecular patterns), prednisolone, high mobility group box-1

INTRODUCTION

Kawasaki disease (KD), an acute systemic vasculitis of unknown etiology, commonly occurs in children (1), and can ultimately lead to complex coronary artery abnormalities (CAAs). Despite standard treatment with high-dose intravenous immunoglobulin (IVIG) and aspirin, approximately 10% to 20% of patients experience persistent or recurrent fever and appear to have elevated risk of developing CAAs (2, 3). Notably, combined treatment with prednisolone (PSL) is more effective in preventing CAAs than IVIG alone in non-responders, thereby reducing the need for additional rescue treatments (4). Thus, corticosteroid combination therapy is considered a promising pre-emptive primary treatment (5, 6).

Innate immune activity is integral toward KD vasculitis etiology (7). High mobility group box-1 (HMGB-1), a representative damage-associated molecular pattern (DAMP) protein, plays a central role in regulating programmed cell death and survival (8). As sentinel innate immune cells, endothelial cells release DAMPs as endogenous danger signals that alert the innate immune system to unscheduled cell death, microbial invasion, and stress (9). Extracellular HMGB-1 coordinates cellular responses associated with immune system activation, cell migration, cell growth, and tissue repair and regeneration, and binds to receptors, such as receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLRs) to activate proinflammatory responses. Downstream signaling involving mitogen-activated protein kinase (MAPK) such as extracellular signal-related kinase (ERK)/c-Jun N-terminal kinase (JNK)/p38 and nuclear factor kappaB (NF-κB) facilitates cellular responses including inflammatory cytokine, chemokine, and corresponding receptor expression (10). Injury-evoked increased HMGB-1-mediated inflammatory responses can increase cardiovascular disease severity (11–14). Notably, serum HMGB-1 and S100 protein levels are also elevated in patients in the acute phase of KD (15–17). Moreover, Nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3)-dependent endothelial cell pyroptosis *via* HMGB-1/RAGE/cathepsin B signaling may contribute to coronary artery endothelial cell (CAEC) damage in KD vasculitis (18). Thus, DAMP-mediated innate immune system activation may facilitate pathological inflammatory responses in KD vasculitis.

We hypothesize that PSL treatment, a standard anti-inflammatory therapy, may suppress inflammation in KD by reducing inflammatory cytokines and DAMPs produced by CAECs consequent to acute KD vasculitis. In this study, we clarified the role of HMGB-1 and evaluated the *in vitro* therapeutic effects of PSL on HMGB-1-mediated inflammatory responses in CAECs during acute KD vasculitis.

Abbreviations: KD, Kawasaki disease; HMGB-1, high mobility group box-1; PSL, Prednisolone; sJIA, systemic juvenile idiopathic arthritis; HCAECs, human coronary artery endothelial cells; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; NLRP3, Nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3; IL, interleukin; TNF, tumor necrosis factor; CAA, coronary artery abnormalities; IVIG, intravenous immunoglobulin; DAMPs, damage-associated molecular pattern; RAGE, receptor for advanced glycation end products; TLRs, Toll-like receptors; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription–polymerase chain reaction.

MATERIALS AND METHODS

Patients

The study was approved by the Kagoshima University and Kagoshima City Hospital Ethics Committee and performed in accordance with the International Conference on Harmonization guidelines for Good Clinical Practice and the Declaration of Helsinki (approval number: MD26-156, Approval date: January 13, 2016). We enrolled eight consecutive patients undergoing treatment for KD at the host institution applying the following exclusion criteria: 1) cardiovascular disease, hematological disease, congenital malformations, primary disease of major organs, and genetic/chromosomal abnormalities; 2) bacteremia or sepsis with positive blood culture; 3) recurrent KD symptoms; 4) previous use of corticosteroids or immunosuppressive treatment; 5) development of coronary artery lesions. We used four healthy subjects and four patients with systemic juvenile idiopathic arthritis (sJIA) as vehicle and disease controls, respectively. Written informed consent was obtained from the parents of all study participants and serum samples were collected from the patients and healthy subjects.

KD was defined using the Japanese criteria (19). The first day of illness was defined as the first day of fever. Treatment was initiated when KD was considered highly likely even if all KD criteria were not met. Patients with KD received a single IVIG infusion (2 g/kg) together with aspirin (30 mg/kg/day, decreased to 3 to 5 mg/kg/day if afebrile for ≥ 28 days following fever onset).

Blood-Sample Collection

Serum samples were collected from patients with KD and sJIA before initial treatment and from healthy controls, separated by centrifugation (700 × *g*, 15 min), and stored at −40°C.

Endothelial Cell Culture and Preparation

Primary human CAECs (HCAECs) were purchased from PromoCell (Heidelberg, Germany) and cultured using MV 2 kit endothelial cell growth medium (PromoCell). Medium was changed every 24 h. Cells at 70% to 80% confluence were seeded into 96-, 8-, or 6-well microplates for assays and fluorescence microscopy. Third passage HCAECs were used for experiments. Vehicle (VH) and KD controls comprised HCAECs at 90% confluence incubated for 24 h in MV 2 basal medium (PromoCell) with 7.5% healthy volunteer or KD patient serum, respectively. Serum-activated HCAECs treated with phosphate buffered saline (PBS) and PSL (10^{-6} M/well (20); Shionogi, Osaka, Japan) for 24 h were defined as KD+PBS and KD+PSL HCAECs, respectively. After each experiment, media were replaced with serum-free fresh MV 2 growth media to discriminate serum cytokine effects. ELISA evaluation of final washes demonstrated TNF-α levels (R&D systems, Minneapolis, MN,

USA) below detectable limits (< 5.5 pg/mL) (**Supplementary Figure 1**). Experiments were repeated at least twice and media were maintained between pH 7.2 and 7.4.

Analysis of Serum-Activated HCAEC Viability and Cytotoxicity

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Dojindo, Kumamoto, Japan). HCAECs were cultured in growth medium in 96-well plates (0.5×10^4 cells/well). Following each experiment, medium was replaced with fresh MV 2 basal medium and the final sample volume adjusted to 100 µL/well. Samples were subjected to MTT assay according to manufacturer instructions and measured in duplicate, using Microplate Reader (Tecan Infinite M200).

Cytotoxicity was evaluated *via* fluorescence to measure the activity of dead-cell protease, which is released from cells with impaired membrane integrity, using a CytoTox-Glo cytotoxicity assay (Promega, Madison, WI, USA) according to manufacturer instructions. Briefly, HCAECs (0.5×10^4 cells/well) were cultured in growth medium in 96-well plates. After each experiment, medium was replaced with fresh MV 2 basal medium (100 µL/well final volume). Fluorescence measured using a Tristar multimode microplate reader (LB 941; Berthold Technologies, Oak Ridge, TN, USA), was directly proportional to the number of dead cells. Each sample was measured in duplicate.

Assessment of Extracellular HMGB-1 Released From KD Serum-Activated HCAECs

HMGB-1 content in supernatant released from KD serum-activated HCAECs for 24 h was measured in duplicate using a commercial ELISA kit (Shino-Test Corporation, Tokyo, Japan) according to manufacturer instructions. The minimum HMGB-1 detection value was 1.0 ng/mL.

Quantitative Analysis of Receptors on KD Serum-Activated HCAECs

Total RNA samples were extracted from cell lysates of serum-activated HCAECs using the RNeasy Mini Kit (#74104; QIAGEN, Hilden, Germany) according to manufacturer's instructions. Reverse transcription was performed using a PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan) according to manufacturer's instructions. An equivalent volume of cDNA solution was used for real-time PCR quantification using a Thermal Cycler Dice Real Time System (TaKaRa), with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal standard. At least two biological replicates were performed and specific PCR product amplification was confirmed by melting curve analysis. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. **Table 1** lists primer sequences (*GAPDH*, *RAGE*, *TLR2*, and *TLR4*) and RT-PCR conditions.

Soluble RAGE (sRAGE) Production

Supernatant sRAGE levels released from KD serum-activated HCAECs for 24 h were measured in duplicate using a commercially available ELISA kit (R&D Systems) according to manufacturer instruction. The minimum sRAGE detection value was 4.21 pg/mL.

Immunofluorescence Staining for HMGB-1

HCAECs prepared on 8-well imaging chamber (7.0×10^4 cells/well) were incubated with MV 2 growth medium (37°C, 5% CO_2). VH, KD control, KD+PBS, and KD+PSL HCAECs were washed with PBS, fixed, permeabilized using the Image-iT fixation/permeabilization kit (Invitrogen, Grand Island, NY, USA) and intracellularly stained with Alexa Fluor 594 anti-HMGB-1 (red) in blocking buffer overnight at 4°C. Nuclei were counterstained using ProLong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI, blue) (Life Technologies, Eugene, OR, USA). Randomly selected cells (n = 100) from each group were observed using fluorescence microscopy (Keyence BZ-X700; Carl Zeiss, Oberkochen, Germany). Quantitative analysis of immunofluorescence staining was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (21).

Western Blot Analysis

Third-passage HCAECs were cultured in 6-well plates (4.0×10^5 cells/well) with growth medium. After each experiment, adherent cells were lysed using the total protein extraction kit for animal cultured cells and tissues (Invent Biotechnologies, Plymouth, MN, USA) and prepared for immunoblotting. Each 15 µL sample was subjected to 10% gradient SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane, then immunoblotted using primary antibodies against phosphorylated ERK 1/2 (p-ERK; Cell Signaling Technology (CST), Beverly, MA, USA; 1:2,000 dilution), ERK 1/2 (CST; 1:1,000), phosphorylated stress-activated protein kinase (SAPK)/c-Jun amino terminal kinase (JNK) (CST; 1:1,000), SAPK/JNK (CST; 1:1,000), phosphorylated p38 (CST; 1:1,000), p38 (CST; 1:1,000), anti-NLRP3 (CST; 1:1,000), anti-cleaved Caspase-1, (CST; 1:1,000), IL-1β (CST; 1:1,000), and TNF-α (CST; 1:1,000), followed by horseradish peroxidase-conjugated secondary antibody (Medical & Biological Laboratories, Nagoya, Japan; 1:1,000). For NF-κB p65, nuclear and cytoplasmic proteins were extracted using an extraction kit (SC-003, Invent Biotechnologies) according to manufacturer instructions. Each 15 µL sample was subjected to 10% gradient SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane and immunoblotted using primary antibodies against phosphorylated NF-κB p65 (p-NF-κB p65; CST; 1:1,000) or NF-κB p65 (CST; 1:1,000), followed by horseradish peroxidase-conjugated secondary antibody (Medical & Biological Laboratories; 1:1,000). Housekeeping protein, such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls on the account of their expression levels. Proteins were visualized by chemiluminescence with SignalFire ECL Reagent (CST), and quantified using Fluor Chem FC2 (Alpha Innotech Kasendorf, Germany). All blotting experiments were repeated at least twice.

Statistical Analysis

Continuous variables are reported as median values with interquartile ranges (IQR; 25th–75th percentiles). Categorical variables are presented as frequencies and percentages. Baseline comparisons between patients were performed using Student's t-tests, Mann–Whitney U-tests, or χ^2 analysis (with Yates' correlation or Fisher's exact test, as appropriate). Differences between > 2 groups were evaluated by one-way ANOVA followed

TABLE 1 | Primer sequences and PCR conditions.

mRNA	Primer sequences	Annealing time and temperature (°C)	Cycle no.	Fragment length/base pairs
GAPDH	sense: 5′-GCACCGTCAAGGCTGAGAAC-3′ antisense: 5′-TGGTGAAGACGCCAGTGGA-3′	0.5 min; 95	40	138
RAGE	sense: 5′-GGAAAGGAGACCAAGTCCAA-3′ antisense: 5′-CATCCAAGTGCCAGCTAAGA-3′	1 min; 59	30	166
TLR2	sense: 5′-GGCTTCTCTGTCTTGTGACC-3′ antisense: 5′-GGGCTTGAACCAGGAAGACG-3′	0.5 min; 49	32	294
TLR4	sense: 5′-TTGTATTCAAGGTCTGGCTGG-3′ antisense: 5′-GCAAACCTTTGAAACTCAAGCC-3′	0.5 min; 47	32	438

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RAGE, receptor for advanced glycation end products; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4.
Bold values indicate statistically significance in patient characteristics between KD group and sJIA group.

by the Bonferroni or Games–Howell test and the Kruskal–Wallis test with the Dunn's *post hoc* test. The former was performed when the variables showed a normal distribution; otherwise, the latter was used. All statistical analyses were performed using SPSS statistical software (v.25.0; SPSS Japan Inc., Tokyo, Japan). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics and Laboratory Findings in Patients With KD and sJIA

Sera from eight patients with KD (median, 1.6 years) and four with sJIA (median 7.8 years) were evaluated. **Table 2** lists patient clinical characteristics and laboratory findings. Age and body weight significantly differed between the groups as KD and sJIA patients exhibited different peak ages of onset. Laboratory data, including baseline white blood cell counts, neutrophil counts, and C-reactive protein levels, did not significantly differ with the exception of total protein and sodium levels.

Serum Concentration From Patients With KD Necessary to Serum-Activate HCAECs

To determine the serum concentration from patients with KD necessary to exert cytotoxic effects on HCAECs, we first exposed HCAECs to four independent KD sera concentrations, 0% (untreated), 5%, 7.5%, and 12.5%, for 24 h. KD serum induced significant cytotoxic effects on HCAECs at concentrations ≥ 7.5% (**Supplementary Figure 2A**) while maintaining cellular viability (**Supplementary Figure 2B**). Therefore, 7.5% KD serum was used for subsequent *in vitro* experiments.

Proliferative Activity and Cytotoxicity of Serum-Activated HCAECs

MTT assay revealed that cell proliferation of serum-activated HCAECs from KD controls was significantly higher than that from the VH and sJIA groups (each $P < 0.001$) (**Figure 1A**). In KD, the proliferation of serum-activated HCAECs from the KD+PSL group was significantly lower than those of serum-activated KD controls and KD+PBS group ($P < 0.002$, and $P = 0.012$, respectively) (**Figure 1B**). Cytotoxicity in serum-activated HCAECs from KD controls was significantly higher than those in the VH ($P = 0.007$) and sJIA groups ($P = 0.044$) (**Figure 1C**). In KD, cytotoxicity of serum-activated HCAECs from the KD+PSL group tended to be lower than those of serum-activated KD controls and KD+PBS group but did not achieve statistical significance (**Figure 1D**).

KD Serum-Activated HCAEC Supernatant HMGB-1 and sRAGE Levels and HMGB-1 Receptor Expression

Supernatant HMGB-1 levels (**Figure 2A**) and sRAGE levels (**Figure 2B**) in the KD controls were significantly higher than those in the VH (each $P < 0.001$) and sJIA ($P < 0.001$ and $P = 0.046$, respectively) groups. HMGB-1 levels in the KD+PSL group were significantly lower than those in KD control and KD+PBS (each $P < 0.001$) (**Figure 2C**) groups, whereas sRAGE levels did not significantly differ between the groups (**Figure 2D**).

Basal expression levels of *RAGE*, *TLR2*, and *TLR4* receptors were increased in KD serum-activated HCAECs compared to those in VH serum-activated HCAECs (**Figure 2E**). Of KD serum-activated HCAECs, *RAGE* expression in the KD+PSL group was significantly lower than that in the KD control and KD+PBS groups (each $P = 0.007$); however, *TLR2* and *TLR4* expression did not differ significantly between the groups (**Figure 2F**).

Immunofluorescence Staining for HMGB-1 in Serum-Activated HCAECs

Representative images revealed that serum activation of HCAECs induced HMGB-1 release from the nucleus. Compared with the VH group, KD control and KD+PBS HCAECs showed increased HMGB-1 staining in the cytoplasm or the extracellular space (**Figures 3A, a–c**). Conversely, compared with KD control and KD+PBS HCAECs, KD+PSL HCAECs showed significant reduction in cytoplasmic and extracellular HMGB-1 (**Figures 3A, d**). Quantitative analysis of the fluorescence intensity of HMGB-1 released from nucleus of serum-activated HCAECs revealed significantly lower values for KD+PSL than KD control and KD+PBS HCAECs (each $P < 0.001$) (**Figure 3B**).

Phosphorylation of Mitogen-Activated Protein Kinase and NF-κB, and NLRP3 Inflammasome in Endothelial Cell Lysates From KD Serum-Activated HCAECs

To determine the role of mitogen-activated protein kinase signaling, we evaluated the levels of ERK, pERK, JNK, pJNK, p38, and pp38 in serum-activated HCAEC lysates. The ERK:β-actin, JNK:β-actin, and p38:β-actin ratios did not significantly differ between VH and

TABLE 2 | Patient characteristics between Kawasaki disease (KD) and systemic juvenile idiopathic arthritis (sJIA).

Group	KD (n = 8)	sJIA (n = 4)	*P* value
Male, N (%)	5 (62.5)	2 (50.0)	0.692
Age at onset (years)	1.6 (0.5–2.9)	7.8 (4.6–13.3)	**0.011**
Body weight (kg)	10.8 (7.8–13.0)	17.7 (13.0–25.1)	0.061
White blood cell count ($\times 10^3/\mu L$)	15.8 (11.9–18.1)	14.6 (12.1–24.4)	1.000
Neutrophil count ($\times 10^3/\mu L$)	11.7 (7.9–14.5)	11.7 (10.0–20.0)	0.734
Platelet count ($\times 10^4/\mu L$)	32.8 (28.3–41.5)	59.0 (34.6–66.4)	0.089
Aspartate aminotransferase (IU/L)	45 (26–288)	29 (23–33)	0.202
Alanine aminotransferase (IU/L)	56 (10–336)	14 (10–21)	0.348
Lactate dehydrogenase (IU/L)	336 (273–394)	353 (283–494)	0.610
Total protein (g/dL)	6.6 (6.4–7.0)	7.7 (7.3–7.8)	**0.006**
Albumin (g/dL)	3.4 (3.2–3.7)	3.3 (3.1–3.5)	0.330
Sodium (mEq/L)	134 (129–134)	138 (137–142)	**0.006**
C-reactive protein (mg/dL)	6.9 (4.4–10.5)	6.9 (5.3–9.0)	1.000

Data are expressed as median values and interquartile range (25th, 75th percentile), or number (proportion, %).
Bold values indicate statistically significance in patient characteristics between KD group and sJIA group.

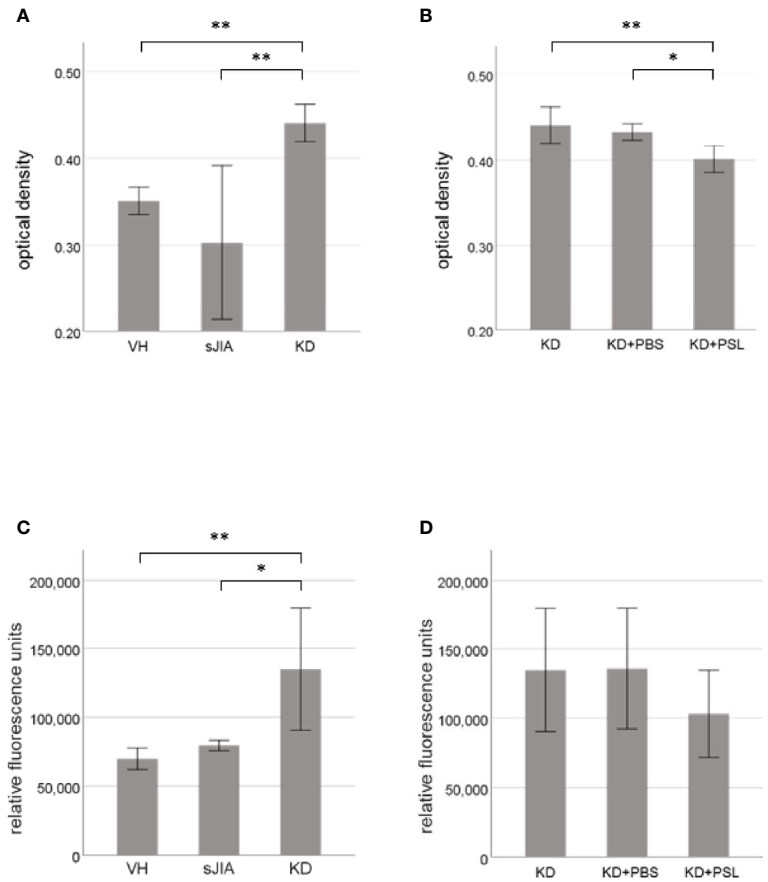

FIGURE 1 | Cell proliferation and cytotoxicity of serum-activated human coronary artery endothelial cells (HCAECs). MTT and cytotoxicity assay results for HCAECs stimulated with sera from healthy controls (VH, $n = 4$) and patients with systemic juvenile idiopathic arthritis (sJIA, $n = 4$) or Kawasaki disease (KD; $n = 8$) for 24 h **(A, C)**, and serum-activated HCAECs from patients with KD ($n = 8$), and treated with PBS or prednisolone (PSL) for 24 h **(B, D)**. $^*P < 0.05$, $^{**}P < 0.01$ [one-way ANOVA followed by Bonferroni post-test **(A–C)** and Games–Howell post-test **(D)**].

KD groups; however, pERK:β-actin, pJNK:β-actin and pp38:β-actin ratios in lysates from KD controls were significantly higher than those in VH lysates ($P < 0.001$). The ERK:β-actin, JNK:β-actin, and p38:β-actin ratios did not significantly differ between KD controls, KD+PBS, and KD+PSL groups, however, pERK:β-actin, pJNK:β-actin, and pp38:β-actin ratios were significantly lower in lysates from KD+PSL groups than in lysates from the KD controls ($P = 0.004$) and KD+PBS groups ($P = 0.006$) (pERK:β-actin; $P = 0.004$

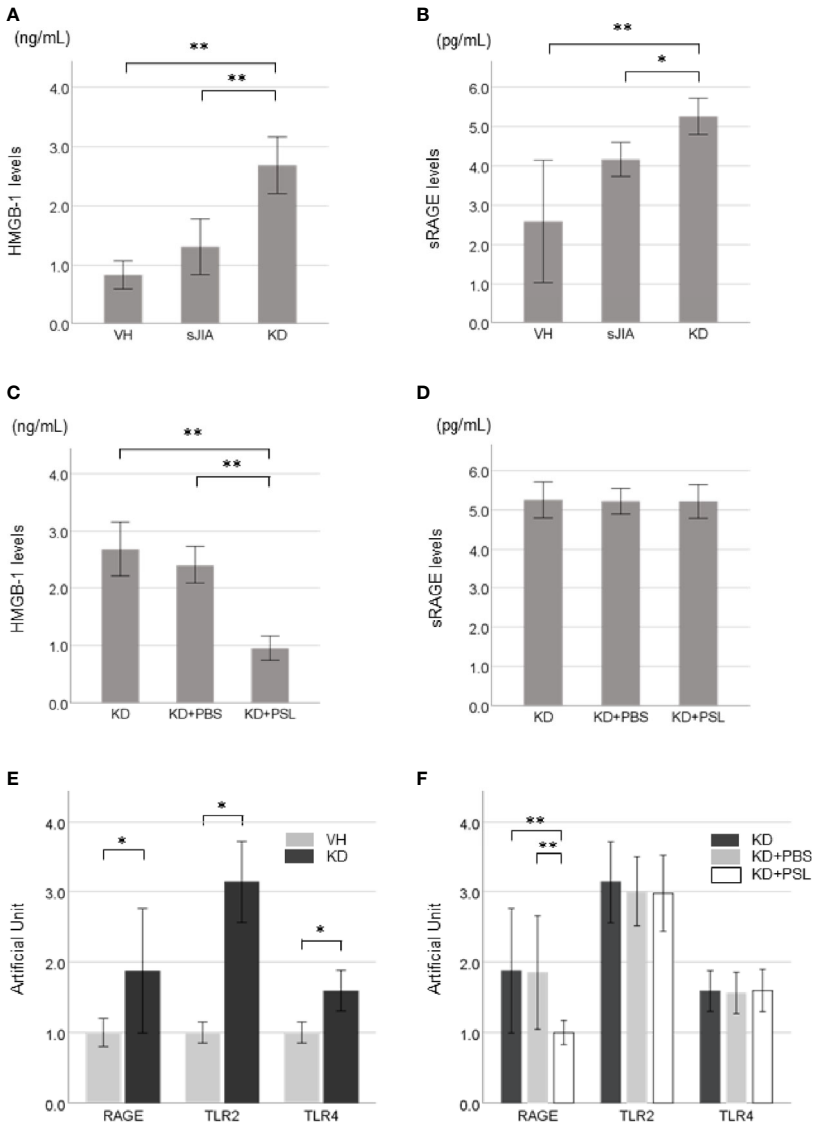

FIGURE 2 | HMGB-1 and sRAGE production in Kawasaki disease (KD)-serum-activated human coronary artery endothelial cells (HCAECs). HMGB-1 levels and sRAGE levels in supernatants from **(A, B)** healthy control (VH, $n = 4$), systemic juvenile idiopathic arthritis (sJIA, $n = 4$), and KD ($n = 8$) serum-activated HCAECs and **(C, D)** KD control, KD+PBS, and KD+prednisolone (PSL) HCAECs. Expression of HMGB-1 receptors in KD serum-activated HCAECs. Artificial Unit of RAGE, TLR2, and TLR4 receptors in **(E)** VH and KD serum-activated HCAECs, and **(F)** KD control, KD+PBS, and KD+PSL HCAECs. $^{*}P < 0.05$, $^{**}P < 0.01$ [one-way ANOVA followed by Bonferroni post-test **(A, B, D, F)** and Games–Howell post-test **(C)**, Mann–Whitney *U*-tests **(E)**].

and $P = 0.006$, pJNK:β-actin; $P < 0.001$ and $P < 0.001$, pp38:β-actin; $P = 0.002$ and $P = 0.004$, respectively) (**Figure 4A**).

The NLRP3:GAPDH and cleaved caspase 1:GAPDH ratios in the lysates from KD controls were higher than those in VH lysates ($P = 0.017$ and $P = 0.018$, respectively), while the NLRP3:GAPDH and cleaved caspase 1:GAPDH ratios were lower in the lysates from KD+PSL groups compared to the lysates from the KD controls and KD+PBS; no significant difference was observed between the groups (**Figure 4B**).

The total NF-κB p65:GAPDH ratio in cell lysates did not significantly differ between the nuclear and cytoplasmic fractions of the VH group, KD controls, KD+PBS group, or KD+PSL group. Conversely, the nuclear p-NF-κB p65:GAPDH ratio in lysates from KD controls was significantly higher than those in lysates from the VH group ($P < 0.001$), whereas it was significantly reduced in the KD+PSL group compared to that in KD control and KD+PBS groups (each $P < 0.001$) (**Figure 4C**).

IL-1β and TNF-α Production in Endothelial Cell Lysates From KD Serum-Activated Hcaecs

Both IL-1β and TNF-α levels in lysates of KD serum-activated HCAECs were significantly higher than those from the VH group (each $P < 0.001$), whereas both were significantly lower

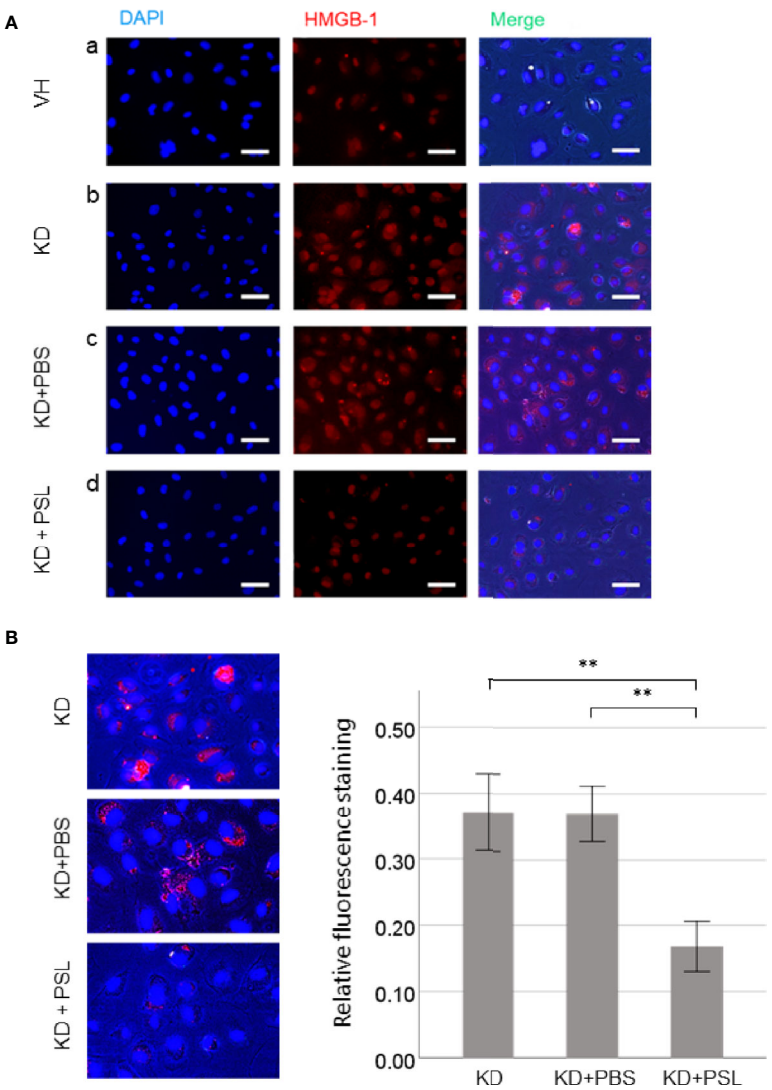

FIGURE 3 | Immunostaining of HMGB-1 in serum-activated human coronary artery endothelial cells (HCAECs) (magnification, ×400). **(A)** Representative images of from healthy control (VH), Kawasaki disease (KD), and KD serum-activated HCAECs treated with PBS and prednisolone (PSL) showing DNA (blue) labelled using DAPI, HMGB-1 (red) labelled using immunofluorescence staining, and merged images. Scale bar = 100 μm. **(B)** Relative fluorescence staining of extranuclear HMGB-1 from 100 cells, selected from KD serum-activated HCAECs alone and treated with PBS and PSL. Scale bar = 100 μm. $^{**}P < 0.01$ (one-way ANOVA followed by Bonferroni post-test).

in endothelial cell lysates from the KD+PSL group than those from KD controls and KD+PBS groups (IL-1β, each $P < 0.001$; TNF-α, $P = 0.001$, and $P = 0.009$. respectively) (**Figures 5A, B**).

DISCUSSION

In this study, we demonstrated that serum obtained from patients with KD prior to IVIG treatment exhibited a cytotoxic effect on HCAECs compared to that from healthy controls and patients with sJIA, in addition to triggering extracellular release of HMGB-1, up-regulating NF-κB-mediated inflammatory responses, and increasing IL-1β and TNF-α production. Although PSL treatment for KD serum-activated HCAECs did not show direct cytoprotective effects, it inhibited endothelial cell proliferation, HMGB-1 translocation, release, and downstream signaling and reduced IL-1β and TNF-α expression. These findings provide a new perspective regarding the anti-inflammatory function of PSL during the acute phase of KD.

The vasculopathic process in the acute phase of KD involves necrotizing arteries progressively destroying the arterial wall from adventia to intima, particularly the coronary arteries (22, 23). Our results are in line with this pathological process in which the KD serum induced stronger cytotoxicity to coronary endothelial cells than sJIA serum, although there was no

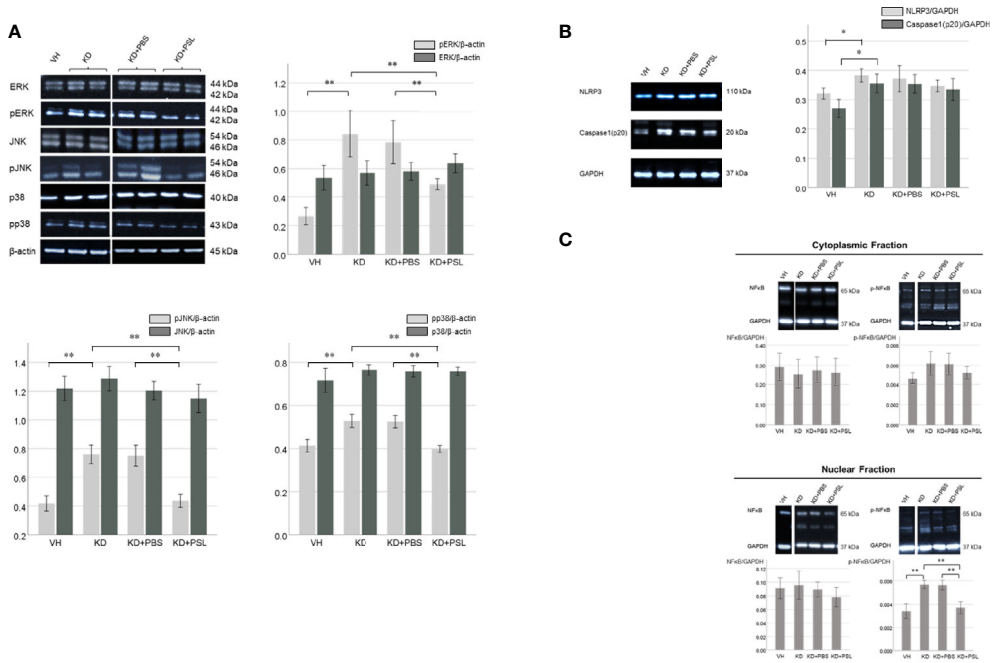

FIGURE 4 | ERK1/2, JNK, p38 and NF-κB levels in serum-activated human coronary artery endothelial cells (HCAECs). **(A)** Western blot analysis of ERK1/2, pERK1/2, JNK, pJNK, p38 and pp38 from healthy controls (VH, $n = 4$), Kawasaki disease (KD, $n = 8$), KD serum-activated HCAECs treated with PBS and prednisolone (PSL) (KD+PBS and KD+PSL, $n = 8$, respectively). Bar graph shows immunoblotting results for the ERK1/2:β-actin, pERK 1/2: β-actin, JNK:β-actin, pJNK: β-actin, p38:β-actin and pp38: β-actin ratio $^{**}P < 0.01$ (one-way ANOVA followed by the Bonferroni, post-test). **(B)** NLRP3 and Caspase-1(p20) in serum-activated HCAECs from healthy controls (VH, $n = 4$), KD ($n = 8$), and KD serum-activated HCAECs treated with PBS and PSL (KD+PBS and KD+PSL, $n = 8$, respectively). Bar graphs show immunoblotting results for NLRP3:GAPDH and Caspase-1(p20):GAPDH ratio in HCAEC lysates. $^{*}P < 0.05$ (Kruskal–Wallis test followed by Dunn's post-test). **(C)** Cytoplasmic and nuclear NF-κB and p-NF-κB in serum-activated HCAECs from healthy controls (VH, $n = 4$), KD ($n = 8$), and KD serum-activated HCAECs treated with PBS and PSL (KD+PBS and KD+PSL, $n = 8$, respectively). Bar graphs show immunoblotting results for NF-κB:GAPDH and p-NF-κB:GAPDH ratio in HCAEC lysates. $^{**}P < 0.01$ (Kruskal–Wallis test followed by Dunn's post-test).

difference in patients' laboratory data between KD and sJIA. Since KD serum also up-regulated DAMP receptors, such as RAGE and TLRs, and induced subsequent intracellular activation of downstream ERK1/2, JNK, p38, and NF-κB signaling pathways in HCAECs, endothelial cell damage consequent to pathogenic proteins in KD serum may thus underlie several of the pathological features of KD and the effects observed during the early stages of disease progression. Specifically, during cytotoxic response to KD serum, HMGB-1 translocated from the nucleus to the extracellular space, where it may function as a DAMP or alarmin to stimulate the innate immune system and mediate inflammation in accordance with its role in the pathogenesis of delayed inflammatory responses and organ dysfunction (24).

Moreover, extracellular HMGB-1 can interact with RAGE or TLRs on the surface of inflammatory endothelial cells. HMGB-1 functional interaction with receptors activates inflammation-associated pathways and triggers a cascade of proinflammatory cytokines, including ILs, TNF-α, and macrophage inflammatory protein-1α and -1β, thereby forming a self-reinforcing inflammatory cycle (25, 26). As serum levels of HMGB-1 and S100 are elevated during the acute KD phase (7, 15–17) and RAGE activation results in up-regulated proinflammatory cytokine expression in patients with KD (18), these processes may stimulate granulocytes or endothelial cells to secrete DAMPs, thereby establishing a self-amplifying positive feedback loop. However, sRAGE, a truncated soluble form of the receptor, acts as a decoy and prevents the RAGE activation-mediated inflammatory response (27). Consistent with our results, Wittkowski et al. (28) found that sRAGE levels in acute KD were significantly lower than those post-IVIG or in the subacute phase, suggesting the potential anti-inflammatory effect of sRAGE on inflammatory vascular disorders. Additionally, subsequent activation of HMGB-1/RAGE-specific downstream signaling pathways and increased levels of IL-1β or TNF-α constitute parallel or consecutive events in response to increased HMGB-1 levels. The HMGB-1/RAGE signaling pathway in endothelial cells also induces cathepsin B activation, subsequently inducing canonical pyroptosis *via* NLRP3 inflammasomes in KD vasculitis (18). Therefore, our study also supports the hypothesis that extracellular HMGB-1 is possibly up-regulated to act as a functional cytokine influencing inflammation and the innate immune response, and that it may thus contribute to the pathogenesis of KD vasculitis (29, 30).

We further revealed that PSL treatment of KD serum-activated HCAECs inhibited extracellular HMGB-1 release, reduced *RAGE* expression, and inhibited NF-κB-mediated inflammatory responses, in addition to reducing IL-1β and

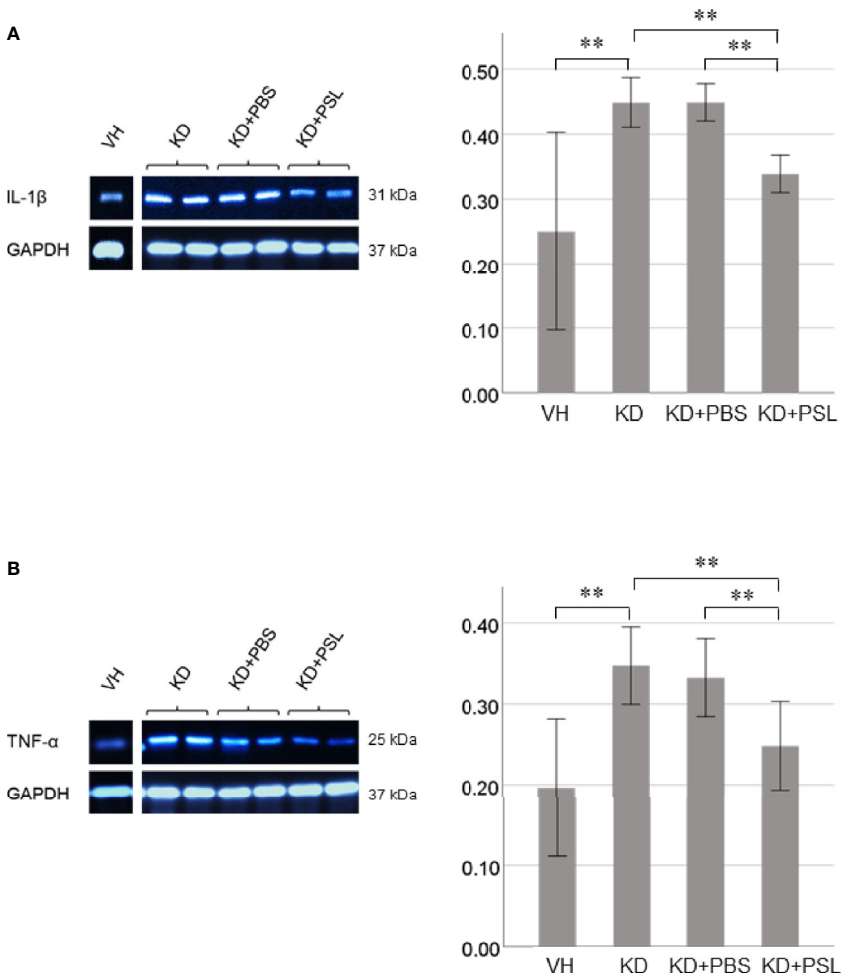

FIGURE 5 | Cytokine production in serum-activated human coronary artery endothelial cell (HCAEC) lysates. Western blot analysis and quantitation of **(A)** IL-1β and **(B)** TNF-α production in serum-activated HCAEC lysates from healthy controls (VH) and Kawasaki disease (KD), and KD serum-activated HCAECs treated with PBS and prednisolone (PSL) (8 independent experiments). GAPDH was used as an internal standard. $^{**}P < 0.01$ (one-way ANOVA followed by Bonferroni post-test).

TNF-α production. Conversely, PSL treatment did not show cytoprotective effects or sRAGE up-regulation in KD-serum-activated HCAECs, supporting that the effects of PSL treatment might occur by directly inhibiting extracellular HMGB-1 release. In general, the anti-inflammatory effects of glucocorticoids are attributable to the transcriptional effects of glucocorticoid-receptor agonism, which alters the transcription of numerous genes both positively and negatively by targeting specific cell populations to combat the immune system hyperactivation or systemic infections (10, 31–33). Glucocorticoid treatment inhibits the expression of proinflammatory genes, including NF-κB and activator protein 1 (10); therefore, the observed PSL-induced inhibition of HMGB-1 release might occur *via* NF-κB signaling.

Another possible mechanism is that glucocorticoids inhibit TNF-α synthesis in activated monocytes/macrophages, a process at least partially involved in the anti-inflammatory effects of TNF-α-induced HMGB-1 secretion from endothelial cells (30). Therefore, PSL treatment of KD serum-activated HCAECs likely has an upstream regulatory component and potentially inhibits the HMGB-1 signaling pathway. CD14 is important in efficient HMGB-1-dependent TLR activation (34, 35), whereas RAGE offers a different transduction pathway in providing a transport route for HMGB-1 and its partner molecule complexes by endocytosis to the endolysosomal component (36). Rather than being degraded in the lysosomes, HMGB-1 transported molecules then leak out from the permeabilized lysosomes into the cytosol to reach and activate cognate cytoplasmic receptors, thereby causing inflammation (36). In the present study, we could not confirm a functional role for the HMGB-1/TLRs signaling pathway and HMGB-1 induced NLRP3 inflammasome activation through the serum stimulation experiments using cultured HCAECs owing to the lack of lymphocytes and macrophages. However, our findings suggest that PSL application during acute KD has a distinct potential to also ameliorate HMGB-1/RAGE-mediated inflammatory responses in KD vasculitis, which is borne out by the efficacy of glucocorticoids at reducing the incidence of CAA and the number of IVIG non-responders in KD (4–6).

Nevertheless, there were several limitations in this study with respect to the effects of PSL treatment in KD vasculitis that remain unaddressed. Serum samples from patients with KD used in this study were limited; hence, we did not measure cytokine and inflammatory markers other than those from routine blood testing. Glucocorticoids affect virtually all immune cells and their precise effects depend on the differentiation and activation state of the cell, making interpretation of *in vivo* effects in specific populations difficult. Glucocorticoids inhibit neutrophil-dependent endothelial cell injury (37) or platelet–neutrophil aggregate formation (38), thereby reducing cytokine-induced adhesion and inhibiting amplified reciprocal vascular inflammatory activation. However, we could not examine the contribution of neutrophil-, monocyte-, and platelet-dependent endothelial cell activation since our serum-stimulation experiments were performed using cultured coronary endothelial cells. Given the differences in glucocorticoid-receptor levels between endothelial cells and various vascular beds, the relative proportion of specific glucocorticoid-receptor isoforms in tissues and cells may influence their responses to glucocorticoid treatment (39–41). IVIG is the standard and most effective treatment for KD, however, the present study focused on the mechanism of action of corticosteroids in KD; and hence no examination or analysis of the effects of IVIG was conducted in this study.

In conclusion, our findings suggest that extracellular HMGB-1 is potentially up-regulated to act as a functional cytokine with roles in both inflammation and the innate immune response, thereby mediating KD pathogenesis. Treatment with PSL during the acute phase of KD ameliorates HMGB-1/RAGE-mediated inflammatory responses and reduces IL-1β and TNF-α production. Inhibiting extracellular HMGB-1 may also inhibit the over-activated innate immune system, thus offering potential relief from or prevention of severe KD vasculitis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repositories and accession numbers can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kagoshima University and Kagoshima City Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KU, YN and YK conceived and designed the experiments. KU and YM performed the experiments. KU and YN analyzed the data and designed the figures. KU performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported financially by a Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP16K19657 and JP19K08302.

ACKNOWLEDGMENTS

We wish to express our appreciation to the patients and their families for their commitment to this project. We thank the laboratory technicians Junko Hirose, Chizuru Tamari, and Emiko Miyahara for their help with our experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.640315/full#supplementary-material

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 June 2021
doi: 10.3389/fimmu.2021.584660

Heat Shock Causes Lower *Plasmodium* Infection Rates in *Anopheles albimanus*

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The immune response of *Anopheles* mosquitoes to *Plasmodium* invasion has been extensively studied and shown to be mediated mainly by the nitric oxide synthase (NOS), dual oxidase (DUOX), phenoloxidase (PO), and antimicrobial peptides activity. Here, we studied the correlation between a heat shock insult, transcription of immune response genes, and subsequent susceptibility to *Plasmodium berghei* infection in *Anopheles albimanus*. We found that transcript levels of many immune genes were drastically affected by the thermal stress, either positively or negatively. Furthermore, the transcription of genes associated with modifications of nucleic acid methylation was affected, suggesting an increment in both DNA and RNA methylation. The heat shock increased PO and NOS activity in the hemolymph, as well as the transcription of several immune genes. As consequence, we observed that heat shock increased the resistance of mosquitoes to *Plasmodium* invasion. The data provided here could help the understanding of infection transmission under the ever more common heat waves.

Keywords: ***Anopheles albimanus***, ***Plasmodium berghei***, **heat shock, immunity, infection resistance**

OPEN ACCESS

Edited by:
Massimo E. Maffei, University of Turin, Italy

Reviewed by:
Nora Kristin Elisa Schulz, Vanderbilt University, United States
Maria Luisa Simões, Johns Hopkins University, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *17 July 2020*
Accepted: *08 June 2021*
Published: *24 June 2021*

Citation:
Condé R, Hernandez-Torres E, Claudio-Piedras F, Recio-Tótoro B, Maya-Maldonado K, Cardoso-Jaime V and Lanz-Mendoza H (2021) Heat Shock Causes Lower Plasmodium Infection Rates in Anopheles albimanus. Front. Immunol. 12:584660. doi: 10.3389/fimmu.2021.584660

INTRODUCTION

Insects are commonly stressed by pathogens (1), toxic compounds, dietary factors, temperature (2), and hypoxia (3). These stressors share commonalities in their effects on the molecular components of cells, and therefore much of the transcriptional response they elicit is conserved (4). There is significant crosstalk in the signalling cascades that regulate each of the various stress-specific transcription factors (5, 6) and so any stressor has the potential to, and generally does, affect the transcription of genes that are not directly related to the specific stress applied (7). When microorganisms challenge insects, molecular patterns on the pathogen trigger alterations in transcription *via* Toll, IMD, and the JAK-STAT pathway, ultimately activating the Rel1, Rel2, and STAT transcription factors. These transcriptional factors control the expression of hundreds of genes, including many immune response genes. The immune response has been shown to be intertwined with the general stress response in several insect models, and a number of genes activated by pathogen invasion are not directly linked to anti-pathogenic functions. For instance,

LPS injection of the *Tribolium castaneum* beetle also induces heat shock stress response genes (HSP 68 and HSP 27) and hypoxia-inducible gene transcription (7). Conversely, in the same insect, heat shock triggers the transcription of the immune genes *tlr6*, *pgrp2*, *defensin1*, and *defensin2* (8). In *Galleria mellonella* larvae, heat shock alters the expression of antimicrobial peptide genes. For example, apoLp-III gene expression is transiently inhibited after heat shock but, when an infection occurs 96 h after the heat shock, it is induced at higher levels than in the absence of prior heat shock (9). Some *Drosophila melanogaster* immune genes are regulated by the heat shock factor, and in fact, most Heat shock Factor Binding Sites in the genome are found in Non-Heat shock Genes (10). The regulatory cascades of Heat shock Factor 1 and NF-kB share some components, such as Droj2 (HSP40) that functions downstream of or directly regulates Relish and DNAJA3 which are required for IκB phosphorylation (11).

Interestingly, heat shock-mediated immune activation can be transgenerational, hence affecting immune homeostasis over a long period of time (12). Upon stress, the beetle *Nicrophorus vespilloides* initiates a transposon-mediated genomic reorganization of its immune genes, suggesting that this reorganization might constitute a common pathway to enhance survival during protein damage (13). This reorganization could occur because stress or infection mobilizes HSP 90 protein, impairing its mutation-dampening function (14). Aside from heat shock direct transcriptional effects, or genomic reorganization, the temperature also affects the function of enzymes that modify genomic DNA such as the Ten eleven translocation (TET) dioxygenase, providing another level of transcriptional regulation (15).

HSP70 also protects the midgut of *Aedes aegypti* mosquitoes against the dramatic increase in body temperature after ingesting a hot blood meal (16), considering that the mosquito ingests its own weight of blood at 37°C (17). Though immune signaling pathways of anopheline mosquitoes have been scrutinized in great detail, little is known about their interactions with hypoxia (HIF) and heat shock factor (HSF) mediated transcriptional regulation. The blood-feeding of female mosquitoes implies that a heat shock is produced in the midgut and that HSP70 proteins are transiently expressed (16). In anopheline mosquitoes, Toll and Imd pathways show a certain amount of cross signaling (18); opening the possibility of a cooperative effect of bacterial infection response and *Plasmodium* mediated response, as shown in *Anopheles gambiae* by Ramirez et al. (19). The gut tissues of hematophagous insects are subjected to large temperature changes when feeding. As a consequence, HSP 82, HSP 90, and HSP 105 of *A. gambiae* are up-regulated during blood-feeding (20, 21). Later, *ex vivo* analysis of the *A. gambiae* HSC70B promoter revealed that the transcription was influenced by immune activation (5), proving the existence of a cross-talk between immune and heat shock activation cascades. In *Anopheles stephensi*, the rearing temperature alters the transcription levels of immune effectors differentially. While the nitric oxide synthase (NOS) expression peaked at 30°C, the humoral melanization, phagocytosis, and defensin expression were maximum at 18°C (22), though the melanization reaction has been described as a minor factor in resistance to *P. falciparum* (23). In general, phenoloxidase (PO) and NO have been implicated in the immune response to *Plasmodium* infection in *Anopheles*. In *A. stephensi*, the metabolites of NOS activity increase nitric oxide concentrations and subsequent ookinete death (24). The enzymatic cleavage regulating NOS activity is temperature-dependent (25). Some heat shock proteins (HSPs), such as *A. gambiae* HSC70B have shown antiviral activities capable of suppressing *O'nyong'nyong* viral infection (26). In turn, this observation opens the possibility that the HSPs could affect the microorganisms directly, once released in the hemolymph. In particular, heat shock triggers a general damage response in mosquitoes, including immune gene transcription. Though *P. berghei* does not constitute a natural *A. albimanus* parasite, this model has proven an invaluable tool for the study of the immune response of this insect. Here, we use this model to investigate the effects of heat shock on the expression of immune-related genes and the subsequent effect on susceptibility to infection.

MATERIALS AND METHODS

Mosquito Rearing and Infection With *P. berghei*

A *Plasmodium*-susceptible strain of *A. albimanus* females (27) were obtained from the insectary of the National Institute of Public Health (INSP) in Cuernavaca, Mexico. Mosquitoes were bred under a 12:12 photoperiod at 28°C and 70–80% relative humidity. At four-days post-emergence, mosquitoes were infected with *P. berghei* ANKA strain expressing the green fluorescent protein (GFP) (28) (kindly donated by Robert E. Sinden, Imperial College, U.K.). Ookinetes were produced by culturing gametocyte-infected mouse blood, as described previously (29). Groups of 300 female mosquitoes were fed for 1 h using artificial membrane feeders with: (i) mouse blood + GFP ookinetes (infected group, with approximately 900 ookinetes per µl), or (ii) mouse blood only (control group). Unfed mosquitoes were removed, and the engorged ones maintained at 21°C to allow for parasite invasion and interaction with the mosquito midgut. Three experimental repetitions were performed.

Mosquito Heat Shock

Since in *Drosophila* HSF binding reaches a maximum level following a 30-minute heat shock at 36.5°C (30), and mosquitoes endure a thermic shock while feeding on mammals blood (16), we exposed the mosquitoes to 30 min of 37°C heat shock, with 80% humidity and availability of 10% sugared water. In nature, the blood feeding is concomitant to the heat shock in naturally infected mosquitoes, and lead to the infection of the mosquito (16). Since the objective of our experiments is to test the effect of abiotic stress on the susceptibility to *P. berghei* infection of *A. albimanus*, we challenged them thermally 6 h before the infective blood meal.

Mosquito Protein Extract Preparation for Acrylamide Electrophoresis

About 10 mosquitoes per sample were homogenized in 100 µl of lysis solution (8 M urea, 2 M thiourea, 1% Chaps, 13 mM DTT, and 4 µl of protease inhibitor cocktail (Sigma, P2714)). The resulting solution was cleared by 15 min centrifugation at 14,000*g* and 4°C. The total protein content of the supernatant was determined according to Lowry et al. (31). Samples were obtained before the heat shock, immediately after, and at 2 and 6 h after 30 min of exposure at 37°C.

Acrylamide Electrophoresis and Western Blot Assay of the Mosquito Protein Extracts

Some 25 µg of mosquito protein extract were separated in SDS-PAGE (10% acrylamide) and transferred to Immobilon-P membrane. The protein transfer on the membrane was assessed by Ponceau S (P7170 Sigma-Aldrich) staining. For the Hsp-70 immunodetection, anti-Hsp70 monoclonal antibody [3A3] Thermo Scientific MA3-006 was used as primary antibody at a final dilution of 1:1,000, and goat anti-mouse-IgG-horseradish peroxidase (Abcam) diluted 1:1,000 as secondary antibody. Development of immunoblots was performed with an ECL kit from Amersham. Fluorescence was developed on a Kodak BioMax ML-2 film for capturing chemiluminescent data (Catalog Number Z370428) using Kodak Developer (Catalog Number P7042) and Kodak Fixer (Catalog Number P7167).

Mosquito Hemolymph Collection

Hemolymph was obtained by perfusion from 30 control and heat-shocked mosquitoes (at six hours post-heat shock), and 24-hours post-*P. berghei* infection (30-hours post-heat shock) as described elsewhere (32). Three experimental repetitions were performed.

Mosquito RNA Extraction

Total RNA from 10 whole female mosquitoes (without head) and midguts only, were obtained by Trizol method (Invitrogen) and then re-purified using RNA Clean-Up Kit (Zimo Research). cDNA was synthesized by reverse transcription using 1 µg of RNA, 100 ng of oligonucleotide dT, and 200 U of the enzyme reverse transcriptase RNase H-SuperScript II (Gibco BRL). Three experimental repetitions were performed, each counting three samples per conditions.

RT-qPCR Amplification

The amplification of genes of the *An. albimanus* immune response was carried out with previously recovered genetic material. Specific primers were used for each gene (**Table 1**).

The samples were run in a real-time thermal cycler (viiA7; Applied Biosystems) under optimal running conditions, according to the manufacturer's recommendations. Samples were incubated at 60°C in a master mix containing SYBR Green (Maximum SYBR Green/Rox qPCR Master Mix; Thermo Scientific), primers, and cDNA of each of the samples, set to a volume of 20 µl with water free of nucleases (Thermo Scientific). The relative expression was quantified by normalizing the expression of immune response genes with the S7 ribosomal gene.

TABLE 1 | Oligonucleotides used to amplify the mRNA transcripts of *A. albimanus* genes.

ppo1 F	5'-GGCGGACCAAATCAAGCAG-3'
ppo1 R	5'-CGATTGCCCGATTCGTCAAC-3'
tet 2 F	5'-TCCTCCGATCCGAGGATCAGGT-3'
tet 2 R	5'-GTACCTTGCTGTTGCTGGGCA-3'
dnmt2 F	5'-GAGCCATCTTTTCCGATTCGTC-3'
dnmt2 R	5'-GAGCCATCTTTTCCGATTCGTC-3'
CECA-R	5'-ATTTGCCAAGTGCCTTCAC-3'
CECA-F	5'-AGTGGACGCTGGTTTTCTCAAG-3'
Gam F	5'-CGCTTATGCTTCGACTTGC-3'
Gam R	5'-AATCATCGTCTGACCATCGC-3'
GNBPA F	5´-CACTCGATACGGAGTCGGC-3´
GNBPA R	5'-AACTAATCTGGGCTCATCGTG-3'
duox F	5'-CTCTCTCTGTTGCAGAATCCAG-3'
duox R	5'-TGGTGTGAGATGGTTATCGACT-3'
hsp70 F	5'-CCAGCATGGAAAGGTGGAGA-3'
hsp70 R	5'-CCATCCATCAGGGCGTCAAT-3'
frep3 F	5'-CAGTGCGTGTCGTGCAAT-3'
frep3 R	5'-AACCGTTTGAGAATCTGTAGCA-3'
S7 F	5'-AACAACAAGAAGGCCATCGTC-3'
S7 R	5'-GGCTTGGGCAGAATACGA-3'

ppo1, Prophenol oxidase 1; tet2, ten-eleven translocation methylcytosine dioxygenases; dnmt2, DNA methyltransferase 2; duox, Dual oxidase; hsp70, heat shock protein 70; frep3, Fibrinogen related protein 3, cecropin, gambicin and GNBP4.

Assays were performed three times in different batches of 10 mosquitoes and three times in different batches of five mosquitoes' midguts. The control and experimental tests were made at the same time. For real-time PCR, 2.5 µl of cDNA was used in SYBR Green I Kit (Applied Biosystems) following the kit instructions. The primers used are described in **Table 1**. The fold changes in expression were calculated using the comparative "delta delta Ct" (Ct) method against the blood-fed control (33) using three replicates per sample. Three independent experiments were done. The data represents the average fold-change relative to the control group. The amplification efficiency was similar between the test and control genes.

Phenoloxidase (PO) Activity

PO activity was measured as described (34). Three pools of 30 female mosquitoes were macerated and centrifuged at 10,000*g* for 10 min at 4°C. L-DOPA was used as the substrate for PO, which is transformed into the dye dopachrome. Auto-oxidation controls (L-DOPA only) and blanks (macerated mosquitoes) were included. PO activity was measured every minute for 30 min at 490 nm in a microplate reader (ELISA iMark, BIO-RAD).

NO Quantification

Nitrites (NO_2^-) and nitrates (NO_3^-) were evaluated by the Griess assay (35). Pools of 30 female mosquitoes per treatment were macerated and centrifuged twice at 10,000*g* for 10 min at 4°C. Proteins were eliminated with $ZnSO_4$. Nitrates were reduced into nitrites using VCl_3 immediately followed by the addition of sulfanilamide and NED. The reaction was incubated for

15 min at R.T., and the absorbance was measured at 490 and 630 nm in a microplate reader.

Statistical Analysis

Data were analyzed and graphed in Prism v6.01 statistical software. qPCR results were evaluated by one way ANOVA followed Tukey's test (Whole body RT-qPCR), and unpaired t test with Welch's correction (Midgut RT-qPCR). The infection parameters were analyzed through Mann–Whitney. Considering that three independent repetitions of the experiment were performed, we applied a log-like generalized lineal model with random effect to determine the difference in ookinete prevalence between control and heat shocked mosquitoes. We used the individual experimental repetitions as categorical variable, this with the objective of measuring the effect of the individual repetitions on the mean differences between the two conditions. PO and NO results were analysed by Student's t-test comparing the heat shock and control groups for the non-infected and infected mosquitoes.

RESULTS

Heat Shock Diminishes Infection of an *A. albimanus* Susceptible Strain

To assess the global effects of heat shock response on parasite development, female *A. albimanus* (susceptible strain) mosquitoes were heat-shocked at 37°C for half an hour, 6 h before infective blood-feeding (900 ookinetes/µl) in three separate experiments with three replicates. Oocyst numbers were assessed five days post blood meal. The prevalence and the intensity were significantly diminished in the heat-shocked mosquitoes (control 77.27%, n = 198 vs HS 67%, n = 184 with $X^2 = 4.7$ and $p = 0.0102$). A median of three oocyst per mosquito midgut were found in control mosquitoes while a median of two oocysts per midgut were found in the heat shocked mosquitoes midguts (**Figure 1A**). A log-like generalized lineal model with random effect showed that oocyst prevalence is 83% smaller in the heat-shocked mosquitoes than in the control (95% IC 80–87%) with p = 0.00. The individual experiments performed (considering each experiment separately) do not affect the outcome of the analysis (P = 0.98).

Anti-HSP-70 western-blot analysis of the mosquito protein extracts shown in **Figure 1B** reveal an increase in expression of HSP 70 at both 2 and 6 h after heat shock. The protein profile of the mosquito's midguts was altered by the treatment, demonstrating the impact of the heat stress on the mosquito cells 2 and 6 h post heat shock (**Figure 1C**, black arrows).

Hemolymph and Body From Heat-Shocked *A. albimanus* Susceptible Strain Mosquitoes Present Higher Phenoloxidase Activity

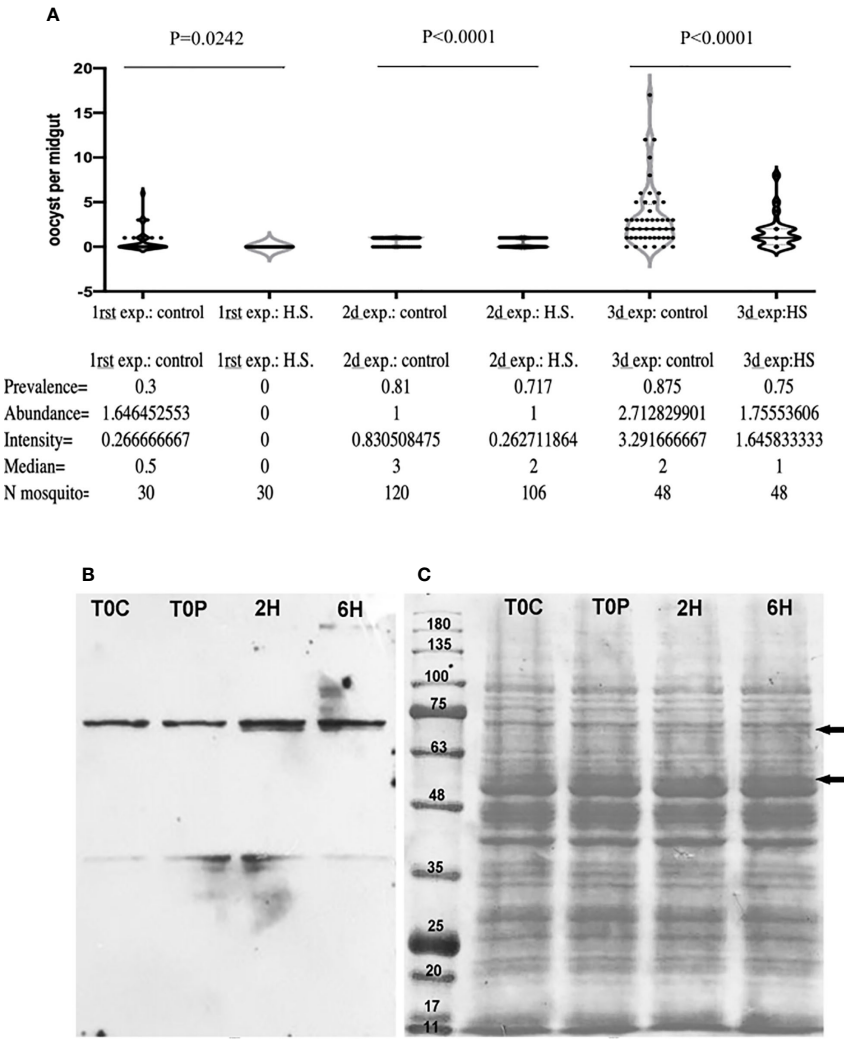
Phenoloxidase activity has been previously described as an important factor limiting the *Anopheles* infection by *Plasmodium* (36). The effect of heat shock upon the enzyme activity could be key to understanding the reduction of the mosquito's susceptibility to the parasite.

Therefore, we measured the PO activity in the hemoplymph and full mosquito body during the heat shock. In three separate experiments, the hemolymph from 30 female *A. albimanus* susceptible strain that were heat-shocked at 37°C for 30 min was collected. The sampled hemolymph was obtained at 6 h post-heat shock, and 24 h post-*P. berghei* infection (30 h post-heat shock). Samples were tested for phenoloxidase activity using the colorimetric L-DOPA assay. As can be seen in **Figure 2**, phenoloxidase activity was altered by the heat shock regime. The heat-shock by itself did not increase the phenoloxidase activity significantly in the hemolymph. As shown previously (37, 38), *P. berghei* infection increased hemolymph phenoloxidase activity, mainly through enzymatic activation by proteolysis and secretion of prophenoloxidase. While heat shock alone did not increase hemolymph phenoloxidase activity, heat shock increased the hemolymph phenoloxidase activity (**Figure 2**) in the *P.berghei* infected mosquito hemolymph when compared to the heat-shocked non infected mosquitoes hemolymph and relative to non-heat shocked infected mosquitoes.

The phenoloxidase activity encountered in the whole mosquito body increased upon heat shock. However, when comparing both control/Pb and HS/HS Pb conditions, the activity diminishes upon infection. When considering the whole mosquito body mRNA, the heat shock did alter the PPO gene transcription at 6 h post-heat shock (**Figure 4**), and showed a tendency to diminish in the midgut (**Figure 5**). Considering that, in insects, the central organ of phenoloxidase production are the hemocytes (39) this result was expected.

Hemolymph From Heat-Shocked *A. albimanus* susceptible Strain Mosquitoes Present Higher Nitric Oxide Concentration

Another key factor involved in the immune response of *A. albimanus* to *Plasmodium* infection occurs through NO synthesis (32). The only stable product of NO, which decays in seconds, is nitrite (NO_2^-), however, the more oxidized nitrate (NO_3^-) can also be produced. Therefore, to measure the total NO production, one must measure the total NOx derivatives. The NO_2^- found in the hemolymph of the heat-shocked mosquitoes diminished (**Figure 3A**) while NO_3^- levels increased when compared with their control (**Figure 3B**), indicating that total NOx production was not changed. This may, however, indicate more oxidizing conditions in the hemolymph after heat shock. In whole body extracts, we observed that the combination of HS and infection increased NO_2^- production above either heat shock or infection alone (**Figure 3**). NO_3^- production in the body of mosquitoes was decreased by heat shock both in uninfected and those infected by *P. berghei*. From these results we suggest that *P. berghei* infection creates reducing conditions in the mosquito body that limits the full oxidation of nitrites into nitrates (**Figure 3B**).



	1rst exp.: control	1rst exp.: H.S.	2d exp.: control	2d exp.: H.S.	3d exp: control	3d exp:HS
Prevalence=	0.3	0	0.81	0.717	0.875	0.75
Abundance=	1.646452553	0	1	1	2.712829901	1.75553606
Intensity=	0.266666667	0	0.830508475	0.262711864	3.291666667	1.645833333
Median=	0.5	0	3	2	2	1
N mosquito=	30	30	120	106	48	48

FIGURE 1 | Effect of Heat shock on *P. berghei* infection in *A. albimanus*. **(A)** *A. albimanus* susceptible strain oocyst infection prevalence expressed as the ratio of infected mosquitoes over total mosquitoes sampled with (0.67 on average) and without previous heat shock (0.77 on average). **(B)** Western blot using the anti-HSP70 antibody of a 10% acrylamide gel of control 0, 2, and 6 h post-heat shock *A. albimanus* body protein extracts. **(C)** Protein profile of the *A. albimanus* body control T0C (control), T0P (Time zero post heat-shock), 2H (2 h post-heat shock), 6H (6 h post-heat shock). Black arrows indicate differential bands appearing in the ponceau red staining of protein profile of the 2 h post heat shock and 6 h post heat shock sample.

Heat Shock Response Affects Gene Transcription in the Whole Mosquito Body

To follow the transcription of inducible heat shock response, RT-qPCR was performed on female *An. albimanus* cDNA. Three independent experiments were performed with three groups of five mosquitoes per condition, per experiment, and the S7 gene cDNA was used to normalize the RT-qPCR. We observed an increase of *ppo* and *hsp70* transcripts upon heat shock. Decreases in *tet* gene transcription were observed immediately after heat shock while *dnmt2* increased two hours post-heat shock, and continued to increase thereafter. The *hsp70* gene showed a transcriptional upsurge after the heat shock, as expected. The transcription of effector molecules commonly considered central to the early response to *P. berghei* infection, such as *duox* and *ppo*, also increased following heat shock. *ppo* transcription increased significantly 6 h post-heat shock, with *duox* increasing slightly (**Figure 4**, **Supplementary Information**).

Heat Shock Alters Antimicrobial Peptide Genes Transcription in the Mosquito Midgut

Control and heat-shocked mosquito midguts were extracted 6 h post-treatment, mRNA was extracted and cDNA synthetized. *Cecropin*, *gambicin*, *GNBP4B* and *ppo* genes were analyzed by RT-qPCR. Transcription of *ppo* was diminished at 6 h post-heat shock (**Figure 5**). This observation is in contradiction with whole body *ppo* transcription results. Inhibition of *ppo* transcription in the midgut could result from a specific sensitivity of this organ to heat shock or may involve a negative feedback loop resulting

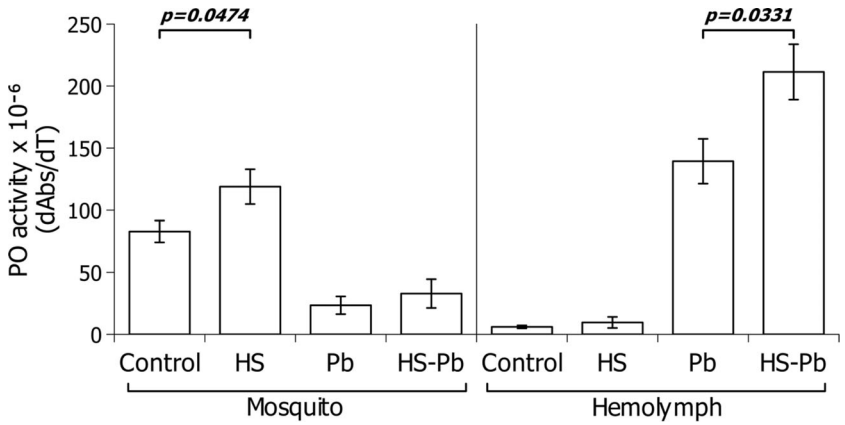


FIGURE 2 | Effect of heat shock on the phenoloxidase response of female *A. albimanus* during a *P. berghei* infection. Phenoloxidase activity in macerated body extracts (right) and hemolymph (left). Control, Mosquitoes without heat shock; HS, heat shock mosquitoes; Pb, *Plasmodium berghei* fed mosquitoes. Data of three independent experiments were analyzed using Student's t-test and are represented with their standard deviations.

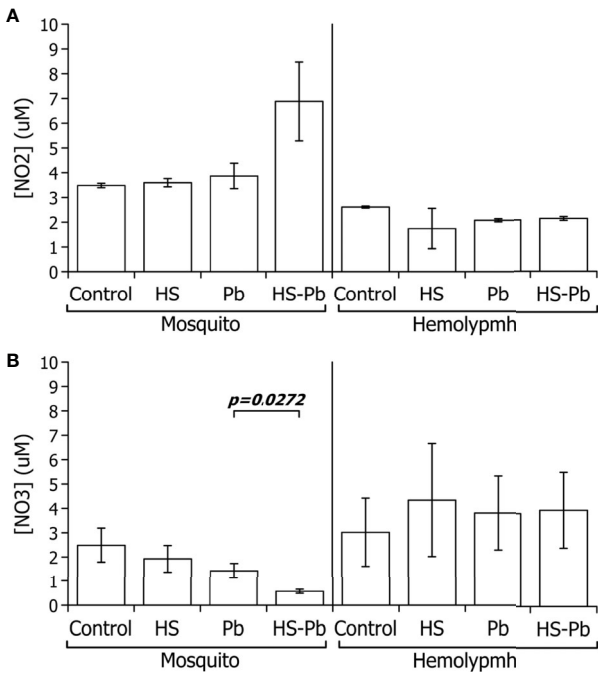


FIGURE 3 | Effect of heat shock on the nitric oxide derivatives of *A. albimanus* during a *P. berghei* infection. **(A)** NO_2^- production **(B)** NO_3^- production. Nitric oxide derivatives present in the hemolymph and body of *A. albimanus* female mosquitoes upon *P. berghei* (Pb) infection and 6 h post 30 min at 37°C heat-shock (heat shock) and the respective control samples. Data of three independent experiments were analyzed using Student's t-test.

from proteolytic phenoloxidase activation. Transcription of the *cecropin*, *gambicin*, and *GNBP4B* genes increased upon heat shock (**Figure 5**, **Supplementary Information**) while *ppo* transcription slightly decreased, indicating again a crosstalk between heat shock and the immune response.

DISCUSSION

Stress history drastically influences the mosquito immune system. The response to heat shock is generally characterized by heat shock protein expression. This response is, in the case of *A. gambiae*, protective from *O'nyong'nyong* virus infection (26). The experimental heat shock scheme chosen allowed us to track the effect of heat stress upon the mosquito immune system, although without a direct relation with the natural circumstances encountered by the mosquito in the wild, with the exception of the ever more common heat waves that are occurring worldwide due to climate change. Heat shock induces a state of increased resistance that lasts for at least 6 h. The heat shock possibly poises the immune system of the insect to respond faster and more intensively to the infection. For this to happen, the relevant changes must persist through the subsequent changes in the cellular environment. Methylation, demethylation, and other alterations to DNA and chromatin constitute a good theoretical mechanism for this ongoing physiological change. In mammals, the differentiation and specification of hematopoietic stem cells is mediated by *tet2* gene transcription. This gene codes for a methylcytosine dioxygenase that is required for activation of genes associated with differentiation (40, 41). In *A. albimanus*, the *tet2* gene could be involved in alteration of transcriptional activity upon *P. berghei* exposure (42). In this article, it was demonstrated that *P. berghei* infection of *A. albimanus* mosquitoes results in alterations in both DNA and mRNA methylation. The resulting methylation in turn alters the transcriptional pattern of the insect cells, suggesting longer term biological accommodation when challenged with *P. berghei* (42). In *Tribolium* beetles, general stress conditions do also lead to alterations in DNA methylation, allowing for wide spread transcriptional reprogramming (15). In insects, heat shock leads to substantial changes in the transcription, particularly in the expression of the Bt DNMT2 (DNA methyltransferase) gene, a part of the DNA methylation system. In the white fly *Bemisia tabaci* DNMT3 inhibition leads to an

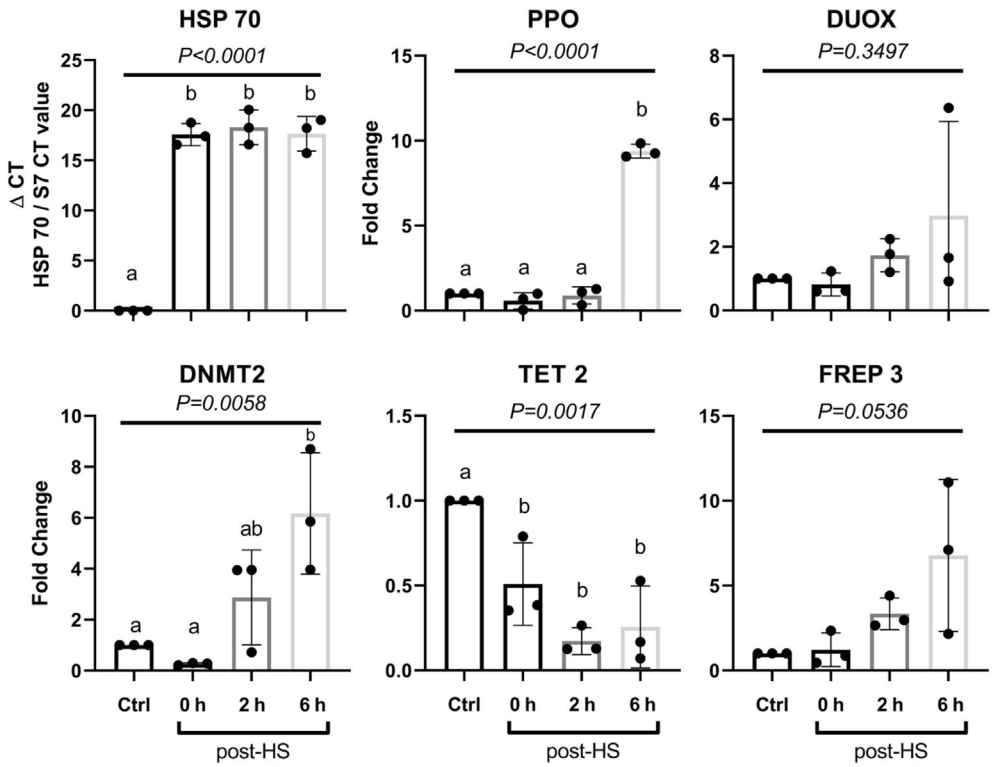


FIGURE 4 | Gene expression at different times post-heat shock in whole body. Gene expression was evaluated immediately (0 h), at 2, and 6 h after 30 min of exposure at 37°C (heat shock) in whole mosquitoes body. Data are indicated as mean ± SD. Data of three independent experiments were analyzed using ANOVA-one way followed by Tukey's test. Different letters indicate statistical significance.

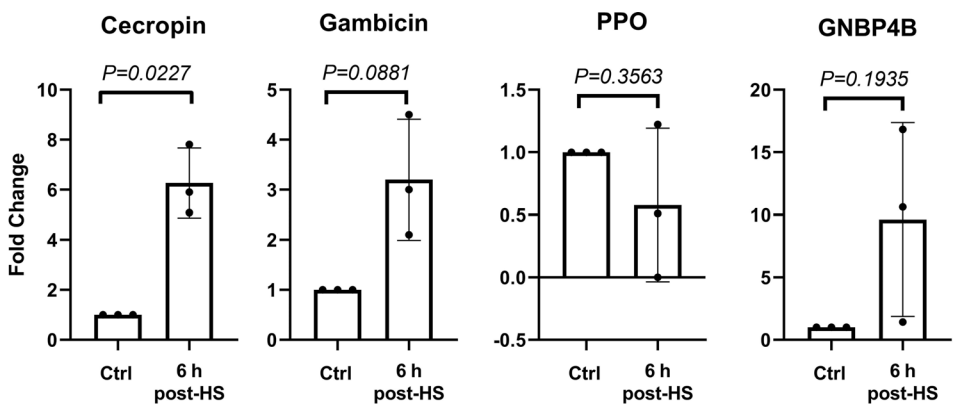


FIGURE 5 | Gene expression at 6 h post heat shock in midguts. Gene expression was evaluated at 6 h after 30 min of exposure at 37°C (heat shock-HS) in midguts. Data of three independent experiments were analyzed using unpaired t test with Welch's correction. Data are indicated as mean ± SD. Cecropin 6.2 ± 1.4, Gambicin 3.2 ± 1.2, PPO 0.5 ± 0.6, and GNBP4B 9.6 ± 7.7.

increase in heat susceptibility (43). Here, we observed that when aseptic stress in the form of heat shock is applied to *A. albimanus*, it enhances resistance to *P. berghei* infection. After heat shock, the activity of oxidative enzymes present in the mosquito hemolymph increased, potentially providing an explanation for the resistance observed (**Figure 6**). When analyzing the effect of heat shock on immunity gene transcription, we observed that genes related to DNA methylation modification (*tet*, *dnmt2*) showed the largest effect. In summary, heat shock alters the expression of many genes and induces the activation of phenoloxidase enzyme as well as

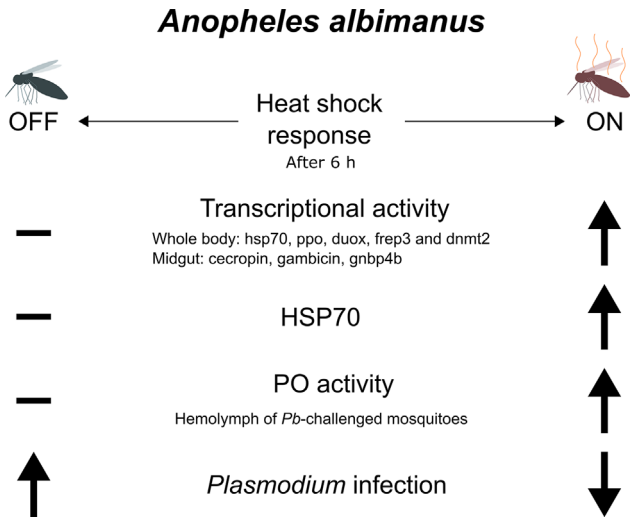


FIGURE 6 | Schematic summary. After a 6 h of a 30 min heat shock at 37°C, several immune genes are upregulated in the mosquitoes. These include genes that have been shown responsible for limiting a *Plasmodium* infection such as *ppo*, *duox*, *frep3* in the whole mosquito, and antimicrobial peptides in the midgut. *hsp70* is also upregulated along with *dnmt2*, a gene responsible for the methylation of DNA and RNA in mosquitoes. This might indicate an increment in methylcytosines in the nucleic acids, which is also supported by the down regulation of the enzyme that removes these epigenetic marks, *tet2* (not shown). The PO activity also increases in the hemolymph of the *Plasmodium*-challenged and heat-shocked mosquitoes, although the total amount of NO produced did not changed (not shown). This overall response caused the mosquitoes to become less infected with the parasite. Only statistically significant results are portrayed.

increasing transcription of its gene. Infection also increased the activation of phenoloxidase.

The heat shock had little effect on NO_2^- production, a species resulting from oxidation of the highly reactive NO. A slight increase in NO_3^- production was detected. Altogether, the results obtained demonstrate that immune alteration induced by heat shock is sufficient to decrease *P. berghei* infection without requiring a prerequisite bacterial immune challenge as reported by Dieme et al. (44). The resistance of *Anopheles* to *Plasmodium* infection induced by high temperature has been reported elsewhere (45), though the mechanism underlying the phenomenon was not described. Here we observed that thermal stress affected the transcription of both heat shock proteins and elements of the anti-pathogenic immune response. Given the recent increases in temperature in the tropical regions, the effect of heat shock on malaria transmission is relevant to future disease tends.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RC and HL-M conceived the presented idea. RC developed the theory and performed the statistics. EH-T, FC-P, BR-T, KM-M, and VC-J carried out the experiments. RC and HL-M wrote the manuscript with support from BR-T and FC-P. FC-P, KM-M, and VC-J fabricated the mosquito samples. EH-T, FC-P, BR-T, KM-M, and VC-J processed and analyzed the samples. HL-M supervised the project and planned the experiments. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.584660/full#supplementary-material

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