# Death Is Coming and the clot thickens, as pyroptosis feeds the fire

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**SUMMARY**
Pyroptotic cell death during endotoxemia causes death via unknown mechanisms. In this issue of *Immunity* **Wu et al**. (2019) show that T3SS rod proteins or LPS induces inflammasome activation, macrophage pyroptosis and accompanying tissue factor release, directly connecting inflammation to coagulation.

**MAIN TEXT**
Assembly of the inflammasome causes activation of inflammatory caspases, leading to proteolytic maturation and release of interlukein-1β (IL-1β) and IL-18 and pyroptotic cell death. Although inflammasome activation and pyroptosis play key roles in host defence, excessive activation during experimental endotoxemia or sepsis leads to coagulation and death by ill-defined mechanisms (Kayagaki et al., 2011). The work by **Wu et al** in this issue shows that it is tissue factor (TF) release after inflammasome activation that triggers systemic coagulation and lethality (Wu et al., 2019). Using an elegant experimental approach they have shown that either active caspase-1 or -11 leads to gasdermin D (GSDMD)-dependent lytic pyroptosis of macrophages and an accompanying release of microvesicle-associated TF. Importantly, inhibition of TF or pre-depletion of macrophages prevented coagulation and lethality **(Figure 1)**.

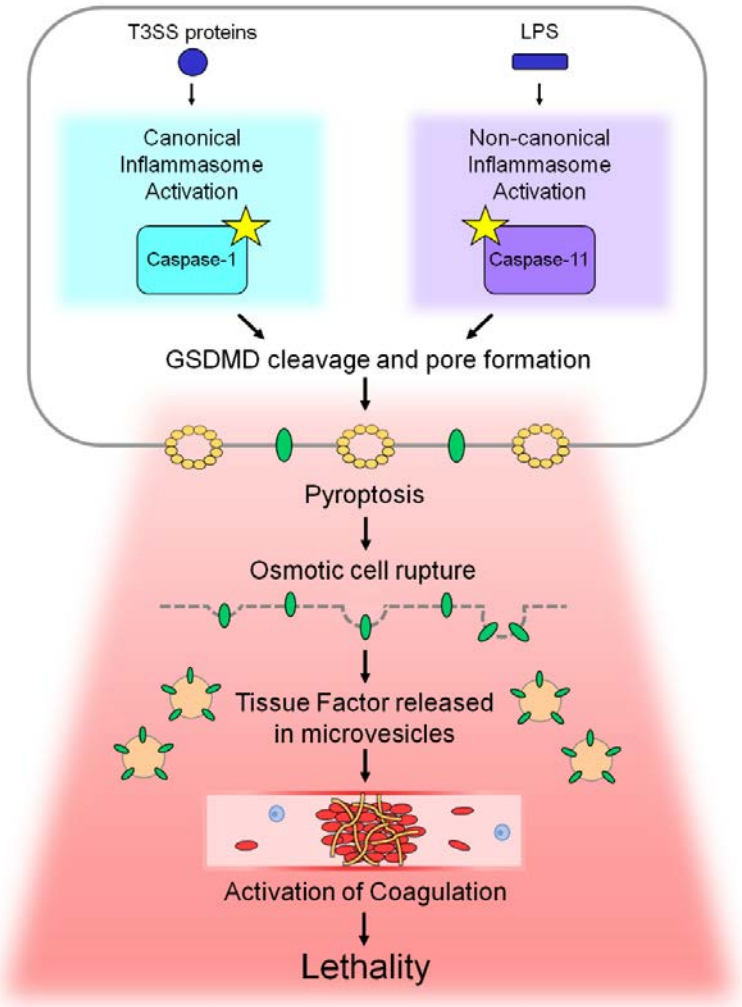


**Figure 1 – Caspase-activated GSDMD drives pyroptosis with tissue factor release, leading to coagulation**. Overt inflammatory responses during experimental sepsis leads to coagulation and death, but the mechanism linking these has remained elusive. Inflammasome assembly leads to activation of caspase-1 or caspase-11, which cleave GSDMD to the functional form. Initial GSDMD insertion into the plasma membrane forms pores, but subsequently drives pyroptotic death and cell rupture. During pyroptotic cell death microvesicles containing tissue factor are released, resulting in systemic activation of the coagulation cascade, occlusion of blood vessels within organs and death.

Inflammasomes are intracellular multiprotein complexes that show stimulus-specific assembly in response to bacteria, viruses, fungi, insoluble aggregates, and environmental factors (Broz & Dixit, 2016). Canonical activation occurs after sensing of factors leads to assembly of a pattern-recognition receptor, caspase-1 and ASC complex, leading to caspase-1 activation and cleavage of pro-IL-1β and pro-IL-18. Non-canonical activation occurs after direct binding of intracellular LPS to caspase-4, caspase-5 (human) or caspase-11 (mice) induces their activation, with subsequent cleavage of GSDMD (Shi et al., 2015). The now functional N-terminus of GSDMD forms pores in the plasma membrane to enable release of IL-1 and IL-18, but also feeds back to activate NLRP3 or AIM2 inflammasomes. Although cytokine release through GSMD pores can occur without cell lysis, too many pores leads to pyroptotic cell death via osmotic lysis, with release and thus exposure of intracellular pathogens to immune cells (Broz & Dixit, 2016). These mechanisms are essential for host defence, but over activation can cause multiple organ failure and death by undefined mechanisms.

Blood coagulation is essential for cessation of bleeding following injury, and is mediated by a serine protease signalling cascade that is tightly regulated to ensure coagulation only occurs when necessary, and to the appropriate extent (Esmon, 2005). In pathophysiological states, such as sepsis, coagulation is inappropriately activated, leading to systemic thrombosis and potentially disseminated intravascular coagulation (DIC), where occluding clots form throughout blood vessels. As TF (an integral membrane protein) initiates activation of pro-thrombin, it is central for initiation of coagulation. Inflammation (e.g. via TNFα, IL-1) drives tissue factor expression and thus pro-thrombin activation, while thrombin activates protease-activated receptors, leading to expression of inflammatory cytokines (Esmon, 2005). However, although these links between coagulation and immunity are known, they don't currently explain the lethality seen after experimental endotoxemia or sepsis (Kayagaki et al., 2011).

Within this context, **Wu** and colleagues have robustly investigated how bacterial products that lead to inflammasome activation might drive coagulation. The authors began by developing a model system utilising the *E.coli* T3SS rod protein EprJ fused to anthrax lethal toxin translocation domain, which when administered with anthrax protein protective antigen (PA) resulted in translocation into the cytosol of macrophages and activation of pyroptosis via caspase-1. EprJ administration to mice resulted in haematological parameters indicative of prior coagulation, with increased prothrombin time (a measure of how long clotting takes), thrombin-anti-thrombin complexes and $TF^{+}$ microvesicles, and decreased fibrinogen and platelets, along with fibrin deposition in liver, spleen and blood vessels – changes consistent with clinical DIC. Importantly, EprJ-induced coagulation was prevented in *Casp1/11*$^{-/-}$ mice, but not in *Casp11*$^{-/-}$ or *Tlr4*$^{-/-}$, and lethality was also averted in *Casp1/11*$^{-/-}$ mice, consistent with EprJ activation of caspase-1 via the NLRC4 inflammasome.

The pivotal finding in this model was that caspase-1 acted via the effector GSDMD, with *Gsdmd*$^{-/-}$ mice similarly protected from the haematological abnormalities and lethality, suggesting EprJ-induced coagulation and fatality were likely the result of either GSDMD-dependent cytokine release or pyroptosis. As coagulation still occurred in *Il1r1*$^{-/-}$ and *Il18r*$^{-/-}$ mice after EprJ, this indicated that it was pyroptosis that was required, and indeed less monocytes and macrophages were present in blood, lung and spleen after EprJ, along with a concomitant increase in cell death. Similar results were obtained upon *E.coli* infection or with other T3SS rod proteins, such as BsaK and PrgJ. Given the propensity of monocytes and macrophages to undergo pyroptosis in response to bacterial products, and their inducible expression of TF, the authors explored if they contributed to EprJ-induced coagulation. Pre-depletion of monocytes and macrophages with clodronate liposomes corrected the EprJ-induced coagulation and partially protected against lethality, while EprJ-treated macrophages released TF during pyroptosis in a caspase-1 and GSDMD-dependent manner. Importantly, because TF release was prevented by the osmoprotectant glycine, this indicated TF did not exit through

GSDMD pores, but instead required cell lysis and integration of TF into the membrane of forming microvesicles. In keeping with this, both *Casp1/11*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice showed reduced TF$^{+}$ microvesicles in plasma after EprJ. Interestingly, clodronate liposomes induce apoptosis of macrophages, emphasizing that the mode of cell death is key to the downstream consequences. Finally, blockade of TF in vivo with either neutralizing antibodies or a *Tf*$^{flox/flox}$/ubiquitous *Ubc* Cre$^{ERT2}$ mouse both showed reduced coagulation and lethality after EprJ treatment. Essentially similar results were found after administration of lethal doses of LPS, but the effects were not as well-defined, likely reflecting the more stochastic nature of caspase-11 activation after LPS internalization and release from endosomes, compared to the model system with EprJ fusion proteins.

Together, these results reveal an additional paradigm for how overt canonical or non-canonical inflammasome activation can drive pathophysiological changes and death. Despite the known reciprocal connections between coagulation and inflammation, and expression of TF by macrophages, no link between the violent pyroptotic death of macrophages and the uncontrolled release of TF has previously been made. Thus, these findings represent a fundamental connection for how inflammatory processes can rapidly drive coagulation. However, the mark of an important paper is that it raises as many questions as it answers. For example, what population of macrophages is pyroptosis-competent and representative enough to release sufficient TF into the circulation for systemic coagulation and DIC? Clodronate liposomes cross vascular barriers poorly and thus readily deplete Kupffer cells and red pulp macrophages. Indeed, bone marrow chimeras with *Casp11*$^{-/-}$ host and wild-type myeloid cells are protected from endotoxemic lethality (Cheng et al., 2017), suggesting a resident macrophage population is perhaps the responder. However, data on inflammasomes in resident macrophage populations is sparse. Interestingly, as EprJ-induced DIC occurs within 90 mins, this indicates the monocyte or macrophage population must already express TF on the surface, and thus must be separated from the blood by a vascular endothelium to prevent TF-induced coagulation. Although artificial, the use of an EprJ fusion protein administered with anthrax PA was pivotal to reveal this important pathway, owing to its acquired ability to enter cells and massively activate caspase-1-driven pyroptosis. Similarly, typical endotoxemia models use LPS at levels ~200,000 fold higher than those seen in humans with severe sepsis, which likely pushes LPS internalization and activation of caspase-11. However, in septic humans would macrophage pyroptosis-released TF reach systemic levels able to mediate DIC alone, would it act in concert with, for example, activated monocyte TF, or would it act during local inflammatory reactions? Similarly, to what degree do other pathways shown to be important during endotoxemia (e.g. caspase-8, TNFα, complement) converge on TF release via pyroptosis?

Thrombin has recently been shown to directly cleave and activate IL-1α (Burzynski et al., 2019), linking the key effector of coagulation to inflammation, while ***Wu et al*** show active inflammasomes can directly cause TF release, linking the key effectors of inflammation to coagulation. The host benefit of coagulation-activated inflammation is direct, as breach of external barriers opens the risk of infection and thus instigating an immune response alongside haemostasis would safeguard against this. However, the host advantage of inflammation activating coagulation is more complex. Many reports show fibrin clots physically trap bacteria (Dunn & Simmons, 1982) to assist their clearance by immune cells (the process used by ancient arthropods, such as horseshoe crabs, where clotting both entraps bacteria and stops haemolymph loss), and indeed the ‘clot busting’ streptokinase is a bacterial protein that likely evolved to evade this trapping. At a tissue level if a severe infection, or the host’s response to it, causes haemorrhage, inflammation-driven coagulation that stops bleeding would be beneficial. Granulomas form in an attempt to wall off infectious agents that cannot be eliminated by the inflammatory response, with fibrin-ring granulomas appearing in a variety of infections. These are characterised by a dense fibrin ring with epithelioid macrophages, again suggesting inflammation-driven coagulation that deposits a fibrin barrier is beneficial. However, as with all immune responses

that intend to be protective, maladaptation can easily drive these toward situations that are injurious to the host.

Inflammasome activation is an essential defence against pathogens, but how excessive activation leads to activation of coagulation and death was unknown. Thus, **Wu et al** have now solved the mystery that during DIC the clot thickens because pyroptosis feeds the fire.

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