Programmed Cell Death in Immune Modulation: Insights from Antimicrobial and Immunosuppressive Responses

Dissertation

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By

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Abstract

My research focuses on the diverse roles of programmed cell death (PCD) in host innate immune responses. My dissertation explores how PCD assists host antimicrobial defense, as well as contributes to the progression of sepsis-induced immunosuppression. The dissertation could be divided into two main sections and one derivative section. Firstly, I have identified a novel interaction between *Pseudomonas aeruginosa* and host necroptosis during *in vitro* and *in vivo* infection. Secondly, I have characterized the impact of a novel PCD regulator, NINJ1, in improving sepsis-induced immunosuppression by partially restoring the host defense to secondary infections.

Quorum sensing (QS), a communication system evolved by *Pseudomonas aeruginosa* to monitor its density, is well-acknowledged to be involved in multiple activities during bacterial infection. Recent studies have revealed clues about link between *Pseudomonas aeruginosa* QS and host programed cell death. However, it remains limited understanding whether QS plays a role in host PCD process during the infection. In this study, I used *rhl* mutants of *Pseudomonas aeruginosa* to *in vitro* challenge multiple genetic knockout macrophages to explore the connection between QS and programmed cell death. According to the data from cell death assays and immunoblotting, I discovered these *rhl* mutants significantly promoted necroptosis which was unknown in this field. Additionally, I found that the increased necroptosis activation was caused by the upregulation of another QS subsystem, *pqs*, because the deletion of *pqs* in *rhl*-deficient

Pseudomonas aeruginosa abolished macrophage necroptosis *in vitro* and *in vivo*. Therefore, this study revealed a novel *rhl-pqs*-necroptosis pathway.

Sepsis is characterized by two dynamic stages occur during the initiation and progression, which are system inflammatory response syndrome (SIRS) in the acute phase and compensatory anti-inflammatory response syndrome (CARS) in the later phase. Recent study revealed that inhibiting HMGB1 release reduced pyroptosis-related effector cell loss in septic mice. As a newly discovered PCD component, NINJ1-mediated plasma membrane rupture has becoming increasingly important in preclinical studies. However, it is totally unclear whether NINJ1 plays a role in sepsis-induced immunosuppression. In this study, I obtained *Ninj1*$^{-/-}$ mice and induced sepsis through cecal ligation and puncture (CLP) surgery. I discovered that NINJ1 deficiency significantly reduced the mortality of septic mice. In addition, by using a two-hit model, I found that NINJ1 deficiency also partially restored host defense to bacterial infection under immunosuppression circumstance. These findings provide us with novel therapeutic target in developing clinical treatments for patients suffered with sepsis.

Inspired by the findings and approaches of my second study, I extended my research to investigate whether NINJ1 has an impact on tumor-mediated immunosuppression. The immunosuppressive status in TME is mainly due to type 2 transition of immune effector cells regulated by tumor cells and immune regulator cells. By exerting the function of NINJ1 in modulating membrane rupture, we hypothesize that targeting NINJ1 in tumor cells can inhibit the pro-tumor transition of immune cells in TME. To test the hypothesis, I collaborated with Dr. Xiang Chen to generate *Ninj1*$^{-/-}$ tumor cells and reconfirmed NINJ1's function in tumor cells. By co-culturing macrophages with supernatants from WT and *Ninj1*$^{-/-}$ tumor cells, I found both the M2-

associated gene expression and M2-related signaling pathway were remarkably suppressed in macrophages treated with *Ninj1*$^{-/-}$ sup, indicating loss of NINJ1 in tumor cells restrains the transition of pro-tumor immune cells and can be function as a potential therapeutic target.

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Fields of Study

Major Field: Molecular Cellular and Developmental Biology Program

Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

1.1 Review of Programmed Cell Death

Innate Immunity functions as the first immune barrier in defending pathogens invasion as well as tissue damage. To exercise its role of protection, the innate immune system has evolved multiple strategies to detect, respond and eliminate any threats to host homeostasis. Innate immunity relies on a limited number of receptors to recognize either highly conservative structures or common metabolic consequences of infection, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (1). Upon activation, these receptors trigger the downstream signaling pathways to produce pro-inflammatory cytokines to eliminate the invading microbes. However, due to its limited recognition spectrum, the innate immunity is unable to detect and intercept all kinds of microorganisms during infection, resulting in break-in of first defense and infected immune cells. To expose the infected pathogens and call "reinforcement", the innate immunity has evolved a pivotal strategy of cell suicide, which is programmed cell death (PCD).

Cell death is recognized as the result of one of two distinct processes, controlled cell death and uncontrolled cell death. The morphological changes of the cell structures, like devastating the plasma membrane and spilling the cellular contents into surrounding environment, were prevalent in a vast number of cell types during cell death, which led to the belief that cell death was

uncontrolled and necrotic. Thus, this type of cell death is also named as necrosis. However, in 1960s, Lockshin and Williams first discovered a kind of specific cell death in silkworm that was executed in a controlled manner and named this type of cell death as "programmed" (2). Later in 1972, Kerr *et al*. also observed programmed cell death in human tissues based on cell morphological changes that the cells and nuclei became condensed and fragmented during death, and they named this PCD as "apoptosis" (3). Apoptosis was then referred as the PCD over decades. It took a long time until "programmed necrosis" was accepted and recharacterized as a member of PCD (4).

1.1.1 Apoptosis

As the first identified PCD, apoptosis is the most classical and well characterized cell death in the field. It is initiated by the activation of a series of cysteine-aspartic proteases, known as Caspases. Recent research has identified the caspase-8 and 9 are the two main initiators to trigger apoptosis upon receiving the cell damage signals (5, 6). Following the activation of Caspase-8 and 9, the executioner caspases, including caspase-3, 6 and 7, are activated to mount a series of events that lead to the destruction of nuclei, DNA fragmentation, expression of ligands for phagocytic cells and formation of apoptotic bodies (5, 7, 8). The apoptotic bodies, which contain the contents of dead cells, can be phagocyted by surrounding innate immune cells, especially macrophages (5, 6). The apoptosis has two activation mechanisms, one is through intrinsic pathway and the other one is from extrinsic pathway. The differences lie between the two signaling pathways are based on how the apoptotic triggers are sensed and integrated by cells as only the extrinsic pathway requires the involvement of death receptors localized on the plasma membrane (9).

The intrinsic apoptosis, which is also characterized as mitochondrial pathway of apoptosis, can be activated through a variety of stimuli, such as DNA damage, lack of growth factor and mitochondrial damage (5, 9, 10). The B cell lymphoma 2 (BCL-2) family of proteins are the pivotal upstream regulators of intrinsic apoptosis in mammalian cells, including both pro-apoptotic and anti-apoptotic members (9, 11, 12, 13). High level of anti-apoptotic BCL-2 proteins can result in the inhibition of apoptosis within cells. While under the presence of apoptotic stimuli, the pro-apoptotic BCL-2 proteins are activated to suppress the anti-apoptotic BCL-2 members and drive the pore formation on mitochondrial outer membrane through oligomerization (9, 13). These pores on mitochondrial membrane led to the release of pro-apoptotic proteins, including cytochrome c and Smac/Diablo, into the cytoplasm and initiation of apoptosis (4, 5, 9, 13). Cytochrome c binds to the apoptotic protease activating factor 1 (APAF1) to form a multiprotein complex, also known as apoptosome (14). During complex formation, APAF1 undergoes a major conformational change to expose its caspase activation and recruitment domain (CARD) and then recruit pro-caspase-9 to the apoptosome for activation (13-15). The activated caspase-9 mediates the cleavage of downstream caspase-3 and 7, which in turn cleave hundreds of cellular substrates to execute the final apoptosis.

The extrinsic apoptosis is activated by the death receptors that are localized on cytoplasmic membrane. These death receptors, including Fas, TNFR1, TRAIL and TRAIL-R2, exhibit similar characteristics by the presence of an intracellular protein-protein interaction domain, also known as death domain (DD) (9, 16). And each of receptors has a corresponding ligand that is produced by surrounding NK cells or macrophages. Upon activation by death ligand, the death receptor recruits procaspase-8 through its death-inducing domain to initiate a death-inducing signal

complex (DISC) at the intracellular domain of the receptor (5). The DISC has been identified to have an important component which is FAS-associated death domain (FADD) or tumor necrosis factor receptor (TNFR)-associated death domain (TRADD) to facilitate the recruitment of pro-caspse-8 to the complex (17-19). The procaspase-8 get activated at DISC and cleaves the downstream executioner caspases, like caspase-3 and caspase-7, to elicit the apoptosis.

Though intrinsic and extrinsic apoptosis differs from the initiator caspases and activation mechanisms, they still share some feathers, for example the same downstream executioner caspases. Both cleaved caspase-8 and caspase-9 will target and cleave caspase-3 and caspase-7 to trigger the apoptosis. Apart from the execution mechanism, both apoptosis pathways have similar inhibition strategy. A group of inhibition apoptosis proteins (IAPs) exist within cells to restrict both caspase-8 and caspase-9 under normal conditions (5, 9). With the presence of IAPs, the cleavage of caspase-3 and 7 by activated caspase-8 or 9 is blocked and the apoptosis is interrupted. This blockage exists until the IAPs are inhibited by the Smac/Diablo which are released via mitochondrial membrane pores (20).

Table 1-1 caspases and their roles in PCD

Caspase	PCD type	Functions in PCD
Caspase-1	pyroptosis; apoptosis	Cleaves and activates GSDMD, IL-1β, IL-18, caspase-7; PARP1
Caspase-2	apoptosis	Cleaves and activates caspase-3 and caspase-7
Caspase-3	apoptosis; pyroptosis	Cleaves and inactivates GSDMD, cleaves and activates GSDME, executes apoptosis through cleavage of other substrates

Caspase-4	pyroptosis	Cleaves and activates GSDMD
Caspase-5	pyroptosis	Cleaves and activates GSDMD
Caspase-7	apoptosis; pyroptosis	Cleaves and inactivates GSDMD, cleaves and activates GSDME, executes apoptosis through cleavage of other substrates
Caspase-8	apoptosis; necroptosis	Cleaves and activates GSDMD, GSDME, IL-1β, IL-18, caspase-3, caspase-7, caspase-9, RIPK1 and RIPK3
Caspase-9	apoptosis	Cleaves and activates caspase-3, caspase-7
Caspase-10	apoptosis	Cleaves and activates caspase-3 and caspase-7
Caspase-11	pyroptosis	Cleaves and activates GSDMD

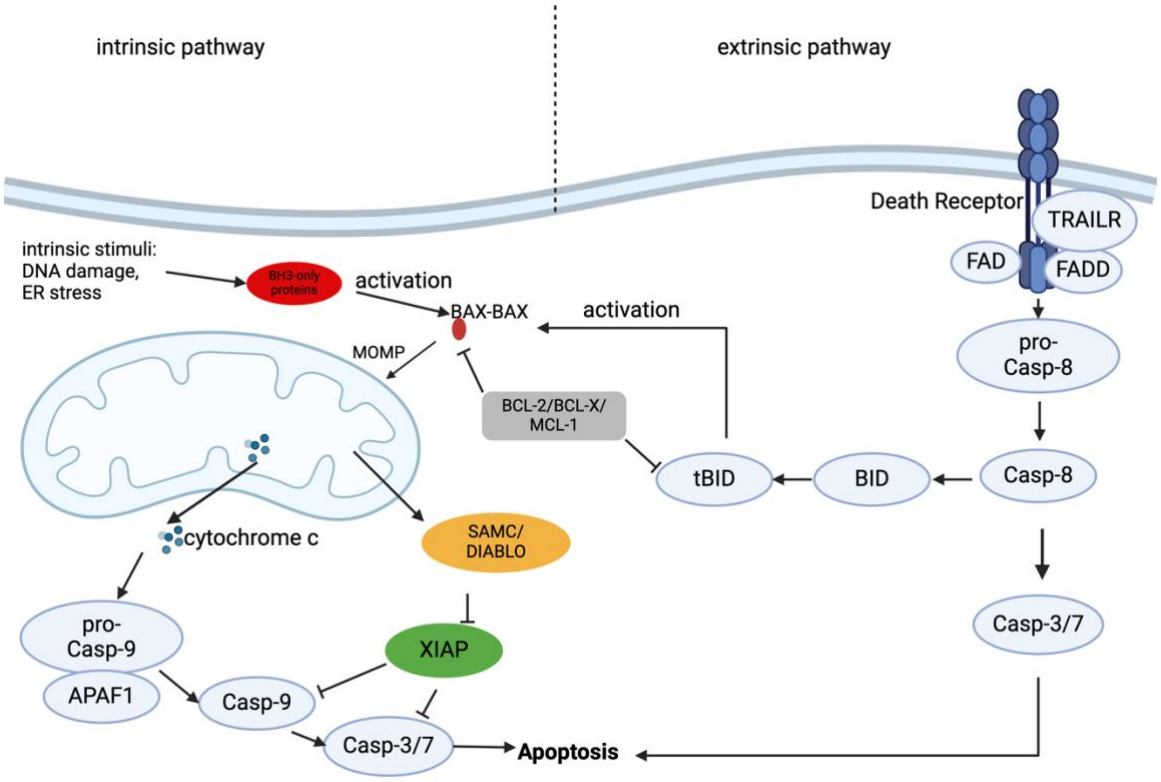


Figure 1-1 Schematic of intrinsic and extrinsic apoptosis pathway. (Created at Biorender)

1.1.2 Necroptosis

Necrosis was traditionally characterized as an uncontrolled cell death with the loss of cytoplasmic membrane integrity and passive leakage of intracellular contents. However, the groundbreaking discoveries made in 2000s overturned such rigid belief by defining the receptor-interacting protein kinase 1 (RIPK1), RIPK3 and mixed lineage kinase domain-like protein (MLKL) together mediated a necrotic PCD pathway, which known as necroptosis (21-24). Necroptosis is the first identified PCD that regulates necrosis and is found by the inhibition of caspase-independent necrosis under the treatment of necrostatin-1 (Nec-1) (22).

Necroptosis can be induced through the activation of various receptors, including death receptors (TNFR1, Fas, TRAIL and TRAIL-R2), toll-like receptors (TLR4 and TLR3) and cytosolic nucleic acid sensors (RIG-I and STING) under apoptosis-deficient conditions (9, 25-30). Among the receptors, TNFR1/TNFα is the most well characterized activation mechanism to trigger necroptosis. After binding with TNFα, TNFR1 recruits RIPK1 and TRADD to form a complex, named as complex I (31). Under the presence of caspase-8, the complex I then recruits caspase-8 and FADD to promote apoptosis in either RIPK1-independent manner (complex IIa-RIA) or RIPK1-dependent manner (complex IIa-RDA) (32). With the absence of caspase-8, RIPK1 is activated in complex I and interacts with RIPK3 to form complex IIb to induce necroptosis.

The activation of RIPK1 is controlled by multiple factors. At the presence of caspae-8, the cleavage mediated by caspase-8 separates the N-terminal kinase domain from the intermediate domain, resulting in the inhibition on RIPK1 activation (33). When the function of caspase-8 is suppressed, the activation of RIPK1 is then modulated by various types of ubiquitin modifications (32). After the initiation of complex I, TRADD then recruits the adapter proteins TRAF2/5 and E3 ubiquitin ligases cIAP1/2 to complex I which in turn modify RIPK1 with K63 ubiquitin (34). K63 ubiquitination of RIPK1 mediates the recruitment of TAB2/3 and the linear ubiquitination (M1-Ubi) assembly complex (LUBAC) to complex I, the former "player" facilitates the activation of transforming growth factor-β-activated kinase 1 (TAK1) and the latter one introduces another type of ubiquitin modification, M1, to RIPK1, which impedes the activation of RIPK1 (32). Along with the recruitment of LUBAC to complex I, two deubiquitinating complexes are also assembled into the complex I, which are CYLD/SPATA2 and A20 (TNFAIP3) (35, 36). Though both of deubiquitinating complexes aim to remove the ubiquitin modifications of RIPK1, the

consequences are different. CYLD/SPATA2 predominately remove the M1-Ubi on RIPK1 which strongly promotes the activation of RIPK1, and deficiencies of CYLD/SPATA2 protect cells from TNFα-induced necroptosis and apoptosis (32, 35, 37). A20 impedes the activation of RIPK1 by removing K63 ubiquitin of RIPK1 through its OTU domain and catalyzing K48 ubiquitin on RIPK1 to promote its degradation (36, 38). Loss of A20 not only increases K63 ubiquitin on RIPK1, but also reduces the M1 ubiquitin levels of RIPK1, resulting in highly sensitivity to TNFα-induced necroptosis (39). Apart from the factors mentioned above, a recent study has demonstrated that the dimerization of RIPK1 can also facilitate its activation (40). The death domain is essential for the complex I formation and RIPK1 recruitment upon TNFR1 activation. Meanwhile, the death domain of RIPK1 also mediates its dimerization during the transition from complex I to complex II. Mutation of K584 (murine RIPK1) / K599 (human RIPK1) that blocks homodimerization of RIPK1 has no influence on recruitment of RIPK1 to complex I but impedes the activation of RIPK1 and formation of complex II, suggesting the dimerization of RIPK1 is required in TNFα-induced necroptosis and RIPK1-dependent apoptosis (40).

RIPK1 is activated by phosphorylation from TAK1 and binds with RIPK3 to form complex IIb to induce necroptosis. As mentioned before, K63 ubiquitin of RIPK1 recruits TAB2/3 to complex I, which in turn activates TAK1. TAK1 later phosphorylates multiple sites of the intermediate domain of RIPK1, including S321, S332 and S334 (41). The phosphorylation on S321 of RIPK1 particularly restrains its binding to FADD and inhibits the following RIPK1-dependent apoptosis, which boosts RIPK1-RIPK3 interaction and necroptosis. Both RIPK1 and RIPK3 contain a same motif named as the RIP homotypic interaction motif (RHIM) which is required for the binding process (42). The RHIM is located within the intermediate domain of RIPK1 that is

inhibited under the presence of caspase-8. The activated RIPK1 recruits and binds to RIPK3 through RHIM and directly leads to the formation of complex IIb, which is the core step on execution of necroptosis. Evidence shows that mouse embryonic fibroblasts (MEFs) with mutated RHIM of RIPK1 have a strong resistance to necroptosis induction, manifesting the crucial role of RHIM in necroptosis activation (43, 44). Notably, ubiquitination of RIPK1 also plays a key role in the formation of complex IIb. Pellino 1 (PELI1), an E3 ubiquitin ligase, mediates the transition from complex I to complex IIb through catalyzing K115 of activated RIPK1 with K63 ubiquitin (45). *Peli1*$^{-/-}$ MEFs exhibit normal activation of RIK1 while failing to form complex IIb, suggesting that PELI1 has no influence on ubiquitination status of complex I but is required for initiation of complex IIb (32, 45).

The execution step of necroptosis is the activation and phosphorylation of MLKL. After activation, RIPK1 recruits and phosphorylates RIPK3 to form the complex IIb. The activated RIPK1-RIPK3 complex then recruits MLKL to form the final complex, named as necrosome, to trigger necroptosis (46). MLKL is a pseudokinase that has a N-terminal four-helical bundle domain (4HBD) and a C-terminal pseudokinase domain. The phosphorylation of MLKL mediated by activated RIPK3 leads to a major conformational change which exposes the 4HBD (47,48). This change makes it available for phosphorylated MLKLs to form disulfide bond with each other and facilitates the oligomerization of MLKL. The phosphorylation on T357/S358 and four cysteines within 4HBD of MLKL is responsible for the disulfide bond formation (49). The oligomerized MLKLs then translocate to plasma membrane with the help of a specific inositol phosphate (IP) code (50). Upon reach the plasma membrane, MLKL compromise the membrane integrity through two nonexclusive models: the oligomerized MLKLs interact with the phosphatidylinositol

phosphates (PIPs) present at the membrane to form pores, and MLKL itself constitutes a platform for the opening of ion channels at the plasma membrane, enabling ion efflux, cell swelling and membrane rupture (51, 52).

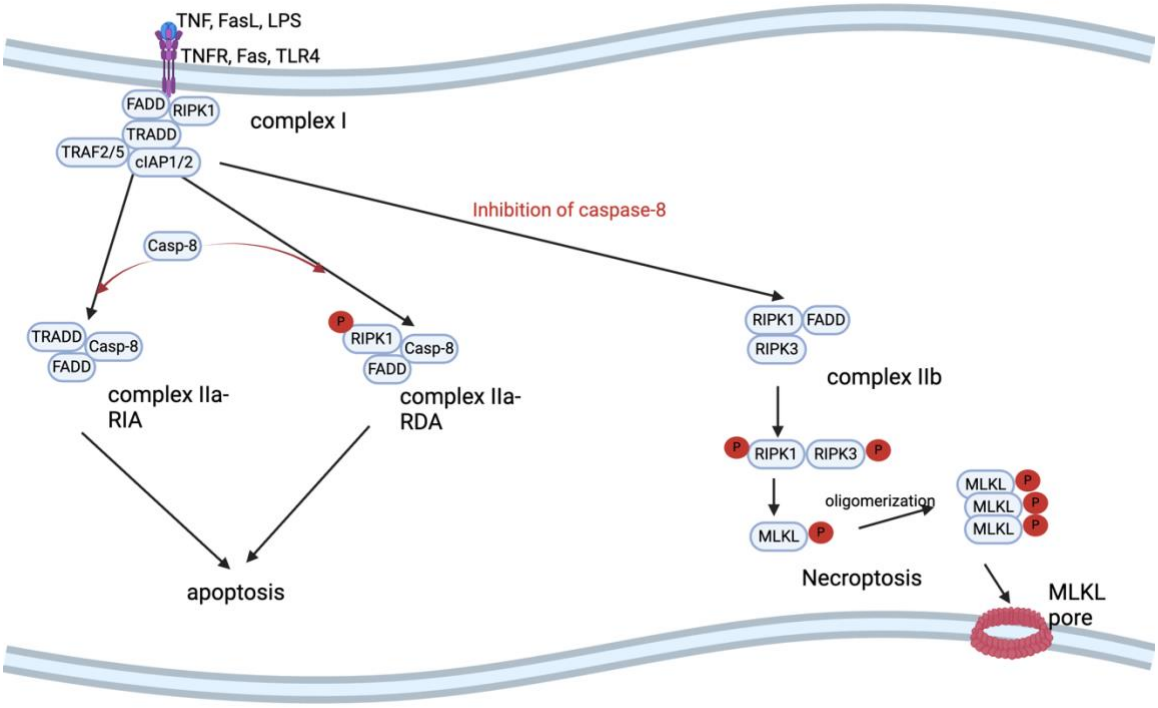


Figure 1-2 Diagram of necroptosis pathway (created at Biorender)

1.1.3 Pyroptosis

Caspases were believed to be involved in only apoptosis activation. However, evidence emerged show that caspases also mediate a form of programmed necrosis, named as pyroptosis (53, 54). Thus, pyroptosis is another type of programmed necrotic cell death which is mediated by dedicated genetic pathways. As both pyroptosis and apoptosis are induced by caspases, they share some similarities. Both PCD pathways lead to cell swelling and bubble-like protrusions appear on

the membrane surface before its rupture, nuclear condensation and oligonucleosomal DNA fragmentation (55-57). However, the main difference between pyroptosis and apoptosis is the morphological change during cell death. Unlike apoptosis, pyroptosis eventually devastates the plasma membrane integrity and spills the intracellular contents into surrounding environment, which easily cause inflammation. Upon receiving extracellular or intracellular stimuli, the caspases get activated and cleave the downstream executioner gasdermin proteins, resulting in the N-terminal of gasdermin oligomerization and translocation to plasma membrane to form pores (58). These gasdermin pores allow ion and water efflux, causing cell swelling and eventually membrane rupture.

Multiple pathways have been identified to trigger pyroptosis, the canonical pathway is the first well characterized mechanism that induce pyroptosis. Upon recognizing PAMPs and DAMPs, the pattern recognition receptors (PRRs) like Nod-like receptor family pyrin domain containing 3 (NLRP3), NLR family caspase activation and recruitment domain (CARD) containing 4 (NLRC4) and NLR family pyrin domain -containing 1 (NLRP1) are activated and initiate the assembly of a multiprotein complex, known as inflammasome (59). NLRs contain a leucine-rich repeat (LRR), a nucleotide-binding oligomerization domain (NACHT), and a pyrin domain or CARD which is located at N-terminal to divide the NLRs to either NLRPs or NLRCs (60). These NLRPs sense a variety of stimulus, NLRP1 has been reported to recognize toxoplasma gondii and the Bacillus anthracis anthrax lethal toxin, while NLRP3 senses toxins, pathogens, metabolites, crystalline substances, nucleic acids, and ATP (61-63). While for NLRC4, its activation requires the initial recognition of ligands by multiple neuronal apoptosis inhibitory proteins (NAIPs) (60). The NAIPs recognize can directly bind to flagellin, proteins of type 3 secretion system (T3SS), the needle of

T3SS and the inner rod of T3SS (64-66). Apart from NLRs, AIM2 can also be activated to induce inflammasome by binding to electronegative double-stranded DNA (dsDNA) (67). During the inflammasome formation, apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 are recruited to the hub. The CARD is essential for recruitment and cleavage of pro-caspase-1 in NLRPs, while NLRC4 can directly recruit and activate caspase-1. The mature caspase-1 in turn cleaves the executioner, gasdermin D (GSDMD), at the Asp275 site to form the N-terminus fragment and C-terminus fragment (60). N-GSDMD perforates the cell membrane to form pores, leading to cell swelling and eventually cell death. Additionally, mature IL-1β and IL-18, which require the cleavage of activated caspase-1 on pro-IL-1β and pro-IL-18, are released through the pores formed by N-GSDMD (68).

The caspases can induce the pyroptosis alone when the upstream sensory complexes are absent, such pyroptosis pathway is named as noncanonical pyroptosis. It's reported that human caspase-4/5 (mouse caspase-11) can directly bind to intracellular lipopolysaccharide (LPS) through the N-terminal CARD (69). Activated caspase-4/5/11 can directly cleave GSDMD, leading to the oligomerization and translocation of N-GSDMD on cell plasma membrane to form pores (70). However, caspase-4/5/11 cannot cleave pro-IL-18 and pro-IL-1β, but can mediate maturation of IL-18 and IL-1β through NLRP3 pathway (71). Notably, one recent research has found that Pannexin-1 is also highly involved in the caspase-11-mediated noncanonical pyroptosis, which can be modified by activated caspase-11 to release cellular ATP and induce pyroptosis (72).

Apart from the canonical and noncanonical pyroptosis pathway, additional pyroptosis induction pathways have been discovered in recent years, including caspase-3/8-mediated pathway and granzyme-mediated pathway. Caspase-3 and caspase-8 were believed to be only apoptosis-

related caspases and were not able to cleave gasdermin proteins. However, it's been demonstrated that the effector protein YopJ expressed by *Yersinia* can suppress TAK1 and induce caspase-8-mediated cleavage of GSDMD to trigger pyroptosis in mouse bone marrow macrophages (BMMs) (73). Moreover, another report exhibits that chemotherapeutic drugs can induce caspase-3-related GSDME cleavage under high expression level of GSDME and elicit pyroptosis in tumor cells (74). Additionally, under the stimulation of TNFα, caspase-8 can specifically cleave GSDMC to produce N-GSDMC and form pores on plasma membrane when the expression level of GSDMC is promoted by hypoxia and PD-L1 (75). In 2020, a study had reported that CAR T cells rapidly activated caspase-3 in target cells by releasing granzyme-B (GzmB), which in turn triggered the caspase-3/GSDME-mediated pyroptosis (76). Additional studies are performed to further improves and expands the understanding of pyroptosis.

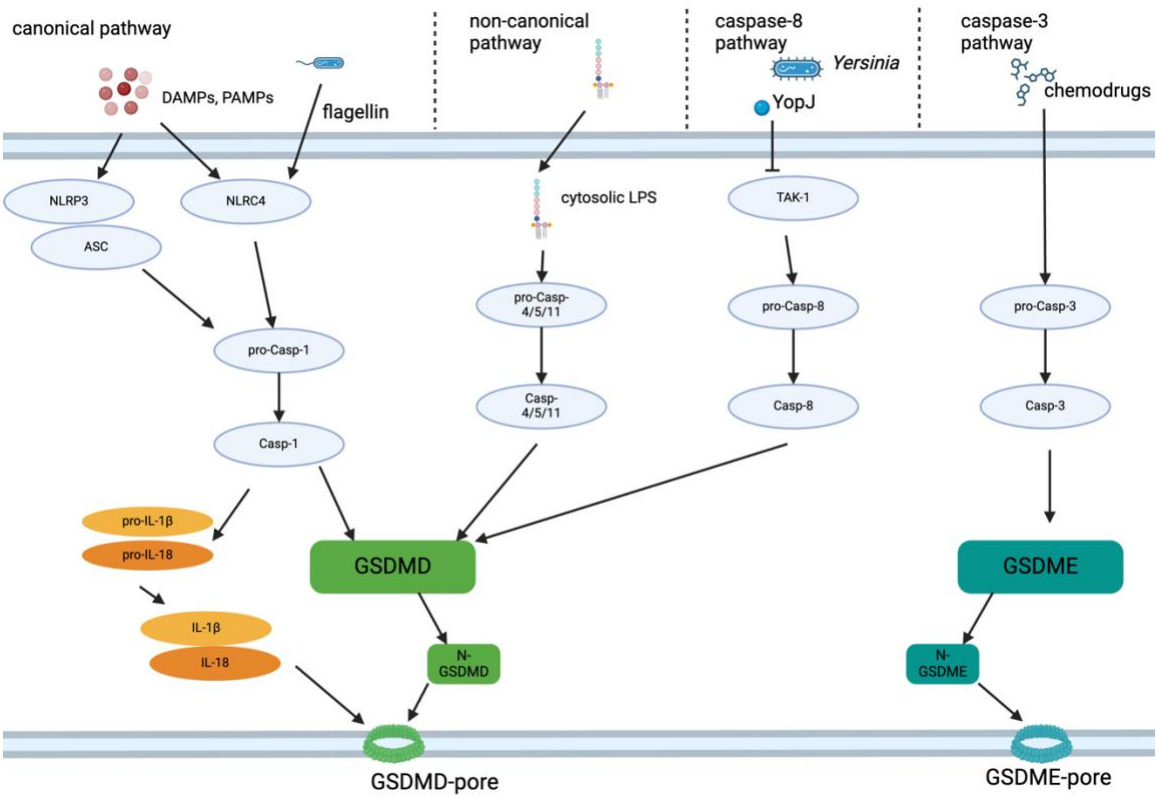


Figure 1-3 Diagram of four pyroptosis pathways (created at Biorender).

1.1.4 Crosstalk of PCD

Though the mechanisms are considered distinct, it is wide accepted that extensive crosstalk exists among the three types of PCDs. As mentioned above, both apoptosis and pyroptosis pathways require the involvement of caspases. Caspase-7, the executioner of apoptosis which was thought to be activated by caspase-8 or caspase-9 through two apoptosis pathways, is confirmed to be cleaved by caspase-1 during *Salmonella Typhimurium* infection (77). Additionally, caspase-1-mediated pyroptosis can also trigger apoptosis through mitochondrial damage. This has been demonstrated that N-GSDMD could transfer to mitochondrial outer membrane and form MOMP, resulting in leakage of cytochrome c and apoptosome formation (78). Caspase-1 can still activate the intrinsic apoptosis even under the absence of GSDMD, as it can directly cleave BCL-2 family protein BID to promote MOMP formation and later release of mitochondrial contents (79). Intriguingly, components of pyroptosis can induce apoptosis in a caspase-1-independent manner. By activating AIM2 through dsDNA electroporation, ASC recruited by AIM2 can interact with caspase-8 to induce the extrinsic apoptosis (80).

The aforementioned caspase-3/8-mediated pyroptosis pathway is an example shows how apoptotic components regulate pyroptosis pathway. Caspase-8, the initiator of extrinsic apoptosis, is reported to cleave GSDMD in response to TAK-1 inhibition induced by *Yersinia* infection. Additionally, caspase-8, along with FADD, can also be recruited to NLRP3 or NLRC4 inflammasomes under bacterial infection (81, 82). The apoptosis executioner, caspase-3, is

confirmed to cleave GSDME in response to multiple apoptotic triggers including chemotherapeutic drugs, TNFα and iron-activated ROS (74, 83).

Except for regulating both apoptotic and pyroptotic components, caspase-8 is also involved in regulation on necroptosis. As mentioned above, after activation by TNFα, TNFR1 binds to RIPK1 and TRADD to form complex I. Caspase-8 functions as a "switch" to decide the direction of complex I transition to either complex IIa or complex IIb. With the presence of caspase-8, complex I recruits caspase-8 and FADD to form complex IIa-RIA or complex IIa-RDA and elicit apoptosis. While under the absence of capase-8, RIPK1 recruits RIPK3 to form complex IIb and trigger necroptosis.

The crosstalk also exists between necroptotic and pyroptotic activation. It is widely accepted that efflux of potassium leads to NLRP3 inflammasome activation (84). MLKL-mediated necroptosis form pores on plasma membrane and eventually rupture causes efflux of potassium which in turn activates NLRP3 and initiates inflammasome (84, 85). Necroptosis can be triggered as a consequence of pyroptosis. For example, N-GSDMD directly mediates depolarization of mitochondrial membrane following AIM2 activation, releases mitochondrial ROS and triggers RIPK1-RIPK3-MLKL dependent necroptotic cell death under the function mutation of *Lrrk2*^{G2019S} (leucine-rich repeat kinase 2) in macrophages (86).

Crosstalk among the PCD pathways has important impacts on host innate immune responses. For instance, a couple of viruses encode caspase-8 inhibitors during infection, like CrmA from the cowpox virus or B13R from the vaccinia virus, facilitating the evasion of exposure by host apoptosis (84, 87). However, this apoptosis inhibition switches the transition of complex I to complex IIb and induces RIPK1-RIPK3 dependent necroptosis, resulting in viral exposure and

clearance. Another research also points out that necroptosis induced under pan-caspase inhibition could be used as an immunotherapy against bacterial infection including *Staphylococcus aureus* (88). Understanding the regulatory connections of PCD will provide a more comprehensive picture of disease processes and insights on development of new therapeutic strategies. Given the physiologically relevant observations highlighting the extensive crosstalk between PCD pathways, a conceptualization of an integrated cell death modality called "PANoptosis" was formed.

Table 1-2 comparison of features in three types of programmed cell death

	apoptosis	pyroptosis	necroptosis
Cell membrane	Plasma membrane blebbing; Rounding-up of the cell	Pore formation and rupture of plasma membrane	Pore formation and rupture of plasma membrane
Cytoplasm	Retraction of pseudopods; Cell volume reduction	Cytoplasmic swelling	Swelling of the cytoplasm and cytoplasmic organelles
Nucleus	Nuclear fragmentation; Chromatin condensation	Nuclear fragmentation; Chromatin condensation	Moderate chromatin condensation
Cellular morphology	Shrink	Increase in size and deformity	Increase in size and deformity

Biochemical features	Activation of caspases	Caspase-1/4/5/11 activation; Caspase-7 activation; Secretion of IL-1β and IL-18	Death receptor signaling; Caspase inhibition; Activation of RIP1, RIP3, and MLKL
Immune features	anti-inflammatory and immunological-silent; in certain situations, provoking an immune response as a result of DAMP exposing and releasing (ex, DNA, histone and HMGB1)	pro-inflammatory due to release of IL-1β and IL-18	Pro-inflammatory in most situations because DAMPs are released (ex, HMGB1); anti-inflammatory in other instances

1.1.5 NINJ1-mediated plasma membrane rupture (PMR) as a pivotal component of PCD

PMR is the hallmark and last step of PCD, leading to release of intracellular contents. Previously, the PMR was thought to be a passive process as it was a consequence of by osmotic pressure change followed by efflux of ions and water through GSDMD pores located on plasma membrane. However, recent research has challenged such perspective. Kayagaki et al. discovered the PMR followed by lytic cell death was actually mediated by a membrane protein, NINJ1 (89). In the research, NINJ1 deficiency macrophages exhibited inhibition on the release of larger cellular

molecules, including lactate dehydrogenase (LDH) and HMGB-1, in response to inflammasome activation (89). The oligomerization of NINJ1 was required to induce PMR after N-GSDMS formed pores on plasma membrane. These findings collectively suggest that NINJ1 functions as the downstream of GSDMD to mediate PMR. Moreover, another study further identified that NINJ1 was essential for the early loss of plasma membrane integrity during lytic cell death, while loss of NINJ1 maintained membrane integrity (90).

NINJ1 is a plasma membrane protein containing two transmembrane regions with both extracellular N-terminus and C-terminus. According to the research by Kayagaki et al., NINJ1 undergoes a conformational change during activation, resulting in oligomerization and large pores formation on plasma membrane. These oligomers appear as rings or branched filaments on membrane, which are observed by fluorescence microscopy and cryo-electron microscopy (91). During oligomerization, the hydrophilic domians of NINJ1 oligomers are inserted into the hydrophobic core of plasma membrane, leading to loss of membrane integrity and pores appearance (91). Though it is still unclear what factors induce NINJ1 activation, a potential assumption has been proposed by researchers that alteration of membrane structure caused by osmotic pressure changes activates the amphipathic helices of NINJ1 (92). Additionally, further evidence demonstrated that NINJ1-mediated PMR promoted the release of pro-inflammatory microvesicles, highlighting NINJ1 as a potential target for reducing inflammatory damage (93).

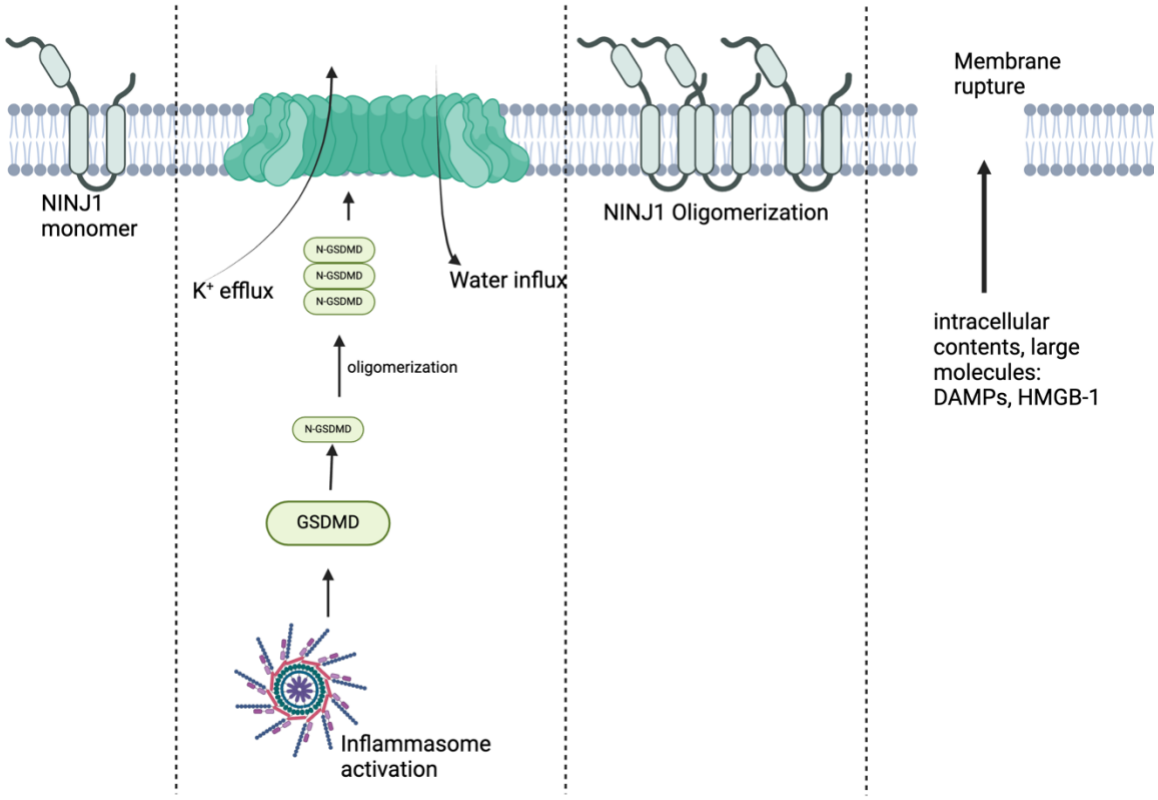


Figure 1-4 NINJ1-mediated PMR during lytic cell death (created at Biorender).

1.2 Role of PCD in different immune responses

1.2.1 PCD in anti-microbial responses

The removal of infected immune cells by PCD was thought to be benefit for the infectious pathogens, as this may compromise the ability of host anti-pathogen responses. However, PCD is becoming more and more accepted as a beneficial strategy developed by host immune systems to eliminate the intracellular niche of certain pathogens. Besides, the cellular debris left by PCD promotes the resolution of infection through mounting an appropriate innate immune response. Here we discuss how the host utilizes PCD to defend against bacterial and viral infections, the

crosstalk between PCDs in response to pathogen infections and how the physical properties of apoptotic bodies, pore-induced intracellular traps (PITs) and neutrophil extracellular traps (NETs) benefit host immune responses (94).

Emerging evidence has demonstrated that pyroptosis is highly involved in clearing bacterial or viral infections. For instance, *in vitro* studies exhibited that deficiency of caspase-1 compromised the killing ability of macrophages infected with a variety of microbial pathogens, including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Franciscella tularensis* and influenza virus (95). Additionally, compared with wildtype strain, engineered *Salmonella enterica* with persistent expression of flagellin were easily detected and cleared by NLRC4-mediated inflammasome during *in vivo* infection (96). And the clearance of flagellin-engineered bacteria still existed in mice with IL-1β or IL-18 deficiency. Another research also pointed out that caspase-11 could activate pyroptosis in response to macrophages infected with *Salmonella enterica* strains which had lost the ability to maintain the integrity of the *Salmonella*-containing vacuole (97). Apart from defense against bacterial infection, pyroptosis is also triggered following the detection of viral infection. For example, activation of AIM2 was induced by detection on cytosolic double-stranded viral DNA released by murine cytomegalovirus (MCMV), leading to removal of infected immune cells through pyroptosis activation (98). Parallel *in vivo* studies also revealed that *Aim2*$^{-/-}$ mice were susceptible to MCMV infection (98). Together, these pieces of evidence have strongly emphasized the function of pyroptosis in host anti-microbial defense.

Similar to pyroptosis, the role of necroptosis is well defined in both viral and bacterial defense. It is reported that *Ripk3*$^{-/-}$ mice have increased mortality during vaccinia virus infection or adapted influenza infection (99, 100). Moreover, the antiviral role of necroptosis is further

supported by another study that mouse RIPK3 can detect and bind to ICP6 released by human-adapted herpes simplex virus 1 (HSV-1) and trigger necroptosis to prevent viral replication (101). As a key regulator of necroptosis and inflammation, RIPK3 is identified to be significantly elevated in patients with *Streptococcus pneumoniae* (102). Further studies reveal that RIPK3 combines with the mitochondrial calcium uniporter (MCU) to form complexes during *Streptococcus pneumoniae* infection, inducing mitochondrial calcium uptake and mROS production, which in turn initiates host necroptosis to protect against *Streptococcus pneumoniae* invasion (103). Besides, necroptosis functions as an important part of host responses to *Mycobacterium tuberculosis* (*Mtb*) infection. It is reported that the signal regulatory protein alpha (SIRPα), which is mainly expressed in macrophages and dendritic cells, promotes macrophage necroptosis upon detection on *Mtb* invasion (103, 104). Additional studies have observed that nonimmune cells can also trigger necroptosis in response to bacterial infections. *Staphylococcal* superantigen-like protein-10 (SSL-10), which is secreted by *Staphylococcus aureus*, can be recognized by TNFR1 on the cell membrane, leading to necroptosis activation in HEK293T cells (105). Furthermore, *Staphylococcus aureus* disrupts plasma membrane integrity and causes calcium ions influx, resulting in increased necroptosis within goat endometrial epithelial cells (106). Though elevated necroptosis may exacerbate inflammatory status during anti-microbial responses, the elimination of bacteria/viruses leads to an overall reduction in inflammation and contributes to host recovery.

As the first identified PCD, the role of apoptosis in host anti-pathogen defense is well characterized. A clear example of this is apoptosis in *Mtb* infection. Although virulent *Mtb* strains predominantly induce necrosis, apoptosis is also detected among macrophages infected with

virulent *Mtb* to control bacterial replication (107). Apoptosis in macrophages can be triggered through both extrinsic and intrinsic pathways is response to *Mtb* infection. It has been identified that following infection with *Mtb,* TNF production induces the phosphorylation of $FLIP_s$ to activate caspase-8, leading to caspase-3 and 7 activation and apoptosis (108). Upon entering cytosol of macrophages, virulent *Mtb* induces MOMP, resulting in mitochondrial swelling and release of cytochrome c, which in turn activates caspase-9 and apoptosis (107). Apart from bacterial defense, apoptosis is also critical in host response to viral infection. Emerging evidence show that *bax*$^{-/-}$ mice have increased susceptibility to multiple viral infections, including influenza virus A, Sendai virus, vesicular stomatitis virus or encephalomyocarditis virus (109, 110). Therefore, the apoptotic response is highly involved in host anti-microbial responses.

1.2.2 PCD in sepsis-induced immunosuppression

Sepsis is a severe, life-threatening organ dysfunction caused by a dysregulated host response to infection. It is a global public health problem because of its high fatality, disability rates, and high disease burden (59). Clinically, two dynamic stages occur during the initiation and progression of sepsis, which are a system inflammatory response syndrome (SIRS) in the acute phase and a compensatory anti-inflammatory response syndrome (CARS) in the later phase (111). The SIRS, which is characterized as excessive inflammatory responses and cytokine storms, has long been considered major causes of high mortality in patients suffered with sepsis (112). However, the latter phase, marked by immune exhaustion and an inability to combat both the initial infection and subsequent opportunistic infections, represents a major challenge in the clinical management of sepsis. And data from clinical studies also supports that imbalance of innate

immune response together with persistent immunosuppression drive multiple organ damage and septic death (112). Therefore, understanding the pathological initiation and progression of sepsis-induced immunosuppression is vital for disease prevention and treatment.

PCD has been increasingly recognized as a central factor contributing to immune dysfunction in sepsis. Although PCD pathways, including apoptosis, necroptosis, and pyroptosis, are essential for immune system balance, the dysregulation of PCD pathways during sepsis results in deteriorated immune consequences and impeded immune balance restoration. Under normal circumstances, PCD helps to control infection and inflammation. However, in sepsis, the dysregulation PCD contributes to a pathological state where immune cells are lost prematurely, leading to immunosuppression and the inability to effectively clear pathogens.

As an important part of PCD, apoptosis has a complex role in sepsis progression. It is reported that 50% of resting neutrophils exhibited apoptotic morphological changes after 24 hours of *in vitro* culture, while the corresponding percentage for septic neutrophils was only 5 - 10% (113). Additionally, one clinical study reveals that neutrophil apoptosis is negatively associated with the severity of sepsis in patients (114). Besides, by using an acute lung injury model, researchers find that lung neutrophil apoptosis is remarkably reduced within 24 hours after injury following ligation and puncture (CLP) (115). Together, these data suggest that apoptosis is inhibited in innate immune cells during early systemic inflammation phase of sepsis. As mentioned above on apoptosis mechanism, the activation of apoptosome leads to formation of apoptotic bodies, which retains the intracellular contents within plasma membrane and avoids the induction of inflammation on surrounding environment. Due to the crosstalk of PCD, inhibition of apoptosis leads to the switch of cell death to pyroptosis or necroptosis two lytic cell death, which in turn

releases the cellular contents and exacerbates the inflammation status. However, apoptosis also functions as the key player in depletion of immune effector cells. Emerging evidence shows that the expression of pro-apoptotic factors, including cytochrome C, Bim, caspase-3, caspase-8, and caspase-9 are significantly increased in T lymphocyte from CLP-treated mice, resulting in promoted T-cell apoptosis (116). In addition, IL-33 restricts T-cell apoptosis and improves the survival rate of the sepsis-induced mice by reducing the expression of Fas and upregulating the expression of Bcl-2 (117). Clinical studies also point out that Fas expression is prominently elevated in peripheral blood mononuclear cells of patients suffered with sepsis (118). Collectively, these pieces of evidence indicate the role of apoptosis during sepsis is complex and further studies are required to obtain a comprehensive view of the interaction between apoptosis and sepsis.

Necroptosis has been widely accepted as a critical process of inflammation-associated cell death. Many sepsis-related studies have revealed that inhibition of necroptosis reduces the severity of sepsis-induced lung injury, kidney injury and hepatic injury (119-121). Additionally, studies have confirmed that deletion of RIPK3 protects against CLP-treated mice, highlighting the importance of RIPK kinase inhibition during sepsis (122, 123). Blocking of RIPK1 or RIPK3 reduces systemic inflammation and organ damage in septic neonatal mice (119, 124). Moreover, one clinical study exhibits that the expression of RIPK3 and MLKL is positively correlated with plasma HMGB-1 level in patients suffered with sepsis (125). However, whether the necroptosis is activated in neutrophils due to the inhibition on apoptosis during sepsis is still unclear. This is because signaling of necroptosis, apoptosis, and pyroptosis can overlap and the precise mechanisms that determine whether neutrophil dies through apoptosis or necroptosis or pyroptosis

remain yet unknown (126). Thus, targeting the necroptosis pathway can be an efficient strategy to ameliorate the hyperinflammation and improve survival rate in patients with sepsis.

As another form of lytic cell death, pyroptosis has been recognized in contributing the initiation and progression of sepsis. A recent study shows that increased expression of caspase-1 is correlated with elevated pro-inflammatory cytokine level, and that that caspase-1/11 ablation improves survival rate in septic mice and neutrophil phagocytosis (127). Another *in vivo* study exhibits that gene deletion or antagonism of GSDMD with disulfiram reduces organ damage, leading to enhanced survival rate in septic mice (128). Additionally, blocking both necroptosis and pyroptosis by RIPK3 and GSDMD ablation, $Ripk3^{-/-}\&Gsdmd^{-/-}$ mice show increased resistance to tissue injury and bacterial infection post CLP surgery (129). Compared with that in normal condition, the expression of caspase-1 and IL-18 in multiple immune cells is increased in septic patients (130). In addition to caspase-1, increased expression and activity of caspase-11 in macrophages are observed in LPS-treated septic mice (131). Together, these data suggest the role of pyroptosis in the initiation and acceleration of sepsis and provide therapeutic direction for the development of drugs for sepsis.

One of the most severe consequences of sepsis is its shift from an inflammatory to an immunosuppressive state. This immunosuppressive phase is marked by a profound depletion of immune effector cells through all three types of PCD and compromised lymphocyte proliferation. Dendritic cells, monocytes, and other antigen-presenting cells undergo extensive apoptosis during sepsis, leading to diminished antigen presentation and T-cell activation. As a result, the capacity of host immune system to react to infections becomes severely impaired, increasing susceptibility to secondary infections, which finally results in high mortality in patients suffered with sepsis.

PCD is a crucial but double-edged sword in sepsis: while essential for controlling infection, its dysregulation leads to immune effector cell depletion and contributes to a state of long-term immunosuppression. This immunosuppressive state increases the risk of secondary infections and complicates clinical outcomes, underscoring the need for therapeutic approaches that can modulate PCD pathways. In sum, the connection between PCD and immunosuppression in sepsis highlights a high potential strategy for therapeutic intervention. Through targeting pathways involved in PCD, researchers aim to preserve immune effector cell populations and maintain immune function in septic patients.

1.2.3 PCD in tumor-mediated immunosuppression

Cancer is a complex disease characterized not only by unchecked cellular proliferation but also by sophisticated mechanisms of immune evasion. Tumors can evade immune surveillance through various strategies, including the induction of immune cell death, the suppression of immune checkpoints, and the alteration of cytokine profiles within the tumor microenvironment (TME) (132). Among these strategies, tumor-mediated immunosuppression via programmed cell death (PCD) plays a critical role in allowing cancer cells to escape immune detection. The three main forms of PCD—apoptosis, necroptosis, and pyroptosis—not only serve as intrinsic tumor-suppressive mechanisms but, when dysregulated by tumors, contribute to a local immunosuppressive environment that fosters cancer progression (133).

Apoptosis is a non-inflammatory, caspase-dependent pathway that is commonly exploited by tumors to reduce immune cell infiltration in the TME. Apoptosis in immune cells, especially T cells, dendritic cells, and natural killer (NK) cells, can be triggered by tumor-secreted factors or

by immune checkpoint pathways such as the programmed death-ligand 1 (PD-L1) interaction with the PD-1 receptor on T cells (134). Tumor cells overexpressing PD-L1 can induce T cell apoptosis, effectively suppressing the anti-tumor immune response and enabling cancer cells to persist and grow unchecked (135). Additionally, tumors can exploit the Fas/FasL pathway to induce apoptosis in tumor-infiltrating lymphocytes, further weakening immune surveillance and contributing to tumor progression (136).

Necroptosis is another form of programmed cell death that is caspase-independent and mediated by receptor-interacting protein kinases RIPK1 and RIPK3. Although necroptosis is typically associated with inflammation, its role in cancer immunosuppression is complex. Necroptosis can promote the release of damage-associated molecular patterns (DAMPs), which should theoretically stimulate an immune response. However, in the TME, necroptosis often leads to the suppression of immune responses by inducing a pro-tumor inflammatory environment that supports cancer progression rather than immune clearance (137). Furthermore, tumor cells can induce necroptosis in immune cells, particularly in dendritic cells and macrophages, dampening their ability to present antigens and activate T cells (138). This selective induction of necroptosis in immune cells allows tumors to maintain an immunosuppressive TME while evading immune detection.

Pyroptosis is a highly inflammatory form of cell death, mediated by caspase-1 activation and the formation of gasdermin pores that allow the release of inflammatory cytokines such as IL-1β and IL-18 (139). While pyroptosis in cancer cells can activate anti-tumor immune responses, in the context of the TME, it often plays an immunosuppressive role. Tumor cells can manipulate the pyroptotic pathway in macrophages and other immune cells to promote a chronic inflammatory

state that paradoxically favors tumor growth and immune evasion. For instance, macrophages undergoing pyroptosis within the TME release pro-inflammatory cytokines that attract immunosuppressive myeloid cells, further enhancing tumor-mediated immunosuppression (140).

The manipulation of these PCD pathways by tumors to foster immunosuppression highlights a significant challenge but also an opportunity for therapeutic intervention. Strategies that inhibit apoptotic pathways in immune cells or modulate necroptosis and pyroptosis within the TME could enhance anti-tumor immunity. Emerging therapies targeting PCD pathways, such as inhibitors of the PD-1/PD-L1 axis and RIPK1/RIPK3 inhibitors, offer promising avenues to disrupt tumor-driven immunosuppression and reinvigorate immune responses against cancer (141,142).

In conclusion, apoptosis, necroptosis, and pyroptosis are key pathways through which tumors exert immunosuppressive effects in the TME, thereby evading immune surveillance and supporting tumor progression. Understanding the intricate roles of these PCD mechanisms in tumor immunology is essential for the development of innovative therapies aimed at overcoming tumor-mediated immunosuppression and restoring effective anti-tumor immunity.

In summary, to address the multiple roles of PCD in host innate immune response, my graduate research work has addressed the following issues:

Role of PCD in antibacterial responses

Role of PCD in sepsis-induced immunosuppression

Role of PCD in tumor-mediated immunosuppression

Chapter 2. *Pseudomonas aeruginosa* mediates host necroptosis through *rhl-pqs* quorum sensing interaction

2.1 ABSTRACT

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that can cause serious infections in immunocompromised patients. Quorum sensing (QS), a communication system evolved by *P. aeruginosa* to survey its density, is well-acknowledged to be involved in various activities during bacterial infection. Recent studies have revealed the link between *P. aeruginosa* QS and host innate immune response. Previous evidence suggests programmed cell death (PCD) exists in response to *P. aeruginosa* infection. However, it remains unclear whether QS plays a role in host PCD process during the infection. In this study, we found that the deficiency of one of QS subsystems, *rhl*, markedly increased mouse bone marrow macrophage cell death induced by *P. aeruginosa*, which was accompanied by elevated phosphorylation of RIPK3 and MLKL. This highly increased necroptosis activation was caused by the upregulation of another QS subsystem, *pqs*, because the deletion of *pqs* in *rhl*-deficient *P. aeruginosa* abolished macrophage necroptosis *in vitro* and *in vivo*. In sum, our data highlight the crosstalk between *P. aeruginosa* QS and host necroptosis, which is executed through the *rhl–pqs* axis.

2.2 INTRODUCTIOPN

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative and ubiquitous bacterium (143). It is a leading cause of severe acute and chronic infections such as pneumonia, sepsis, urinary tract infections, respiratory tract infections in immunocompromised patients and the major cause of morbidity and mortality in patients with cystic fibrosis (CF) (144-147). As one of the most versatile and opportunistic bacteria, *P. aeruginosa* possesses a broad range of virulence factors which facilitate its infections and adaption to host immune defenses (148). Owing to its intrinsic resistance to a wide spectrum of antibiotics, as well as acquisition of adaptive mutations during chronic infections, *P. aeruginosa* becomes one of the most difficult bacteria to be eradicated in patients (145-147). Thus, novel treatments and antibiotics development are urgently needed to treat infections induced by *P. aeruginosa* (146).

P. aeruginosa has multiple mechanisms to facilitate its survive and rapid adaption to various conditions. Quorum sensing (QS) is one of such mechanisms that allows bacteria to dynamically coordinate their behavior in response to changes in cell density and surrounding environments (143, 149, 150). *P. aeruginosa* uses multiple types of autoinducers and related receptors in QS system to regulate this communication system (151, 152). QS is involved in various *P. aeruginosa* activities, including biofilm formation, virulence factors production and subsequent release (144). Recently, the relationship between QS and host immune response has become increasingly critical in antimicrobial research. Three hierarchical *P. aeruginosa* QS systems are extensively investigated, including *las*, *rhl* and *pqs* (151, 152). Recent study revealed that the mammalian aryl hydrocarbon receptor (AhR) can recognize the autoinducers released by

las and *pqs* system to adjust host immune response upon *P. aeruginosa* infection (143). Another study reported that *las*-regulated protease, LasB, impairs host inflammatory responses by targeting lung epithelial cystic fibrosis transmembrane regulator signaling (153). Therefore, accumulative evidence indicates a critical role of QS in regulating host immune response.

Programmed cell death (PCD) is a pivotal innate immune mechanism in protecting against acute bacterial infection, thus limiting their spread by removing infected cells (154, 155). Apoptosis, necroptosis and pyroptosis are three most common types of PCD, known as PANoptosis, which plays a critical role in host defense during bacterial infection (155). Through recognizing pathogen-associated molecular patterns (PAMPs) released by bacteria with multiple sensors, a protein complex formation, the inflammasome, will be initiated and functions as a hub to interact and activate PCD machinery (155, 156). It has been reported that the NLR family apoptosis inhibitory protein 5 (NAIP5) is activated by flagellin and induces NLR family CARD domain containing 4 (NLRC4) to initiate inflammasome activation, leading to pyroptosis (157-159). Additional studies reported that QS molecule N-(3-oxododecanoyl) homoserine lactone (C_{12}-HSL) released by *P. aeruginosa* triggers apoptosis in human macrophages (160, 161). Despite the well-known function of PCD in anti-*P. aeruginosa* response, whether QS affects host PCD during *P. aeruginosa* infection remains unclear.

In this study, we discovered that *rhl*-deficient *P. aeruginosa* caused an enhanced PCD in mouse macrophages during infection. Both pyroptosis and necroptosis were robustly elevated as evidenced by the cleavage of gasdermin D (GSDMD) and phosphorylation of receptor interacting protein kinase-3 (RIPK3) and mixed lineage kinase like protein (MLKL), respectively. In addition, we identified that necroptosis increased by *rhl*-deficient *P. aeruginosa* was caused by the upregulation of the downstream *P. aeruginosa pqs* system. *Rhl*-deficient *P. aeruginosa*-induced

necroptosis was abolished by depleting the *pqsA*, suggesting a novel *rhl* – *pqs* axis controlling macrophage necroptosis, which may represent a therapeutic target for anti-*P. aeruginosa* treatment strategies.

2.3 MATERIALS AND METHODS

Animals

C57BL/6J (000664) mice were obtained from the Jackson Laboratory. *Gsdmd*$^{-/-}$ (162), *Ripk3*$^{-/-}$ (163, 164), *Nlrp3*$^{-/-}$ (165), *Nlrc4*$^{-/-}$ (159), *Casp1/11*$^{-/-}$ (166), and *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ (167) mice have been previously described. All mice were housed in SPF facilities at The Ohio State University (OSU). 8-12 weeks old mice with sex-matched were used in animal experiments. All *in vivo* experiments were performed in according with the guidelines established by The OSU and National Institute of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (IACUC) (Protocol: 2018A00000022-R2).

Cell culture

Bone marrow-derived macrophages (BMMs) were generated from indicated genotypes of mice in the presence of L-929 conditional medium, as described in our previous studies (164, 168). After culture of 6 days, BMMs were then harvested and seeded at a concentration of 0.2×10^6 cells into 96-well plates or 1×10^6 cells into 12-well plates and incubated overnight for subsequent assays.

Bacterial strains and growth condition

All bacterial strains, plasmids and primers are listed in Table 1. Gene-deletion constructs were incorporated into the *P. aeruginosa* genome using homologous recombination as previously described (169). *P. aeruginosa* was grown in 5 ml Luria broth (LB) overnight at 37°C with 200 rpm shaking, diluted 1:5 (volume/volume), and grown for 2 h to reach the exponential phase. Bacteria

were washed and resuspended with Dulbecco's Phosphate-Buffered Saline (DPBS) for subsequent experiments.

Bacterial stimulation

Resuspended *P. aeruginosa* were diluted and applied to BMMs at a multiplicity of infection (MOI) of 10 for 2 h. BMMs were then washed with sterile DPBS twice to remove excess *P. aeruginosa*. The medium was replaced with 200 µg/ml gentamicin-containing medium. After additional indicated periods, cells and supernatant were collected for further experiments. For autoinducers co-stimulation assays, BMMs were pre-treated with N-butyryl-L-Homoserine lactone (C_4-HSL) (Sigma-Aldrich, SML3427, 100 µM), 2-heptyl-3-hydroxy-4(1H)-quinolinone (PQS) (Cayman, 29186, 100 µM), 2-Heptyl-4-quinolone (HHQ) (Sigma-Aldrich, SML0747, 100 µM) 2 h before *P. aeruginosa* stimulation.

Phagocytosis assay

BMMs seeded in 96-well plates were infected with different strains of *P. aeruginosa* at a MOI of 10 for 2 h. The cells were then washed twice with DPBS and lysed in 200 µl 0.1% Triton X-100 in DPBS. Tenfold serial dilutions of the lysates were mixed with DPBS and 10 µl was applied on an antibiotic-free LB agar plate. Colonies were counted after 8h incubation at 37°C. Each experiment was conducted in triplicate wells and repeated three times.

LDH release assay

Supernatants were collected from 96-well plates of bacterial stimulation experiments and LDH activity was determined with the Cytotoxicity Detection Kit (LDH) (11644793001, Roche). Cells

left untreated or treated with 1% Triton X-100 were used as negative and positive controls, respectively.

Immunoblotting

For immunoblotting, cells were collected and lysed with RIPA buffer containing protease inhibitor cocktail. Electrophoresis of proteins was performed by using the NuPAGE system (Invitrogen) according to the manufacturer's protocol. Proteins were transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies were applied to membranes after 1 h of blocking with 5% skim milk for overnight incubation at 4°C. Appropriate HRP-conjugated secondary Abs were used and proteins were detected using the Enhanced Chemiluminescent (ECL) reagent (Thermo Scientific). The images were acquired with the ChemiDoc MP System (Bio-Rad). Primary Abs for immunoblotting included: GSDMD (10026, 1:1000) from Genentech, caspase-1 (AG-20B-0042, 1:1000) from Adipogen, caspase-8 (8592, 1:1000), Caspase-7 (9492, 1:1000), p-RIPK3 (57220, 1:1000), p-MLKL (62233, 1:1000) from Cell Signaling Technology, total MLKL (AP14272b, 1:1000) from Abgent, total RIPK3 (NBP1-77299, 1:1000) from Novus, and HRP-conjugated anti–β-actin (sc-47778, 1:3000) from Santa Cruz Biotechnology. Secondary Abs for immunoblotting included: anti-rabbit HRP-linked IgG (7074, 1:2000) and anti-mouse HRP-linked IgG (7076, 1:2000) from Cell Signaling Technology, anti-rat HRP-linked IgG (sc-2006, 1:3000) from Santa Cruz Biotechnology.

Statistical analysis

All experiments were performed at least three independent replications. GraphPad Prism v9.0 software was used for data analysis. Data were analyzed as mean ± SD. The means of two groups

were compared with Student's unpaired t-test. Comparisons between multiple groups were analyzed by repeated-measures ANOVA with Bonferroni post tests. Survival analyses were performed by Kaplan-Meier method and log-rank (Mantel-Cox) test for significance. *P* values of less than 0.05 were considered statistically significant.

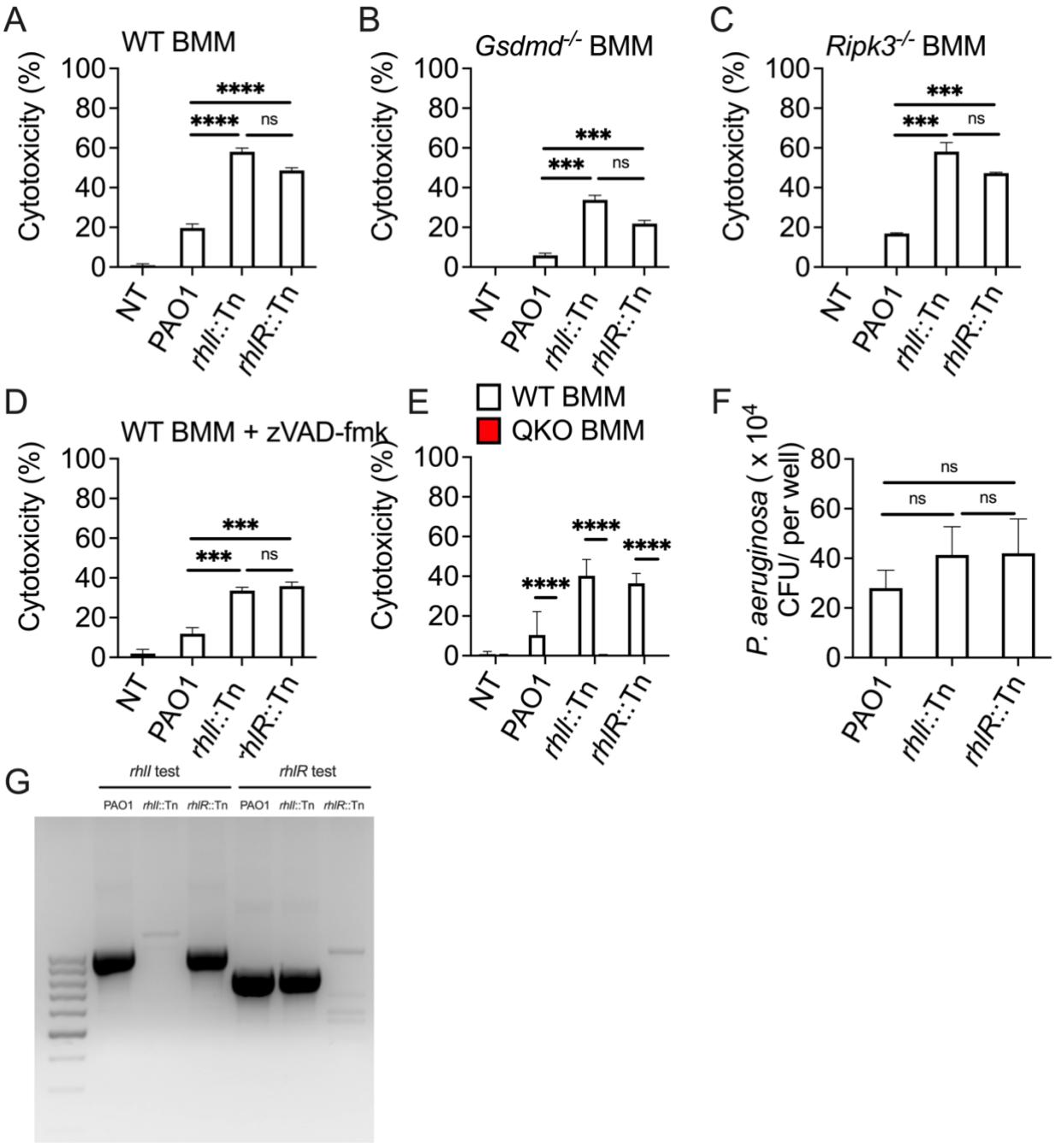
2.4 RESULTS

Enhanced cell death induced by rhl-deficient P. aeruginosa

To better define the role of *P. aeruginosa rhl* in macrophage cell death, we employed two *P. aeruginosa rhl* transposon mutation strains (*rhl* mutant), *rhlI*::Tn and *rhlR*::Tn, derived from the comprehensive mutant library (170). These strains, which were confirmed by genotyping (Fig. 2-1G), along with the isogenic wild type (WT) strain PAO1, were used to infect mouse BMMs. We observed a significant increase of cell death induced by *rhl* mutants compared to PAO1, while no difference existed between the two transposon mutants (Fig. 2-1A). To further investigate if cell death promoted by *rhl* mutants was specific to pyroptosis, we conducted the stimulation experiments with BMMs generated from *Gsdmd*$^{-/-}$ mice, which are deficient in the execution of pyroptosis (162, 171, 172). Deletion of GSDMD in macrophages failed to rescue the increased cell death induced by *rhl* mutants (Fig. 2-1B). Additional gene-deletion cells including *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, and *Casp1/11*$^{-/-}$ BMMs could not reverse the enhanced cell death induced by *rhl* mutants (Fig. 2-7). These results suggest that increased cell death stimulate by *rhl* mutants was not specific to pyroptosis. We next examined *Ripk3*$^{-/-}$ BMMs and obtained similar results indicating that this cell death promoted by *rhl* mutants was not specific to necroptosis (Fig. 2-1C). Furthermore, we pretreated BMMs with zVAD-fmk, a pan-caspase inhibitor, before *P. aeruginosa* infection, and cell death still existed (Fig. 2-1D). To test that the cell death stimulated by *rhl* mutants was PANoptosis (155), we repeated those experiments with *Casp1/11*$^{-/-}$*Casp8*$^{-/-}$*Ripk3*$^{-/-}$ BMMs and observed a completely suppressed cell death induced by both WT and *rhl* mutant *P. aeruginosa*, indicating that cell death induced by *rhl*-deficient *P. aeruginosa* was PANoptosis (Fig. 2-1E)

(155). We also examined macrophage phagocytosis of bacterial and found no difference between PAO1 and *rhl* mutants engulfed by macrophages after 2 h of incubation (Fig. 2-1F). These results suggest that increased PANoptosis induced by *rhl*-deficient *P. aeruginosa* was not caused by altered bacterial phagocytosis. In sum, we found that *rhl* deficiency in *P. aeruginosa* induce an elevated PANoptosis, which could not be blocked in macrophages with the deficiency in any single PCD pathway. *Gsdmd*$^{-/-}$ macrophages blocked pyroptosis pathway elicited by *P. aeruginosa*, while necroptosis and apoptosis remain activation during infection. As a pan-caspase inhibitor, zVAD-fmk greatly impeded the caspase proteins cleavage, which in turn suppressed both apoptosis and pyroptosis pathway during infection, resulting in the decreased cell death.

We next sought to examine individual cell death pathways upon *P. aeruginosa* infection with immunoblotting. Increased cleavage of caspase-1 (P20) and cleavage of GSDMD (P30) were detected in BMMs with *rhl* mutants compared to those with PAO1 bacteria (Fig. 2-2A). We also detected the cleaved caspase-8 (P18) and cleaved caspase-7 (P20) in both PAO1 and *rhl* mutant group. Stimulation with *Rhl* mutants elevated the cleavage of these apoptosis-related caspases, showing that apoptosis was also increased with a low extent (Fig. 2-2A). In addition, we found markedly enhanced phosphorylation of RIPK3 and MLKL under the stimulation of *rhl*-deficient *P. aeruginosa* compared to PAO1 (Fig. 2-2B). Together, these results demonstrate that *rhl*-deficient *P. aeruginosa* promotes host PCD, especially pyroptosis and necroptosis.


A
WT BMM
Cytotoxicity (%)
100
80
60
40
20
0
****
****
ns
NT
PAO1
rhlI::Tn
rhlR::Tn
B
Gsdmd-/- BMM
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C
Ripk3-/- BMM
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***
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D
WT BMM + zVAD-fmk
***
***
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E
WT BMM
QKO BMM
****
****
****
F
P. aeruginosa (x 10^4 CFU/ per well)
ns
ns
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G
rhlI test
rhlR test
PAO1
rhlI::Tn
rhlR::Tn
PAO1
rhlI::Tn
rhlR::Tn

Figure 2-1 *rhl*-deficient *P. aeruginosa* promotes macrophage programmed cell death.

(A-D) Cell death assessed by LDH release in WT BMMs (A), *Gsdmd*$^{-/-}$ BMMs (B), *Ripk3*$^{-/-}$ BMMs (C) and WT BMMs with 2 h pre-treatment of zVAD-fmk (2μM) (D) infected with PAO1, *rhlI*::Tn and *rhlR*::Tn (MOI=10) for 4 h. (E) Quantification of cell death by LDH release in WT and *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMMs challenged with *P. aeruginosa* (MOI=10) for 4 h. (F) Phagocytosis of PAO1, *rhlI*::Tn and *rhlR*::Tn assessed by colonies counting on LB agar plates with a dilution of 2×10^4 after 2 h incubation with wildtype BMMs (MOI=10). (G) Genotyping test on *rhlI*::Tn and *rhlR*::Tn transposon mutation. Data are representative of at least three independent experiments. Data in (A) – (F) are shown as mean ± SD. Each symbol represents a technical replicate. ***$p < 0.001$, ****$p < 0.0001$, by Student's unpaired t-test and one-way ANOVA.

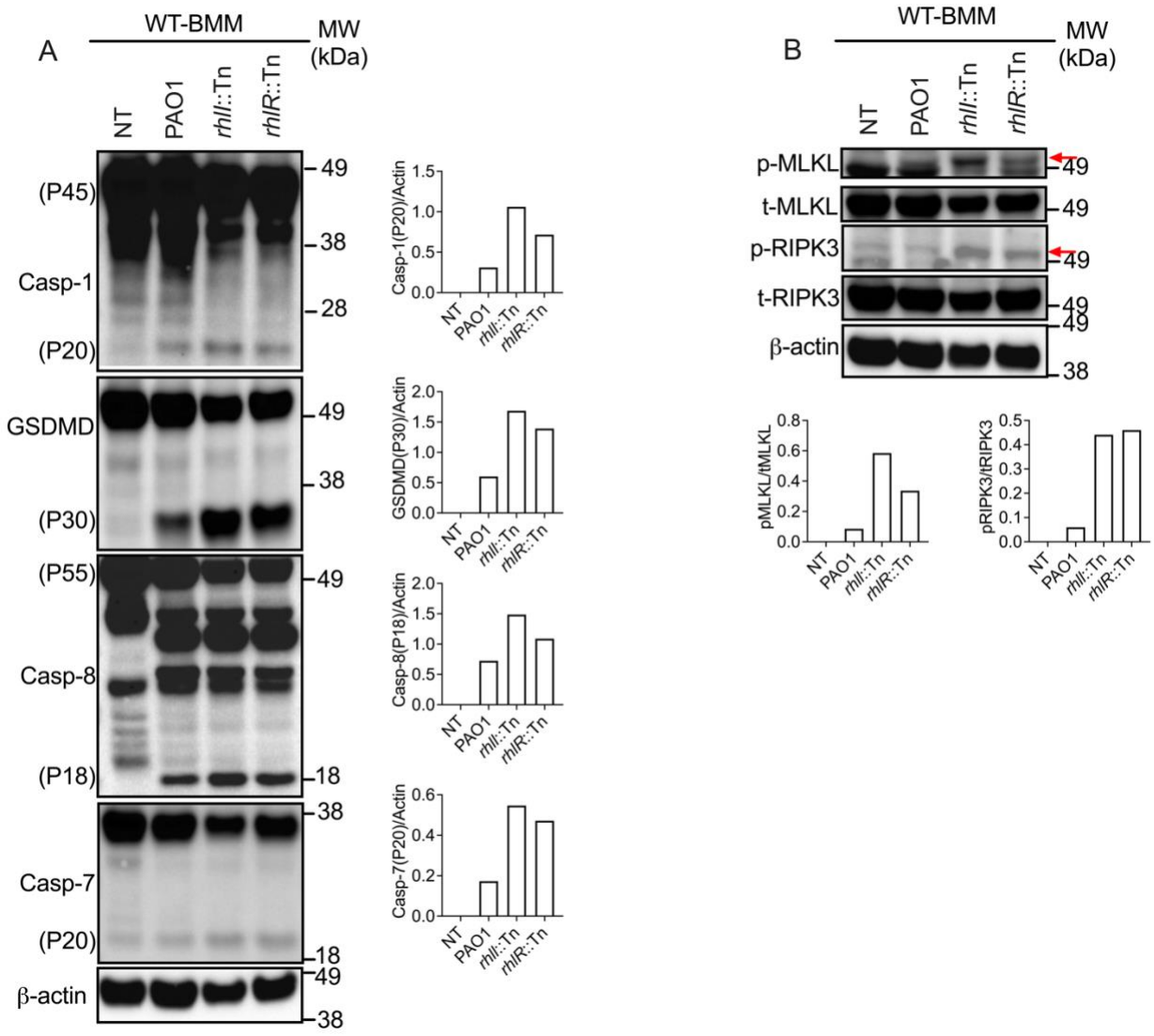

Figure 2-2 *rhl*-deficient *P. aeruginosa* increases pyroptosis and necroptosis.

(A) Immunoblotting and densitometric analysis of pro- (P53) and activated (P30) gasdermin D (GSDMD), pro- (P45) and activated (P20) caspase-1 (Casp-1), pro- (P55) and cleaved (P18) caspase-8 (Casp-8), and pro- (P35) and cleaved (P20) caspase-7 (Casp-7). The relative levels of the indicated proteins were normalized to actin and adjusted with value from non-treated group. (B) Immunoblotting and densitometric analysis of pMLKL, total MLKL (tMLKL), pRIPK3 and total RIPK3 (tRIPK3). The relative levels of phosphorylated proteins were normalized to total

proteins respectively and adjusted with value from NT group. β-Actin was used as the loading control. (A) - (B) All wildtype BMMs were collected after 6h stimulation experiments with PAO1, *rhlI*::Tn and *rhlR*::Tn (MOI=10). Data are representative of at least three independent experiments.

Upregulation of pqs in rhl-deficient P. aeruginosa causes an increased necroptosis

We next sought to determine the mechanism by which *rhl* mutants promoted macrophage PCD. Previous study showed that *rhl* deficiency caused an increased production and secretion of flagellin in *P. aeruginosa*, which triggered NLRC4 inflammasome activation and pyroptosis in macrophages (173, 174). However, the mechanism by which *rhl* mutants promote necroptosis is still unclear. We next asked whether enhanced PCD caused by *rhl* mutants were due to the lack of C_4-HSL, an autoinducer produced by *rhl* system (152). Pre-treatment with C_4-HSL caused no significant reduction of cell death induced by WT or *rhl*-deficient *P. aeruginosa*, suggesting no involvement of C_4-HSL in PCD regulation (Fig. 2-3A). Previous studies have documented that *rhl* negatively regulates the downstream *pqs* system, thus repressing the production of the autoinducer PQS and HHQ (173). Pre-treatment with PQS or HHQ resulted in a significantly increased cell death under the stimulation with PAO1 and abolished cell death difference induced by WT and *rhl* mutants (Fig. 2-3B-C). Additionally, our MS data on gene expression patterns on PAO1, *las* mutants, *rhl* mutants and *las&rhl* double mutants suggested that only *rhl* mutants had elevated *pqs* expression (Fig. 2-9). These results suggest that increased PCD by *rhl* mutants are associated with increased production of PQS and HHQ.

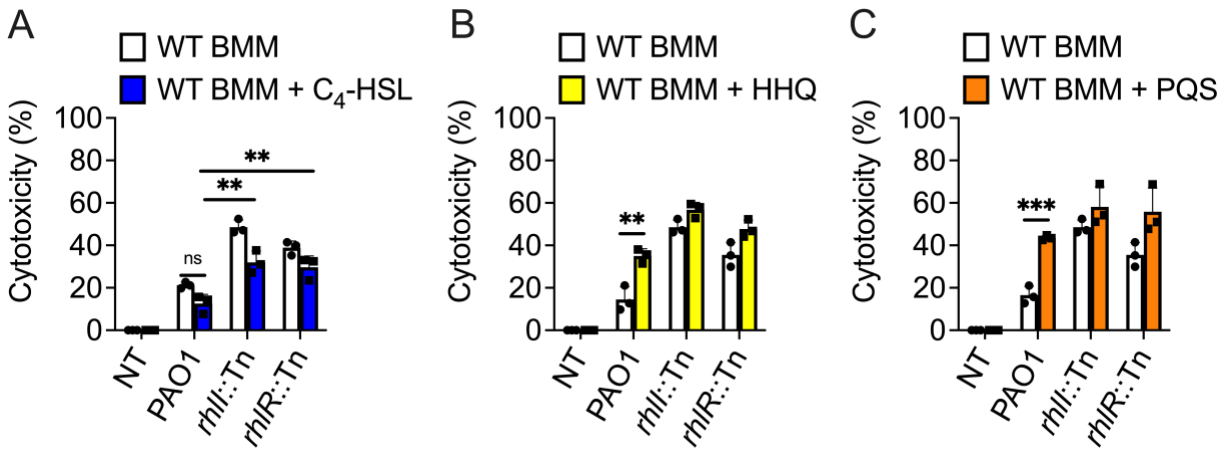

Figure 2-3 Complementarity of autoinducers fails to recover the promoted cell death induced by *rhl* mutants.

(A-C) Quantification of cell death by LDH release in wildtype BMMs and wildtype BMMs with 2h pre-treatment of C4-HSL (100 µM) (A), HHQ (100 µM) (B) or PQS (100 µM) (C) infected with P. aeruginosa (MOI=10) for 4h. Data is shown as mean ± SD. Each symbol represents a technical replicate. **p < 0.01, ***p < 0.001, by Student's unpaired t-test and ANOVA.

Depletion of pqs inhibits necroptosis

To further examine the causal relationship between increased production of PQS and HHQ and elevated PCD induced by *rhl*-deficient *P. aeruginosa*, we deleted *pqsA* gene in *rhl*-deficient background through homologous recombination (Fig. 2-8). Since *PqsA* regulates the transcription of downstream *pqsB/C/D/E* genes in the *pqs* system, deletion of *pqsA* leads to defective PQS and HHQ production (150, 175). We next examined whether double-knockout (DKO) mutants, *rhlI*::TnΔ*pqsA* and *rhlR*::TnΔ*pqsA*, could rescue macrophage cell death. Upon *P. aeruginosa* challenge in BMMs, we observed that DKO mutants only partially reduced cell death increased by *rhl* deficiency, while still exhibited higher level of cell death than that of PAO1, indicating that

pqs depletion indeed suppressed cell death promoted by *rhl* deficiency. No difference of cell death existed between PAO1 and Δ*pqsA* (Fig. 2-4A). To further identify the type of cell death reduced by DKO mutants, we tested *P. aeruginosa* challenge in *Gsdmd*$^{-/-}$ BMMs. DKO mutants failed to trigger cell death when pyroptosis was blocked, suggesting that *pqs* depletion rescued the necroptosis induced by *rhl* deficiency. We also observed that Δ*pqsA* diminished cell death mounted by PAO1 (Fig. 2-4B). To determine whether *pqs* depletion could inhibit pyroptosis, we re-conducted the challenge with *Ripk3*$^{-/-}$ BMMs. Cell death induced by DKO mutants was slightly lower than that by *rhl* mutants, and no difference existed between PAO1 and Δ*pqsA* two groups (Fig. 2-4C). Additionally, depletion of *pqs* in *rhl* mutants had no effect on phagocytosis (Fig. 2-4D). To further examine whether DKO mutants suppressed necroptosis, we sought to investigate signaling pathways through immunoblotting. We observed that deletion of *pqs* did have some influence on pyroptosis as the cleavage of Casp-1 (P20) and GSDMD (P30) were decreased in DKO groups compared with *rhl* mutant groups. While depletion of *pqs* seemed to have no effect on cleavage of Casp-8 (P18) (Fig. 2-5A). We next detected the phosphorylation of RIPK3 and MLKL were completely suppressed by DKO mutants, which was consist with our previous cytotoxicity data that depletion of *pqs* led to the inhibition of necroptosis in macrophages (Fig. 2-5B). Taken together, we concluded that *rhl*-deficient *P. aeruginosa* enhanced host necroptosis through upregulation of *pqs* system.

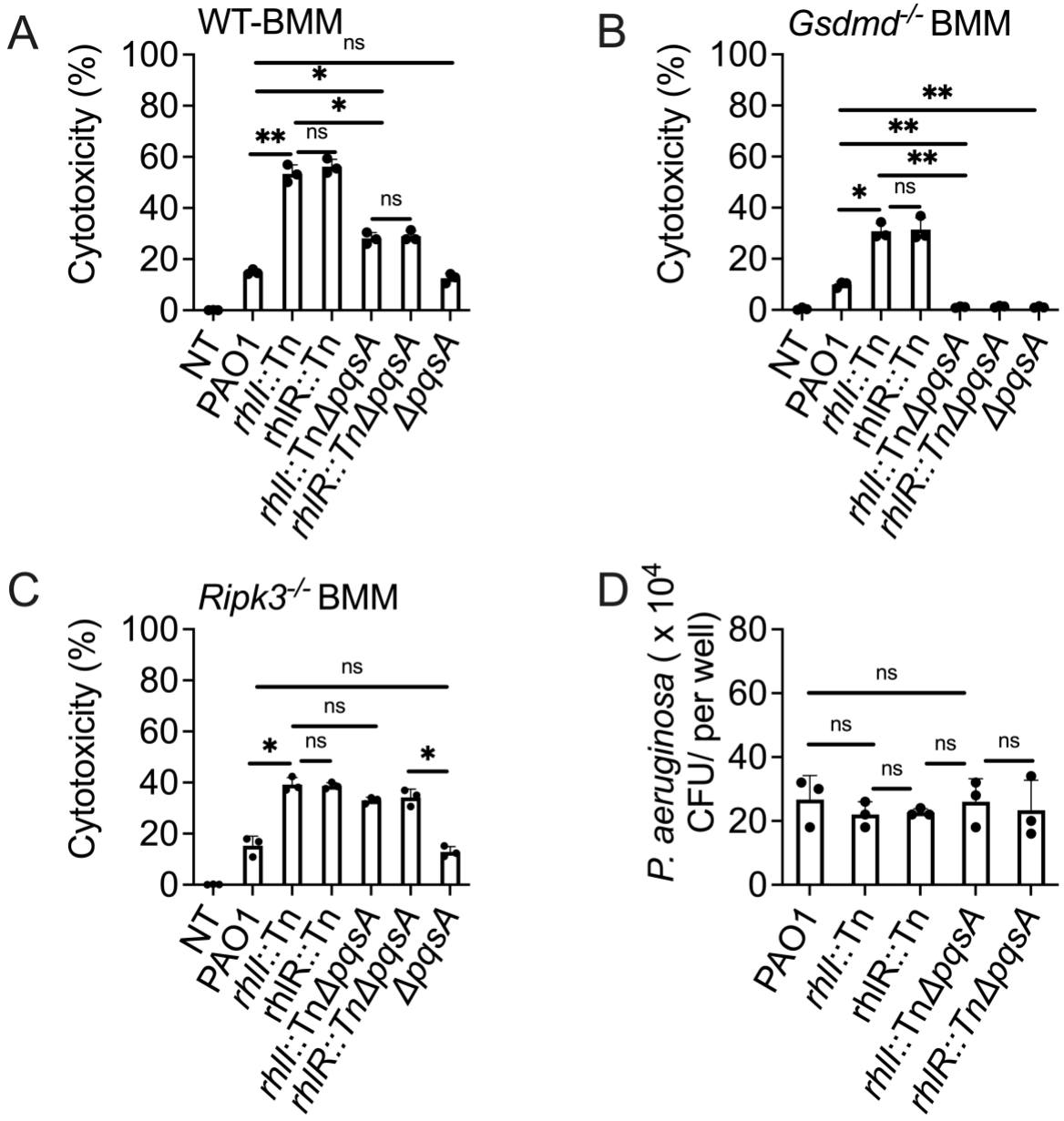

Figure 2-4 *pqs* depletion in *P. aeruginosa* partially rescues cell death mounted by *rhl* deficiency.

(A-C) Quantification of cell death by LDH release in wildtype BMMs (A), *Gsdmd*$^{-/-}$ BMMs (B) and *Ripk3*$^{-/-}$ BMMs (C) infected with PAO1, *rhlI*::Tn, *rhlR*::Tn, *rhlI*::TnΔ*pqsA*, *rhlR*::TnΔ*pqsA* and Δ*pqsA* (MOI=10) for 4h. (D) Phagocytosis of PAO1, *rhlI*::Tn, *rhlR*::Tn, *rhlI*::TnΔ*pqsA* and

rhlR::TnΔ*pqsA* assessed by colonies counting on LB agar plates with a dilution of 2×10^4 after 2h incubation with wildtype BMMs (MOI=10). Data are representative of at least three independent experiments. Data in (A) – (D) are shown as mean ± SD. Each symbol represents a technical replicate. *$p < 0.05$, **$p < 0.01$, by Student's unpaired t-test and one-way ANOVA.

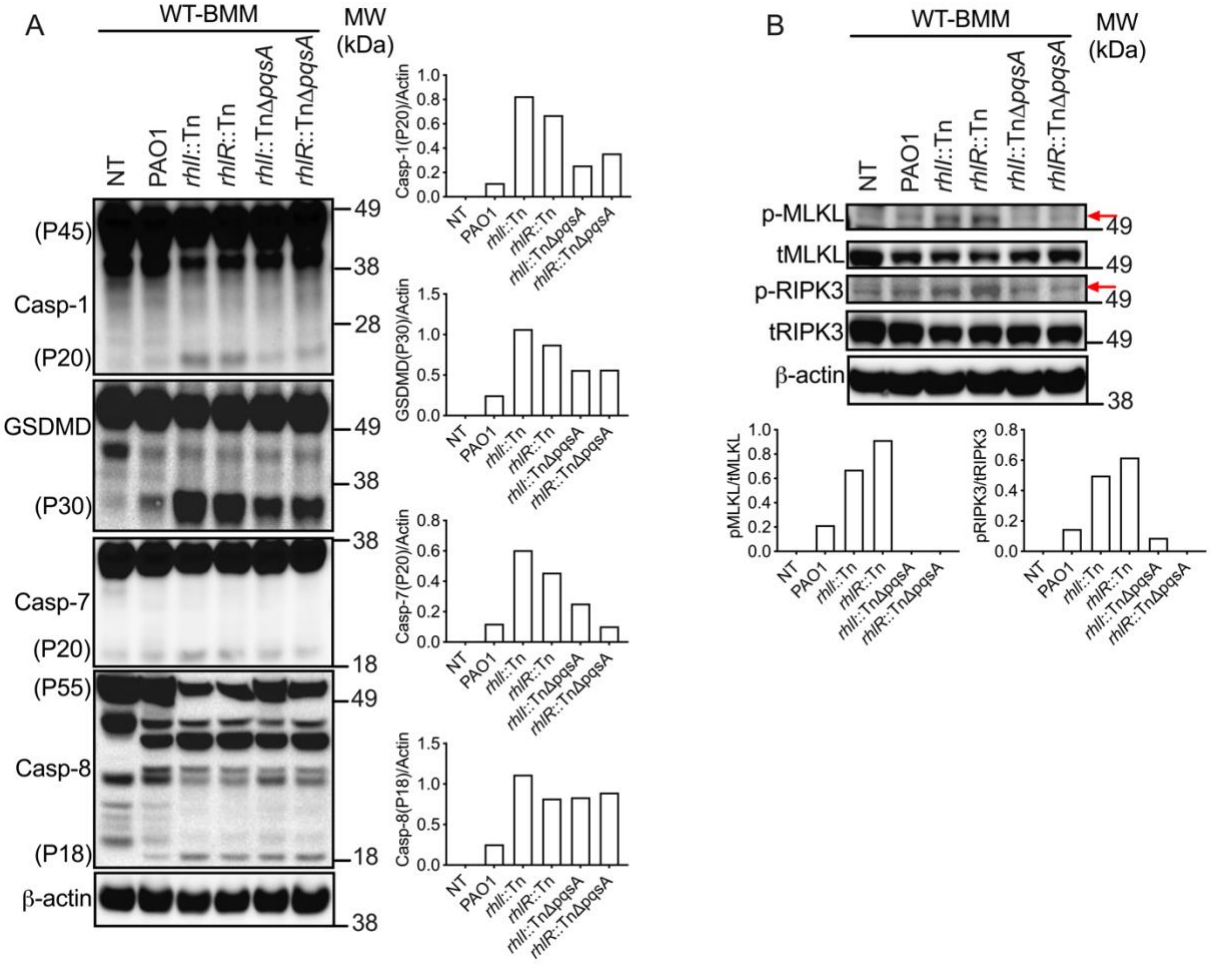

Figure 2-5 *pqs* suppression blocks necroptosis induced by *P. aeruginosa.*

(A) Immunoblotting and densitometric analysis of pro- (P45) and activated (P20) Casp-1, pro- (P53) and activated (P30) GSDMD, pro- (P55) and cleaved (P18) Casp-8, and pro- (P35) and cleaved (P20) Casp-7. The relative levels of the indicated proteins were normalized to actin and

adjusted with value from non-treated group. (B) Immunoblotting and densitometric analysis of pMLKL, total MLKL (tMLKL), pRIPK3 and total RIPK3 (tRIPK3). The relative levels of phosphorylated proteins were normalized to total proteins respectively and adjusted with value from NT group. β-Actin was used as the loading control. (A) - (B) All wildtype BMMs were collected after 6h stimulation experiments with PAO1, *rhlI*::Tn, *rhlR*::Tn, *rhlI*::TnΔ*pqsA* and *rhlR*::TnΔ*pqsA* (MOI=10). Data are representative of at least three independent experiments.

Depletion of pqs protects mice from rhl deficiency during P. aeruginosa challenge

To examine the susceptibility of mice to *P. aeruginosa* with *rhl* deficiency or *rhl&pqs* deficiency, we employed acute infection model with PAO1, *rhlR*::Tn and *rhlR*::TnΔ*pqsA* to challenge C57BL/6 mice. All bacteria were resuspended from overnight culture to achieve exponential phase, and then were centrifuged and washed with DPBS. Each mouse was administrated with 3×10^6 CFUs of *P. aeruginosa* intranasally. After 9 days of observation, all mice from *rhlR*::Tn group failed to survive, whereas all mice challenged with PAO1 successfully survived over the same period (Fig. 2-6A). Besides, mice infected with *rhl* mutant exhibited fast loss of body weight and failure in recovery (Fig. 2-6B). However, mice administrated with DKO mutant significantly improved mice survival even with *rhl* deficiency as 80% of mice survived after 9 days observation. Additionally, the body weight loss of DKO had no significant difference with PAO1 group and recovered 2 days post administration (Fig. 6A-B). In sum, these findings collectively demonstrated that repression of *pqs* could reduce susceptibility of mice during *P.*

aeruginosa challenge.

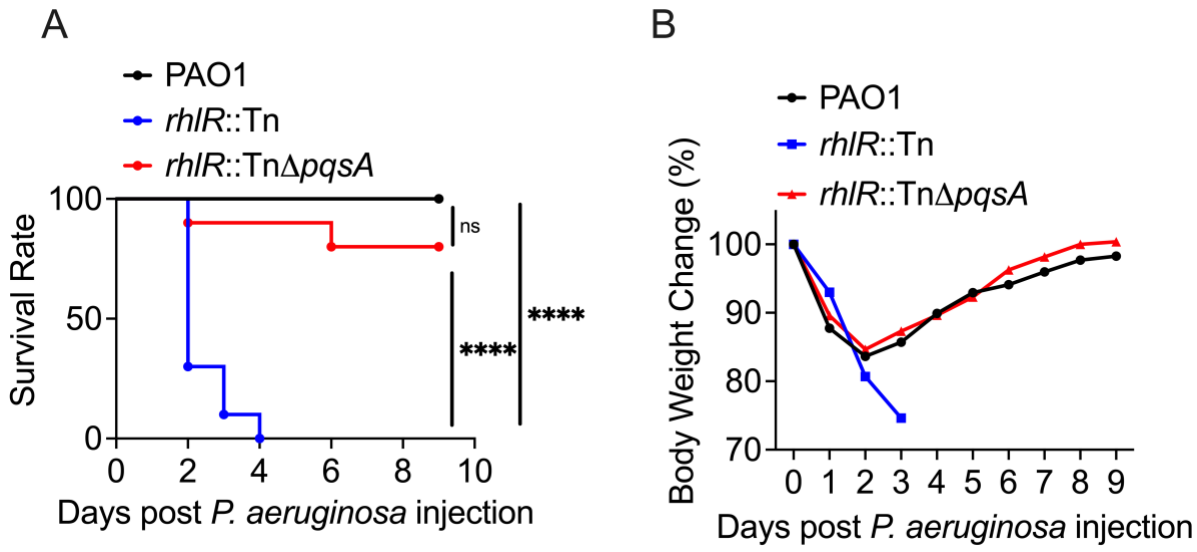

Figure 2-6 Inhibition of pqs ameliorates survive of mice from *rhl*-deficient *P. aeruginosa in vivo* challenge.

(A-B) 8-12 weeks, sex-matched C56BL/6 mice were administrated intranasally 3×10^6 CFUs per mouse with PAO1, *rhlR*::Tn and *rhlR*::TnΔ*pqsA*, respectively (n = 10 to 12 for each group). Survival (A) and body weight loss (B) were recorded for 9 days. ****$p < 0.0001$, by Kaplan-Meier method and log-rank (Mantel-Cox) test.

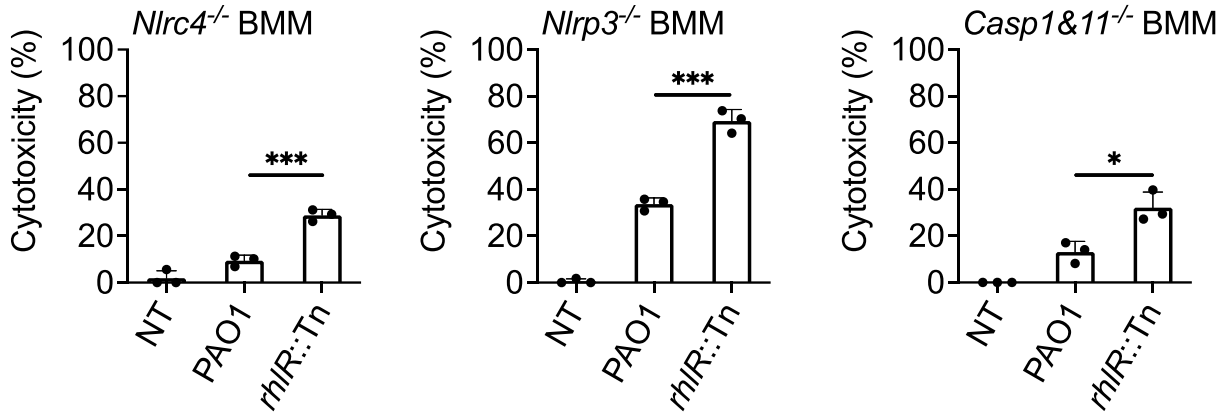

Figure 2-7 *rhlR*::Tn increases cell death without inflammasome activation.

Cell death assessed by LDH release in *Nlrc4*$^{-/-}$ BMMs, *Nlrp3*$^{-/-}$ BMMs and *Casp1&11*$^{-/-}$ BMMs infected with PAO1 and *rhlR*::Tn (MOI=10) for 4h. Data is shown as mean ± SD. **p* < 0.05, ****p* < 0.001, by Student's unpaired t-test.

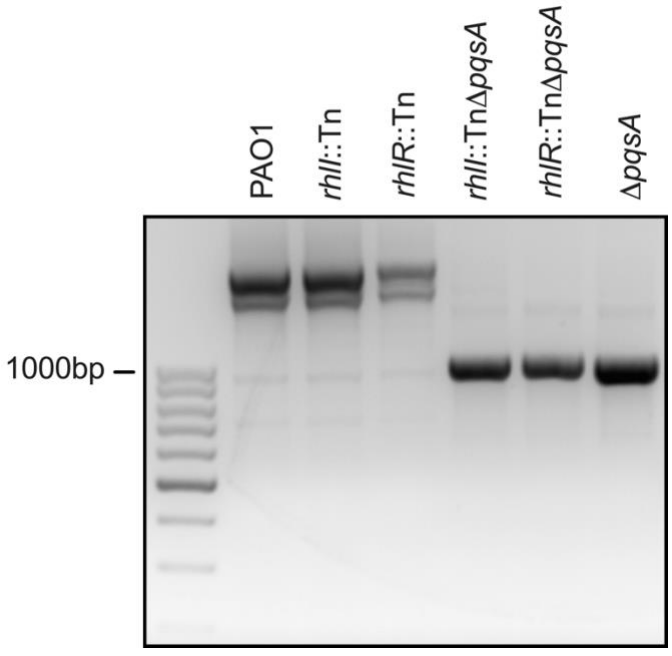

Figure 2-8 Genotyping of DKO mutants *P. aeruginosa* confirms deletion of *pqsA*.

Genotyping of P. aeruginosa with pqsA primers that cover 500bp from both upstream and downstream of *pqsA* gene. PAO1 and *ΔpqsA* were used as positive and negative control, respectively.

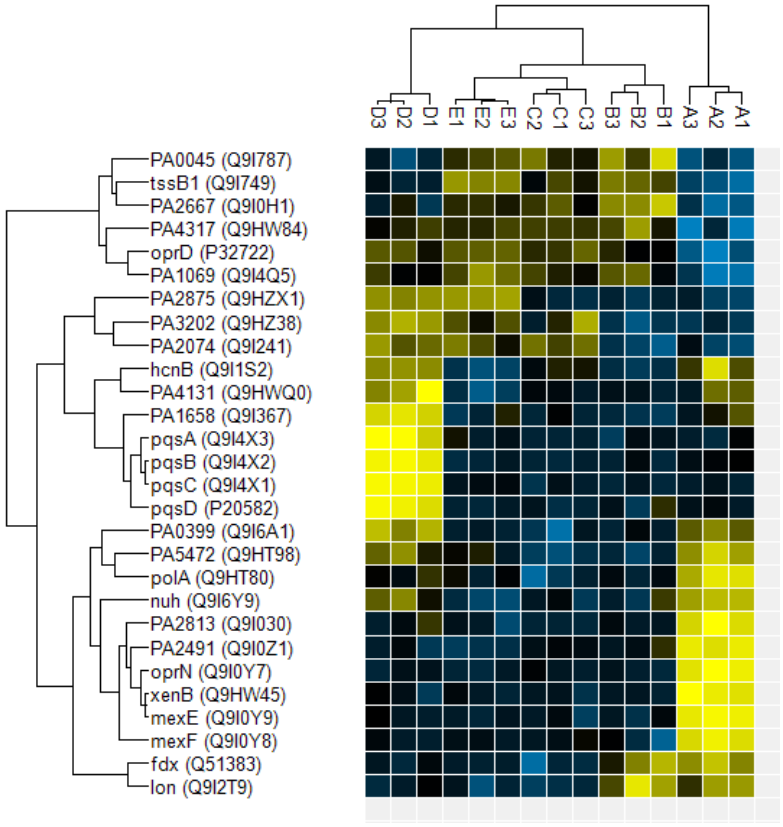

Figure 2-9 Mass spectrometry analysis on *P. aeruginosa* gene expression pattern.

Five groups of Pseudomonas aeruginosa strains were harvested and conduced MS analysis, each group had three replicates. A: PAO1, B: PAO1 Δ*lasI*, C: PAO1 Δ*lasR*, D: *rhlR*::Tn, E: PAO1 Δ*lasR&rhlR*.

2.5 DISCUSSION

The crosstalk between *P. aeruginosa* and PCD in host cells has become increasingly critical in both laboratorial and clinical research. Due to the characteristics like fast adaption to environment and wide range of antibiotic resistance, seeking the "arsenals" from host innate immunity becomes a new strategy in battling against *P. aeruginosa* infection diseases. QS functions as a pivotal mechanism that facilitates *P. aeruginosa* adaption has been reported to entangle with host PCD in previous studies (160, 161, 173, 174, 176). Nevertheless, most of these studies demonstrate the interaction of QS with apoptosis and pyroptosis in innate immune cells. As for another type of PCD, necroptosis, the connection between QS and necroptosis is pretty much sparse. In this study, we identified *P. aeruginosa rhl* mediated macrophage necroptosis through regulation on downstream *pqs*. *Rhl*-deficient *P. aeruginosa* significantly enhanced macrophage PCD during *in vitro* challenge, which were identified as pyroptosis and necroptosis via immunoblotting. It has been known that *rhl* deficiency caused an increased production and secretion of flagellin in *P. aeruginosa*, which triggered NLRC4 inflammasome activation and pyroptosis (173, 174). Indeed, we found that in addition to Ripk3-mediated necroptosis, *rhl* deficiency also caused a small increase in cell death that was independent of *pqsA* or Ripk3, which is consistent with previous finding on increased pyroptosis due to excessive flagellin production in *rhl* deficient *P. aeruginosa* (173, 174). *RhlR* had been demonstrated to suppress downstream *pqs* genes, and our results showed that *pqs* autoinducers, PQS and HHQ, robustly facilitated cell death induced by *P. aeruginosa*, even PAO1 (177, 178). After generating *rhl*/*pqs* DKO mutants, we observed the overall cell death was reduced by DKO mutants compared with *rhl* mutants. Moreover, necroptosis was inhibited under the challenge of DKO mutants. We therefore

demonstrate that *rhl*-deficient *P. aeruginosa* upregulates downstream *pqs* is an important mechanism underlying the activation of necroptosis.

Necroptosis is considered as the best-characterized form of regulated necrosis mediated by RIPK3 and its phosphorylation substrate MLKL, which is also a major player of PCD (179, 180). Upon activation of Tumor necrosis factor receptor 1 (TNFR1), the downstream effector RIPK1 will be deubiquitinated and activated under the inhibition of Casp-8, which then leads to the activation and phosphorylation of RIPK3 and MLKL to finally execute necroptosis (181). Clinical studies reveal that necroptosis plays a double-edge role in diseases as both to eliminate infected cells and to contribute to pathogenesis (181, 182). In this study, mice infected with DKO mutant significantly improved the survival outcome when compared with mice challenged with *rhl* mutant. *rhl* mutant induced high intensity of cell death in macrophages as well as other immune cells, resulting in tissue damage associated hyperinflammation and low survival rate in mice. Through depletion of *pqs*, DKO mutant reverted the outcome caused by *rhl* deficiency. However, further studies that identify the host receptors monitor the elevation of *pqs* and *pqs*-related virulence factors will be important to better understand how exactly *pqs* triggers host necroptosis. In sum, our results provide a novel aspect of link between *P. aeruginosa* QS and host necroptosis. Targeting the *rhl-pqs*-necroptosis axis presents a potential therapeutic strategy for combating multiple *P. aeruginosa* involved diseases, especially cystic fibrosis.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors have no financial conflicts of interest.

Table 2-1 Strains, plasmids and primers used in this study

Strain, plasmid or primer	**Description**
P. aeruginosa	
PAO1	wildtype *P. aeruginosa*
rhlI::Tn	*rhlI* transposon mutant (PA3476::ISlacZ/hah)
rhlR::Tn	*rhlR* transposon mutant (PA3477::ISlacZ/hah)
Δ*pqsA*	*pqsA* deletion mutant
rhlI::TnΔ*pqsA*	*rhlI* and *pqsA* double-deletion strain
rhlR::TnΔ*pqsA*	*rhlR* and *pqsA* double-deletion strain
Plasmids	
pEX18Gm	For allelic exchange in *P. aeruginosa*
pΔ*pqsA*	For *pqsA* deletion
Primer	
pqsA-up_fwd	taaaacgacggccagtgccaGAAGCCTGCAAATGGCAG
pqsA-up_rev	acagcctgaaGACAGAACGTTCCCTCTTC
pqsA-down_fwd	acgttctgtcTTCAGGCTGTGGGGGTGAACC
pqsA-down_rev	gctcggtacccggggatcctCGGATCACCGCCCAGCGC

Chapter 3 PCD in sepsis induced immunosuppression

3.1 Introduction

Sepsis, which is defined as a life-threatening organ dysfunction caused by host pathological dysfunctional response to infection, has become increasingly significant in both biological and clinical research (112). According to Global Burden of Diseases report, sepsis affected more than 30 million people annually worldwide, with a mortality rate of 22.5%, accounting for nearly 20% of all global deaths (183, 184). Although considerable progress has been made in intensive care units (ICU) and medical technologies, the incidence of sepsis still rises at rates between 1.5% and 8% each year (185). In addition to the high health-related burden, septic also brings a major burden to national health care system, with an estimated annual cost of $16.7 billion to extend the hospitalization of septic patients in United States (185). The mortality of sepsis is believed to be caused by either sepsis-induced immunosuppression or reactivation of overwhelming inflammation (112). With additional evidence emerges, the immunosuppression has been recognized as the major reason to induce mortality, and attracted increasing attention from researchers. Thus, having a complete understanding on the pathological role of sepsis-induced immunosuppression is pivotal for developing therapeutic treatments.

There are two dynamic stages occur during the initiation and progression of sepsis, which are a system inflammatory response syndrome (SIRS) in the acute phase and a compensatory anti-inflammatory response syndrome (CARS) in the later phase (111). In normal condition, the immune balance can be quickly restored if the invading pathogens are cleared properly during

early stage of systemic inflammatory response. However, if the inflammation persists due to failure in pathogens removal, the immune balance is then devasted, leading to SIRS and following CARS. One prominent characteristic of SIRS is the overproduction of pro-inflammatory cytokines and chemokines, which is named as “cytokine storm”, leading to organ failure, tissue damage and eventually death. The uncontrolled inflammatory response also has paradoxical effects, like the promotion on the activation of the coagulation system and the formation of intravascular micro thrombosis, leading to disseminated intravascular coagulation (186, 187). Such high level of pro-inflammatory responses and cytokine storm are previously believed to be the major causes of high mortality. Nevertheless, clinical studies have revealed that by targeting these pro-inflammatory cytokine production fails to achieve significant outcomes in improving the survival rate of patients with sepsis (188, 189). In addition, recent preclinical and clinical studies have indicated that imbalance of innate immune response together with persistent immunosuppression drive multiple organ damage and septic death (190, 191). Collectively, these pieces of evidence suggest that targeting CARS can be an effective therapeutic method to treat sepsis.

A high mortality rate is often found during the acute phase of severe clinical sepsis, which is associated with the development of a sustained immunosuppressive state. The hallmark of this persistent immunosuppression is the inability to clear primary infections and/or secondary infections. Though the mechanisms of sepsis-induced long-term immunosuppression is still under investigation, several perspectives have been proposed in recent studies, including increased expression of anti-inflammatory cytokines, expansion of regulator cells and loss of immune effector cells due to PCD (112). The anti-inflammatory cytokines including IL-4, IL-10 and IL-37 are mainly secreted by multiple immune cells, like macrophages, Th2 cells and mast cells during sepsis-induced immunosuppression (112). The function of these anti-inflammatory cytokines can

be divided into three parts: inhibition on effector cells' proliferation and function; suppression on pro-inflammatory cytokine production; promotion on proliferation of immunosuppressive cells. Due to the excessive level of anti-inflammatory cytokines, the immune regulator cells expand quickly, leading to further deterioration on homeostasis. Tregs have been well characterized as an indispensable role in sepsis, like secreting anti-inflammatory cytokines TGF-β and IL-10; enhancing their stability through epigenetic modifications on *Foxp3* gene; increasing the suppressive capacity by metabolic shift glycolysis to oxidative phosphorylation (192- 194). Another group of regulator cells are myeloid-derived suppressor cells (MDSCs), including monocytes/macrophages, neutrophils and dendritic cells. In response to sepsis stimulation, MDSCs are released from bone marrow and migrate to lymph nodes to inhibit the proliferation and function of effector lymphocytes (195). Clinical studies have reported that patients with sepsis-induced chronic immune suppression are associated with significantly elevated Tregs and MDSCs, suggesting the potential therapeutic targets to treat the persistent immunosuppression (196, 197). The PCD has a crucial role in mediating the loss of effector cells during sepsis-induced immunosuppression. It is reported that the expression levels of cytochrome c, caspase-3, caspase-8, and caspase-9 are significantly increased in the sepsis-induced mouse model, resulting in increased T-cell apoptosis (116). Moreover, elevation of Fas expression and apoptosis activation are observed in peripheral blood monocytes of patients with sepsis (118). Additional evidence emerges that increased expression and activity of caspase-1 in macrophages are observed in LPS-treated septic mice, and knockout of caspase-1 or GSDMD reduces septic mortality of mice (131). The large molecules released by pyroptosis are also involved in the sepsis-induced immunosuppression. For example, HMGB1 released by hepatocytes is critical in caspase-11-dependent pyroptosis and lethality in LPS-mediated endotoxemia and bacterial sepsis (198).

Besides, inhibiting hepatocyte HMGB1 release reduces pyroptosis-related effector cell loss in sepsis (198). Together, these findings suggest that targeting PCD can be an effective strategy in developing clinical treatments for sepsis.

As mentioned in chapter 1, NINJ1-mediated PMR functions as the final part of PCD, which regulates the release of large molecules into surrounding environment during lytic cell death. NINJ1 deficiency macrophages exhibited inhibition on the release of larger cellular molecules, including lactate dehydrogenase (LDH) and HMGB-1, in response to inflammasome activation. The aforementioned evidence has demonstrated blocking HMGB-1 in hepatocytes mitigates sepsis through inhibition on pyroptosis-induced effector cells loss, manifesting the potential function of NINJ1 in alleviate sepsis-induced immunosuppression. Besides, absence of NINJ1 can significantly decrease the DAMPs release during early pro-inflammatory phase, resulting in suppressed inflammatory intensity, pro-inflammatory cytokine release and PCD activation. Therefore, NINJ1 may function as a novel therapeutic target in treating sepsis.

In this study, we discovered that NINJ1 deficiency significantly improved the mortality of mice with cecal ligation and puncture (CLP)-induced sepsis. Additionally, when challenged the septic mice with secondary infection, *Pseudomonas aeruginosa*, *Ninj1*$^{-/-}$ mice exhibited better bacterial clearance than wildtype mice, suggesting NINJ1 deficiency also reduced sepsis-induced immunosuppression. Collectively, we have identified the positive role of NINJ1 in improving the outcomes brought by sepsis through mitigating the overheated inflammation and the following sepsis-induced immune suppression, which provides solid evidence to support that NINJ1 can function as a potential therapeutic target for clinical treatments on sepsis.

3.2 Materials and Methods

Mice

C57BL/6J (000664) mice were obtained from the Jackson Laboratory. *Ninj1*$^{-/-}$ mice are obtained through heterologous breeding of *Ninj1*$^{+/-}$ mice (purchased from Genentech, lnc.) and examined through genotyping. All mice were housed in SPF facilities at The Ohio State University (OSU). 8-12 weeks old mice with sex-matched were used in animal experiments. All *in vivo* experiments were performed in according with the guidelines established by The OSU and National Institute of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (IACUC) (Protocol: 2018A00000022-R2).

Experimental sepsis induced by CLP

Mice were anesthetized with an intraperitoneal injection of 2.25 mg of ketamine HCL (Abbott Laboratories, Chicago, IL) and 150 μg of xylazine (Lloyd Laboratories, Shenandoah, IA). Under sterile surgical condition, a 1cm midline incision was made to the ventral surface of the abdomen, and the cecum was exposed. The cecum was partially ligated at its base with a 3.0 silk suture and punctured four times with 21-gauge needle. The cecum was returned to the peritoneal cavity, and the abdominal incision was closed using surgical staple. Mice were rehydrated with 1 ml saline water and placed on a heating pad until they recovered from anesthetic.

Survival studies following CLP

The first set of survival studies was performed to determine the effect of the presence of NINJ1 on survival in CLP-induced sepsis animal model. WT and *Ninj1*$^{-/-}$ mice were subjected to CLP surgery. Survival was monitored for 6 days following surgery. The second set of survival studies

focus on the impact of NINJ1 on host antibacterial defense during sepsis-induced immunosuppression. At day 7 post-surgery, both surviving CLP mice and sham-operated mice were anesthetized and intranasally injected with *P. aeruginosa* at a CFU of 3×10^6. 24h post *P. aeruginosa* challenge, all mice were sacrificed to harvest lung tissue samples and Bronchoalveolar lavage (BAL) fluid for following *ex vivo* assays.

Bacterial load in sham and CLP-treated mice

Lung tissue samples collected from each mouse were first weighted and recorded. 500 μl DPBS was added to each sample in 2ml microtube under sterile conditions. Lung tissue was grinded by sonicating to release the *P. aeruginosa* phagocyted by alveolar immune cells. Tenfold serial dilutions of the lysates were mixed with DPBS and 10 μl was applied on an antibiotic-free LB agar plate. Colonies were counted after 8h incubation at 37°C.

ELISA

Lung tissue samples collected post *P. aeruginosa* infection were mixed with 500 μl DPBS and grinded by sonicating under sterile conditions. Both lung tissue sample mixer and BAL fluids were then centrifuged at 360×g, 10min to separate cell pellets and supernatants. Concentrations of IL-6, TNFα and IL-1β were measured in cell-free BAL fluid and supernatants of lung tissue. The whole protein concentrations of all samples were determined by Bradford Agent (BD, lnc.). Using a standardized sandwich ELISA technique. Briefly, 96-well polystyrene high bind plates (corning, 9018) were coated with 1-5 μh/ml capture Abs in DPBS overnight at 4 and washed with PBS containing 0.05% Tween 20. IL-6 and TNFα Abs were purchased from BD company, and IL-1β was purchased from R&D company. Nonspecific binding sites were blocked with 5% FBS in

DPBS for 1h at RT. Plates were rinsed three times with wash buffer and cell-free supernatants were loaded and incubated for 2h at RT. After five washings, a biotinylated detection polyclonal Ab mixed with peroxidase-conjugated streptavidin were added to the well for 1h at RT. Plates were washed, and after the addition of TMB substrate (BD, lnc.), OD readings were measured at 450 nm using an ELISA plate reader. Recombinant murine cytokines were used to generate the standard curves from which the concentrations present in the samples were calculated.

Statistics analysis

GraphPad Prism v9.0 software was used for data analysis. Data were analyzed as mean ± SD. The means of two groups were compared with Student's unpaired t-test. Comparisons between multiple groups were analyzed by repeated-measures ANOVA with Bonferroni post tests. Survival analyses were performed by Kaplan-Meier method and log-rank (Mantel-Cox) test for significance. *P* values of less than 0.05 were considered statistically significant.

3.3 Results

NINJ1 deficiency improves the mortality of mice with sepsis

In order to obtain a comprehensive understanding on the mechanisms of clinical sepsis, effective experimental models are needed to replicate what occurs in patients with sepsis. A couple of models, including establishment of abscesses in the extremities, intravenous endotoxin challenge, injection of live organisms into the peritoneal cavity and the induction of polymicrobial peritonitis via cecal ligation and puncture (CLP) have been utilized in studies that focus on sepsis (199-201). Among these models, the CLP has been acknowledged as the most suitable model to replicate the initiation and progression of clinical sepsis due to its reproducibility and capacity to modify the severity of sepsis through modulation on needle size, number of cecal punctures and antibiotic utilizations (202).

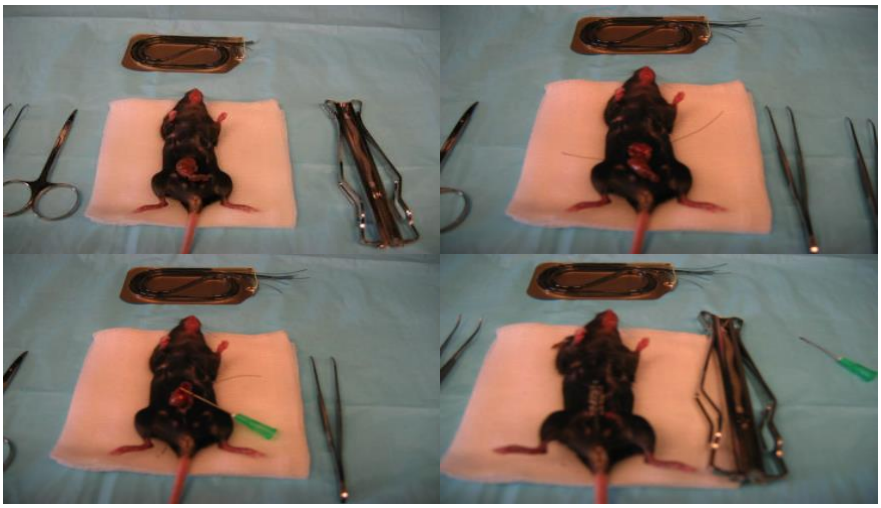

Figure 3-1 Cecal ligation and puncture Surgery on mice.

After anesthesia, a 1cm midline incision was made to the ventral surface of the abdomen, and the cecum was exposed. The cecum was punctured four times with 21-gauge needle. The cecum was returned to the peritoneal cavity, and the abdominal incision was closed using surgical staple.

To examine the function of NINJ1 in modulating the mortality caused by sepsis, we conducted CLP surgery to induce sepsis on wildtype and *Ninj1*$^{-/-}$ mice (Fig 3-1). Both genotypes of mice were divided into non-treated (sham) and treated (CLP) groups. While for sham groups, the mice were processed with incision on the ventral surface of the abdomen and ligated quickly after exposure of the cecum. For CLP groups, four times of punctures were applied to each mouse to induce a moderate intensity of sepsis in mice. After 7 days of observation, *Ninj1*$^{-/-}$ mice treated with CLP exhibited significantly reduced mortality when compared with wildtype mice with CLP, indicating NINJ1 is positively associated with the progression of sepsis (Fig 3-2A). Besides, ELISA on peritoneal fluids collected from all four groups of mice exhibited significant reduction of IL-6 release in CLP-treated *Ninj1*$^{-/-}$ mice compared with CLP-treated WT mice, suggesting NINJ1 deficiency suppressed PCD-induced hyperinflammation during the early stage of sepsis to improve the survival rate.

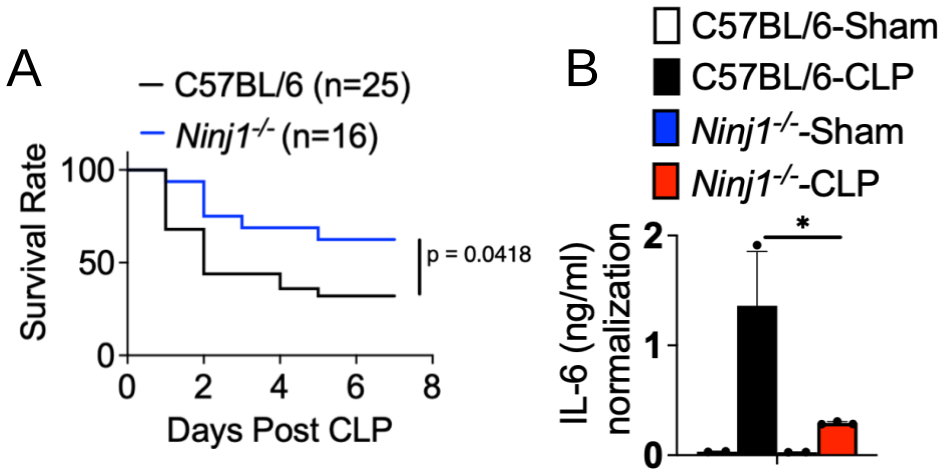

Figure 3-2 NINJ1 deficiency improves survival rate of mice post CLP surgery.

(A) 8-12 weeks C56BL/6 and *Ninj1*$^{-/-}$ mice were treated with CLP surgery. Survival was recorded for 7 days post-surgery. $p < 0.05$, by Kaplan-Meier method and log-rank (Mantel-Cox) test. (B) ELISA on IL-6 release of peritoneal fluid collected from sham and CLP-treated WT and *Ninj1*$^{-/-}$ mice 24h post CLP.

NINJ1 deficiency improves the bacterial resistance of mice with sepsis

As mentioned above, patients with sepsis are highly vulnerable to secondary pathogen infections which results as the major cause of death. In order to identify whether NINJ1 has any influence on the immune response to secondary infections, we have utilized a two-hit model system to test the assumption (Fig 3-3). Followed by the CLP surgery, both sham and CLP groups of mice were monitored for 7 days to guarantee the mice survived from primary hit. 7 days post CLP surgery, all groups of mice were administrated with 3×10^6 CFUs/mouse of *P. aeruginosa* intranasally as the secondary hit. 24h after *P. aeruginosa* challenge, all mice were sacrificed to collect the BAL fluid and lung tissue for *ex vivo* experiments. A proportion of lung tissue samples were weighted and processed for the evaluation of bacterial load. We could easily discover that CLP-treated WT mice have weaker bacterial clearance than that of non-treated WT mice, supporting that CLP-induced sepsis impairs the defense capacity of host in response to secondary infections (Fig 3-4). When compared with CLP-treated WT mice, *Ninj1*$^{-/-}$ mice with CLP-induced sepsis exhibit significantly improved bacterial resistance. Besides there is no significant difference of bacterial load exists between non-treated WT mice and CLP-treated *Ninj1*$^{-/-}$ mice, suggesting NINJ1 deficiency may rescue the inability of septic mice in pathogen defense. Notably, during non-treated condition, both WT and *Ninj1*$^{-/-}$ mice show similar bacterial removal, indicating the NINJ1 may not be involved in host antimicrobial responses. In sum, these pieces of evidence

demonstrate that NINJ1-mediated PMR plays a crucial role in compromising host pathogen defense ability under CLP-induced sepsis condition and deletion of NINJ1 could mitigate sepsis intensity and restore host anti-microbial response.

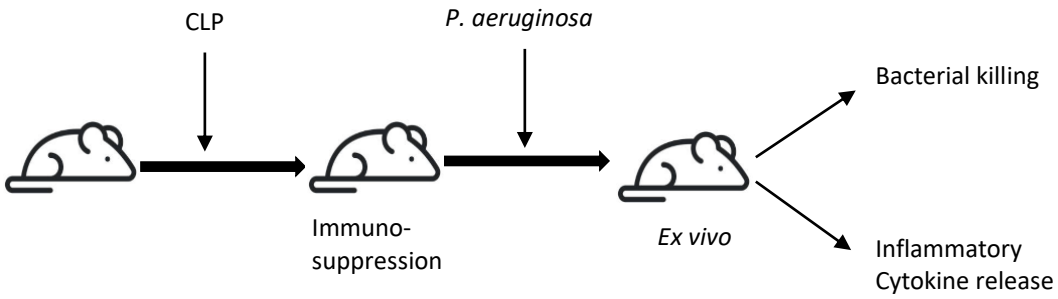

Figure 3-3 Schematic of two-hit model system.

C56BL/6 and *Ninj1*$^{-/-}$ mice are treated with CLP to initiate sepsis. 7 days after CLP, a compensated long-term immunosuppression appears in mice that survive from hyperinflammation. *P. aeruginosa in vivo* challenge functions as secondary hit. After certain time course of *P. aeruginosa* infection, mice are sacrificed to collect lung samples for *ex vivo* assays to evaluate the impact of NINJ1 on sepsis-induced immunosuppression.

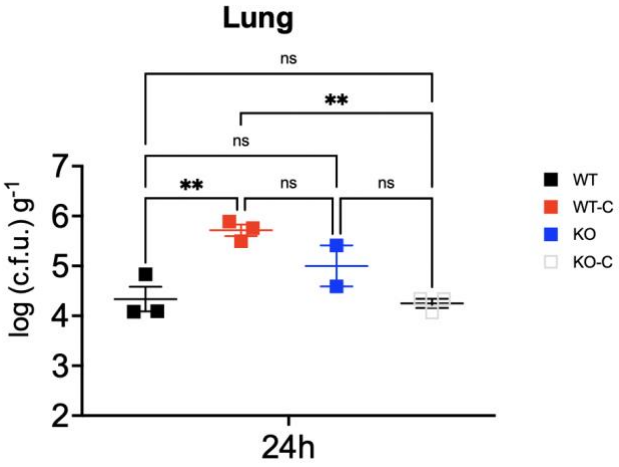

Figure 3-4 Bacterial load of WT and *Ninj1*$^{-/-}$ mice post CLP surgery.

Bacterial load were performed to evaluate the resistance of four groups, WT-sham, WT-CLP, *Ninj1*$^{-/-}$-sham, *Ninj1*$^{-/-}$-CLP, to secondary infection. The number of colonies of each sample was normalized to sample weight.Each symbol represents a technical replicate. *$p < 0.05$, **$p < 0.01$, by Student's unpaired t-test and one-way ANOVA.

NINJ1 deficiency improves the sepsis-induced immunosuppression

To further explore the role of NINJ1 on sepsis-induced immunosuppression, we performed ELISA on pro-inflammatory cytokines after the secondary infection. BAL fluids and lung tissue were collected to evaluate the cytokine secretion at different parts of lung. We observed that IL-6 released by CLP-treated *Ninj1*$^{-/-}$ mice was significantly reduced when compared with CLP-treated WT mice, which was probably due to the low bacterial load at the 24h time point (Fig 3-5A). However, though deficiency of NINJ1 rescued the bacterial clearance impaired by sepsis-induced immunosuppression, CLP-treated *Ninj1*$^{-/-}$ mice released lower level of IL-6 than that of non-treated WT mice, indicating that sepsis-induced immune suppression still existed under the NINJ1 absence condition. This might also explain the difference of IL-6 release between sham and CLP *Ninj1*$^{-/-}$ mice. The secretion patterns of TNF-α and IL-1β were similar to those of IL-6 in WT and *Ninj1*$^{-/-}$ mice under the absence/presence of CLP surgery (Fig 3-5BC). Notably, both IL-6 and TNF-α were highly detected in BAL fluids, while IL-1β released from PCD was highly found in lung tissue, suggesting that IL-6 and TNF-α were constantly secreted by immune effector cells in response to bacterial infection and IL-1β was released after PCD triggered by bacterial virulence factors. Due to PCD is only one of the mechanisms involved in sepsis initiation and following immunosuppression, we propose that NINJ1 deficiency partially rescue the sepsis-induced immunosuppression.

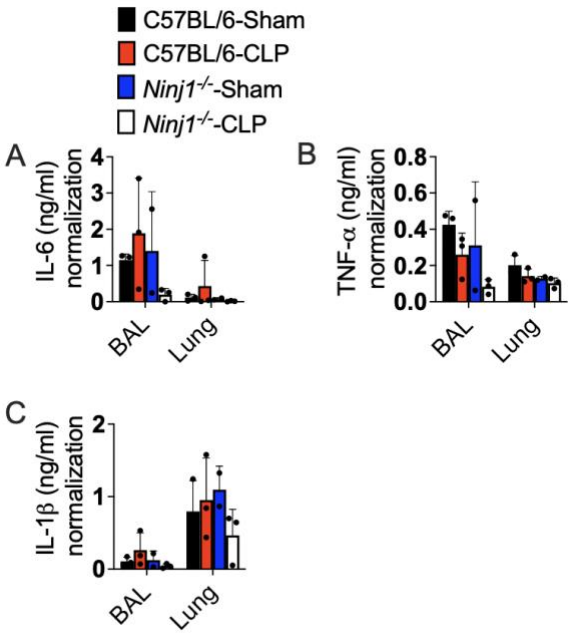

Figure 3-5 NINJ1 deficiency ameliorates sepsis-induced immunosuppression.

(A-C) BAL fluids and supernatants of lung samples from each group were collected and centrifuged. Pro-inflammatory cytokines IL-6, TNFα and IL-1β were measured by ELISA to evaluate inflammatory response to secondary infection. The cytokine concentrations were normalized to total protein concentrations determined by Bradford.

3.4 Discussion

The function of PCD in sepsis has becoming widely studied due to its crucial role in loss of immune effector cells. In order to restore the balance of homeostasis and reduce the intensity of "cytokine storm" and its following tissue damage, targeting the PCD has been recognized as an efficient strategy in developing treatment for patients with sepsis. As a novel discovered PCD regulator, NINJ1 plays a vital role in spreading the consequences brought by PCD to surrounding environment and cells through mediating the PMR to control the release of DAMPs. Therefore, we hypothesized NINJ1 might function as an important factor to affect the severity of sepsis-induced immunosuppression. In this study, we identified that loss of NINJ1 improved the survival rate of mice post CLP-induced sepsis. Additionally, through a two-hit model system, we found NINJ1 deficiency significantly restored host immune response to *P. aeruginosa* infection during sepsis-induced immune suppression. However, the ELISA results of pro-inflammatory cytokines release indicated that deletion of NINJ1 might not completely prevent the presence of immunosuppression but only alleviate its suppression on host immune responses.

The long-term immunosuppression induced by sepsis is reported to be the major cause of mortality in patients that encounter secondary pathogen infections. Though we have confirmed that loss of NINJ1 reduces this immune suppression, further studies need to be conducted to explore how NINJ1 deficiency affects the immunosuppression. For example, rather than collect lung tissue samples only 24h post *P. aeruginosa* infection, multiple time courses should be applied to the sample collection process to obtain a comprehensive pattern of cytokine release in response to secondary hit. Besides, additional biochemistry experiments should be performed to determine the activation of both pro- and anti-inflammatory signaling pathways. Future studies that focus on the role of NINJ1 in modulating immune regulator cells and anti-inflammatory cytokine

production will bring us a more detailed association between NINJ1 and sepsis-induced immunosuppression, extending the function of NINJ1 in other types of immune suppression, like tumor-associated immunosuppression. In summary, our results provide a novel and feasible therapeutic target to develop clinical treatments for patients suffered with sepsis.

Chapter 4. Conclusions and future directions

4.1 Conclusion

The general aim of this study is focus on the dual roles of programmed cell death in innate immune response to diverse infections. From the first discovery of programmed cell death in silkworm back in 1960s, thousands of hundreds of studies have been conducted to explore the underlying mechanisms of PCD and their positions in the interaction between host immune system and "bad guys" that threaten homeostasis (2). The impact on host immune responses varies dramatically by the type of PCD signaling that is engaged in the infections (203). For instance, apoptosis is thought to be a "silent" cell death which prevents inflammatory responses due to apoptotic bodies formation but also contributes to long-term immunosuppression in sepsis through depletion of adaptive immune effector cells. Lytic cell death, including necroptosis and pyroptosis, is highly inflammatory due to the release of DAMPs and other immunogenic signals (204). Given these diverse outcomes, the signaling that orchestrates PCD is tightly regulated by host cells to remove exogenous or endogenous pathogens and restore immune balance.

PCD has been well-demonstrated to be a crucial part of host antimicrobial responses. The death of infected cells is mostly concomitant with the death of the infecting agents due to increased exposure of these agents, resulting in efficient pathogen clearance. Destruction of infected tissues may also eliminate a pathogenic niche, thereby hampering microorganism replication and dissemination (205). Activation of macrophage PCD eliminates a potential site for future proliferation and destroys the infecting bacteria (206). Besides, phagocytosis of apoptotic bodies

by surrounding monocytes/macrophages leads to increased fusion of phagosome with lysosome and accelerated digestion of pathogens (207). In addition, engulfment of apoptotic bodies by dendritic cells promotes antigen presentation to T cells, linking innate and adaptive immunity (208-210). Another research reported that *Streptococcus pneumoniae* triggered macrophage apoptosis during infection, resulting in bacteria elimination rather than evasion of the immune system (211). It has demonstrated that interferon induces ZBP1-mediated PANoptosis (convergence of apoptosis, necroptosis, pyroptosis) in human and mouse macrophages during SARS-CoV-2 infection (219). And patients suffered with SARS-CoV-2 infection exhibit increased expression of ZBP1 in immune cells compared with those recovered, indicating an important link between PANoptosis and disease progression (219).

Interestingly, pathogens have also developed diverse strategies to inhibit PCD for evasion from host clearance. For example, viable host cell is required for bacteria to replicate and thrive during *Rickettsia rickettsii* infection. To achieve the goal of successfully survival, *Rickettsia rickettsii* activates NF-κB signaling to prevent host PCD and avoid from exposure in host defense system (212). Similarly, *Chlamidiae spp.* can suppress the activation of apoptosis in infected cells through blocking cytochrome *c* release from the mitochondria (213). Together these pieces of evidence suggest the importance of PCD in elimination of infection.

Our observation of the crosstalk of *P. aeruginosa* quorum sensing and host PCD indicates a novel strategy in battling against *P. aeruginosa* infections. Our findings reveal that *P. aeruginosa rhl* system mediates host macrophage necroptosis through its regulation on downstream *pqs* system. Previous studies have already exhibited the connection between *P. aeruginosa* and other types of PCD, including apoptosis and pyroptosis. For instance, quorum sensing autoinducer N-(3-oxododecanoyl) homoserine lactone is identified to activate apoptosis during *P. aeruginosa*

invasion (160). And it is reported that *rhl* system of *P. aeruginosa* also regulates flagellin production and translocation into host macrophages through T3SS system (173). Intracellular flagellin is detected and recognized by NLRC4, which in turn recruits caspase-1 to form inflammasome and activation of pyroptosis. Our study has filled the gap by uncovering the underlying mechanism of necroptosis activation by *P. aeruginosa* infection. However, the further mechanism that upregulated *pqs* promotes the activation of necroptosis remains unclear, and additional studies are needed for obtain a better comprehensive understanding of *P. aeruginosa* and host necroptosis. Notably, during *P. aeruginosa* infection, we observed all three types of PCD, apoptosis, necroptosis and pyroptosis are activated based on the results from immunoblotting, suggesting that multiple types of PCD will be involved in host defense in response to bacterial infection.

Dysregulation or aberration in the induction of PCD is closely linked to the development of several diseases, including neurological, metabolic, autoimmune, and infectious diseases and cancer (133, 214-218). For example, mutations in TNF receptor superfamily member 6 (*TNFRSF6)*, TNFRSF6 membrane-bound ligand and caspase 10 cysteine proteases also influence apoptosis of lymphocytes, resulting in massive accumulation of mononuclear cells within lymphoid tissue and subsequent failure to delete autoreactive cells (220). Additionally, it is reported that the key regulator of pyroptosis, NLRP3, is highly involved in accumulating MDSCs in tumors and inhibiting antitumor T-cell immunity after dendritic cell vaccination (221). Besides, NLRP3 signaling in macrophages drives immunosuppressive $CD4^+$ T-cell polarization in the TME of pancreatic ductal adenocarcinoma (PDA) via IL-1β (222). Clinical study also discovered that excessive production of apoptotic bodies and increased calcification of cartilage tissue induced pyroptosis and, thereby, exacerbated osteoarthritis in patients (223). Collectively, these data

suggest the negative role of disordered PCD in destroying homeostasis and accelerating disease progression.

Our data indicates a novel therapeutic target of PCD in resolving sepsis-induced long-term immunosuppression. During the progression of sepsis, dysregulated PCD keep release of intracellular contents to surrounding environment, escalating the inflammation and cytokine storms. Besides, rapid loss of immune effector cells by disordered PCD leads to profound immunosuppressive condition and inability of host defense against secondary infections. NINJ1 plays a vital role in spreading the consequences brought by PCD to surrounding environment and cells through mediating the PMR to control the release of DAMPs and HMGB-1, which functions as a switch to restrain the severity of PCD dysregulation. Our findings reveal that loss of NINJ1 improves the survival rate of mice post CLP-induced sepsis. Additionally, we also notice that loss of NINJ1 can restore host immune response to *P. aeruginosa* infection during sepsis-induced immune suppression.

In summary, programmed cell death plays a pivotal role in both antibacterial defense and disease-induced immunosuppression, highlighting its dual impact on immune homeostasis. While PCD is essential for controlling infections and mobilizing immune responses, its dysregulation in sepsis contributes to immune exhaustion and susceptibility to secondary infections, complicating recovery. The therapeutic modulation of PCD represents a promising approach to addressing the immunosuppressive phase of sepsis, but requires careful balancing to avoid unintended side effects. Further research into the molecular underpinnings of PCD in the context of sepsis and infection will be crucial for developing targeted treatments that can harness the beneficial aspects of cell death while minimizing its detrimental effects.

4.2 Future direction

PCD in tumor immunotherapy

It is now acknowledged that cancer-related chronic inflammation induces myeloid immunosuppression. In the early acute systemic inflammation phase, a quick shift from steady-state hematopoiesis to emergency granulopoiesis is required in hematopoietic system to meet the high demand for granulocytes and monocytes (224). However, cancer-related inflammation can hijack this emergency granulopoiesis process and drives the generation and expansion of heterogeneous immature myeloid cells with immunosuppressive activities (225, 226).

Tumor-associated macrophages (TAMs) are a major type of immunosuppressive myeloid cells in the tumor microenvironment (TME), and big-data analysis indicates that a high density of TAMs is connected with deterioration in multiple types of cancer (227). Though TAMs are mainly involved in various protumor activities, including TME immunosuppression, angiogenesis, promoting drug-resistance in tumor cells, they can still contribute to antitumor immunity under certain conditions by phagocytosis, antigen presentation, and direct tumoricidal activity (228). For examples, activation of Notch1 signaling pathway was identified to drive the polarization of TAMs into the classically activated phenotype (M1 macrophages), leading to enhanced mitochondrial ROS and upregulated M1-associated genes (229, 230). Nevertheless, the transition between anti-tumor (M1) and pro-tumor (M2) can be regulated by tumor immune microenvironment (TIME) or therapeutic interventions. It is reported that hepatocellular carcinoma (HCC)-derived exosomes could reshape macrophages to M2-polarized TAMs via inducing pro-inflammatory factors and activating NF-κB signaling (231). One of the major factors in promoting TAMs M2-polarization are tumor cell-derived soluble molecules, which in turn promotes tumor progression and metastasis. Tumor cells secrete the sonic hedgehog (SHH) to drive TAM M2 polarization, which

then suppresses the recruitment of $CD8^+$ T cells to TME through inhibiting CXCL9 and CXCL10, mediating TAM immunosuppression mechanism (232). However, the mechanisms of tumor cells-mediated TAM polarization remain unclear.

In chapter 3, we have discussed about our research on role of NINJ1 in sepsis-induced immunosuppression. From both *in vivo* and *ex vivo* experiments, we found that NINJ1 deficiency significantly increased the survival rate of mice post CLP surgery, as well as rescued host anti-bacterial response under sepsis-induced immunosuppression. These findings strongly proposed that NINJ1 has a crucial position in the initiation and progression of sepsis and its following immune suppression. To extend our knowledge of NINJ1's function on immune balance, it attracts our attention that how NINJ1, the newly discovered downstream part of PCD, would function in tumor-mediated immunosuppression.

Loss of NINJ1 maintains the membrane integrity of tumor cells

To examine whether NINJ1 still mediates the plasma membrane rupture during lytic cell death in tumor cells, we engineered mouse *Ninj1* gene deletion using CRISPR-Cas9 method in two lung tumor models, Lewis lung carcinoma (LLC1) and Mouse lung carcinoma (CMT167). Guide-RNA for mouse *Ninj1* gene was designed and purchase from IDT, lnc. and the gene deletion was conducted through electroporation. After monoclones selection, we confirmed the successful deletion of *Ninj1* in both LLC1 and CMT167 cells with immunoblotting, as no NINJ1 protein bands were detected (Fig 4-1AC). Nutrition depletion is often utilized as a method to induce tumor cells PCD during *in vitro* cell experiments, which is also the strategy for chemodrugs in treating various types of tumors. Under the doxorubicin treatment, we found that deletion of NINJ1 significantly reduced release of large molecules in LLC1 cells, like LDH, suggesting NINJ1-

mediated PMR still function in tumor cells (Fig 4-1B). Similarly, under the treatment of etoposide, *Ninj1*$^{-/-}$ CMT167 cells exhibited decreased LDH release when compared with WT CMT167 cells, further supporting that NINJ1-mediated PMR is prevalent in different tumor models (Fig 4-1D).

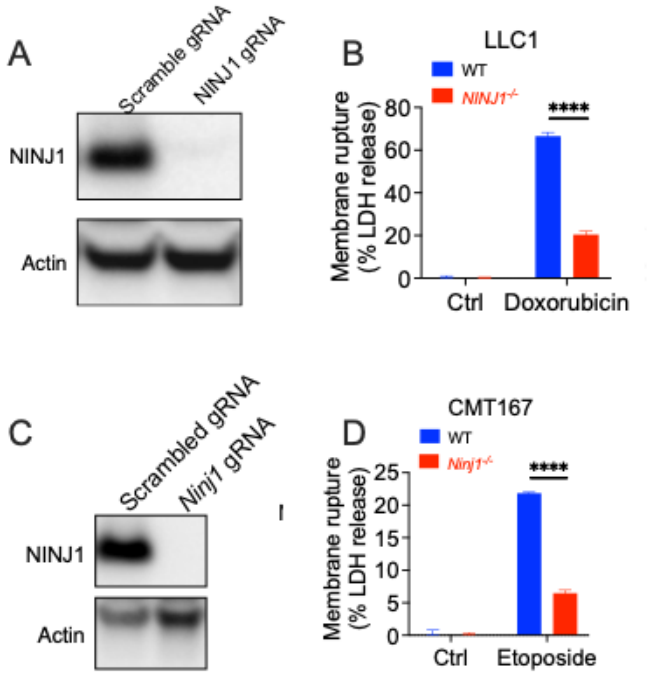

Figure 4-1 Genetically generation and function test of *Ninj1*$^{-/-}$ LLC1 and CMT167 tumor cells.

(A) *Ninj1*$^{-/-}$ LLC1 cells were generated through electroporation-based CRISPR-Cas9 technique. Immunoblotting was performed to determine the results of genetic knockout. (B) WT and *Ninj1*$^{-/-}$ LLC1 cells were treated with Doxorubicin to induce cell death. LDH release assay was conducted to evaluate impact of NINJ1 on LLC1 membrane rupture. (C) *Ninj1*$^{-/-}$ CMT167 cells were generated through electroporation-based CRISPR-Cas9 technique and examined with immunoblotting. (D) WT and *Ninj1*$^{-/-}$ CMT167 cells were treated with Etoposide to induce cell death. LDH release assay was conducted to evaluate impact of NINJ1 on CMT167 membrane

rupture. ****$p < 0.0001$, by Student's unpaired t-test and one-way ANOVA. (Data generated from collaboration with Dr. Xiang Chen)

Loss of NINJ1 in tumor cells suppresses M2 TAMs polarization

As mentioned above, TAMs play a vital role in promoting tumor growth under M2 polarization which is induced by tumor cells and/or immunosuppressive TME. Thus, we hypothesized that NINJ1 regulated M2-polarization of TAMs through modulating the secretion molecules of tumor cells during PCD. To test our hypothesis, we examined the M2 polarization of TAMs under the stimulation of supernatant from WT and *Ninj1*$^{-/-}$ LLC1/CMT167 cells. In order to mimic the TME under tumor cell death by nutrition depletion, we treated both genotypes of LLC1/CMT167 cells with respective chemodrugs and collected the supernatant to co-culture with primary BMMs. The M2 polarization of TAMs was evaluated through RT-qPCR on M2-associated genes expression (Fig 4-2AB). We observed that BMMs treated with *Ninj1*$^{-/-}$ LLC1 sup revealed significantly decreased expression of M2-associated genes, like *Arginase-1*, *Thbs-1*, *Mmp-8*, *Il4ra*, *Nrg-1* (Fig 4-2A) (233-237). While we found BMMs treated with *Ninj1*$^{-/-}$ CMT167 sup exhibited reduced expression of *Arginase-1*, *Thbs-1*, *Mmp-8*, *Il4ra* (Fig 4-2B). According to the gene expression of two different tumor models, deletion of NINJ1 in tumor cells enormously inhibited *Arginase-1* expression in macrophages, which nearly prevents the transition to M2 TAMs. Collectively, we demonstrated that NINJ1 deficiency within tumor cells inhibited M2 polarization of TAMs.

In addition to gene expression results, we also examined the activation of M2-related signaling pathway by immunoblotting. Primary BMMs were co-cultured with sup from WT and

Ninj1$^{-/-}$ LLC1 cells treated with doxorubicin. After 4, 8 and 12h of co-culture, BMMs were collected and processed for immunoblotting. We clearly observed that BMMs treated with sup of *Ninj1*$^{-/-}$ LLC1 cells had prominent decreased Arginase 1 production, which was consistent with previous results from RT-qPCR (Fig 4-2A). Moreover, we also found another key regulator of M2 signaling, p-STAT3, was remarkably inhibited in macrophages with *Ninj1*$^{-/-}$ LLC1 sup. Emerging evidence has demonstrated that phosphorylation of STAT3 is highly involved in macrophage differentiation and promotes M2 polarization (238). While for the key regulator of M1 TAMs, p-STAT1, there is no significant difference exists between BMMs treated with WT LLC1 sup and *Ninj1*$^{-/-}$ LLC1 sup, suggesting NINJ1 deficiency of tumor cells has no influence on M1 polarization of TAMs (Fig 4-3). It is reported that activation of STAT5 is involved in both M1 and M2 TAMs differentiation (239). Therefore, further research needs to be performed to figure out the reduction of p-STAT5 under *Ninj1*$^{-/-}$ LLC1 sup treatment. Notably, in the WT LLC1 sup group, the activation of STAT3 and expression of Arginase 1 are positively associated with the increase time period of co-culture. Interestingly, we also found that the M1 mark, p-STAT1 and pro-inflammatory response, MAPK pathway, had no difference between WT and Ninj1-KO groups, suggesting NINJ1 deficiency had no influence on M1 TAM polarization. In sum, these findings strongly support our hypothesis that NINJ1 located in tumor cells regulates M2-polarization of TAMs through modulating the secretion molecules of tumor cells during PCD.

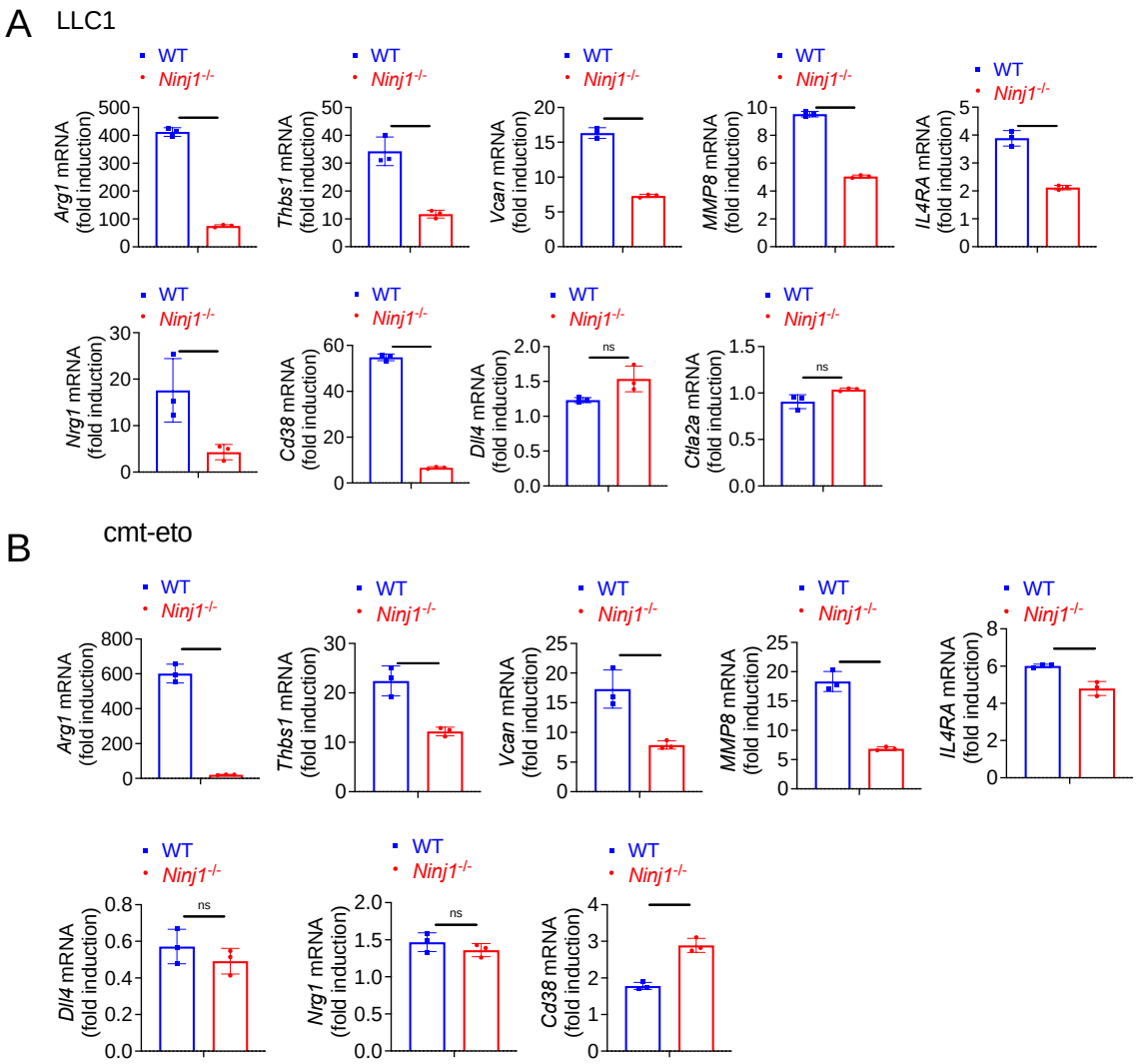

Figure 4-2 NINJ1 deficiency suppresses M2-associated genes expression in TAMs.

(A) Genes associated with M2 TAMs polarization were measured by RT-qPCR on TAMs treated with supernatants of WT and *Ninj1*$^{-/-}$ LLC1 cells (cell death induced). (B) Genes associated with M2 TAMs polarization were measured by RT-qPCR on BMMs treated with supernatants of WT and *Ninj1*$^{-/-}$ CMT167 cells (cell death induced). Data in (A) – (B) are shown as mean ± SD. Each symbol represents a technical replicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, by Student's unpaired t-test and one-way ANOVA.

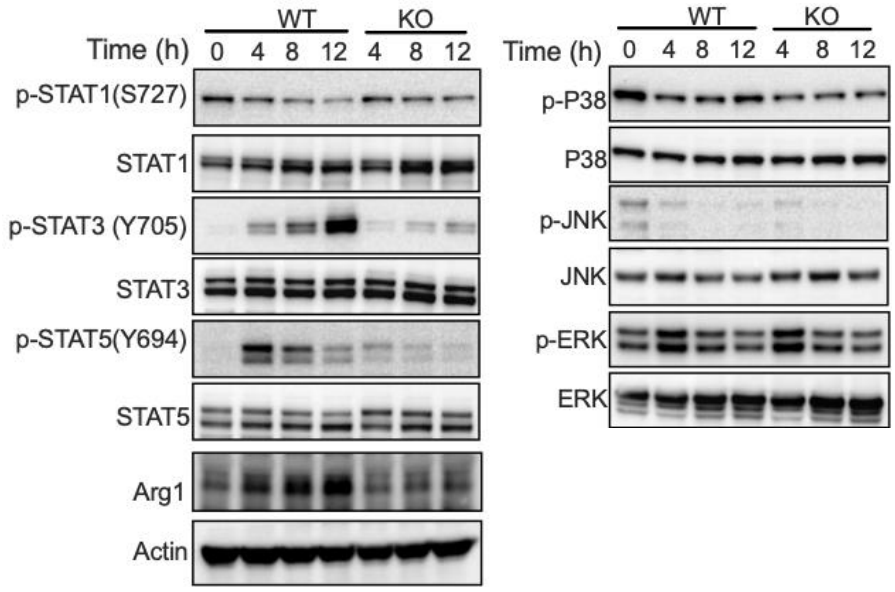

Figure 4-3 Loss of NINJ1 inhibits M2-related signaling activation in TAMs.

Immunoblotting of p-STAT1 and STAT1, p-STAT3 and STAT3, p-STAT5 and STAT5, Arginase 1, p-P38 and P38, p-JNK and JNK, p-ERK and ERK. TAMs were collected after 4h, 8h and 12h co-culture with supernatants of WT and *Ninj1*$^{-/-}$ LLC1 cells (cell death induced). Data are representative of at least three independent experiments.

RBM22 functions as the key factor in promoting M2 polarization in TAMs

As mentioned above, NINJ1 mediates the tumor cell membrane rupture after nutrition depletion treatment, controlling the proteins release which contribute to the immunosuppressive status of TME and TAMs M2 polarization. In order to narrow the range of target proteins, we collected the supernatant from WT and *Ninj1*$^{-/-}$ LLC1 cells with doxorubicin treatment and separated the sup with 10Kda dialysis tubes. Primary BMMs were co-cultured with different groups of supernatant and collected for RT-qPCR to evaluate M2-related gene expression. We clearly found that BMMs exhibited almost no Arginase 1 expression when treated with supernatant

contained small size proteins (<10Kda) from either WT or *Ninj1*$^{-/-}$ LLC1 cells (Fig. 4-4A). However, the difference of Arginase 1 expression appeared and was consistent with previous results when the BMMs were treated with supernatant contained large size proteins (>10Kda), suggesting the protein(s) that promotes M2 macrophage differentiation is/are large size protein(s).

After Secretomics analysis of WT and *Ninj1*$^{-/-}$ tumor models including LLC1 and CMT167 through mass spectrometry, we have identified a bunch of molecules are retained in tumor cells with the absence of NINJ1, including RBM22, RBM25 and RBM39 (Fig. 4-4B). To examine which molecule is the key factor regulated by NINJ1 in promoting M2 polarization of TAMs, we generated *Rbm22*$^{-/-}$, *Rbm25*$^{-/-}$ and *Rbm39*$^{-/-}$ LLC1 and CMT167 cells with CRISPR-Cas9 technique. Later, we treated each genotype-knockout LLC1 tumor cells with respective chemo drugs and collected the supernatant to co-culture with primary BMMs. The M2 polarization of TAMs was evaluated through RT-qPCR on M2-associated genes expression (Fig. 4-4C). Additionally, we found that only *Rbm22*$^{-/-}$ group exhibited the same phenotypes with *Rbm22*$^{-/-}$ group, as remarkable reduced expression of M2-asscoiated genes, *Arginase-1*, *Thbs-1*, *Nrg-1*, *Il4ra* (Fig. 4-5). Therefore, we proposed that Rbm22, whose release is mediated by NINJ1, functions as the key factor in promoting M2 TAMs.

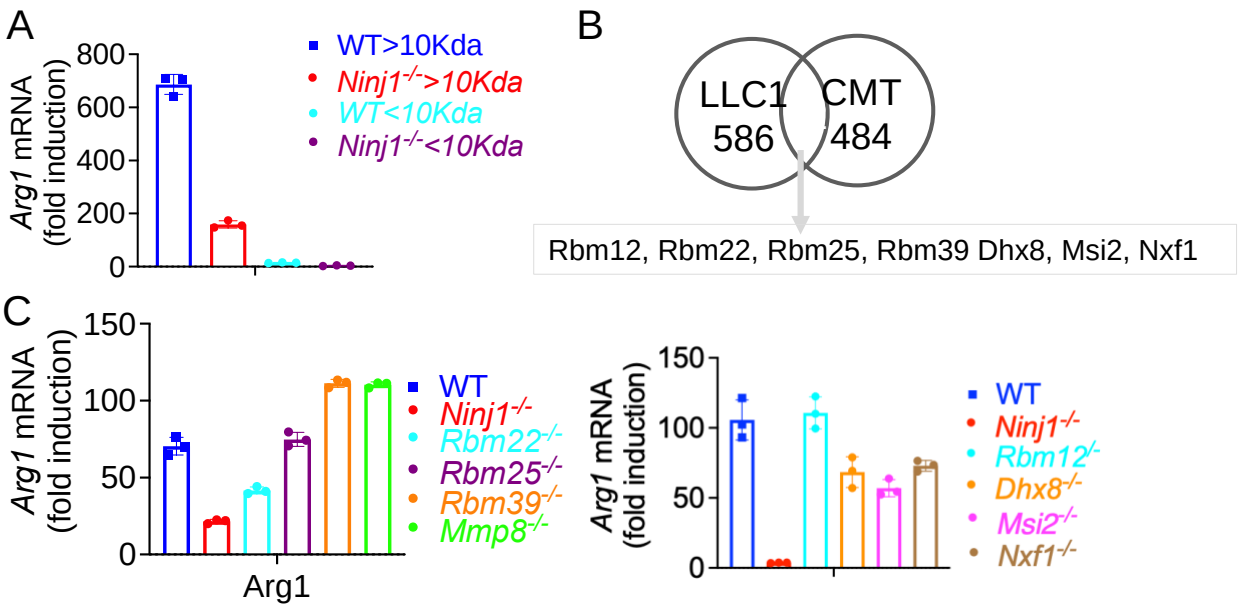

Figure 4-4 NINJ1 regulates M2 TAM polarization through mediating release of RBM22.

(A) Expression of TAM M2 differentiation gene, Arginase 1, was measured by RT-qPCR. Macrophages were co-cultured with dialysis-treated supernatants of WT or *Ninj1*$^{-/-}$ LLC1 cells (cell death induced). (B) Secretomics analysis on WT and *Ninj1*$^{-/-}$ LLC1 and CMT167 cells. (C) Expression of Arginase 1 was measured by RT-qPCR. Macrophages were co-cultured with supernatants of WT, *Ninj1*$^{-/-}$, *Rbm22*$^{-/-}$, *Rbm25*$^{-/-}$, *Rbm39*$^{-/-}$, *Mmp8*$^{-/-}$, *Rbm12*$^{-/-}$, *Dhx8*$^{-/-}$, *Mis2*$^{-/-}$, *Nxf1*$^{-/-}$ LLC1 cells (cell death induced). Data is shown as mean ± SD. Each symbol represents a technical replicate.

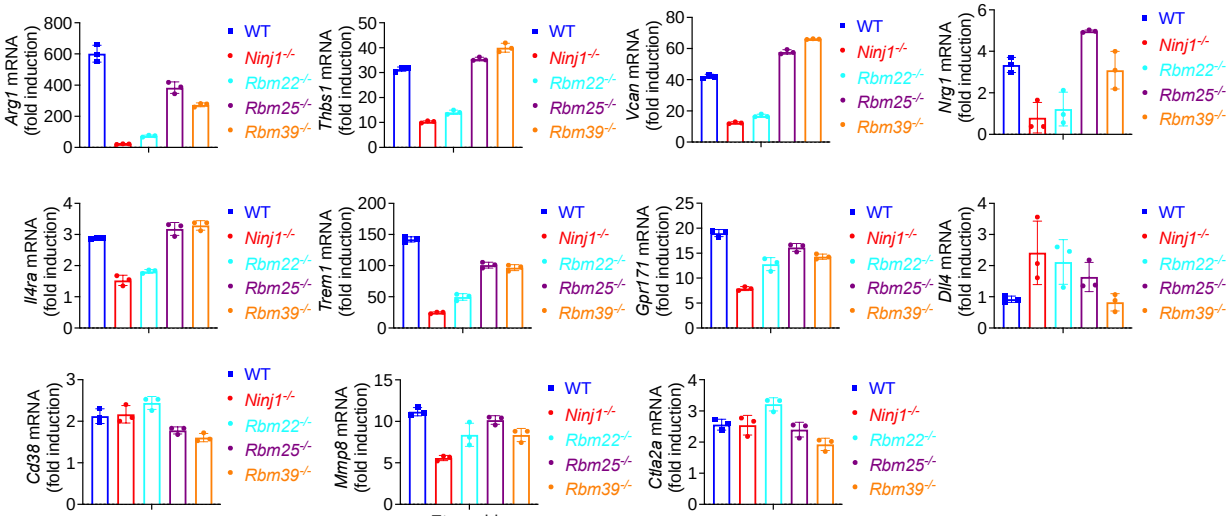

Figure 4-5 Release of RBM22 mediated by NINJ1 promotes M2 TAM differentiation.

Genes associated with M2 TAM polarization were measured by RT-qPCR on TAMs co-cultured with supernatants of WT, *Ninj1*$^{-/-}$, *Rbm22*$^{-/-}$, *Rbm25*$^{-/-}$ and *Rbm39*$^{-/-}$ LLC1 cells (cell death induced). WT and *Ninj1*$^{-/-}$ groups functioned as positive and negative control, respectively. Data is shown as mean ± SD. Each symbol represents a technical replicate.

Recombination, Expression and purification of RBM22

In order to further identify RBM22 is the key factor regulated by NINJ1 that promotes M2 TAMs differentiation in TME, we plan to complement RBM22 protein into supernatant from *Ninj1*$^{-/-}$ LLC1/CMT167 cells and then co-culture with primary BMMs to evaluate M2-associated genes expression. To obtain RBM22 protein, we first constructed pET-24b with mouse *Rbm22* gene and transfected *E.coli* with the plasmid. Through the induction of IPTG, we successfully expressed mouse RBM22 protein and confirmed with electrophoresis. RBM22 band was detected at the presence of IPTG in both supernatant and pellet samples, while no RBM22 was expressed

without IPTG induction (Fig 4-6A). Later, we purified RBM22 protein with dialysis (Fig 4-6B). Based on the results from Coomassie blue staining, we confirmed to obtain purified RBM22 protein for following both *in vitro* and *in vivo* complementarity experiments.

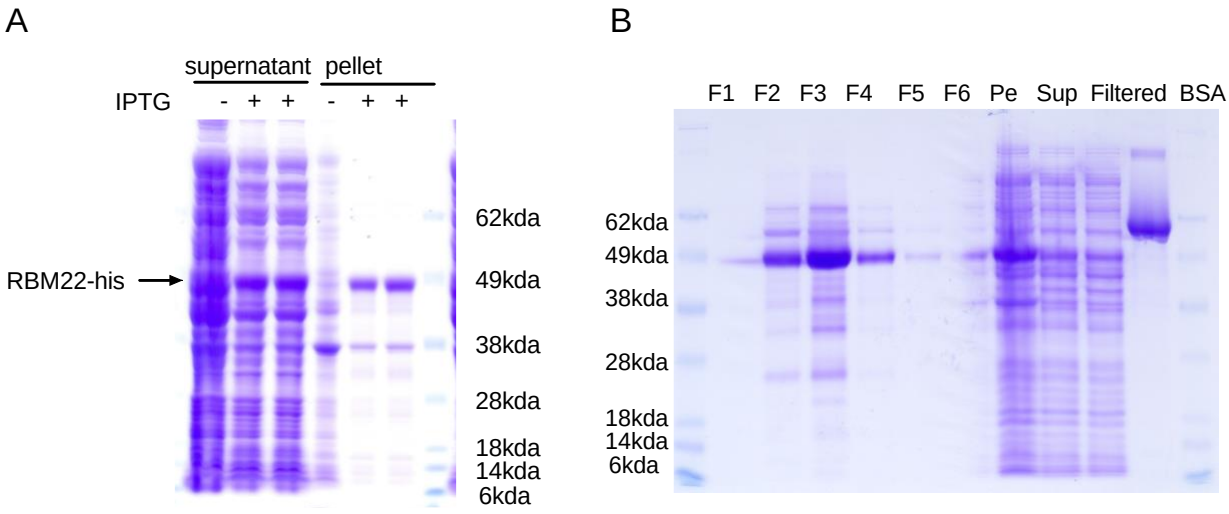

Figure 4-6 Expression and purification of RBM22.

(A) RBM22 expression plasmid was constructed in pET-24b and expressed in *E.coli* under the induction of IPTG. Coomassie blue staining was performed to determined expression of RBM22. (B) RBM22 was purified through multiple times of dialysis and confirmed with Coomassie blue staining.

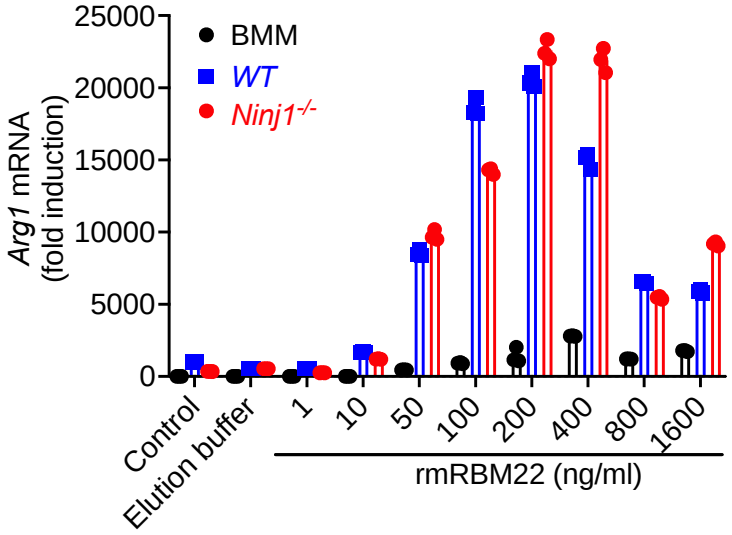

Figure 4-7. Recombination of RBM22 on TAM M2-related gene expression.

Recombination of multiple concentrations of RBM22 with co-culture of TAMs and sup from WT and *Ninj1*$^{-/-}$ LLC1 cells. M2 mark gene, Arginase 1, was evaluated with RT-qPCR. (Data generated from collaboration with Dr. Xiang Chen)

Collectively, we have identified the crucial impact of NINJ1 on tumor-mediated immunosuppression. Through deletion of NINJ1 in tumor cells, large pro-tumor molecules including RBM22 are retained within tumor cells after cell death induction, resulting in prominent inhibition on pro-tumor (M2) TAMs differentiation and immunosuppressive atmosphere in TME. After successfully expression and purification of RBM22, we added back RBM22 on co-culture of TAMs and sup of LLC1. We found that recombination of RBM22 recovered the difference of Arginase 1 expression in TAMs treated with WT and *Ninj1*$^{-/-}$ LLC1 sup. Besides, 200ng/ml of RBM22 generated the highest expression level of Arginase 1 in TAMs (Fig. 4-7). Future works will focus on the *in vivo* complementarity experiment to further confirm that RBM22 is the major factor released during tumor cell death to promote type 2 transition of immune effector cells in TME. This study will provide us with novel therapeutic targets in developing clinical tumor treatments.

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