ZBP1-Mediated Necroptosis

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Contributor: Xin-yu Chen, Ying-hong Dai, Xin-xing Wan, Xi-min Hu, Wen-juan Zhao, Xiao-xia Ban, Hao Wan, Kun Huang, Qi Zhang, Kun Xiong

Cell death is a fundamental pathophysiological process in human disease. The discovery of necroptosis, a form of regulated necrosis that is induced by the activation of death receptors and formation of necrosome, represents a major breakthrough in the field of cell death in the past decade. Z-DNA-binding protein (ZBP1) is an interferon (IFN)-inducing protein, initially reported as a double-stranded DNA (dsDNA) sensor, which induces an innate inflammatory response. ZBP1 was identified as an important sensor of necroptosis during virus infection. It connects viral nucleic acid and receptor-interacting protein kinase 3 (RIPK3) via two domains and induces the formation of a necrosome.

Keywords: ZBP1 ; PANoptosis ; pyroptosis

1. Introduction

Cell death is a fundamental pathophysiological process in various diseases. According to the type of death process, cell death can be divided into two major groups: programmed cell death (PCD), a precise and genetically controlled cellular death process, and non-PCD, also called necrosis. In past decades, PCD has been indicated to play important roles in the development of human diseases and immune response [1].

Apoptosis is the first programmed cell death pathway to be identified [2][3]. This cell death mainly occurs in the process of development and aging, while it can occur under a variety of pathological stimuli in the immune defense [4]. When apoptosis occurs, it shows cell shrinkage, condensation of the chromatin, formation of an apoptosome, and phagocytosis [5]. The execution of this pathway is considered to be related to the Bcl-2 protein family and the Cysteinyl aspartic acid protease (Caspase) family [6][7].

Necrosis, as opposed to apoptosis, refers to a passive death when cells are injured, which is characterized by cytoplasmic swelling, membrane rupture, and the release of intracellular contents [8]. Necroptosis is a form of regulated necrosis controlled by receptor-interacting protein (RIP) kinases (RIPKs) [9]. However, it is found that the tumor necrosis factor (TNF) pathway, which induces apoptosis, can also mediate the occurrence of necroptosis under certain conditions [10]. In addition, other PCD pathways can also occur along with necroptosis [11].

Pyroptosis is a new type of PCD found in recent years, which is a type of typical inflammatory cell death. It mostly occurs in infectious diseases [12]. Morphologically, the formation of membrane pores, breaking of the plasma membrane, and release of cell content cause a strong inflammatory response in pyroptosis [13]. Inflammasomes play a major role in the process of pyroptosis, which activates Caspase family members to promote the activation of pro-inflammatory cytokines IL and gasfermin protein. In recent years, it has been found that there is crosstalk between various PCD pathways, and the discovery of key factors that can widely regulate these processes is a research hotspot.

2. ZBP1, the Innate Sensor

2.1. Structure of ZBP1

ZBP1 contains two N-terminal ZBDs (Zα1 and Zα2), at least two RIP homotypic interaction motif domains (RHIM1 and RHIM2), and one C-terminal signal domain (SD) [14]. Zα2 domain plays a key role in sensing Z-DNA and Z-RNA. Relevant studies demonstrated that specific mutations in this region effectively block the recognition of ZBP1 with vRNA or endogenous Z-NA, thereby inhibiting subsequent cell death and inflammation [15]. This domain is also the target of many ZBP1 inhibitors, including vaccinia virus (VACV) E3 protein and ADAR1 [16][17]. The RHIM domain mediates cell death. ZBP1 combines with receiver interacting protein kinase 3 (RIPK3) via the RHIM domain [18]. ZBP1 promotes RIPK3 autophosphorylation and induces phosphorylation of mixed linear kinase domain-like (MLKL), the downstream necroptosis executor, to induce necroptosis. In the presence of RIPK1, a protein with the same RHIM domain, the binding of ZBP1 to

RIPK3 is inhibited by RIPK1 competition [19]. Murine cytomegalovirus (MCMV) M45 protein, which is a co-purified protein in virus and host immune defense system, also carries an N-terminal RHIM domain. It inhibits necroptosis by simulating the interaction between RIPK1 and RIPK3 to form a heterogeneous amyloid structure [20]. The SD domain of ZBP1 recruits TANK-binding kinase-1 (TBK1) and IFN regulatory factor 3 (IRF3) to activate type I IFN synthesis and other inflammatory reactions [21]. However, the ZBP1-IRF3 axis also mediates the proliferation of myeloma cells [22].

As a sensor of Z-NA, ZBP1 mainly relies on its Zα domain to identify ligands. In the middle part of ZBP1, there are at least two RHIM domains, which can bind with other RHIM-containing proteins (such as RIPK1, RIPK3, and TRIF) and mediate downstream signal transduction. These two special domains may also become targets for ZBP1 inhibition. For example, the M45 protein of MCMV can inhibit ZBP1-mediated cell death with its RHIM domain. While ADAR1-P150 is an inhibitor with ZBP1 by the Zα domain hindering the activation of ZBP1, it has a unique extra Zα domain, compared with the invalid subtype ADAR1-P110. Zα1, Zα2, Z-α, and Z-β are Z-DNA binding domains. SD: Signal domain; KD: Kinase domain; ID: Intermediate domain; DD: Death domain; TIR: Toll/interleukin-1 receptor domain; RNR-LIKE: Ribonucleotide reductase-like domain.

2.2. ZBP1 Binds Viral Z-NA to Mediate Inflammatory Response and Host Defense Response

The molecule most closely relevant to ZBP1 is undoubtedly IFN. ZBP1 expression is induced by IFN and also induces IFN responses [23]. This association with IFN suggests that ZBP1 plays an indispensable role in the inflammatory response and host defense [24]. Since ZBP1 contains ZBD, studies investigated the type of Z-DNA it binds to and the induced immune response [25]. Preliminary studies reported that both B-DNA and Z-DNA derived from multiple sources (synthetic DNA or DNA of bacterial, viral, or mammalian origin) induce strong expression of ZBP1 and IRF to mediate IFN expression and antiviral response [26]. The recognition of Z-RNA by ZBP1 of influenza virus (IAV) resulted in necroptosis [27]. Here, ZBP1 acted as an innate sensor of IAV recognizing Z-RNA in the viral ribonucleoprotein (vRNP) complex to induce necroptosis to resist virus infection. ZBP1 also induced interleukin-1α (IL-1α) in IAV via NOD-like receptor (NLR) family pyrin domain-containing 3(NLRP3) and recruited pulmonary neutrophils, resulting in inflammation [28]. Further studies proved that defective viral genes (DVGs) of IAV and other orthomyxoviruses produced Z-DNA, which were sensed by ZBP1, and induced cell death and inflammatory responses [29]. In addition, ZBP1 sense endogenous Z-NA in mice to induce cell death and skin inflammation, especially in the case of RIPK1 and Caspase-8 mutations [30]. ZBP1 acts as a cytoplasmic DNA receptor in many types of pathogenic infections, including *Toxoplasma gondii* infection [31][32], Fungi [33], and *Yersinia pseudotuberculosis* [34]. However, it remains to be confirmed whether Z-NA can be produced in these pathogens and other viruses for ZBP1 sensing.

2.3. ZBP1 Senses Endogenous Z-NA and Induces Cell Death

For a long time, studies have focused on the role of ZBP1 in sensing viral nucleic acid in virus-induced cell death, but whether ZBP1-mediated cell death in non-viral infections can detect endogenous ligands remains to be explored [35]. Jonathan et al. reported the recognition of endogenous nucleic acids in noninfectious cells with high expression of ZBP1 [36]. Further, photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) analysis demonstrated that ZBP1 binds to RNA rather than DNA, and these nucleic acids may be in the Z-conformation.

New progress was made in 2020 [30]. The team found that ZBP1 recognition of endogenous Z-NA triggered inflammation and cell death in RIPK1-deficient mice, which led to skin inflammation. In addition, ZBP1 can also sense endogenous ligands to trigger cell death resulting in colitis in mice by inhibiting FADD-Caspae-8 signal transduction [37]. ZBP1 binds to endogenous dsRNA via the Zα domain, which is most likely mediated by endogenous retroelements (ERE). In EREs, B2 and Alu SINEs have the greatest potential to form dsRNA [38]. They were specifically expressed in the epidermis and formed dsRNA to induce cell death and skin inflammation in RIPK1-deficient mice [39]. ADAR1 carries a Zα domain, which can edit dsRNA produced by ERE, suggesting that ADAR1 may play an important role in mediating the recognition of endogenous nucleic acid by ZBP1. In recent years, some studies reported the regulatory effect of ADAR1 on ZBP1-mediated cell death and inflammation and identified ADAR1 as a negative regulator of ZBP1 [40].

ADAR1 can be classified into two subtypes, P110 and P150. P150 can be induced by IFN and plays a major role in regulating ZBP1 [41]. Compared with P110, P150 contains additional Zα domains and nuclear output signals (NESs), which determine its ability to translocate into the cytoplasm and interact with ZBP1. The negative regulation of ADAR1 on ZBP1 occurs via inhibition of Z-RNA- and ZBP1-dependent cell death by preventing the accumulation of mRNA transcripts, which form Z-RNA [42]. However, it is directly associated with ZBP1 Zα domain interactions, which hinder the recognition of endogenous Z-NA. In ADAR1-deficient mice, ZBP1 mediates RIPK3-dependent cell death and MAVS-dependent pathogenic type I IFN response [43]. Further, Caspase-8-dependent apoptosis also contributes to the disease under ADAR1 deficiency, which is induced by the constitutive combination of ZBP1 and RIPK1 [44]. Caspase-8 also

inhibits the ZBP1-mediated nuclear factor-kappaB (NF-κB) inflammatory pathway. Further investigations suggested that endogenous Alu dsRNA may be the ligand recognized by ZBP1 in the case of ADAR1 deficiency [45]. Nonetheless, a related study also identified and confirmed a small molecule, CBL0137, which promoted the utilization of Z-DNA conformation by the genome sequence [44]. Therefore, CBL0137 generates a large amount of endogenous Z-DNA and induces ZBP1-dependent cell death in tumor stromal fibroblasts during ADAR1 inhibition.

Both ADAR1 and ZBP1 are induced by IFN, but ADAR1-P150, one of its subtypes, can inhibit the function of ZBP1. ADAR1-P150 attenuates the synthesis of endogenous Z-RNA in the nucleus and inhibits the recognition of Z-NA of ZBP1 by combining with it in the cytoplasm. A small molecule drug CBL0137 promotes the synthesis of endogenous Z-DNA in the nucleus and plays an important role in inducing the ZBP1-mediated signal pathway. When ADAR1 is defective, ZBP1 mainly causes two forms of cell death: necroptosis, and apoptosis, which depend on the recognition of the Zα2 domain. Necroptosis is mainly caused by the ZBP1-mediated activation of the RIPK3-MLKL signal axis, while apoptosis is caused by the constitutive combination of ZBP1 and RIPK1 to induce the activation of Caspase-8. Caspase-8 can also inhibit the effects of ZBP1 and RIPK3 to inhibit necroptosis. In addition, ZBP1 also promotes type I IFN responses by inducing the mitochondrial antiviral-signaling (MAVS) pathway.

3. ZBP1 Mediates Necroptosis

In previous studies, necrosis was considered to be a passive and unregulated form of cell death [3][46][47]. However, in recent years, a special form of programmed cell death, namely necroptosis, has been reported [48][49][50][51]. It is characterized by necrotic death and is also regulated by related molecules, including RIPK1/3 [52][53][54][55]. This kind of programmed cell death can be induced by multiple factors, including TNF, IFN, LPS, dsRNA, DNA damage, and endoplasmic reticulum stress [56][57].

Necroptosis is caused by a combination of different ligands with TNF family death domain receptors, pattern recognition receptors, and virus sensors via an independent and unified downstream pathway [58][59][60]. TNF-induced necroptosis is the most studied and classic pathway, which is mediated by RIPK1, RIPK3, and MLKL [61][62][63]. TNF binds to the corresponding receptor (TNFR1), and its death domain TRADD binds and activates RIPK1. In the absence of Caspase-8, FADD is further recruited to form a complex, which acts on RIPK3 to activate phosphorylation and oligomerization [64][65][66][67]. Finally, the necrosome composed of RIPK3 activates the MLKL protein. MLKL is activated by phosphorylation at different sites in different species [68][69][70]. Human MLKL is phosphorylated at Thr357, Ser358, Ser345, and Ser347, whereas mouse MLKL is phosphorylated at Thr349 and Ser352 [71]. As an executor, MLKL changes its conformation after activation via RIPK3 phosphorylation. It releases four helical bundle domains, followed by translocation from the cytoplasmic matrix to the cell membrane, leading to structural disintegration of the plasma membrane [57][72][73]. The leaked cellular components may bind to the original and surrounding cells as ligands to further induce necroptosis.

ZBP1 is the master regulator of one of the induction pathways of necroptosis, which is mainly caused by virus infection [74]. It is associated with the induction and execution of necroptosis. The biggest difference between this pathway and the classical pathway lies in the role played by RIPK1, which often exists as a negative regulator of necroptosis in the ZBP1-mediated pathway [19][39][75]. RIPK3 and MLKL mediate the signal transduction at the final stages of necroptosis by integrating different signals to determine the extent of necrosis.

3.1. ZBP1 Interacts with Key Molecules in Necroptosis Signal Transduction via RHIM Domain

The signal transduction of necroptosis involves four proteins carrying RHIM domains, namely ZBP1, RIPK1, RIPK3, and TRIF [76]. The role of TIR domain-containing adaptor inducing interferon-β (TRIF) is similar to that of ZBP1 in necroptosis. As an adapter of Toll-like receiver 3/4 (TLR3/4), it interacts with RIPK3 to mediate necroptosis [77]. ZBP1 is associated with another initiating pathway, which induces necroptosis by combining the RHIM domain with RIPK3. RIPK1 also regulates this process via the RHIM domain.

3.1.1. ZBP1 Combines with RIPK3 during the Formation of Necrosome

The necrosome was first proposed in the typical necroptosis pathway induced by TNF [78]. It is a cytoplasmic amyloid complex, mainly composed of activated RIPK1, RIPK3, and MLKL, which trigger necroptosis [79]. The core function of the necrosome is to promote the recruitment and phosphorylation of RIPK3 and MLKL. In the TNF pathway, RIPK1 promotes the autophosphorylation and activation of RIPK3. While in ZBP1-mediated necroptosis, ZBP1 induces the autophosphorylation of RIPK3. The interaction between ZBP1 and RIPK3 is also sufficient to generate another type of necrosome and activate MLKL. RIPK1 plays the opposite role in this pathway and inhibits necroptosis. During mouse development, the deletion of RIPK1 induces ZBP1-mediated necroptosis and apoptosis, resulting in perinatal death [19][75]

[80]. The loss of keratinocyte RIPK1 triggers skin inflammation and necroptosis [39]. RIPK1 has no kinase activity without induction of TNF and other factors. However, it can bind to RIPK3 via the RHIM domain, and it cannot promote RIPK3 phosphorylation. In this case, other proteins that activate necroptosis, such as TRIF and ZBP1, cannot bind to RIPK3, suggesting that RIPK1 inhibits ZBP1-mediated necroptosis.

When viruses or endogenous Z-NA are accumulated, ZBP1 plays a critical role in the induction of necroptosis. After its Zα2 domain sensed Z-NA, ZBP1 can phosphorylate and activate RIPK3 by directly binding to it, which depends on their RHIM domains. The activated RIPK3 spontaneously oligomerizes to form necrosomes and induces activation and oligomerization of MLKL to carry out necroptosis. Therefore, the function depending on this domain is inhibited by other proteins with the RHIM domain, including RIPK1 and M45 protein in MCMV. In addition, LPS produced in other pathogenic infections can also recognize TLR4 receptors on the cell membrane to induce the activation of RIPK3 to form necrosomes, and the connection between this receptor and RIPK3 is also realized by the protein with RHIM domain, TRIF. Another classic pathway of necroptosis is mediated by TNF, which can recognize more abundant pathogenic signals. Excessive TNF binds to TNFR, which can combine with FADD and RIPK1 to form a complex which activates RIPK3 to promote the formation of necrosomes.

3.1.2. Combination of ZBP1 and RIPK1

Both RIPK1 and ZBP1 contain RHIM domains, suggesting direct interaction [81]. ZBP1, as an RHIM protein, not only participates in necroptosis but also regulates apoptosis using Caspase-8 as the main executor by controlling the formation of a complex called TRIFosome [34]. TRIFosome is composed of ZBP1, RIPK1, FADD, and Caspase-8. In the case of LPS induction, TLR4 recruits RIPK1 via TRIF bound to ZBP1, resulting in the assembly of TRIFosome, followed by the activation of Caspase-8, resulting in apoptosis [26]. In addition, the formation of this complex is also crucial to inflammasome activation. In another study, the interaction between ZBP1 and RIPK1 also activated the NF-κB pathway [18], which led to the activation of type I IFN and other cytokines.

3.2. ZBP1 Mediates the Activation of NLRP3 Inflammasome to Induce PANoptosis

NLRP3 is a typical inflammasome sensor, which initiates the assembly of the inflammasome in the innate immune system [46][82]. NLRP3 inflammasome mediates the activation of Caspase-1 to induce pyroptosis during IAV infection, which is a typical inflammatory cell death [74]. NLRP3 recruits and activates Caspase-1 via apoptosis-associated speck-like protein containing a CARD (ASC) carrying the Caspase recruitment domain. The activated Caspase-1 enables the pro-inflammatory factor pro–IL-1 by cleaving β pro-IL-18, resulting in the activation of pro-IL-18. The N-terminal of the pole-forming protein gasdermin D (GSDMD) is released, which simultaneously induces a pro-inflammatory reaction and pyroptosis [83]. The upstream of NLRP3 is regulated by the RIPK1-RIPK3-Caspase-8 axis mediated by ZBP1 [84].

However, the role of NLRP3 inflammasome mediated by ZBP1—extends to PANoptosis, which was first proposed in 2019 to describe cell death and inflammation caused by IAV infection [85]. PANoptosis represents a combination of pyroptosis, apoptosis, and necroptosis, which are common regulatory mechanisms with mutual crosstalk [86][87]. The three play a redundant role in the initiation and amplification of inflammation [88]. When one of the pathways is blocked, an antiviral inflammatory reaction can still occur. ZBP participates in PANoptosis by triggering the assembly of a signal conduction complex. ZBP1-NLRP3 inflammasome may be assembled with ZBP1-RIPK3-FADD-cassase-8 complex to form large multi-protein complexes constituting a PANoptosome [89]. This complex is involved in NLRP3 inflammasome-dependent pyroptosis, Caspase-8-mediated apoptosis, and MLKL-driven necroptosis [90]. This concept has been extended from IAV to HSV1, coronavirus [91], Fungi [33], *Yersinia* [92], tumor [93], and nerve injury [94] and is under continuous development.

PANoptosis represents a combination of pyroptosis, apoptosis, and necroptosis, which is mediated by ZBP1 following IAV infection and other viral infections and inflammation. After sensing a large amount of IAV Z-RNA, ZBP1 can combine with RIPK1, RIPK3, Caspase-8, FADD, NLRP3, ASC, and Caspase-1 to form a giant complex called the PANoptosome. Among these molecules, RIPK1, RIPK3, FADD and caspase-8 are related to apoptosis. The activation of the molecules ultimately induces activation of caspase-8, which acts on the executor Caspase-3/6/7 and leads to apoptosis. While RIPK3 is mainly related to necroptosis, RIPK1 and FADD are also considered to play a positive role in the occurrence of necroptosis. The activation of RIPK3 directly activates and oligomerizes MLKL, the executor of necroptosis, to form an ion channel that destroys the plasma membrane. NLRP3, ASC, and Caspase-1 are key molecules in the occurrence of pyroptosis. They can form NLRP3 inflammasomes to promote the generation of final executors of pyroptosis. NLRP3 is responsible for sensing the corresponding stimulus. ASC has a PYD domain and a CEAD domain that can be recruited by NLRP3 and then recruit Caspase-1. Caspase-1 cleaves and activates the final executor, GSDMD. Pyroptosis is mainly caused by the N-terminal domain of GSDMD, which can transfer to the cell membrane and promote pore formation, leading to the release of pro-inflammatory cytokines IL-1 β and IL-18.

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