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Exploring the Roles of RIPKs in Immune Responses, Inflammation, and Cell Death: Recent Findings

Introduction

The RIPK family comprises seven proteins (RIP1, RIP3, RIP4, RIP5, RIP6, RIP7, and DIK) that are involved in cytokine stimulation, pathogen infection, DNA damage, and inflammation signaling pathways. They help to initiate various responses, from immune cell activation to cell death. They are also essential sensors of intracellular and extracellular stresses, regulating programmed necrosis, apoptosis, and other cell death pathways. RIPKs are critical for proper immune function and modulating the NF-kB pathway and cell death program [1,2,3].

The ProSci portfolio brings more than 25 years of antibody expertise in developing high quality single domain Variable Heavy domain of Heavy chain (VHH), recombinant, monoclonal, and polyclonal antibodies. In addition, our custom antibody services provide the flexibility to tailor antibody development and production for almost any need. This white paper provides examples of the use of ProSci RIPK antibodies and highlights recent findings on the roles of RIPKs in signaling pathways involved in immune responses, inflammation, and cell death.

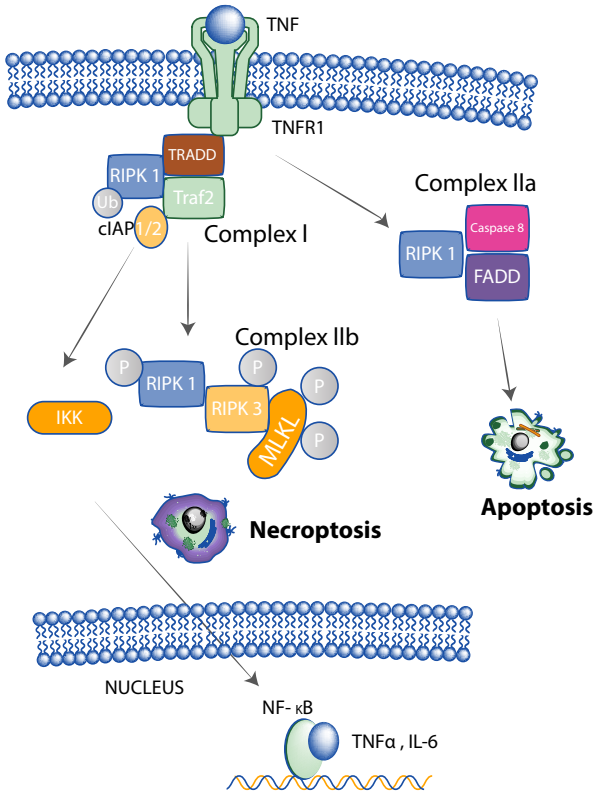

Fig. 1 RIPK1's influence on apoptosis vs. necroptosis.

RIPK3-ZBP1 Interaction Facilitated by Caspase-6:

Essential Role in Inflammasome Activation and Host Defense Against Influenza A Virus

The paper published in 2020 by Zheng et al. titled "Caspase-6 Is a Key Regulator of Innate Immunity, Inflammasome Activation, and Host Defense" demonstrated that caspase-6 modulates innate immunity, inflammasome activation, and multiple cell death pathways [4].

Caspases are a family of cysteine proteases essential in regulating various biological processes such as host cell death, innate immune responses, and homeostasis [5]. Caspases are categorized into inflammatory caspases (caspase-1, 4, 5, 11, and 12) and apoptotic caspases (caspase-2, 3, 6, 7, 8, 9, and 10). The apoptotic caspases can be further categorized into the initiator caspases (caspase-2, 8, 9, and 10) and the effector (or executioner) caspases (caspase-3, 6, and 7) [6]. The significance of caspase-6 in innate immunity remains an essential focus of the investigation, as its precise mechanisms in regulating cell death, inflammasome activation, and host defense mechanisms are still not fully understood.

Caspase-6 plays a crucial role in defending the host against Influenza A virus (IAV) infection. To examine the impact of caspase-6 deficiency, both Casp6+/– and Casp6–/– littermate mice were infected with IAV via the intranasal route. The findings revealed that a higher number of Casp6–/– mice succumbed to IAV infection. The authors conducted further investigations to understand the involvement of caspase-6 in cell death pathways induced by IAV, including pyroptosis, apoptosis, and necroptosis. They observed that Casp6–/– mice exhibited decreased levels of IL-1β in bronchoalveolar lavage fluid (BALF) on day seven post-infection and a significant increase in viral titer in their lungs compared to wild-type (WT) mice.

Interestingly, caspase-6 appears to function independently of priming, STAT1 activation, IFN signaling, and ZBP1 expression. Moreover, the results demonstrated that caspase-6 is not essential for canonical NLRP3 inflammasome activation, NLRC4 inflammasome activation, AIM2 inflammasome activation, or PYRIN inflammasome activation in response to pathogen infections. The CRISPR gene editing technique was employed to knockout caspase-6 in mouse embryonic fibroblasts (MEFs) to validate these findings at the cellular level further. The researchers observed reduced cell death following IAV infection compared to WT MEFs.

Furthermore, WT and caspase-6-deficient bone marrow-derived macrophages (BMDMs) were stimulated with ATP or nigericin or infected with various bacteria and viruses. It was found that the reintroduction of caspase-6 into Casp6-/- BMDMs led to increased caspase-1 activation induced by IAV infection both in its WT and catalytically dead forms. This suggests that the caspase activity of caspase-6 is not necessary for inflammasome activation.

The ProSci rabbit anti-RIPK3 antibody (ProSci Cat #: 2283) was used to immunoprecipitate RIPK3 during IAV infection to determine the role of RIPK3 and caspase-8 in caspase-6 activation during IAV infection. Rabbit anti-RIPK3 antibody was also used in co-immunoprecipitation to investigate the interaction between caspase-6 and RIPK3 and their interaction with ZBP1 and RIPK1.

These findings suggest that caspase-6 plays a vital role in regulating multiple cell death pathways in response to DAMPs or PAMPs and protecting the host against IAV infection.

RIPK3's Role in Balancing Necrosomal Degradation and Maturation:

A New Mechanism Mediated by Triad3a with Implications for Controlling Inflammation and Necroptosis

The study published in 2018 by Alturki et al. titled "Triad3a induces the degradation of early necrosome to limit RipK1-dependent cytokine production and necroptosis" investigated the role of E3-ubiquitin ligase Triad3a in regulating cell death during infections. They demonstrated that Triad3a plays a key role in regulating cell death during infections by mediating the degradation of RIPK3 interacting proteins and inducing inflammatory cell death, a new mechanism of shutting off necrosome signaling [8].

TLR signaling induces MyD88-dependent and TRIF-dependent signaling, which leads to the production of cytokines and chemokines and the recruitment of myeloid cells [9,10]. Necrosis is a pathway of regulated necrosis, called "necroptosis," and is induced by TNF-R or IFN-IR engagement. Necrosome signaling involves RIPK1-FADD-caspase-8 interaction and leads to the phosphorylation of RIPK3 [11,12]. Macrophage-like cells are present throughout the body and play a significant role in initiating inflammatory responses to control pathogens.

The authors investigated the role of RIPK1 and RIPK3 in necrosome signaling and the impact of deubiquitinating enzyme CLYD and ubiquitin-editing enzyme A20 knockdown on the degradation of RIPK1. It was found that an inhibitor of necroptosis necrostatin 1 (Nec-1) completely nullified the phosphorylation of RIPK3, degradation of RIPK1, and cell death by necroptosis. Loss of RIPK3 or mixed lineage kinase domain-like pseudokinase (MLKL) led to a decrease in RIPK3 phosphorylation and protection against necroptosis. CYLD deficiency had little effect on RIPK1 degradation but led to decreased phosphorylation of RIPK3 and necroptosis. Stimulation of cells with lipopolysaccharide (LPS) and pan-caspase inhibitor zVAD had a marginal impact on cell death at earlier points in time.

Co-immunoprecipitation with an anti-RIPK3 antibody showed that Nec-1 completely abrogated the phosphorylation of RIPK3, degradation of RIPK1, and cell death by necroptosis. The combined stimulation of toll-like receptor 4 (TLR4) by LPS and caspase inhibition by zVAD caused the phosphorylation of RIPK1 and RIPK3. Caspase-8 inhibitor zIETD failed to induce the loss of RIPK1 expression or necroptosis of macrophages. K48-ubiquitination of RIPK1 and caspase-8 following necrosome activation was significantly reduced in RIPK3-deficient macrophages. In macrophages, CYLD and A20 knockdown enhanced the degradation of RIPK1. The results revealed that early degradation of the necrosome was a mechanism of cellular escape from necroptosis and cytokine expression. Knockdown of Triad3a resulted in reduced degradation of RIPK1, FADD, and caspase-8 and enhanced the expression.

In this study, ProSci's rabbit anti-RIPK3 antibody (ProSci Cat #: 2283) antibody was used for western blot and immunoprecipitation to investigate its role in the degradation of necrosomal proteins during necroptosis.

This study investigated a new mechanism of self-regulation built into necrosome signaling, which involves the expression and phosphorylation of CYLD. The results show that forming an early necrosome platform triggers a decision between degradation via K48-ubiquitination or cell death via RIPK3 kinase activity and MLKL recruitment. The study also found that the kinase function of RIPK3 is necessary to activate necroptosis, but its interaction with RIPK3 leads to the degradation of RIPK1 and other necrosomal proteins. Finally, applying small molecule inhibitors of RIPK3 can prevent activation of the late necrosome, leading to cells that are resistant to necroptotic challenges. The study reveals that necrosome signaling is regulated by a complex mechanism involving multiple kinases and ubiquitin ligases.

Conclusion:

This white paper presents studies that underscore the crucial role played by the RIPK family—specifically RIPK3—in regulating immune responses, inflammation, and cell death processes.

Zheng et al. highlight how caspase-6 has a significant role in activating the NLRP3 inflammasome through ZBP1 during IAV infection. The authors emphasize the importance of caspase-6 activation and how it relies on interactions with RIPK3 and caspase-8. Such findings hold promise for potential therapeutic approaches targeting caspase-6 along with its interplay with RIPK3, ZBP1, and RIPK1 to address IAV infection effectively [4].

In addition, Alturki et al. revealed an innovative mechanism where Triad3a mediates downregulation of the necrosome—a central signaling complex tied to inflammation and necroptosis processes. This process involves degrading key proteins such as RIPK1 and other necrosomal proteins. Balancing the degradation and maturation of the necrosome allows for better control over the extent of necroptotic cell death. Consequently, this Triad3a-dependent mechanism presents novel therapeutic strategies that can be explored to influence inflammatory responses and regulate necroptosis [8].

Overall, these studies contribute to a more comprehensive understanding of the complex role played by the RIPK family in modulating immune responses, inflammation, and cell death processes. The knowledge gained from these studies offers the potential for developing innovative therapeutic interventions targeting various diseases, including those related to viral infections and inflammatory conditions.

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References

1. *Zhang D, Lin J, Han J. Receptor-interacting protein (RIP) kinase family. Cell Mol Immunol. 2010 Jul;7(4):243-9. doi: 10.1038/cmi.2010.10. Epub 2010 Apr 12. PMID: 20383176; PMCID: PMC4003224.*

2. *Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. Cell. 2009 Jul 23;138(2):229-32. doi: 10.1016/j.cell.2009.07.006. PMID: 19632174.*

3. *Dondelinger Y, Jouan-Lanhouet S, Divert T, Theatre E, Bertin J, Gough PJ, Giansanti P, Heck AJ, Dejardin E, Vandenabeele P, Bertrand MJ. NF-κB-Independent Role of IKKα/IKKβ in Preventing RIPK1 Kinase-Dependent Apoptotic and Necroptotic Cell Death during TNF Signaling. Mol Cell. 2015 Oct 1;60(1):63-76. doi: 10.1016/j.molcel.2015.07.032. Epub 2015 Sep 3. PMID: 26344099.*

4. *Zheng M, Karki R, Vogel P, Kanneganti TD. Caspase-6 Is a Key Regulator of Innate Immunity, Inflammasome Activation, and Host Defense. Cell. 2020 Apr 30;181(3):674-687.e13. doi: 10.1016/j.cell.2020.03.040. Epub 2020 Apr 15. PMID: 32298652; PMCID: PMC7425208.*

5. *Van Opdenbosch N, Lamkanfi M. Caspases in Cell Death, Inflammation, and Disease. Immunity. 2019 Jun 18;50(6):1352-1364. doi: 10.1016/j.immuni.2019.05.020. PMID: 31216460; PMCID: PMC6611727.*

6. *Man SM, Kanneganti TD. Converging roles of caspases in inflammasome activation, cell death and innate immunity. Nat Rev Immunol. 2016 Jan;16(1):7-21. doi: 10.1038/nri.2015.7. Epub 2015 Dec 14. PMID: 26655628; PMCID: PMC4915362.*

7. *Kuriakose T, Man SM, Malireddi RK, Karki R, Kesavardhana S, Place DE, Neale G, Vogel P, Kanneganti TD. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Sci Immunol. 2016 Aug 5;1(2):aag2045. doi: 10.1126/sciimmunol.aag2045. Epub 2016 Aug 12. PMID: 27917412; PMCID: PMC5131924.*

8. *Alturki NA, McComb S, Ariana A, Rijal D, Korneluk RG, Sun SC, Alnemri E, Sad S. Triad3a induces the degradation of early necrosome to limit RipK1-dependent cytokine production and necroptosis. Cell Death Dis. 2018 May 22;9(6):592. doi: 10.1038/s41419-018-0672-0. PMID: 29789521; PMCID: PMC5964080.* **9.** *Van Opdenbosch N, Lamkanfi M. Caspases in Cell Death, Inflammation, and Disease. Immunity. 2019 Jun 18;50(6):1352-1364. doi: 10.1016/j.immuni.2019.05.020. PMID: 31216460; PMCID: PMC6611727.*

9. *Akira S. TLR signaling. Curr Top Microbiol Immunol. 2006;311:1-16. doi: 10.1007/3-540-32636-7_1. PMID: 17048703.*

10. *Eckmann L, Kagnoff MF. Cytokines in host defense against Salmonella. Microbes Infect. 2001 Nov-Dec;3(14-15):1191-200. doi: 10.1016/s1286-4579(01)01479-4. PMID: 11755407.*

11. *He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell. 2009 Jun 12;137(6):1100-11. doi: 10.1016/j.cell.2009.05.021. PMID: 19524512.*

12. *Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol. 2010 Apr;22(2):263-8. doi: 10.1016/j.ceb.2009.12.003. Epub 2010 Jan 4. PMID: 20045303; PMCID: PMC2854308.*