Co-transfection Analysis of Putative RHIM Interacting Partners

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Abstract

The process of cell death, which regulates cellular homeostasis, occurs in unicellular organisms and multicellular organisms. Necroptosis—a programmed form of necrosis or inflammatory cell death that can be stimulated by various stimuli, such as TNF, lipopolysaccharides (LPS), type I and type II interferons (IFNs), and viral infection—is one such response. However, the mechanism of LPS-dependent necroptosis is not so clear. Previous pull-down experiments have shown that RBPJ, a transcriptional regulator in the Notch signaling pathway, binds to RIPK1 in LPS-induced necroptosis in RAW264.7 cells. Here, we amplified mutated FLAG tagged RIPK1, RBP-J, HA tagged RIPK1 and transfected 239T cells with them. After pulldown experiments, we found that the binding sites on RIPK1 are between 540 and 550 amino acids, and the β-trefoil domain is the binding domain on RBP-J. In addition, the RHIM domain possibly does not have effect on interaction between RIPK1 and RBP-J. Since ZBP1 is a key mediator of IFN-beta-induced necroptosis and it contains 2 RHIM domains. We treated RAW264.7 cells with IFN-beta and performed pulldown, which showed that ZBP1 binds to RBP-J/RIPK1 complex during IFN-beta-induced necroptosis. In conclusions, specific binding sites of RIPK1/RBP-J complex were investigated and ZBP1/RIPK1/RBP-J complex might form on cell death pathways

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List of Abbreviations

IDN - IDN-6556, caspase inhibitor
IP - Immunoprecipitation
IRAK - IL-1 receptor-associated kinase
LI - LPS+IDN-6556
LIN - LPS+IDN-6556+Nec-1
LPS - Lipopolysaccharides
MLKL - Mixed lineage kinase domain-like pseudokinase
MyD88 - Myeloid differentiation primary-response protein 88
NF-κB - nuclear factor-κB
RBP-J - Recombination Signal Binding Protein for Immunoglobulin Kappa J Region
RHIM - Rip homotypic interaction motif
RIPK - Receptor-interacting serine/threonine-protein kinase
TLR - Toll-like receptors
TNF - tumor necrosis factor
TRAF - TNF Receptor-Associated Factor
TRIF - TIR-domain-containing adaptor inducing interferon
IFNb - interferon-beta

Chapter 1: Introduction

1.1. TLR4 pathway.

Toll-like receptors (TLRs) are proteins playing crucial roles in the innate and adaptive immune systems through their modulatory effects on various immune cells[1].Members of the TLR family can be classified according to their localization in the cell. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are typically located on the cell surface, identifying components of the pathogen envelope, whereas TLR3, TLR7, TLR8, and TLR9 function on the endoplasmic reticulum membrane or on the endosomal/lysosomal membrane, recognizing nucleic acids of these infectious agents[2].

The protein TLR4 is a transmembrane protein that has been linked to the pathophysiology of many human diseases, including asthma, cardiovascular disorder, diabetes, obesity, metabolic syndrome, autoimmune disorders and neuroinflammatory disorders[3, 4].

The Toll-like receptor 4 (TLR4) is a membrane protein that recognizes lipopolysaccharides (LPS), a component of many Gram-negative bacteria. When stimulated by LPS, TLR4 recruits the glycosylphosphatidylinositol-anchored protein CD14 to transfer LPS to the next protein in the signaling cascade: myeloid differentiation primary response protein 2 (MD-2)[5, 6]. In this way, TLR4 can stimulalte two different intracellular pathways: MyD88-dependent and MyD88-independent pathways. On its MyD88-dependent pathway, TLR4 recruits IRAK4 (IL-1 receptor associated kinase 4), IRAK1, and IRAK2. These proteins are phosphorylated and detached from their complex with MyD88/IRAK and bind TRAF6 (Tumor Necrosis Factor receptor associated factor 6). TRAF6 activates TAK1 (TNF receptor associated factor 6) which leads to activation

of NF-κB and MAPKs activity resulting in inflammatory cytokine production[7-9]. In the MyD88-independent pathway, also called TRIF-dependent pathway, TRIF activates transcription factor interferon regulatory factor 3 (IRF3), which in turn mediates the late phase of NF-κB activation by inducing the expression of type I IFN-inducible genes[1].

1.2. LPS induced cell death.

Cell death regulates cellular homoeostasis via cellular response in unicellular or multicellular organisms and is typically discussed dichotomously as either apoptosis or necroptosis[10, 11]. Apoptosis is defined as a active, caspase-dependent form of programmed cell death its dysregulation underlies various pathological and physiological processes, including cell homeostasis, tissue remodelling, and tumorigenesis[10, 12, 13].

RIPK1 is an important mediator of multiple signaling pathways as it performs pro-cell-death and pro-inflammatory activities. It has three major domains called the N-terminal kinase domain, the intermediate domain (containing RHIM) and the C-terminal death domain. RIPK1 interacts with RIPK3 via their common homotypic interaction motifs (RHIM) to form a hetero oligomer Phosphorylation of RIPK1 in multiple residues, especially Ser166, and phosphorylation of RIPK3 on Ser227 will eventually induce the phosphorylation of mixed lineage kinase domain-like pseudokinase (MLKL) on Thr357 and Ser358. Phosphorylation leads to oligomerization and membrane translocation of MLKL, causing loss of membrane integrity. Fas-associated death domain protein (FADD) can also be recruited to RIPK1 by interacting with the C-terminal death domain. The complex of RIPK1/RIPK3/FADD/MLKL/caspase-8 is termed as necrosome. Necroptosis can be inhibited by Necrostatin-1 (Nec-1), a selective allosteric inhibitor of RIPK1[14].

1.3. ZBP1 in cell death signaling pathway

Z-DNA binding protein 1 (ZBP1), also known as DNA-dependent activator of IFN-regulatory factors (DAI), is identified as a cytosolic DNA sensor which plays an essential role in such host response in neoplasia and DNA-mediated activation of innate immune responses[15]. Recently, ZBP1 is recognized another host protein as an innate immune sensor regulating activation of both programmed cell death and inflammation in diverse conditions including infection and embryonic development[16]. ZBP1 encodes two N-terminal Z-DNA binding domains, which is reported to bind with Z-DNA, B-DNA and RNA. ZBP1 also has two RHIM domains at the center part that facilitate interactions with other RHIM domain-containing proteins. These RHIM domains are important in mediating ZBP1—dependent cell death and inflammatory responses. The conserved C-terminal domains of ZBP1 were reported to interact with TBK1 and IRF3 to induce type I IFN responses to immunostimulatory DNA[16]. ZBP1 can drive necroptosis during development by interacting with RIPK3 through RHIM domain, and previous research showed that mutation of the RIPK1 RHIM promotes RHIM-dependent interactions between ZBP1 and RIPK3, resulting in RIPK3 autophosphorylation and MLKL-dependent necroptosis [17]. J. P. Ingram et al. found that ZBP1 can work as the dominant activator of IFN-driven RIPK3 activation and perinatal lethality in the absence of RIPK1[18]. Recently, researchers found that constitutive binding between ZBP1 and RIPK1 is essential for the initiation of TRIFosome interactions, caspase-8-mediated cell death and inflammasome activation, thus positioning ZBP1 as an effector of cell death in the context of bacterial blockade of pro-inflammatory signaling[19]. Also, J. Y. Baik et al.

reported that ZBP1, not RIPK1, mediates tumor necroptosis during tumor development in preclinical breast cancer models, which showed the important role ZBP1 playing in cell death signaling pathways[20].

1.4. RBP-J and Notch signaling pathway and its connections to TLR4 signaling

Notch signaling pathway is a highly conserved cell signaling system present in most species, which is a key pathway for cell growth, differentiation, survival and cell-cell communication including gene regulation mechanisms[21]. It plays a key role in human disease by regulating angiogenesis and arteriogenesis, modulating neuronal function and development, influencing the differentiation of hemogenic endothelial cells and regulating binary fate decisions of cells of the gut, etc[22-25].

Humans and other mammals possess four receptor proteins of the Notch family, which include NOTCH1, NOTCH2, NOTCH3, and NOTCH4[26]. The Notch intracellular domain (NICD) binds transmembrane proteins characterized by three motifs: DSL, DOS and EGF repeats[27]. When Notch binds ligands and causes the release of the NICD, it acts as a switch to turn on the downstream events. This fragment enters the cell nucleus so that it interacts with CSL, an alias for Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ) in Mus musculus, to modulate transcription. RBPJ is indispensable in activating the Notch signaling pathway[28]. A NICD/RBPJ module is recognized by Mastermind (MAM)/Lag-3 protein and this complex can recruit a positive regulator to assemble an active transcription complex to switch on the expression of Notch target genes such as hairy and enhancer of split family, Cyclin D1, p21, glial fibrillary acidic protein (GFAP) and Nodal.[29, 30]. Previous studies had

shown RBPJ is involved in regulating many diseases, like malignancy of glioblastoma, inflammatory bone resorption, lung cancer and prostate cancer[31-34].

Recent research has shown that the activation of the Toll-like receptor (TLR) leads to up-regulation of the expression of Notch ligands, receptors and RBPJ[35]. Specifically, LPS activates Notch signaling and its target gene by JNK-dependent pathway. Full activation of Notch signaling has been reported to increase transcription of p52, p50, p65/RelA, RelB, and c-Rel as well as IκBα , thus potentially stimulating NF-κB activity; NF-κB induces the expression of various pro-inflammatory genes including those encoding cytokines and chemokines which could potentially induce inflammation. Moreover, NF-κB has also been reported to increase transcription of Notch ligand Jagged-1, as well as Notch targets Deltex-1 and HES-5.[36] Significantly, RBPJ is indispensable in regulating NF-κB and affecting inflammation; Xiaozhi et al reported that RBPJ knockout led to decreased inflammation by reducing p65 and IκBα activation[37].

Much is known about the detailed relationship and underlying mechanisms between Notch and TLR4 pathway, however, the connections between Notch, especially RBPJ, and LPS-dependent necroptosis remain far from being understood. Our previous study shows that RBP-J binds to RIPK1 under LPS-induced cell death, including apoptosis and necroptosis, and could negatively regulate LPS-induced necroptosis by affecting necrosome formation. In this paper, we determined the possible binding domain on RBP-J and RIPK1, respectively, and what possible role does the RHIM domain of RIPK1 play in protein-protein interactions of RBP-J and RIPK1.

Chapter 2: Methods

2.1. Cell Culture

RAW (RAW264.7) cell lines were cultured in DMEM medium (Corning) containing 10% Fetal Bovine Serum (FBS) (Sigma) and 1% PSA (penicillin/ streptomycin/ antimycotic solution) (Gibco).

Fetal liver macrophages were grown in DMEM medium (Corning) supplemented with 20% FBS (Sigma), 1% PSA (Gibco). All cells were cultured at 37 °C with 5% CO2.

293T cells were grown in DMEM medium (Corning) supplemented with 10% FBS (Sigma), 1% PSA (Gibco),1:10000 Blasmocure (VivoGen)

2.2. Reagents and antibodies

Lipopolysaccharide (LPS) Escherichia coli 0111: B4 (10 ng/ml, L4391), IDN6556, IFN-beta. Opti-MEM™ (Gibco), Opti-Plex™ Complexation Buffer (Gibco), PEI PrimeTM linear polyethylenimine (Sigma-Aldrich), NEB® Stable competent E. coli (High Efficiency) (NEW ENGLAND BioLabs), NEB® 10-beta/Stable Outgrowth Medium (NEW ENGLAND BioLabs), Q5® Hot-Start High-Fidelity 2X Master Mix (NEW ENGLAND BioLabs), NEBuilder® HiFi DNA Assembly Master Mix M5520AA (NEW ENGLAND BioLabs), Pierce™ Anti-DYKDDDDK Magnetic Agarose (Themo Scientific), QIAGEN® Plasmid Midi Kit, GeneJET Plasmid Miniprep Kit (thermos scientific)

The following antibodies acquired from Cell Signaling Technology were used: RIPK1 (3493), RBPSUH (D10A4) XP(R) (5313), DYKDDDDK Tag (D6W5B) Rabbit mAb**,** HA-Tag (C29F4) Rabbit mAb #3724, anti-rabbit IgG (H+L) Dylight™ 800 4X

PEG Conjugate, anti-mouse IgG (H+L) Dylight™ 800 4X PEG Conjugate. ZBP-1 mAb (Zippy-1) was bought from AdipoGen Life Science. Alpha tubulin polyclonal antibodies (11224-1-AP) was bought from ProteinTechs. Rabbit Polyclonal Antibody to RBP-J used for RBP-J pulldown was bought from Abcam.

2.3. PCR, agarose gel electrophoresis and Gibson Assembly

Designed and ordered the primers from Integrated DNA Technologies, Inc. After receiving the primers, dissolved theprimers to 100mM concentration by adding specific volume of nuclease-free water. 0.5µl of front and reverse primer (100mM), respectively, 4µ of template DNA, 50µl of Q5® Hot-Start High-Fidelity 2X Master Mix (NEW ENGLAND BioLabs), and 45µl of nuclease-free water were added to a PCR tube and aliquot to two tubes. Tubes were centrifuged for 3 seconds and placed in the 96-well T100 PCR thermal cycler. The setting of PCR cycle is showed on Table 2.1. After 2-hour cycle, mixtures in two tubes were combined to one tube and 300µl ethanol was added to the mixture. The tube was put into freezer. The following day, the mixture was transferred to 1.5ml tube and centrifuged at 4℃, 14000rpm for 10 minutes. The ethanol was removed from the tube and 100µl 70% ethanol added to DNA sample. Sample was centrifuged at 14000rpm and air dried for 5 minutes. 20µl of nuclease-free water was added to DNA sample, and 5µl of dye was added to the sample. The samples and ladder were loaded to 1.5% agarose gel and the gel was run at 75V for 10 minutes, then 120V for 20 minutes. The isolation of DNA was cut from gel and solved in another 1.5ml tube. GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used to extract the DNA. The volume of DNA fragment and vector are calculated by using the Gibson spread sheet.

The PCR tube containing DNA fragments, vector and NEBuilder® HiFi DNA Assembly Master Mix M5520AA (NEW ENGLAND BioLabs) was placed in PCR cycler at 55℃ for 15 minutes. Finally, the DNA plasmids were harvested.

2.4. Western blotting

After the indicated treatments, cells were lysed and harvested in RIPA buffer (Cell Signaling) supplemented with PMSF (5 mg/ml), leupeptin (1 mg/ml), pepstatin (1 mg/ml), and aproprotinin (1 mg/ml), sonicated twice. Supernatant was collected for protein concentration measurement with Bio-Rad Protein Assay reagent. After protein concentration normalization, 4x SDS sample loading buffer was added. All samples were heated at 98 °C for 5 minutes and stored at -20 °C. After loading the same value of samples on SDS-PAGE gel, the gel was run at 30 mA and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After transfer, membranes were blocked in Protein-free T20 (TBS) blocking buffer (Thermo Fisher Scientific) at room temperature for 1 hour. After one hour, membranes were rinsed with TBST (0.1%) and incubated at 4°C overnight with corresponding primary Abs (1:1000 dilution). Membranes were washed with 1x TBST three times for 10 min and incubated with secondary Ab (1:5000 dilution) at room temperature for 1 hour. Subsequently, membranes were washed with 1x TBST three times for 45 minutes each with constant rocking. After washing, membranes were brought to Li-Cor Odyssey CLx imaging system and output the developed film images.

2.5. Bacterial transformation

The E. coli was placed on ice from -80 freezer till they were completely thawed. 25 uL of E. coli were transferred to 1.5 ml Eppendorf and added 1uL of indicated DNA plasmid. The solution was then mixed manually. The mixture was incubated on ice for 30 minutes. Simultaneously, the bacterial medium was thawed in the warm bath. The heating block was turned on to 42℃ and the mixture was heated for 30 seconds. After that, the mixture was incubated on ice for 5 minutes. 300 uL bacterial medium was added in the mixture of E. coli and plasmid and the mixture was shaken at 30℃ for 90 minutes. The agarose medium with ampicillin added was warmed in incubator for 1 hour. 30-100 uL of bacterial mixture was transferred in prepared medium and the medium was shook horizontally to separate the bacteria. All media were incubated in the bacterial incubator 37℃ overnight. Next day, single colony of E. coli was picked by a 200 ul pipet and transferred to bottle of 100 mL of ampicillin-added PI medium. Each bottle was shaken in shaker at 37℃, 220 rpm, overnight. The bacteria were transferred to 50 ml tube and centrifuged at 4℃, 30 minutes, then removed the supernatant. The harvested bacteria were applied to Midi/Mini-Prep or stored them in -80℃ freezer for following experiments.

2.6. Cells transfection

2×10^6 of 293T cells were seeded in 100mm tissue culture plate for 24 hours. The 1x DMEM medium with 10% FBS added without PSA was changed and incubated with cells in incubator at 37℃, 5% of CO2, for 2-4 hours. The plasmids and PEI were thawed in the heating block at 37℃ and transferred to laboratory hood. 1.2 ml of Opti-MEM was

added to 1.5 ml Eppendorf. Subsequently, 18ug of DNA plasmid was mixed to Opto-MEM and vortex the tube at 4.5 setting of speed for 3-5 seconds. After it, 54uL of PEI were pipetted in the tube and incubate at room temperature for 15 minutes. After that, the mixture was transferred in the prepared 293T cells, and the cells were cultured in incubator for 24 hours. The following day, the 1x DMEM medium with 10% FBS, 1% PSA, 1:10000 Plasmocure was changed to transfected 293T cells, and the cells were cultured for another 24 hours.

2.7. Co-immunoprecipitation

After treating cells with the indicated conditions, cells were harvested. Media was removed from samples, and cell pellets were dissolved in lysis buffer. After one hour of end-over-end rotating, supernatants were collected and be normalized. For RBP-J pulldown, lysates were incubated with antibodies overnight with the concentration of 5 μg/ml. For FLAG pulldown, after beads washed three times with lysis buffer, lysates were incubated with anti-FLAG beads overnight with the concentration of 20μg/μl. On the next day for RBP-J pulldown, after washing Pierce® Protein A/G Magnetic Beads (VK308442) (Thermo Fisher Scientific) three times with lysis buffer containing NP-40, beads were evenly added into each sample and rotated for 2 hours at 4 °C. in terms of both RBP-J pulldown and FLAG pulldown, Bound material was washed 3× with lysis buffer. Half samples were boiled for 15 min.

2.8. Inducing cell death and IFN-beta treatment

RAW264.7 cells were seeded at 2×10^6 in 100mm tissue culture plate two days before treatment. LPS (1:10000) was added to induce cell death, and IDN6556 (1:1000) was added to inhibit apoptosis. IFN-beta was added to induced ZBP1 expression. After 4.5-hour treatment, cells were harvested. Medium were removed from samples, and cell pellets were dissolved in lysis buffer. Cell lysates can be applied for western blot or co-immunoprecipitation.

2.9. Cell viability

RAW264.7 cells were seeded at 1.5×10^4 per well in 96-well plates on the day before treatment. The test compounds were added to wells and incubated in corresponding hours. ATP viability assay was performed according to the protocol of CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega). Each independent experiment was performed in duplicate and repeated three times.

2.10. Statistical analysis

All data were analyzed by Li-Cor Odyssey CLx imaging system and Image Studio Lite.

2.11. Collaboration

All methods were performed by Zhenyu Chang.

Chapter 3: Results

3.1. The binding site of RBP-J and RIPK1

In the previous research, we identified RBPJ protein constitutively bound to RIPK1 (Figure 3.1 A). We did the RBPJ pull-down and confirmed that RIPK1 was bound to RBPJ in unstimulated, LPS+IDN6556 (LI, induces necroptosis) and LI+Nec-1 (rescue from necroptosis by RIPK1 inhibitor) treated RAW264.7 cells (Figure 3.1 B). To identify the specific binding sites on RBP-J and RIPK1, respectively, we utilized DNA plasmids containing different sequences of RBP-J and RIPK1 to transfect HEK293T cells and applied co-immunoprecipitation on these cells.

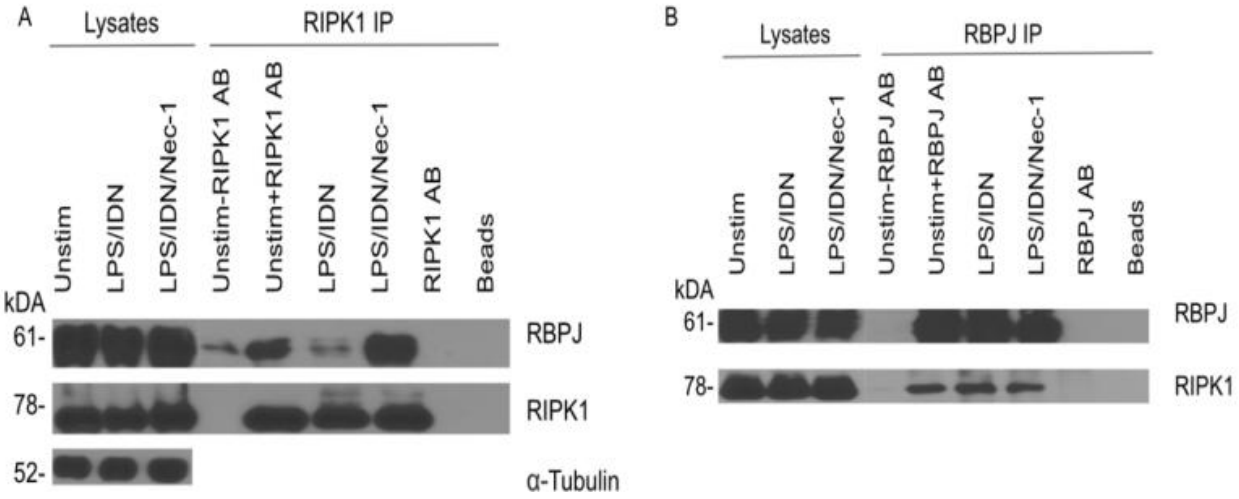

Figure 3.1 The combination of RBPJ and RIPK1 in RAW264.7 Macrophages (A) Pulldown RBPJ with RIPK1 antibody in RAW264.7 wildtype cell lines. RAW264.7 cells were treated with 10 ng/ml LPS, 20 μM IDN and 10 μM Nec-1 for 4 hours. (B) Pulldown RIPK1 with RBPJ antibody in RAW264.7 wildtype cell lines. RAW264.7 cells were treated with 10 ng/ml LPS, 20 μM IDN and 10 μM Nec-1 for 4 hours.

We designed primers for PCR and generated different length of plasmids, 1-327, 1-480, 1-559 amino acids of human RIPK1 and inserted 3X FLAG tag with them to easily detect the expression of hRIPK1. The immunoblotting results of RBP-J pulldown showed a clear band on lane of 1-559 hRIPK1, whereas there was no band on lane of 1-480 and

1-327 hRIPK1 (Figure 3.2 A), indicating that on hRIPK1, the binding sites of RBP-J and hRIPK1 is between 480-559 amino acids. To identify the binding sites between 480 amino acid and 559 amino acids, we applied three plasmids, pcDNA3.1-EcoR1-3X FLAG tagged 1-480, 1-500, 1-530 hRIPK1 sequence,

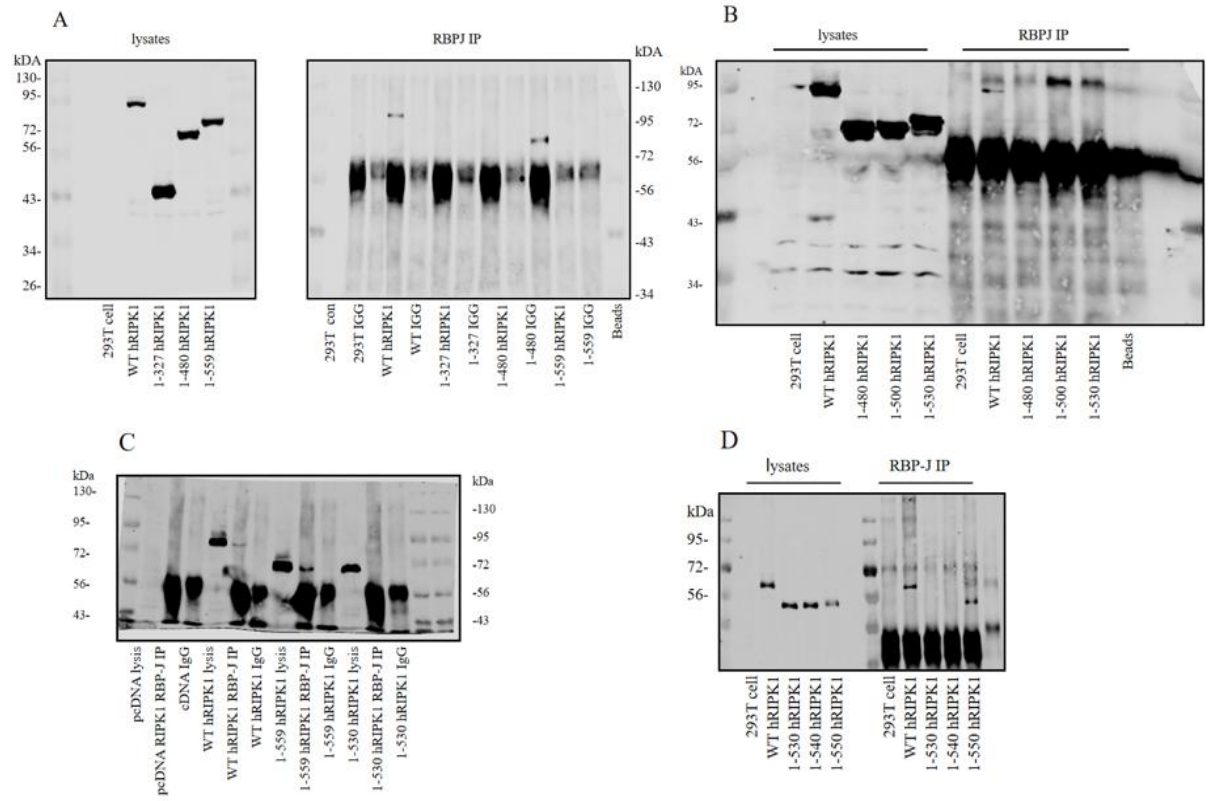

Figure 3.2 RIPK1 binds to RBP-J between 530 and 540 amino acids of RIPK1. Transfection of 293T cells by (A) 3Xflag tagged 1-327, 1-480, 1-559 hRIPK1 plasmids, (B) 3Xflag tagged 1-480, 1-500, 1-530 hRIPK1 plasmids, (C) 3Xflag tagged 1-530, 1-559 hRIPK1 plasmids, (D) 3Xflag 1-530. 1-540, 1-550 hRIPK1 plasmids. 293T cells transfected with 1x flag WT hRIPK1 were arranged as a positive control, and 239T cells without transfection and incubated with IgG mouse as a negative control. Pulldown hRIPK1 with RBP-J antibody in transfected 293T cells in all experiments. All co-immunoprecipitations were analyzed by western blot.

on HEK293T cells comparing with cells transfected by pcDNA 3.1 -EcoR1- 3X FLAG tagged WT hRIPK1 plasmids (Figure 3.2 B). The results of co-immunoprecipitation showed that no immunoblotting band on every plasmid transfected cells compared to cell

lysates, which indicates that the binding sites are not between 480-530 amino acids. It may exist on the region between the 530 amino acids and 550 amino acids. In order to confirm this finding, we used pcDNA 3.1- EcoR1- 3X FLAG tagged 1-530 and 1-559 sequences to transfect the HEK293T cells. From the immunoblotting results, there is a clear band on the lane of 1-559 hRIPK1 and not in the lane of 1-530 hRIPK1 (Figure 3.2 C), indicating that the binding site is between 530- 559 amino acids of hRIPK1 which is consistent with the results of previous experiments. On the next experiment, we designed primers and constructed pcDNA 3.1 – EcoR1-3X FLAG tagged 1-530, 1-540, 1-550 plasmids of DNA then transfected HEK293T cells to overexpress the hRIPK1 with different length of amino acids (Figure 3.2 D). The immunoblotting results showed that a band generated on the lane of 1-550 hRIPK1 RBP-J pulldown sample and not in 1-540 and 1-530 hRIPK1 lanes. Thus, the binding site on hRIPK1 is between 540 amino acids and 550 amino acids.

After we confirmed the binding site on hRIPK1 is between 540 amino acids and 550 amino acids, we went on to identify the binding site on RBP-J. RBP-J contains three domains that mediate contacts with coactivators and corepressors: the N-terminal domain (NTD), the β-trefoil domain (BTD), and the C-terminal domain (CTD)[38]. We hypothesized that the binding site on RBP-J to hRIPK1 is on one of those domains. In order to test our hypothesis, we designed three primers which can amplify the sequences, full length of RBP-J, BTD + CTD of RBP-J, and BTD of RBP-J. To detect the expression of RBP-J and hRIPK1 more easily, we inserted HA tag on hRIPK1 and 3X FLAG tag on RBP-J. A FLAG pulldown experiment was performed to test whether HA tagged hRIPK1

and 3X FLAG tagged RBP-J bound together (Figure 3.3 A and B).

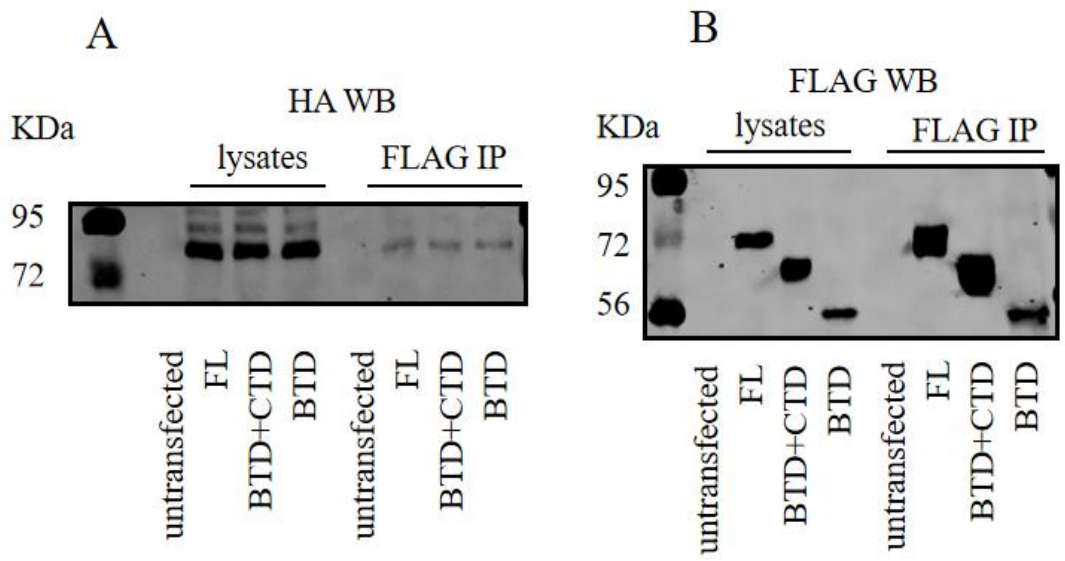

Figure 3.3 RBP-J binds with hRIPK1 on BTD other than CTD or NTD. 239T cells were co-transfected by HA tagged hRIPK1 and 3x flag tagged RBP-J (FL, BTD+CTD, BTD). Pulldown of hRIPK1 with RBP-J by using Anti-DYKDDDDK Magnetic Agarose in transfected 293T cells. (A) Overexpressed and bound HA tagged hRIPK1 were detected by immunoblotting. (B) Expression level of flag tag was detected by immunoblotting. 293T cells were set as negative control.

The result of immunoblotting showed three clear bands in three samples of cell transfection; therefore, this results supports that RBP-J binds with hRIPK1 through some sites on BTD other than CTD or NTD.

3.2. Mutation of RHIM domain core structure on RIPK1 has no effects on binding

Thought we have already identified the potential binding sites on RBP-J and hRIPK1, we still want to determine the exact binding sites on hRIPK1 or RBP-J. According to the previous research, The RHIM domain is involved in virus recognition. It is necessary for the recruitment of RIP and RIP3 by the IFN-inducible protein Z-DNA

binding protein (ZBP1), also known as DNA-dependent activator of IRFs (DAI). Both RIP kinases contain RHIM domains and contribute to ZBP1-induced NF-kappaB activation[39].As a result, the RHIM domain plays an important role in protein-protein interactions in cell death signaling pathways. We hypothesized that RHIM domain may also contribute to the binding of RBP-J and hRIPK1, it perhaps is the core domain for protein interaction of RBP-J and hRIPK1. In order to test our hypothesis, we mutated the core sequence of RHIM domain on hRIPK1, IQIG, to four alanine (AAAA) and inserted it with 3X FLAG tag. After the RBP-J pulldown experiment,

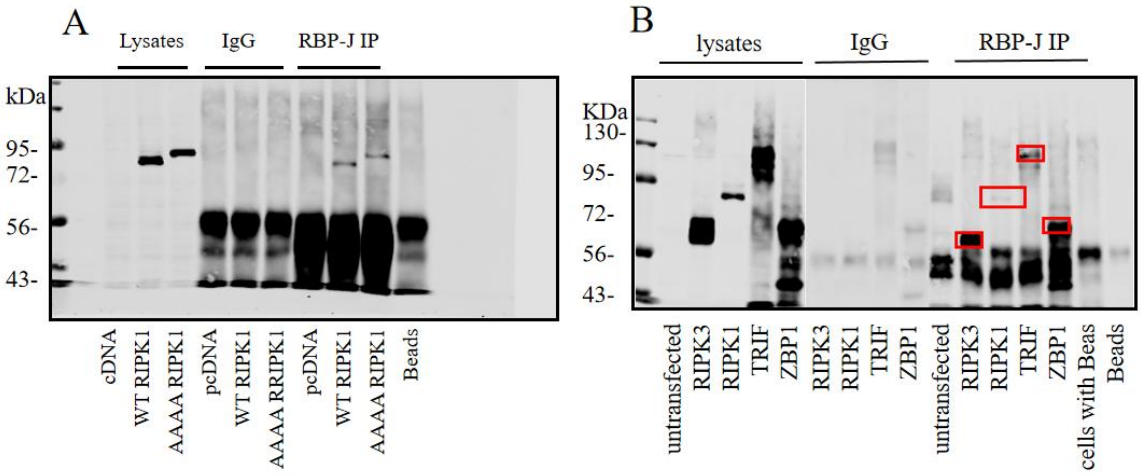

Figure 3.4 Mutation of RHIM domain core structure on RIPK1 has no effects on binding and every RHIM domain protein binds to RBP-J. (A) 293T cells were transfected by 3x flag tagged mutated hRIPK1, 1x flag tagged WT hRIPK1 and vector only. Pulldown hRIPK1 with RBP-J antibody in 293T cells and the binding results were represented by immunoblotting. (B) 293T cells transfected by 3x flag tagged RIPK1, RIPK3, TRIF and ZBP1 plasmids. Pulldown hRIPK1 with RBP-J antibody in 293T cells and the immunoblotting showed the binding situation of RHIM domain proteins and RBP-J.

we tested the binding situation of hRIPK1 and RBP-J by using immunoblotting (Figure 3.4 A). The results represented that a band on the lane of mutated hRIPK1 transfected 293T cells sample, substantiating hRIPK1 and RBP-J were still bound even with core of RHIM domain mutation. From our experiments, we conclude that RHIM domain may not

be essential for proteins interaction of RBP-J and hRIPK1, or perhaps it contributes to the binding of these proteins in a certain extent. There might be other factors that affect the interactions of RBP-J and hRIPK1.

RHIMs are conserved protein sequences located within disordered regions in four multi-domain immunity-associated proteins. Not only RIPK1 contains a RHIM domain, but other proteins, including TRIF, ZBP1, RIPK3, which are important components in necroptosis signaling pathways contain RHIMs domain as well. RHIMs contain a highly conserved core tetrad of residues with the consensus sequence (V/I)Q(V/I/L/C)G. To investigate whether the RHIM domain proteins interact with RBP-J, we used plasmids of ZBP1(mouse) and TRIF(?), RIPK3(mouse), and RIPK1(human) to transfect 293T cells and performed RBP-J pulldown by using the cell lysates. The immunoblotting results show that on every lane of samples, a clear band was detected (Figure 3.4 B, indicated in the red boxes). In conclusion, four proteins containing RHIM domain can bind to RBP-J in overexpressed 293T cells. Therefore, whether RHIM domain has affects on RIPK1 and RBP-J binding and what role it plays in RIPK1 and RBP-J interactions, are questions that need to be solved in future.

3.3. RBP-J binds to ZBP1 on BTD domain and they bind in the RAW264.7 cells

From previous findings, each protein containing RHIM domain binds to RBP-J which supports the idea that the RHIM domain plays an important role in protein-protein interactions with RBP-J. To address whether RHIM domain proteins bind to the BTD domain on RBP-J. Here, we chose ZBP1 as RHIM domain protein for our following experiments. 293T cells were co-transfected by plasmids of HA tagged ZBP1 and 3X

FLAG tagged full length, BTD+CTD, BTD of RBP-J. Then we performed FLAG pulldown on the transfected 293T cells. The results of immunoblotting show that three bands were found on three lanes of the IP treatment (Figure 3.5 A, B). This suggest that ZBP1 binds to RBP-J through the BTD domain on RBP-J which is consistent with the previous finding above. However, the interaction with the BTD domain alone is considerably weaker than with the BTD+CTD construct suggesting other sequences may also contribute to binding. Since IFNs can induce the expression of ZBP1, and the RAW264.7 cells cannot express ZBP1 in normal situation. Thereby, we added IFN-beta to the RAW cells and performed RBP-J pulldown to investigate whether ZBP1 binds to RBP-J under IFN-beta induction with LPS-induced cell death. From the results of immunoblotting, there is a band in each two lanes in the middle of IP group (Figure 3.5 C), whereas, there is no band showed after treated with IDN6556 in the right lane of IP group (Figure 3.5 C), which indicates that RBP-J binds to ZBP1 and RIPK1 spontaneously after the raw cells treated by either IFN-beta alone or both IFN-beta and LPS, and RIPK1/ZBP1/RBP-J complex might constructs in apoptosis signaling pathway not necroptosis. However, the distribution of construction RIPK1/ZBP1/RBP-J complex remains question and needs futher investigation.

3.4 Collaboration

Figure 3.1, Figure 3.2 A,B were finished by Zixuan Hao. Rest of figures were presented by Zhenyu Chang.

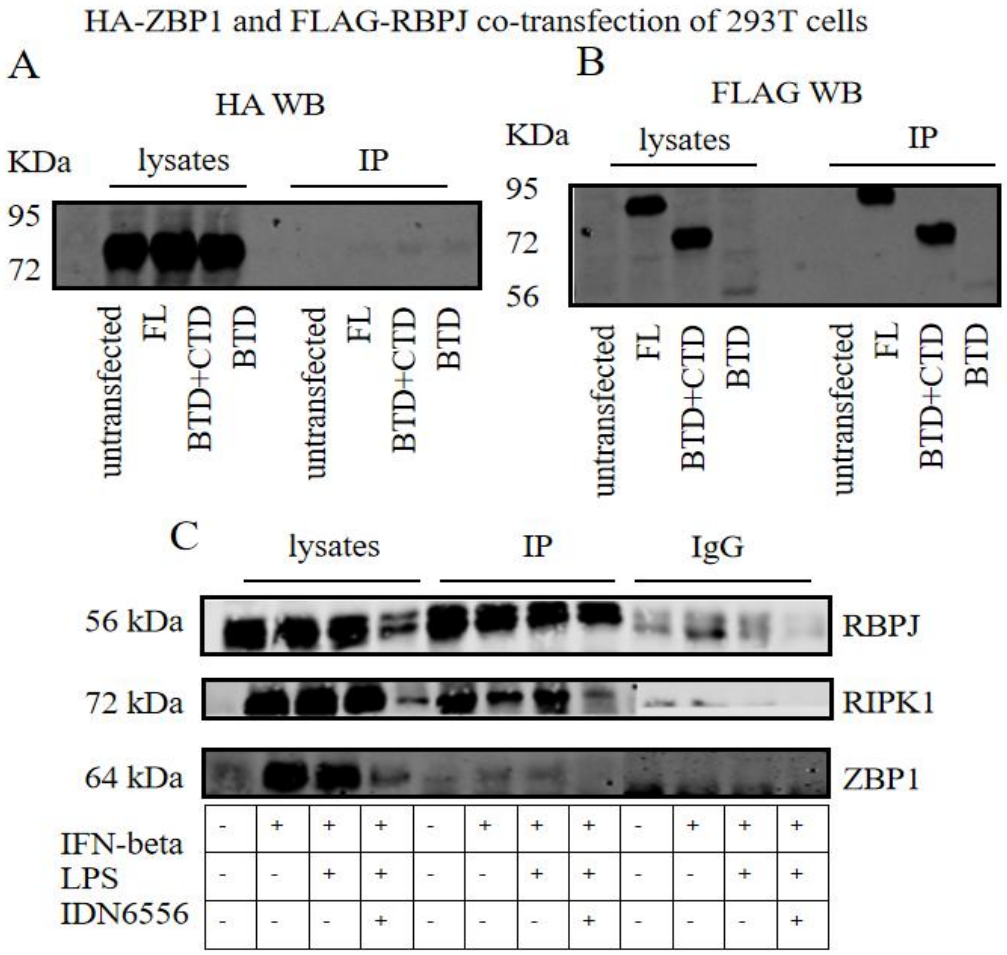

Figure 3.5 RBP-J binds with ZBP1 on BTD other than CTD or NTD. 239T cells were co-transfected by HA tagged ZBP1 and 3x flag tagged RBP-J (FL, BTD+CTD, BTD). Pulldown of ZBP1 with RBP-J by using Anti-DYKDDDDK Magnetic Agarose in transfected 293T cells. (A) Overexpressed and bound HA tagged ZBP1 were detected by immunoblotting. (B) Expression level of flag tag was detected by immunoblotting. 293T cells were set as negative control. (C) Pulldown RIPK1 and ZBP1 with RBP-J antibody in RAW264.7 cells, and cells were treated with 100 ng/ml IFN-beta, with or without 10ng/ml LPS and 20μM IDN6556 for 5 hours. The binding results were analyzed by immunoblotting.

Chapter 4: Discussion

4.1. The specific binding sites on RIPK1 and RBP-J

In previous study, the binding of RBP-J and RIPK1 has been determined. To find out how RBP-J and RIPK1 affect each other, we performed experiments to investigate the specific binding sites on RIPK1 and RBP-J. Since the structure of RIPK1 has been fully determined, we first focused on RIPK1. As RIPK1 has three main domains, N-terminal kinase domain, intermediate domain and C-terminal death domain. We transfected 293T cells with different lengths of these domains and performed RBP-J pulldowns. The results showed that the binding sites on RIPK1 are between 540-550 amino acids. The core of RHIM domain of RIPK1 (IQIG) is exactly on 540-550 amino acids. Therefore, we mutated IQIG to AAAA and transfected 293T cells. After the pulldown experiments, we found the mutation of core of RHIM domain did not affect the binding of RIPK1 and RBP-J. To determine whether the RHIM domain of RIPK1 have effect on binding, more experiments remain to be performed.

For the binding site on RBP-J, we repeated the RIPK1 pulldown several times and the background of western blotting were too dark to recognize the results (Data not shown). To find out the specific binding site on RBP-J, we transfected 293T cells with plasmids containing various domains (BTD, CTD+BTD, FL) and used FLAG pulldown to determine the sites. From this, it appears that BTD is the key domain contributing to the binding of RBP-J and RIPK1. In the future, we could focus on the BTD of RBP-J and mutate the sequence of RBP-J to investigate the specific binding sites on RBP-J.

4.2. ZBP1 possibly binds to RIPK1/RBP-J complex in TLR4 responses

In IFNb-induced necroptosis, ZBP1 serves as a regulator by interaction with RIPK3 through RHIM domain, resulting in RIPK3 autophosphorylation and MLKL-dependent necroptosis. Moreover, evidence was shown that ZBP1 interacts with RIPK1 in caspase-8 mediated cell death and constitutive bind to RIPK3 in IFN-induced cell death. In this paper, we found that ZBP1 and RIPK1 binds to RBP-J spontaneously in RAW cells under both LPS-induced and IFN-beta-induced cell death. This suggests that ZBP1, RIPK1 and RBP-J combine to form a complex. However, whether ZBP1/RIPK1/RBP-J complex serve as a mediator in IFN-beta-induced necroptosis needs more experiments to be tested.

Since ZBP1 binds to RIPK1 and RIPK3 on RHIM domain, we assumed ZBP1 binds to RBP-J through RHIM domain as well. There are two RHIM domains on ZBP1 and we mutated either and both, then transfect 239T cells. After RBP-J pulldown, we found that the signals of bands in IP group are as same level as IgG group, which means non-specific bands in the IP groups. However, whether ZBP1 binds to RBP-J through RHIM domains following the IFN-beta or LPS induced necroptosis and how extent RHIM domains contribute to the binding of RBP-J and ZBP1 remaining questions.

4.3. Notch pathway and necroptosis

Since RBPJ is an important transcription factor in Notch signaling pathway, it's very necessary to know whether Notch signaling pathway is being activated in LPS-induced necroptosis. If Notch target genes such as hairy and enhancer of split family, Cyclin

D1, p21, glial fibrillary acidic protein (GFAP) and Nodal upregulated a lot in LI group, Notch signaling may affect necroptosis by regulating RBPJ/RIPK1 complexes. We can also inhibit Notch pathway by DBZ, an inhibitor of γ-secretase, which cleaves transmembrane proteins including Notch and test whether it will affect necroptosis.

Moreover, as only RBPJ knockout Raw264.7 cells were sensitive to LPS-induced necroptosis, we want to know if RBPJ regulating necroptosis is cell type specific or not. In the further experiments, we plan to knockout RBPJ in RIPK1 wildtype and knockout fetal liver macrophages to determine the remaining questions. Whether this phenomenon is related to Notch signaling activation is also an interesting point. More works need to be done to test that.

Chapter 5: Bibliography

1. Ashayeri Ahmadabad, R., et al., *Toll-Like Receptor Signaling Pathways: Novel Therapeutic Targets for Cerebrovascular Disorders.* Int J Mol Sci, 2021. **22**(11).
2. Kutikhin, A.G., *Association of polymorphisms in TLR genes and in genes of the Toll-like receptor signaling pathway with cancer risk.* Hum Immunol, 2011. **72**(11): p. 1095-116.
3. Lucas, K. and M. Maes, *Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway.* Mol Neurobiol, 2013. **48**(1): p. 190-204.
4. Roy, A., et al., *Potential therapeutic targets for inflammation in toll-like receptor 4 (TLR4)-mediated signaling pathways.* Int Immunopharmacol, 2016. **40**: p. 79-89.
5. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-82.
6. Vives-Pi, M., et al., *Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to endotoxin (LPS) in islet beta cells.* Clin Exp Immunol, 2003. **133**(2): p. 208-18.
7. Lim, J., et al., *Autophagy regulates inflammatory programmed cell death via turnover of RHIM-domain proteins.* Elife, 2019. **8**.
8. Najjar, M., et al., *RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4.* Immunity, 2016. **45**(1): p. 46-59.
9. Tanimura, N., et al., *Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling.* Biochem Biophys Res Commun, 2008. **368**(1): p. 94-9.
10. Schulze-Osthoff, K., et al., *Apoptosis signaling by death receptors.* Eur J Biochem, 1998. **254**(3): p. 439-59.
11. Tang, D., et al., *The molecular machinery of regulated cell death.* Cell Res, 2019. **29**(5): p. 347-364.
12. Fadeel, B. and S. Orrenius, *Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease.* J Intern Med, 2005. **258**(6): p. 479-517.
13. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
14. Degterev, A., et al., *Identification of RIP1 kinase as a specific cellular target of necrostatins.* Nat Chem Biol, 2008. **4**(5): p. 313-21.
15. Fu, Y., et al., *Cloning of DLM-1, a novel gene that is up-regulated in activated macrophages, using RNA differential display.* Gene, 1999. **240**(1): p. 157-63.
16. Kuriakose, T. and T.D. Kanneganti, *ZBP1: Innate Sensor Regulating Cell Death and Inflammation.* Trends Immunol, 2018. **39**(2): p. 123-134.
17. Newton, K., et al., *RIPK1 inhibits ZBP1-driven necroptosis during development.* Nature, 2016. **540**(7631): p. 129-133.
18. Ingram, J.P., et al., *ZBP1/DAI Drives RIPK3-Mediated Cell Death Induced by IFNs in the Absence of RIPK1.* J Immunol, 2019. **203**(5): p. 1348-1355.
19. Muendlein, H.I., et al., *ZBP1 promotes LPS-induced cell death and IL-1beta release via RHIM-mediated interactions with RIPK1.* Nat Commun, 2021. **12**(1): p. 86.

20. Baik, J.Y., et al., *ZBP1 not RIPK1 mediates tumor necroptosis in breast cancer.* Nat Commun, 2021. **12**(1): p. 2666.
21. Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake, *Notch signaling: cell fate control and signal integration in development.* Science, 1999. **284**(5415): p. 770-6.
22. Bolos, V., J. Grego-Bessa, and J.L. de la Pompa, *Notch signaling in development and cancer.* Endocr Rev, 2007. **28**(3): p. 339-63.
23. Gaiano, N. and G. Fishell, *The role of notch in promoting glial and neural stem cell fates.* Annu Rev Neurosci, 2002. **25**: p. 471-90.
24. Liu, Z.J., et al., *Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis.* Mol Cell Biol, 2003. **23**(1): p. 14-25.
25. Sander, G.R. and B.C. Powell, *Expression of notch receptors and ligands in the adult gut.* J Histochem Cytochem, 2004. **52**(4): p. 509-16.
26. Kumar, R., L. Juillerat-Jeanneret, and D. Golshayan, *Notch Antagonists: Potential Modulators of Cancer and Inflammatory Diseases.* J Med Chem, 2016. **59**(17): p. 7719-37.
27. Talora, C., et al., *Notch signaling and diseases: an evolutionary journey from a simple beginning to complex outcomes.* Biochim Biophys Acta, 2008. **1782**(9): p. 489-97.
28. Miele, L., *Transcription factor RBPJ/CSL: a genome-wide look at transcriptional regulation.* Proc Natl Acad Sci U S A, 2011. **108**(36): p. 14715-6.
29. D'Souza, B., L. Meloty-Kapella, and G. Weinmaster, *Canonical and non-canonical Notch ligands.* Curr Top Dev Biol, 2010. **92**: p. 73-129.
30. Pursglove, S.E. and J.P. Mackay, *CSL: a notch above the rest.* Int J Biochem Cell Biol, 2005. **37**(12): p. 2472-7.
31. Xue, L., et al., *Inhibition of Recombining Binding Protein Suppressor of Hairless (RBPJ) Impairs the Growth of Prostate Cancer.* Cell Physiol Biochem, 2015. **36**(5): p. 1982-90.
32. Zhang, G., et al., *RBPJ contributes to the malignancy of glioblastoma and induction of proneural-mesenchymal transition via IL-6-STAT3 pathway.* Cancer Sci, 2020. **111**(11): p. 4166-4176.
33. Zhang, Q., et al., *RBPJ polymorphisms associated with cerebral infarction diseases in Chinese Han population: A Clinical Trial/Experimental Study (CONSORT Compliant).* Medicine (Baltimore), 2018. **97**(31): p. e11420.
34. Zhao, B., et al., *TNF-induced osteoclastogenesis and inflammatory bone resorption are inhibited by transcription factor RBP-J.* J Exp Med, 2012. **209**(2): p. 319-34.
35. Tsao, P.N., et al., *Lipopolysaccharide-induced Notch signaling activation through JNK-dependent pathway regulates inflammatory response.* J Biomed Sci, 2011. **18**: p. 56.
36. Osipo, C., et al., *Off the beaten pathway: the complex cross talk between Notch and NF-kappaB.* Lab Invest, 2008. **88**(1): p. 11-7.
37. Bai, X., et al., *Acetylation-Dependent Regulation of Notch Signaling in Macrophages by SIRT1 Affects Sepsis Development.* Front Immunol, 2018. **9**: p. 762.

38. Tabaja, N., et al., *Structure-function analysis of RBP-J-interacting and tubulin-associated (RITA) reveals regions critical for repression of Notch target genes.* J Biol Chem, 2017. **292**(25): p. 10549-10563.
39. Rebsamen, M., et al., *DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-κB.* EMBO reports, 2009. **10**(8): p. 916-922.