Summer 2023

CUL3 Negatively Regulates NLRP12-Mediated Inhibition of the Canonical NF-κB Signaling Pathway

Inyeong Lee
Missouri State University, Inyeong814@live.missouristate.edu

CUL3 NEGATIVELY REGULATES NLRP12-MEDIATED INHIBITION OF THE CANONICAL NF-κB SIGNALING PATHWAY

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Natural and Applied Sciences, Biology

By

Inyeong Lee

August 2023

CUL3 NEGATIVELY REGULATES NLRP12-MEDIATED INHIBITION OF THE CANONICAL NF-κB SIGNALING PATHWAY

Biology

Missouri State University, August 2023

Master of Science

Inyeong Lee

ABSTRACT

Nod-like receptor family pyrin domain-containing protein 12 (NLRP12) is mainly known for its inhibitory function on NF-κB signaling in innate immune cells, and more recently, for its ability to regulate chemokine signaling and ubiquitination of the immune receptor RIG-I. Through a yeast 2-hybrid screen, the Lupfer lab previously discovered that NLRP12 interacts with other ubiquitin-associated proteins including Cullin 3 (CUL3) and RING finger protein 2 (RNF2). This research was conducted to mainly investigate the interaction between NLRP12 and CUL3 in human cells and examine the role in regulating NF-κB signaling. Previously, co-immunoprecipitation, followed by western blot analysis, and confocal microscopy confirmed the interaction in HEK293T cells. In this research, NF-κB activation was examined during the interaction. HEK293T cells that express TLR2 were co-transfected with NLRP12 and CUL3 and treated with peptidoglycan (1 μg/mL) for 0, 0.25, 0.5, 1, and 4 hours to examine NF-κB activation. Then, NF-κB activation was assessed by western blot for IκBα phosphorylation. Although NLRP12 alone suppressed NF-κB activation, the co-transfected cells did not show a significant difference from the control transfected cells. These data suggest that CUL3 negatively regulates NLRP12, preventing it from inhibiting NF-κB signaling by ubiquitinating NLRP12 itself.

KEYWORDS: NOD-like Receptors, NLRP12, Inflammasomes, Inflammation, Autoinflammatory Disease, Ubiquitination, RING Finger Protein 2, Cullin 3

CUL3 NEGATIVELY REGULATES NLRP12-MEDIATED INHIBITION OF THE CANONICAL NF-κB SIGNALING PATHWAY

By

Inyeong Lee

A Master's Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
Master of Natural and Applied Sciences, Biology

August 2023

Approved:

Christopher Lupfer, Ph.D., Thesis Committee Chair

Kyoungtae Kim, Ph.D., Committee Member

Paul Durham, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to thank the Missouri State Graduate College and the Biology Department. I truly am thankful for the guidance provided by my advisor Dr. Christopher Lupfer, as well as my other graduate thesis committee members Dr. Kyoungtae Kim and Dr. Paul Durham. This appreciation also extends to my previous advisor Dr. Joshua Smith. Without the help of all these individuals, I wouldn't have come this far. Thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Immune System

In our everyday life, our body is exposed to various foreign pathogens or cellular stress, such as bacteria, viruses, parasites, fungi, toxins, or UV radiation. Pathogens that cause infections or cellular damage can replicate in our body quickly. Our innate immune system is the first line of defense against those invading pathogens and endogenous danger molecules. It has evolved to recognize the structure of antigens that are conserved among pathogens and respond accordingly through germline-encoded pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), whose structures are also conserved (Mogensen, 2009). Since those receptors are limited in number and recognize broadly shared patterns among pathogens, the system does not respond differently based on the specific invader that it detects (Vivier and Malissen, 2005). This provides a quick, generalized response to pathogen invasion without the need for prior exposure to the pathogen. Furthermore, information from the innate immune system is required for the adaptive immune response to be mobilized (Alberts et al., 2002). When an innate immune system fails to eliminate a new infection, the innate immune system can activate and coordinate with the adaptive immune system, which provides a more specific and long-lasting response (Janeway et al., 2001). Thus, the innate immune system is a crucial component of the overall immune response, protecting the body from harmful pathogens and preventing infections from taking hold.

Immune Disorders

One in fifteen people in the United States are suffering from autoimmune diseases, compared to five in 10,000 people in the states that have autoinflammatory diseases (Ciccarelli et al., 2014). Traditionally, researchers believed that autoimmune diseases were caused by adaptive immune cells, such as T or B cells, that mistakenly attack our normal healthy cells, whereas autoinflammatory diseases were caused by dysregulation of the innate immune system that causes hyperinflammation without an apparent trigger (McGonagle and McDermott, 2006). Autoinflammatory and autoimmune diseases have common features as the activation against self, with subsequent systemic inflammation, and without any external causal factors trigger these attacks (Georgin-Lavialle et al., 2018). Although some autoinflammatory disorders can resemble autoimmune diseases and sometimes serve as a prelude to autoimmunity, the underlying pathogenesis is mostly distinct. Autoinflammatory diseases can result from gene mutations that are directly or indirectly involved in the regulation of inflammasomes or the interleukin 1 (IL-1) cytokine signaling pathway (Alsharief et al, 2020; Georgin-Lavialle et al., 2018). IL-1β is vital in liking the innate immune response mediated by PRRs, such as NOD-like receptors (NLRs), or IL-1, and the adaptive immune responses of T and B cells. IL-1 has also a role in the activation of IFN-γ in memory T cells, T cell proliferation, increased B cell proliferation, and increased antibody synthesis, thus connecting the inflammasome-driven responses to the adaptive immune system in response to exogeneous and endogenous signals (Bendtzen, 2011).

Although autoimmune diseases are still considered adaptive immunity-mediated disorders, there is increasing proof that innate immunity and inflammasomes are also included (Georgin-Lavialle et al., 2018; Zhang and Lu, 2018; El-Shebiny et al., 2021; Arakelyan et al., 2017; Hachim et al., 2020; Marshak-Rothstein, 2006). For example, dysregulation of NLRP3 can lead to a development of an autoimmune diseases, such as experimental autoimmune

encephalomyelitis (EAE), arthritis (Place and Kanneganti, 2020; Davis et al., 2011; Duhen, 2014). Furthermore, studies have shown that NLRP3 also contributes to shaping adaptive immune responses through production of the innate cytokines interleukin-1β (IL-1β) and IL-18, which promote the differentiation of Th1/17 cells that are specific for host protection against microbes. Researchers are exploring ways to target inflammasomes to control inflammation and tissue damage in these diseases. For example, some studies have shown that blocking the inflammasome protein NLRP3 can reduce inflammation in models of gout, multiple sclerosis, diabetes, ulcerative colitis, and Alzheimer's disease (Bai and Zhang, 2021).

Pattern Recognition Receptors (PRRs)

PRRs are located either on the surface of the cells or in the cytoplasmic compartment of the cell. There are five types of PRRs categorized based on their central protein domain: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs). Here, I will focus on those receptors important for my research, which are the TLRs and NLRs. Signaling pathways mediated by TLRs and NLRs work together to provide strong and effective immune responses. Toll-like receptors (TLRs) are present in the plasma membrane of various cell types, including innate and adaptive immune cells and non-immune cells, for example, macrophages, monocytes, dendritic cells, neutrophils, B cells, T cells, fibroblasts, endothelial cells, and epithelial cells (Duan et al., 2022). NOD-like receptors (NLRs) are located in the cytosol of innate immune cells, but also in that of B cells and epithelial cells.

Toll-like Receptors (TLRs). Our innate immune system can immediately respond to both PAMPs and DAMPs of broad invaders using the receptors TLRs and NLRs along with other PRRs. When a TLR binds its ligand, it forms either a homo- or hetero-dimer with another TLR. Dimerization allows for cytoplasmic tails of the TLRs to recruit and bind to TIR domain-containing adaptor proteins, for example, MyD88, TRIF, TRAM, or Mal. TLR signaling pathways result in different outcomes depending on the adaptor proteins. There are 10 TLRs in humans, which are numbered in TLR1 through TLR10. TLR1, TLR2, TLR4, TLR5, and TLR6 are located on the surface of the cells, and TLR3, TLR7, TLR8, and TLR9 are endosomal transmembrane proteins. TLR2 heterodimerizes with TLR1 or TLR6, and the dimers detect exogenous bacterial lipoproteins, lipoteichoic acid, or peptidoglycan (PepG). TLR4 and TLR5 are homodimerized and detect extracellular lipopolysaccharide (LPS) and flagellin, respectively. The endosomal TLRs detect single or double stranded RNA or unmethylated DNA that entered the endosome, as a mechanism to defend against foreign viruses and abnormal self-molecules.

NOD-like Receptors (NLRs). NOD-like receptor family pyrin domain-containing protein or nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins are a family of intracellular PRRs that play a critical role in innate immune responses. There are more than 20 NLRs in humans, and each NLR has its own mode of regulation and function (Wilmanski et al., 2008). An NLR is a multi-domain protein made up of three functional domains: (1) a central nucleotide-binding and oligomerization domain (NOD; also known as NBD and NACHT) that allows ATP binding and NLR oligomerization, (2) the central leucine-rich repeat (LRR) domain that is responsible for ligand recognition and functional regulation, and (3) the N-terminal signaling/effector domain involved in signal transduction through interactions with downstream adaptor proteins (Trindade and Chen, 2020). There are four types

of N-terminal domains: PYD (pyrin domain), CARD (caspase recruitment domain), BIR (baculovirus inhibitory repeat domain), and acidic transactivation domain (AD). Based on the N-terminal domains, NLRs can be classified into four subfamilies: NLRP (PYD-containing; formerly known as NALPs), NLRC (CARD-containing; formerly known as NODs), NLRB (BIR-containing; formerly known as NAIP), and NLRA (AD-containing) (Franchi et al., 2009). Among them, NLRP is the most extensively studied subfamily.

Some NLR proteins form immune protein complexes that play important roles in the innate immune system called inflammasomes. An inflammasome is composed of an NLRP, an adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and a pro-caspase-1 enzyme (Figure 1). The most well studied inflammasome contains NLRP3. NLRP3 recognizes various intracellular PAMPs or DAMPs and oligomerizes with ASC and pro-caspase-1. Then, pro-caspase-1 is cleaved, which induces the production of an active caspase-1. The active caspase-1 then cleaves pro-inflammatory cytokines pro-IL-1β and pro-IL-18 to release an active form of those cytokines, IL-1β and IL-18. Caspase-1 activation also cleaves gasdermin D, which triggers a highly inflammatory mode of programmed cell death called pyroptosis (Coll et al., 2022).

Because of their role in inflammation, autoimmune and inflammatory diseases and even cancer can be caused by dysregulation of NLR proteins (Péladeau and Sandhu, 2021). For example, when NLRP12 is genetically deleted, an intestinal inflammatory disease called experimental colitis and colon cancer can be caused (Gharagozloo et al., 2018). Also, deficiencies in NLRP3 are reported to cause susceptibility to colitis and colitis-associated cancer (CAC) (Davis et al, 2011).

NF-κB Signaling Pathway

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that plays an important role in regulating transcription, protein expression, and signaling pathways. The NF-κB signaling pathway is a key pathway that regulates the survival, activation and differentiation of innate immune cells and inflammatory T cells. Consequently, deregulated NF-κB activation contributes to the pathogenic processes of various inflammatory diseases. The pathway is activated by a variety of stimuli, including cytokines, bacterial and viral components, and stress signals. Activation of the pathway leads to the translocation of NF-κB transcription factors from the cytoplasm to the nucleus, where they induce the expression of genes involved in the immune response (Oeckinghaus and Ghosh, 2009).

There are two types of NF-κB signaling pathways: canonical and non-canonical (Figure 1). The canonical NF-κB pathway is activated by TLRs when they sense bacterial antigens like LPS or PepG. A dimerized set of ligand-bound TLRs recruits adapter proteins like MyD88, which in turn activate downstream signaling kinases, including IRAK1 and TRAF6. These kinases then activate the IKK complex, which phosphorylates the inhibitor of NF-κB (IκB), leading to its degradation and the subsequent translocation of NF-κB to the nucleus (Kawai and Akira, 2007; Oeckinghaus and Ghosh, 2009). The non-canonical NF-κB pathway is activated when tumor necrosis factor (TNF) receptors (TNFRs) are activated by its ligand. The receptors recruit adaptor proteins like TRAF1 or TRAF2 and activate downstream signaling proteins like TRAF3, NIK, IKK, and NF-κB in a similar manner. Eventually, NF-κB transcription factors are localized to the nucleus and activate genes that the canonical pathway does not activate. Importantly, NLR proteins have been found to regulate the NF-κB pathway through a variety of mechanisms. For example, some NLRs, such as NLRP3, can activate the NF-κB pathway, while

others, such as NLRP12, which is the protein of interest in this thesis project, can negatively regulate the pathway by inhibiting IRAK1 phosphorylation (Rothschild et al., 2018).

NLRP12 as a Negative Regulator

NLRP12, also known as Monarch-1 or PYPAF7, is a member of the NLR protein family, which plays critical roles in the innate immune response to microbial infection and cellular stress. It is expressed in various tissues including immune cells such as macrophages, dendritic cells, and T cells, as well as in non-immune cells, such as epithelial cells and fibroblasts (Davis et al., 2011).

Like other NLR proteins, NLRP12 is comprised of three main domains: an N-terminal pyrin domain (PYD), a central NACHT domain (also known as NOD domain), and a C-terminal leucine-rich repeat (LRR) domain. The PYD domain is responsible for protein-protein interactions and mediates the recruitment of downstream effector proteins, such as caspase-1, to the NLRP12 complex. The NACHT domain is a nucleotide-binding domain that undergoes conformational changes upon activation, leading to the formation of higher-order oligomers. The LRR domain is involved in ligand recognition and signal transduction (Tuladhar and Kanneganti, 2020).

NLRP12 is mainly known to negatively regulate the innate immune signaling pathways in a pathogen-specific manner (Tuladhar and Kanneganti, 2020). NLRP12 inhibits NF-κB activation by several mechanisms, although the exact mechanisms still remain to be determined. (1) NLRP12 is known to negatively regulate the canonical NF-κB signaling pathway by inhibiting the hyperphosphorylation of the kinase IRAK1, which is a downstream target of Toll-like receptors (TLRs) involved in the activation of NF-κB (Lich et al., 2007; Rothschild et al.,

2018). (2) NLRP12 also negatively regulates the non-canonical NF-κB signaling pathway by interaction with TRAF3 and NF-κB-inducing kinase (NIK) resulting in degradation through a proteasome-dependent pathway (Lich et al., 2007; Allen et al., 2012). This pathway was shown to be important for the inhibition of the non-canonical NF-κB signaling pathway during colon inflammation (Allen et al., 2012). (3) NLRP12 also inhibits the MAPK/ERK signaling pathway, which plays a crucial role in various cellular processes such as cell proliferation, differentiation, survival, and apoptosis (Tuladhar and Kanneganti, 2020). In addition, during vesicular stomatitis virus (VSV) infection, NLRP12 interacts with the E3 ubiquitin ligase TRIM25 to reduce polyubiquitination of RIG-I, resulting in reduced type I IFN production (Chen et al., 2019).

Unlike many other NLRP proteins, NLRP12 does not typically form an inflammasome. However, it has been reported that during *Yersinia pestis* and *Plasmodium chabaudi* infection, NLRP12 forms the NLRP12 inflammasome, which can lead to caspase-1 activation and IL-1β and IL-18 release (Vladimer et al., 2012). Taken together, it is interesting that NLRP12 does almost the opposite of what other NLRP proteins do even though it shares very similar structures. Scientists are still unclear how exactly NLRP12 regulates various cellular processes including signaling pathways, inflammation, and cellular homeostasis.

Ubiquitination

Ubiquitination is a tightly regulated post-translational modification process where one or more ubiquitin molecules are attached to a protein that needs to be regulated. Ubiquitin is a small and highly conserved protein that consists of 76 amino acids and is found in all eukaryotic cells. Ubiquitination occurs through a series of enzymatic cascades utilizing three enzymes: E1, E2, and E3 ubiquitin ligases (Song and Luo, 2019). The E1 enzyme forms a thioester bond with the

C-terminus of ubiquitin to activate a ubiquitin molecule. Then, the activated ubiquitin is transferred to the active site of the E2 enzyme. Finally, the E3 ubiquitin ligase transfers the ubiquitin from the E2 enzyme to a target protein (Guo et al., 2023). This process is involved in targeting proteins for either regulation of protein activity and localization through protein-protein interactions or degradation through the proteasome. In the innate immune system, ubiquitination can regulate the activation of NF-κB by promoting the degradation of the NF-κB inhibitor, IκB (Chen, 2005).

Ubiquitination takes several forms. It could be monoubiquitination, where only one ubiquitin is added to a protein, or multiubiquitination where more than one ubiquitin molecule is attached to the same lysine residue of a substrate protein, or polyubiquitination where an elongated ubiquitin molecule is attached to the substrate protein (Sadowski and Sarcevic, 2010). The most common types of polyubiquitination are K48-linked and K63-linked polyubiquitination. K48-linked polyubiquitination signals mainly target proteins for proteasome-dependent protein degradation, whereas K63-linked ubiquitination acts as a scaffold for protein/protein interactions that regulate the localization and activity of protein kinases (Wang et al., 2012).

Ring finger protein 2 (RNF2). RNF2, also known as Ring1B, acts as a ubiquitin E3 ligase to ubiquitinate histone H2A for its monoubiquitination. RNF2 plays a pivotal role in early development as its deficiency causes early embryonic lethality. RNF2 polyubiquitinates tumor suppressor TP53 that leads to tumor formation, and DNA replication inhibitor Geminin to regulate the activity of hematopoietic stem cells. RNF2 is also known for K48-linked ubiquitination of AMBRA1 as an E3 ligase, leading to downregulation of autophagy (Xia et al., 2014).

Cullin 3 (CUL3). Cullin 3 is a part of an E3 ubiquitin ligase complex called Cullin-RING ubiquitin ligase (CRL), which is the most extensive group of ubiquitin E3 ligases in eukaryotic cells. CUL3 serves as scaffold through the C-terminal domains tightly associates with the RING-H2-domain proteins RBX1 or RBX2 where an activated ubiquitin molecule is transferred to the active site of the E2 enzyme (Figure 2). CUL3 interacts the substrate adaptor and receptor proteins called BTB through N-terminal domains. The BTB domain mediates CUL3 binding and an adjacent protein-interaction domain recruits substrates for ubiquitylation. The BTB domain of the CUL3-RING ubiquitin ligase includes one or more Kelch repeats that are responsible for substrate binding and involved in CUL3-dependent ubiquitination (Dubiel et al., 2018).

Scientific Questions and Hypothesis

Lich et al. (2007) demonstrated that NLRP12 negatively regulates the canonical NF-κB signaling pathway by inhibiting the hyperphosphorylation of the kinase IRAK1. According to personal communication with Dr. Christopher Lupfer, he has observed that NLRP12 negatively regulates the signaling pathway through the ubiquitination and degradation of IRAK1. How NLRP12 accomplishes this ubiquitination of IRAK1 is still unknown. NLRP12 does not have intrinsic activity as a ubiquitin ligase, protease, kinase, or phosphatase. Therefore, it is likely that NLRP12 requires interaction with additional proteins in order to become activated.

Previously, the Lupfer lab found that NLRP12 interacts with several ubiquitin-associated proteins including RNF2 (RING finger protein 2) and CUL3 (Cullin 3) through yeast 2-hybrid screening. In addition, they observed that NLRP12 interacts with CUL3 in the human cell line HEK293T cells.

Based on this, I hypothesized that first, RNF2 may also interact with NLRP12 in HEK293T cells. Second, since RNF2 and CUL3 are ubiquitin-associated proteins, NLRP12 may regulate ubiquitination and degradation of IRAK1 via RNF2 or/and CUL3 to regulate NF-kB signaling.

MATERIALS AND METHODS

Cell Culture and Maintenance

TLR2-negative HEK293T and TLR2-positive HEK293T cells were purchased from BEI Resources. Cells were cultured in DMEM (Table 1) supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin solution (Cytiva, Table 1) in a T75 flask and incubated at 37°C in 5% CO_2 until a desired confluence was reached. Once 80% confluence was observed under the microscope, the old serum media was aspirated. Then, 10 mL 1 X PBS (Table 1) was slowly added to the cells and swirled over the cells. Then, they were treated with 1 mL 0.25% Trypsin, 2.21 mM EDTA (Table 1) for up to 10 minutes in the 37°C 5% CO_2 incubator. The trypsin was then neutralized and diluted in 10 mL DMEM with 10% v/v FBS and 1% v/v penicillin-streptomycin solution. Cells were either counted and replated for experiments or passaged for continued growth.

Transfection

The DNA plasmids that were used in this thesis were designed and cloned previously (Rippe, 2023). HEK293T cells were seeded in 6-well plates at a density of 4.5×10^5 per well or seeded in 12-well plates at a density of 2.5×10^5 per well. The plates were left in the 37°C 5% CO_2 incubator until the cells reached at least 50% confluence. Then, for 12-well plates, old serum media in each well was removed and replaced with 0.75 mL serum-free DMEM. In a sterile 1.5 mL microcentrifuge tube, 1.2 µg DNA was diluted in 80 µL serum-free DMEM. Then, 3.6 µL PEI MAX (1 µg/µL, Table 1.) was added to the diluted DNA. For 6-well plates, old serum media was removed and replaced with 1.5 mL serum-free DMEM. Then, in a sterile 1.5 mL microcentrifuge tube, 3 µg DNA was diluted in 200 µL serum-free DMEM, followed by the

addition of 9 µL of PEI MAX (1 µg/µL) to the diluted DNA. The DNA/PEI mixture was vortexed for 10 seconds and incubated for 15 minutes at room temperature and then added to cells. The cells were incubated with the mixture for 4 hours in the 37°C 5% CO_2 incubator. The serum-free media with reagents was replaced with 1 mL DMEM with 10% v/v FBS and 1% v/v penicillin-streptomycin solution after 4 hours of incubation, because cells that were transfected with NLRP12 were dying quicker in the media. The cells were incubated for 32 more hours to allow plasmid gene expression and protein translation.

Western Blot

Samples were collected and lysed using either RIPA lysis buffer (Table 1) for NF-κB activation assay or 1% NP-40 lysis buffer Table 1) for co-immunoprecipitation and ubiquitin assay. Then, 4X SDS loading dye was added to the lysates. Samples were heated using a heat block at 95°C for 10 minutes. Then, the samples were removed from the heat and separated using electrophoresis on a 6 or 8% sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gel that was made for the assay in lab. The proteins were then transferred onto a PVDF (polyvinylidene difluoride) membrane. After the transfer was complete, the membrane was blocked in blocking buffer (5% w/v nonfat dry milk in 1X TBST; 1X TBS + 0.5% Tween-20; wash buffer) for 30 minutes. The blocking buffer was then removed, and the primary antibody diluted in fresh blocking buffer (1:1000) was added and allowed to incubate while slowly rocked at 4°C overnight. The next day, the primary antibody was removed, and the membrane was washed with 1x TBST three times before the secondary antibody was added (1:5000 dilution in blocking buffer) and incubated for 1 hour at room temperature. The membranes were then washed five

more times with the wash buffer. Blots were imaged using Radiance Q substrate (Table 1) on an Azure c300 digital gel imager.

NF-κB Activation Assay

Four groups of HEK293T cells were transfected: one group with GFP as a positive control, one group with NLRP12, another group with both NLRP12 and CUL3, and a fourth group with just CUL3. After 38 hours post-transfection, each group was treated with peptidoglycan (1 μg/mL) for 0, 0.25, 0.5, 1, and 4 hours in the 37℃ 5% CO_2 incubator. After incubation, the cells were lysed using RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were collected and analyzed by western blot using anti-Myc tag, anti-Flag tag, anti-β-actin, anti-IκB alpha, and anti-phospho-IκB alpha antibodies in fresh blocking buffer (1:1000) as primary antibodies, and HRP antibodies in fresh block buffer (1:5000) as secondary antibodies. β-actin was used as a loading control to normalize differences in protein between different groups. ImageJ was used to quantify the intensity of the protein bands and the intensity values of pIκBα and IκBα were normalized to the intensity of the control. The intensity of the pIκBα was divided by that of the total IκBα; This ratio represents the relative intensity of pIκBα compared to total IκBα and is used to measure IκBα phosphorylation levels. GraphPad Prism 9 was used to plot the data. The whole assay was performed in duplicate.

Co-Immunoprecipitation

Thirty-six hours post-transfection, cells were lysed in 0.5 mL cold 1% NP-40 lysis buffer for 30 minutes on a shaker at 4℃. Next, the lysates were collected and disrupted using Dounce homogenizers on ice. The disrupted lysates were transferred into 1.5 mL tubes. The tubes were

centrifuged at 5,000 x *g* for 10 minutes at 4℃. Then, 350 µL supernatant of each sample was placed into a new tube and 2 µg of IP antibody was added for the ubiquitin assay. The mixture was incubated on a shaker at 4℃ for 1 hour. Then, 40 µL protein A/G agarose beads (Table 1) were added to the mixture and incubated on the shaker at 4℃ overnight. The following day, the beads were washed five times by centrifuging at 2,000 x *g* for 1 minute at 4℃, removing the supernatant, and adding 1 mL cold PBS. After the final wash step, 50 µL 4X SDS loading dye was added to the immunocomplex. Samples were vortexed for 10 seconds and denatured using a heat block at 95℃. After about 10 minutes, the samples were then removed from the heat and electrophoresed on a 6-8% SDS-PAGE gel to perform western blot.

Ubiquitination Assay

Three groups of HEK293T cells were transfected: one group with GFP as a positive control, one group with just NLRP12, and the other group with both NLRP12 and CUL3. The samples were co-immunoprecipitated with anti-Myc antibody. Then, the samples were collected and analyzed by western blot using an anti-ubiquitin antibody in fresh blocking buffer (1:1000) as a primary antibody, and an HRP antibody in fresh block buffer (1:5000) as a secondary antibody (Table 2). The whole assay was performed in duplicate.

Statistical Analysis

Two-Way ANOVA was performed using GraphPad Prism 9 to evaluate statistical differences between groups. A p-value of <0.05 was considered statistically significant (Table 3).

RESULTS

RNF2 May Not Interact with NLRP12 in HEK293T Cells

Given that the Lupfer lab previously discovered that NLRP12 interacts with RNF2 via yeast-2-hybrid experiment, co-immunoprecipitation was performed to investigate the potential interaction between NLRP12 and RNF2 in HEK293T cells. Results from previous western blots (Figure 3A) once showed a strong interaction between NLRP12 and RNF2. The result alone provides evidence that the two proteins may interact, but this could not be consistently reproduced (Figure 3B). As the interaction with NLRP12 and RNF2 was questionable, I pursued other experiments with NLRP12 and CUL3.

NF-κB Activation Was Enhanced by the Interaction between NLRP12 and CUL3

Prior to this research, the Lupfer lab found that NLRP12 interacts with CUL3 in both yeast and HEK293T cells. Based on that information, an NF-κB activation assay was performed to examine whether there is any change in NF-κB activation during the interaction in HEK293T cells. Four different transfection groups (GFP, NLRP12, NLRP12 + CUL3, and CUL3) of HEK293T cells expressing TLR2 were treated with peptidoglycan (1 μg/mL) for 0, 0.25, 0.5, 1, and 4 hours to examine the canonical NF-κB signaling pathway (Figure 4A). As expected, overexpression of NLRP12 alone resulted in a decrease in NF-κB activation. However, co-transfection of HEK293T cells with NLRP12 and CUL3 surprisingly resulted in an increase in NF-κB activation compared to NLRP12 alone and showed a similar pattern to that of the positive control (Figure 4B). Interestingly, NLRP12 expression was increased when both NLRP12 and CUL3 were overexpressed at all time periods (0-4 hours). When CUL3 alone was overexpressed

in HEK293T cells, an increase in NF-κB activation was observed from 0.5 hours to 4 hours (Figure 4B).

CUL3 Mediates Ubiquitination of NLRP12

Based on the NF-κB activation assay results, it was proposed that CUL3 prevents NLRP12 from inhibiting the canonical NF-κB signaling pathway. Since CUL3 is an E3 ubiquitin ligase, it was also proposed that NLRP12 may be ubiquitinated by CUL3. HEK293T cells were co-transfected with NLRP12 and CUL3. Then, co-immunoprecipitation was conducted to perform a ubiquitin assay using the anti-ubiquitin antibody. Western blot analysis of the assay showed a smeared band of ubiquitin molecules, revealing that NLRP12 was ubiquitinated when both NLRP12 and CUL3 were overexpressed as expected (Figure 5).

DISCUSSION

The overall goal of my thesis project was to investigate the potential interaction between NLRP12 and CUL3 in HEK293T cells. Previously, the Lupfer lab demonstrated that NLRP12 is involved in the degradation of IRAK1 through ubiquitination. In addition, they also reported that some ubiquitin-associated proteins including RNF2 and CUL3 interact with NLRP12 in yeast through a yeast 2-hybrid screen. The initial protein of interest for my thesis project was RNF2, and one of the original goals was to determine if there was an interaction between RNF2 and NLRP12 in a human cell line. However, the co-immunoprecipitation experiments yielded inconsistent results. This could be that the interaction in HEK293T cells may not normally occur, or they interact indirectly, or only under certain conditions. It is possible that transfecting the cells to overexpress the proteins may have caused an abnormal interaction between the proteins. In order to know whether the two proteins interact indirectly or not, confocal microscopy can be used to observe the localization of the proteins, while fluorescence resonance energy transfer (FRET) can be performed to both measure the distance between the proteins and observe their interactions. Although I could not confirm the interaction between NLRP12 and RNF2, it still needs to be determined if there is any change in NF-κB activation when both NLRP12 and RNF2 were overexpressed. If there is no change, that would confirm that there is no interaction between NLRP12 and RNF2 in HEK293T cells. If there is any change, there must be other factors that are involved in the interaction. Since I was unable to confirm the interaction of RNF2 with NLRP12, the focus was shifted to CUL3 as the Lupfer lab previously found evidence of CUL3 interacting with NLRP12 in HEK293T cells.

I decided to examine whether there is any change in NF-κB activation during the interaction of CUL3 and NLRP12 in HEK293T cells. Initially, it was expected that co-transfection of NLRP12 and CUL3 would result in lower levels of NF-κB activation compared to NLRP12 alone. However, the level of activation was actually found to be higher in the presence of both NLRP12 and CUL3. This finding suggested that the overexpression of CUL3 resulted in the loss of NLRP12's function as a negative regulator of the canonical NF-κB signaling pathway. Given that CUL3 is an E3 ubiquitin ligase that is involved in the ubiquitination process, it was proposed that CUL3 mediates the ubiquitination of NLRP12, thereby leading to the loss of its negative regulatory function. Furthermore, it was observed that the overexpression of CUL3 alone resulted in a decrease in NF-κB activation. This finding is consistent with the results of an IL-8 enzyme-linked immunosorbent assay (ELISA) conducted by another graduate student in the Lupfer lab (Rippe, 2023). It is possible that the overexpression of CUL3 may have led to the activation of other signaling pathways that indirectly influenced the canonical NF-κB pathway. Alternatively, CUL3 may have other targets besides NLRP12 that could affect NF-κB activation.

In order to further investigate the implications of the NF-κB activation assay results, I aimed to confirm whether NLRP12 is ubiquitinated by CUL3. Through western blot analysis, I observed that NLRP12 was ubiquitinated, when HEK293T cells were co-transfected with NLRP12 and CUL3. This finding does not support our initial thought that NLRP12 negatively regulates NF-κB activation through CUL3. Based on the previous findings that NLRP12 is involved in the ubiquitination of IRAK1 (Rothschild et al., 2018), and CUL3 interacts with NLRP12 in HEK293T cells (Rippe, 2023), it was believed that NLRP12 promotes ubiquitination of IRAK1 by CUL3. However, my results showed that CUL3 does not participate in the NLRP12-mediated inhibition of the NF-κB signaling pathway, as the overexpression of CUL3

led to the ubiquitination and loss of NLRP12's function as a negative regulator of the signaling pathway. Interestingly, although NLRP12 lost its function as a negative regulator when both NLRP12 and CUL3 were overexpressed, the protein expression of NLRP12 was increased. This suggests that the ubiquitination of NLRP12 may possibly be K63-linked polyubiquitination, but further experiments need to be performed to validate this hypothesis. Statistical analysis was performed to see if the differences between the treatment groups were significant. However, the p-value of treatments was 0.7132, indicating that this experiment needs more repeats to support the data stronger. Altogether, I conclude that CUL3 negatively regulates the NLRP12-mediated inhibition of the canonical NF-κB signaling pathway, likely through the ubiquitination of NLRP12.

For future directions, several approaches can be pursued to understand the interaction between NLRP12 and the two ubiquitin-associated proteins, RNF2 and CUL3, in HEK293T cells. For RNF2, although it did not appear to interact with NLRP12 in HEK293T cells, it is still worth investigating whether co-transfection of these cells results in any changes in NF-κB activation. That way, if there is no change, we can confirm that the two proteins do not interact to regulate this pathway. For CUL3, one promising approach would be to investigate the specific type of ubiquitination that occurs on NLRP12 when CUL3 is present. Co-immunoprecipitation using anti-K48-linked or anti-K63-linked polyubiquitination antibodies could be utilized to determine the exact type of ubiquitination that is occurring (Wang et al., 2012). Furthermore, the effects of CUL3 alone on the NF-κB signaling pathway needs to be evaluated, as this protein appears to have an interesting effect on pathway activation. Knocking out CUL3 and overexpressing NLRP12 simultaneously can help us understand the roles of each protein in the NF-κB signaling pathway by allowing us to observe the effect of each protein in isolation,

without the potential confounding effects of the other protein. By comparing the level of NF-κB activation in cells where CUL3 has been knocked out and NLRP12 has been overexpressed to the level of NF-κB activation in control cells, we can determine the effect of NLRP12 overexpression on the NF-κB signaling pathway independent of any potential effect of CUL3. Similarly, by comparing the level of NF-κB activation in cells where NLRP12 has been knocked out and CUL3 has been overexpressed to the level of NF-κB activation in control cells, we can determine the effect of CUL3 overexpression on the NF-κB signaling pathway independent of any potential effect of NLRP12. By combining these results with the results obtained from the co-transfection experiments, we can gain a more comprehensive understanding of the roles of CUL3 and NLRP12 in the NF-κB signaling pathway.

In conclusion, my findings suggest that NLRP12 promotes the ubiquitination of IRAK1 by CUL3. Instead, CUL3 negatively regulates NLRP12-mediated inhibition of the canonical NF-κB signaling pathway. This research needs to be done with more repeats to support the data I currently have and solve the detailed molecular mechanisms that can explain the observed changes in NF-κB activation, hoping to improve our understanding of the complex interplay between the various components of the NF-κB signaling pathway.

REFERENCES

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Innate Immunity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26846/

Allen, I. C., Wilson, J. E., Schneider, M., Lich, J. D., Roberts, R. A., Arthur, J. C., Woodford, R. M., Davis, B. K., Uronis, J. M., Herfarth, H. H., Jobin, C., Rogers, A. B., & Ting, J. P. (2012). NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-κB signaling. *Immunity*, *36*(5), 742–754. https://doi.org/10.1016/j.immuni.2012.03.012

Arakelyan, A., Nersisyan, L., Poghosyan, D., Khondkaryan, L., Hakobyan, A., Löffler-Wirth, H., Melanitou, E., & Binder, H. (2017). Autoimmunity and autoinflammation: A systems view on signaling pathway dysregulation profiles. PloS one, 12(11), e0187572. https://doi.org/10.1371/journal.pone.0187572

Bai, H., & Zhang, Q. (2021). Activation of NLRP3 Inflammasome and Onset of Alzheimer's Disease. *Frontiers in immunology*, *12*, 701282. https://doi.org/10.3389/fimmu.2021.701282

Chen G. Y. (2014). Role of Nlrp6 and Nlrp12 in the maintenance of intestinal homeostasis. *European journal of immunology*, *44*(2), 321–327. https://doi.org/10.1002/eji.201344135

Chen, S. T., Chen, L., Lin, D. S., Chen, S. Y., Tsao, Y. P., Guo, H., Li, F. J., Tseng, W. T., Tam, J. W., Chao, C. W., Brickey, W. J., Dzhagalov, I., Song, M. J., Kang, H. R., Jung, J. U., & Ting, J. P. (2019). NLRP12 Regulates Anti-viral RIG-I Activation via Interaction with TRIM25. *Cell host & microbe*, *25*(4), 602–616.e7. https://doi.org/10.1016/j.chom.2019.02.013

Chen Z. J. (2005). Ubiquitin signalling in the NF-kappaB pathway. *Nature cell biology*, *7*(8), 758–765. https://doi.org/10.1038/ncb0805-758

Coll, R. C., Schroder, K., & Pelegrín, P. (2022). NLRP3 and pyroptosis blockers for treating inflammatory diseases. *Trends in pharmacological sciences*, *43*(8), 653–668. https://doi.org/10.1016/j.tips.2022.04.003

Davis, B. K., Wen, H., & Ting, J. P. (2011). The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annual review of immunology*, *29*, 707–735. https://doi.org/10.1146/annurev-immunol-031210-101405

Duan, T., Du, Y., Xing, C., Wang, H. Y., & Wang, R. F. (2022). Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. Frontiers in immunology, 13, 812774. https://doi.org/10.3389/fimmu.2022.812774

Dubiel, W., Dubiel, D., Wolf, D. A., & Naumann, M. (2018). Cullin 3-Based Ubiquitin Ligases as Master Regulators of Mammalian Cell Differentiation. Trends in biochemical sciences, 43(2), 95–107. https://doi.org/10.1016/j.tibs.2017.11.010

Franchi, L., Warner, N., Viani, K., & Nuñez, G. (2009). Function of Nod-like receptors in microbial recognition and host defense. *Immunological reviews*, *227*(1), 106–128. https://doi.org/10.1111/j.1600-065X.2008.00734.x

Gharagozloo, M., Gris, K. V., Mahvelati, T., Amrani, A., Lukens, J. R., & Gris, D. (2018). NLR-Dependent Regulation of Inflammation in Multiple Sclerosis. *Frontiers in immunology*, *8*, 2012. https://doi.org/10.3389/fimmu.2017.02012

Guo, H. J., Rahimi, N. R., & Tadi, P. (2023). Biochemistry, Ubiquitination. In *StatPearls*. StatPearls Publishing.

Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. Principles of innate and adaptive immunity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27090/

Jesus, A. A., & Goldbach-Mansky, R. (2014). IL-1 blockade in autoinflammatory syndromes. *Annual review of medicine*, *65*, 223–244. https://doi.org/10.1146/annurev-med-061512-150641

Kawai, T., & Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. *Trends in molecular medicine*, *13*(11), 460–469. https://doi.org/10.1016/j.molmed.2007.09.002

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International journal of molecular sciences*, *20*(13), 3328. https://doi.org/10.3390/ijms20133328

Li, X., Yang, K. B., Chen, W., Mai, J., Wu, X. Q., Sun, T., Wu, R. Y., Jiao, L., Li, D. D., Ji, J., Zhang, H. L., Yu, Y., Chen, Y. H., Feng, G. K., Deng, R., Li, J. D., & Zhu, X. F. (2021). CUL3 (cullin 3)-mediated ubiquitination and degradation of BECN1 (beclin 1) inhibit autophagy and promote tumor progression. *Autophagy*, *17*(12), 4323–4340. https://doi.org/10.1080/15548627.2021.1912270

Lich, J. D., Williams, K. L., Moore, C. B., Arthur, J. C., Davis, B. K., Taxman, D. J., & Ting, J. P. (2007). Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. *Journal of immunology (Baltimore, Md. : 1950)*, *178*(3), 1256–1260. https://doi.org/10.4049/jimmunol.178.3.1256

Marshak-Rothstein A. (2006). Toll-like receptors in systemic autoimmune disease. *Nature reviews. Immunology*, *6*(11), 823–835. https://doi.org/10.1038/nri1957

McGonagle, D., & McDermott, M. F. (2006). A proposed classification of the immunological diseases. *PLoS medicine*, *3*(8), e297. https://doi.org/10.1371/journal.pmed.0030297

Mogensen T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews*, *22*(2), 240–273. https://doi.org/10.1128/CMR.00046-08

Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor perspectives in biology*, *1*(4), a000034. https://doi.org/10.1101/cshperspect.a000034

Péladeau, C., & Sandhu, J. K. (2021). Aberrant NLRP3 Inflammasome Activation Ignites the Fire of Inflammation in Neuromuscular Diseases. *International journal of molecular sciences*, *22*(11), 6068. https://doi.org/10.3390/ijms22116068

Place, D. E., & Kanneganti, T. D. (2020). The innate immune system and cell death in autoinflammatory and autoimmune disease. *Current opinion in immunology*, *67*, 95–105. https://doi.org/10.1016/j.coi.2020.10.013

Rippe, C., Examining the Immune Regulation of NLRP12 Through Novel Protein Interactions, The Department of Biology, Missouri State University: MSU Graduate Theses, 2023

Rothschild, D. E., McDaniel, D. K., Ringel-Scaia, V. M., & Allen, I. C. (2018). Modulating inflammation through the negative regulation of NF-κB signaling. *Journal of leukocyte biology*, 10.1002/JLB.3MIR0817-346RRR. Advance online publication. https://doi.org/10.1002/JLB.3MIR0817-346RRR

Sadowski, M., & Sarcevic, B. (2010). Mechanisms of mono- and poly-ubiquitination: Ubiquitination specificity depends on compatibility between the E2 catalytic core and amino acid residues proximal to the lysine. *Cell division*, *5*, 19. https://doi.org/10.1186/1747-1028-5-19

Song, L., & Luo, Z. Q. (2019). Post-translational regulation of ubiquitin signaling. *The Journal of cell biology*, *218*(6), 1776–1786. https://doi.org/10.1083/jcb.201902074

Ting, J. P., Lovering, R. C., Alnemri, E. S., Bertin, J., Boss, J. M., Davis, B. K., Flavell, R. A., Girardin, S. E., Godzik, A., Harton, J. A., Hoffman, H. M., Hugot, J. P., Inohara, N., Mackenzie, A., Maltais, L. J., Nunez, G., Ogura, Y., Otten, L. A., Philpott, D., Reed, J. C., … Ward, P. A. (2008). The NLR gene family: a standard nomenclature. Immunity, 28(3), 285–287. https://doi.org/10.1016/j.immuni.2008.02.005

Trindade, B. C., & Chen, G. Y. (2020). NOD1 and NOD2 in inflammatory and infectious diseases. *Immunological reviews*, *297*(1), 139–161. https://doi.org/10.1111/imr.12902

Tuladhar, S., & Kanneganti, T. D. (2020). NLRP12 in innate immunity and inflammation. *Molecular aspects of medicine*, *76*, 100887. https://doi.org/10.1016/j.mam.2020.100887

Vivier, E., & Malissen, B. (2005). Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nature immunology*, *6*(1), 17–21. https://doi.org/10.1038/ni1153

Vladimer, G. I., Weng, D., Paquette, S. W., Vanaja, S. K., Rathinam, V. A., Aune, M. H., Conlon, J. E., Burbage, J. J., Proulx, M. K., Liu, Q., Reed, G., Mecsas, J. C., Iwakura, Y., Bertin, J., Goguen, J. D., Fitzgerald, K. A., & Lien, E. (2012). The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity*, *37*(1), 96–107. https://doi.org/10.1016/j.immuni.2012.07.006

Wang, G., Gao, Y., Li, L., Jin, G., Cai, Z., Chao, J. I., & Lin, H. K. (2012). K63-linked ubiquitination in kinase activation and cancer. *Frontiers in oncology*, *2*, 5. https://doi.org/10.3389/fonc.2012.00005

Wilmanski, J. M., Petnicki-Ocwieja, T., & Kobayashi, K. S. (2008). NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. *Journal of leukocyte biology*, *83*(1), 13–30. https://doi.org/10.1189/jlb.0607402

Xia, P., Wang, S., Huang, G., Du, Y., Zhu, P., Li, M., & Fan, Z. (2014). RNF2 is recruited by WASH to ubiquitinate AMBRA1 leading to downregulation of autophagy. Cell research, 24(8), 943–958. https://doi.org/10.1038/cr.2014.85

Table 1. Reagents Used in This Resaerch

Reagents	Manufacturer	Product Number
DMEM, 1X	Corning	10-013-CV
DPBS, 1X	Corning	1X 21-031-CM
Penicillin - Streptomycin Solution	Cytiva	SH40003.01
0.25% Trypsin, 2.21 mM EDTA, 1X [-] sodium bicarbonate	Corning	25-053-CI
Peptidoglycan	Sigma	69554
RIPA Lysis Buffer	Santa Cruz Biotechnology	sc-24948
NP-40	US Biologicals	N3500
Phosphatase Inhibitor Cocktail 2	Sigma	P5726-1ML
PEI MAX	Polysciences	24765-100
Protein A/G PLUS-Agarose Beads	Santa Cruz Biotechnology	SC2003
Accu Prestained Protein Ladder II	Lamda Biotech	G02
Q Radiance Substrate	Azure Biosystems	AC2101

Table 2. Antibodies Used in Western Blot Analysis

Antigen	Type	Host	Manufacturer	Product Number	Dilution
Anti-Myc tag	Monoclonal	Mouse	Invitrogen	MA1-21316	1:1000
Anti-Flag tag	Monoclonal	Mouse	Invitrogen	14-6681-82	1:1000
Anti-β-actin	Polyclonal	Rabbit	Invitrogen		1:1000
Anti-IκB-alpha	Polyclonal	Rabbit	Cell Signaling Technology	92242	1:1000
Anti-P-IκB-alpha	Monoclonal	Rabbit	Cell Signaling Technology	2859	1:1000
Anti-ubiquitin	Monoclonal	Rabbit	Cell Signaling Technology	43124	1:1000
Anti-IgG	Secondary	Rabbit	Sigma	I8140	1:1000
Anti-mouse IgG-HRP	Secondary	Goat	R&D Systems	HAF007	1:5000

Anti-rabbit IgG-HRP	Secondary	Rabbit	Jackson Immuno Research	111-035-003	1:5000

Table 3. Two-Way ANOVA

	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Time x Treatment	0.3200	12	0.02667	F (12, 16) = 0.7483	P = 0.6903
Time	0.1115	4	0.02787	F (1.533, 6.134) = 0.7819	P = 0.4655
Treatment	0.07405	3	0.02468	F (3, 4) = 0.4807	P = 0.7132
Subject	0.2054	4	0.05135	F (4, 16) = 1.441	P = 0.2661

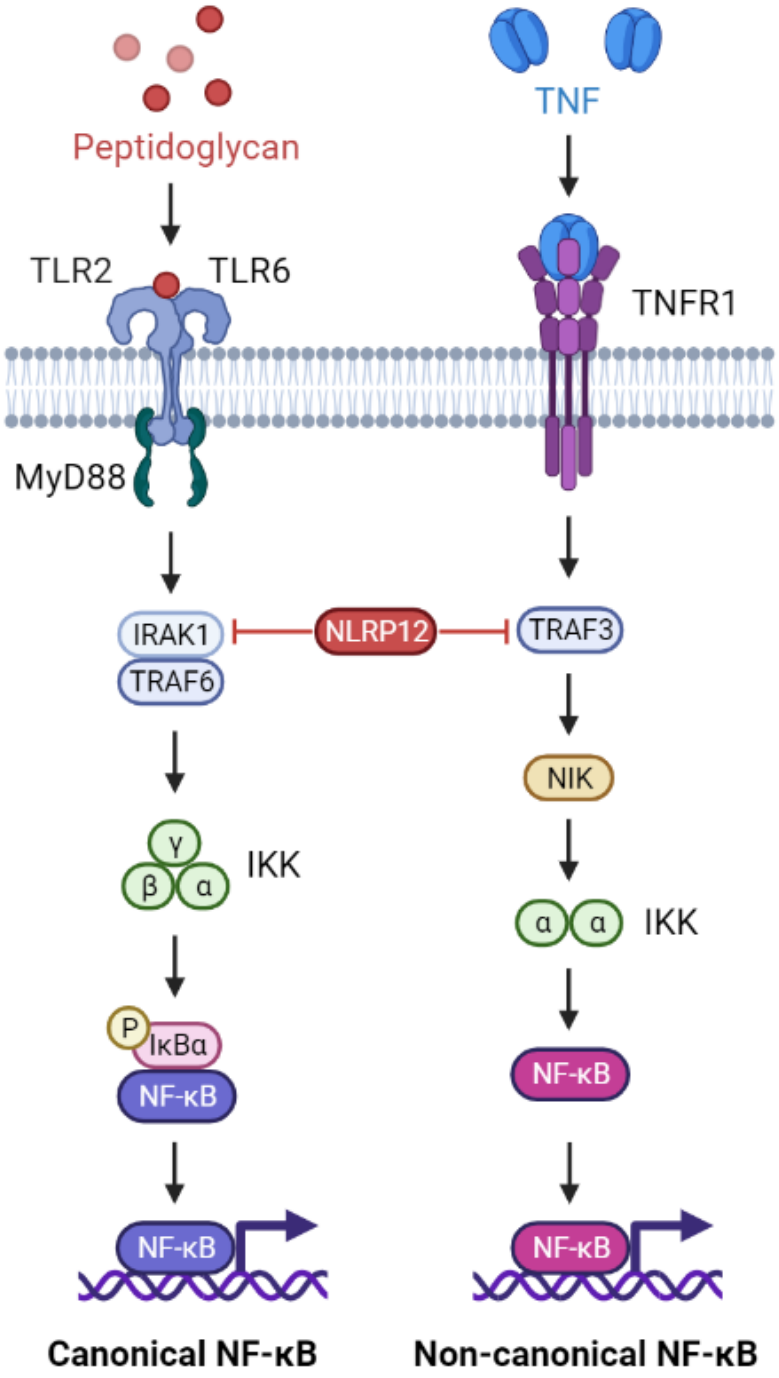


Figure 1. NLRP12 as a Negative Regulator in the NF-κB Signaling Pathways. NLRP12 acts as a negative regulator in both canonical and non-canonical NF-κB signaling pathways by interacting with IRAK1 and TRAF3 respectively.

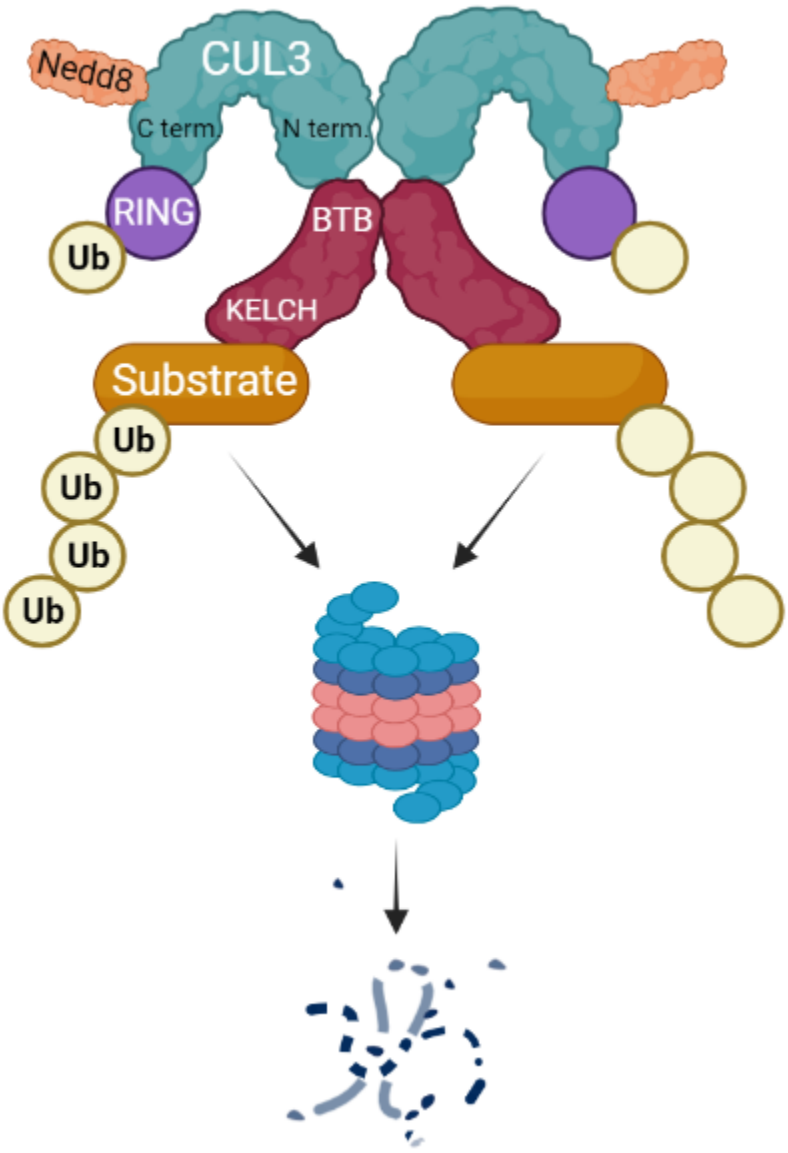


Figure 2. Structure of Cullin 3-RING complex. The complex consists of the CUL3 protein, RING-box protein 1 (RBX1), which recruits E2 ubiquitin-conjugating enzyme, and a Bric-a-brac/Tramtrack/Broad (BTB) protein, which recognizes a substrate to be ubiquitinated.

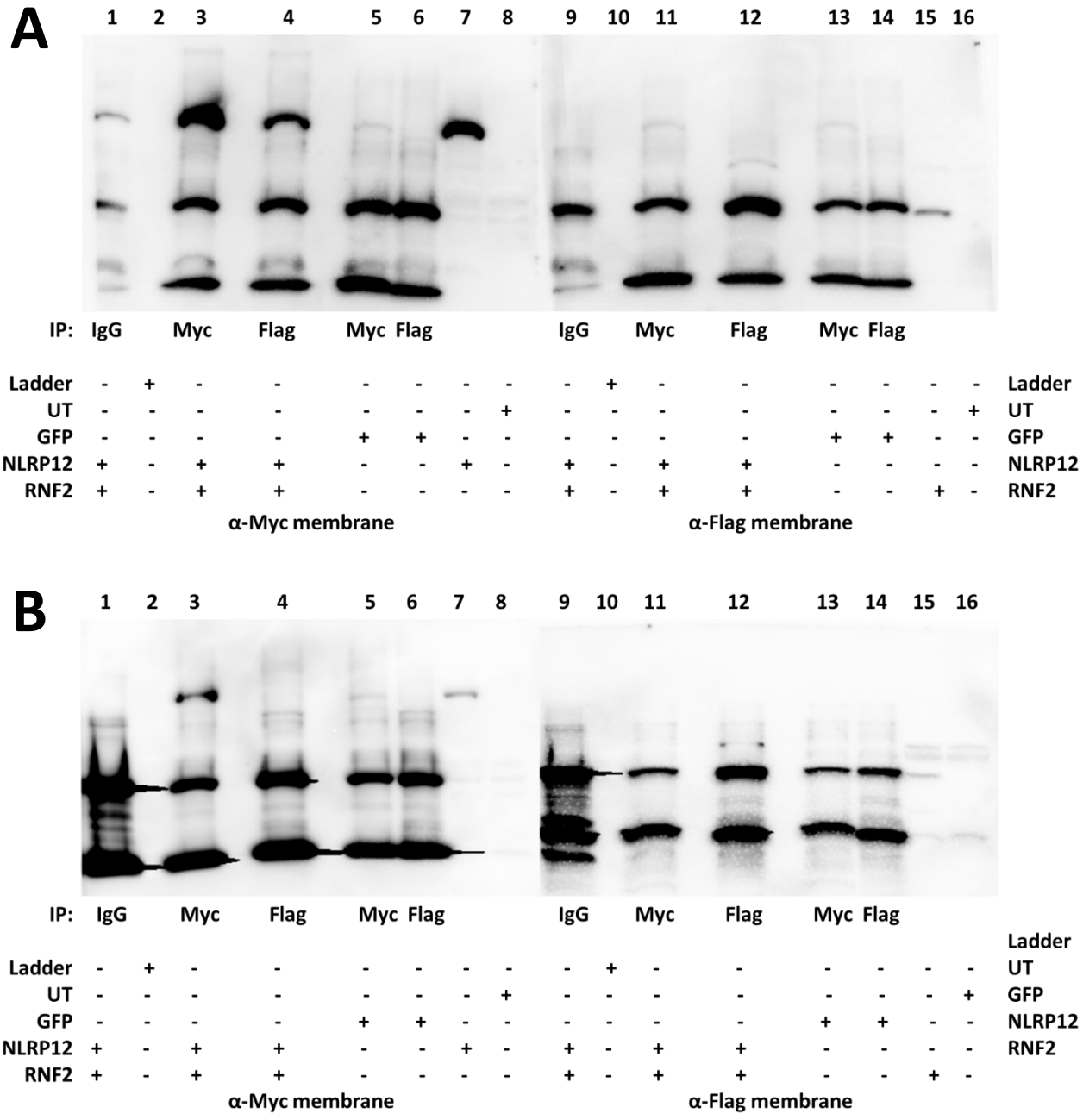
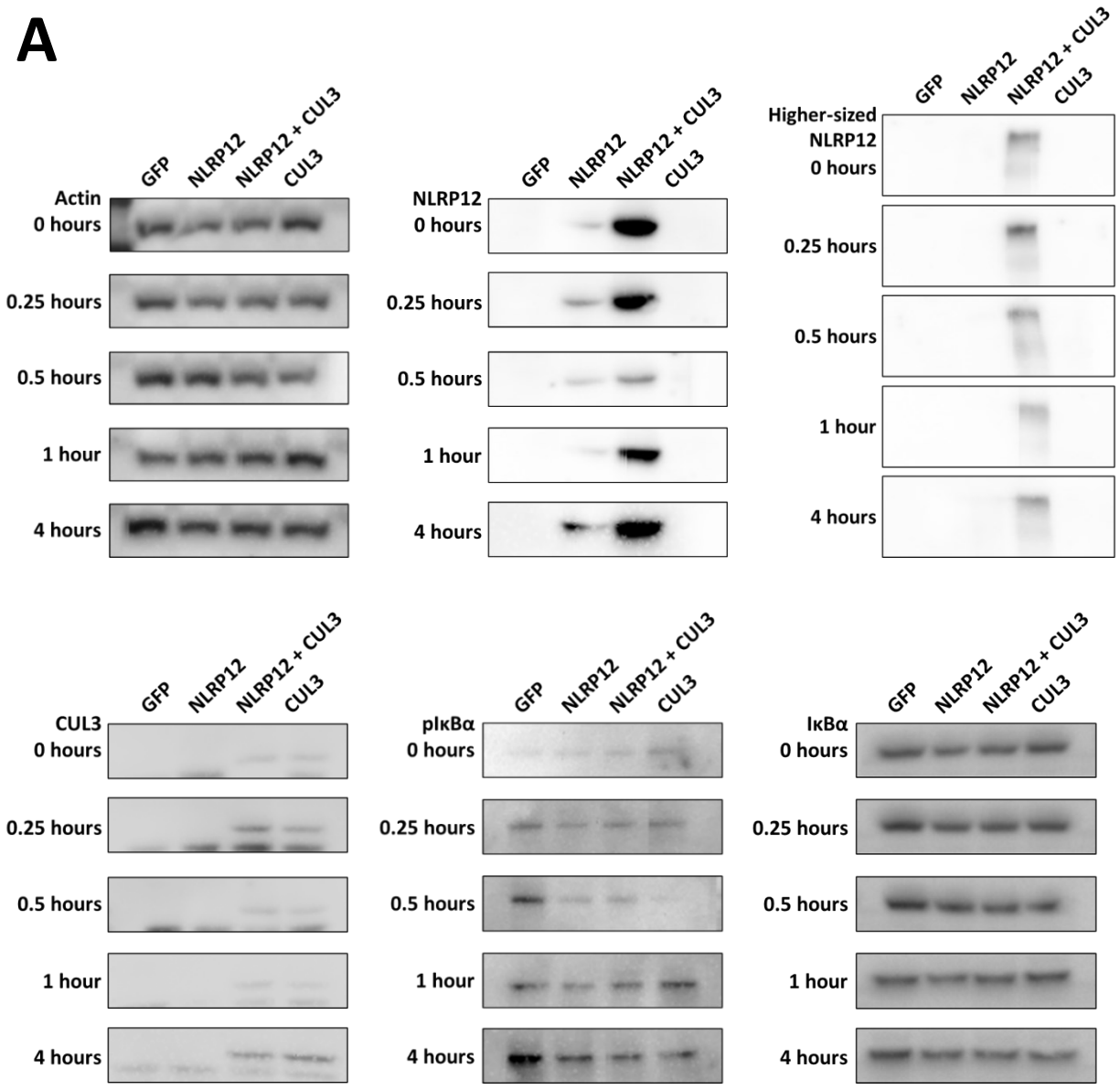


Figure 3. NLRP12 may not interact with RNF2 in HEK293T cells. A co-immunoprecipitation experiment once showed a strong interaction between NLRP12 (120 kDa) and RNF2 (39 kDa) (A). However, this result could not be reproduced (B). HEK293T cells were co-transfected with NLRP12 and RNF2 (lanes 1, 3, 4, 9, 11, and 12) and co-immunoprecipitated with anti-IgG (lanes 1 and 9), anti-Myc (lanes 3 and 11), and anti-Flag (lanes 4 and 12) antibodies. HEK293T cells were transfected with GFP (lanes 5, 6, 13, and 14) as negative controls and immunoprecipitated with anti-Myc (lanes 5 and 13) and anti-Flag (lanes 6 and 14) antibodies. UT: untransfected cells; TC: transfection controls.


A
GFP
NLRP12
NLRP12 + CUL3
CUL3
Actin
0 hours
0.25 hours
0.5 hours
1 hour
4 hours
NLRP12
Higher-sized
CUL3
pIκBα
IκBα

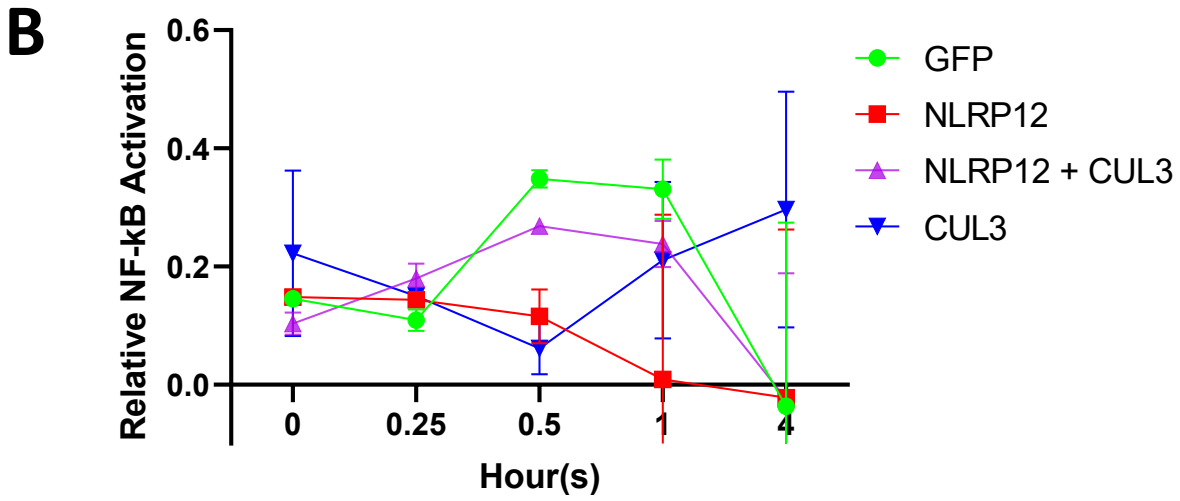


Figure 4. The NF-κB activation assay, followed by western blot analysis, revealed that overexpressed NLRP12 and CUL3 resulted in increased NF-κB activation. (A) Western blot results of the assay showed higher NF-κB activation levels in HEK293T cells transfected with both NLRP12 and/or CUL3 compared to those transfected with NLRP12 alone. (B) Relative intensity of pIκBα was normalized to the actin protein of the same sample and plotted using GraphPad Prism 9.

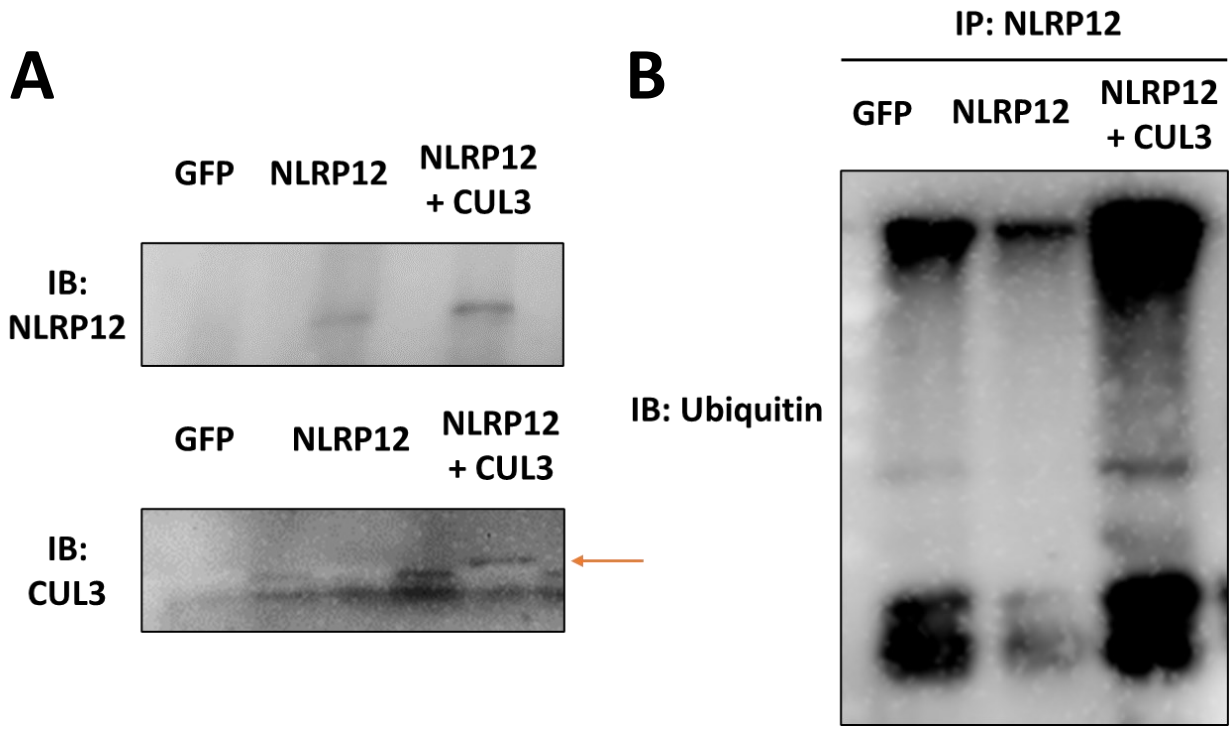


Figure 5. The ubiquitin assay, followed by co-immunoprecipitation and western blot analysis, demonstrated that NLRP12 was ubiquitinated when CUL3 co-existed alongside. (A) Successful

transfection of plasmids was confirmed by western blot results (NLRP12 at 120 kDa and CUL3 at 89 kDa). The orange arrow points to the band corresponding to CUL3. (B) Co-transfection of HEK293T cells with NLRP12 and CUL3 showed resulted in ubiquitination of NLRP12.

APPENDIX: IBC APPROVAL

IBC approved 4/28/2022 to 4/27/2024

Missouri State University

MEMORANDUM OF UNDERSTANDING & AGREEMENT (MUA) FOR RECOMBINANT DNA EXPERIMENTS

All MUA'S can be submitted electronically to researchadministration@missouristate.edu or submitted as a hard copy to the ORA in Carrington 405. A signed copy must be provided. The *NIH Guidelines for Research Involving Recombinant DNA Molecules* should be used as a reference when completing this MUA (see http://oba.od.nih.gov/rdna/nih_guidelines_oba.html).

A. General Information

Date: April 22, 2022

Researcher Name: Christopher Lupfer

Researcher Title: Assistant Professor

Phone: 417-836-6887

Department: Biology

Office Bldg & Room #: Temple Hall, Room 254

Laboratory Bldg & Room #: Temple Hall, Room 232

Granting Agency: Planned grant applications to the National Institutes of Health

Grant Number (if applicable):

Title of Grant or Project: Mechanisms regulating NOD-like receptor function

B. Project Information

1. Describe the experiments involving recombinant DNA techniques. Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it. A summary or abstract of your methods and materials section may also be provided if needed for clarity.

NOD-Like receptors are a class of innate immune receptors that have diverse functions in fighting infectious disease, cancer, and during fetal development. For example, NLRP3 and NLRC4 trigger the formation of the inflammasome, a macromolecular protease containing complex that cleaves the inactive forms of several inflammatory cytokines, including pro-IL-1β and pro-IL-18, into their active forms. Although the function of the inflammasome has been studied in numerous models of infection or inflammation, inflammasome activation in the absence of inflammation has never been examined. Furthermore, the mechanism of action of other NLR proteins like NLRP6 and NLRP12 have not been examined. Finally, NLRP2 and NLRP7 are structurally related to other NLRs, but appear to be important in fetal development and not during infection. To better understand the functions of NLRs, my lab will generate conventional overexpression plasmids (pCDNA3.1 or pCMV-Myc or similar vectors) and plasmids using the iDimerize™ Inducible Homodimer System (Clontech) or similar plasmids that have an inducible dimerization domain cloned in place of the protein interaction domains of caspase-1, its adaptor protein ASC or the pathogen sensing molecules NLRP3, NLRP6, NLRC4, NLRP12, NLRP2, NLRP7 and other NLRs as needed. Human and mouse cell lines such as HEK 293T cells, HeLa cells or RAW 264.7 macrophage cells (or similar cell lines) will be transfected with these plasmids and NF-kB signaling, inflammasome formation and caspase-1 activation examined by western blot. Cell death will also be determined as will the production of active IL-1β and IL-18 and other cytokines. Finally, novel protein interactions will be examined by immunoprecipitation and Western blot analysis of these tagged proteins that are activated in this system in the presence or absence of inflammation. Confocal microscopic imaging will elucidate the location and timing of the interactions. To examine protein interactions, the interacting protein will also need to be expressed in (pCDNA3.1 or pCMV-Myc or similar vectors). Current interactions of interest include Cul3, RPS8, COPS5, COPS6, IFIT1, SNAPIN, and many more. Ongoing studies will elucidate other novel interactions that cannot yet be listed, but will be human genes that interact with NLRs listed above.

In addition to these experiments, we will also generate vectors containing NLRP3, NLRC4, NLRP6, NLRP2, NLRP7 or NLRP12 for use in yeast-2-hybrid assays for the screening of novel interactions with these proteins and a library of cDNA from Clontech. The Clontech Match Maker 2-hybrid system or other similar plasmid system will be used.

2. Provide an assessment of the physical containment required for the experiments.

The DNA sequences to be cloned into the vectors are native to human or mice. The cells to be used in these experiments are of mouse (e.g. RAW 264.7) or human origin (e.g. HEK293T and HeLa cells). For bacterial propagation of the DNA, the cloned genes are not under control of bacterial promoters. For the yeast-2-hybrid system, all samples will be handled and disposed of as indicated below. Antibiotic or other selection markers are common to the field (e.g. Ampicillin for bacterial plasmids or histidine for yeast selection). Furthermore, the overexpression of these proteins is associated with increased cell death and therefore they are unlikely to provide any selective advantage. Therefore, standard precautions and decontamination procedures should be sufficient.

3. Describe the facilities and specific procedures which will be used to provide the required levels of containment.

Our laboratory in Temple 232 will be the site for all experiments. All cell culture equipment, bacterial culturing equipment, growth media and sterilization equipment are present in Temple 232. An autoclave is available in the microbiology prep room across the hall for decontamination.

All personnel working with recombinant DNA will wear disposable latex or nitrile gloves, a laboratory coat and eye protection. These must be worn at all times. Long pants and close toed shoes are also required. No eating (including chewing gum), drinking, applying cosmetics or contact lenses is allowed in the laboratory even when work with recombinant DNA is not taking place.

All purified recombinant DNA, transfected cells or transformed bacteria and yeast will be disposed of by incubation in a 10% bleach solution (final concentration) for a minimum of 5 minutes and/or by autoclaving the sample for 30 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. All work surfaces will be decontaminated prior to and following each procedure using 10% bleach with a contact time of at least 5 minutes. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

4. Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.

If purified DNA plasmid or bacteria expressing the plasmids need to be transported, then samples must be sealed in a shatter resistant container (such as a threaded-cap polypropylene plastic test tube) and the outside of the container decontaminated with 10% bleach. The shatter resistant container must then be placed in a leak proof secondary container with absorbent material (small paint can filled with paper towels etc. and then properly sealed). Alternatively, a drop of purified DNA can be placed on a piece of filter paper and air dried. This filter paper can then be placed in a plastic bag and the bag placed in a shipping envelope. This has the advantage of avoiding the shipment of liquid cultures or samples.

5. Describe the waste disposal procedures expected to be used during this experiment.

All purified recombinant DNA, transfected cells or transformed bacteria and yeast will be disposed of by incubation in a 10% bleach solution (final concentration) for a minimum of 5 minutes and/or by autoclaving the sample for 30 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. All work surfaces will be decontaminated prior to and following each procedure using 10% bleach with a contact time of at least 5 minutes. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

6. Will this project involve environmental release? If yes, please provide a description of the release.

No.

7. Please list all students, staff and faculty involved with this project. CITI Biosafety training is mandatory for all personnel working with biohazards prior to final IBC approval.

Dr. Lupfer
Nayeon Son
Catherine Rippe
Rakshya Bhatta

8. The undersigned agree to certify the following conditions of the proposed research:

a. The information above is accurate and complete.
b. We agree to accept responsibility for training of all laboratory workers involved in the project.
c. We agree to comply with all appropriate requirements pertaining to shipment and of hazardous biological and recombinant DNA materials.
d. We are familiar with and agree to abide the provisions of the Missouri State University policies and procedures applicable to experiments involving recombinant DNA, the provisions of the current *NIH Guidelines for Research Involving Recombinant DNA Molecules,* and any other specific instructions pertaining to the proposed project.

Via email 4/28/2022			
Principal Investigator	Date	Department Head	Date

9. The Institutional Biosafety Committee has determined, based on information provided the principal investigator, that:

a. No special medical surveillance (other than usual University health programs) is required for the project described in this MUA

b. The following specific medical surveillance procedures must be carried out, for individuals listed by name, before commencing the project described in this MUA:

10. We certify that the Missouri State University Institutional Biosafety Committee has reviewed the proposed project for recombinant DNA experiments for compliance with the current *NIH Guidelines for Research Involving Recombinant DNA Molecules* and Missouri State University's policies and procedures applicable to experiments involving recombinant DNA. The MSU IBC will monitor throughout the duration of the project the facilities, procedures, and the training and expertise of the personnel involved in the recombinant DNA activity.

MSU IBC Chair or Representative _____ Date _____