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EXAMINING THE IMMUNE REGULATION OF NLRP12 THROUGH NOVEL PROTEIN INTERACTIONS

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Catherine Rippe

May 2023

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EXAMINING THE IMMUNE REGULATION OF NLRP12 THROUGH NOVEL PROTEIN INTERACTIONS

Biology

Missouri State University, May 2023

Master of Science

Catherine Rippe

ABSTRACT

NOD-like receptors (NLRs) are intracellular proteins that play an important role in the regulation of the innate immune response to pathogens. Since being identified, various functions for NOD-like receptor pyrin domain containing 12 (NLRP12) have been suggested. It has been shown to negatively regulate the inflammatory response through canonical and noncanonical NF-κB signaling pathways, control tumorigenesis and gut homeostasis and exacerbate inflammation through the formation of a multi-protein complex called an inflammasome. Due to the varying roles established for NLRP12, the mechanisms by which it functions remain poorly understood. In this study, I sought to confirm a novel protein-protein interaction between NLRP12 and CUL3 to aid in better understanding the mechanisms in which NLRP12 is activated and functions. CUL3 is an E3 ligase involved in the ubiquitin-proteasome system (UPS) that targets proteins for degradation in the 26S proteosome. I found that NLRP12 and CUL3 interact and as a result IL-8 chemokine is downregulated indicative of downregulation of the inflammatory response. Thus, this study provides insight into a possible mechanism that controls NLRP12 function through ubiquitination.

KEYWORDS: NOD-like receptors, inflammation, ubiquitin, NF-κB, innate immunity

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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INTRODUCTION

The Immune System

Overview. The immune system is responsible for distinguishing “self” antigens from “non-self” antigens, which is accomplished through organized lymphoid tissues, immune cells, chemical messengers, and proteins. “Non-self” antigens include antigenic components of bacteria, fungi, viruses, parasites, cancer cells and toxins; all of these can cause illness and disease. In response to antigenic stimulation, the immune system responds in two distinct ways. The innate immune system responds quickly and non-specifically, while the adaptive immune system is slower to respond but is more targeted in the response. The two systems work in tandem to recognize “non-self” antigens and eliminate or contain them, thus providing protection to the host against disease. My thesis research focuses on the regulation of innate immune signaling pathways.

Innate Immunity. Innate immunity is considered the first line of defense against invading pathogens. It is a fast-acting, non-specific defense mechanism without immunologic memory, unlike the adaptive immune system which can recall immune responses to previous pathogens, enabling it to illicit a more rapid and robust immune response upon re-exposure to pathogens. Exposure to pathogens can occur via breaking skin, inhalation, or ingestion, and therefore, components of the innate immune system include physical and anatomical barriers, such as skin and mucous membranes, as well as effector cells, antimicrobial peptides, soluble mediators, and cell receptors [1]. Innate immune cells derived from hematopoietic stem cells in the bone marrow include macrophages, neutrophils, and dendritic cells (DC). Macrophages and neutrophils have phagocytic functions, while DCs are involved in antigen presentation. A vital

family of cellular receptors for pathogen sensing and recognition during the innate immune response is pattern recognition receptors (PRRs).

Pattern Recognition Receptors. PRRs are expressed on a variety of innate immune cell types including macrophages, neutrophils, and dendritic cells. PRRs are a large class of germline-encoded proteins responsible for recognition of a large variety of molecules in pathogens, called pathogen associated molecular patterns (PAMPs) or molecules produced as a result of damage to cells, damage associated molecular patterns (DAMPs) [2]. Generally, PAMPs are microbial components such as proteins, lipids, and nucleic acid structures that are not present in host cells, while DAMP's can be ATP, uric acid, and ion influx triggered by damage to host cells. These PRR proteins are classified into five families: Toll-like receptors (TLRs), nucleotide-binding and 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 (AIM)-like receptors (ALRs) [3]. The transmembrane receptors, TLRs and CLRs, recognize extracellular microbial components or microbial components in endosomal compartments. Alternatively, NLRs, RLRs and ALRs are located in the cytosol and recognize intracellular infection. These proteins are essential and provide the scaffolding for how the innate immune system functions. Excessive activation or mutation of PRRs could lead to disruption/malfunction of innate immunity and cause inflammatory diseases. For example, mutations in the genes encoding NLRP3 or NLRP12 lead to hereditary periodic fever syndromes, while mutations in the *CARD15* gene that encodes the NOD2 protein are linked to Crohn's disease or Blau's syndrome [4].

NOD-like Receptors (NLRs). NLRs are a large class of intracellular proteins involved in the innate immune response to pathogens in plants and animals. Over 20 human NLRs have been

discovered; however, many of them have not been fully characterized [5]. Based on the effector domain responsible for mediating protein interactions and effector function, NLRs are classified into four subfamilies: NLRA, NLRB, NLRC and NLRP. Common NLR effector domains are caspase activation and recruitment domains (CARDs) and pyrin domains (PYDs), present in the NLRC and NLRP families, respectively [6]. PYDs and CARDs are involved in homotypic protein interactions and allow for the recruitment of downstream effector molecules, which allow for pathogen sensing [7]. NLRs sense pathogens and/or damage and modulate the immune response through signaling, transcriptional control of inflammation, autophagy and inflammasome formation [6].

A well-known member of the NLRP family is NLRP3, which is extensively studied due to its involvement in inflammasome formation. Inflammasomes are multi-protein complexes that form due to stimulation by various PAMPs and DAMPs, causing caspase-1 driven inflammatory responses[8]. Inflammasomes generally consist of an NLR protein associated with apoptosis speck protein (ASC) and pro-caspase-1. Briefly, stimulation of NLRP3 causes association with ASC via PYD-PYD interactions [9]. Pro-caspase-1 then associates with ASC via CARD-CARD interactions, and is cleaved into its active form, caspase-1 p20. Active caspase-1 subsequently functions to cleave the proinflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL-18, and cause pyroptosis, a type of inflammatory cell death (**Figure 1**) [10, 11]. Additionally, other NLRs such as NLRP1, NLRC4, NLRP6 and NLRP12, have also been reported to form inflammasomes through similar mechanisms [12, 13].

NLRP12

Overview of Structure and Function(s). NLR pyrin domain containing 12 (NLRP12) is a member of the NLRP family of NLRs. NLRP12 consists of an N-terminal PYRIN domain, a

central NACHT binding domain and a C-terminal leucine-rich repeat (LRR) region, domains shared amongst members of the NLRP family (**Figure 2**) [14]. The PYRIN domain is responsible for effector functions, while the LRR domain is necessary for ligand binding and the NACHT domain is involved in oligomerization [15]. Expressed primarily in myeloid lineage immune cells, its functions in the inflammatory response are largely associated with regulation of pro- and anti-inflammatory pathways. NLRP12 has been shown to negatively regulate inflammation through canonical and non-canonical Nuclear factor-κB (NF-κB) pathways, control tumorigenesis and aid in the maintenance of intestinal homeostasis and regulate chemokine signaling [16-20]. Moreover, mutations in NLRP12 in humans can lead to an autoinflammatory disease known as Familial Cold Autoinflammatory Syndrome 2 [21]. Much debate still surrounds these functions and the mechanisms involved.

Inflammasome Activation. Much debate has surrounded the functions of NLRP12 in the last two decades since the protein's discovery. One proposed function is the involvement of NLRP12 in the formation of inflammasomes. NLRP12, also known as PYPAF-7, was the first NLR shown to interact with ASC to form an active IL-1β-maturing inflammasome [22]. The role of NLRP12 in inflammatory diseases has been studied; however, the role of NLRP12 in infectious diseases has not been elucidated conclusively. During *Yersinia pestis* infection, it was revealed that an upregulation of NLRP12, through TLR4 stimulation, limits the susceptibility to the pathogen through inflammasome assembly and pro-inflammatory cytokine production [23]. However, an inflammasome independent role for NLRP12 has been established in inflammatory disease. Silencing NLRP12 caused a significant increase in proinflammatory cytokines while knockdown had no effect on ATP-induced caspase-1 activation and IL-1β levels, with NF-kB activation increasing, thus suggesting an inflammasome independent NLRP12 function [24].

Mice Vs. Humans. In a mouse model of LPS-induced inflammation and *Klebsiella pneumoniae* infection, NLRP12 deficiency causes decreased chemokine levels and impaired neutrophil recruitment and migration, implying a role of NLRP12 in neutrophil migration in mice [25, 26]. In contrast, humans with NLRP12 mutations have increased systemic inflammation and autoinflammation. In peripheral blood mononuclear cells (PBMCs) of systemic lupus erythematosus patients, low levels of NLRP12 were associated with type I interferon production, and NLRP12 deficiency in lupus-prone mice augmented inflammation [27]. These differences between mice and human NLRP12 suggests that it has varying roles depending on the organism, thus, mice may not be the ideal model organism for human translational studies.

NF-κB. NF-κB activation is inflammasome independent. NF-κB is a group of transcription factors, composed of five members, p50/p105, p52/100, RelA (p65), RelB and c-Rel, that together mediate immune and inflammatory responses [28]. Two major pathways have been identified that contribute to NF-κB activation, canonical and non-canonical, or alternative pathways [29].

<u>Canonical NF-κB.</u> The canonical NF-κB pathway elicits an immune response via stimulation of several diverse receptor signals, including those from PRRs and T-cell receptors (TCR) [30]. NF-κB proteins are transcription factors that are confined to the cytoplasm and released into the nucleus following stimulation of NF-κB activating pathways [31]. Canonical NF-κB proteins are held in the cytoplasm by inhibitor of κB (IκB) [32]. Canonical activation results in activation of inhibitor of κB kinase (IKK), composed of catalytic IKKα and IKKβ, and NF-kB essential modulator (NEMO/IKKγ), which phosphorylates IκBα/β, leading to their dissociation from p50/RelA and p50/c-Rel dimers [33, 34]. IκBα/β are subsequently

ubiquitinated, leading to degradation in the proteasome, allowing p50/RelA and p50/c-Rel dimers to translocate to the nucleus and induce proinflammatory gene transcription (**Figure 3**) [33, 34]. While canonical NF-κB activation is driven by degradation of IκB, activating the noncanonical NF-κB pathway relies on other proteins.

<u>Noncanonical NF-κB.</u> The noncanonical NF-κB pathway is more selective, responding to limited stimuli including signals from a subset of TNF receptors [30]. An important upstream kinase for this pathway is NF-κB inducing kinase (NIK), a member of the mitogen-activating protein 3 (MAP3) kinase family. Noncanonical NF-κB activation is characterized by activation of NIK, which activates IKKa and recruits p100, a precursor to p52 and inhibitor of RelB translocation. p100 is phosphorylated, resulting in ubiquitination and processing by the proteosome of p100 into p52, allowing p52/RelB to translocate to the nucleus and induction of proinflammatory gene transcription (**Figure 3**) [35, 36]. Modulation of both pathways is important to regulate signals and prevent excessive inflammation. A major regulator of both canonical and noncanonical NF-κB pathways is ubiquitination.

Ubiquitination and NF-κB. Ubiquitination is a form of post-translational modification that involves tagging a substrate protein with ubiquitin; a small 8.6Kd protein [37]. Post-translational modification with ubiquitin can have varying effects depending on the linkage of poly-ubiquitin chains. In all, there are seven lysine residues and the N-terminus in ubiquitin that serve as sites for poly-ubiquitination chain formation with various functional consequences [38]. Poly-ubiquitin chains created by adding ubiquitin to the lysine 48 residue in ubiquitin target the tagged protein for degradation via the 26S proteasome. However, ubiquitination can also alter protein-protein interactions or protein activation when the poly-ubiquitin chains are linked at lysine 63. Ubiquitination controls many cellular functions including cell division, apoptosis,

DNA repair, immune responses, and protein processing for destruction [39]. The process for adding ubiquitin is a three-step biochemical process involving three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). In brief, E1, using an ATP dependent process, forms a bond between ubiquitin and itself, E2 then binds to this complex, transferring ubiquitin to its active site, and E3 forms the bond between ubiquitin and the target protein forming a ubiquitin-substrate complex (**Figure 4**) [40-42]. Importantly, K48 poly-ubiquitination of IκBα/β results in the degradation of these proteins via the 26S proteasome and facilitates release of NF-κB transcription factors and inflammatory gene expression in response to infection or disease [37].

In addition to regulating the degradation of IκB, several studies have elucidated other roles for poly-ubiquitination in NF-κB signaling. A recent study showed that TRIM25, an E3 ligase, interacted with TRAF2 and potentiated K63-linked polyubiquitination thereby promoting tumor necrosis factor-α (TNF-α)-induced NF-κB activation [43]. TRIM25 has also been shown to enhance K63-ubiquitination of TRAF6 through antiviral signaling. TRAF6, upon association with ubiquitin-conjugating enzymes, forms polyubiquitin chains which have been shown to be required for IKK activation [44]. This highlights the role that ubiquitination has on the activation of NF-κB through many axes and provides insight into what role NLRP12 may play in NF-κB regulation.

NLRP12 and NF-κB Regulation. NLRP12 appears to regulate both canonical and noncanonical NF-κB signaling pathways, thus suggesting some effect on ubiquitin activity or a ubiquitin-related function. It is mostly thought that NLRP12 negatively regulates NF-κB. NLRP12 has been shown to attenuate colon inflammation and subsequent tumorigenesis [16, 17]. Bone marrow-derived macrophages from NLRP12-deficient mice stimulated with LPS

showed enhanced NF-κB activation indicated by increased IκBα degradation and phosphorylation [17]. On the contrary, while investigating the role of the NLRP12 inflammasome in tolerance to *Y. pestis*, NF-κB signaling following bacterial challenge appeared normal in NLRP12-deficient cells [23]. Additionally, NLRP12-deficient mice, infected with either *Klebsiella pneumoniae* or *Mycobacterium tuberculosis*, showed no significant difference in NF-κB activation, suggesting that there may be contradictory roles for NLRP12 and its regulatory function of NF-κB [45]. Thus, additional research is needed to understand the diverse roles the NLRP12 gene plays in disease and infection.

E3 Ligases

Types and Structure. As previously mentioned, ubiquitination is a biochemical process carried out by three enzymes, E1, E2 and E3 (**Figure 4**). There are 2 E1 enzymes, ~40 E2 enzymes and an estimated 600 or more E3 enzymes present in humans [46]. E3 ligases are of particular importance because it is thought that they account for the diversity of ubiquitination patterns and functions due to their large numbers [41]. E3 ligases are categorized into 4 groups based on their structure and function: HECT type, U-box type, RING-finger type and RBR type [40]. RING-finger type E3 ligases are of interest here. These can be part of monomeric or multi-subunit complexes. An example of a multi-subunit RING-type E3 ligase is the Cullin RING Ligases (CRLs) [42]. Generally, these consist of a cullin protein, a small RING protein (RBX1 or RBX2) and an adaptor protein, but in the case of CRL3, instead of adaptor proteins, there are proteins that can bind to the cullin protein (CUL-3) and the substrate [40].

CUL3. *CUL3* is a gene responsible for producing the protein cullin-3, a member of a large subfamily of Cullin-RING E3 ubiquitin ligases responsible for degradation of proteins via the ubiquitin-proteasome system (UPS) [47]. The complex consists of CUL3 and BTB-domain

containing proteins that bind substrates and a catalytic RING protein[48]. BTB-domain containing proteins contributes to the difference between the CUL3 E3 ligase complex and other cullin containing E3 ligase complexes. They function as substrate-specific adaptors and are unique because they are capable of recruitment of two CUL3 subunits upon dimerizing and possess a second protein-protein interacting domain which allows the subunit to function as both an adaptor and in substrate recognition [49]. BTB-domain containing proteins in the Kelch or Kelch-like protein family are well known to interact with CUL3 [49]. CUL3 complexes and mutations in interacting proteins have been shown to be involved in cancer, antioxidant responses, cardiovascular disease, cytokinesis, and protein trafficking [50-54].

Disease and Pathology

Cryopyrin-Associated Fever Syndromes. The dysregulation of NLRs is known to cause autoinflammatory diseases, such as Crohn's disease (i.e., NOD2 mutations) [4]. Mutations in NLRP3 are involved in a group of rare diseases called cryopyrin-associated periodic syndromes (CAPS) [55]. CAPS include Muckle-Wells syndrome, familial cold autoinflammatory syndrome (FCAS) and neonatal-onset multisystem inflammatory disease (NOMID), generally due to a gain-of-function mutation in NLRP3, leading to excessive production of IL-1 [56, 57]. NLRP12 mutations are associated with a similar condition, familial cold autoinflammatory syndrome type 2 (FCAS2).

Familial Cold Autoinflammatory Syndrome Type 2 (FCAS2). Nonsense and splice-site mutations in NLRP12 were first recorded in 2008 to cause hereditary periodic fever syndromes, which includes FCAS. The inhibitory properties of NLRP12 were shown to be diminished with these mutations, indicating enhanced NF-κB signaling [58]. Missense and nonsense mutations identified in NLRP12 have also been shown to lead to periodic fever

syndromes and atopic dermatitis [59-61]. However, in some of these cases, NF-κB signaling was unaffected. Common clinical manifestations of FCAS(2) are fever, rash, joint pain upon exposure to the cold, diarrhea, headache and even hearing loss [62]. FCAS2, although rare, can be debilitating. The goal for treatment of these inflammatory conditions is to suppress the hyperinflammatory response caused by the excessive production of IL-1b, which is difficult since the pathological mechanism is still unclear [21]. In adults and children over 2 years of age, canakinumab, which blocks the IL-1 receptor, is FDA approved to treat periodic fever syndromes including CAPS. However, some patients receiving IL-1 inhibitors have returned within 18 months with drug resistance [4].

Research Question and Hypothesis

Elucidating protein-protein interactions can aid in understanding protein function and the signaling pathways involved in disease development and facilitate development or modification of drugs and therapeutics for disease. Research into protein-protein interactions is limited for NLRP12. Previous research has identified IRAK1 and other proteins as interacting proteins with NLRP12 [19, 63]. Preliminary data, via a yeast-2-hybrid screen performed in the Lupfer lab, has identified at least 6 proteins that potentially interact with NLRP12 [64]. Among these proteins were CUL3 and RNF2, both of which are components of ubiquitin ligase complexes and/or have some ubiquitin ligase activity. Here, I sought to confirm the novel protein interaction between NLRP12 and CUL3 and determine whether there is an effect on the inflammatory response through evaluation of proinflammatory cytokines and chemokines. I am interested in understanding the functional significance of the CUL3-NLRP12 interaction. I hypothesize that this interaction can regulate NF-κB signaling due to the role for ubiquitination in this pathway and this interaction may regulate NF-κB activation and inflammatory cytokine production.

MATERIALS AND METHODS

Cloning CUL3 into Mammalian Expression Vector

pcDNA3-myc-CUL3 was a gift from Yue Xiong (Addgene plasmid # 19893; http://n2t.net/addgene:19893; RRID:Addgene_19893). pcDNA3-myc-CUL3 was used to clone the CUL3 gene into a mammalian expression vector, pcDNA 3.1 3xFlag. CUL3 primers were designed using NEBuilder Assembly Tool and listed in **Table 1**. CUL3 gene was amplified via PCR using Phusion High-Fidelity PCR kit (Cat. F553L, ThermoScientific) according to the manufacturer's protocols. PCR conditions are listed in **Table 2**. pcDNA3.1 3xFlag IFIT1 was a gift from Kathleen Collins (Addgene plasmid # 53554; http://n2t.net/addgene:53554; RRID:Addgene_53554). Institutional Biosafety Committee approval for the use of recombinant plasmids can be found in **Appendix: IBC Approval**. Fast Digest enzymes (Cat. K1991, ThermoScientific) *Xho1* and *BamH1* were used to digest the mammalian expression vector pcDNA 3.1 3xFlag to remove IFIT1 via the manufacturer's protocol and observed via gel electrophoresis. pcDNA 3.1 3xFlag was extracted using GenScript QuickClean II Gel Extraction Kit (Cat. L00418) following the manufacturer's protocol. Following the manufacturer's protocol, CUL3 and pcDNA 3.1 3xFlag were ligated together using HiFi Gibson cloning kit (Cat. E2621S/L/X, NEB). The assembled product was transformed into DH5α Competent Cells (Cat. E5520S, NEB) and plated on LB agar plates containing 100μg/mL Ampicillin. DreamTaq polymerase (Cat. EP0701, ThermoScientific) was used according to the manufacturer's protocols to screen colonies using T7/BGH and CUL3 specific primers (**Table 1**). PCR conditions are shown in **Table 2**. To confirm the proper insertion of the CUL3 gene into the pcDNA 3.1 3xFlag

plasmid, the cloned plasmid was sequenced via Sanger sequencing using T7/BGH primers at Eurofins.

Transfection into HEK 293T Cells

Escherichia coli containing pcDNA 3.1 3xMyc human NLRP12 was obtained from a frozen glycerol stock and plated, then grown in LB broth containing 100µg/mL Ampicillin overnight at 37°C and the plasmid DNA purified with ZymoPURE II Plasmid Midiprep Kit (Cat. 4201, Zymo Research) according to the manufacturer's protocols. *E. coli* containing pcDNA 3.1 3xFlag CUL3 was also incubated overnight in LB broth containing 100µg/mL Ampicillin and the plasmid DNA purified. Human embryonic kidney (HEK) 293T cells were purchased from B.E.I. Resources. HEK 293T cells cultured with DMEM + 10% fetal bovine serum (FBS), 100U Penicillin and 100U Streptomycin were seeded at 150,000 cells/mL in a 12-well tissue culture plate. After the cells reached 50-70% confluency, 2.5 µg/µL of plasmid DNA was transfected into the cells using XFECT (Cat. 631324, Clontech) transfection reagent according to the manufacturer's protocols. Plates were incubated at 37ºC, 5% CO_2 for 24-48 hours to allow observation of protein expression.

Western Blot

Western blotting was performed to confirm individual protein expression of CUL3 and NLRP12 in HEK 293T cells. After transfection, cells were washed with PBS and lysed with RIPA buffer. 4X loading dye was added and the samples boiled for 20 minutes. Samples were separated into an 8% acrylamide gel, transferred to PVDF membrane, and blocked in 5% non-fat dried milk in TBST. The immunoblot was probed with the appropriate target specific primary

antibody (1:1000) diluted in blocking buffer overnight at 4°C. The next day, the blots were washed with TBST wash buffer, then probed with the appropriate secondary antibody (1:5000). Antibodies used are denoted in **Table 3**. The immunoblots were developed using chemiluminescence (Radiance Q, Cat. AC2101, Azure Biosystems) and visualized with an Azure C300 digital imager.

Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) was performed to capture and purify protein of interest and their interacting proteins. Target-specific antibodies to detect the proteins of interest and SDS-PAGE gel electrophoresis along with Western blotting was used to visualize the interaction. HEK 293T cells were seeded at 450,000 cells/mL supplemented with DMEM +10% FBS, 100U Penicillin and 100U Streptomycin in a 6-well tissue culture plate. Co-transfection with 2.5 µg/µL of each plasmid was performed when the cells reached 50-70% confluency with XFECT transfection reagent (Cat. 631324, Clontech) according to the manufacturer's protocols. Co-immunoprecipitation was performed after 48 hours. Cells were lysed using 0.5 mL ice-cold 1% NP-40 plus protease inhibitor (Cat. A32953, Pierce) and phosphatase inhibitor (Cat. P2850-1ml, Sigma-Aldrich). Cells were incubated on a shaker at 4ºC for 30 minutes and kept on ice. After incubation, cells were further disrupted by passing lysate through a Dounce homogenizer. Samples were collected in 1.5 mL microcentrifuge tubes and centrifuged at 5,000xg for 10 minutes at 4ºC. 250 µL of the supernatant was then placed into a new 1.5 mL microcentrifuge tube and 2 µg of the appropriate antibody (**Table 3**) was added and incubated for 1 hour at 4ºC. After incubation, 40 µL of protein A/G PLUS-Agarose beads (Cat. Sc-2003, SCBT) was added and samples incubated overnight at 4ºC. The next day, samples were washed 4 times with 1 mL

of ice-cold PBS and centrifuged at 2,000xg for 1 minute at 4ºC. After the wash step, 50 μL of 4X loading dye was added to samples and boiled for 5 minutes. Duplicate samples were separated on two 8% acrylamide gels, transferred to PVDF membrane, and blocked in 5% milk in TBST. The two immunoblots were probed with the appropriate target specific primary antibody (1:1000) followed by the appropriate secondary antibody (1:5000). Antibodies used are denoted in **Table 3**.

Confocal Microscopy and Immunostaining

HEK 293T cells were plated and transfected as previously described with one exception: a clean, sterilized glass cover slip was added to each well before plating. After 48 hours cells were fixed to the coverslip with 4% paraformaldehyde in PBS for 15 minutes at room temperature and washed 3 times with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 1 hour at 37ºC. Cells were blocked with 1X ELISA diluent for 30 minutes at room temperature. Cells were incubated with primary antibody (anti-myc/anti-flag, 1:1000) in 1X ELISA diluent and incubated overnight at 4ºC. Afterward, the cells were washed 3 times with PBS and incubated in secondary antibody (FITC/Rhodamine, 1:1000) in PBS for 40 minutes at 37ºC. FITC has excitation and emission spectrum peak wavelengths of approximately 495 nm and 519 nm; the excitation and emission of rhodamine peak at 546 nm and 568 nm, respectively. All antibodies used are denoted in **Table 3**. Cells were washed 4 times with PBS and allowed to air dry. A drop of DAPI Fluoromount-G® medium (Cat. 0100-20, SouthernBiotech) was added to a microscope slide and coverslip placed on top. Visualization of cells was performed using Olympus FV1000 Confocal IX81 Microscope (Olympus) and SlideBook 6 (Intelligent Imaging Innovations, Denver, CO.).

Enzyme-Linked Immunosorbent Assay (ELISA)

HEK 293T TLR-2^{YFP}/MD-2 and HEK 293T cell lines purchased from B.E.I Resources were seeded at 150,00 cells/mL and transfected with the appropriate plasmid(s) using P.E.I Max (Cat. 24765-100, Polysciences) transfection reagent according to the manufacturer's protocols. After 24 hours, the cells were stimulated with peptidoglycan (PGN) at 1 µg/mL and samples were collected after 4 hours. The samples were analyzed for IL-8 using an ELISA kit (Cat. 431504, BioLegend) according to the manufacturer's protocols. Plates were read at 450 nm on a microplate reader (BioTek ELx808).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). Statistical differences between groups were evaluated using one-way ANOVA, correcting for multiple comparisons. A p-value of <0.05 was considered statistically significant.

RESULTS

CUL3 is Cloned into a Mammalian Expression Vector

A previous yeast-2-hybrid screen performed in the Lupfer lab identified a potential interaction between CUL3 and NLRP12 prompting further investigation to confirm this protein-protein interaction[64]. To facilitate identification of the interaction, the CUL3 gene was cloned into a mammalian expression vector containing a Flag tag; thus, allowing for confirmation of the interaction between CUL3 and NLRP12, which possesses a myc tag. After digestion of a plasmid containing the Flag tag (**Figure 5A**) and amplification of CUL3, the colony PCR of the ligated product showed the appropriate band size for the CUL3 gene (**Figure 5B**). Sanger sequencing of the plasmid identified the whole CUL3 gene with the appropriate tag, lacking mutations, which was used for further downstream applications.

CUL3 and NLRP12 Interact in HEK 293T Cells

To confirm the interaction between CUL3 and NLRP12, we first needed to confirm that each protein is individually expressed in human embryonic kidney (HEK) 293T cells. Via western blotting, CUL3 and NLRP12 showing the appropriate band size were observed to be overexpressed in HEK 293T cells (**Figure 6A and 6B**, respectively). Secondly, co-immunoprecipitation and western blotting were performed to confirm the interaction in HEK 293T cells. When co-transfected, CUL3 and NLRP12 were observed to interact (**Figure 6C**). Confirming this interaction led to an investigation of colocalization and the resulting inflammatory response.

CUL3 and NLRP12 Colocalize in the Cytoplasm of the Cell

To further confirm the interaction between NLRP12 and CUL3, we utilized fluorescent staining and confocal microscopy. This revealed that NLRP12 and CUL3 are present in the cytoplasm when individually transfected (**Figure 7A**). Excitingly, when the proteins are co-transfected, they appear to be co-localized in the cytoplasm in punctate structures (**Figure 7A**), identified as a yellow color from the fluorophores overlapping. This further indicates that, in co-transfected cells, NLRP12 and CUL3 interact. This led us to further investigate the inflammatory response in HEK 293T cells.

CUL3 and NLRP12 Downregulate the Inflammatory Response

NLRP12 is known to downregulate NF-κB signaling and is involved in neutrophil recruitment, therefore IL-8 levels were examined via ELISA. Therefore, HEK293T cells expressing TLR-2 were stimulated with peptidoglycan (PGN). Compared to untransfected controls, cells transfected with NLRP12 showed significant downregulation of IL-8 upon stimulation (368.7±83.54, $p=0.01$) (**Figure 8**). No significance was seen when comparing transfected groups, but there was a trend towards significance in the co-transfected cells compared to the NLRP12 transfected cells (473.0±43.07, $p=0.0889$) (**Figure 8**). This coincides with previous research suggesting NLRP12 downregulates the inflammatory response.

DISCUSSION

Investigating protein-protein interactions aids in our understanding of the functions of proteins and the processes they are involved in. NLR proteins are members of the innate immune receptors and mutations are linked to several inflammatory conditions, suggesting a role in inflammation and host-pathogen interactions [65]. Understanding the functions of key effector molecules in the inflammatory response and host-pathogen interactions is important when treating and preventing disease.

NLRP12, while not widely studied since its discovery over two decades ago, has some allure to it because of its wide array of potential functions. Conversely, it has been shown to modulate intestinal inflammation and tumorigenesis, some believe it acts as part of an inflammasome and it has been shown to negatively regulate the inflammatory response. Many studies have investigated the role of NLRP12 in mice *in vivo* and *in vitro*, but few have specifically investigated proteins that interact with human NLRP12. Examining novel protein interactions in human cells may help elucidate the mechanisms by which NLRP12 performs its various functions and could lead to important discoveries for human disease.

I have investigated the novel protein interaction between NLRP12 and CUL3 and determined the effect it has on the inflammatory signaling in response to PGN, a TLR2 ligand. In agreement with my hypothesis, NLRP12 and CUL3 were found to interact in HEK 293T cells via co-immunoprecipitation and western blotting. It was also found that both proteins localize in the cytoplasm of the cell independently and colocalize upon co-transfection. Both independently transfected and co-transfected proteins decreased IL-8 levels, indicating downregulation of the inflammatory response. However, contrary to my initial hypothesis, the decrease in IL-8 was

more pronounced in NLRP12 transfected cells, than in CUL3 alone or co-transfected cells. In fact, co-transfected cells had more IL-8 than NLRP12 transfected cells alone, suggesting that CUL3 may actually inhibit NLRP12 from suppressing NF-κB signaling.

Since CUL3 is an E3 ubiquitin ligase, my initial hypothesis was that it worked with NLRP12 to inhibit NF-κB signaling and suppress inflammation. However, the IL-8 data suggest that CUL3 could be negating NLRP12's function by ubiquitinating NLRP12 itself instead of working in conjunction with NLRP12 to further downregulate inflammation as initially hypothesized. This doesn't account for why downregulation of inflammation was also seen with CUL3 independently. The mechanism(s) that drive these varying results should be further examined. It would also be of interest to investigate the effect of the interaction and individually expressed proteins on NF-κB levels directly instead of the cytokines produced. This could provide insight into which NF-κB pathway is being affected since NLRP12 is known to affect both the canonical and non-canonical pathways.

It is important to note that preliminary data in the Lupfer lab suggested that NLRP12 interacts with many ubiquitin proteins and complexes, including RNF2 and COPS5 [64]. RNF2 is a RING finger protein and part of the Polycomb group (PcG) of proteins known to repress genes involved in development, differentiation, cancer transformation, and cell cycle [66, 67]. RNF2 possesses ubiquitin ligase activity, targeting proteins for degradation via the ubiquitin-proteosome system (UPS). RNF2 is known to promote progression of numerous cancers including breast cancer, colon cancer and melanoma [68]. In a study that identified p53 as an interacting partner with RNF2, p53 was ubiquitinated and protein expression was downregulated in selective cell lines promoting tumor development [66]. Another study found that

overexpression of RNF2 in SW480 cells regulates the ubiquitination of interferon regulatory factor 4 (IRF4), allowing invasion and migration of colon cancer in mice [69].

Intriguingly, NLRP12 is known to control tumorigenesis and gut homeostasis. During the recovery phase in NLRP12 deficient mice with DSS-induced colitis, deficient mice continued to lose weight and had shorter and enlarged colons compared to WT mice, suggesting continued colon inflammation even after DSS treatment was halted and upon tumor induction, NLRP12 deficient mice had increased tumor burden, with tumors throughout the entire colon, and proinflammatory cytokines levels were also increased in colon tissue [17]. In NLRP12 deficient macrophages, it was found that NF-κB and ERK activation was enhanced [17]. All of this suggests that NLRP12 controls tumorigenesis and colitis via suppression of proinflammatory cytokines and chemokines through NF-κB and ERK signaling. Other studies have found that NLRP12 maintains intestinal homeostasis through control of the gut microbiome. It was found that NLRP12 deficient mice had reduced microbial diversity and dissimilarity in community composition compared to WT, indicating that NLRP12 deficiency alters the microbiome [70]. DSS-induced colitis was ameliorated upon co-housing with WT mice, fecal transplantation, and inoculation of *Lachnospiraceae*, a family of bacteria shown to be decreased in NLRP12 deficient mice [70]. Overall, this indicates that NLRP12 limits intestinal inflammation by maintaining gut microbiota. It would be interesting to further investigate the interaction between NRLP12 and RNF2 to establish a link to inflammation, the microbiota, and cancer.

Outside of the Lupfer lab, NLRP12 has also been shown to interact with TRIM25, another E3 ubiquitin ligase. NLRP12 reduced RIG-I signaling and it was determined that it interacted with TRIM25, an adaptor protein for the RIG-I signaling pathway. The interaction cannot occur with the LRR or pyrin domain alone; the pyrin-NBD, NBD alone or NBD-LRR

associated with TRIM25 and while TRIM25 continued to associate with RIG-I, the resulting interaction with NLRP12 decreased this association and TRIM25-mediated Lys63 ubiquitination of RIG-I [71]. This is notable because Lys63 ubiquitination doesn't target proteins for degradation via the proteosome. Thus, the NLRP12 interaction with TRIM25 prevented the activation of RIG-I, suppressing inflammation.

In summary, NLPR12 interacts with CUL3, diminishing its effect on downregulation of the inflammatory response. NLRP12 also appears to interact with many ubiquitin ligase proteins and complexes to fulfill its role in regulating inflammation, cancer development and gut homeostasis. This highlights the need for further investigation into NLRP12 protein-protein interactions, especially with ubiquitin proteins and the role they play in the inflammatory response and disease.

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Table 1. The following is a list of primer sequences used for all cloning experiments.

Primers	Sequences
hCUL3 for	GAA GCT TGG TAC CGA GCT CGG ATC CAT GTC GAA TCT GAG CAA AG
hCUL3 rev	TTT AAA CGG GCC CTC TAG ACT CGA GTT ATG CTA CAT ATG TGT ATA CTT TG
T7 for	TAA TAC GAC TCA CTA TAG GG
BGH rev	TAG AAG GGC ACA GTC GAG G

Table 2. The following are the protocols for all PCR experiments.

Protocol	Denaturation	Annealing	Extension	Cycle #	Final Extension
Cloning PCR	98°C for 10 sec	55°C for 10 sec	72°C for 90 sec	35	72°C for 10 min
Colony PCR	95°C for 30 sec	53°C for 30 sec	72°C for 3 min	34	72°C for 5 min

Table 3. The following table consists of all antibodies used in western blotting, Co-IP, and staining for confocal microscopy.

Antibody	Detects	Catalog #	Company
Anti-DYKDDDDK Tag	DYKDDDDK Flag Epitope (Mouse)	14-6681-82	Invitrogen
c-Myc-Tag Monoclonal Antibody	c-Myc protein (Mouse)	631206	Clontech
Myc Tag Polyclonal Antibody	Myc-Tag EQKLISEEDL (Rabbit)	PA1-981	Invitrogen
Goat Anti-Mouse IgG-HRP Conjugate	Mouse IgG	170-6516	BioRad
Conjugated FITC	Mouse IgG	62-6511	Invitroge
Conjugated Rhodamine	Rabbit IgG	A10040	Invitrogen

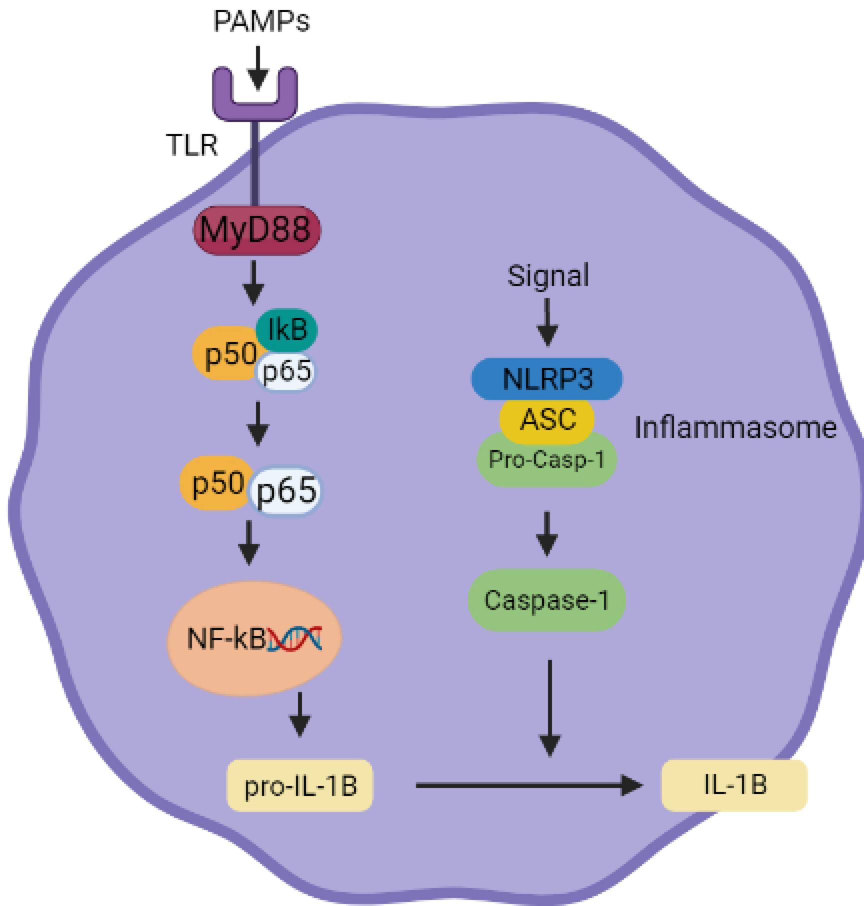


Figure 1. Diagram of inflammasome activation via NLRP3.

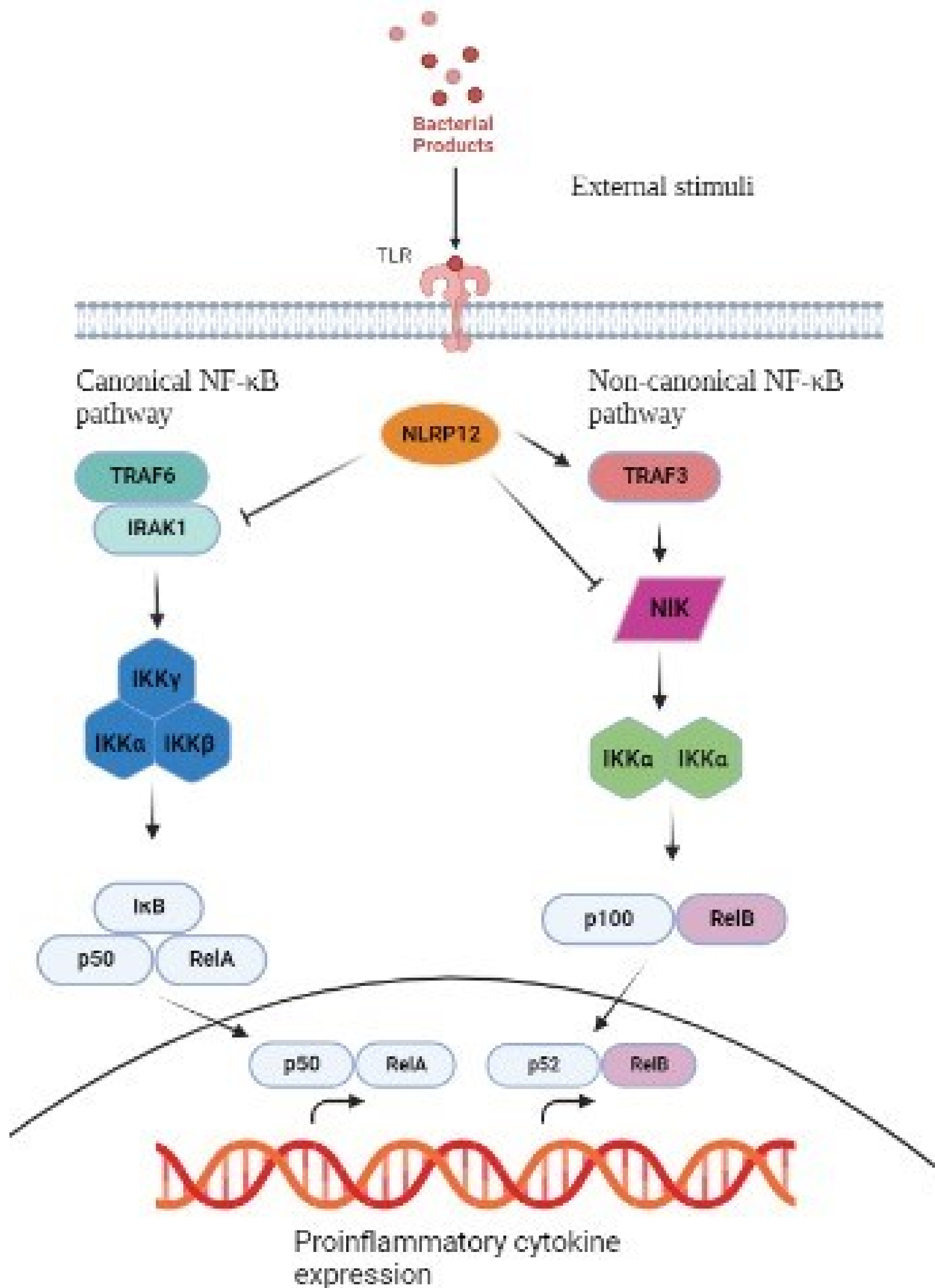


Figure 2. Diagram of NLRP12s activation and inhibition of canonical and noncanonical NF-κB pathways. Arrowheads indicate continuation of the pathway. Bar lines indicates inhibition of the pathway.

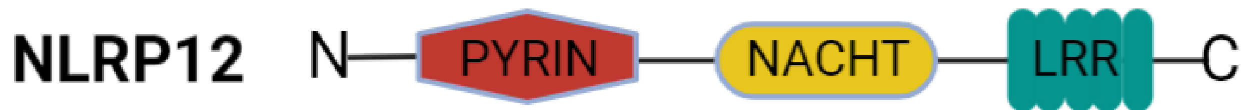


Figure 3. Structure of NLRP12 showing the N-terminal PYRIN domain, NACHT binding domain and C-terminal Leucine-Rich Repeat (LRR) domain.

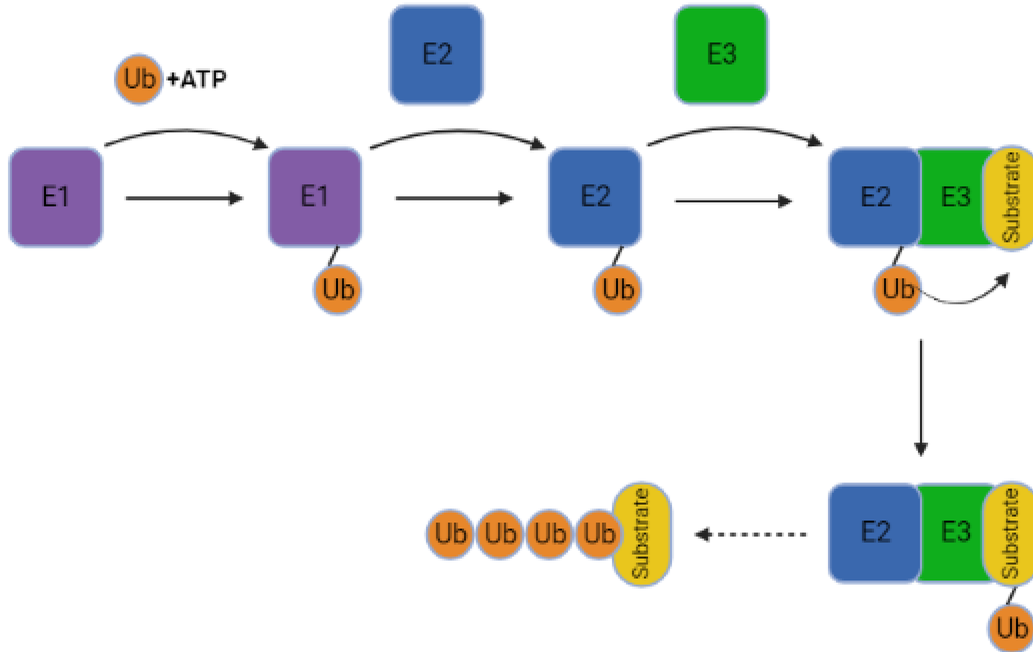


Figure 4. Diagram of the process of tagging proteins with ubiquitin for degradation by the Ubiquitin-Proteosome system (UPS) with E1, E2 and E3 ligases.

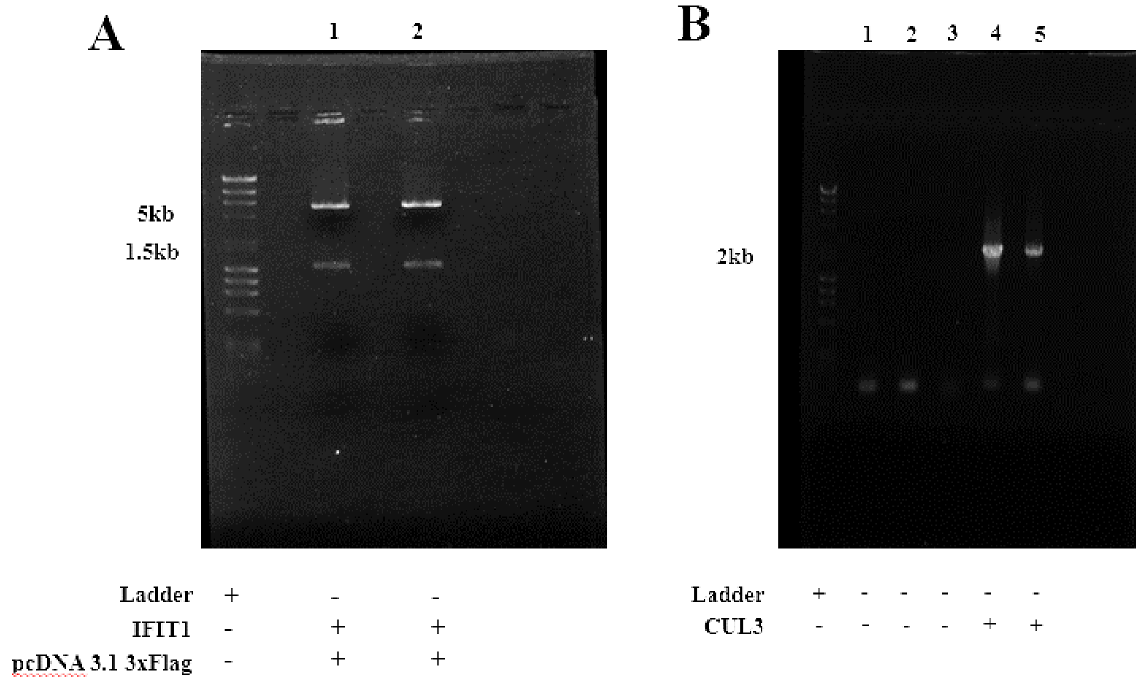


Figure 5. (A) Gel electrophoresis showing digestion of pcDNA 3.1 3xFlag IFIT1 with vector backbone pcDNA 3.1 (top) at the appropriate band size (5.4kb). (B) Colony PCR of sequenced CUL3 clones (lanes 4 and 5) showing appropriate band size (2.3kb).

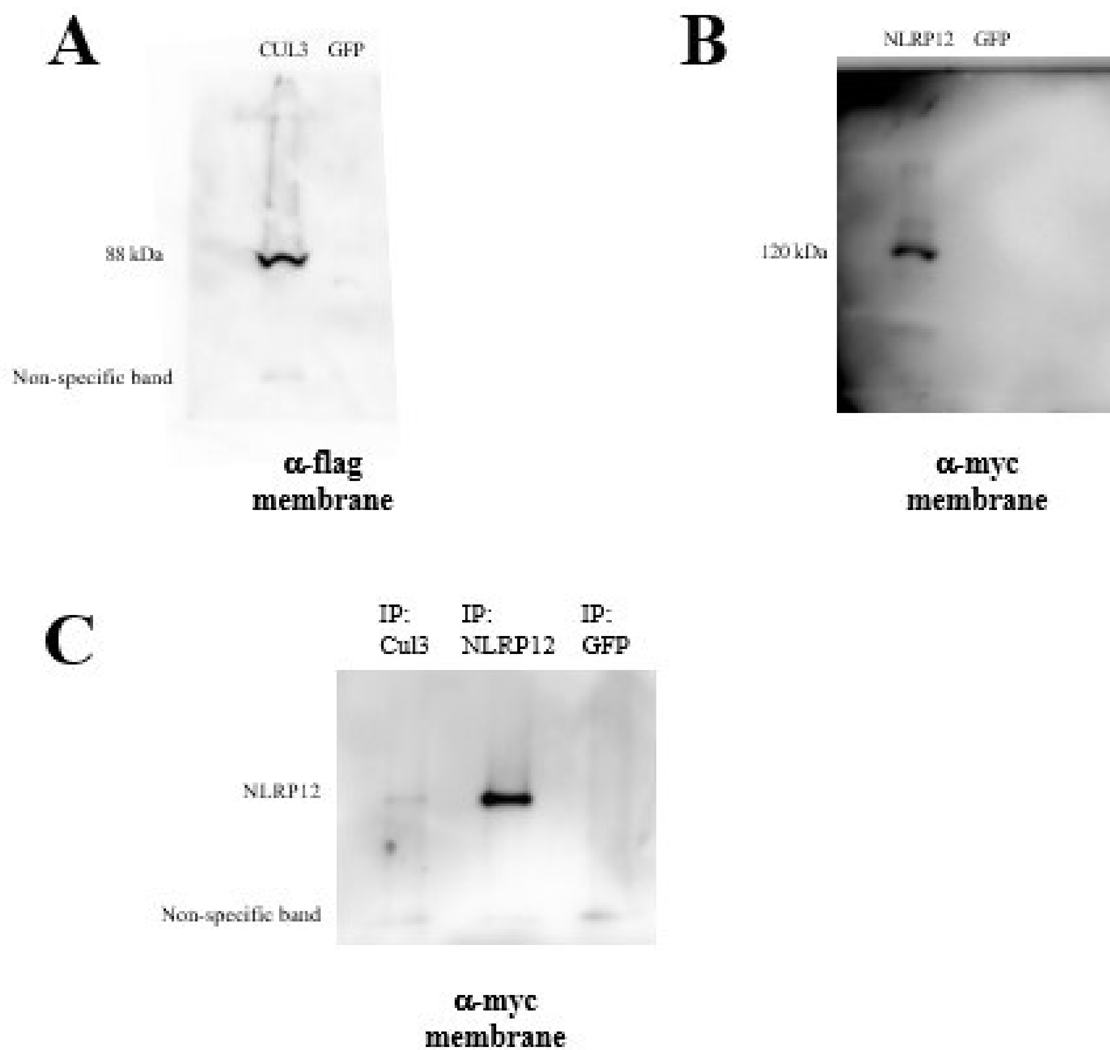


Figure 6. (A) Western blot showing overexpression of CUL3 (flag-tagged). (B) Western blot showing overexpression of NLRP12 (myc-tagged). (C) Co-Immunoprecipitation of co-transfected NLRP12 and CUL3 in HEK 293T cells. GFP transfected cells were used as controls. Anti-myc and anti-flag specific antibodies were used for co-immunoprecipitation and western blot was performed with myc-epitope tag antibody.

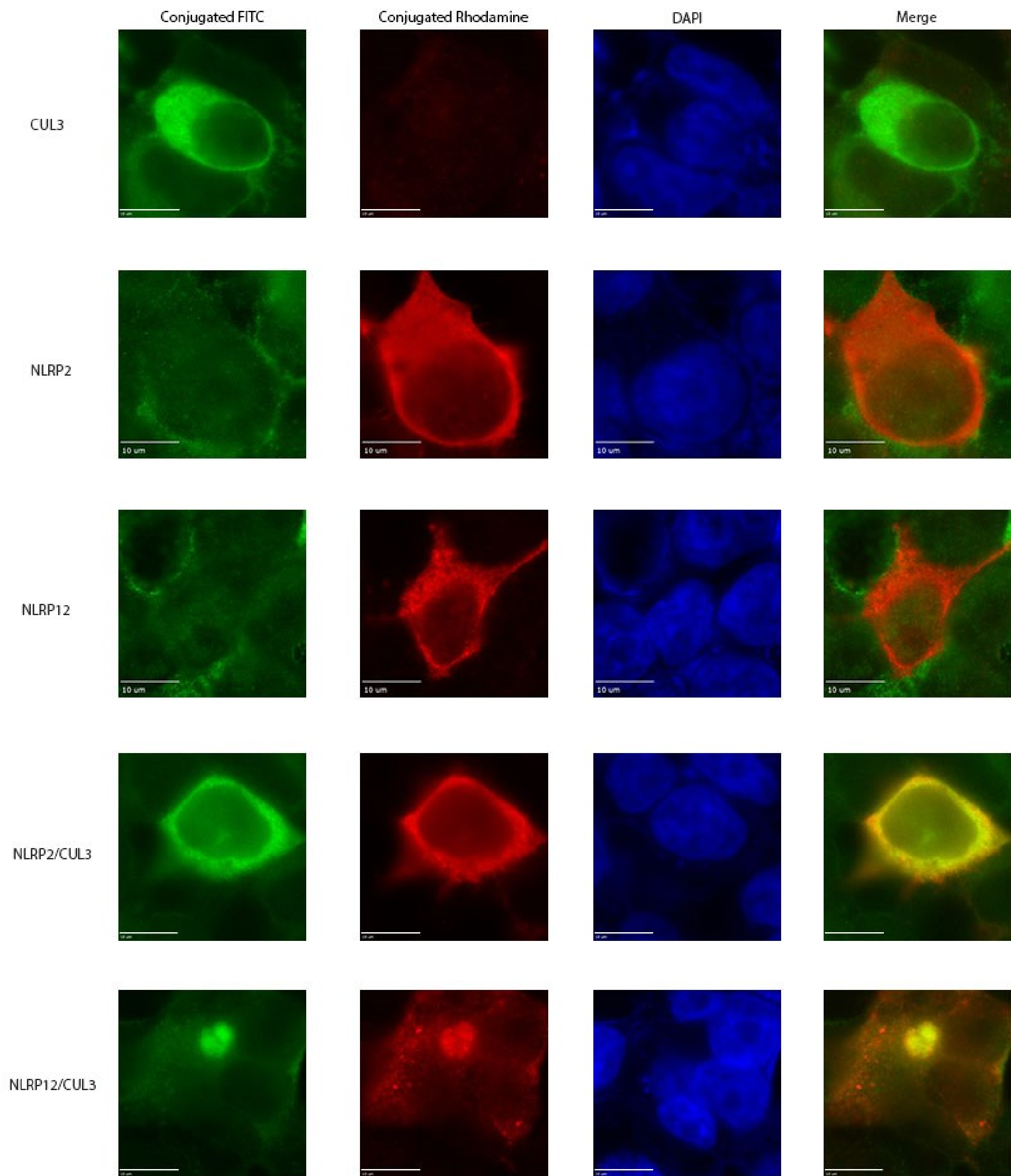
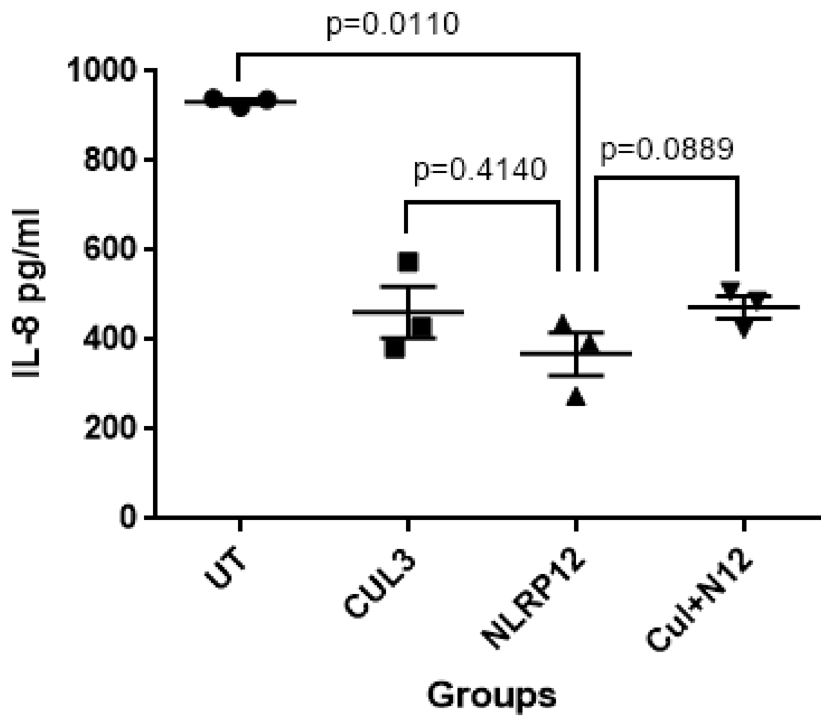


Figure 7. Co-localization of transfected NLRP12 and CUL3 in HEK 293T cells. Scale bar is 10 μm. NLRP2 was used as a control.



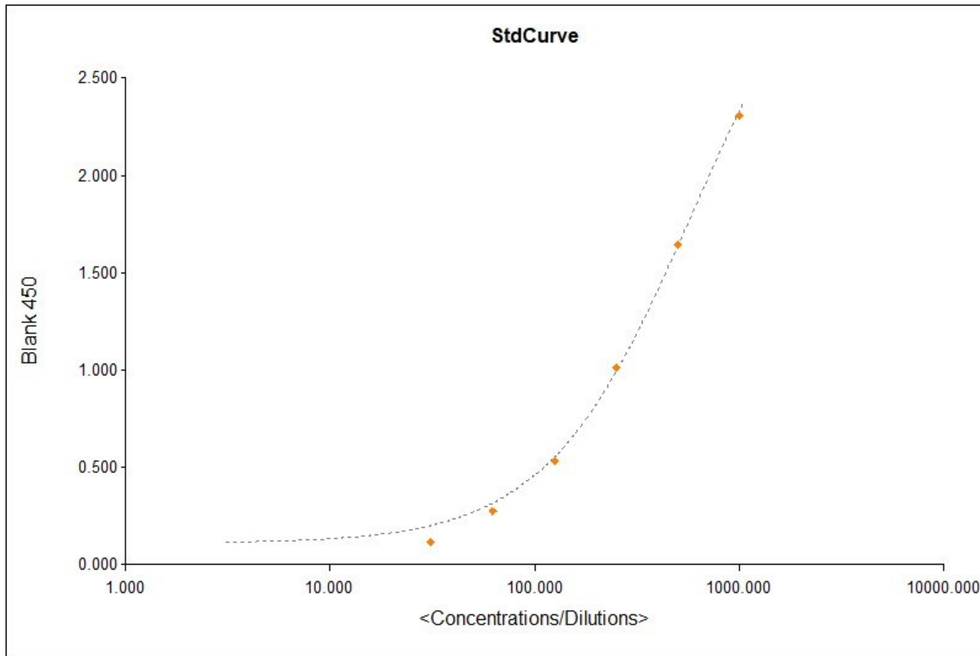


Figure 8. IL-8 ELISA of transfected NLRP12 and CUL3 in HEK 293T TLR-2^{YFP}/MD-2 cells. Data are representative of three individual experiments performed in duplicate. Statistical significance was determined using one-way ANOVA using Dunnett's multiple comparison test. A p value of 0.05 was considered statistically significant.

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