Abstract

The signal transducing ATPases with numerous domains (STAND) proteins are members of the extended ATPase associated with diverse cellular activities (AAA+) superfamily of P-loop NTPase proteins. These proteins act as molecular switches that cycle between ADP-bound and ATP-bound states, which determines their activation and inactivation. Members of the nucleotide-binding and leucine-rich repeat containing (NLR) protein family have a similar nucleotide-binding oligomerization domain (NOD) domain structure as these STAND ATPases. We examined this model in the context of the NLR family protein, nucleotide-binding and oligomerization domain containing 2 protein (NOD2), a protein that binds cytosolic muramyl dipeptide (MDP) and activates NF-κB, MAPK and autophagy signaling pathways. We mutated the NOD2 Walker A motif, which is known to bind nucleotides, to disrupt nucleotide binding in NOD2 and pathogenic alleles. These mutations caused a decrease in NOD2 binding to (CARD)-containing serine/threonine kinase (RIP2K), as well as a decrease in NF-κB activity in HEK293T cells. Interestingly, mutation of the NOD2 Walker A motif did not seem to abrogate NOD2-ATG16L binding or NOD2 homo-oligomerization, suggesting that while nucleotide-binding is necessary for NOD2-RIP2K hetero-oligomerization and NOD2-mediated NF-κB signaling, it is not necessary for NOD2-ATG16L binding and NOD2 homo-oligomerization. These data suggest that nucleotide binding is only required for interactions with some proteins, but not others. In light of these new data, we suggest an alternative model for NOD2 activation.
Introduction

The primary function of the immune system is to protect the body from pathogens. This system performs this task through a variety of complex mechanisms, and all of these rely primarily on the body’s ability to detect pathogens. To quickly and efficiently promote pathogen detection, two complementary signaling networks within the immune system (the innate and adaptive immune system) promote the recognition of a pathogen, facilitating its clearance, and the ability to mount a more effective secondary response. The innate immune system serves as the body’s first line of defense against pathogens and is comprised primarily of phagocytic cells as well as specific proteins that quickly recognize conserved features of invading pathogens. Alternatively, the adaptive immune system serves as a mechanism to recognize and clear a specific pathogen more efficiently following a previous exposure to this pathogen. The adaptive immune system is comprised primarily of a network of cells that patrol the body and can recognize key signatures of that specific pathogen. Thus, the innate immune system functions as a primary, general response to pathogens while the adaptive immune system serves to efficiently identify pathogens to which the body has already been exposed.

Proper activation of innate immune signaling is essential to protection of the body against invading pathogens. The innate immune system relies on the function of a series of key receptors known as pattern recognition receptors (PRRs) that recognize evolutionarily conserved motifs in a variety of potential invading pathogens, known as pathogen-associated molecular patterns (PAMPs). In response to PAMP recognition, these PRRs will initiate signaling cascades that result in changes in gene expression that stimulate an immune response, characterized by antimicrobial and inflammatory activity.
Due to the importance of the recognition of these PAMPs, there are a variety of PRRs that often play redundant roles in this process. For example, three types of receptors known as the Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding and leucine-rich repeat containing proteins (NLRs) recognize key motifs in viruses, bacteria and protozoa.\textsuperscript{1-3} These three receptor types are essential to properly mounting an innate immune response, and are known to localize to distinct subcellular locations, with TLRs serving as cell surface and endomembrane receptors while RLRs and NLRs act as cytosolic sentinels for PAMPs.\textsuperscript{3} Although these receptors often serve redundant functions, both the recent discovery that certain NLRs can form complexes known as inflammasomes\textsuperscript{4}, and the identification of mutant NLRs that confer susceptibility to the development of inflammatory disease\textsuperscript{5-8} in humans highlight the importance of these proteins not only in pattern recognition, but also for the maintenance of immune signaling homeostasis.

Within the past fifteen years, several human NLRs have been identified and linked to essential regulatory functions in immunity. For example, class II transactivator (CIITA), which functions specifically as a transcriptional co-activator of genes involved in antigen processing and presentation, such as class II major histocompatibility complex (MHC), human leukocyte antigen-DM and invariant chain.\textsuperscript{9} CIITA consists of a tripartite domain structure, with a N-terminal effector domain, a central nucleotide-binding domain and a series of C-terminal leucine-rich repeats. Given the important function that CIITA was shown to play in the regulation of antigen presentation, and immune signaling, as well as the association of loss of function mutants with bare lymphocyte syndrome\textsuperscript{9}, researchers hoped to discover similarly important immune system regulators through the
identification of proteins with a similar domain organization to CIITA. Using the domain organization and sequence of CIITA, researchers were able to identify 22 other proteins that contained the conserved domain organization of CIITA. These proteins came to be termed the nucleotide-binding and leucine-rich repeat containing (NLR) protein gene family.\(^{10}\) These NLRs contain a conserved N-terminal effector domain, a central nucleotide-binding domain (NBD), and a series of C-terminal leucine-rich repeats (LRRs).

Since their discovery, it has been shown that NLRs can be distinguished from each other by their N-terminal effector domains, which generally govern the protein-protein interactions that ultimately serve to initiate signaling cascades that regulate an immune response following pathogen detection (Figure 1).\(^{11}\) Although these proteins contain a conserved domain structure, they have diverse functions. For example, while a subset of NLRs such as NLRC4, NLRC5, NLRP1, NLRP3 and NLRP10 are known to form inflammasomes, which activate caspase and process pro-inflammatory cytokines such as IL-1\(\beta\).\(^{39-40}\) These NLR proteins have also been implicated in the regulation of a variety of other processes. For example, other NLRs have been shown to promote negative regulation of the inflammatory response (NLRC3, NLRP12), and pathogen sensing (NOD1/2, NAIP). Despite recent advances in the characterization of these diverse functions, however, the molecular mechanisms that control their activation remain largely unknown. Although the activation mechanism for NLRs is not well understood, their conserved domain organization groups them into the STAND (signal transducing ATPases with numerous domains) protein family, which may reveal a potential mechanism for their activation and inactivation.
The STAND proteins are known to play a role in immune system signaling through their functions in processes such as transcriptional regulation\(^{12,13}\), apoptosis\(^{12,14-15}\) and disease-resistance\(^{12,16-17}\). Specifically, the STANDs comprise a specific clade of P-loop NTPases known as the ATPase associated with diverse cellular activities (AAA+) superfamily of P-loop NTPase proteins. Like NLRs, STAND proteins are typically comprised of an effector domain, a nucleotide binding domain and an auto-inhibitory, regulatory domain. Within the STAND nucleotide binding domain, two key motifs govern nucleotide binding and nucleotide hydrolysis, giving STANDs the ability to cycle between nucleotide-binding states (which is thought to function as a regulation mechanism to control their activation). The first of these motifs, the Walker A region, regulates nucleotide binding, which has been shown to activate STANDs. This motif consists of the following amino acids: GXXXXGK[S/T] (where X is any residue). The glycine residues of this sequence are involved in hydrogen bonding interactions with the phosphoryl groups of the bound nucleotide, and their torsional angles provide the flexibility necessary to adopt a loop structure consistent with other P-loop containing nucleotide binding proteins (Figure 2).\(^{38}\) Additionally, the positively charge lysine in this motif interacts with and stabilizes the β- and γ-phosphoryl groups of the bound nucleotide.\(^{18,19}\) Finally, the serine/threonine residue of this motif has been shown to promote hydrogen-bonding with key intra-protein residues and the β-phosphate of the bound nucleotide.\(^{20}\) Thus, the Walker A motif regulates STAND nucleotide binding, which is thought to facilitate the activation and downstream signaling of STAND proteins.
While nucleotide binding is believed to activate STAND proteins, nucleotide hydrolysis is thought to facilitate STAND inactivation. The region known to catalyze nucleotide hydrolysis (known as the Walker B motif) is also found in the STAND nucleotide binding domain. The canonical Walker B motif in STAND proteins is comprised of the following amino acids: hhhhd[d/e] (where ‘h’ represents a hydrophobic amino acid) (Figure 2). The first acidic residue of this motif is believed to conjugate the catalytic Mg\(^{2+}\) ion in the nucleotide binding pocket of STANDs while the second acidic residue serves to prime a water molecule for nucleophilic attack of the \(\gamma\)-phosphate group of the bound triphosphate moiety.\(^{21-24}\) Overall, the conjugation of the \(\text{Mg}^{2+}\) ion and the priming of a water molecule serve to hydrolyze ATP. Thus, these Walker A and Walker B motifs allow for STANDs to cycle between activation states through regulation of their nucleotide binding state. Specifically, nucleotide binding serves as a mechanism for STAND activation while nucleotide hydrolysis functions as a mechanism for STAND protein inactivation.

To better characterize NLR function and regulation, we evaluated this model for STAND activation within the context of the NLR family protein, NOD2 (nucleotide-binding and oligomerization domain containing 2 protein). NOD2 is an ATPase that functions as an intracellular PRR for muramyl dipeptide (MDP), a cell wall component of both gram-negative and gram-positive bacteria (Figure 3).\(^{35}\) NOD2 is known to activate NF-\(\kappa\)B, MAPK signaling (Figure 4) and the autophagy pathways (Figure 5).\(^{25-26}\) Additionally, increased NOD2 expression has been observed in myeloid- and lymphoid-derived cells as well as in intestinal epithelial cells and Paneth cells of the intestinal crypts (Figure 6).\(^{27,28}\) Due to the expression of NOD2 in cell types that frequently
encounter bacterial pathogens, functional NOD2 plays an important role in bacterial recognition and clearance within the vertebrate host. Genetic aberrations in NOD2 are associated with structural changes in the NOD2 protein and susceptibility to the development of various diseases in humans. For example, not long after the initial characterization of NOD2 as a NLR family member, intracellular pathogen recognition receptor (PRR) and activator of NF-κB signalling, mutations in NOD2 were linked to both Crohn’s Disease (CD) and Blau Syndrome (BS).

Structurally, wild type NOD2 consists of 1040 amino acids and is comprised of two N-terminal caspase activation and recruitment domains (CARDs), a central nucleotide-binding and oligomerization domain (NOD) and a series of C-terminal leucine-rich repeats (LRRs). However, the first-identified CD-disease association mutation was determined to be a cytosine insertion at nucleotide 3020 (exon 11), resulting in a frameshift of the codon 1007, causing a Leu→Pro mutation, which is immediately followed by a premature stop codon, which results in a truncation in the LRR (Figure 7). Functional study of NOD2 L1007fsinsC (also known as NOD2 Δ32) mutants revealed that are deficient in their ability to induce NOD2-mediated NF-κB activity.

This NOD2 Δ32 mutation is associated with Crohn’s Disease, an inflammatory bowel disorder that is characterized by discontinuous inflammation that can manifest in any part of the gastrointestinal tract. While the Δ32 mutation is the most common CD-susceptibility mutation, other CD susceptibility mutations have been identified in NOD2 as well (ie. R702W and G908R, respectively). All of these CD susceptibility mutations result in a signaling-defective NOD2. For example, it has been shown in vitro that these
mutants fail to activate NF-κB signaling, even in the presence of MDP. Additionally, patient cohorts with the Δ32 mutation (of Western European descent) have been shown to be deficient in the production of IL-1β, an important pro-inflammatory cytokine. Although it is not explicitly clear how these NOD2 polymorphisms contribute to CD development, several hypotheses have been suggested. For example, it has been proposed that these mutations in the NOD2 LRR lead to a mutant protein that is unable to sense MDP due to its mutated LRR domain, and is thus unable to properly recognize bacteria, become activated, and consequently clear bacteria. This hypothesis is supported by the observation that NOD2 CD mutants are deficient in stimulating NF-κB activity and secretion of the pro-inflammatory cytokine, IL-1β. However, recent work has demonstrated that MDP binding is facilitated by the NOD2 NBD, and not the LRR region. Thus, while these CD mutants are, indeed, signaling deficient, the mutation of the NOD2 LRR region might not prevent NOD2-MDP recognition. As such, the mechanism by which these mutations result in NOD2 signaling deficiency remains unclear.

Blau Syndrome is a rare, autosomal dominant disorder that is characterized by uveitis, early-onset granulomatous arthritis, severe skin rash and camptodactyly. These symptoms can manifest as the product of a sporadic form of this disease as well, known as Early Onset Sarcoidosis (EOS). This disease usually starts to present within the first 2-4 years of life, and skin rash is commonly the first observed symptom. During the course of the disease, patients often experience moderate to severe joint pain (due to chronic inflammatory activity) that can lead to severe impairment in a significant portion of patients. Additionally, almost all documented cases of Blau Syndrome patients
experience visual impairment of some form, with approximately two-thirds experiencing complete visual loss.\textsuperscript{36,37} Like CD, Blau Syndrome is a chronic inflammatory disorder. However, while NOD2 expression has been observed in colonic epithelial and Paneth cells (which may be directly involved in the manifestation of the CD phenotype), NOD2 is not known to be as highly expressed in tissues that are directly affected by BS (Figure 7). Additionally, while mutations that lead to CD susceptibility are hypoactive, NOD2 R334W is a hyperactive mutation. The difference in NOD2 activity but similar chronic inflammatory phenotype within these two disease states, has led to a greater investigation of the structure NOD2 R334W mutant, so as to better inform our understanding of the signaling aberrations that may lead to this tissue-specific BS phenotype.

Most mutations leading to Blau Syndrome susceptibility lie within the NOD2 NBD, with the most common mutation being an Arginine$\rightarrow$Tryptophan change at amino acid 334 (R334W). Molecular modeling of NOD2 Blau Syndrome mutations (based on NOD2 homology with the crystallized STAND proteins Apaf-1 and mouse Nlrc4) has suggested that all SNPs mapped to the NOD2 ATP/Mg$^{2+}$ patch result in a hyperactive NOD2 following mutation.\textsuperscript{41} It is hypothesized that amino acid changes in this location of the NBD disrupt the function of the catalytically active Mg$^{2+}$ ion within this patch, which is necessary for ATP hydrolysis. These findings support a model for NOD2 deactivation whereby NOD2 is returned to its monomeric, autoinhibited and ADP bound state following ATP hydrolysis. Thus, ATP hydrolysis may function as a mechanism for deactivation of signaling competent NOD2.\textsuperscript{41}

Despite our understanding of the downstream outcomes of NOD2-mediated signaling, and the pathological consequences of disruptions in this signaling, the
characterization of the molecular and structural events critical to NOD2 activation remain poorly elucidated. Multiple groups have proposed models for NOD2 activation that, although they differ slightly in the timing of the molecular events required for activation, typify the general mode of activation of STAND proteins. One such model, proposed by Mo et al. (2012) hypothesizes that NOD2 remains in a monomeric, autoinhibited and ADP-bound basal state until ADP is exchanged for ADP, which causes a conformational change that allows for homo-oligomerization between monomers and NOD2 MDP binding. Binding of MDP then further stabilizes this ATP-bound, open NOD2 conformation, and thus enables the formation of higher-order oligomers and promotes NOD2-mediated signaling. Finally, ATP hydrolysis is hypothesized to return NOD2 to its monomeric, autoinhibited state (Figure 8). This hypothesized model posits that several key NOD2-mediated interactions are critical to the proper activation of NOD2 and NOD2-regulated downstream signaling.

Following this model NOD2-mediated homo-oligomerization is the apical step in this process. Recent work has demonstrated that NOD2-NOD2 interactions are mediated through the NOD2 NBD, and it has been suggested that this homo-oligomerization is required for NOD2-mediated immune signaling. Specifically, it has been shown that following NOD2 homo-oligomerization and subsequent activation, a fraction of cytosolic NOD2 becomes re-distributed within the cell, and can localize both to the plasma membrane and to endosomes. After this re-distribution event, membrane localized NOD2 can recruit RIP2K to the plasma membrane, promoting NOD2 activation of RIP2K, and thus NF-κB and MAPK signaling. As such, it has been hypothesized that NOD2 homo-oligomerization is a key step in the activation of NOD2. Based on our
current model for NOD2 activation, NOD2-homo-oligomerization is thought to lie downstream of nucleotide binding (Figure 8), suggesting that this event may be dependent on NOD2 nucleotide-binding.

Additional NOD2-mediated interactions are known to be essential to the signal transducing properties of NOD2 and the initiation of NOD2-mediated NF-κB and MAPK activity. For example, NOD2 interacts directly with RIP2K through homotypic CARD-CARD interactions.\textsuperscript{44,45} RIP2K is located in the cytosol, and is essential for the activation of NF-κB activity following MDP stimulation. It is hypothesized that within the cell, in the absence of MDP, RIP2K is sequestered by MEKK4 through a competitive binding process.\textsuperscript{46} Without MDP present, MEKK4 outcompetes NOD2 for RIP2K, which can RIP2K-mediated NF-κB activation.\textsuperscript{46} However, in the presence of MDP, NOD2 is able to outcompete MEKK4, thereby promoting NOD2-RIP2K binding and NF-κB activation. Following NOD2-RIP2K binding, RIP2K auto-phosphorylates\textsuperscript{45}, which promotes its K63 ubiquitination (an event required for effective RIP2K signaling). Active RIP2K then mediates the K63-linked ubiquitination of NF-κB essential modulator (NEMO).\textsuperscript{47} Active NEMO triggers the activation of IκB kinase (IKK), which phosphorylates IκB, the negative regulatory subunit of the inhibitor of κB kinase (IKK) complex. This phosphorylation mediated its proteasomal degradation.\textsuperscript{48,49} Following destruction of IκB, transforming growth factor-β activating kinase 1 (TAK1) is recruited and activates mitogen-activated protein kinases (MAPKs). Simultaneously, the destruction IκB allows for nuclear translocation of the NF-κB transcription factor, which initiates the transcription and expression of proinflammatory cytokines, chemokines and adhesion molecules.\textsuperscript{49}
NOD2 also plays a key role in autophagy through its association with ATG16L. Autophagy is a key process in innate immune signaling as can promote pathogen clearance, and organelle and protein repair in response to damage.\textsuperscript{50} ATG16L is expressed in the colon, small intestine, intestinal epithelial cells, leukocytes and the spleen.\textsuperscript{50,51} During the activation of the autophagocytic machinery, autophagy-related protein 12 (ATG12) will form an isopeptide linkage to an internal lysine residue of autophagy-related protein 5 (ATG5) via a series of ubiquitin-like reactions that are mediated by E1 and E2 ligase-like proteins (ATG7 and ATG10, respectively).\textsuperscript{52} This ATG12-ATG5 heterodimer then forms a complex with ATG16L. These multiprotein complexes associate with LC3 and transport LC3 to lipidation sites where phosphatidylethanolamine (PE) is attached to LC3 through formation of an amide bond between the head group amine of PE and the C-terminal carboxyl base of LC3.\textsuperscript{50,51} This lipidation serves as a key marker for autophagosome formation, but the exact function of this step within the process of autophagy has not yet been identified. However, NOD2 activation promotes the association of ATG16L with the ATG12-ATG5, thus promoting autophagosome formation.

Specifically, NOD2 is hypothesized to target ATG16L to the plasma membrane at entry points of cyto-invasive bacteria. This hypothesis is supported by research that demonstrates that MDP stimulation of fibroblasts, dendritic cells and epithelial cells has been shown to induce autophagosome formation.\textsuperscript{53} Additionally, NOD2-mediated autophagosome formation is a thought to be a RIP2K-dependent process whereby activation of RIP2K promotes the activation of p38 and MAPK signaling, which then activates the serine/threonine protein kinase Ulk1 and simultaneously inactivates the
protein phosphatase 2A (PP2A) complex (a negative regulator of NOD2-mediated autophagy). However, aside from the fact that MDP stimulation of certain cell types seems to increase autophagosome formation, the exact mechanism by which NOD2 supposedly targets ATG16L to the cell membrane whereby it stimulates the formation of autophagosomes remains to be elucidated.

Given the relationship between NOD2 and STAND ATPases, the association between NOD2 dysfunction and disease susceptibility, and the multifaceted role NOD2 plays in pathogen recognition and clearance, we investigated the prevailing model of NOD2 activation (Figure 8). Based NOD2 NOD domain homology with other STAND AAA+ ATPases, we hypothesized that NOD2 nucleotide binding would play an essential role in NOD2 activation. To test this hypothesis, we mutated the NOD2 Walker A motif \((G^{304}K^{305}S^{306} \rightarrow AAA)\) in an effort to abrogate NOD2 nucleotide binding. Specifically, we predicted that the non-conservative amino acid substitutions of \(G^{304} \rightarrow A\) (which is a change from a hydrophilic hydrogen R-group to a hydrophobic R-group at this residue) would eliminate the possibility for hydrogen bonding interactions between the phosphoryl groups of the bound nucleotide and this amino acid residue. Another non-conservative substitution at \(K^{305} \rightarrow A\) would eliminate the presence of a charged, ammonium ion in the R-group at this residue to stabilize the negatively charged \(\beta\)- and \(\gamma\)-phosphoryl groups of ATP. Finally, mutation of \(S^{306} \rightarrow A\) would result in another hydrophilic to hydrophobic non-conservative amino acid substitution, preventing the stabilization of the NOD2 nucleotide binding pocket through interaction with the Walker B motif as well as interaction with the catalytically active \(Mg^{2+}\) ion in the nucleotide-binding pocket and the \(\beta\)-phosphate of the bound nucleotide. Similar mutations have
shown that proteins containing mutations that abrogate this motif demonstrate decreased nucleotide binding.\textsuperscript{9,35}

Based on our hypothesis that nucleotide binding is required for NOD2 activation and our prediction that mutation of the Walker A motif would disrupt proper ATP binding, we predicted that the disruption of NOD2 nucleotide binding would impair NOD2 activation, and subsequently, NOD2-mediated downstream signaling. We tested this hypothesis in wild type NOD2 (WT), as well as in two pathogenic NOD2 alleles—the hyperactive R334W BS-associated mutation and the hypoactive Lfs1007insC (Δ32) CD-associated mutation. We assessed NOD2 function through its ability to hetero- and homo-oligomerize with its known binding partners, RIP2K, ATG16L and itself via co-immunoprecipitation. We predicted that if nucleotide binding is required for NOD2 activation, mutation of the NOD2 Walker A motif would lead to decreased binding between NOD2 and its direct interactors (RIP2K, ATG16L and NOD2). It should be noted that our examination of NOD2 homo-oligomerization following Walker A mutation examined interactions between wild type NOD2 and all of our constructs as well as the interactions between each construct and itself. This experimental set-up was designed to examine NOD2 homo-oligomerization in individuals that are both heterozygous and homozygous for NOD2 pathogenic alleles.

After investigating NOD2 protein-protein interactions following disruption of nucleotide binding, we wanted to examine whether the induction of a Walker A mutation would lead to NF-κB dependent transcription-level changes. We used a luciferase assay system specific for NF-κB signaling.\textsuperscript{55} NOD2-RIP2K interaction is known to initiate NOD-mediated NF-κB signaling. Thus, we predicted that mutation of the NOD2 Walker
A motif would lead to decreased NF-κB signaling in Walker A mutants due to the disruption of NOD2-RIP2K binding, which is the product of a disruption in NOD2 nucleotide binding. As expected, disruption of NOD2 nucleotide binding abrogated NOD2-mediated signaling with RIP2K, both at the level of NOD2 protein-protein interactions as well as at the level of NOD2-mediated NF-κB activity. However, we saw deviations from our predictions when examining NOD2 interaction with ATG16L and homo-oligomerization following mutation of the Walker A motif. Specifically, our results suggest that nucleotide-binding may not be necessary for all NOD2 protein-protein interactions.
**Materials & Methods**

*Constructs.* NOD2 constructs were generated as previously described (Mo et al., 2012). Briefly, NOD2-encoding cDNA and mutations of the NOD2-encoding cDNA were inserted in the mammalian expression vector, pcDNA™3.1D/V5 His TOPO® (Invitrogen) according to the manufacturer’s protocols. This process was repeated (using RIP2K-encoding cDNA) to generate V5-tagged RIP2K, which served as the template from which the full length RIP2K open reading frame was amplified using Platinum Pfx polymerase (Life Technologies). This open reading frame was then cloned into the pCR8/TOPO/TA base vector (Life Technologies) by TOPO TA cloning. A recombination reaction was performed into a modified FLAG Gateway destination vector (previously described) using LR clonase (Life Technologies). All vector sequences were verified by bidirectional sequencing.

*Cell culture and Transfection.* Human embryonic kidney (HEK293T) and HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with a final concentration of 9% heat activated fetal bovine serum (Invitrogen), 1X sodium pyruvate (Gibco), 20 units of penicillin, 20 µg streptomycin and 146 mg L-glutamine (Gibco). Cells were grown at 37 °C with 5% CO₂. For transient transfections, both HEK293T and HeLa cells were plated at 5.0 x 10⁵ cells per well in six-well plates and grown overnight. Transfections were then performed with Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol.

*Co-immunoprecipitation and Immunoblot Analysis.* Immunoprecipitation was carried out in 50 mM Tris pH 7.7, 150 mM NaCl, 1% Igepal and 0.2% deoxycholate supplemented with a protease inhibitor cocktail, and cells were lysed in this solution for
30 minutes on ice. Lysates were then immunoprecipitated with agarose-conjugated anti-FLAG-M2 beads (Sigma) for twenty-four hours at 4°C. Following three washes with (0.5% Igepal in 1X PBS), immunoprecipitation samples as well as total cell lysates were analyzed using SDS-polyacrilamide gel electrophoresis (using 4-15% Mini-PROTEAN® TGX™ gels). Gels were transferred to a nitrocellulose membrane (BIO-RAD) and incubated in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20 containing 5% nonfat milk for one hour at room temperature. The membranes were then probed with anti-V5 (Invitrogen) and anti-FLAG M2 (Sigma-Aldrich) primary-conjugated horseradish peroxidase antibodies.

**Luciferase Assay.** Luciferase Assays were performed using the Dual Glo Luciferase System (Promega), according to the manufacturer’s protocol. HEK293T cells were transiently co-transfected with a NOD2 construct, a positive control (p65) or a negative control (CIITA) construct in combination with firefly luciferase and Renilla luciferase reporter vectors containing NF-κB specific promoters. MDP-simulated cells were incubated in 10ng/mL muramyl dipeptide (Invivogen) for 8 hours post-transfection. After 24 hours post-transfection, cells were washed twice with 1X PBS and frozen at -80°C. At 48 hours post-transfection, cells were lysed in hypotonic 0.1X PBS. All samples were measured in triplicate using a Perkin-Elmer EnSpire plate reader and normalized to Renilla signal.
Results

*NOD2 Walker A mutants show decreased binding to RIP2K*

To investigate if nucleotide binding is required for NOD2-mediated signaling, we assayed mutant NOD2 proteins for the ability to interact with RIP2K, ATG16L and itself. First, we examined the interaction between NOD2 and RIP2K, a direct downstream binding partner, which is necessary for the induction of NOD2-mediated NF-κB and MAPK signaling. Walker A (WA) mutants of NOD2 wild type (WT), R334W and Δ32 proteins and are expected to disrupt NOD2 nucleotide binding (Figure 9). Following generation of these Walker A mutants, NOD2 WT, R334W and Δ32 along with their corresponding Walker A constructs were assayed for RIP2K binding through co-immunoprecipitation and visualized using SDS-PAGE. While all non-Walker A mutant constructs were able to bind RIP2K, NOD2 WA (Figure 10), R334W WA (Figure 11) and Δ32 WA (Figure 12) displayed a decreased RIP2K binding. Although this pattern was observed consistently throughout multiple experiments, the amount of RIP2K binding in WA mutants was variable across experiments (Figures 10B, 11B and 12B). This observed decrease NOD2-RIP2K binding in WA mutants suggests that the interaction between NOD2 and RIP2K requires proper NOD2 nucleotide binding upstream of this event, which is consistent with our prediction and our current model for NOD2 activation (Figure 8).

*NOD2 Walker A mutation disrupts NOD2-mediated NF-κB Activity*

Given our results that nucleotide binding is necessary for proper NOD2-RIP2K interaction, and the knowledge that RIP2K directly facilitates the induction of NOD2-
mediated NF-κB and MAPK signaling, we wanted to determine whether NOD2 nucleotide-binding disruption would have an effect further downstream of NOD2-RIP2K binding. Since RIP2K activation following its binding to NOD2 activates NF-κB signaling, we predicted that disruption of NOD2-RIP2K binding in the presence of the Walker A mutation, would decrease NF-κB signaling in our NOD2 Walker A mutants. We tested this prediction using a NF-κB specific luciferase assay system. Thus, an increase in NF-κB transcriptional activity would lead to an increase in luciferase production and vice versa. In the absence of MDP stimulation, NOD2 WA (Figure 13A), R334W WA (Figure 14A) and Δ32 WA (Figure 15A) were not significantly different in NF-κB signaling as compared to their non-Walker A counterparts. Upon MDP stimulation, however, these Walker A variants demonstrated significantly decreased NF-κB activation (Figures 13C, 14C and 15C). Overall, non-Walker A NOD2 Walker A variants activated NF-κB significantly less than their non-Walker A counterparts in the presence of MDP, suggesting that nucleotide binding is necessary for NOD2-mediated NF-κB signaling, as was predicted.

**NOD2 Walker A mutants Demonstrate Increased Binding to ATG16L**

We investigated the interaction between NOD2 and the autophagy-related protein ATG16L, which is necessary for the induction of NOD2-mediated autophagy. Here we show that NOD2 WA (Figure 16A), R334W WA (Figure 17A) and Δ32 WA (Figure 18A) mutants (as well as NOD2 Δ32) are all able to interact with ATG16L, whereas decreased binding was observed between NOD2 WT (Figure 16A) and R334W(Figure15A) and ATG16L. These results suggests that in contrast to the NOD2-
RIP2K interaction, NOD2-ATG16L does not require NOD2 nucleotide binding. In fact, all NOD2 constructs known to be effective activators of NOD2 downstream signaling such as NF-κB activation demonstrated decreased NOD2-ATG16L interaction. This decreased interaction may suggest that the regulation of the NOD2-ATG16L binding is distinct from that of NOD2-RIP2K binding, and may even point to a different mechanism to promote induction NOD2-mediated autophagy as compared to NOD2-mediated NF-κB and MAPK signaling. As our data have shown, surprisingly, the NOD2-ATG16L interaction does not require nucleotide binding.

**NOD2 Nucleotide Binding Does Not Disrupt NOD2 Homo-oligomerization or Allele Specific Interactions**

Since NOD2 homo-oligomerization is predicted to act along with NOD2 nucleotide binding to facilitate the activation of NOD2 (Figure 8), we tested the effect of disruption of NOD2 nucleotide binding on NOD2 homo-oligomerization. Additionally, we wanted to test this effect within the context of hetero- and homozygotes in NOD2 pathogenic disease states. Co-immunoprecipitations were carried out between wild type NOD2 and each NOD2 construct as well as between each NOD2 construct and itself to simulate homo-oligomerization of heterozygous and homozygous NOD2 variants, respectively.

It was observed that in the co-immunoprecipitations examining NOD2 homo-oligomerization with NOD2 wild type, the interaction between NOD2 WA (Figure 16B), R334W WA (Figure 17B) and Δ32 WA (Figure 18B) was not substantially different than that between NOD2 and non-Walker A constructs. These results suggests that in the case
of individuals heterozygous for these pathogenic NOD2 alleles, NOD2 nucleotide binding does not seem to affect homo-oligomerization between the pathogenic variant and wild type NOD2. This result was unexpected given our prediction that aberration of NOD2 nucleotide binding would disrupt downstream NOD2-mediated protein-protein interactions.

Additionally, the co-immunoprecipitation between each NOD2 construct and itself revealed that each NOD2 variant was able to homo-oligomerize appropriately, regardless of the presence of a Walker A mutation in the NOD2 NBD (Figure 16C, Figure 17C, Figure 18C). This observation was inconsistent with our prediction that disruption of NOD2 nucleotide binding would disrupt downstream protein-protein interactions, including NOD2 homo-oligomerization. These results are surprising given the role that nucleotide binding is predicted to play in NOD2 activation—suggesting the possibility that NOD2 nucleotide binding may, in fact, lie upstream of nucleotide binding in the NOD2 activation pathway. In all, we have shown that nucleotide binding seems to be necessary for NOD2-RIP2K interaction, and NOD2-RIP2K mediated NF-κB signaling. However, nucleotide binding does not seem to be necessary for NOD2-ATG16L binding or for NOD2 homo-oligomerization, suggesting that nucleotide binding may not play a role in the mediation of all NOD2 protein-protein interactions.
Discussion

Pathogen detection and clearance are essential functions of the immune system that serve to protect the body from pathogenic insult. The body’s first line of defense in combating pathogens is the activation of the innate immune system, which serves as a general mechanism to quickly facilitate an immune response and clearance of the pathogen. Members of the NLR protein family have been shown to play essential roles in innate immune system signaling and regulation. However, the specific mechanism for the regulation of the activation and function of these proteins remains poorly characterized. Here, we tested a prevailing model of activation for a NLR protein family member known as NOD2 in an effort to better elucidate a general mechanism for NLR signaling regulation. Specifically, we examined the impact of nucleotide binding on NOD2 activation. Current models for NOD2 activation posit that nucleotide binding and exchange initiates NOD2 signaling (Figure 8). However, the cellular and molecular events that control NOD2 activation are not well understood. Through analysis of NOD2 protein-protein interactions and signal transduction following mutation of the NOD2 Walker A motif, our data suggest that proper nucleotide binding is necessary for NOD2-mediated RIP2K activation and downstream signaling. In contrast, nucleotide binding is not necessary for NOD2-ATG16L activation and NOD2 homo-oligomerization, suggesting that NOD2-ATG16L binding and NOD2 homo-oligomerization may lie upstream of NOD2 nucleotide binding in the NOD2 activation pathway.

Characterization of the interaction between NOD2 and RIP2K following disruption of nucleotide binding demonstrated that mutation of the Walker A motif in wild type NOD2 as well as pathogenic NOD2 variants resulted in decreased NOD2-
RIP2K binding. Interestingly, while decreased NOD2-RIP2K interaction was consistently observed following mutation of the NOD2 Walker A motif, the relative decrease in NOD2-RIP2K interaction did seem to vary between experiments. This diminished, but observable interaction between Walker A mutants and RIP2K may be due a nucleotide binding capability that is disrupted, but not eliminated in NOD2. Thus, the $G^{304}K^{305}S^{306} \rightarrow AAA$ mutation may not completely abrogate NOD2 nucleotide binding, allowing for a fraction of these Walker A mutants to become activated and signal normally. This incomplete loss of nucleotide binding could be tested through incubation of radioactively labeled nucleotide with purified recombinant Walker A mutant NOD2 variants as well as their non-Walker A counterparts, followed by visualization of the amount of radioactively-labeled nucleotide bound to these NOD2 proteins.

Alternatively, the Walker A mutation may fully inhibit NOD2 nucleotide binding, resulting in the adoption of a closed, monomeric conformation. However, it is possible that this inactive conformation is not completely signaling incompetent. While the NOD2 LRR domains are predicted to fold back over the NBD to promote the formation of autoinhibited NOD2 monomers, since NOD2-RIP2K binding is mediated by CARD-CARD interactions, it is possible that adoption of this monomeric conformation does not entirely eliminate the possibility of homotypic CARD-CARD interactions between NOD2 and its downstream binding partners. The facilitation of CARD-CARD interaction even in this closed NOD2 conformation may allow for tuning of innate immune system signaling. Specifically, these CARD-CARD interactions would promote a basal level of RIP2K-mediated NF-κB and MAPK activation, which would lead to the transcription of both cytokines and negative NOD2 regulators, such as SOCS-3. As such, a basal level
of transcription of these immune response modulators and NOD2 negative regulators may function to inhibit NOD2 signaling in the absence of *bona fide* bacterial detection, while simultaneously allowing for a quick immune response following detection of bacterial pathogens. Despite the observation that the Walker A mutation did not completely abrogate NOD2-RIP2K binding and the potential biological implications of this observation, our results indicate that nucleotide binding is necessary for NOD2-RIP2K interaction and downstream signaling. These results support our current model for NOD2 activation (Figure 8), suggesting that not only is nucleotide binding necessary to promote NOD2-RIP2K interaction, but also that nucleotide binding and exchange may, indeed, lie upstream of hetero-oligomerization with RIP2K.

Further analysis of NOD2-mediated signal transduction after disruption of NOD2 nucleotide binding revealed that the nucleotide-binding ability of NOD2 plays an important role in the transcription of NF-κB downstream targets. Specifically, mutation of the NOD2 Walker A motif resulted in decreased NF-κB signaling in wild type NOD2 as well as in both pathogenic alleles. Although this result supported our prediction that nucleotide binding would result in disruption of NOD2 downstream signaling, NF-κB activation by the NOD2Δ32 mutant, both in the presence and absence of MDP was not consistent with past literature. Although this mutant is a hypoactive NOD2 protein, we observed that NOD2Δ32 was able to activate NF-κB signaling at comparable levels to wild type NOD2 in the absence of MDP stimulation. However, in the presence of MDP, NOD2Δ32-mediated NF-κB signaling was significantly than that stimulated by NOD2 wild type protein. This result directly contradicts the results of multiple studies investigating the ability of NOD2Δ32 to elicit NF-κB signaling. These studies
demonstrating ineffective NOD2 Δ32-mediated NF-κB signaling have been done in a variety of cell types, including HEK293T cells (which were used in our luciferase assay experiments). Thus, it does not appear that our choice of cell-type would explain these results. However, in these luciferase assay experiments, NOD2 was greatly over-expressed. This overexpression is a caveat of the luciferase assay system, and this alteration in NOD2 Δ32 NF-κB signaling has been observed in the past by other studies using a similar experimental approach.55

Nevertheless, our comparison of NF-κB activity in Walker A and non-Walker A NOD2 variants supports our hypothesis that nucleotide binding is necessary for NOD2 activation and NOD2-mediated signaling. These results were also consistent with our current model for NOD2 activation, in which nucleotide binding and exchange lies upstream of NOD2 hetero-oligomerization in the NOD2 activation pathway (Figure 8). Thus, it seems as if nucleotide binding is necessary for NOD2-RIP2K interaction and downstream signaling.

Interestingly, the interaction between ATG16L and NOD2 following mutation of the NOD2 Walker A motif did not appear to follow our prediction that disruption of NOD2 nucleotide binding would decrease the interaction between NOD2 and its downstream binding partners. Through co-immunoprecipitation, it was observed that NOD2-ATG16L binding was increased in Walker A mutants as compared to their corresponding non-Walker A constructs. While this result was not expected, there are examples of NOD2 regulating proteins that do demonstrate binding to inactive NOD2 protein. Two such proteins, heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) both play important roles in the modulation of NOD2 signaling. For example,
inactive NOD2 is hypothesized to be constitutively bound to HSP90. Following MDP exposure, NOD2 is predicted to undergo a conformational change that results in its dissociation from HSP90, and allows for NOD2 activation.\textsuperscript{54} Upon its activation, NOD2 can then initiate NF-κB and MAPK signaling through its association with RIP2K, and promote transcriptional changes that mediate pathogen clearance. Similarly to HSP90, the chaperone protein, heat shock protein 70 (HSP70), was recently found to bind to NOD2 independent of MDP.\textsuperscript{56} Interestingly, it was shown that over-expression of HSP70 further activated NOD2-mediated NF-κB signaling in the presence of MDP, and that the half-life of NOD2 was dramatically increased in the presence of HSP70 (and dramatically decreased in its absence) suggesting that HSP70 positively regulates NOD2 signaling through stabilization of inactive NOD2. Thus, binding to these two proteins may serve to modulate NOD2 activity through stabilization of inactive NOD2.

The results of our NOD2-ATG16L co-immunoprecipitation experiment may suggest that ATG16L can bind NOD2 in a manner similar to these chaperone proteins. For example, as is observed with HSP90 and HSP70-NOD2 interaction, it is possible that NOD2 and ATG16L are constitutively bound in the absence of MDP (when NOD2 is in its inactive state). In a manner similar to HSP70-NOD2 interaction, ATG16L and NOD2 could remain bound following MDP recognition and NOD2 activation. As active NOD2 is thought to undergo re-distribution from the cytosol to the plasma and endosomal membranes\textsuperscript{27,62,63}, this re-distribution event could then lead to NOD2-mediated trafficking of ATG16L to the plasma membrane, where NOD2 and ATG16L would then dissociate following ATG16L binding to ATG12-ATG5 heterodimers. NOD2 mutants that are unable to become properly activated are presumably unable to traffic to the
plasma membrane, which has been observed in the signaling deficient NOD2 Δ32 mutant. Based on the observation of decreased NOD2-RIP2K binding and decreased NF-κB signaling following mutation of the NOD2 Walker A motif, it is reasonable to conclude that these Walker A mutants are signaling deficient. As such, it is likely that these mutants not undergo redistribution to the plasma membrane. Without the ability to traffic to the plasma membrane whereby ATG16L could be released, these mutants could remain bound to ATG16L, even in the presence of MDP. While the role NOD2 plays in the promotion of autophagy is still not well characterized, this type of mechanism would serve to regulate NOD2-mediated autophagy such that induction of autophagosome formation would only result following NOD2 activation and membrane redistribution.

Conversely, if membrane redistribution following NOD2 activation is required to promote dissociation of ATG16L and induction of autophagy signaling, hyperactive NOD2 mutants such as NOD2 R334W (which are known to undergo increased redistribution to the plasma membrane) could promote increased autophagy signaling. While recent work has linked deficiency in autophagy with susceptibility to the development of inflammation-associated diseases such as diabetes, Crohn’s Disease, and cystic fibrosis, no explicit association with increased autophagy signaling and disease susceptibility has been found to date. However, given the importance of autophagy in the immune response, it is not unreasonable to postulate that increased autophagy signaling promoted by the constitutive activation of hyperactive NOD2 mutants may lead to dysregulation of immune signaling, and more specifically, increased dissociation of NOD2 and ATG16L due to increased NOD2 plasma membrane trafficking and release of ATG16L.
To more explicitly test the hypothesis that interaction between NOD2-ATG16L is dependent on NOD2 activation state, we could use membrane-localization as a proxy for the activation state of NOD2 and engineer a NOD2 construct that preferentially localizes to the plasma membrane (ie. through myristoylation of NOD2). We could then assess NOD2-ATG16L interaction through co-immunoprecipitation and co-localization studies of NOD2 and ATG16L. Additionally, to investigate the induction of autophagy signaling, we could attempt to quantify the amount of lipid conjugated LC3, to serve as a proxy for autophagosome formation. Alternatively, we could examine autophagy activity by assessing pathogen clearance in cells containing this membrane-linked NOD2, following infection.

Despite the role that NOD2 activation may play in the induction of autophagosome formation and autophagy signaling, our results suggest that nucleotide binding is not necessary for NOD2-ATG16L interaction. These results contradict those observed for NOD2-RIP2K binding and downstream signaling. Additionally, the observation that mutation of the Walker A motif does not abrogate NOD2-ATG16L interaction suggests that our current model for NOD2 activation may not be accurate for the interaction between NOD2 and ATG16L, and that nucleotide binding may be required for interactions with some proteins (RIP2K), but not others (ATG16L).

Finally, it was observed that mutation of the NOD2 Walker A motif did not seem to decrease homo-oligomerization between wild type NOD2 and each NOD2 construct. These results contradict our prediction that disruption of nucleotide binding would disrupt NOD2 homo-oligomerization, which is predicted to follow NOD2 nucleotide exchange by the current model for NOD2 activation (Figure 8). Through our
observations, it seems as if NOD2 homo-oligomerization between wild type isotypic NOD2 mutants is not dependent on nucleotide binding, suggesting that NOD2 homo-oligomerization may lie upstream of nucleotide binding in the NOD2 activation pathway.

Additional co-immunoprecipitation experiments examined the interaction between each NOD2 protein and itself, and found that each NOD2 protein was able to self-associate independent of its ability to bind nucleotides, further suggesting that NOD2 homo-oligomerization may lie upstream of nucleotide binding in NOD2 activation. Interestingly, it has been observed that compound heterozygous NOD2 mutations and homozygous mutant NOD2 genotypes occur more frequently in Crohn’s Disease patients than would be expected. Additionally, it has been observed that homozygosity for NOD2 CD mutations increases susceptibility to CD development at a disproportionally large rate as compared to susceptibility for heterozygotes. Our results do not seem to indicate any difference in NOD2 homo-oligomerization of wild type NOD2 with NOD2 variants in comparison to these NOD2 variants with themselves. While this observation may be due to the fact that these NOD2 variants were highly over-expressed to perform these co-immunoprecipitation studies, it may also mean that this homozygosity effect comes into play further downstream in the NOD2 activation pathway. For example, heterozygous NOD2 alleles may be able to homo-oligomerize at comparable levels to homozygous alleles, however the ability of homozygous alleles to hetero-oligomerize with downstream effector proteins may be significantly impaired as compared to heterozygous alleles.

Overall, the results of this study suggest that nucleotide binding does play a critical role in the activation and downstream interaction between NOD2 and RIP2K.
Additionally, we demonstrated that disruption in nucleotide binding decreases NOD2-mediated NF-κB activity. This nucleotide binding dependent decrease in signaling supports the hypothesis that NOD2 uses an ATP binding cycle to mediate its activation state. While our work does not necessarily elucidate the specific sequence of structural and molecular events that lead to NOD2 activation, this study does confirm the prediction that nucleotide binding plays a role in this process early on, specifically in regards to the initiation of downstream interactions between NOD2 and RIP2K as well as the activation of the NF-κB signaling cascade, which is dependent on NOD2-RIP2K interaction. However, our results do call into question the timing of this nucleotide-binding step in relation to NOD2 homo-oligomerization. Further work will be necessary to elucidate the cellular events that govern this nucleotide-binding cycle, and how they may be altered in NOD2-associated pathologies.

Specifically, our results do not seem to be entirely consistent with our current model for NOD2 activation. This model predicts that NOD2 exists basally in an autoinhibited, ADP-bound monomeric state that is released from autoinhibition following ADP-ATP exchange, allowing NOD2 monomers to homo-oligomerize. This homo-oligomerization event is predicted to stabilize this open NOD2 conformation, which then allows NOD2 to bind up its ligand, MDP. This step would then facilitate hetero-oligomerization between itself and downstream signaling molecules, such as RIP2K and ATG16L—eliciting the formation of higher-order oligomers (termed the NODosome), which initiates NOD2-mediated signaling (Figure 8). From this constructive model for NOD2 activation, we predicted that NOD2 nucleotide binding would lie upstream of any homo- or hetero-oligomerization steps, and that disruption in nucleotide binding would
disrupt both NOD2 homo- and hetero-oligomerization. This is not what we observed experimentally, however—suggesting the necessity for revision of this model.

For example, it is possible that rather than beginning the activation process as autoinhibited ADP-bound monomers, NOD2 begins this process as inactive, homo-oligomers that are able to bind negative regulators such as HSP90 and HSP70 (and perhaps, ATG16L). Following a nucleotide exchange step, these homo-oligomers could dissociate, forming NOD2 monomers that bind up and are stabilized by MDP. These stabilized NOD2-monomers could then hetero-oligomerize with signaling effector proteins such as RIP2K, which could then lead to the formation of NODosome complexes, and the induction of downstream signaling. In this model, NOD2 homo-oligomerization events and specific hetero-oligomerization events would lie upstream of nucleotide binding and exchange in the NOD2 activation pathway, which would be more consistent with our observed results (Figure 19).

Our results suggest a key role for the proper function of the Walker A motif and nucleotide binding in the NOD2 activation process. This observation leads to the question: Would changes in the NOD2 nucleotide binding state also govern NOD2 inactivation? Along these lines, recent work by Monie and colleagues hypothesizes that NOD2 ATP hydrolysis may be an essential step in the process of NOD2 inactivation. In this work, Monie and colleagues hypothesize that the NOD2 R334W mutation and other hyperactive NOD2 mutations map to the ATP/Mg$^{2+}$ binding patch of the NOD2 nucleotide-binding domain. They suggest that mutations of the amino acid residues in this region may affect NOD2 hydrolytic activity, and that this disruption in nucleotide-hydrolysis traps NOD2 in the constitutively active state that is observed for these BS-
associated mutants. Thus, they propose that NOD2 nucleotide hydrolysis serves as a mechanism to inactivate NOD2, which would explain why disruption of this activity would result in a constitutively active protein.\textsuperscript{41} Mutations in other NLR proteins that also confer a hyperactive phenotype and disease-susceptibility (such as in NLRP3)\textsuperscript{64} have also been predicted to map to this same region of the nucleotide-binding domain, and thus inhibit proper nucleotide hydrolysis.\textsuperscript{41} These observations further support the hypothesis that nucleotide-hydrolysis may be essential to the inactivation of NOD2, and NLRs in general. Due to the potential relevance to the R334W NOD2 phenotype, and the association of this phenotype with the development of Blau Syndrome, it would be valuable to examine the hypothesis that proper nucleotide hydrolysis is necessary for the inactivation of NOD2. To do this, a similar approach to our methodology in this study could be taken. Namely, the Walker B motif of the NOD2 nucleotide-binding domain (which is known to regulate nucleotide hydrolysis) could be mutated such that it should impair NOD2 ATPase activity. Following this, NOD2 signaling activity could be assayed. Based on the observed activity of R334W mutants, as well as the results of Monie and colleagues, we would predict that mutation of the Walker B motif would result in a constitutively active NOD2 due to its inability to become inactivated.

Here, we demonstrate that NOD2 nucleotide binding is necessary for the interaction between NOD2 and RIP2K as well as for the activation of NOD2-mediated NF-κB activity. These results were consistent with our current model for NOD2 activation. However, the interaction between NOD2 and ATG16L as well as between NOD2 and itself (in a homo-oligomeric fashion) did not seem to follow the same pattern as NOD2-RIP2K interactions following disruption of NOD2 nucleotide binding,
suggesting that nucleotide binding may not be necessary for NOD2-ATG16L interaction or for NOD2 homo-oligomerization. These results are not consistent with our current model for NOD2 activation, and may suggest that rather than following a constructive scheme, NOD2 activation may be a destructive mechanism whereby NOD2 homo-oligomers function as the inactive, signaling-incompetent NOD2 molecules. Overall, our study is the one of the first to examine the hypothesized importance of nucleotide binding on NOD2 activation, and we have demonstrated that it does, indeed, play an important role in the proper induction of NOD2-mediated NF-κB downstream signaling.
**Figure 1. NOD2 is a NLR family member.** NOD2 contains the conserved tripartite domain structure of the NLR family—a N-terminal effector domain, central nucleotide binding domain, and a series of C-terminal leucine-rich repeats. Specifically, NOD2 is a member of the NLRC proteins, which all contain N-terminal caspase activation and recruitment domain (CARD) domains (adapted from: Zhang, Y., et al. 2013. *Frontiers in Immunology*: 4(333): 10.3389/fimmu.2013.00333.).
Figure 2. STAND ATPases have a conserved domain structure containing nucleotide binding and nucleotide hydrolyzing motifs. A) STAND ATPases have a conserved nucleotide binding and oligomerization domain (NOD) domain, a sensor domain, and an effector domain. B) The STAND NOD domain structure, including the conserved Walker A and Walker B motifs (adapted from: Danot, O., et al. 2009. Structure. 17: 172-182.).
Figure 3. NOD2 recognizes MDP, a constituent of both gram-positive and gram-negative bacterial cell walls. A) Both gram-positive and gram-negative cell walls contain peptidoglycan (PGN). B) MDP is a constituent of PGN. C) Chemical structure of active L-MDP stereoisomer.
Figure 4. NOD2 activates NF-κB signaling through its association with RIP2K. Active NOD2 hetero-oligomerizes with RIP2K, promoting RIP2K auto-phosphorylation and K63 ubiquitination, which activate RIP2K. Active RIP2K then stimulates further activation of NF-κB signaling, which eventually results in the degradation of the negative regulatory subunit of the IKK complex, translocation of NF-κB and the transcription of cytokines, chemokines and cell adhesion molecules that initiate an immune response (adapted from: Damgaard, R.B. 2011. Discovery Medicine. 60.)
Figure 5. NOD2 stimulates autophagy through its association with ATG16L.
Active NOD2 recruits ATG16L to the plasma membrane whereby ATG16L can associate with the ATG5-ATG12 complex. This newly formed complex then stimulates the formation of a double membrane autophagosome which will eventually fuse with a lysosome to degrade the contents enclosed within the autophagosome (adapted from: Shin, J.N. et al. 2010. *Immunology and Cell Biology.* 88: 343–345).
Figure 6. Relative expression of NOD2 in various tissues. Expression levels in different tissue types were compared to median NOD2 expression across all tissues. Data were provided by NextBio Body Atlas.
Figure 7. NOD2 1007fsinsC (Δ32) is a truncated NOD2 protein. A) An insertion of a cysteine in exon 11 of NOD2 results in a premature stop codon at amino acid 1007. B) The premature stop codon in NOD2 Δ32 results in the premature truncation of this protein and the deletion of the final 33 amino acids in the NOD2 LRR (adapted from: Ogura, Y. et al. 2001. *Nature*. 411: 603-606.).
Figure 8. Model for NOD2 activation. NOD2 exists basally in a monomeric, autoinhibited ADP-bound form. Following nucleotide exchange, NOD2 will undergo a conformational change that allows the protein to adopt an “open” conformation, and to associate with other NOD2 open monomers to stabilize this conformation. NOD2 can then directly bind to MDP, further stabilizing NOD2 homo-oligomers, and allowing NOD2 to hetero-oligomerize with effector proteins such as RIP2K or ATG16L, which initiate signal transduction and result in the production of an immune response.
Figure 9. Structure of NOD2 and locations of NOD2 mutations. The Walker A mutation is displayed at amino acids 304, 305 and 306. The R334W mutation is displayed at amino acid 334. The Δ32 mutation is displayed at amino acid 1007. Sequence comparisons between wild type and the Walker A mutations and between the wild type, R334W and Δ32 mutations are shown. The Δ32 mutation is depicted in reverse complement form.
Figure 10. NOD2-RIP2K interaction is reduced in wild type NOD2 Walker A (WA) mutants. A) HEK293T cells were co-transfected with either wild type NOD2-V5 or NOD2 WA-V5 and RIP2K-FLAG. Following lysis, RIP2K was immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP. B) Results of replicate co-immunoprecipitation experiment with HEK293T cells co-transfected with NOD2-V5 and RIP2K-FLAG.
Figure 11. NOD2-RIP2K interaction is reduced in NOD2 R334W Walker A (WA) mutants. A) HEK293T cells were co-transfected with either R334W NOD2-V5 or R334W WA-V5 and RIP2K-FLAG. Following lysis, RIP2K was immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP. B) Results of replicate co-immunoprecipitation experiment with HEK293T cells co-transfected with NOD2-V5 and RIP2K-FLAG.
Figure 12. NOD2-RIP2K interaction is reduced in Δ32 NOD2 Walker A (WA) mutants. A) HEK293T cells were co-transfected with either NOD2 Δ32-V5 or NOD2 Δ32 WA-V5 and RIP2K-FLAG. Following lysis, RIP2K was immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP. B) Results of replicate co-immunoprecipitation experiment with HEK293T cells co-transfected with NOD2-V5 and RIP2K-FLAG.
Figure 11. NOD2 WA mutants show a decreased activation of NF-κB signaling. A) HEK 293T cells were transfected with 25 ng of wild type or WA NOD2. B) HEK293T cells were transfected with 25 ng of CIITA or p65. C) HEK 293T cells were transfected with 25 ng of wild type or WA NOD2 and stimulated with MDP. D) HEK293T cells were transfected with 25 ng of CIITA or p65 and stimulated with MDP. For all cells, NF-κB activity was investigated by a Dual-Luciferase® Reporter Assay system (Promega); (**) = p<0.001.
Figure 14. NOD2 R334W WA mutants show a decreased activation of NF-κB signaling. A) HEK 293T cells were transfected with 25 ng of NOD2 R334W or NOD2 R334W WA. B) HEK293T cells were transfected with 25 ng of CIITA or p65. C) HEK 293T cells were transfected with 25 ng of NOD2 R334W or NOD2 R334W WA and stimulated with MDP. D) HEK293T cells were transfected with 25 ng of CIITA or p65 and stimulated with MDP. For all cells, NF-κB activity was investigated by a Dual-Luciferase® Reporter Assay system (Promega); (**= p<0.001).
Figure 17. NOD2 Δ32 WA mutants show a decreased activation of NF-κB signaling. A) HEK 293T cells were transfected with 25 ng of NOD2 Δ32 or NOD2 Δ32 WA. B) HEK293T cells were transfected with 25 ng of CIITA or p65. C) HEK293T cells were transfected with 25 ng of NOD2 Δ32 or NOD2 Δ32 WA and stimulated with MDP. D) HEK293T cells were transfected with 25 ng of CIITA or p65 and stimulated with MDP. For all cells, NF-κB activity was investigated by a Dual-Luciferase® Reporter Assay system (Promega); (**= p<0.001).
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**Figure 12.** Wild type NOD2-ATG16L interaction and homo-oligomerization is not dependent on nucleotide binding. A) HEK293T cells were co-transfected with either wild type NOD2-V5 or NOD2 WA-V5 and ATG16L-FLAG. B) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with either wild type NOD2-V5 or NOD2 WA-V5 and wild type NOD2-FLAG. C) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with wild type NOD2-V5 and wild type NOD2-FLAG and HEK 293T cells co-transfected with NOD2 WA-V5 and NOD2 WA-FLAG. Following lysis, FLAG-tagged constructs were immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP.
Figure 15. NOD2 R334W-ATG16L interaction and homo-oligomerization is not dependent on nucleotide binding. A) HEK293T cells were co-transfected with either NOD2 R334W-V5 or R334W WA-V5 and ATG16L-FLAG. B) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with either NOD2 R334W-V5 or R334W WA-V5 and wild type NOD2-FLAG. C) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with NOD2 R334W-V5 and R334W-FLAG and HEK293T cells co-transfected with R334W WA-V5 and R334W WA-FLAG. Following lysis, FLAG-tagged constructs were immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP.
**Figure 18. NOD2 Δ32-ATG16L interaction and homo-oligomerization is not dependent on nucleotide binding.** A) HEK293T cells were co-transfected with either NOD2 Δ32-V5 or Δ32 WA-V5 and ATG16L-FLAG. B) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with either NOD2 Δ32-V5 or Δ32 WA-V5 and wild type NOD2-FLAG. C) Results of co-immunoprecipitation experiment with HEK293T cells co-transfected with NOD2 Δ32-V5 and Δ32-FLAG and HEK293T cells co-transfected with Δ32 WA-V5 and Δ32 WA-FLAG. Following lysis, FLAG-tagged constructs were immunoprecipitated with agarose-conjugated anti-FLAG M2 beads. Co-immunoprecipitates and total cell lysates were subjected to SDS-PAGE and blots were then probed with anti-V5-HRP and anti-FLAG M2-HRP.
Figure 19. Constructive vs. destructive model for NOD2 activation. This destructive model for NOD2 activation may initiate NOD2 signaling through the destruction of NOD2 homooligomers which can hetero-oligomerize following nucleotide exchange.
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44. Ogura Y. *et al.* 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB. *Journal of Biological Chemistry.* 276: 2551-2554.


