Modulation of Innate Immunity by Nucleotide Binding
-Biochemical and Functional Characterization of a CATERPILLER/NLR Protein,
Monarch-1/NLRP12

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ABSTRACT
Zhengmao Ye: Modulation of Innate Immunity by Nucleotide Binding
-Biochemical and Functional Characterization of a CATERPILLER/NLR Protein,
Monarch-1/NLRP12
(Under the direction of Dr. Jenny P.-Y. Ting)

The recently discovered Nucleotide Binding Domain-Leucine Rich Repeat (NLR) gene family is conserved from plants to mammals and several members are associated with human autoinflammatory or immunodeficiency disorders. This family is defined by a central nucleotide binding domain that contains the highly conserved Walker A and Walker B motifs. Although the nucleotide binding domain is a defining feature of this family, it has not been extensively studied in its purified form. In this thesis, we show that purified Monarch-1/NLRP12, an NLR protein that negatively regulates NF-κB signaling, specifically binds ATP and exhibits ATP hydrolysis activity. Intact Walker A/B motifs are required for this activity. These motifs are also required for Monarch-1 to undergo self-oligomerization, TLR- or CD40L- activated association with NIK and IRAK-1, degradation of NIK, and inhibition of IRAK-1 phosphorylation. Stable expression of a Walker A/B mutant in THP-1 monocytes results in increased production of proinflammatory cytokines and chemokines to an extent comparable to cells in which Monarch-1 is silenced via shRNA. In addition, the functional role of conserved motifs in Monarch-1 NBD domain is examined. The results of this study are consistent with a model wherein ATP binding regulates the anti-inflammatory activity of Monarch-1.
To my Grand parents

To my mom and my dad

To my wife Dongmei

To my daughter Katelyn
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>ATPases associated with various cellular activities</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>avr</td>
<td>avirulence</td>
</tr>
<tr>
<td>BAC</td>
<td>bacteria artificial chromosome</td>
</tr>
<tr>
<td>BIR</td>
<td>baculovirus IAP repeat</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC class II transactivator</td>
</tr>
<tr>
<td>CINCA</td>
<td>chronic infantile neurological cutaneous and articular syndrome</td>
</tr>
<tr>
<td>CPPD</td>
<td>calcium pyrophosphate dihydrate</td>
</tr>
<tr>
<td>dADP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence Activated Cell Sorter</td>
</tr>
<tr>
<td>FCAS</td>
<td>familia cold autoinflammatory</td>
</tr>
<tr>
<td>FMF</td>
<td>familial Mediterranean fever</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HM</td>
<td>hydatidiform mole</td>
</tr>
<tr>
<td>HR</td>
<td>hypersensitive response</td>
</tr>
<tr>
<td>iE-DAP</td>
<td>g-D-glutamyl-meso-diaminopimelic acid</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>The interleukin-1 (IL-1) receptor-associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal kinase</td>
</tr>
<tr>
<td>KDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>LT</td>
<td>lethal toxin</td>
</tr>
<tr>
<td>LTβ</td>
<td>lymphotoxin-β</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>MSU</td>
<td>monosodium urate</td>
</tr>
<tr>
<td>MWS</td>
<td>Mukcle-Wells syndrome</td>
</tr>
<tr>
<td>NACHT</td>
<td>NAIP,CIITA,HER-E,TP-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NAD</td>
<td>NACHT associated domain</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucelotide binding domain</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide binding domain-leucine rich repeat</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOMID</td>
<td>Neonatal-onset multisystem inflammatory disease</td>
</tr>
<tr>
<td>Pam3Cys4</td>
<td>S-[2,3-bis-(palmitoyloxy)-2(2-RS)-propyl]-N-palmitoyl-(R)-cys-(s)-Ser-Lys-4-OH tryhydrochloride</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>ShRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded ribonucleic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTSS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Chapter 1  INTRODUCTION
1.1 The Introduction of NLR protein family

Vertebrates rely on a robust immune system to combat pathogen invasion. The immune response of jawed vertebrate is comprised of two arms: the adaptive and the innate immune response. The adaptive immune response is mainly conducted by T and B cells that utilize somatically rearranged receptors to specifically recognize antigen. Upon activation, T and B cells undergo clonal expansion to achieve effective immune protection. In the primary adaptive immune response, the activation, expansion and differentiation of T and B cells often require several days, during which the fast acting innate immune system exerts its critical role for the eradication or at least the containment of the invading pathogen. The innate immune system is mainly mediated by macrophages, mast cells, neutrophils, and natural killer cells, which do not possess somatically rearranged receptors. Then, how does the innate immune system recognize invading pathogens and distinguish them from our own body? It has been predicted by Charles Janeway nearly 18 years ago that the innate immune system must bear a battery of germline encoded receptors, which he termed pathogen-recognition receptors (PRRs), to specifically detect certain invariant pathogen derived products, which he termed pathogen-associated molecular pattern (PAMP) (1). In addition, Matzinger also predicted that the invading pathogen could induce certain perturbation of normal physiological condition. This perturbation servers as the ‘danger signal’ that alert the innate immune system (2).

In 1997, the discovery and characterization of the first mammalian Toll-like receptor (TLR): TLR4 (3), significantly advanced our knowledge of the innate immune system. Toll-like receptors are germline encoded type I transmembrane glycoproteins that specifically detect PAMPs. So far, there are 10 and 11 TLRs identified in humans and mice,
respectively. Each TLR recognizes various PAMPs derived from bacteria, viruses, fungi, and/or protozoa: TLR4 recognizes lipopolysaccharide (LPS), a major component of Gram negative bacteria cell wall; TLR2 heterodimerizes with TLR1 to sense bacterial triacylated lipopeptides; TLR2 also forms heterodimer with TLR6 to detect bacteria diacylated lipopeptides. Other TLRs can respond to pathogen-derived nucleic acid. TLR3 detects viral derived double-stranded RNA (dsRNA); TLR7 and 8 sense viral single-stranded RNA (ssRNA) and TLR9 recognizes unmethylated CpG DNA. TLRs also detect pathogen-derived proteins. TLR5 recognizes bacteria flagellin and murine TLR11 recognizes a profilin-like protein from *Toxoplasma gondii* (4). In addition to the detection of pathogen derived products, TLR2 and TLR4 have been demonstrated to sense fragments of the extracellular matrix component hyaluronan, highlighting the role of TLR in recognizing self-body derived danger signal (5).

All TLR contains a C-terminal extracellular leucine-rich repeats (LRR) domain, a transmembrane domain, and a cytosolic TIR (Toll/IL-1β Receptor) domain. The LRR domain is responsible for the pathogen detection. Upon receiving stimulations from their cognate ligands, TLRs transmit the signals via a myriad of intracellular adaptors including MyD88, MAL (MyD88-adaptor-like, also know as TIRAP), TRIF (TIR-domain-containing adaptor protein inducing IFN-β, also known as TICAM1), TRAM (TRIF-related adaptor molecule, also known as TICAM2) and SARM (armadillo-motif-containing protein), to activate the nuclear factor-κB (NF-κB), mitogen-activated protein Kinase (MAP Kinase), and interferon(IFN) signaling pathways. The activation of those pathways results in the secretion of proinflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-8, and type I IFNs and the upregulation of costimulatory molecules,
which facilitate the activation of the adaptive immune system. The overall effect of TLR signaling not only controls the pathogen spreading but also serves as a key bridge between the innate and the adaptive immune response. TLRs mainly reside on the cell surface. Some TLRs localize in the endosomal compartment. However, many bacteria and all viruses are cytosolic pathogen. Thus, it was postulated that there must be cytosolic molecules that are able to sense the intracellular PAMPs or danger signals.

During the search for the MHC class II transactivator (CIITA) homologs in the human genome, our group first discovered a novel protein family, which is now thought to mainly mediate and regulate intracellular inflammatory response. We christened this protein as CATERPILLER (CARD (caspase recruitment domain), transcription enhancer, purine binding, pyrin, lots of leucine repeats) gene family (6-8). Subsequently, another group identified a similar class of genes called NOD (nucleotide oligomerization domain) (9) or NOD-LRR (10), while others reported a subset of the family called PYP AF (11), PAN (12), and NALP (NACHT leucine-rich repeat and PYD containing protein) (13) according to their own specifications. Currently, according to the recommendation of human Genome Organization (HUGO), the CATERPILLER gene family along with other similar gene families are unified as NLR (nucleotide-binding domain, leucine-rich repeats containing) family. (See table 1 and http://www.genenames.org/genefamily/nacht.html). This nomenclature is recommended to be used by the scientific community in the future publications. Because this nomenclature has not been formally announced, I will still use the most common gene names in this thesis, but use the proposed nomenclature for genes that are not well characterized.
The NLR proteins are defined by their tripartite domain architecture that contains a nucleotide binding-domain (NBD also known as NACHT (NAIP, CIITA, HER-E, TP-1) domain) (14), at the center of the protein. The NBD domain has been implicated to mediate self-oligomerization and other regulatory functions. At their C-terminal, most NLRs contain LRR domain that are variable in the repeat composition and number. The LRR domain has been implicated in mediating autoregulation, protein-protein interaction and ligand detection. For example, the LRR of NOD1 and NOD2 are thought to sense peptidoglycan, the breakdown product of bacteria cell wall component. The N-terminal domain of NLRs varies within the NLR protein and is the basis for the definition of NLR subgroups. The largest subgroup (14 members) contains an N-terminal pyrin domain. The rest of NLR subfamilies contain a CARD, BIR (baculoviral inhibitory repeat), transactivator domain or uncharacterized domains. The N-terminal domain has been demonstrated to recruit downstream effector molecules, therefore also known as effector domain.

To date, no crystal structure of a full length NLR protein is reported. Nevertheless, the pyrin domains of NALP1 and ASC are elucidated by nuclear magnetic resonance (NMR) analysis (15, 16). The crystal structure of NOD1 CARD domain has also been depicted recently (17). Pyrin and CARD domains belong to the death domain superfamily, hallmarked by the presence of the six-helical bundle fold structure. The pyrin domain from NALP1 and ASC both obtain a classical six-helical bundle fold but with a unique H3 helix (15, 16). The LRR domain has been proposed to mediate ligand sensing. So far, direct evidence demonstrating the binding of the NLR LRR to its cognate ligand is lacking. However, the crystal structure of LRR domain of TLR may shed some light on this issue.
The crystal structure of the ectodomain of TLR3 displays a horseshoe-shaped array of LRRs that is formed by the interconnected β-strand of each LRR facing the concave side and α-helix of each repeats forming a out-facing convex side (18). The binding pocket for the dsRNA on TLR3 is postulated to be located at the convex side of LRR. This binding activates the homodimerization of TLR3. The exciting breakthrough in understanding the role of LRR in PAMP detection was reported by two groups. They showed the crystal structure of TLR4 with its cognate ligand LPS (19) and TLR1/2 heterodimer with its synthetic ligand Pam3Cys4 (20). Although the overall shapes of LRR domain of TLR4 and TLR1/2 heterodimer are generally similar, the ligand binding mechanisms are dramatically different. While the TLR1/2 synthetic ligand Pam3Cys4 directly binds to the convex side of TLR1/2 LRR domain, the TLR4 agonist LPS binds to the concave side of TLR4 LRR indirectly through an intermediate protein MD2. For the first time, these studies not only provide us the direct evidence of receptor-ligand binding of TLR but also elucidate the potential binding mechanisms. In the light of these findings, the resolution of structure of NLR proteins with their interacting partners would be the next breakthrough.

NLRs are highly conserved through evolution with orthologs found throughout vertebrates. In contrast to the evolution of TLR, exhaustive database searches of Drosophila and Caenorhabditis elegans genomes do not yield significant hits with typical NLR tripartite domain structure. Interestingly, a recent database search in Sea Urchin genome yields 203 putative NLRs in contrast with 22 members in humans (21, 22). This result indicates that the size of NLR pool might reflect the different selective pressures maintained on each population.
NLRs are structurally and functionally related to the plant disease resistant protein (R protein) family that confers major anti-microbial response in plants. Plants have a large family of R proteins. For example, Arabidopsis has 140 R proteins (23), rice has over 500 R proteins (24). Structurally similar to NLR protein, plant R proteins contain a tripartite domain structure that consists of a C-terminal LRR, a central NBS (Nucleotide binding site) domain and an N-terminal domain that contains either a TIR or a coil-coil domain. R proteins sense and respond to pathogen-derived molecules that are encoded by pathogen \textit{avr} (avirulence) genes. However, in contrast to certain TLRs, in most cases, R protein mainly respond to the stress or the perturbation that is caused by the intrusion of pathogen-derived effector on a ‘one-for-one’ fashion, instead of direct recognizing the pathogen-derived molecules. This theory is called the "Guard Hypothesis" (25). Upon activation by their cognate ligands, R proteins elicit swift biological responses that lead to the localized cell death and confinement of pathogen spreading. The R protein mediated protective response is often called Hypersensitive Response (HR). Interestingly, two recent reports highlight that R protein mediated anti-pathogen response and mammalian NLR mediated inflammatory response might share certain common grounds. To exert its function, plant R protein interacts with heat-shock protein 90 (HSP90) and an ubiquitin ligase-associated protein SGT1. Similar to plant R protein, Mayor et al reported that many NLRs such as cryopyrin interact with SGT1 and HSP90. The interactions between cryopyrin, SGT1 and HSP90 are required for cryopyrin mediated inflammasome activation (26). In another study, Correia et al demonstrated that NLR protein NOD1 associates with SGT1 and HSP90. This interaction is essential for the NOD1 activation (27). Very recently, Arthur et
al also demonstrated that Monarch-1 interacts with HSP90 and this interaction is important for Monarch-1 induced NF-κB inducing kinase degradation (28).

The importance of NLR proteins in the regulation of the innate immune response is manifested by its association with a variety of inherited immunologic disorders. CIITA is the funding member of CATERPILLER family. Mutations in this gene result in the loss or reduction of MHC class II expression that causes type II Bare lymphocyte Syndrome (BLS); Mutations in CIAS1 gene, which encodes protein cryopyrin, cause a trio of autoinflammatory diseases (29): FCAS (familia cold autoinflammatory syndrome), MWS (muckle-wells syndrome), and NOMID/CINCA (neonatal onset multisystemic autoinflammatory disease/chronic infantile neurological cutaneous and articular syndrome); NOD2 mutations are associated with Crohn's disease (30, 31) and Blau syndrome (32). Crohn’s disease is one of two forms of inflammatory bowel disease and Blau syndrome is a rare autosomal inflammatory disease characterized by fever, arthritis, uveitis, and dermatitis. Moreover, mutations in NAIP gene are thought to be associated with spinal muscular atrophy (33), although more recent evidence suggests otherwise. Nonetheless, NAIP is important for the recognition of Legionella flagellin (34). Finally, although not a member of the NLR family, the mutations in the gene Pyrin, which shares the pyrin domain with NLRP subfamily, are associated with familial Mediterranean fever (FMF) (11).

NLRs display diverse expression patterns. As expected, many NLRs are expressed within the innate immune system. For example, human cryopyrin is expressed mainly in monocytes/macrophages, although the expression pattern in mouse extends to chondrocytes and lymphocytes. Some NLRs are more ubiquitously expressed. Interestingly,
NALP5/NALP5/MATER presents an oocyte restricted expression pattern. Mouse Nlrp5 has been demonstrated to play an important role in embryonic development (35). Furthermore, the maternal mutation of human NLRP7 has been recently linked to hydatidiform mole (HM) further implicating that certain NLRs might play important roles in the reproduction systems (36).

1.2 The role of NLR in the control of IL-1β secretion

IL-1β, also known as the endogenous pyrogen, is a major proinflammatory cytokine that mediates host responses to microbial infection and tissue injury. Once secreted, it exerts multiple biological activities on host cells bearing its receptor. IL-1β has been shown to induce a plethora of systemic and immunological responses including fever, rashes, peripheral neutrophil accumulation, increased cytokine production (IL-2,-3,-4,-5,-6,-7,-10,-12), elevated antibody production and enhanced infiltration of immune effector cells into inflammatory sites, etc. Mice deficient in IL-1β are completely resistant to LPS-induced endotoxin shock. In humans, overproduction of IL-1β plays a critical role in sepsis. Along the same line, dysregulated secretion of IL-1β causes devastating systemic inflammatory disease such as SoJIA (also known as systemic onset juvenile idiopathic rheumatoid arthritis). Moreover, the symptoms of SoJIA patient can be rapidly and sustainably resolved by the administration of IL-1β receptor antagonist (IL-1Ra, anakinra) (37). This evidence highlights the critical role of IL-1β in systemic inflammatory response and the importance of tight regulation of its secretion.

The immune system imposes several regulatory mechanisms to prevent deleterious effects induced by the over-secretion of IL-1β. First, the transcription and translation of IL-
IL-1β are kept at extremely low levels and can only be drastically increased upon the cell receiving extracellular signals such as TLR signaling. Second, the IL-1β is synthesized and stored in the cytoplasm and secretory lysosomal vesicles are inactive precursors (proIL-1β, or IL-1βp35) that must be proteolytically cleaved into its active form (IL-1βp17). Third, the secretion of leaderless IL-1β is strictly controlled by a still poorly defined mechanism. Finally, a constitutively secreted naturally occurring IL-1β receptor antagonist (IL-1Ra) binds type I IL-1β receptor with an affinity higher than that of IL-1β. Among these regulatory steps, the most critical one is the process that converts proIL-1β to IL-1β. This process is mediated by an enzyme identified as caspase-1 (also known as IL-1β-converting enzyme (ICE)).

Caspase-1 is an aspartate-specific cysteine protease that consists of an N-terminal CARD domain and a C-terminal caspase domain. Caspase-1 has been demonstrated to play an vital role in the cleavage of proIL-1β and proIL-18. Recently, IL-33, a novel cytokine that is involved in generating a T helper type 2 cell response, has also been shown to be cleaved by caspase-1 (38). The evidence obtained in caspase-1 null mice definitely reveals the essential role of caspase-1 in the proIL-1β and proIL-18 processing. Namely, caspase-1 null mice have a defect in the maturation of proIL-1β and proIL-18 and are resistant to a lethal dose of endotoxin treatment. Furthermore, the caspase-1 activity is also regulated by numerous endogenous proteins, including COP (Pseudo-ICE) (39), DASC (POP1), ICEBERG (40), and human caspase-12. Caspase-1 itself exists in the cytosol as a monomeric zymogen (pro-caspase-1, p45). Upon certain stimulation, pro-caspase-1 undergoes dimerization and autocatalytic processing that forms an active caspase-1. Interestingly, in addition to its well established role in the IL-1β maturation, a recent study
reveals a novel function of caspase-1 by demonstrating that the caspase-1 activates the central regulators of membrane biogenesis: the Sterol Regulatory Element Binding Proteins (SREBPs) (41), which in turn promote cell survival upon toxin challenge possibly by facilitating membrane repair.

What are the external or internal signals that lead to the activation of caspase-1? How is caspase-1 activated? Towards answering the first question, there is evidence that LPS, a Gram-negative cell wall components and a major bacteria PAMP, can activate caspase-1 in phagocytes (42, 43). There are also extensive data to demonstrate that exposing cells to extracellular ATP in vitro leads to caspase-1 activation (44, 45). Extracellular ATP binds to P2X7 receptor, which is a membrane ion channel responsible for the potassium efflux (46). Thus, the extracellular ATP, released by membrane-compromised cells, might serve as a ‘danger signal’ to alert neighboring cells.

Towards answering the second question, the major breakthrough comes from a seminal biochemical study demonstrating that caspase-1 is activated by a multi-protein complex termed ‘inflammasome’ (modeled from Apoptosome). This complex is comprised of caspase-1, caspase-5, ASC (apoptosis-associated speck-like protein containing a CARD; also known as CARD5, Pycard, and TMS1), and NALP1/NLRP1 (47). Upon stimulation, NALP1 recruits adaptor protein ASC. The NALP1/ASC interaction initiates a self-oligomerization process that forms a five or seven-fold symmetry structure that allows the further recruitment of caspase-1 and caspase-5. The exact mechanism regarding how caspase-1 becomes activated within this protein complex is still unknown but a close proximity-induced activation has been proposed. This study first demonstrates the inflammasome as the major molecular platform for caspase-1 activation and further
implicates the existence of inflammasomes formed by other NLR proteins, especially for those that are expressed highly in monocytes/macrophages such as cryopyrin/NLRP3 and Ipaf/NLRC4. Indeed, the cryopyrin and Ipaf inflammasomes are all demonstrated recently. However, the identification and characterization of those NLR inflammasomes in intact cells remain to be demonstrated and should drastically advances our knowledge in understanding the regulation of IL-1β secretion.

1.2.1 The cyropyrin inflammasome

Cryopyrin is a 118 KDa NLR protein encoded by gene CIAS1. Cryopyrin is comprised of an N-terminal pyrin domain, a central nucleotide binding domain and a C-terminal LRR domain. In humans, mutations in CIAS1 are responsible for several autosomal dominant auto-inflammatory disorders. The initial overexpresison study indicated that human cryopyrin forms a inflammasome with ASC, Cardinal, and caspase-1 (48). A subsequent study has shown that the bacterial peptidoglycans (PGN) product muramyl diapeptide (MDP) induces cryopyrin mediated activation of caspase-1 and maturation of proIL-1β in human THP-1 monocytic cell line (49). However, the role of MDP in cryopyrin-mediated IL-1 production was not found under physiologic conditions when CIAS1−/− mice were studied.

The physiological role of cryopyrin in caspase-1 activation is not firmly defined until recently by several studies using genetic approaches. First, it was reported that cryopyrin-deficient and ASC-deficient macrophages fail to activate caspase-1 in response to the treatment of LPS plus ATP. This result indicates that cryoprin is vital in LPS plus ATP stimulated caspase-1 activation (50) (51). Extracellular ATP binds its receptor P2X7, a potassium channel, and induces its rapid opening. The activated P2X7 also recruits a
hemichannel, pannexin-1, that gradually forms a larger membrane pore upon activation. Indeed, pannexin-1 has been recently demonstrated to play an essential role in cryopyrin induced caspase-1 activation (52). Furthermore, nigericin, a bacterially derived potassium ionophore and maitotoxin, a shellfish toxin, which induces the formation of plasma membrane pore and rapid potassium efflux, can activate caspase-1 in macrophages from WT mice but not from cryopyrin deficient mice (50). Along the same line, bacteria pore-forming toxins such as *Staphylococcus* α-toxin and *Listeria* aerolysin have been demonstrated to activate caspase-1 in a cryopyrin dependent manner in human THP-1 cells (41). Collectively, these data firmly demonstrate that ATP or pathogen induced potassium efflux are major stimuli in cryopyrin mediated inflammasome formation and caspase-1 activation. Notably, it is initially thought that TLR signaling is critical for the ATP induced, cryopyrin dependent, caspase-1 activation due to the clear requirement of LPS. However, TLR4<sup>−/−</sup>, TLR2<sup>−/−</sup>, MyD88<sup>−/−</sup> and Ticam1<sup>−/−</sup> mice are all exhibit normal caspase-1 activity upon the treatment of heat killed bacteria and ATP (52). These data indicate that cryopyrin-dependent caspase-1 activation is independent of TLR signaling. However, TLR signaling might enhance the transcription of proIL-1β; therefore, priming the cells for ATP induced caspase-1 activation and IL-1β maturation.

At least two additional stimuli can lead to the cryopyrin mediated IL-1β secretion. Martinon et al. reported that gout and pseudogout-associated uric acid crystals, namely, monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD), activate caspase-1 and induce maturation of IL-1β and IL-18 in a cryopyrin and ASC dependent manner (53). Blocking ATP receptor P2X<sub>7</sub> has no effect on MSU and CPPD induced IL-1β secretion, suggesting this pathway acts independent of the ATP induced pathway.
Interestingly, colchicines, a tubulin polymerization inhibitor and a drug frequently used for the treatment of gout, completely blocks MSU induced maturation of IL-1β indicating that the activation of cryopyrin inflammasome might be mediated by microtube formation. Finally, cryopyrin inflammasome also responds to bacterial RNA and antiviral imidazoquinoline compounds R837 and R848, which are known TLR7 and TLR8 agonists (54). This R837 and R848 induced cryopyrin inflammasome activation is completely independent of TLR7 and TLR8 signaling. However, the role of ATP is dispensable. TLR ligands LPS and Pam3csk4 alone can activate cryopyrin inflammasome without the addition of ATP (54) whereas, other groups demonstrates ultrapure LPS fails to do so. This discrepancy may be due to the subtle difference in cell preparation and stimulation protocol.

Previously, MDP has been shown to activate caspase-1 through cryopyrin inflammasome in human monocytic cell line (49). However, all studies of mice macrophages fail to confirm this result. Thus, the role of MDP in cryopyrin-mediated caspase-1 activation remains to be seen. In addition, intracellular bacteria *Listeria monocytogenes* and *Staphylococcus aureus* induce cryopyrin dependent caspase-1 activation and IL-1β maturation whereas *Salmonella* and *Francisella* do not. These results highlight the specificity of cryopyrin inflammasome (50).

What is the physiological role of cryopyrin in *vivo*? In a lethal LPS induced endotoxin shock model, while all wt mice succumbed to death with 48 hr, only 30% of cryopyrin−/− mice died after 72 hr. Correlating with their enhanced survival, cryopyrin deficient mice had significant less serum IL-1β and IL-18 than the wild-type mice (50). This result is echoed in another report which showed that cryopyrin deficient mice and
ASC deficient mice are protected from lethal dose of LPS (51). Interestingly, cryopyrin mice only show enhanced survival in a low lethal dose of LPS treatment (9.38mg/Kg), however, ASC deficient mice are resistant to death even with high-end lethal dose of LPS (37.5mg/Kg) indicating adaptor ASC might participates in additional inflammatory response. In these studies, ATP was not used. It is still not clear why cryopyrin-dependent inflammasome can be activated \textit{in vivo} without the addition of ATP. It is possible that LPS treatment elicits ATP release from other cell types. Nevertheless, these results clearly demonstrate the role of cryopyrin activation and IL-1β maturation \textit{in vivo}. Finally, the cryopyrin inflammasome has also been demonstrated to play an important role in mediating contact hypersensitivity, a T cell mediated cellular immune response to repeated epicutaneous exposure to contact allergens. Suttutterwala et al sensitized and challenged wild type and cryopyrin deficient mice with trinitrophenylchloride (TNP-Cl). Significant ear swelling is evident in wt type mice but not in cryopyrin deficient mice and ASC deficient mice (51).

All together, these results reveal the critical role of cryopyrin in caspase-1 activation and IL-1β maturation in response to a variety of stimuli ranging from endogenous danger signals to pathogen derived products. However, the fact that cryopyrin deficiency only partially protects mice from lethal dose of LPS challenge, whereas ASC\textsuperscript{-/-} and caspase-1\textsuperscript{-/-} completely protect mice suggests the existence of other inflammasome activation pathways.

1.2.2 \textbf{The Ipaf inflammasome}

NLR protein NLRC4/Ipaf (ICE-protease activating factor also know as CARD12 and CLAN) contains a CARD domain at its N-terminal. Ipaf deficient mice fail to activate
caspase-1 in response to Salmonella and Pseudomonas aeruginosa infection but remain responsive to LPS plus ATP induced caspase-1 activation. This result indicates that Ipaf inflammasome functions independent of cryopyrin inflammasome (55). It is has been known for a long time that the activation of caspase-1 by Salmonella infection requires Salmonella SipB gene, a translocase of Salmonella type III secretion system (TTSS). The TTSS is encoded in the Salmonella pathogenicity island1 (SP1) and delivers effector proteins to the eukaryotic cell cytosol. Previously, it has been demonstrated that SipB protein directly binds and activates caspase-1 upon Salmonella infection (56). However, TTSS might inject a pathogenic effector into host cytosol that directly activates Ipaf inflammasome. Recently, two groups independently demonstrated that infecting mouse macrophage with Salmonella mutants that are deficient in flagellin fails to activate caspase-1. This result suggests that flagellin is the agonist for Ipaf (57, 58). Importantly, the flagellin-dependent activation of caspase-1 is not depend on TLR5 but requires the TTSS components SipB. In addition, direct intracellular deliver of flagellin by protein transfection activates caspase-1. Although flagellin relies on its own secretion system to assemble cell surface flagellum, it is tempting to speculate that TTSS accidental delivers a small amount of flagellin into the cytoplasm of infected cells. However, the detailed mechanism of how Ipaf inflammasome senses flagellin requires further elucidation.

1.2.3 The NALP1 inflammasome

NALP1 (CARD7, DEFCAP, NLRP1) inflammasome is the first prototypical inflammasome discovered five years ago. By using THP-1 cell lysate, Martinon et al demonstrated that human NALP1 inflammasome consists of NALP1, ASC, caspase-5 and caspase-1 (47). However, the cell lysate system used by this study cannot preclude the
possibility that additional components participate in the NALP1 inflammasome. To define the minimal components of NALP1 inflammasome and to further characterize its biochemical properties, Faustin et al. reconstituted the NALP1 inflammasome in vitro by using purified recombinant proteins (59). They demonstrated that the minimal components of NALP1 inflammasome are NALP1 and caspase-1. More importantly, they showed that activation of NALP1 inflammasome requires the presence of ribonucleotide, especially, ATP. This result indicates that ATP binding and hydrolysis might play a critical role in the inflammasome activation (59). Since NALP1 is the only protein in this complex that possesses a conserved NTP binding domain, we can hypothesize that the ATP binding to NALP1 plays a critical role in the NALP1 oligomerization and activation. This proposed mechanism of inflammasome formation is reminiscent of apoptosome formation. However, evidence for the direct binding of nucleotides to NALP1 is absent in their study. Faustin et al also demonstrated that the addition of other nucleotides such as GTP and CTP activate the inflammasome. It is currently unknown why the binding of NALP1 to nucleotide shows no specificity. Moreover, Faustin et al demonstrated that bacteria cell wall component MDP strongly activates caspase-1 in the reconstituted NALP1 inflammasome (59). However, direct binding of MDP to NALP1 was not demonstrated. Previously, MDP has been shown to activate human cryopyrin in THP-1 cell lysate system, although this is controversial. MDP is also a well-known agonist for NOD2. The underlying mechanisms of how MDP stimulates each pathway require more detailed biochemical analysis and the use of physiologic systems similar to what has been achieved for NOD2.

What is the physiological role of NALP1 inflammasome in vivo? In genetic analysis, Boyden and Dietrich demonstrated that a genetic region containing the mouse
NALP1b gene is associated with the susceptibility to *Bacillus anthracis* lethal toxin (LT) (60). *Bacillus anthracis* is the causative agent of anthrax. *Bacillus anthracis* secretes several virulence toxins including lethal toxin that is thought to be responsible for the mortality in systemic anthrax episodes. Macrophages from different inbred mice have different susceptibilities to anthrax LT. The LT susceptibility has been mapped to *Ltxs1* locus. There are three tandem Nalp1 paralogues (Nalp1a, 1b, 1c) in *Ltxs1* locus, but only Nalp1b is actively transcribed in mice macrophages. Nalp1b itself is highly polymorphic with 5 distinct Nalp1b alleles identified. Strikingly, eight of nine LT-sensitive strains carry only one of these five alleles, designed allele 1, whereas the nine LT-resistant strains carry allele 2, 3 or 4. BAC transgenic mice that carry the sensitive allele 1 on a resistant background confer LT sensitivity. Furthermore, upon toxin exposure, caspase-1 is only activated in LT sensitive strain and transgenic mice carrying allele1, but not in LT resistant strain. This study strongly demonstrates the role of NALP1 in anthrax pathogenesis. Intriguingly, the locus responsible for familial vitiligo has been demonstrated to contain NALP1 (61). Vitiligo is a chronic skin condition that causes loss of pigment, resulting in irregular pale patches of skin. Although the etiology of the disease is complex and not fully understood, there is some evidence suggesting it is caused by a combination of autoimmune, genetic, and environment factors. It is now highly possible that specific mutants of NALP1 are responsible for vitiligo.

### 1.2.4 NAIP inflammasome

NAIP (Baculovirus IAP containing 1, also known as Birc1, Baculovirus inhibitor of apoptosis repeat-containing 1) is an unique NLR protein that contains three N-terminal BIR domains in its N-terminal instead of more commonly presented pyrin or CARD
domain. Early high-resolution genetic and physical mapping have implicated mouse Naip2 and Naip5 in *Legionella pneumophila*-induced pathogenesis (62), while others used a BAC rescue approach and found that Naip5 is the gene that confers resistance or susceptibility to the pathogen (63, 64). Later, it was found that Naip5 is required for the restriction of *Leginella* replication in macrophages, in a caspase-1 dependent fashion, and this restriction requires the *Legionella* flagellin (34, 65, 66). However, another group has found that Naip5 restricted *Legionella pneumophila* proliferation by Naip5 is caspase-1 independent while its restriction by Ipaf is caspase-depent and flagellin-dependent (67, 68). The conflicting nature of these data remain to be solved, however they point to the roles of NAIP and Ipaf inflammasome in host defense against *L.pneumophila*

Besides the inflammasome pathways described here, there are likely other inflammasome pathways. Indeed, the NLR protein that accounts for *Francisella tularensis* induced IL-1β activation remains unknown. *Francisella tularensis* is Gram-negative coccobacillus and the causative agent for the zoonotic disease tularaemia or ‘rabbit fever’. It has been demonstrated that *F. tularensis* infection results in the activation of caspase-1 and maturation of IL-1β in an ASC dependent fashion (69). Caspase-1 deficient and ASC deficient mice were highly susceptible to the *F.tularensis* infection in vivo (69). However, *F.tularensis* induced caspase-1 has not been shown to depend on cryopyrin and Ipaf. Therefore, an unidentified NLR molecule might link the *F.tularensis*-derived signals to an ASC containing inflammasome in response to cytosolic *F.tularensis*.

### 1.2.5 Summary

In the last three years, we have witnessed a tremendous progress in understanding the regulation of IL-1β secretion. To date, the essential role of NLRs (including cryopyrin,
Ipaf, NALP1) in the assembly of inflammasome and the activation of caspase-1 is defined. The field has also identified numerous agonist/stimuli that lead to the activation of inflammasome. What we have not learned is the detail mode of actions. For instance, we do not know how decreased intracellular potassium level is sensed by cryopyrin inflammasome. We do not know how Ipaf recognizes flagellin. Is it through a direct binding or through an intermediate protein? Furthermore, are there undetermined inflammasome pathways? Do different cell types preferentially use particular inflammasome pathway? Is individual NLR mediated inflammasome functionally redundant or overlapping during pathogen infection? How is caspase-1 cleaved after being recruited to the inflammasome? What is the role of nucleotide binding/hydrolysis in inflammasome assembly? These questions cannot be answered only by using genetic approaches. The field needs elaborate biochemical studies to define the mechanisms. Moreover, most genetic studies of inflammasome activation are done in murine monocytes/macrophages. So, can human primary cells recapitulate the results obtained in mice? Future studies need to focus on primary human cell culture or even humanized mice to address these questions.

1.3 The role of NLR in cell death

Cell death exists in multiple forms, the most prominent being apoptosis or necrosis (70). Apoptosis is a morphological and biochemical distinct death process and has been shown to play an essential role in cell differentiation and tissue development. Cells undergoing apoptosis display distinct morphologies such as extensive chromatin condensation, nuclear fragmentation, cleavage of chromosomal DNA into nucleosomal fragments and forming of the dead cell into apoptotic bodies without cell membrane
breakdown. The signaling pathways that lead to apoptosis are now detailed studied and are mediated by the activation of caspase cascade. On the other hand, necrosis, or more accurately ‘oncosis’, is described as an accident and non-programmed event that is triggered by a sudden external insult. Cells undergo necrosis often display cell and organelle swelling and rapid loss of membrane permeability(70, 71).

Bacteria infection not only leads to inflammatory response but also cell death. These two processes are not isolated events but are interconnected phenomena. Inflammation induced cell death, especially necrosis, causes the release of a large amount of cellular substances that are strong stimuli of inflammation. Therefore, it forms a positive feed back loop that boosts the magnitude and sustains the duration of an inflammatory response. Inflammation induced cell death can be beneficial to the host because it removes the host cells that are infected by microbial pathogen, therefore, eliminating the host environment that propagates the pathogen. On the other hand, inflammation induced cell death can be detrimental to the host, because an overzealous inflammatory response might results in significant damage to the host, as in the case of sepsis.

*Salmonella typhimurium and Shigella flexneri*, two enteric bacteria with distinct characteristic and pathogenic outcome, are the prototypic bacteria that induce cell death (72). Initial studies showed that *Salmonella* infection leads to a caspase-1 dependent cell death both in vitro and in vivo. This death process is unique in that it morphologically differs from the classical apoptosis and is often manifested by a rapid loss of membrane integrity, a hallmark of necrosis. This necrosis like, caspase-1 dependent death process is subsequently termed ‘pyroptosis’(71). Furthermore, the *Salmonella* induced cell death has been shown to require the *Salmonella* SipB protein, a component of type III secretion
system. An initial study demonstrated that SipB directly binds caspase-1 and mediates its activation (56). A subsequent study revealed that NLR protein Ipaf and adaptor ASC are required to mediated Salmonella induced caspase-1 activation and cell death. The later study formally delineates the important role of NLR protein in initiating pathogen induced death process. However, a Salmonella induced, caspase-1 independent cell death is also reported (73).

Early studies revealed that Shigella infection triggers caspase-1 dependent apoptosis while other groups subsequently described a form of Shigella induced death that is caspase-1 dependent and necrotic in nature. How Shigella infection leads to the activation of caspase-1 is largely unknown until a recent study by Suzuki et al showed that Ipaf and ASC are required for Shigella induced caspase-1 activation (74). Interestingly, while Ipaf is required for both Shigella induced caspase-1 and initial cell death process, ASC is completely dispensable in the Shigella induced cell death, yet it is absolutely required for caspase-1 activation (74). In addition, Suzuki et al demonstrated that Ipaf deficient mice and caspase-1 deficient mice still undergo cell death even though the initial death progress is significantly delayed (74). This data clearly demonstrates the existence of a caspase-1 independent death process in Shigella infection that happens concurrently along with the caspase-1 dependent death. Indeed, Willingham et al recently reported that Shigella infection leads to a rapid necrosis-like cell death in mice macrophages that is independent of caspase-1 but dependent on cryopyrin and ASC. They termed this caspase-1 independent necrosis like cell death as ‘pyronecrosis’. This result is in line with a previous study showing that the cytosolic Shigella induced a caspase-1/TLR4 independent necrosis-like cell death in macrophages (75). In sharp contrast to study from Suzuki et al,
Willingham et al also demonstrated that *Shigella* induced caspase-1 activation is depend on cryopyrin instead of Ipaf. This is in sharp contrast to Suzuki et al ‘s study. This discrepancy might be attributed to the subtle difference of bacteria strain and experiment conditions. Therefore, both forms of inflammasome might be produced upon *Shigella* infection.

CIAS1/Cryopyrin mutations are associated with several autoinflammatory disorder: FCAS, MWS and CINCA/NOMID. To evaluate the potential role of cryopyrin induced cell death in the pathogenesis of those diseases, Fujisawa et al transfected human THP-1 monocytic cell line with disease associated *CIAS1* mutants. They demonstrated that overexpression of these mutants induced a rapid necrosis-like cell death (76). This necrosis-like cell death can be blocked by the inhibitor for cathepin B, a lysosomal enzyme that nonspecifically digests protein within the lysosomal compartment. This result indicated that the cryopryin induced necrosis-like death undergoes a cathepsin B dependent pathway.

Subsequently, Willingham et al demonstrated a similar necrosis-like death process in THP-1 cells infected by adenovirus encoding CIAS1 disease-associated mutants. Similar to the *Shigella* induced death, this CIAS1 mutants induced necrosis-like cell death is independent of caspase-1. Importantly, the authors also showed that peripheral blood mononuclear cells (PBMC) from FCAS patients undergo significant cell death under LPS treatment. Together, these data combined with previous studies demonstrate that cryopyrin disease-associated mutants not only enhance the caspase-1 activation but also induce a rapid necrosis-like cell death, both processes might all contribute to disease state in patients.
In summary, accumulating evidences demonstrate the important role of NLRs in mediating caspase-1 dependent or independent cell death. Of note, a recent study demonstrated that activated ASC oligomerized into a 1μm supermolecular complex termed ‘pyroptosome’, which can mediate caspase-1 dependent cell death (77). Nevertheless, how the activation of caspase-1 leads to cell death remains elusive.

### 1.4 The role of NLR in the intracellular sensing of pathogen-derived molecules

The NLRs that have been first demonstrated to detect intracellular PAMP are NOD1 (CARD14) and NOD2 (CARD15). NOD1 and NOD2 both have typical NLR protein domain arrangement that consists of C-terminal LRR, N-terminal CARD domain and central NBD domain. NOD1 have one CARD domain at its N-terminal whereas NOD2 have 2 tandem CARD domains. NOD 1 and NOD2 are required for cellular response to peptidoglycan, a component of both Gram positive and negative bacteria. Specifically, the ligands for NOD1 and NOD2 is \( \gamma \)-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. Of note, iE-DAP is only derived from Gram negative bacteria, whereas MDP is derived from both Gram positive and negative bacteria suggesting that NOD1 only senses products from Gram-negative bacteria and NOD2 detects both type of bacteria. Nevertheless, evidence demonstrating the direct binding of NOD1, NOD2 by their cognate ligands are still lacking. NOD1 has a wide tissue distribution while NOD2 are primary expressed by macrophages and dendritic cells. Although NOD1 and NOD2 mainly reside in cytosol, NOD2 has been demonstrated to associate with plasma membrane in epithelial cells (78).
Upon their cognate ligand stimulation, NOD1 and NOD2 activate a CARD containing serine/threonine kinase RIP2 (also known as RICK). The activation of RIP2 leads to a K63-linked polyubiquitylation of NEMO (IKKγ), which triggers the activation of IKKβ and NF-κB translocation (79). In addition to the activation of NF-κB, NOD1 and NOD2 signaling also activate MAP Kinase pathways such as JNK (JUN amino-terminal kinase), ERK (extracellular-signal-regulated kinase (ERK) and p38. Previous study demonstrates that RIP2 is also used by TLR signaling pathway due to the data showing that RIP2 deficient mice show reduced cytokine secretion upon LPS and lipoteichoic acid stimulation (80). However, by using highly purified and synthetic TLR ligand, Park et al showed that the TLR signaling pathways are mostly intact in RIP2 deficient mice suggesting that RIP2 is only used by NOD1 and NOD2 signaling pathway (81). In addition, NOD2 mediated signaling is regulated by its binding partners. For example, GRIM-19, a protein with homology to the NADPH dehydrogenase complex, interacts with NOD2 and is required by NOD2 mediated NF-κB activation (82). Erbin, a member of LRR and PDZ domain-containing family, has been shown to interact with NOD2. Interestingly, Erbin functions as a negative regulator of NOD2 mediated signaling(83) (84).

NOD1 and NOD2 are thought to be the intracellular bacteria sensor. The activation of NOD1 and NOD 2 elicit a strong production of proinflammatory cytokine such as IL-6, TNF-α and the induction of antimicrobial peptides such as β-defensin and cryptdins. Kobayashi et al reported that mice deficient in NOD2 fail to respond to MDP indicating the specificity of NOD2/MDP detection (85). Moreover, NOD2 deficient mice are susceptible to oral infection of Listeria Monocytogenes suggesting the critical in vivo role of NOD2 in anti bacteria host defense (85). This study also implies that the Crohn’s
disease associated NOD2 mutations are loss-of-function mutations and that the nonfunctional NOD2 mutants might result in defective response to commensal and pathogenic bacteria. However, Watanabe and Strober et al have shown that, in NOD2 deficient mice, the secretion of IL-12, a cytokine critical in the development of T help cells type 1 response, is significantly increased upon TLR2 stimulation. This result suggests that NOD2 actually functions as a negative regulator of TLR2 signaling (86). Furthermore, in a subsequent study, Watanabe et al demonstrated that NOD2 deficient mice develop colitis in an E.coli induced colitis model (87) implying that the Crohn’s disease-associated mutants fail to control TLR2 signaling leading to an elevated inflammatory response. Thus this also suggests disease-associated mutants as loss-of-function mutant, but the underlying mechanism is drastically different. This discrepancy awaits further clarification in future study.

Finally, Maeda et al generated a Crohn’s disease (CD) mutant knock-in mice and demonstrated that the CD mutant Knock-in mice exhibits elevated NF-κB activation in response to MDP and enhanced secretion of IL-1β (88). This study indicates the CD associated mutant is a gain-of-function mutant and seemingly fits the massive inflammation observed in CD patient. However, this study is in sharp contrast to the in vitro studies from human monocytes bearing NOD2 disease associated mutant, which show abolished MDP sensing and synergy with TLRs. Clearly, more efforts need to be made to address the mechanisms of NOD2 mutants in Crohn’s disease pathogenesis.

1.5 The biological function of NLR protein, Monarch-1/NLRP12

1.5.1 Canonical and non-canonical NF-κB signaling pathway
NF-κB is a dimeric transcription factor consisting of p50, p52, Rel-A (also known as p65), Rel-B and c-Rel. p50 and p52 are derived from the larger precursors p105 (NF-κB1) and p100 (NF-κB2), respectively, through proteolytic processing by the proteasome. The most common dimeric forms of NF-κB are p50/Rel-A and p52/Rel-B.

In the resting state, the NF-κB proteins are sequestered in the cytoplasm by the IκB (Inhibitor of κB) proteins that include IκBα, β and ε. Upon activation, The IκB is subjected to K48-linked ubiquitination and subsequent degradation by proteosome. This process liberates the NF-κB dimers that then translocate into the nucleus to initiate gene transcription. The NF-κB signaling pathway can be generally divided into the canonical and the non-canonical pathway.

The TLR signaling leads to the activation of canonical NF-κB pathway. Upon engaged by its cognate agonist, TLR dimerizes and recruits adaptor protein such as MyD88 through TIR-TIR domain interaction. The recruitment of MyD88 activates kinase IRAK-4 (interleukin-1 receptor-associated kinase-4) that subsequently phosphorylates its downstream target IRAK-1. The phosphorylated IRAK-1 undergoes further self-phosphorylation and activates an ubiquitin E3 ligase TRAF6. Activated TRAF6 recruits ubiquitin E2 complex consisting of Ubc13 and an Ubc-like Uev1A. This process synthesizes a K63-linked polyubiquitin chain on IKKγ (NEMO) and TRAF6 itself. The ubiquitinated TRAF6 recruits TAB2 and activate the TAB2-associated TAK1 kinase. Activated TAK1 then phosphorylates, and activates IKKβ, which phosphorylates IκB protein leading its degradation. The ubiquitination plays a critical role in the activation of canonical NF-κB pathway and therefore is tightly regulated by the deubiquitination process mediated by CYLD (cylindromatosis tumor suppressor protein) and A20.
The non-canonical NF-κB pathway operates in response to stimulation of a subset of the TNF receptor superfamily, including receptors for lymphotoxin-β (LTβ), BAFF and CD40 ligand. Stimulation of these receptors activates the protein kinase NIK (NF-κB inducing Kinase), which in turn activates IKKα. IKKα then phosphorylates p100 leading to the selective degradation of its IκB-like domain by the proteasome. The mature p52 subunit and its binding partner Rel-B translocate into the nucleus to regulate gene expression.

1.5.2 Monarch-1 is a negative regulator of both canonical and non-canonical NF-κB pathway

By data mining the human genome, our group first identified a novel gene family termed CATERPILLER, which now named as NLR protein family. One of the novel members of this protein family is Monarch-1, which was cloned by our group using 5' and 3' RACE reaction that amplified cDNA from human U937 monocytic cell line (89). Concurrently, another group also identified the same cDNA sequence named PYPAF7 (90). The full-length cDNA of Monarch-1 is 3731-bp long with a 220-bp 5'UTR, a 323-bp 3'UTR and a 3189-bp open reading frame encoding a 118 KDa Monarch-1. It is located on human chromosome 19q13.4.

Monarch-1 is expressed predominantly in cells of the myeloid lineage, including monocytes and granulocytes. Furthermore, single nucleotide polymorphism analysis has recently demonstrated a genetic link between Monarch-1 and atopic dermatitis (91). However, in contrast to most NLR proteins that promote inflammation, Monarch-1 functions as an attenuator of inflammatory responses. Monarch-1 inhibits TLR-mediated hyperphosphorylation of IRAK-1, a necessary step in TLR signaling pathways (92). In
addition, Monarch-1 binds to and destabilizes the MAP3 kinase, NIK and blocks NIK-mediated processing of NF-κB2/p100 to p52 (93). The p52 subunit is an important downstream mediator of signaling by TLRs as well as TNF family receptors including CD40 and lymphotixin-β receptor. The introduction of shRNA specific for Monarch-1 greatly enhances NF-κB activation and the transcription of NIK-dependent genes induced by TLR and TNF family receptor activation. These earlier studies suggest that Monarch-1 performs important anti-inflammatory roles as an inhibitory molecule of innate immune activation (Figure 1-2).

### 1.6 The role of Nucleotide binding in the biological function of NLR

The nucleotide binding domain of NLRs belongs to the AAA+ (ATPases associated with various cellular activities) ATPase protein family that also includes plant R protein and mammalian apoptotic protein Apaf-1 (apoptotic protease-activation factor 1) (94). AAA+ ATPase family is a functionally diverse group of enzymes that are able to induce conformational changes in a wide range of substrate proteins. The defining feature of AAA+ protein family protein is the structurally conserved ATP-binding and hydrolysis domain (AAA domain) that generally contains 200-250 amino acids. This domain contains two well-conserved motifs called Walker A and Walker B motifs (95). The structure of AAA domain comprises a compact core structure with five β-sheets sandwiched by six α-helix fold. This core structure is followed by a C-terminal α-helical subdomain. For example, the crystal structure of a AAA+ protein NSF (N-ethylmaleimide-sensitive factor) AAA domain is shown in (Figure 1-3A) (96). The Walker A motif (also called p-loop) comprises the consensus sequence GxxxxxxGK[T/S] (where x is any amino acid and has been implicated to interact with the phosphate moiety of ATP directly. The lysine residue
in the consensus sequence is essential in coordinating the $\beta$ and $\gamma$ phosphates of ATP. Mutation of this key residue typically eliminates nucleotide binding activity (97). The Walker B motif also contacts with nucleotide and is crucial for ATPase activity. The Walker B motif contains a consensus sequence hhhhhDE (h represents a hydrophobic amino acid). The aspartic acid residue in the Walker B motif coordinates $\text{Mg}^{2+}$ that is required for stable ATP binding and the glutamate residue is thought to be responsible for $\beta$-$\gamma$ phosphodiestor bond breakage during ATP hydrolysis (97, 98). AAA$^+$ proteins also contain a conserved region that is positioned C-terminal of the Walker-B motif and is named the second region of homology (SRH). The SRH comprises several conserved motifs including Sensor 1, Sensor 2, and arginine fingers, which all have been proposed to coordinate nucleotide hydrolysis and conformational changes. AAA$^+$ protein typical oligomerized into a six or seven-fold symmetry multimer. It is clear now that the conformational changes required for oligomerization are driven by ATP binding and/or hydrolysis (97).

Due to the sequence similarity between the NLR protein family and AAA$^+$ ATPase family, I hypothesized that NLR protein binds and hydrolyze ATP and this process plays a critical role for the biological function of NLR.

Although, there is no evidence that clearly demonstrates nucleotide binding and its role in NLR protein function when this thesis study is initiated, studies on nucleotide binding of closely related protein Apaf-1 and plant R protein strongly support my hypothesis.

For example, the functional role of ATP binding and hydrolysis is clearly demonstrated in the study of mammalian Apaf-1 that plays a vital role in the execution of
intrinsic apoptosis pathway (99-101). Structurally similar to NLR protein, Apaf-1 is composed of an N-terminal CARD domain, a central nucleotide binding and oligomerization domain (called NB-ARC) (102) and a C-terminal multiple WD40 repeats. In a non-apoptotic condition, Apaf-1 resides in cytosol as an inactive monomer. During early apoptosis process, mitochondria cytochrome C specifically binds Apaf-1. In the presence of the dATP, the binding of cytochrome C induces a conformational change that triggers the oligomerization of Apaf-1/cytochrome C to form a wheel-like heptomeric structure termed ‘Apoptosome’. The core of this wheel-like structure contains seven Apaf-1 N-terminal CARD domains. The formation of this complex allows interaction of pro-caspase-9 with Apaf-1 through the interaction of its own CARD with the CARD of Apaf-1, thus placing individual pro-caspase-9 molecules in close proximity with one another and promoting their activation (101, 103-105).

Recent detailed biochemical and structural studies start to reveal the detailed mode of action in the assembly of Apoptosome and the critical role of ATP binding/hydrolyzing in mediating this process (106, 107). In the non-apoptotic state, WD40 domain of Apaf-1 folds back on the NBD and CARD domain, resulting in an inactive monomer. ESI-Mass spectrometry analysis of purified Apaf-1 protein generated from insect cells reveals that inactive Apaf-1 is bound by dATP. Upon the induction of apoptosis, the binding of cytochrome C to the WD40 domain liberates the NBD domain from the inhibition of WD40 domain. Apaf-1 then hydrolyzes the bound dATP and undergoes a conformational change. Critically, only if the bound dADP is subsequently exchanged with exogenous dATP, does form the active Apoptosome with ordered structure. If the concentration of cellular dATP is insufficient for nucleotide exchange after hydrolysis, the
dADP bound Apaf-1 oligomerizes into an inactive aggregates (Figure 1-3B). Currently, we do not yet know the precise binding site of cytochrome c on Apaf-1 nor do we know how dADP is exchanged for dATP after the initial hydrolysis of dATP. Furthermore, the crystal structure of WD-40-truncated Apaf-1 reveals that the inactive Apaf-1 binds an ADP but not dADP. This crystal structure is obtained from bacteria derived Apaf-1 protein. It is unknown why the bacteria-derived Apaf-1 prefers ATP instead of dATP as cofactor. We also do not know whether endogenous Apaf-1 in mammalian cells also exclusively binds dATP. Nevertheless, the ATP hydrolysis/exchange driven oligomerization shown in Apaf-1 provides us a prototype for understanding the role of nucleotide binding in NLR function.

Plant disease resistant R proteins are also highly homologous to NLR protein. Like NLR protein, Plant R protein also contains a central nucleotide binding domain (called NBD-ARC) and C-terminal LRR. Plant R protein mediates Hypersensitivity Reaction (HR) upon encountering their cognate agonist. The nucleotide binding and its functional role in R protein are recently revealed by studies on tomato R protein I-2 that confers resistance to the fungal pathogen *Fusarium oxysporum*. It has been demonstrated that purified LRR truncated I-2 protein binds ATP. The binding is abolished when mutation is introduced to Walker A motifs (108). Furthermore, purified I-2 protein also exhibits ATP hydrolysis activity. This result shows that I-2 is a *bona fide* ATPase. Two auto-activation mutants of I-2 protein are identified that lead to HR in the absence of an effectors. In a subsequent study, when a Walker A mutation was introduced in the context of autoactivation mutant, the double mutant completely abolished the HR reaction caused by overexpression of autoactivation mutant (109). This result indicates that the nucleotide binding is required for the plant hypersensitivity reaction.
Before the discovery of the CATERPILLER protein family, our lab also discovered that the founding member of CATERPILLER protein family CIITA binds GTP, albeit the GTP binding activity is demonstrated by CIITA protein immunoprecipitated from an in vitro protein translation system. Importantly, the disruption of nucleotide binding pocket completely rescinds the CIITA nuclear import highlighting the critical role of GTP binding in mediating CIITA nucleus import (110).

1.7 Summary and future direction

Great strides are made in understanding the function of NLR protein in the past few years. The entire family has been identified and the key functions of several prominent NLR family proteins have been defined by over-expression, gene deletion and gene knockdown approaches. Several agonists and stimuli that lead to the activation of NLR have been identified and several key adaptors that are critical in the NLR signaling pathway have been found. The majority of NLR proteins appears to specialize as intracellular pathogen sensors and can mount strong inflammatory response upon the recognition of their cognate agonists; We also can raise several examples to show that NLR protein can also dampen the inflammatory response.

However, many key questions regarding to the function of NLR protein are still left unanswered. To date, we have not obtained any crystal structure of NLR protein. Equally lacking is the experimental data demonstrating the biochemical mode of action regarding the “sensing” of pathogen products, proposed nucleotide-driven conformation change and signaling complex assembly. This knowledge cannot come solely by well-tailored genetic approaches but can only be obtained by sophisticated biochemical approaches.
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Table 1-1 The Nomenclature of NLR protein family
Table 1-1 The HUGO recommended Nomenclature for NLR protein family.

The gene names used in this thesis are highlighted. The domain structures of each gene are listed. PYD: Pyrin domain; NACHT: NAIP, CIITA, HET-E and TP1; CARD: caspase-recruitment domain; NAD: NACHT associated domain; LRR: Lucine rich repeats.
Figure 1-1 The biological function of NLR proteins
**The role of NLR protein in the secretion of proinflammatory cytokine IL-1β and IL-18.** Several NLRs can form multi-protein complex called ‘inflammasome’. The inflammasome complex recruits and activates caspase-1 that converts pro-IL-1β and pro-IL-18 to biological active IL-1β and IL-18, respectively. NLR protein cryopyrin senses the intracellular potassium efflux that is caused by the engagement of extracellular ATP on cell surface receptor P2X7. The activation of P2X7 receptor also recruits cell membrane forming protein Pannexin-1, which further facilitates the potassium efflux. Bacteria pore forming toxin, viral RNA mimics R848, R837, gout-associated crystals as well as bacteria *Listeria monocytogenes* and *Staphylococcus aureus* all have been shown to activate cryopyrin inflammasome. Upon activation, Cryopyrin interacts with adaptor protein ASC through Pyrin-Pyrin domain interaction. ASC then recruits pro-caspase-1 through CARD-CARD domain interaction. This process leads to oligomerization and the activation of caspase-1. The bacteria product flagellin from *Salmonella typhimurium* and *Shigella Flexneri* activate Ipaf inflammasome. Flagellin produced by *Legionella pneumonphila* activate NAIP5, which does not possess CARD or Pyrin domain, but is predicted to associates with Ipaf. It is not clear whether the Ipaf mediated inflammasome requires ASC. Anthrax lethal toxin activates NALP1 inflammasome, which requires adaptor ASC. **B. NOD2 mediated NF-κB activation.**

NOD2 can directly or indirectly detect bacteria cell wall components MDP. Upon detection, NOD2 recruits kinase RIP2/RICK, which subsequently ubiquitinates NEMO (IKKγ). The ubiquitination of NEMO results in the phosphorylation of IKKβ. This signal induced phosphorylation targets IκB for polyubiquitination and subsequent degradation by the 26S proteasome, thus releasing NF-κB. The p65/RelA dimer then translocates into the
nucleus and initiates the transcription of proinflammatory cytokines such as TNF-α and IL-6.
Figure 1-2 The function of Monarch-1 and the proposed role of nucleotide binding in NLR function.
Figure 1-2 The canonical and non-canonical NF-κB pathway and the biological function of Monarch-1. The TLR2 signaling leads to the activation of canonical NF-κB pathway. Upon engaged by its cognate agonist, TLR2 recruits adaptor protein such as MyD88 through TIR-TIR domain interaction. The recruitment of MyD88 activates kinase IRAK-4 that subsequently phosphorylates its down stream target IRAK-1. The phosphorylated IRAK-1 undergoes further self-phosphorylation and activates an ubiquitin E3 ligase TRAF6. TRAF6 recruits additional E2 ubiquitin ligase and synthesize a K63-linked polyubiquitin chain on IKKγ (NEMO) and TRAF6 itself. The ubiquitinated TRAF6 recruits TAB2 and activate the TAB2-associated TAK1 kinase. Activated TAK1 then phosphorylates, and activates IKKβ, which phosphorylates IκB protein leading its degradation. The ubiquitination is tightly regulated by the deubiquitination process mediated by CYLD (cylindromatosis tumor suppressor protein) and A20. In human monocytes, Monarch-1 block the hyperphosphorylation of IRAK-1 therefore inhibits the canonical NF-κB pathway.

The non-canonical NF-κB pathway operates in response to stimulation of a subset of the TNF receptor superfamily, including receptors for lymphotoxin-β (LTβ), BAFF and CD40 ligand. Stimulation of these receptors activates the protein kinase NIK, which in turn activates IKKα. IKKα then phosphorylates p100 leading to the selective degradation of its IκB-like domain by the proteasome. The mature p52 subunit and its binding partner Rel-B translocate into the nucleus to regulate gene expression. Monarch-1 interacts with NIK and decreases the stability of NIK, thus negative regulates the noncanonical NF-κB pathway.
Figure 1-3 The AAA+ ATP binding structure and Apoptosome formation
Figure 1-3 A. The crystal structure of AAA+ family protein NSF (N-ethylmaleimide-sensitive factor). The PDB file of NSF (accession number:1D2N) was obtained from protein data bank. The crystal structure was rendered in King 2.12. Core structure for the nucleotide binding consists of five β-sheets sandwiched by six α-helixes. The position of Walker A, B motif and the bounded ATP and Mg$^{2+}$ were indicated. B. The formation of Apoptosome. The release of cytochrome C initiates the apoptosome formation. In non-apoptotic state, Apaf-1 exists as a dATP bound monomer. The CARD domain and WD40 domain folds back on the NBD domain of Apaf-1 render it inactive. The release cytochrome C interacts with WD40 domain leading to a change of conformation. The conformation change results further hydrolysis of bound ATP to ADP. The ADP bound Apaf-1 has to undergo one round ADP-ATP exchange to become fully activated. Activated Apaf-1 oligomerized into a wheel-like heptamer structure with CARD domain presented in the center of the structure. The structure then recruits and activates caspase-9, thus activates apoptosis process.
Chapter 2  ATP binding by Monarch-1/NLRP12 is critical for its inhibitory function
ABSTRACT

The recently discovered Nucleotide Binding Domain-Leucine Rich Repeat (NLR) gene family is conserved from plants to mammals and several members are associated with human autoinflammatory or immunodeficiency disorders. This family is defined by a central nucleotide binding domain that contains the highly conserved Walker A and Walker B motifs. Although the nucleotide binding domain is a defining feature of this family, it has not been extensively studied in its purified form. In this report, we show that purified Monarch-1/NLRP12, an NLR protein that negatively regulates NF-κB signaling, specifically binds ATP and exhibits ATP hydrolysis activity. Intact Walker A/B motifs are required for this activity. These motifs are also required for Monarch-1 to undergo self-oligomerization, TLR- or CD40L-activated association with NIK and IRAK-1, degradation of NIK, and inhibition of IRAK-1 phosphorylation. Stable expression of a Walker A/B mutant in THP-1 monocytes results in increased production of proinflammatory cytokines and chemokines to an extent comparable to cells in which Monarch-1 is silenced via shRNA. The results of this study are consistent with a model wherein ATP binding regulates the anti-inflammatory activity of Monarch-1.
2.1 INTRODUCTION

Nucleotide Binding Domain-Leucine Rich Repeat (NLR) proteins share strong structural homology to the largest subgroup of plant disease resistance (R) proteins. These proteins share a trimeric domain architecture consisting of an N-terminal effector domain, a central nucleotide binding domain (NBD) and C-terminal leucine rich repeats (LRR). Mounting evidence suggests that NLR genes are important for the host response to pathogens and the regulation of inflammation. Interest in these genes has been further propelled by the realization that mutations in certain NLR genes are linked to human autoinflammatory and immunodeficiency diseases. For example, mutations in \textit{CIITA}, the MHC class II transactivator, lead to a severe immunodeficiency disease, Bare Lymphocyte Syndrome (111). Mutations in \textit{NOD2/CARD15} are associated with Crohn's disease and Blau syndrome, two human disorders with hyperinflammatory manifestations (30-32, 112). Finally, mutations in the \textit{Cold-Induced Autoinflammatory Syndrome-1} gene (\textit{CIAS1}, also \textit{NALP3}) is associated with a spectrum of autoinflammatory disorders which likely represent similar diseases with varying severity: Familial Cold-Induced Autoinflammatory Syndrome (FCAS), Muckle–Wells Syndrome (MWS) and Neonatal-Onset Multisystem Inflammatory Disease (NOMID)/Chronic Infantile Neurologic, Cutaneous, Articular Syndrome (CINCA) (29, 113-116). Most notably, the majority of known disease-associated mutations within these NLR genes reside within the NBD domain. However, the influence of these mutations on the nucleotide binding activity remains poorly understood.

Several recent studies have provided greater detail regarding the mechanism and biological significance of nucleotide binding by NLR and NLR-related proteins.
Apoptotic protease activating factor-1 (APAF-1) is a protein that contains a central NBD and C-terminal WD-40 repeats and thus is closely related to the NLR family. Under apoptotic conditions, APAF-1 binds cytochrome C. This interaction stimulates APAF-1 binding to dATP leading to the formation of an APAF-1 heptamer that activates caspase-9. Thus dATP binding by APAF-1 is a key regulatory step in the apoptotic process (106, 117).

Similar to APAF-1, the NLR protein NLRP3 (previously known as cryopyrin, CIAS1 or NALP3) also requires nucleotide binding for its activity. Our group has recently demonstrated that, in the absence of a functional NBD, NLRP3 cannot form an active inflammasome. This results in reduced IL-1β processing and decreased cell death (118). Most importantly, inactivation of the NBD of NLRP3 abolishes the hyper-reactive phenotype of naturally occurring disease-associated mutations of this NLR protein. This demonstrates that nucleotide binding is required for the inflammatory phenotype of NLRP3 linked diseases.

In light of these findings, we examined the contribution of nucleotide binding to the functional role of an NLR protein, Monarch-1/NLRP12. Monarch-1 is expressed predominantly in cells of the myeloid lineage, including monocytes and granulocytes. Furthermore, single nucleotide polymorphism analysis has recently demonstrated a genetic link between Monarch-1 and atopic dermatitis (91). However, in contrast to most NLR proteins that promote inflammation, Monarch-1 functions as an attenuator of inflammatory responses. Monarch-1 inhibits TLR-mediated hyperphosphorylation of IRAK-1, a necessary step in IRAK-1 signaling pathways (92). In addition, Monarch-1 binds to and destabilizes the MAP3 kinase, NF-κB inducing kinase (NIK) and blocks NIK-mediated processing of NF-κB2/p100 to p52. The p52 subunit is an important downstream mediator.
of signaling by TLRs as well as TNF family receptors including CD40 and lymphotoxin-beta receptor. The introduction of shRNA specific for Monarch-1 greatly enhances NF-κB activation and the transcription of NIK-dependent genes induced by TLR and/or TNF family receptor activation (93). These earlier studies suggest that Monarch-1 performs an important anti-inflammatory role as an inhibitory molecule of innate immune activation.

In this report, we explore the role of nucleotide binding in the anti-inflammatory activity of Monarch-1. Herein, we demonstrate that Monarch-1, purified to homogeneity, specifically binds ATP. Nucleotide binding is indispensable for the biological function of Monarch-1, as an NBD mutant form of Monarch-1 does not inhibit IRAK-1 hyperphosphorylation nor does it inhibit NIK-dependent p52 production. Moreover, THP-1 monocytes stably expressing the NBD mutant form of Monarch-1 secrete elevated levels of proinflammatory cytokines and chemokines. These results open the door for the characterization of the nucleotide binding properties of other NLR members and will facilitate the design of pharmacological agents that modulate the functions of this family of proteins.

2.2 Materials and Methods

2.2.1 Reagents

The TLR2 agonist, the synthetic lipoprotein S-[2,3-bis-(palmitoyloxy)-2(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys-4-OH trihydrochloride (Pam3Cys4) was obtained from Invivogen. CD40L was obtained from PeproTech. Anti-His-HRP conjugates were obtained from (Santa Cruz) and anti-Flag M2-HRP was obtained from Sigma. THP-1 cell lines stably expressing empty vector (THP-EV), Ha-tagged WT Monarch-1 (THP-WT) or shRNA targeting Monarch-1 (THP-shMon) have been described
The THP-1 cell line stably expressing Ha-tagged Monarch-1 containing the Walker A/B mutation was generated by the same procedure (THP-mutA/B).

2.2.2 Expression and purification of bacterial MBP-Monarch-1-NBD fusion protein

The cDNA sequence encoding amino acids 188-448 of Monarch-1 that includes the Walker A/B motifs was amplified by PCR using PFU Turbo polymerase (Stratagene). Restriction enzyme sites for HindIII and BamHI were incorporated into the 5’ and 3’ end of PCR product respectively. DNA sequence encoding a 6xHis tag was also introduced in the 3’ reverse primer. The amplified product was digested by HindIII and BamHI (New England Biolabs) and cloned into the C-terminus of the maltose binding protein (MBP) in the vector pMAl-c2E (New England Biolabs). Mutations in both Walker A and Walker B were generated by site-direct mutagenesis (Stratagene). All constructs were confirmed by DNA sequencing. The MBP-Monarch-1-NBD fusion plasmids were transformed into the *Escherichia coli* strain Rosetta-Origami B (EMB bioscience). One liter of LB with 100 μg/ml ampicillin was inoculated with 5 ml overnight bacteria culture. The culture was grown at 37 °C to a density of OD₆₀₀ = 0.8 and then isopropylthio-β-galactoside (IPTG) was added to a final concentration of 0.3 mM to induce the expression of the MBP fusion proteins. After 3 hr of induction at 25 °C, the cells were harvested by centrifugation at 6000 rpm. Cell pellets were washed once with cold PBS and resuspended in ice cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10 mM β-ME and Roche protease inhibitor cocktail). Resuspension was facilitated by sonication for 2 min. Bacteria were then lysed with recombinant lysozyme (EMB bioscience) followed by benzonase (EMB Bioscience) treatment to degrade bacterial DNA and RNA. Bacterial
lysates were clarified by centrifugation twice at 15,000 g for 30 min. The supernatant was filtered through a 0.2 µm low-protein binding filter. Amylose resin (New England Biolabs) was washed twice with column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM β-ME) and added directly to the bacterial lysate. The lysate/resin mix was rotated at 4°C for 1 hr and then transferred into an empty column. The resin was washed with 10 volumes of column buffer and eluted with five volumes of column buffer containing 10 mM maltose. The eluate containing MBP fusion protein was concentrated with Amicon centrifugal filter device. The partially purified MBP-Monarch-1-NBD fusion proteins were further purified over an FPLC size-exclusion column (Biosilect 400, Bio-Rad). Each fraction was tested for nucleotide binding activity. The fractions with high nucleotide binding activity were pooled and subsequently purified on a cobalt based metal affinity column (Sigma) and eluted with 300 mM imidazole.

2.2.3 Expression and purification of mammalian cell-derived Monarch-1ΔLRR.

cDNA encoding Monarch-1 amino acids 1-686, which correspond to the pyrin and NBD domain, was PCR amplified and cloned into the pCEP4 vector (Invitrogen) by standard molecular cloning procedures. This expression construct was introduced into the HEK293EBNA cell line (ATCC CRL10852) using polyethyleneimine (PEI; Polyscience). The transfected HEK293EBNA cells were then harvested and lysed in hypotonic lysis buffer (25 mM Hepes/KOH, pH7.5, 10 mM KCl, 5 mM MgCl2, 0.1mM PMSF and Roche protease inhibitor cocktail) for 15 min on ice followed by a brief sonication for 40 seconds. Lysates were cleared by centrifugation at 20,000 rpm for 30 min and filtered through 0.45 µm filter. The lysate was then subject to cobalt metal affinity resin purification (Clontech).
The eluate was further purified over an anti-Flag affinity matrix and eluted with excess Flag peptide (Sigma). These eluates were resolved by SDS-PAGE and proteins visualized with Coomassie blue stain.

### 2.2.4 Nucleotide binding assay

A rapid filter binding assay was developed to measure nucleotide binding to Monarch-1 fusion proteins. \( [\gamma^{35}\text{S}]\text{ATP} \) (1250 Ci/mmole) (Perkin Elmer Life and Analytical Sciences) was mixed with the indicated amount of recombinant Monarch-1 in a final volume of 100 µl of binding buffer (50 mM TrisHCl, 150 mM NaCl, 20 mM MgCl\(_2\), 2 mM DTT, 5% Glycerol, pH 7.5) and was incubated at 30°C for 1 hr. After this incubation, the samples were filtered through a 96-well nitrocellulose plate (Millipore) and immediately washed twice with 200 µl of ice-cold binding buffer by vacuum filtration (Millipore). The filter plate was then air-dried and radioactivity was measured using a scintillation counter. For homologous competition assays, Monarch-1-NBD fusion protein (2 µg) or Monarch-1ΔLRR (450 ng) was incubated with 90 nM \( [\gamma^{35}\text{S}]\text{ATP} \) and increasing concentrations of unlabeled \( [\gamma\text{-S}]\text{ATP} \). Curves represent non-linear regression fit to single site completion model. Nucleotide binding specificity was determined by incubating Monarch-1-NBD fusion protein with \( [\gamma^{32}\text{S}]\text{ATP} \) and 10 µM of the indicated unlabeled competitor nucleotide.

### 2.2.5 ATPase assay

ATP hydrolysis was measured by visualizing the conversion of \( ^{32}\text{P-ATP} \) to \( ^{32}\text{P-ADP} \) using thin layer chromatography. 5 µg of purified Monarch-1ΔLRR was incubated with 10 µM ATP and 0.1 µM \( ^{32}\text{P-ATP} \) (3000 Ci/mmol, Perkin Elmer life and Analytical Science) in a total volume of 40 µl reaction buffer (25 mM TrisHCl, pH 7.5, 150 mM NaCl,
10 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF) for 2 hours. The reaction was quenched by adding an equal volume of TLC development solvent (1 M formic acid, 0.5 M LiCl). 2 μl of the reaction was spotted on a PEI cellulose TLC plate (Sigma) and developed with 1 M formic acid with 0.5 M LiCl in a TLC chamber. The TLC plate was then exposed to X-ray film.

2.2.6 ELISA

THP-1 derived cell lines were stimulated with conditions indicated. Cytokine and chemokine levels in cell supernatants were analyzed by sandwich ELISA using antibody pairs and protocols as recommended (R&D systems).

2.2.7 Immunoprecipitation and western blot analysis

HEK293T and THP-1 cell lines, stimulated with conditions indicated, were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM NaF, 2 mM EDTA, plus a protease inhibitor cocktail (Roche). Immunoprecipitates were washed four times in lysis buffer and eluted by boiling in reducing sample buffer. Samples were fractionated by SDS-PAGE and transferred to nitrocellulose. Western blots were probed with the indicated antibodies then visualized by enhanced chemiluminescence (Pierce) and exposure to photographic film (Genesee Scientific). The films were scanned into Adobe Photoshop and whole images were adjusted for brightness. The images were cropped and formatted in Adobe Illustrator. Nuclear/cytoplasmic fractions were prepared using the Pierce NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit. The following antibodies were used: anti-Ha 12CA5 (Roche), anti-IRAK-1 (C-20), anti-NIK (H-248), anti-CagA (b-300, isotype control) (Santa Cruz Biotechnology).
2.3 Results

2.3.1 Enrichment and characterization of recombinant Monarch-1

Initial attempts to express full-length Monarch-1 protein using *Escherichia coli* and baculovirus expression systems failed to yield a sufficient quantity of soluble protein for biochemical analysis despite extensive testing and optimization of expression conditions and parameters. This is a common problem encountered in the study of this family of proteins (119). Therefore, to improve solubility, we employed a maltose binding protein (MBP) expression system, in which MBP was fused to the NBD of Monarch-1.

The NBD can be divided into a NACHT domain (NAIP, CIITA, HET-E and TP1) (14) and NACHT-associated domains (NAD) (120). The NACHT domain of NLR proteins contains well conserved nucleotide binding structures including the ATP/GTP-specific phosphate binding loop called Walker A and an Mg$^{2+}$ coordination site called Walker B (95, 121, 122). We generated a fusion protein containing MBP fused to amino acids 188-448 of Monarch-1 (Figure 2-1A). In addition to the N-terminal MBP moiety, a 6xHis tag was added to the C-terminus of the Monarch-1-NBD fusion proteins to facilitate purification by dual-affinity chromatography. The aa188-448 region of Monarch-1 comprises the NACHT domain, NAD1. We predicted this region to have nucleotide binding properties based upon: 1) the crystal structure of the related dATP binding protein Apaf-1 and, 2) a recent molecular modeling study in which the previous broadly defined NBD domain was subdivided to the NACHT domain and three subsequent NAD sequences (120).

NACHT domains consist of six $\alpha$-helices and $\beta$-sheet core structure, which contains the highly conserved Walker A and Walker B motifs responsible for nucleotide binding.
The Walker A motif contains the consensus sequence GxxGxGK[T/S], wherein the conserved lysine residue is responsible for coordination of the α and β-phosphate moieties of ATP. The Walker B motif, DxxDE, contains two well-conserved aspartic acid residues; the first of which is involved in coordination of Mg$^{2+}$ and the second aspartic acid and the third glutamate are involved ATP hydrolysis. To ensure complete disruption of nucleotide binding, mutations were introduced within both the Walker A and Walker B motifs and will be referred to hereafter as mutA/B.

Plasmids encoding the wild type (WT) or mutA/B Monarch-1-NBD fusion proteins were transformed into E. coli and were predominantly expressed as soluble proteins of approximately 80 kDa (Figure 2-1B). An affinity based amylose resin targeting MBP was used to enrich the fusion proteins and yielded a partially purified product (Figure 2-1C). Although MBP increases the solubility of its cargo protein, a large portion of the fusion protein may remain in a misfolded, high molecular weight soluble aggregate that is biochemically inactive. To separate functionally active protein from these soluble aggregates and further purify the protein, we applied the amylose resin-enriched product to a size exclusion column. Figure 2-1D depicts the FPLC elution profile of WT Monarch-1-NBD and the Coomassie blue stained SDS-PAGE gel containing the size exclusion fractions. The mutA/B Monarch-1-NBD fusion protein exhibited identical expression and chromatographic profile as the WT protein (data not shown). A large percentage of the protein (70-80%) eluted in fractions 12-14. These fractions consisted of the excluding volume of column (>700 kDa) and represent proteins existing in a high molecular weight aggregation state. Fractions 15-19 contained the Monarch-1-NBD fusion protein existing in lower molecular weight states (~80-300 kDa), which likely represented monomeric,
dimeric and trimeric complexes. Fractions 20-22 contained degradative products (Figure 2-1D). The presence of intact Monarch-1-NBD was confirmed by Western blot analysis of fractions 17-21 using antibodies against N-terminal MBP and C-terminus 6xHis (Figure 2-1E). The MBP antibody detected full-length Monarch-1-NBD protein as well as smaller molecular weight moieties. The anti-6XHis antibody only detected full-length Monarch-1-NBD proteins. This suggests that the smaller moieties detected by the MBP antibody in fractions 20-21 were likely MBP fusion proteins missing an intact C-terminus.

To determine if the gel filtration fractions contained biochemically active Monarch-1-NBD, an ATP binding assay was performed. In this assay, recombinant proteins from each fraction were incubated with radio-labeled $[^\gamma-^{35}S]ATP$, a nonhydrolysable ATP analog, and then loaded onto a nitrocellulose filter plate. Protein-bound nucleotides were trapped on the membrane while free nucleotides passed through the membrane by repeated buffer washes. Fractions 12-14, corresponding to the high molecular weight aggregates, exhibited poor ATP binding activity (Figure 2-1F). This suggests that these high molecular weight soluble aggregates of Monarch-1-NBD likely remained in a misfolded, inactive state. In contrast, ATP binding activity significantly increased in the smaller molecular weight fractions, with fractions 18 and 19 exhibiting the highest ATP binding activity. To assess if the Walker A and B motifs are required for nucleotide-binding, we performed this ATP binding assay using size exclusion chromatography fractions containing mutA/B Monarch-1-NBD. Mutations within the Walker A/B sequences dramatically reduced the nucleotide binding activity of the protein (Figure 2-1F), demonstrating their importance for Monarch-1 nucleotide binding. The greatest difference was observed in fraction 19, where the ATP-binding activity of WT Monarch-1-NBD was
5-fold higher than the corresponding mutA/B form of the protein. Together these results indicate that Monarch-1 binds ATP, and the Walker A/B motifs are required for nucleotide binding.

2.3.2 **Monarch-1 specifically binds ATP.**

To more accurately determine both the affinity and specificity of ATP binding, we further purified WT Monarch-1-NBD by taking advantage of the C-terminal 6xHis tag. Fractions 18 and 19, which demonstrated the highest level of nucleotide binding (Figure 2-1F), were pooled and applied to a cobalt-based, metal affinity purification column. The resulting product was highly enriched and migrated as a single band at ~80kDa as examined by Coomassie blue staining (Figure 2-2A). To determine the affinity of ATP binding, highly enriched WT Monarch-1-NBD was incubated with radio-labeled [γ-35S]ATP along with increasing concentrations of unlabeled [γ-S]ATP. The dissociation constant (Kd) of [γ35S]ATP binding was determined to be 100 nM (Figure 2-2B). Next we tested the nucleotide binding preference of WT Monarch-1-NBD. The fusion protein was preincubated with radio-labeled ATPγS for 2 hr and then unlabeled [γ-S]ATP, ATP, GTP, or CTP was added to compete for WT Monarch-1-NBD binding. Unlabeled [γ-S]ATP and ATP displaced radio-labeled [γ35S]ATP, while CTP and GTP had no such effect, demonstrating that WT-Monarch-1-NBD specifically binds ATP (Figure 2-2C).

2.3.3 **Recombinant Monarch-1 derived from mammalian cells binds ATP.**

The experiments described above employed WT Monarch-1-NBD fusion proteins derived from bacteria to demonstrate ATP binding activity. However, these fusion proteins lacked the N-terminal Pyrin domain and C-terminal LRR domain found in the full length protein. The crystal structure of Apaf-1 indicates that its N-terminal effector
domain folds back on the ATP binding pocket within the NBD (107). Although this does not affect dATP binding by Apaf-1, this observation led us to question if the Pyrin or LRR domains of Monarch-1 affect nucleotide binding.

To determine the influence of these domains on ATP binding activity, we purified full length Monarch-1 using a mammalian expression system. In order to enhance protein expression, we employed an Epstein - Barr virus (EBV) based episomal replication system. Sequences encoding WT and mutA/B Monarch-1 were cloned into EBV expression vectors and an N-terminal 10xHis tag and a C-terminal Flag tag were added to facilitate dual-affinity purification. Again, we were unable to obtain sufficient quantities of full length Monarch-1. However, soluble Monarch-1 protein was successfully obtained after deleting the LRR domain (Monarch-1ΔLRR) (Figure 2-3A). WT and mutA/B Monarch-1ΔLRR were first partially purified by cobalt metal affinity. The recombinant proteins in this eluate were then further purified by anti-Flag affinity chromatography. Coomassie blue staining along with anti-His and anti-Flag western blotting revealed a single band that corresponded to purified WT and mutA/B Monarch-1ΔLRR (Figure 2-3B and C).

To determine if Monarch-1ΔLRR exhibited nucleotide binding activity, an ATP binding assay was performed. As seen in the bacterial expression system, purified WT Monarch-1ΔLRR exhibited strong ATP binding activity while this binding activity was dramatically reduced in the mutA/B form of the protein (Figure 2-3D). Nucleotide competition assays determined the binding affinity to be 84 nM (Figure 2-3E). This was comparable to the binding affinity observed with the WT Monarch-1-NBD fusion protein derived from bacterial expression. In addition, similar to the bacterial-derived WT Monarch-1-NBD fusion protein, unlabeled ATP successfully competed for [γ-35S]ATP.
binding while unlabeled CTP and GTP failed to do so even at 100 fold higher concentrations.

2.3.4 Recombinant Monarch-1 derived from mammalian cells possesses ATPase activity.

To determine if WT Monarch-1ΔLRR possessed ATP hydrolysis activity, the purified protein was incubated with radio-labeled $^{32}$P-ATP and the reaction mixture was analyzed by thin layer chromatography. Whole cell lysate was used as a positive control to show the conversion of radio-labeled ATP to ADP, while bovine serum albumin, a non-ATPase, was used as a negative control. Purified WT Monarch-1ΔLRR exhibited ATPase activity as shown by the conversion of ATP to ADP (Figure 2-3G). Together, these results clearly demonstrate that Monarch-1 binds ATP and possesses ATPase activity.

2.3.5 Nucleotide binding regulates Monarch-1 self-association.

The oligomerization of NLR proteins has been shown to be important for their activity (117, 123). To determine if nucleotide binding is required for Monarch-1 self-association, full-length forms of WT or mutA/B Monarch-1 were co-transfected into HEK293T cells. Protein complexes were immunoprecipitated with anti-Flag antibody and Western blots probed with anti-Ha antibody to detect homomeric Monarch-1 complexes. As expected, WT Monarch-1 exhibited self-association (Figure 2-4 lane 3). In addition, WT Monarch-1 also associated with mutA/B Monarch-1. However, mutA/B Monarch-1 failed to associate with another mutA/B molecule. This suggests that complex formation among Monarch-1 molecules requires nucleotide binding, but that not every member of the complex must bind nucleotide. This phenomenon was also observed for the NLR protein, CIITA, suggesting it may be a common feature of NLR proteins (123).
2.3.6 Nucleotide binding regulates the ability of Monarch-1 to inhibit NIK activity.

Recently, we demonstrated that Monarch-1 suppresses activation of non-canonical NF-κB by associating with NF-κB inducing kinase, NIK. To determine the role of nucleotide binding in the formation of this molecular complex, HEK293 cells were co-transfected with NIK and full length forms of Ha-tagged WT or mutA/B Monarch-1. Cell lysates were immunoprecipitated with anti-NIK antibodies and Western blots were probed with anti-Ha antibody to detect Monarch-1. As previously reported, WT Monarch-1 co-precipitated with NIK (Figure 2-5A). Similarly, mutA/B Monarch-1 also associated with NIK, suggesting that nucleotide binding is not a requirement for complex formation in this overexpression model. To further assess the role of nucleotide binding in Monarch-NIK complex formation, THP-1 monocytes stably expressing Ha-tagged WT or mutA/B Monarch-1 were treated with CD40L to induce activation of endogenous NIK. Cell extracts were immunoprecipitated with anti-NIK antibodies and Western blots were performed to detect co-precipitating Monarch-1. As previously described, CD40L treatment enhanced the association of WT Monarch-1 with endogenous NIK (Figure 2-5B). In contrast, the association between mutA/B Monarch-1 and NIK failed to increase upon activation. Thus, while nucleotide binding is not an absolute requirement for NIK binding, it is required for activation-induced complex formation.

NIK activates noncanonical NF-κB by inducing proteolytic processing of NF-κB2/p100 to p52. This smaller active form of NF-κB2 rapidly translocates to the nucleus to regulate transcription of inflammatory genes. In contrast, the unprocessed form, p100, functions as an inhibitor of NF-κB activity (124). We recently demonstrated that the
association of Monarch-1 with NIK results in suppression of p100 processing. To
determine the role of nucleotide binding in this function, THP-1 monocytes expressing an
empty vector, WT Monarch-1 or mutA/B Monarch-1 were stimulated via TLR2 to induce
p100 expression. The cells were then treated with CD40L to induce NIK-dependent p100
processing. As previously reported, WT Monarch-1 suppressed p100 processing as
demonstrated by a sharp reduction in nuclear p52 in THP-WT Monarch-1 cells (Figure
2-5C, lane 4). Moreover, this reduction in nuclear p52 occurred in the presence of elevated
levels of cytoplasmic p100 in these cells. Finally, the accumulation of p100 in the nucleus
was consistently detected in THP-WT Monarch-1 cells, further emphasizing the role of
Monarch-1 in inhibiting p100 processing. In contrast to WT Monarch-1, mutA/B
Monarch-1 did not affect CD40L induced activation of noncanonical NF-κB, as nuclear
p52 levels were comparable to those seen in control THP-EV cells. Thus, consistent with
the inability of mutA/B Monarch-1 to bind NIK following activation, the NBD mutant did
not inhibit p100 processing. These results demonstrate that nucleotide binding is required
for Monarch-1 to suppress noncanonical NF-κB.

2.3.7 Nucleotide binding regulates the ability of Monarch-1 to inhibit
IRAK-1 activation.

In addition to NIK, we have also shown that upon TLR stimulation, Monarch-1
binds IRAK-1 and inhibits its hyperphosphorylation (92). To determine if nucleotide
binding is required for the association of Monarch-1 with IRAK-1, THP-1 cells expressing
WT or mutA/B Monarch-1 were stimulated with the TLR2 agonist, Pam3Cys4.
Endogenous IRAK-1 complexes were captured by immunoprecipitation and Western blots
performed to detect Monarch-1. In agreement with our previous findings, complex
formation between WT Monarch-1 and IRAK-1 strengthened upon TLR stimulation (Figure 2-6A). However, similar to NIK, mutA/B Monarch-1 associated more strongly with IRAK-1 in resting cells, and activation-induced association was abrogated by Walker A/B mutations. Accordingly, TLR2 stimulation resulted in the hyperphosphorylation of IRAK-1 in THP-mutA/B Monarch-1 at levels comparable to control THP-EV cells (Figure 2-6B). In agreement with our previous findings, WT Monarch-1 suppressed the accumulation of these hyperphosphorylated forms of IRAK-1 (Figure 2-6B) (93). These data confirm the requirement for nucleotide binding in Monarch-1 mediated suppression of inflammatory signaling.

2.3.8 Nucleotide binding regulates the anti-inflammatory activity of Monarch-1.

Previously, we demonstrated that Monarch-1 suppresses the production of proinflammatory cytokines and chemokines in stimulated monocytic cells (92, 93). To determine the role of nucleotide binding in this anti-inflammatory activity, THP-1 cells stably expressing full length WT or mutA/B Monarch-1 were stimulated and supernatants were applied to a cytokine antibody array (data not shown). THP-1 cells stably transfected with empty vector (THP-EV) were used as controls. In agreement with our previous reports, THP-1 cells expressing WT Monarch-1 produced lower levels of inflammatory cytokines and chemokines as compared to THP-EV control samples. These included IL-6, CXCL12 and CXCL13, which have been previously shown to be suppressed by WT Monarch-1 (92, 93). In contrast, THP-1 monocytes expressing mutA/B Monarch-1 produced increased levels of cytokines and chemokines as compared to control
samples. Of the 79 cytokines/chemokines examined, we found the greatest differences in IL-6, CXCL6, and CXCL13 and these values are reported in Figure 2-7A.

To confirm the results from the array, we performed ELISA experiments. In these experiments, THP-1 cells expressing shRNA targeting endogenous Monarch-1 (THP-shMon) were incorporated into the experiments. As expected, the expression of WT Monarch-1 resulted in decreased production of IL-6, CXCL13, and CXCL6 as compared to THP-EV cells (Figure 2-7B). In contrast, elevated levels of these cytokines/chemokines were detected in supernatants from THP-shMon cells, confirming our earlier reports demonstrating that silencing endogenous Monarch-1 results in a hyper-inflammatory response (92, 93). Similar to THP-shMon cells, THP-mutA/B Monarch-1 cells also produced increased levels of these inflammatory mediators. These results demonstrate that nucleotid binding is required for the anti-inflammatory activity of Monarch-1. In addition, these results suggest that the presence of a nucleotid binding deficient form of Monarch-1 can block the activity of endogenous Monarch-1.

2.4 Discussion

Despite the general perception that members of the NLR family function as nucleotid-binding proteins, this property has not been extensively studied. Among plant R proteins, one group has shown data derived from R proteins that demonstrate nucleotid binding properties of these proteins (108). Furthermore, despite this lack of convincing data, the assumption has been that nucleotid binding is required for the function of these proteins. This concept is largely modeled after the analysis of Apaf-1 (125). Thus, a key finding in this report is that highly enriched Monarch-1 specifically binds ATP and this is
required for its anti-inflammatory activity. Monarch-1 is also unique in that it functions as a brake of innate immune activation, as opposed to several of the other NLR proteins.

The requirement of nucleotide binding for Monarch-1 activity supports a recent report by our group that focused on the NLR protein, cryopyrin/NLRP3. In that report, we demonstrated that NLRP3 binds ATP and this binding activity is required for NLRP3-mediated IL-1β processing (118). Prior to these studies, only two other reports suggested nucleotide binding as a regulatory step in NLR function. An earlier report from our group suggested that CIITA binds GTP and mutations within the Walker A/B motifs block CIITA-mediated transcriptional activation (110). In that study, however, nucleotide binding assays were performed by analyzing CIITA proteins that had been immunoprecipitated from transfected cells. Since CIITA forms large protein complexes, the nucleotide binding activities of co-precipitating proteins could not be ruled out. More recently, GST-fusion proteins containing the NBD of the NLR protein Ipaf were shown to bind ATP (119). Mutations with the Walker A motif of Ipaf result in reduced caspase-1 activity. However, similar to the CIITA study, protein purity remains an issue in this study.

In this report, we produced highly enriched Monarch-1 fusion proteins derived from both prokaryotic and eukaryotic expression systems to conclusively demonstrate the nucleotide binding activity of Monarch-1. The importance of Monarch-1 nucleotide binding in suppressing NF-κB activation and inflammatory signaling has been clearly demonstrated in this report. Expression of a nucleotide binding deficient form of Monarch-1 (mutA/B Monarch-1) in monocytes resulted in dramatically increased production of proinflammatory mediators. These levels were comparable to those observed in THP-shMon cells in which endogenous Monarch-1 expression was silenced.
This hyper-inflammatory phenotype of cells expressing mutA/B Monarch-1 correlates well with the inability of this mutant to suppress NF-κB2/p100 processing and the inability of this mutant to efficiently bind NIK and IRAK-1 following stimulation.

Interestingly, an association was detected between these kinases and mutA/B Monarch-1 in resting cells. This suggests that the mutations within the Walker A/B sequences are subtle enough not to disrupt complex formation in resting cells but render Monarch-1 unable to bind these kinases upon stimulation. This loss of activation-induced NIK and IRAK-1 binding by mutA/B Monarch-1 may be due to its inability to form homomeric structures, a property required for the activity of CIITA, APAF-1 and NALP3. A second possibility relates to the functional dissection of ATP binding and ATP hydrolysis. For instance, it has been reported that ATP binding is required for the activity of the plant R protein I-2, yet the hydrolysis of this bound ATP moiety suppresses I-2 function (108, 109). Thus, binding and hydrolysis ATP may represent an on/off switch of NLR protein function. Future studies will be conducted to fully characterize the ATP hydrolysis cycle of Monarch-1 and to determine its contribution to the anti-inflammatory role of Monarch-1.

Despite exhaustive attempts to optimize conditions, we were not able to produce sufficient quantities of soluble, full-length purified protein. Instead, we generated recombinant Monarch-1 in mammalian cells by deleting the LRR domain. Thus, while it is clear that Monarch-1 binds ATP, it remains uncertain whether the LRR domain regulates this activity. In our recent report describing ATP binding by cryopyrin, purified proteins contained intact LRR sequences, and these domains did not affect ATP binding. Therefore,
due to the sequence similarities between Monarch-1 and NALP3, it is unlikely that the LRR domain disrupts nucleotide binding.

The nucleotide binding property of NLRs lends these proteins to pharmacologic intervention, as there are abundant nucleotide and nucleoside analog libraries available for drug screening. Thus, the possibility now emerges to screen for nucleotide analogs that can bind to Monarch-1, cause sustained blockage of its function, and potentiate innate immune responses. In summary, our work demonstrates that Monarch-1 is an ATP binding protein and this ATP binding activity is essential for Monarch-1 to perform its inhibitory role in innate immune signal.

2.5 Acknowledgments

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Figure 2-1 The generation and purification of Monarch-1–NBD fusion proteins
Figure 2-1 **The generation and purification of Monarch-1-NBD fusion proteins.** (A) Schematic diagram of domain structure of full length Monarch-1 and the WT and mutA/B Monarch-1-NBD fusion proteins. The positions of the Walker A and Walker B motifs are depicted as solid bars within the NACHT domain. The conserved amino acids within both motifs are shown and the underlined residues were substituted with alanine by site-directed mutagenesis. (B) The pMAL-c2E vector encoding WT and mut A/B Monarch-1-NBD were transformed into *E. coli* strain Rosetta origami B. Soluble bacterial extracts before and after IPTG induction were analyzed by SDS-PAGE and Coomassie blue staining. *Arrowhead*, Monarch-1-NBD fusion protein; *U*, uninduced; *I*, IPTG-induced. (C) Bacterial lysates were passed over an amylose resin column targeting MBP. Eluted proteins were resolved by SDS-PAGE and visualized with Coomassie blue stain. (D) Eluates from the amylose resin column were further purified by size exclusion chromatography. The absorbance profile of size exclusion purification is depicted (upper panel) and the size exclusion fractions were analyzed by SDS-PAGE and visualized by Coomassie blue staining (lower panel). (E) Western blots from size exclusion fractions 17-21 were probed with an anti-MBP antibody to verify the intactness of the N-terminus and anti-His to determine the intactness of the C-terminus of the Monarch-1-NBD fusion protein. (F) Size exclusion fraction from WT or mutA/B Monarch-1-NBD were tested for ATP binding activity using [γ-32S]ATP and specific binding was normalized to the protein concentration of each fraction.
Figure 2-2 Purified Monarch-1-NBD fusion protein specifically binds ATP
Figure 2-2 Purified Monarch-1-NBD fusion protein specifically binds ATP. (A) Gel filtration fractions 18-19 (Fig.1) were pooled and further purified by cobalt metal affinity chromatography. Eluates were concentrated and the buffer exchanged over a PD10 column. The purity of the final purification product was assessed by SDS-PAGE followed by visualization with Coomassie blue stain. Arrowhead, Monarch-1-NBD fusion protein. (B) Homologous competition assays were performed to assess the ATP binding affinity of Monarch-1-NBD. (C) Specificity of ATP binding was determined by incubating Monarch-1-NBD fusion protein with [γ-32S] ATP and 10 μM of the indicated unlabeled competitor nucleotide.
Figure 2-3 Mammalian cell-derived recombinant Monarch-1ΔLRR binds and hydrolyzes ATP
Figure 2-3 Nucleotide binding is required for Monarch-1 to suppress NIK-mediated p100 processing. (A) HEK293T cells were co-transfected with NIK and Ha-tagged WT or mutA/B Monarch-1. Cell lysates were immunoprecipitated with anti-NIK antibodies and western blots probed with anti-Ha antibodies to detect Monarch-1. (B) THP-WT or THP-mutA/B cells were stimulated with 250 ng/ml CD40L for indicated times. Endogenous NIK was immunoprecipitated with anti-NIK antibodies and western blots probed with anti-Ha to detect co-precipitating Monarch-1. Control immunoprecipitations were performed with an isotype control antibody to monitor specificity. Control western blots were performed to monitor expression of Monarch-1 and NIK in cellular lysates. (C) THP-EV, THP-WT or THP-mutA/B cells were stimulated with 200 ng/ml Pam3Cys4 for 18 h to induce p100 expression. The cells were then treated with 250 ng/ml CD40L for an additional 5 h to induce p100 cleavage to p52. Cells were fractionated into nuclear and cytoplasmic fractions and proteins from each fraction were separated by SDS-PAGE. Western blots were probed with anti-p100 to detect p100 and its cleaved form, p52. Anti-Ha was used to monitor Monarch-1 expression.
Figure 2-4 Nucleotiding binding is required for Monarch-1 self association.
Figure 2-4 Mammalian cell-derived recombinant Monarch-1ΔLRR binds and hydrolyzes ATP. (A) Diagram depicting full length Monarch-1 and the WT and mutA/B Monarch-1ΔLRR proteins produced in HEK293EBNA cells. (B) Soluble extracts of HEK293EBNA cells expressing WT and mutA/B Monarch-1ΔLRR were enriched for Monarch-1ΔLRR using a cobalt metal affinity column. Eluates were resolved by SDS-PAGE, and visualized with Coomassie blue staining (lanes 1-2). The cobalt eluate was further purified over an anti-Flag affinity matrix and the purity was evaluated by Coomassie blue staining (lanes 3-4). (C) The double purified Monarch-1ΔLRR proteins were analyzed by western blot using anti-His antibody and anti-Flag antibody. (D) ATP binding activity of purified WT and mutA/B Monarch-1ΔLRR was determined by incubating 500 ng purified protein with 90 nM [γ-32S]ATP. Error bars represent the standard deviation of ATP binding measurements in triplicate. (E) The ATP binding affinity of WT-Monarch-1ΔLRR was determined by homologous competition binding assays. (F) The nucleotide binding preference of WT-Monarch-1ΔLRR was determined by incubating WT-Monarch-1ΔLRR with [γ-32S]ATP and increasing concentrations of unlabeled nucleotide. (G) The ATPase activity of purified WT-Monarch-1ΔLRR was measured by visualizing the conversion of 32P-ATP to 32P-ADP by thin layer chromatography followed by autoradiography. HEK293EBNA lysate and purified bovine serum albumin (BSA) were used as positive and negative controls, respectively. Arrowhead, Monarch-1-NBD fusion protein.
Figure 2-5 Nucleotide binding is required for Monarch-1 to suppress NIK-mediated p100 processing.
Figure 2-5 Nucleotide binding is required for Monarch-1 to suppress NIK-mediated p100 processing. (A) HEK293T cells were co-transfected with NIK and Ha-tagged WT or mutA/B Monarch-1. Cell lysates were immunoprecipitated with anti-NIK antibodies and western blots probed with anti-Ha antibodies to detect Monarch-1. (B) THP-WT or THP-mutA/B cells were stimulated with 250 ng/ml CD40L for indicated times. Endogenous NIK was immunoprecipitated with anti-NIK antibodies and western blots probed with anti-Ha to detect co-precipitating Monarch-1. Control immunoprecipitations were performed with an isotype control antibody to monitor specificity. Control western blots were performed to monitor expression of Monarch-1 and NIK in cellular lysates. (C) THP-EV, THP-WT or THP-mutA/B cells were stimulated with 200 ng/ml Pam3Cys4 for 18 h to induce p100 expression. The cells were then treated with 250 ng/ml CD40L for an additional 5 h to induce p100 cleavage to p52. Cells were fractionated into nuclear and cytoplasmic fractions and proteins from each fraction were separated by SDS-PAGE. Western blots were probed with anti-p100 to detect p100 and its cleaved form, p52. Anti-Ha was used to monitor Monarch-1 expression.
Figure 2-6 Nucleotide binding by Monarch-1 is required for the suppression of IRAK-1 hyperphosphorylation.
Figure 2-6 Nucleotide binding by Monarch-1 is required for the suppression of IRAK-1 hyperphosphorylation. (A) THP-WT or THP-mutA/B cells were stimulated for the indicated times with 200 ng/ml Pam3Cys4. Endogenous IRAK-1 was immunoprecipitated with anti-IRAK-1 antibodies and western blots were probed with anti-Ha to detect Monarch-1. Control samples were immunoprecipitated with an isotype matched antibody. Control western blots were performed on cellular lysates to monitor the levels of Monarch-1 and IRAK-1. (B) THP-EV, THP-WT and THP-mutA/B cells were stimulated with 200 ng/ml Pam3Cys4 for the indicated times. Lysates were separated by SDS-PAGE and western blots probed with anti-IRAK-1 antibodies. Control western blots were performed and probed with anti-Ha to monitor Monarch-1 expression.
Figure 2-7 Nucleotide binding is required for Monarch-1-mediated suppression of proinflammatory cytokine and chemokine production.
Figure 2-7 Nucleotide binding is required for Monarch-1-mediated suppression of proinflammatory cytokine and chemokine production. (A) THP-1 cells expressing empty vector (EV), full length WT Monarch-1 (WT) or full length mutA/B Monarch-1 (mutA/B) were stimulated with 200ng/ml Pam3Cys4 for 18 h and then 250 ng/ml CD40L for an additional 5 h. Supernatants were applied to a RayBiotech cytokine/chemokine array. The values for the three cytokines/chemokines that demonstrated the most dramatic differences among the cell lines are shown as fold induction over unstimulated samples. (B) THP-EV, THP-WT, THP-mutA/B and THP-shMon were stimulated as described above. Cell culture supernatants were harvested and cytokine/chemokine levels determined by ELISA.
Chapter 3  Investigating the role of conserved NBD motifs in mediating Monarch-1 function
Abstract

The recently discovered Nucleotide Binding Domain-Leucine Rich Repeat (NLR) gene family has emerged as key player in the induction and modulation of the innate immune response. This family is defined by a central nucleotide binding domain that contains 9-12 conserved motifs including Walker A and Walk B, which has been demonstrated to play a vital role in mediating nucleotide binding activity. In the previous chapter, I examined the nucleotide binding property and its biological function in NLR protein, Monarch-1. In this chapter, I sought to determine the biological function of the remaining conserved regions within the NBD domain of Monarch-1. Nine THP-1 cell lines stably expressing WT and Monarch-1 conserved NBD domain mutants have been established and their negative regulatory role of cytokine/chemokine secretion has been examined. Among all conserved motifs, Walker A (motif 1), Walker B (motif 2), Motif 6 and Motif 9 have been identified to play a critical role in mediating the negative regulating cytokine/chemokine IL-6, BLC and GCP-2. This result further highlights the role of nucleotide binding in the in vivo function of Monarch-1 and reveals novel functional motifs in the NBD domain of NLR proteins.


3.1 Introduction

The newly discovered NLR protein family is critically involved in mediating inflammatory response against a variety of pathogens (7, 8). A number of NLRs function as intracellular PAMP sensor. Upon directly or indirectly engagement by their cognate agonists, NLRs swiftly activate NF-κB signaling pathway leading to the secretion of proinflammatory cytokines such as TNF-α and IL-6. The activation of some NLRs results in the formation of a multiprotein complex called 'inflammasome' (47), which eventually leads to the activation of caspase-1 and IL-1β and IL-18 secretion. The activation of NLRs also elicits a host cell death program manifested by either pathogen induced apoptosis or necrosis. Finally, a group of NLR proteins have negative regulatory roles in the control of inflammatory molecules such as chemokines and interferons.

One of the hallmarks of the NLRs is the central localized nucleotide binding domain that is also named as NACHT (14) or NOD domain. The NBD domain itself is highly homologous to an ancient AAA+ ATPase family, which has been shown to exert diverse biological functions (94). The major mechanism by which the AAA+ ATPase functions is its oligomerization upon ATP binding and hydrolysis. However, the precise biochemical action steps from ATP binding/hydrolysis to oligomerization have not been fully characterized (97).

Given sequence similarity between NLR protein and AAA+ ATPase family, the NLRs have been presumed to bind/hydrolyze nucleotide since the indetification of its first family member, CIITA. However, the definitive biochemical proof of nucleotide binding of NLR family has only recently provided by our group and others (118, 119). The nucleotide binding of NLR protein is predicated to result in a conformational change that eventually leads to the oligomerization of the protein. This notion is largely modeled on the mechanism of Apaf-1
mediated Apoptosome formation. Apaf-1 is a critical mediator of intrinsic apoptosis pathway and possesses a central nucleotide binding domain called NB-ARC that is also belongs to the AAA⁺ ATPase super family. Upon binding its cognate ligand cytochrome C, Apaf-1 rapidly hydrolyze its bound (d)ATP. This process results in a conformational change that eventually leads to the formation of heptomeric structure named Apoptosome. Apoptosome recruits and activates caspase-9, a key caspase in mediating intrinsic apoptosis pathway. Largely analogous to this process, a group of activated NLR protein also has been shown to oligomerize into a large molecular complex termed ‘inflammasome’, and we have shown that nucleotide binding/ hydrolysis is important in mediating this oligomerization process ((118) and chapter 2).

The difficulties in the generation and purification of recombinant NLR severely impeded the structural study of NLR protein. However, the newly identified Apaf-1 crystal structure has provided us a suitable template to perform molecular modeling of the NBD domain of NLR. The NBD-ARC domain of Apaf-1 contains an ATP binding α/β fold followed by three distinct helices domains. Based on the sequence similarity between Apaf-1 and NLRs, the NBD domain of NLR has also been bioinformatically divided into a NATCH domain and three NAD domains, which correspond to the respective NBD domains in Apaf-1 (120). Additionally, there are nine to twelve evolutionary conserved motifs encoded by the exon that contains the NBD (6, 102). In the previous chapter, we studied the function of Walker A and Walker B, however the function of the remaining sequence motifs remain largely elusive. In this report, we sought to use NLR protein Monarch-1 as an example to elucidate the biological significance of its conserved motifs. This study provides us insight
regarding the structure-function relationship of NLR NBD domain and facilitates the understanding of biochemical mechanism of NBD domain function.

3.2 Materials and methods

3.2.1 Multiple sequence alignment of NLR NBD domains

The protein sequences of selected NLR proteins were obtained from Genebank using the following accession numbers: CIAS1, NP_00488; Monarch-1, NP_653288; CIITA, NP_000237; NALP1, NP_127497; NALP2, NP_060322; NALP6, NP_612202; NALP7, NP_996611; NOD1, NP_006083; NOD2, NP_071445; The alignment was performed by using the Align module of the Vector NTI 10 (Invitrogen) software that is based on the CLUSTAL W multiple sequence alignment algorithm. The alignment of NBD domain of selected NLR proteins were further manually edited by GeneDoc (ver 2.6.003).

3.2.2 Cell lines and reagents

The TLR2 agonist, the synthetic lipoprotein S-[2,3-bis-(palmitoyloxy)-2(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys-4-OH trihydrochloride (Pam3Cys4) was obtained from Invivogen. CD40L was obtained from PeproTech. THP-1 cell lines shRNA targeting Monarch-1 (THP-shMon) have been described (92, 93).

3.2.3 Generation of Monarch-1 NBD mutants

To generate Monarch-1 NBD mutants, a retroviral vector pHSPG (kindly provided by Dr. Lishan Su, University of North Carolina) encoding an HA-tagged Monarch-1 was subjected to site-directed mutagenesis by Quick-change site-directed mutagenesis kit (Strategene). The mutated motifs and its amino acid sequences are shown in Figure 3-1. The mutagenesis primers for each motifs are: Motif 1, Forward: CGC GGC AGG GAT AGC CGC CGC CAT GCT GGC ACA C; Reverse: GTG TGC CAG CAT GGC GGC GGC TAT
Motif 2, Forward: CTC TTC TAC ATC AAC GCC GCC GCC ATG
AAC CAG AGT G; Reverse: CAC TCT GTT TCA TGG CGG CGT TGA TGT AGA AGA G;
Motif 3: Forward: CTT TTC ATC ATC GCC GCC GCC TTC GCT GCC CTC AAG
CCT TCT; Reverse: AGA AGG CTT GAG GGC AGC GAA GCC GGC GAT GAT GAA AAG;
Motif 4: CTA TCT TTG CTC ATC GCC GCA GCC CCC ACG GCT TTG G;
Reverse: CCA AAG CCG TGG GGG CTG CGG CGA TGA GCA AAG ATA G; Motif 5,
Forward: GGA GAT CCT GGG CGC CGC TGC CGC AGA AAG GG;
Reverse: CCT TCC TTT CTG CGG CAG CGG CGC CCA GGA TCT CC;
Motif 6, Forward: CTT CAC CAT GTG CGC CGC AGC CCT GGT GTG CTG G;
Reverse: CCA GCA CAC CAG GGC TGC GGC GCA CAT GGT GAA G;
Motif 7, Forward: CAG ACG TCC AGG GCC GCC GCT GCA GTG TAC ATG;
Reverse: CAT GTA CAC TGC AGC GGC GGC CCT GGA CGT CTG;
Motif 8, Forward: GGG TTG TGC TCC TTG GGG CTC TGG AAT CAG;
Reverse: CTG ATT CCA GAG CCC CAA GGA GCA CAA CCC;
Motif 9, Forward: CAT CCA CTT GAG TGC CGC CGC ATT CTT TGC AGC TAT G;
Reverse: CAT AGC TGC AAA GAA TGC GGC GGC ACT CAA GTG GAT G;
PCR reactions were preformed by using PFU turbo DNA polymerase (Stratagene).
The PCR products were digested by DpnI for 1 hour and transformed into XL1-blue supercompetent cells (Stratagene). All mutants were confirmed by DNA sequencing.

### 3.2.4 Generation of retrovirus

The retroviral vectors encoding WT and mutants HA-Monarch-1 were cotransfected with pVSV-G and pGag-Pol into HEK293T cells by CaPO4 precipitation. Forty eight hours after transfection, the virus containing supernatants were harvested and concentrated by ultracentrifugation (20,000 rpm, sorvall 5Ti) for 3 hours at 4 °C. The virus pellet were
resuspended in culture medium RPMI1640 plus 10% FBS and stored at -80°C. Retrovirus stock was titrated by FACS analysis based on the GFP (Green fluorescence protein) expression upon infection of HEK293T cells. The titer of concentrated retrovirus was typically at 1x10^7/pfu.

3.2.5 Infection of THP-1 by retrovirus.

Human monocytic THP-1 cell line (ATCC TIB-202) were cultured in RPMI 1640 supplemented by 10% fetal calf serum, 2 mM L-glutamine, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and streptomycin-penicillin and were grown at 37 °C with 5% CO₂. THP-1 plated in 6-well culture plate (5x10^5 cells/well) were infected with 100 μl retroviral supernatant (MOI=2) and 8 μg/ml polybrene (Sigma). Seventy-two hours post infection, the GFP positive cells were FACS (Fluorescent-activated cell sorting) (MolFlow, Dako cytation) sorted and further expanded. The purity of retroviral infected THP-1 cells was evaluated by GFP expression by FACS analysis (FACS Calibur, Beckman Dickson).

3.2.6 Western blot

THP-1 cells were lysed in 50mM Tris.HCl (PH7.6), 150mM NaCl, 2mM EDTA, 1x Complete protease inhibitor cocktail (Roche). The lysate were separated on 4-12% gradient mini polyacrylamide gel (Invitrogen) and blot on nitrocelluse membrane. The HA-tagged Monarch-1 were blotted with Anti-HA HRP (12CA5) (Roche) and visualized by pico Western blot substrates (Pierce).

3.2.7 ELISA

Human chemokine BLC, GCP-2 and cytokine IL-6 ELISA (R&D systems) were preformed according to the manufacture’s procedure.
3.3 Results

3.3.1 Multiple alignment of NLR NBD domain

Based on the recently elucidated crystal structure of WD40 truncated Apaf-1, the NBD-ARC domain of Apaf-1 contains four subdomain structures: \(\alpha/\beta\) sandwich fold, Helix I domain, Winged Helix domain and Helix II domain. Based on the sequence similarity, the NBD or the previous NATCH domain of NLR proteins is further divided into four subdomains: NATCH, NAD1, NAD2, NAD3 that are correspondent to the Apaf-1 subdomains respectively. To identify the conserved motifs within the NLR domain and to map those conserved motifs on the newly designated subdomain structure, we preformed a multiple sequence alignment of 9 selected NLR family proteins. Of these selected proteins, nine proteins belong to previously named NALP family that contains N-terminal pyrin domain, currently renamed NLRP. Those proteins were CIAS1, NLRP12, NARP1, 2, 6, and 7. The other four proteins carry either the N-terminal CARD domain (NOD1, NOD2) or Transcriptional activator (CIITA). As shown in Figure 3-1, at least nine conserved motifs are found in the NBD domain of NLR. In those nine conserved motifs, five motifs are mapped in the NACHT domain that is predicted to adopt the \(\alpha/\beta\) sandwich fold. The first domain is well characterized Walker A motif that has an extremely conserved sequence: GxxGxGK[S/T]. The lysine residue of Walker A is shown to coordinate the \(\beta\) and \(\gamma\)-phosphate moiety of ATP. The third domain is the Walker B motifs that contains a conserved sequence: hhhhhhDxx[D/E]. The first aspartic acid residue typically coordinate the \(\text{Mg}^{2+}\) and the second aspartic acid or glutamate acid plays an important role in facilitate ATP hydrolysis. Interestingly, CIAS1 disease associated mutant, D303N maps to the second aspartic acid implying the ATP hydrolysis may plays an important role in CIAS1 associated disease pathogenesis. The
biochemical functions of Motifs 2, 4, 5 are not well understood. However, several disease-associated mutants are mapped in these conserved areas. For example, disease-associated CIAS1 mutant R260W and NOD2 disease associated mutant R334W/Q are both located in the Arginine residue of Motif 2 indicating this region is critical for the function of these proteins. The NAD1 subdomain consists of two motifs and the NAD2 domain contains the rest of two motifs. We did not find significantly sequence homology within the NAD3 domain.

3.3.2 Generation of mutants for conserved motifs in Monarch-1 NBD domain

To further gain insight of the biological functions of NBD motifs, we used NLR protein Monarch-1 to test the function of NBD motifs. Previously, we have shown that Monarch-1 functions as a negative regulator in both canonical and non-canonical NF-κB signaling pathways. Furthermore, we also revealed that purified recombinant Monarch-1 binds and hydrolyzes ATP while protein bearing mutations in both Walker A and Walker B failed to bind ATP. The binding of ATP is critical for the negative regulator function of Monarch-1 as the Walker A and B double mutant functions as a dominant negative mutant. In this report, we sought to further characterize the biological functions of other conserved motifs in the Monarch-1 NBD domain. We introduced triple alanine substitution to the key residues of Monarch-1 NBD motifs shown in Figure 3-1. We then generated retrovirus encoding the corresponding mutants and infected human THP-1 monocytic cell line. The initial infection resulted in approximately 10-30% GFP positive THP-1 cells (Data not shown). We then employed the FACS sorting to further purify the GFP\(^+\) THP-1 cells from GFP negative non-infected parental cell lines. After the expansion of sorted GFP positive
cells, we examined the GFP expression. As shown in Figure 3-2A, all 10 cell lines tested contain >98% GFP positive cells, thus indicating our procedure generated a near homogenous populations that express wild type or mutant Monarch-1. THP-1 cell lines containing Monarch-1 mutant were further expanded and they did not show apparent changes in morphology and growth rate (data not shown). To evaluate the expression of wild type and mutant Monarch-1 in the THP-1 cells, we used western blot analysis to visualize the HA-tagged Monarch-1. As shown in Figure 3-2B, all wild type and mutants were successfully expressed in the stable THP-1 line.

3.3.3 The biological function of Motifs in the NBD domain of Monarch-1

To investigate the biological function of the mutants, we tested the production of cytokine IL-6, chemokines GCP-2 and BLC from both wild type and mutant Monarch-1 expressing THP-1 cells line upon TLR2 and CD40ligand stimulation. We have previously shown that Monarch-1 inhibits the non-canonical NF-κB pathway by affecting NIK stability. The negative effect of Monarch-1 results in the decreased secretion of IL-6, GCP-1 and BLC upon the activation of NF-κB pathway. In the current study, the wild type and all NBD mutant of Monarch-1 were stimulated with TLR2 agonist Pam3Cys4 and CD40L, 48 hour later, the culture supernatant were assayed for the cytokine and chemokines as indicated above. We found that over-expression of Monarch-1 wild type resulted in the reduction of IL-6, GCP-1 and BLC. Reducing endogenous Monarch-1 by shRNA mediated gene ablation led to a marked increased of assayed cytokine and chemokines (Figure 3-3). This result confirms the negative regulator function of Monarch-1.

A testing of the Monarch-1 NBD mutants show that overexpression of Mutant 1 (walker A), 3(Walker B), 6 and 9 resulted a significant increase of IL-6, GCP-1, and BLC
secretion to a extent that was comparable to that of shRNA expression THP-1(Figure 3-3). This result indicates that the mutation of Walker A, Walker B, motif 6, motif 9 not only resulted in a non-functional Monarch-1 but also dominant negative Monarch-1 mutants that interfere with the function of endogenous wild type Monarch-1. Furthermore, the critical roles of NBD motif 6 and 9 for mediating NLR function have not been recognized previously. Taken together, We identified that Walker A (motif 1), Walker B(motif 2), Motif 6 and Motif 9 are critical function structures in the Monarch-1 NBD domain.

3.4 Discussion

In this report, we examined the biological function of nine conserved motifs in the NBD domain of NLR protein Monarch-1. Our previous report indicated that overexpression of Walker A and Walker B double mutant leads to a dominant negative mutant phenotype. In this study, the Walker A or Walker B single mutant was revealed to behave similarly to the Walker and Walker B double mutant. This results implies that severe disruption of either Walker A or Walker B by triple alanine substitution is likely sufficient for the disruption of nucleotide binding. This result was in line with a previous report showing that triple alanine substitution of either Walker A or Walker B motif within the context of the CIAS1/cryopyrin gain-of-function mutants completely abolished the gain-of-function phenotype associated with autoinflammatory diseases. This result also agrees with the finding from a random mutagenesis based study on NOD2 protein, in which the Walker B mutant D379A (the first aspartic acid in Walker B motif) result a loss-of function phenotype (126). Taken together, these results conclusively demonstrated the requirement of ATP binding in NLR function. One caveat of current study is the alanine substitution of Walker A and Walker B mutant did not separates the ATP hydrolysis function from ATP binding. It is of interest to generate a
Monarch-1 mutant that retains ATP binding but losses ATP hydrolysis activity (creating an ATP trap). Such mutant will enable us evaluate the role of ATP hydrolysis in NLR function. It has been shown that the main function of the second aspartic acid of Walker B motif is to attack the water molecular but not ATP binding \textit{per Se}. By selectively mutating this residue, we might create a NLR protein that retains ATP binding but losses ATP hydrolysis activity.

In addition to the Walker A and Walker B, we further revealed that the Motif 6 and 9 is critical for Monarch-1 function. However, the biochemical functions of those motifs are largely unknown. According to a molecular modeling study, the NAD1 and NAD2 are likely folded back on the nucleotide binding core structure (116). It is highly likely that the key residues of NAD1 and NAD 2 domain motifs indirectly contact with bound ATP molecule, therefore stabilize or facilitate the ATP binding/hydrolysis. To test this possibility, the nucleotide binding activities of these mutants have to be measured in vitro. Another possibility is that NAD1 and NAD2 domains have no impact in nucleotide binding, but facilitate or mediate the ATP driven conformation change. The proof of this hypothesis awaits the detailed structural study due to negative functional nature of Monarch-1, our experiment design only selects the dominant negative mutants. The recessive mutant and gain-of-function mutant likely evade our screen strategy. Therefore, further study in evaluating the NBD conserved motifs shall be perform using NLRs such as NLRP3/CIAS1 whereas a positive effect can be easily monitored.
Figure 3-1 The NLR NBD domain structure and multiple sequence alignment.
Figure 3-1. The NLR NBD domain structure and multiple sequence alignment. The homology of NBD domain of selected NLR proteins were aligned using CLUSTAL W multiple sequence alignment algorithm. The black boxes indicate identical residues, the three levels of grey box, which range from dark grey to light grey, depicts residues with most similarity to residues with less similarity. Numbers to the right of the sequences indicate the positions of the last residue shown. Amino acids are showed in the single-letter (code). Text labels denote the function of key residues within the motifs and disease associated mutants. The position of key residues that were mutated to alanine in the Monarch-1 protein are also denoted. The distribution of nine motifs in the Nucleotide binding domain of NLR is depicted in the domains structure diagram. CIAS, cold-induced autoinflammatory syndrome; CINCA, chronic infantile neurologic cutaneous articular; MWS, Muckle-wells syndrome; FCAS, familia cold autoinflammatory syndrome.
Figure 3-2 Generation of THP-1 cells expressing Monarch-1 wild type and mutants.
Figure 3-2  Generation of THP-1 cells expressing wild type and Monarch-1NBD mutants. THP-1 cells were infected by retrovirus encoding wild type and NBD mutants. 3 days post infection, the GFP positive cells were FACS sorted and further expanded. A. the GFP expression of wild type and Monarch-1 NBD mutants stably expressing cells lines. B. Western blot analysis of THP-1 expressing wild type and NBD mutant Monarch-1 using anti-HA antibody.
Figure 3-3 Motifs 1, 3, 6, 9 are required for Monarch-1 mediated suppression of proinflammatory cytokine and chemokine production.
Figure 3-3  THP-1 cells expressing empty vector (Mock), full length WT Monarch-1 (WT), full length Monarch-1 NBD mutants (Motif 1-9 were assigned as M1-9) and THP-1 shRNA Monacrch-1 Knock-down cells were stimulated with 200ng/ml Pam3Cys4 for 18 h and then 250 ng/ml CD40L for an additional 5 h. Cell culture supernatants were harvested and cytokine/chemokine levels determined by ELISA.
Chapter 4  Conclusions and future directions
The newly discovered NLR/CATERPILLER gene family has emerged as a key set of genes that plays a vital role in mediating the innate immune response. By the combination of traditional molecular biology, biochemical and genetic tools such as DNA microarray, yeast two-hybrid, co-immunoprecipitation, gene knockout as well as newly emerged experimental methods including proteomics, siRNA or shRNA based gene ablation, great strides have been made in the last several years in understanding the biological functions of NLR proteins. NLR proteins functions as intracellular bacteria sensor to detect the invading pathogen derived products and to activate a number of proinflammatory signaling pathways including NF-κB signaling pathway. A group of NLRs responds to pathogen-derived products or -induced perturbations and activates caspase-1 leading to the secretion of IL-1β. In addition, NLRs also elicits a rapid cell death program manifested as either apoptosis or necrosis. Finally, several NLRs have been shown to act as negative regulators of inflammatory response by dampening the proinflammatory cytokines and chemokine releases.

The defining feature of NLR protein is the central localized nucleotide binding domain that is extremely conserved through the evolution. The nucleotide binding of NLR proteins is thought to play a key role in their oligomerization and complex formation. However, until the start of this thesis study, definitive evidence for the nucleotide binding capacity of NLR protein has not been shown. The biological role of nucleotide binding also remained elusive. This thesis study aims to characterize the biochemical property of nucleotide binding by the NLR protein, Monarch-1, and to examine the role of nucleotide binding in Monarch-1 function. The results from this study along with a study from a colleague in the lab, demonstrate the nucleotide binding activity of NLR proteins.
These studies show the critical roles of nucleotide binding in regulating the function of NLR proteins.

The daunting difficulties in the generation and purification of NLR protein impede the biochemical and structural study of NLR protein for years. The complex domain structure and pro-oligomerization nature of NLR protein often render the protein insoluble during the generation of recombinant proteins. Through the course of the study, several methods suitable for the generation and purification of NLR proteins have been successfully developed. The MBP fusion protein approach coupled with dual affinity purification strategy is robust and reliable to generate large amount of NLR NBD fusion proteins for the study of nucleotide binding property. The caveat of this approach is that the size restriction of MBP fusion partner precludes the study of full length protein. On the other hand, the episomal based mammalian systems (293EBNA) system generates longer protein product but less quantity than that of the MBP fusion protein systems. Although the tour de force effort has been made to generate and purify the full-length Monarch-1, the result is still disappointing due to the insolubility and cytotoxicity issues. Future studies shall explore novel methods and purification strategies to overcome these challenging problems. Alternatively, we shall not abandon the traditional biochemical purification strategies that aim to purify endogenous protein/complex. With current technological advances in large-scale mammalian cell culture, it is feasible to obtain 10-50 liters of mammalian cell culture (i.e. THP-1 cells). Thus, the purification of NLR protein/complex by traditional biochemical strategy is in the foreseeable future.

In this thesis study, solid evidence demonstrating the nucleotide binding property of NLR protein has been provided. However, several remaining issues still need
to be further addressed in future studies. First, we do not have evidence to demonstrate the prebound status of nucleotide in NLR proteins. Insect cell-derived Apaf-1 is prebound by dATP. It is of interest to examine if this is the case for the NLR proteins. Solving this issue needs a large amount of protein and a sensitive assay such as mass spectrometry to detect the trace amount of prebound nucleotide. Second, the ATPase properties and ATP hydrolysis cycle of NLR protein needs to be fully characterized. The basic enzyme properties such as Vmax and Km needs to be measured. Third, the nucleotide binding activity and the role of nucleotide binding of the NLR protein complex in lieu of a single NLR protein awaits to be examine in *in vitro* reconstituted protein complex. Finally, even though large amount of full length NLR proteins is difficult to obtain, it is still feasible to determine the subdomain structure of NLR protein. The structural study will provide us invaluable knowledge in understanding the structure-function relationship of NLR protein.

Current study examined the biological role of nucleotide binding in NLR function by over-expressing NLR wild type or mutant protein in cell lines. The caveat of this strategy is that the amount of expressed protein often largely exceeds the endogenous protein, thus this artificial condition may not recapitulate physiological conditions. One approach to overcome these drawbacks is to generate BAC transgenic mice bearing the NLR nucleotide binding mutant and/or disease-associate mutants. In light of the current progress of BAC recombineering and BAC transgenic technology (127-130), the generation of BAC transgenic is rapid and efficient. The large size of BAC clone retains all the transcription/epigenetic control elements that allow faithful expression of target protein with precise temporal and spatial control. The BAC recombineering technology also allows extensive manipulation of target sequence. For example, we could built IRES
(internal ribosome entry site)-GFP in 3’ UTR region of NLR protein to trace the NLR protein in vivo. We can tagged targeted NLR protein with small peptide tag to facilitate the detection of transgene. We also could generate point mutations that resemble the disease associated SNP in NLR protein. Furthermore, the BAC transgenic mice could subsequently be intercrossed with NLR gene deficient mice to obtain more physiological-relevant experimental condition. The BAC transgenic mice are powerful tools especially for examining the function of NLR protein cryopyrin, whereas its disease associated mutants are dominant gain-of-function mutants.

The current thesis work establishes the nucleotide binding as a major biochemical and biological property of NLR protein. The findings of this work not only establish the foundation for future detailed biochemical studies, but also provide us with the possible means for pharmacological intervention of NLR functions.
Reference


