NLRP12 REGULATES IMMUNITY BY CONTROLLING CELL MIGRATION

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ABSTRACT

Janelle Corrinne Arthur: NLRP12 regulates immunity by controlling cell migration
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NLRP12 is a member of the NLR family of genes that are responsible for coordinating inflammatory responses upon recognition of invading pathogens and host danger signals. Remarkably, mutations in several NLR genes have been linked to autoinflammatory diseases; greatly expanding our understanding regarding the etiology of these debilitating conditions. NLRP12 is expressed exclusively in innate immune cells and suppresses inflammation by negatively regulating the noncanonical NF-κB pathway. This is achieved by inducing proteasome-mediated degradation of NF-κB inducing kinase (NIK) in response to pathogens and activation through pro-inflammatory receptors. Because NLRP12 functions to dampen these signals, it is clear that NLRP12 must be controlled in order to mount an adequate cellular response to such insults. Here we find that NLRP12 stability is regulated by the evolutionarily conserved molecular chaperone Hsp90. In the presence of Hsp90 inhibitors, NLRP12 protein is rapidly degraded via the proteasome leading to increased NIK stability and function. Thus, Hsp90 activity is a critical regulatory factor for NLRP12 function and is required for NLRP12-induced degradation of NIK and suppression of the noncanonical NF-κB pathway.

Human *NLRP12* polymorphisms have been linked to atopic dermatitis and hereditary periodic fevers with skin urticaria, however the mechanisms by which NLRP12 affects these conditions remain largely unknown. To better understand these mechanisms, we tested

several well defined models of inflammation using *Nlrp12* knockout mice. Remarkably, we found that *Nlrp12* deficient mice failed to mount T cell mediated responses in hapten induced contact hypersensitivity, a model of allergic dermatitis, and EAE, a model of multiple sclerosis. Mechanistically this is due to defective migration of peripheral dendritic cells. These innate immune cells express *Nlrp12* and play a pivotal role in T cell activation. Molecular analysis reveals that in the absence of NLRP12, dendritic cells display an inappropriate activation of NIK, resulting in high levels of NIK dependent gene expression. These findings expand our understanding of NLRP12 function in vivo and provide a rationale for the diseases associated with this NLR. Furthermore our results reveal a novel role for NLRP12 in bridging innate and adaptive immunity.

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LIST OF ABBREVIATIONS

ASC: apoptosis-associated speck-like protein containing a CARD domain

BAFF: B cell activating factor

BALF: Bronchoalveolar lavage fluid

BMDC: bone marrow derived dendritic cells

C-terminal: carboxy-terminal

CAPS: Cryopyrin-Associated Periodic Syndromes

CARD: activation and recruitment domain

CATERPILLER: caspase activation and recruitment domains [CARD], transcription enhancer, R [purine]-binding, lots of leucine repeats

CCL19: chemokine (C-C motif) ligand 19

CCL21: chemokine (C-C motif) ligand 21

CCL5: chemokine (C-C motif) ligand 5

CCR7: chemokine (C-C motif) receptor 7

CD40L: CD40 ligand

CFA: Complete Freund's Adjuvant

CFSE: Carboxyfluorescein succinimidyl ester

CHS: contact hypersensitivity

CIITA: MHC class II transcriptional activator

CINCA: Chronic infantile neurologic cutaneous articular syndrome

COX-2: cyclooxygenase-2

CPPD: calcium pyrophosphate dehydrate

CXCL12: chemokine (C-X-C motif) ligand 12

CXCR4: chemokine (C-X-C motif) receptor 4

DC: Dendritic cell

DSS: dextran sodium sulfate

EAE: experimental autoimmune encephalitis

ERK: extracellular signal-regulated kinase

FCAS: Familial cold auto-inflammatory syndrome

FITC: fluorescein isothiocyanate

GA: Geldanamycin

H&E: hematoxylin and eosin

Ha: hemagglutinin

Hsc70: heat shock cognate 70

Hsp70: heat shock protein 70

Hsp90: heat shock protein 90

i.p.: intraperitoneal

IFN-β: interferon-beta

IKK: IκB kinase

IL-1β: interleukin 1 beta

IκB: inhibitor of NF-κB

IKK: inhibitor of NF-κB kinase

IRAK1: interleukin-1 receptor-associated kinase

IRF: interferon regulatory factor

Lm: *Listeria monocytogenes*

LPS: lipopolysaccharide

LRR: leucine-rich repeats

LTβR: lymphotoxin beta receptor

MAPK: mitogen-activated protein kinase

MAVS: mitochondrial anti-viral signaling protein

MDP: muramyl dipeptide

MOG: myelin oligodendrocyte glycoprotein

MPO: myeloperoxidase

MSU: monosodium urate

MWS: Muckle-Wells Syndrome

N-terminal: amino-terminal

NALP: NACHT domain-, leucine-rich repeat-, and pyrin- containing protein

NBD: nucleotide binding domain

NF-κB: nuclear factor kappa B

NF-κB2: nuclear factor kappa B2, noncanonical NF-κB

NIK: NF-κB inducing kinase

NLR: nucleotide binding domain leucine-rich repeat

NLRP12: nucleotide binding domain leucine-rich repeat containing a pyrin 12

NLS: nuclear localization signal

NOD: nucleotide oligomerization domain

NOMID: Neonatal-onset multisystem inflammatory disease

Ova: Ovalbumin

PGD_2: prostaglandin D_2

PGE_2: prostaglandin E_2

PRR: pattern recognition receptor

R protein: disease resistance protein

RIG-I: retinoic acid inducible gene 1

RIP2: receptor-interacting protein kinase 2

RLH: RIG-like helicases

s.c.: subcutaneous

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAK1: transforming growth factor beta-activated kinase 1

T_H1: T helper type 1

T_H2: T helper type 2

TIR: Toll/IL-1β receptor

TLR: Toll-like receptor

TNFα: tumor necrosis factor alpha

WT: wildtype

XTT: Tetrazolium hydroxide

CHAPTER 1: INTRODUCTION

Portions of section B, part 2 have been adapted from,

Lich JD, Arthur JC, and Ting JP. Cryopyrin: in from the cold. *Immunity* **24**, 241-3 (2006).

A. INTRODUCTION

Vertebrates utilize the immune system to protect themselves against pathogens. Over the course of evolution, they have developed two systems of immune detection – the innate and adaptive immune systems. All vertebrates employ the innate immune system, however, only jawed vertebrates employ both innate and adaptive immune systems. Adaptive immune cells, such as T and B lymphocytes, can express nearly an unlimited number of antigen-specific receptors that are generated through somatic rearrangement. After activation through these receptors, lymphocytes undergo clonal expansion to exponentially increase the number of antigen-specific lymphocytes. This generates effector cells capable of clearing the infection, as well as memory cells that are poised to react quickly upon secondary exposure to the same antigen. Activation and expansion of an adaptive immune response occurs over several days. In contrast, an innate immune response can be initiated within minutes to hours and shapes the ensuing adaptive immune response. Accordingly, the innate immune system is considered the first line of defense against pathogens.

Nearly two decades ago it was predicted that the immune system utilizes germline encoded receptors to rapidly detect and alert the host to invading pathogens. Janeway hypothesized that the immune system employs pattern-recognition receptors (PRR) to detect conserved microbial products, termed pathogen associated molecular patterns (PAMPs), and distinguish "self" from "non-self" [1]. Matzinger hypothesized that invading pathogens cause the host to produce danger signals that alert the adaptive immune system to infection [2,3]. Both theories have proven to be correct.

In 1997, the discovery of a human homolog of the *Drosophila* innate immune receptor Toll launched the field of innate immunology into view [4]. This Toll homolog was

the first member identified in a family of eleven PRR called Toll-like receptors (TLR). TLRs are type I transmembrane glycoproteins that recognize pathogen products through their extracellular leucine-rich repeat (LRR) domain. Their cytosolic TIR (Toll/IL-1β Receptor) domain recruits cytosolic adaptor proteins including MyD88, TIRAP/Mal, TRAM, and TRIF to transduce downstream signals leading to activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways [5-7]. This signaling induces the secretion of pro-inflammatory cytokines and upregulation of costimulatory molecules important for shaping the ensuing adaptive immune response. All members of the TLR family recognize conserved microbial structures such as viral double stranded RNA [8], the Gram negative bacterial cell wall component lipopolysaccharide (LPS) [9-11], flagellin – a component of the bacterial motility apparatus flagellum [12], and unmethylated CpG motifs in bacterial DNA [13]. TLRs localize to the plasma membrane or within endosomes, however, many bacteria and viruses invade the host cell's cytosol. Thus it was believed that there similar molecules might reside in the intracellular and cytosolic compartments.

In 2002, our lab reported the discovery of a large family of intracellular pattern recognition molecules, which we termed CATERPILLER. This family has recently been renamed NLR, for Nucleotide-binding domain, Leucine-rich Repeat containing proteins [14] (see section A.1). Approximately 20 members were identified in humans and were expressed predominantly in immune cells. Genes belonging to this family were predicted to encode proteins with a central nucleotide binding domain and, similar to TLRs, C-terminal leucine-rich repeats [15]. It was soon realized that these mammalian proteins share striking structural homology to a large family of plant disease resistance (R) proteins with nucleotide binding – leucine rich repeat (NB-LRR) architecture[16], hinting that the NLR family represents an

ancient family of immune defense genes. In addition, several members of this newly discovered family had previously been linked to human immune and autoinflammatory diseases [17] (see section A.3). In response to pathogen products and endogenous danger signals, NLR proteins trigger signaling pathways that can enhance or suppress immune responses, such as those mediated by the innate immune Toll-like receptors (TLRs)[18]. However, many questions remain regarding how NLR proteins function at the biochemical level and what physiologic response is evoked by their activation.

In this dissertation I discuss two major findings regarding the NLR protein NLRP12: an evolutionarily conserved mechanism that controls the stability and function of NLRP12, as well as an *in vivo* role for NLRP12 in bridging innate and adaptive immunity by controlling dendritic cell migration.

1. Discovery of the NLR family

Within the past decade, we and others have discovered the NLR family as a large evolutionarily conserved gene family that serves an important role in innate and adaptive immunology [14]. Many NLR family members with known function participate in the innate immune response, such as sensing pathogenic insult and regulating inflammatory signaling and cell death. In addition, several NLR gene products have recently been shown to affect adaptive immune responses. At least half of the NLR genes encode proteins with functions that remain elusive or not yet studied. For this reason it is exciting to consider what will be discovered in the near future within the field of NLR biology.

The NLR family was discovered in our lab by mining the human genome for open reading frames encoding proteins with predicted domain architecture similar to that of

CIITA, the MHC class II transcriptional activator and founding member of the NLR gene family (see section B.1). Approximately 20 genes are present in humans, and all encode a putative central nucleotide binding domain and C-terminal leucine rich repeats (LRR). We named this family CATERPILLER, an acronym for caspase activation and recruitment domains [CARD], transcription enhancer, R [purine]-binding, lots of leucine repeats [15]. Others have named this group or subgroups of these genes NOD (nucleotide oligomerization domain) [19], NOD-LRR [20], PYPAF [21], PAN [22] and NALP (NACHT domain-, leucine-rich repeat-, and pyrin- containing protein) [23]. Recently a unifying nomenclature has been adopted for the NLR family, designated "NLR", as well as individual NLR family members. The gene symbol for each family member begins with "NLR" plus an additional letter signifying the subfamily to which the individual NLR member belongs. The subfamily is based upon the N-terminal effector domain: NLRA, NLR family, acidic domain containing; NLRB, BIR domain containing; NLRC, CARD domain containing; NLRP, pyrin domain containing; NLRX, N terminal domain with no known homology. Within the subfamily, the individual NLRs are numbered sequentially, i.e. NLRP1, NLRP2, etc. CIITA, NAIP, NOD1 and NOD2 have retained their original names in addition to their new NLR designation [14]. Please refer to Table 1.1.

2. NLR domain organization

In general, the N terminal domains of NLR family members consist of six α-helices that adopt a coiled coil structure and can be further classified as either CARD, pyrin, BIR or Activation Domain [14]. It is believed that these domains engage in homotypic interactions to mediate signaling downstream of NLR molecule activation. For example, homotypic CARD

interactions are utilized by NOD1/NLRC1 and NOD2/NLRC2 to bind receptor-interacting protein kinase 2 (RIP2) and elicit downstream NF-κB activation [24-26]. Several NLRs utilize the N terminal pyrin domain in homotypic interactions with the adaptor ASC (apoptosis-associated speck-like protein containing a CARD domain) to activate caspase-1 [27]. The N terminal domain of several NLRs acts as a dominant negative when overexpressed (Lich, J.D. unpublished data), raising the possibility that unidentified splice variants may serve to negatively regulate the activity of these NLRs.

The central nucleotide binding domain (NBD) of the NLRs is required for the activation and oligomerization of the NLR protein [28]. The NBD encoded by NLR family members can be classified with AAA+ (ATPases associated with various cellular activities) ATPases, which are utilized by many plant *R* proteins [29]. AAA+ ATPases contain well-conserved motifs, the Walker A and Walker B motifs, important for nucleotide binding and hydrolysis, respectively [30]. Mutation of one key lysine in the Walker A motif typically abolishes nucleotide binding [31]. This mutagenesis strategy has been used to assess the importance and specificity of nucleotide binding in NLR function. Binding preference of each NLR is generally specific to one nucleotide. The exception thus far is NLRP1, which binds nucleotides indiscriminately to activate caspase-1 [32]. ATP binding is required for NLRP3 and NLRC4-mediated cell death and inflammation, as well as formation of the large multi-protein complex termed the inflammasome (see section B.2) [33,34]. ATP binding is required for NLRP12 self-oligomerization and suppression of NF-κB [35]. GTP binding is required for CIITA self-oligomerization, association with and activation of MHC class II promoters [36-40].

The C-terminus of NLR molecules is comprised of a varying number of leucine rich repeats (LRR), which are involved in autoregulation, ligand recognition, and protein-protein interactions. LRRs are defined by repeating units of LxxRxxL ('x' being any amino acid) and each unit is a structural motif of 20-30 amino acids forming a beta strand-turn-alpha helix [41,42]. In both plant R proteins and mammalian NLRs, truncation of the LRRs can yield a constitutively active molecule, suggesting the LRRs maintain the NLR protein in an auto-inhibited state until an activating stimulus is received [43-46]. Evidence of a direct interaction between the LRRs of NLR proteins and pathogens or pathogen products is sparse [32]. In plants, however, yeast two-hybrid experiments have detected an interaction between the LRR-like region of *Pi-ta*, a rice *R* gene, and its cognate avirulence effector from the rice blast fungus *Magnaporthe grisea* [47]. However, such interactions have only been demonstrated in artificial systems and not verified with endogenous protein. Thus further studies are required to determine whether or not NLRs are activated through direct ligand binding.

3. Associations with human disease

The importance of NLR family members in human immunity is continually highlighted by the discovery of mutations in NLRs that are linked to human immune and auto-inflammatory disorders. Not surprisingly, many of these mutations are located in the region encoding the NBD domain. The first disease linked to an NLR family member was bare lymphocyte syndrome, a severe immunodeficiency disorder caused by the lack of MHC class II expression on the cell surface. Mutations in CIITA, the class II transcriptional activator and founding member of the NLR family, are linked to this disease [48].

Of the CARD-containing NLRs, mutations in NOD1 and NOD2 are the best studied. Mutations in NOD2 have been linked to Crohn's disease, an inflammatory disease of the intestines, and Blau's syndrome, a familial granulomatous disease characterized by inflammation of the eyes, joints and skin [26,49,50]. Mutations in NOD1 are associated with numerous inflammatory disorders such as inflammatory bowel disease (IBD), asthma, and sarcoidosis [51,52].

Several pyrin-containing NLRs have been linked to human inflammatory disorders. Mutations in NLRP3/Cryopyrin are associated with a group of dominantly inherited autoinflammatory disorders that are referred to as cryopyrinopathies or Cryopyrin-Associated Periodic Syndromes (CAPS). CAPS is comprised of three syndromes, listed as least to most severe: Familial cold auto-inflammatory syndrome (FCAS)[53], Muckle-Wells syndrome (MWS)[53,54] and Neonatal-onset multisystem inflammatory disease (NOMID) / Chronic infantile neurologic cutaneous articular syndrome (CINCA)[55,56]. Symptoms of these syndromes include recurrant rash, fever/chills, joint pain, deafness, systemic amyloidosis, central nervous system inflammation, mental retardation, and bone deformities. Recently, mutations in the most closely related NLR to NLRP3, NLRP12, have been linked to hereditary periodic fevers with nearly identical symptoms to CAPS [57]. Mutations in NLRP12 have also been linked to atopic dermatitis, where inflammation manifests in the skin of affected individuals [58]. Other NLRs associated with human autoinflammatory disease include NLRP1, linked to vitiligo [59], Addison's disease and type 1 diabetes [60], and NAIP/NLRB1, linked to spinal muscular atrophy [61].

B. MECHANISM OF ACTION

NLR proteins organize and assemble into multi-protein complexes to assert their function(s). Based upon these functions, NLRs can be categorized into three groups. It should be noted, however, that many NLRs likely have overlapping functions and should only be loosely categorized into these three groups. CIITA is the sole member of the first group and serves as a transcriptional coactivator at the promoter of MHC class II genes (reviewed in [62]). The second group contains NLRP1, NLRP3, NLRC4, and NAIP/NLRB1. These NLRs assemble into multi-protein complexes with ASC and caspase-1 to promote IL-1β processing and secretion (reviewed in [63]). They also perform an important but less studied role in promoting distinct forms of cell death (reviewed in [64]). The third and final group, the signaling NLRs, includes NOD1, NOD2, NLRX1 and NLRP12. These NLR proteins fine-tune inflammation by enhancing or suppressing distinct arms of inflammatory signaling pathways, such as those leading to interferon regulatory factor (IRF) and NF-κB [65-67].

1. CIITA, the MHC class II transactivator

The founding member of the NLR family, CIITA, controls constitutive and cytokine-induced activation of MHC class II genes. However, while CIITA drives expression of MHC class II genes, it does not directly bind DNA [48]. Instead, it acts as a transcriptional coactivator by organizing other proteins in the appropriate spatial orientation to interact with and induce transcription from MHC class II promoters [68]. MHC class II promoter organization is highly conserved and is comprised of a group of *cis* acting elements, the W-X-Y module [69]. This module is recognized and bound by the transcription factors RFX and NFY [62,70-73] and by CREB [74,75]. These DNA-bound transcription factors assemble into a

multi-protein complex and recruit CIITA to MHC class II promoters, forming the MHC class II enhanceosome and promoting MHC class II transcription [76]. While these transcription factors are constitutively expressed, transcription of MHC class II genes is not induced in the absence of CIITA [48]. CIITA organizes and stabilizes this complex of transcription factors, chromatin modifiers (including Brahma-related gene 1 (BRG-1)) and the requisite transcriptional machinery (histone acetyltransferases, CREB binding protein (CBP)/p300, CBP/p300 associated factor (pCAF), steroid receptor coactivator 1 (SRC-1), the TATA-binding protein (TBP), TATA associated factors), and transcriptional elongation factors necessary for MHC class II gene expression [62,77,78].

CIITA is encoded by *MHC2TA,* which was identified by complementation cloning of an MHC class II negative RJ 2.2.5 cell line [48]. *MHC2TA* is expressed in a cell type and differentiation state specific manner that mirrors MHC class II expression. *MHC2TA* is epigenetically activated in response to IFNy through histone acetylation and chromatin remodeling [79,80] and epigenetically silenced through DNA hypermethylation [81]. *MHC2TA* transcription is controlled by at least three different promoters based upon cell type [82].

CIITA activity is primarily controlled through its cellular localization, as CIITA exerts its transactivating function in the nucleus. Two conventional nuclear localization signals (NLS) and a bipartite NLS direct CIITA into the nucleus; two nuclear export motifs direct export via CRM1 [83,84]. The LRRs of CIITA influences both nuclear import and export, as truncating or mutating the LRRs leads to decreased nuclear localization [44,85]. Regions of the LRR are also important for CIITA self-association and transactivating function [37,86,87]. CIITA binds GTP [88], and GTP binding controls both the nuclear import and export of CIITA. Mutation of the GTP binding domain blocks nuclear import and accumulation of CIITA [88].

Furthermore, deletion of 140 amino acids containing the GTP binding domain increases CIITA nuclear export and association with the nuclear export protein CRM1 [89]. GTP binding is also required for CIITA self-association [36,37] and activation of the MHC class II promoter [38-40].

Post-translational modifications are important in modulating CIITA activity, as unmodified CIITA is not recruited to the MHC class II enhanceosome [90]. CIITA activity is inhibited through phosphorylation by ERK1/2 [91] and protein kinase A (PKA), such as upon prostaglandin E (PGE) treatment [92]. Phosphorylation of CIITA may signal its nuclear export, as mutating these phosphorylation sites [93] or inhibiting ERK1/2 causes retention of CIITA in the nucleus and prevents association with and nuclear export via CRM1 [91]. Ubiquitination of CIITA, however, enhances the ability of CIITA to activate MHC class II gene expression [94].

2. Inflammasome-forming NLRs

Similar to the requirement for CIITA to form higher multiprotein structures to coordinate MHC class II expression , several other NLR proteins have also been shown to form protein complexes termed the inflammasome, which functions in inflammatory cytokine activation [95]. Assembly of this molecular platform leads to processing and release of the potent pro-inflammatory cytokine, IL-1β, and related cytokines, IL-18 and IL-33. The inflammasome includes the core components ASC and caspase-1. In the absence of either of these core components, pro-IL-1β is not processed into its active form [95].

Most inflammasomes studied to date contain the core components ASC and caspase-1. However, the particular NLR involved in inflammasome formation appears to be stimulus-specific (reviewed in [63]). For example, NLRP1 forms an inflammasome and

promotes IL-1β processing and secretion in response to muramyl dipeptide and anthrax lethal toxin of *Bacillus anthracis* [96]. The NLRC4 inflammasome is formed in response to multiple Gram-negative bacteria expressing flagellin [97] while NLRP3 inflammasomes have been shown to respond to a variety of pathogen and host derived molecules. Recently an additional layer of complexity has been revealed by the discovery of an inflammasome complex consisting of both NOD2 and NLRP1. This heterogenous inflammasome was formed in response to *B. anthracis* and the bacterial cell wall component muramyl dipeptide (MDP) [98]. Given the well defined role of NOD2 in NF-κB activation, it is tempting to speculate that this heterogeous inflammasome couples transcriptional activation of inflammatory genes with IL-1β production. Further research will likely reveal the existence of other heterogenous inflammasomes and may explain the apparent overlap between certain elicitors and NLR proteins.

The NLRP3 inflammasome

The NLRP3 inflammasome is arguably the best studied among the NLR family. Therefore it provides a model for understanding the molecular mechanisms underlying inflammasome formation and activation leading to IL-1β and IL-18 production. A collection of dominantly inherited human autoinflammatory disorders are associated with mutations in NLRP3. Interestingly, symptoms are relieved by IL-1β neutralization, suggesting that excessive and improperly regulated NLRP3 inflammasome activation and downstream IL-1β production underly these disorders.

IL-1β is a potent pro-inflammatory cytokine that is translated as a leaderless 35 kDa precursor protein that must be processed into its active form. The processing of pro-IL-1β is

mediated predominantly by the IL-1β converting enzyme caspase-1. Treatment of macrophages with LPS results in the production of high levels of pro-IL-1β that accumulate in secretory lysosomal structures [99]. Yet, the release of mature IL-1β is very inefficient in the absence of a second signal. This second signal can be provided by ATP, which activates the ion-gated channel P2X7. This triggers the rapid activation of caspase-1 and subsequent processing and release of bioactive IL-1β [100]. The role of P2X7 in IL-1β release has been well documented over the years. However, it was not until the discovery of NLRP3 that the molecular mechanisms began to be revealed.

NLRP3, formerly known as Cryopyrin, PYPAF1 or NALP3, is encoded by the *NLRP3* gene, formerly known as *CIAS1*, and is a pyrin-containing member of the NLR family of genes [101]. Hoffman et al. first identified point mutations within exon 3 of *NLRP3* that segregate with Muckle-Wells Syndrome (MWS) and Familial Cold Autoinflammatory Syndrome (FCAS) [53], two inflammatory diseases characterized by fever, rash, and excessive IL-1β production. However, until recently, the role of NLRP3 in these disorders has remained elusive. In 2006, four groups described an important role for NLRP3 in promoting IL-1β maturation, leading to the release of this potent pro-inflammatory cytokine [102-105].

Two groups observed that when prestimulated with LPS, wild-type (wt) macrophages activated caspase-1 and released large quantities of IL-1β upon ATP stimulation, whereas macrophages from *Nlrp3*$^{-/-}$ mice did not [102,103]. This indicates that NLRP3 is a key factor in caspase-1 activation and IL-1β secretion after ATP stimulation. Another group determined that NLRP3 is required for IL-1β maturation in response to R848 (TLR7/8 agonist), R837 (TLR7 agonist), bacterial mRNA (TLR7/8 agonist) and viral RNA [105]. Interestingly, these agonists induce NLRP3-dependent IL-1β maturation in the absence of TLR signaling.

Because R848 and R837 are purine analogs, it is possible that these compounds stimulate members of the P2X or P2Y family of purine receptors, similar to ATP stimulation. Alternatively, NLRP3 may "sense" these agonists within the cytoplasm in a manner analogous to the recognition of the bacterial cell wall component muramyl dipeptide (MDP) by NOD2.

Further support for NLRP3 in bacterial recognition lies in the finding that NLRP3 is also required for IL-1β secretion in response to the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes (Lm)*. NLRP3 displays a level of specificity, as it is not required for caspase-1 activation and IL-1β release in response to *Salmonella typhimurium* or *Francisella tularensis* [103]. A previous report demonstrated that NLRC4, another member of the NLR family, is required for IL-1β release in response to *Salmonella typhimurium* [106]. These findings support the general assumptions that distinct NLR family members respond to different stimuli.

The reports described above indicate a role for NLRP3 in caspase-1 activation and IL-1β maturation in response to a variety of stimuli. A question is undoubtedly raised regarding how NLRP3 can respond to such diverse stimuli with no shared molecular structure. A shared characteristic of these stimuli, however, is the ability to induce K^+ efflux. Indeed, others more recently have described a role for K^+ efflux in activating the NLRP3 inflammasome [107]. In the case of ATP, extracellular ATP binds the cell surface receptor P2X7 and induces the rapid efflux of K^+. The depletion of K^+ leads to the activation of calcium-independent phospholipase A2 (iPLA2) [99]. iPLA2 induces the colocalization of caspase-1 and pro-IL-1β within secretory lysosomes [99]; presumably, this leads to caspase-1 activation and processing of pro-IL-1β. Perhaps this iPLA2-mediated mechanism also

permits vesicular import of the NLRP3 inflammasome, thus allowing it to colocalize with caspase-1 and pro-IL-1β. This supports the observation that inflammasome components are released from stimulated macrophages along with IL-1β. Interestingly, iPLA2 activity also leads to the production of cytoplasmic lipids such as lysophospholipids and arachidonic acid. This has prompted the suggestion that NLRP3 may respond to lipid second messengers generated by phospholipase activity [99].

In addition to the stimuli described above, NLRP3 mediates IL-1β maturation and secretion induced by endogenous danger signals and crystalline particles including asbestos [108], silica [108-110], aluminum hydroxide [111-114], and fibrilar amyloid-β [115]. The first of these found to activate the NLRP3 inflammasome are monosodium urate (MSU) and calcium pyrophosphate dehydrate (CPPD) crystals [104], the deposition of which lead to gout. In the case of MSU and CPPD, IL-1β maturation occurred in the presence of a P2X7 inhibitor, suggesting NLRP3 responds to signals other than those initiated from this ATP receptor. IL-1β processing and secretion was blocked by the microtubule inhibitor colchicine, suggesting that cytoskeletal events, such as endocytosis or vesicle trafficking, are required for NLRP3-mediated IL-1β release. This observation was further supported by the finding that destabilization of the lysosomal membrane in response to phagocytosis of crystalline particles activates the NLRP3 inflammasome [109]. Conflicting reports exist regarding the role of reactive oxygen species in activating the inflammasome under these conditions [108,109]. It appears, however, that the lysosomal protease Cathepsin B plays a role in NLRP3-mediated events as it is required for caspase-1 activation by silica, as well as caspase-1 independent NLRP3-induced cell death pathways [116]. Thus, additional studies are needed to fully understand the mechanisms underlying NLRP3 inflammasome activation.

3. Signaling NLRs

In addition to NLR proteins that comprise the inflammasome, a smaller but growing number of NLRs function in controlling pro- and anti-inflammatory signal transduction. Signaling NLRs include NOD1, NOD2, NLRX1, and NLRP12 (see section C for greater detail on NLRP12). These NLRs enhance or suppress inflammation by fine-tuning inflammatory signaling pathways such as NF-κB, MAPK, and IRF3/7 [65-67]. By controlling these inflammatory pathways, signaling NLRs not only control the innate immune response, but also influence the ensuing adaptive immune response [117-120].

NOD1 and NOD2

Both NOD1 and NOD2 were identified before the discovery of the NLR gene family. *NOD1* was cloned in 1999 by two groups interested in identifying CARD-containing proteins resembling the pro-apoptotic protein APAF-1 [24,25]. *NOD2* was identified based upon its homology to *NOD1* [26]. Their importance in human immunity was evident as mutations in these genes could be linked to Crohn's disease, Blau syndrome, and inflammatory bowel disease [26,49,50]. While NOD1 and NOD2 were identified based upon their homology to the pro-apoptotic protein APAF-1, it was soon found that NOD1 and NOD2 act as cytosolic molecular sensors that promote host resistance to a variety of bacteria by activating inflammatory signaling pathways.

NOD1 and NOD2 reside in the cytoplasm and recognize distinct breakdown products of the bacterial cell wall component peptidoglycan. The composition of peptidoglycan differs between Gram positive and Gram negative bacteria, and while NOD2 responds to a

moiety found in both Gram positive and negative bacteria, NOD1 responds to a moiety found predominantly in Gram negative bacteria. The minimal structure required to activate NOD1 is GlcNAc-MurACc-LAla-c-D-Glu-*meso*-diaminopimelic acid (GM-triDAP)[121], whereas NOD2 responds to MDP and the minimal component GlcNAc-MurNAc-LAla-D-isoGlen (GM-Di)[122,123]. NOD1 and NOD2 clearly display a high level of specificity, however, neither have been shown to directly bind these bacterial products.

NOD1 and NOD2 activate multiple pro-inflammatory signaling pathways that result in the secretion of inflammatory cytokines and chemokines like IL-6, IL-8 and TNFα and antimicrobial peptides including defensins. Upon activation, NOD1 and NOD2 are recruited to the plasma membrane [124,125], self-oligomerize and recruit the CARD-containing kinase RIP2 through homotypic CARD interactions [126,127]. RIP2 is activated by K63-ubiquitination which recruits transforming growth factor-β-activated kinase 1 (TAK1) [128-130]. Signaling can proceed through the mitogen activated protein kinases (MAPK) p38, JNK and ERK, as well as to NF-κB via ubiquitination of IKKγ/NEMO, the regulatory subunit of the IKK (inhibitor of NF-κB (IκB) kinase) complex, phosphorylation of IκB, and nuclear translocation of NF-κB [26,131,132]. NOD2-mediated NF-κB activation can be suppressed by A20 [130], an enzyme with both ubiquitinating and de-ubiquitinating machinery that suppresses NF-κB downstream of pro-inflammatory receptors [133].

The importance of NOD1 in protecting the host from bacterial infection is supported by numerous *in vitro* and *in vivo* studies. NOD1 induces an inflammatory response by activating NF-κB upon stimulation with *Bacillus* species[133], *Shigella flexneri*[134], enteroinvasive *Escherichia coli* [135], *Listeria monocytogenes* [127], and *Campylobacter jejuni* [136]. NOD1-deficient mice are more susceptible to infection with pathogenic *cag*PAI-positive

Helicobacter pylori, but not to infection with nonpathogenic *cag*PAI-negative *Helicobacter* [137]. This finding suggests that NOD1 may participate in distinguishing between commensal and noncommensal flora in the gut, where NOD1 is highly expressed in intestinal epithelial cells. NOD1 can also drive antigen-specific T cell responses and antibody responses, but the mechanism involved remains unknown [119].

NOD2 typically activates NF-κB and promotes an inflammatory response upon recognition of pathogens, some of which include *Streptococcus pneumoniae*[138], *Mycobacterium tuberculosis*[139], *Staphylococcus aureus*[140], *and Listeria monocytogenes (Lm)*[117]. Interestingly, NOD2 may also suppress inflammatory pathways under certain conditions. NOD2 suppresses NF-κB activation when stimulated in concert with TLR2, an extracellular TLR that like NOD2, recognizes peptidoglycan. Accordingly, IL-12 secretion is reduced, as is the ensuing T_H1 response[118]. Perhaps NOD2 cooperates with TLR2 to promote a T_H2 response and enhanced clearance of extracellular bacteria. Other groups, however, have found a synergism upon co-stimulation of NOD2 and TLRs [141-143]. Thus *in vivo* studies utilizing whole bacteria may provide a clearer view in characterizing the role of NOD2 in host defense.

NOD2 is highly expressed in Paneth cells located in the intestinal crypts, thus its location is ideal for recognition of ingested pathogens [144]. NOD2-deficient mice have increased susceptibility to *Lm,* not upon intravenous or intraperitoneal infection, but only upon intragastric infection[117]. These findings have direct relevance to human heath as Listeriosis is a foodborne illness caused by oral infection with *Lm*. During *Lm* infection, NOD2-deficient mice fail to release antimicrobial peptides called cryptidins (α-defensins in humans)[117]. Similarly, Crohn's disease patients with *NOD2* mutations are deficient in

intestinal defensin expression [145]. It is not fully understood whether or not decreased defensin expression contributes to the pathology of Crohn's disease, or how mutations in *NOD2* contribute to the pathology of Crohn's disease.

NLRX1

NLRX1 is a newly characterized NLR that suppresses anti-viral signaling. Intracellular viral RNA is sensed by RIG-like helicases (RLH) that associate with mitochondrial anti-viral signaling protein (MAVS) to activate IRF3 and NF-κB. This results in the production of type 1 interferon and inflammatory cytokines that are essential for anti-viral defenses, but when dysregulated can cause excess inflammation and tissue damage in the host [146].

NLRX1 was identified as a member of the NLR family in 2003 [15], however, its physiologic role remained uncharacterized until 2008 [147]. *NLRX1* is ubiquitously expressed in human cells and cell lines and encodes an unclassified N-terminal domain, central nucleotide binding domain, and C-terminal leucine-rich repeats. It contains a mitochondrial targeting sequence in the N-terminus that targets it to the mitochondrial outer membrane[147]. NLRX1 is the first NLR, with the exception of CIITA, that requires localization to a particular organelle.

NLRX1 performs its anti-viral function by associating with MAVS at the mitochondrial outer membrane and preventing its interaction with the viral RNA sensor retinoic acid inducible gene 1 (RIG-I). Consequently, NLRX1 suppresses the release of interferon-β (IFNβ) and inhibits the activation of IRF3 and NF-κB in response to Sendai virus, Sindbis virus, and intracellular poly(I:C), a viral double-stranded RNA analog. This

response is independent of the extracellular viral RNA sensor TLR3 and requires mitochondrial localization of NLRX1[147]. When overexpressed, NLRX1 can cause a modest change in the level of reactive oxygen species[148]. This too may serve to fine-tune antiviral responses. Many questions remain regarding NLRX1 – most notably, the mechanism by which NLRX1 prevents MAVS association with RIG-I, and the *in vivo* role of NLRX1 in host defense against viral infection.

C. NLRP12 / MONARCH-1

1. NLRP12 identification and expression

NLRP12, formerly named RNO, PYPAF7, and Monarch-1, is a pyrin-containing NLR protein expressed in cells of myeloid lineage. A partial 3' portion of the gene encoding *NLRP12* was first identified in the HL60 human leukemic cell line. This gene was upregulated when these cells were stimulated with nitric oxide, thus it was first named *rno* – Regulated by Nitric Oxide [149]. The full-length gene product was subsequently cloned two groups: Our group named this gene *Monarch-1* [150] and the other group named it *PYPAF7* [151]. In 2008, the HUGO Gene Nomenclature Committee approved the designation *NLRP12* for this gene[14].

NLRP12 encodes an intracellular protein with an N-terminal pyrin domain, a central nucleotide binding domain, and C-terminal leucine-rich repeats. The full-length human cDNA has a 3189-bp open reading frame (accession no. AY116204) encoded by 10 exons. There are also four known splice forms of NLRP12 [150], however it remains unknown if these splice forms are differentially expressed and/or serve different functions from the full-length product. In humans, *NLRP12* is expressed exclusively in cells of myeloid lineage – granulocytes including neutrophils and eosinophils, monocytes/macrophages, and immature dendritic cells [149-151]. *NLRP12* expression is upregulated by nitric oxide, yet it is downregulated in response to pathogens, pathogen products, and inflammatory cytokines [149,150,152]. Downregulation of *NLRP12* after TLR stimulation is achieved, at least in part, by binding of B lymphocyte-induced maturation protein-1 (Blimp-1) to the *NLRP12* promoter [153].

2. NLRP12 as a negative regulator of inflammation

The expression of NLRP12 is restricted to immune cells and its expression is downregulated in response to pathogens, pathogen products, and inflammatory cytokines, thus we and others predicted that NLRP12 functions in regulating inflammation and immunity. Early studies, however, describe conflicting roles for NLRP12. One study describes that NLRP12 co-localizes with ASC and activates NF-κB and caspase-1, leading to IL-1β secretion [151]. This is reminiscent of other pyrin-containing NLRs that regulate IL-1β processing by forming an inflammasome with ASC. Another report describes that NLRP12 can control expression of classical and non-classical MHC I genes [150]. Again, this is reminiscent of CIITA, an NLR that is essential for the expression of MHC II genes. However both studies relied upon overexpression in non-immune cells. Thus they provide little information about the function of NLRP12 in cells that naturally express the gene product. They do, however, imply a complex role for NLRP12.

More recent studies utilizing the human THP-1 monocytic cell line have uncovered an important biological function for NLRP12 as a negative regulator of inflammatory signaling in human monocytes. In these studies, endogenous *NLRP12* expression was silenced in THP-1 cells using siRNA. Compared to cells treated with a control siRNA, *NLRP12*-silenced cells displayed a dramatic enhancement of NF-κB activation. Furthermore, *NLRP12*-silenced cells stimulated with TNFα, TLR ligands and whole bacteria produced greater amounts of NF-kB-regulated pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β and TNFα [152]. These observations were some of the first to indicate that NLRP12 serves as a negative regulator of inflammation in human monocytes.

3. Molecular mechanisms of NLRP12-mediated NF-κB suppression

Biochemical studies in our lab have revealed that NLRP12 suppresses pro-inflammatory cytokine and chemokine production downstream of TLRs by targeting multiple points in the NF-κB pathway [152,154]. Stimulation through TLRs leads to the recruitment of cytoplasmic adaptor proteins, such as MyD88, that then recruit the kinase IRAK1. IRAK1 becomes activated through autophosphorylation and accumulates in a hyperphosphorylated form, leading to downstream NF-κB activation [155,156]. Exogenous expression of NLRP12 reduces IRAK1-induced activation of an NF-κB luciferase reporter plasmid, suggesting that NLRP12 may intersect the NF-κB pathway by affecting IRAK1 signaling. Indeed, minutes after TLR stimulation, NLRP12 7associates with IRAK1 and prevents the accumulation of hyperphosphorylated IRAK1 [152]. Current literature support that the loss of hyperphosphorylated IRAK1 would hinder downstream signaling leading to NF-κB [157]. As *NLRP12* expression declines upon TLR stimulation but returns within 24 hours in THP-1 monocytes (Arthur, J.C. unpublished data), this suggests a possible role for NLRP12 in the resolution of inflammation and may even play a role in the transition from innate to adaptive responses.

In addition to suppressing classical NF-κB activation, we have found that NLRP12 inhibits an alternative and tightly controlled pathway, the noncanonical NF-κB pathway. This pathway is activated downstream of TNFR superfamily members [158,159]. In antigen presenting cells, this may occur during the transition from innate to adaptive responses, such as downstream of CD40 after binding to CD40L expressed on T cells. To understand the complexity of the canonical and noncanonical NF-κB pathways, a brief explanation is provided below.

4. Canonical and noncanonical pathways of NF-κB activation

NF-κB represents a family of dimeric transcription factors that mediates cellular responses during inflammatory conditions. NF-κB subunits include RelA (also known as p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) [160]. The latter two are expressed as large precursors that must be proteolytically cleaved to their corresponding smaller and active forms [161,162].

NF-κB activation occurs through two distinct pathways, referred to as canonical and noncanonical (Figure 1.1). The canonical pathway proceeds rapidly upon activation of pro-inflammatory receptors, such as TLRs. In this pathway, RelA/p50 heterodimers are sequestered in the cytoplasm in an inactive state by a family of inhibitors of NF-κB (IκB). Activation is mediated through a large IκB kinase (IKK) complex comprised of the regulatory subunit IKKγ/NEMO, and two catalytic subunits, IKKα and IKKβ. This complex can be activated by a wide range of upstream kinases and serves to phosphorylate IκB, leading to its degradation. Newly liberated RelA/p50 heterodimers then rapidly translocate to the nucleus to regulate the activation of early inflammatory genes. One of these genes is *NF-κB2/p100*, whose gene product must be processed to its active form p52 through the noncanonical pathway [163-165].

In contrast to the canonical pathway, the noncanonical NF-κB pathway displays slower kinetics and tighter regulation. It is commonly activated downstream of TNF receptor superfamily members such as BAFF, LTβR and CD40 [158,159]. While initial canonical NF-κB activation NF-κB1/p105 processing to p50 is constitutive, processing of NF-κB2/p100 to p52 is inducible and relies upon the activity of NF-κB inducing kinase, NIK [166,167]. In this alternate pathway, NIK selectively activates IKKα, leading to phosphorylation and

subsequent processing of p100 to its active form p52. Nuclear translocation of RelB/p52 dimers results in the activation of a different set of inflammatory genes that support the ongoing immune response [168-170].

5. NLRP12 targets NIK to control the noncanonical NF-κB pathway

Detailed analysis of the canonical and noncanonical NF-κB pathways in THP-1 cells has revealed that while elevated expression of NLRP12 moderately suppresses the canonical NF-κB pathway, NLRP12 nearly abolishes activation of the noncanonical NF-κB pathway. In THP-1 cells expressing elevated levels of NLRP12 and stimulated with TLR agonist followed by CD40L, nuclear translocation of the canonical NF-κB subunits RelA and p50 proceeds normally. However, processing of NF-kB2/p100 to p52 and nuclear translocation of p52 is nearly absent in these cells. To exert this effect, NLRP12 targets NIK, the sole kinase responsible for activation of the noncanonical pathway. NLRP12 associates with NIK and induces its degradation via the proteasome, a major protein degradation pathway [154]. Accordingly, in NLRP12-silenced cells, NIK and p52 levels are elevated (Lich, J.D., unpublished data). This results in increased expression p52-regulated cytokines and chemokines including *CXCR4*, *CXCL12* and *CXCL13* [154] (see Appendix 1).

6. Conclusions

Detailed analyses of inflammatory signaling pathways both affected and unaffected by NLRP12 provide us with novel mechanism of regulation that is distinct from other NLR family members. Thus, from this work, it is clear that NLRP12 controls the noncanonical NF-κB pathway by associating with and inducing proteasome-mediated degradation of NIK

(Figure 1.1). Consequently, NF-kB2/p100 to p52 processing is abolished, and p52-regulated genes are suppressed until NLRP12 expression fades. The noncanonical NF-κB pathway is triggered later than the canonical NF-κB pathway, and often in response to a second signal through TNF receptor superfamily members. In this manner, the noncanonical pathway drives later events in innate immunity. In addition, in antigen presenting cells like monocytes/macrophages and dendritic cells, activation of noncanonical NF-κB may come from the interaction with cell-surface molecules on T cells, indicating that this alternative pathway is also intimately involved in the transition from innate to adaptive immunity.

Based on these statements, two key hypotheses are raised.

1). In order for the noncanonical pathway to operate, NLRP12 must be tightly regulated in a manner that allows NIK to function. We hypothesize that NLRP12 activity is controlled at the level of protein stability.

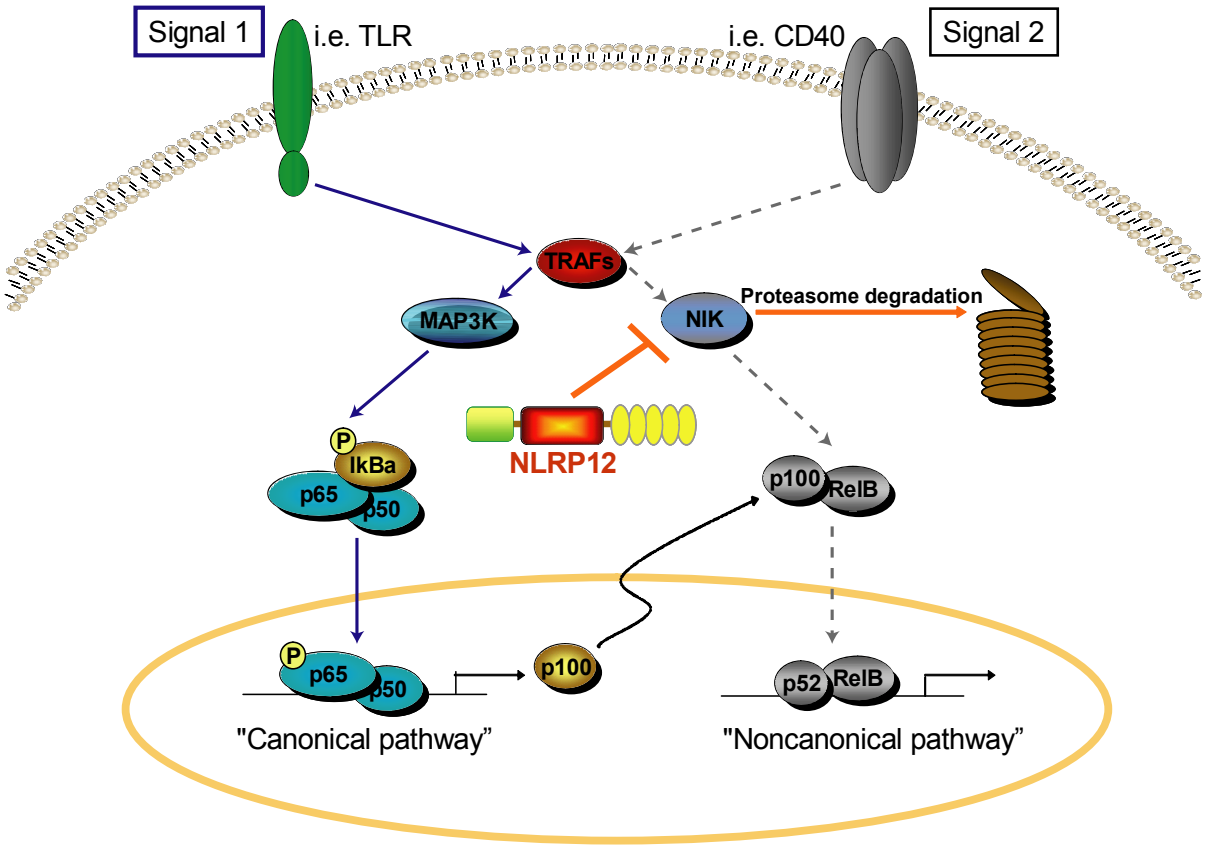
2). Because NLRP12 is expressed in antigen presenting cells, we hypothesize that NLRP12 affects adaptive immune responses in vivo.

In this dissertation we provide support for these two hypotheses. First we provide a mechanism by which the stability of the NLRP12 protein is regulated in human monocytes. This is through an evolutionarily conserved mechanism involving the chaperone Hsp90. Second, we reveal that NLRP12 affects immunity *in vivo* by controlling dendritic cell migration. This is due to the ability of NLRP12 to modulate intracellular signaling pathways, including noncanonical NF-kB, that are integral to dendritic cell function.

Table 1.1. Human NLR family members

NLR name	Alternative names	Protein accession
CIITA	NLRA, MHC2TA, C2TA	NP_000237
NAIP	NLRB1, BIRC1, CLR5.1	NP_004527
NOD1	NLRC1, CARD4, CLR7.1	NP_006083
NOD2	NLRC2, CARD15, CD, BLAU, IBD1, PSORAS1, CLR16.3	NP_071445
NLRC3	NOD3, CLR16.2	NP_849172
NLRC4	IPAF, CARD12, CLAN, CLR2.1	NP_067032
NLRC5	NOD27, CLR16.1	NP_115582
NLRP1	NALP1, DEFCAP, CARD7, CLR17.1	NP_127497
NLRP2	NALP2, PYPAF2, NBS1, PAN1, CLR19.9	NP_060322
NLRP3	CIAS1, PYPAF1, NALP3, CLR1.1, Cryopyrin	NP_004886
NLRP4	NALP4, PYPAF4, PAN2, RNH2, CLR19.5	NP_604393
NLRP5	NALP5, PYPAF8, MATER, PAN11, CLR19.8	NP_703148
NLRP6	NALP6, PYPAF5, PAN3, CLR11.4	NP_612202
NLRP7	NALP7, PYPAF3, NOD12, PAN7, CLR19.4	NP_996611
NLRP8	NALP8, PAN4, NOD16, CLR19.2	NP_789781
NLRP9	NALP9, NOD6, PAN12, CLR19.1	NP_789790
NLRP10	NALP10, PAN5, NOD8, PYNOD, CLR11.1	NP_789791
NLRP11	NALP11, PYPAF6, NOD17, PAN10, CLR19.6	NP_659444
NLRP12	NALP12, PYPAF7, RNO2, PAN6, CLR19.3, Monarch1	NP_653288
NLRP13	NALP13, NOD14, PAN13, CLR19.7	NP_789780
NLRP14	NALP14, NOD5, PAN8, CLR11.2	NP_789792
NLRX1	NOD9, CLR11.3	NP_078894

Figure 1.1. NLRP12 suppresses noncanonical NF-κB activation



CHAPTER 2: HSP90 ASSOCIATES WITH MONARCH-1/NLRP12 AND REGULATES ITS ABILITY TO PROMOTE DEGRADATION OF NF-κB INDUCING KINASE

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A. ABSTRACT

Monarch-1/NLRP12, is expressed in myeloid cells and functions as a negative regulator of inflammation by inducing proteasome mediated degradation of NF-κB inducing kinase, NIK. NLRP12 is a member of the CATERPILLER (CLR) gene family, also known as the Nucleotide Binding Domain-Leucine Rich Repeat gene family (NLR). This family shares strong structural homology to major immune regulators expressed in lower organisms, including plants. In plants, these disease resistance (R) proteins sense pathogenic insult and initiate a protective response to limit pathogen growth. To perform this role, many R proteins require the highly conserved chaperone molecule, heat shock protein 90 (Hsp90). Using a 2-D gel/mass spectrometry system, we detected the association of the NLR protein NLRP12 with heat shock proteins. Further analysis indicates that analogous to plant R proteins, Hsp90 is required for NLRP12 activity. In human monocytes, NLRP12 associates with Hsp90, and these complexes are sensitive to treatment with specific Hsp90 inhibitors. Disruption of these complexes results in rapid degradation of NLRP12 via the proteasome and prevents NLRP12-induced proteolysis of NIK. This demonstrates that Hsp90 is a critical regulator of NLRP12 anti-inflammatory activity.

B. INTRODUCTION

Inflammation is a dynamic protective response that must be controlled at both the initiation and resolution phase as improper regulation underlies many human diseases. Nucleotide Binding Domain- Leucine Rich Repeat (NLR) proteins (previously known as CATERPILLER, NOD-LRR or NACHT-LRR) play a critical role in this regulation [19,23,67,101]. The importance of NLR proteins in controlling inflammation is highlighted by strong linkage of the NLR proteins CIITA, NOD2, and NALP3 to human immunodeficiency and autoinflammatory diseases [171]. Yet despite the critical role of NLR proteins in human health, relatively little is known regarding their molecular regulation.

NLR proteins share a strong structural and functional homology to a class of disease resistance (R) proteins that comprise a major immune response system in the plant kingdom. These plant proteins function as molecular sensors that mediate the containment of a broad range of pathogens including bacteria, viruses, fungi, parasites, nematodes, and insects [16,172]. Recent evidence suggests that a critical component in R protein mediated defense responses is Hsp90. This evolutionarily conserved molecular chaperone associates with a subset of proteins, deemed "client proteins," to promote their maturation and stability

The Hsp90 chaperone cycle is a multi-step process where client proteins first form an early complex with Hsp70. An intermediate complex then forms with the incorporation of Hsp90. Within this intermediate complex, the client protein is transferred from Hsp70 to Hsp90. Finally, Hsp70 dissociates from the complex and the client protein remains bound to Hsp90 in an activation-competent state (reviewed in [173]). Pharmacologic inhibition of Hsp90 prevents the transfer of client proteins to Hsp90 and stalls this chaperone cycle at the intermediate stage. In the absence of Hsp90 binding, the client protein remains bound to

Hsp70 and is degraded by the proteasome [174-176]. Notably, the majority of known Hsp90 client proteins are signaling molecules such as kinases and transcription factors (reviewed in [177]).

NLRP12 is an understudied NLR protein with a unique function. Previously, we demonstrated that NLRP12 serves as a novel attenuating factor of inflammation by destabilizing NIK, which results in suppression of noncanonical NF-κB activation [178]. In the present study, we demonstrate that this activity is regulated by Hsp90. We find that NLRP12 associates with Hsp90 in a stable, functionally competent state. In the presence of Hsp90 inhibitors, this association is lost resulting in degradation of NLRP12 via the proteasome. This rescues NIK from NLRP12-induced proteolysis, demonstrating that Hsp90 performs an integral role in regulating NLRP12 activity.

C. MATERIALS AND METHODS

Cell lines: HEK293T cells (GenHunter) were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 100mg/ml penicillin and 100mg/ml streptomycin. Undifferentiated THP-1 cells were maintained in RPMI (Gibco) supplemented with 10% FCS, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 100mg/ml penicillin and 100mg/ml streptomycin. THP-Ha-NLRP12 and THP-EV cells have been previously described [178].

Primary cell isolation: Peripheral blood mononuclear cells (PBMC) were isolated from whole blood (American Red Cross) using a ficol hypaque gradient (ICN-Cappel). To enrich human primary adherent monocytes, PBMC were plated in serum-free RPMI (Gibco) and allowed to adhere for 2 hours at 37°C; at this time non-adherent cells were removed and adherent cells were washed with sterile PBS in triplicate. Cells were cultured in RPMI supplemented as described above.

Antibodies and Reagents: Anti-Hsp70 (W27), anti-Hsc70 (B-6), anti-Hsp90 (H-114), anti-TAK1 (C-9), anti-NIK (H-248) and anti-actin-HRP (C-11) antibodies were obtained from Santa Cruz. Anti-Ha antibodies (12CA4 and 13F10) were obtained from Roche Applied Science. Purified anti-mouse I-A^d (control Ig) was obtained from Pharmingen. Rabbit polyclonal anti-CagA (b-300, control Ig) was obtained from Santa Cruz. Normal rabbit serum was obtained from Vector Laboratories. Rabbit polyclonal anti-NLRP12 and mouse monoclonal anti-NLRP12 have been described previously [179]. Geldanamycin, Radicicol and MG132 were obtained from Calbiochem. TLR2 agonist Pam3Cys4 was obtained from Invivogen.

Two-dimensional gel electrophoresis and mass spectrometry: HEK293T cells seeded in 10 cm plates were transfected with 5 μg Flag-tagged NLRP12 or pcDNA control vector. Twenty-four hours later the cells were lysed in 0.5% CHAPS and protein complexes immunoprecipitated for 18 hours with M2-agarose. Protein complexes were dissociated in an 8M urea solution and individual proteins separated by two-dimensional gel electrophoresis. Briefly, protein eluate was loaded on 18-cm immobilized pH gradient strips (pH 4–7) and separated by pI for a total of 58,000 V-h. The strips were transferred to SDS-PAGE gels (10%; 19 x 18 cm) and the proteins were then separated by molecular weight. Silver stained gels were analyzed and spots unique to precipitates derived from NLRP12 transfected cells were excised from the gel and analyzed by MALDI-MS as described previously [180]. Protein identities were established using the MASOCT search engine (Matrix Sciences) with the following settings: peptide mass tolerance of 0.1 Da, zero missed cleavage sites, one fixed modification of carboxymethyl cysteine, one variable modification of methionine oxidation, and no restrictions on protein molecular weight or pI. The protein identities reported received a Mowse score greater than the significance threshold ($p<0.05$).

Immunoprecipitations and Western Blot analysis: HEK293T cells seeded in 6-well plates were transfected with 1μg Ha-tagged NLRP12 plasmid described previously using Fugene 6 (Roche). Twenty hours later the cells were lysed in 1% TX-100, 150mM NaCl, 50mM Tris pH 8 supplemented with protease inhibitor cocktail (Roche). Samples were immunoprecipitated with 2μg of the indicated antibody and rotated end-over-end for 18 h. Antibody complexes were captured by the addition of protein A/G agarose beads (Pierce) for an additional 2 h. The beads were washed three times in lysis buffer, eluted into boiling sample reducing buffer and separated by SDS-PAGE. Proteins were transferred to

nitrocellulose (BioRad), probed with the indicated primary antibody and visualized by chemiluminescence (Pierce).

To examine stably expressed proteins in THP-1 cells, THP-Ha-NLRP12 and THP-EV cells were seeded at a density of $8x10^6$ per $25cm^2$ tissue culture flask. To examine endogenous NLRP12 protein, THP-1 cells were seeded at a density of $2x10^7$ in $75cm^2$ tissue culture flasks; primary adherent monocytes were seeded at $1x10^7$ in $25cm^2$ flasks. Samples were immunoprecipitated with 2μg of the indicated antibody or 10μl NLRP12 rabbit anti-sera. Cell lysis and Western blot analysis were performed as described above.

D. RESULTS

NLRP12 interacting proteins include Hsp70.

NLR proteins assemble into large, multi-protein complexes that serve as functional platforms to promote downstream activities such as transcription regulation and IL-1β processing [101]. To begin to identify the protein complexes formed by NLRP12, HEK293T cells were transfected with Flag-NLRP12 or a pcDNA control vector and cellular lysates were immunoprecipitated with anti-Flag antibodies. Captured protein complexes were resolved by two-dimensional gel electrophoresis. The gels were stained and protein spots unique to NLRP12 transfected cells were identified (Figure 2.1A). These spots were excised and processed for MALDI-MS (Table 2.1). Protein identities were determined by comparing the resulting peptide mass fingerprints to the MASCOT search engine. Among the proteins that achieved high confidence scores were members of the Hsp70 family.

Mammalian Hsc/Hsp70 binds to a wide range of newly synthesized proteins in unstressed cells [181]. To confirm the association of NLRP12 with Hsp70, we performed co-immunoprecipitation experiments. HEK293T cells were transfected with Ha-NLRP12 or pcDNA control vector, and endogenous Hsp70 complexes were immunoprecipitated from cellular lysates. Western blots were then probed with anti-Ha to detect NLRP12 (Figure 2.1B, lanes 1-2). In agreement with the results obtained from two-dimensional gels (Figure 2.1A), NLRP12 co-precipitated with endogenous Hsp70. Identical results were obtained from THP-1 monocytes stably transfected with Ha-tagged NLRP12 (THP-Ha-NLRP12) (Figure 2.1B, lanes 3-4). As NLRP12 is expressed exclusively in cells of myeloid lineage [150,151], this is a more physiologically relevant model system. NLRP12 was not detected in control samples that were immunoprecipitated with an isotype matched antibody (Figure

2.1B, lane 5).

NLRP12 associates with endogenous Hsp90.

The molecular chaperone Hsp70 is an essential component of the Hsp90 multi-chaperone complex. This complex aids in the maturation and stabilization of a select set of client proteins, predominantely signaling molecules [177]. We identified Hsp70 as a NLRP12-interacting protein, and combined with the important role of Hsp90 in plant R protein function, we hypothesized that NLRP12 also associates with Hsp90. To test this hypothesis, we transfected HEK293T cells with Ha-NLRP12 or pcDNA control vector and endogenous Hsp90 containing complexes were immunoprecipitated. Western blots were then probed with anti-Ha to detect NLRP12. NLRP12 co-precipitated with endogenous Hsp90 but not in control samples employing an isotype matched antibody (Figure 2.2). Importantly, this association was also found in THP-Ha-NLRP12 monocytic cells, demonstrating that NLRP12 forms molecular complexes with Hsp90 in a more relevant model system (Figure 2.2, lanes 3-4).

Hsp90 inhibition alters the association of NLRP12 with Hsp70 and Hsp90.

Pharmacologic inhibition of Hsp90 prevents the transfer of client proteins to Hsp90. This then leads to the accumulative association of client proteins within Hsp70 complexes [173]. The observation that NLRP12 associated with both Hsp70 and Hsp90 led us to hypothesize that NLRP12 serves as an Hsp90 client protein. If this is the case, treatment of cells with the ansamycin antibiotic geldanamycin (GA), a specific Hsp90 inhibitor, would result in reduced association of NLRP12 with Hsp90 and increased association with Hsp70.

To test this, we treated THP-Ha-NLRP12 cells with GA and then immunoprecipitated endogenous Hsp70 or Hsp90 complexes at multiple time points over a 6 h period (Figure 2.3A). Western blots were probed with anti-Ha to detect co-precipitated NLRP12. After 2 h of GA treatment, NLRP12 was barely detectable in Hsp90-containing complexes. Notably, NLRP12 levels decreased with GA treatment, suggesting that the stability of NLRP12 is dependent upon Hsp90 activity. However, since NLRP12 levels were comparable at 1 h and 6 h post-GA treatment (lanes 2 and 5) the loss of NLRP12/Hsp90 complex formation was due to Hsp90 inhibition and not due to decreased levels of NLRP12 protein.

In sharp contrast to its association with Hsp90, the association of NLRP12 with Hsp70 increased after 2 h of GA treatment and strengthened throughout the 6 h time course (Figure 2.3B). This agrees with other studies where GA treatment causes an increase in association of the Hsp90 client protein with Hsp70 [182-184]. Also in line with previous reports [185-188], a minimal increase in Hsp70 levels was detected upon inhibition of Hsp90. These results indicate that Hsp90 inhibition leads to the accumulation of NLRP12 within Hsp70 containing molecular complexes and supports our hypothesis that NLRP12 is processed through the Hsp90 chaperone cycle.

In addition to Hsp70, we also analyzed the association between NLRP12 and Hsc70. In contrast to Hsp70, which is induced upon cell stress, Hsc70 is constitutively expressed and performs multiple functions distinct from Hsp70 [189]. Also in contrast to Hsp70, the association between NLRP12 and Hsc70 decreased upon treatment of cells with GA (Figure 2.3C). Thus, the pattern of association was similar to that seen with Hsp90. These results suggest different roles for Hsp70 and Hsc70 in NLRP12 function and demonstrate that the

association between NLRP12 and Hsc70 is dependent upon Hsp90 activity. Taken together, these results demonstrate that NLRP12 is a substrate of the Hsp90 multi-chaperone complex.

Endogenous NLRP12 stability in THP-1 cells and primary monocytes is dependent upon Hsp90 activity.

In addition to altering the dynamic association of client proteins with Hsp70 and Hsp90, inhibition of Hsp90 also results in degradation of the client protein [173]. Consistently, Western blot analysis of cellular lysates indicated that NLRP12 levels decreased upon Hsp90 inhibition. In fact, in THP-Ha-NLRP12 cells, NLRP12 protein levels were dramatically reduced after only 1 h of treatment with GA (Figure 2.3). This was also observed upon treatment of THP-Ha-NLRP12 cells with Radicicol, an Hsp90 inhibitor that is structurally dissimilar and chemically unrelated to GA (Figure 2.4A) [190].

To ensure that the effect of Hsp90 inhibition on NLRP12 levels was not due to overexpression of a tagged protein, we next analyzed endogenous NLRP12 levels in wild type THP-1 monocytes. In agreement with the results obtained with tagged NLRP12, endogenous NLRP12 protein levels were significantly reduced after 1 h of treatment with GA (Figure 2.4B), demonstrating that NLRP12 stability is regulated by Hsp90.

Next we examined endogenous NLRP12 levels in the presence of GA in primary human monocytes. Endogenous NLRP12 is expressed at very low levels in monocytes [151]. In these experiments, NLRP12 was first immunoprecipitated with a NLRP12 specific rabbit polyclonal antibody and then Western blots were probed with a NLRP12 specific mouse monoclonal antibody (Figure 2.4C). In agreement with the results obtained from THP-1 cells, NLRP12 levels declined after 1 h of GA treatment and were undetectable following 2.5

h of treatment. Control samples using normal rabbit serum in the immunoprecipitation confirmed the specificity of the NLRP12 band. Together these data demonstrate that in human monocytes, NLRP12 stability is dependent upon Hsp90 activity.

Hsp90 inhibition results in proteasome-mediated degradation of NLRP12.

It is generally believed that Hsp90 inhibition leads to proteasome mediated degradation of Hsp90 client proteins [174-176]. To determine if the reduction of NLRP12 protein levels upon GA treatment was due to proteasome mediated degradation, we treated THP-Ha-NLRP12 cells with GA in the presence or absence of the proteasome inhibitor, MG132. In agreement with the results presented above, NLRP12 protein levels declined after 1 h of treatment with GA. In contrast, however, NLRP12 protein levels remained stable in cells that were pre-treated with proteasome inhibitor (Figure 2.5). No change was observed in the cellular levels of TAK1, demonstrating that these treatments were not globally affecting signaling molecules. In addition, no change was observed in cellular levels of Hsp90 or actin under these treatment conditions. Together, these results demonstrate that Hsp90 controls NLRP12 stability and upon Hsp90 inhibition, NLRP12 is degraded via the proteasome.

Hsp90 regulates NLRP12-induced NIK degradation.

Recently, we demonstrated that NLRP12 suppresses the production of pro-inflammatory cytokines and chemokines [178,179]. One mechanism by which NLRP12 performs this function is by associating with and destabilizing NIK [178]. NIK is degraded when co-expressed with NLRP12, thus providing a measurable function of NLRP12. To examine the

role of Hsp90 in NLRP12-induced NIK degradation, NIK and NLRP12 were co-expressed in HEK293T cells in the presence or absence of GA. NIK levels were then monitored by Western blot analysis. In cells expressing NIK alone, inhibition of Hsp90 caused a slight reduction in NIK levels (Figure 2.6A, lane 2). This agrees with an earlier report indicating that NIK is an Hsp90 client protein and NIK levels are reduced upon Hsp90 inhibition [191]. In agreement with our previous report, co-expression of NLRP12 and NIK resulted in the near ablation of NIK protein (Figure 2.6A, lane 3). However, GA treatment restored NIK levels to those observed in cells treated with GA in the absence of NLRP12 (Figure 2.6A, compare lanes 2 and 4), thus demonstrating that Hsp90 activity is required for NLRP12-induced NIK degradation.

NLRP12 and NIK associate to form molecular complexes [178]. Therefore, we next sought to determine if the inability to induce NIK degradation in the presence of GA was due to a loss of association. NLRP12 and NIK were co-expressed in HEK293T cells and co-immunoprecipitation experiments were performed. Although NLRP12 and NIK protein levels were both reduced by an incubation with GA (see lysate controls, row 2 and 3), there was sufficient residual protein expression that permitted the examination of their interaction (Figure 2.6B), in the absence (lane 2) or presence (lane 3) of GA. The cells were treated with GA for 6 h, NIK complexes were immunoprecipitated and Western blots were probed with anti-Ha to detect associated NLRP12. Interestingly, the association of NLRP12 with NIK was not affected by Hsp90 inhibition, suggesting that this chaperone is not required for NLRP12 to form molecular complexes with NIK. NLRP12-induced NIK degradation, however, was prevented upon Hsp90 inhibition (Figure 2.6A), thus demonstrating that Hsp90 is required for the functional activity of NLRP12.

E. DISCUSSION

NLR proteins are rapidly emerging as important mediators of innate and adaptive immune signaling. Yet, despite recent reports describing the physiologic role of NOD2, NALP3, and IPAF in the response to numerous ligands, relatively little is known concerning the molecular events that regulate these proteins. Recently, we demonstrated that the NLR protein, NLRP12, functions as a negative regulator of NF-κB activation through its association with NIK [178]. In this report we show NLRP12 requires Hsp90 for both its stabilization as well as its negative regulatory activity.

Hsp90 is a highly conserved chaperone molecule that plays a critical role in the stability and function of many signaling proteins. These client proteins generally follow a pathway where upon translation they associate with Hsp70 to achieve proper folding conformation. The client protein is then transferred to Hsp90 where it is held in a functionally active state. Upon the addition of Hsp90 inhibitors, the client protein no longer associates with Hsp90, but instead remains in a complex with Hsp70 and undergoes proteasome-mediated degradation [177]. In this study, we show that NLRP12 follows the same mechanism as reported Hsp90 client proteins. We found that upon Hsp90 inhibition with GA, NLRP12 proteins dissociated from Hsp90 and, simultaneously, accumulated within Hsp70 complexes. As a result, NLRP12 protein levels rapidly decreased due to degradation via the proteasome.

These results are analogous to those reported for NLR structural homologs found in the plant kingdom. In *Arabidopsis,* inhibition of Hsp90 reduces steady-state levels of the R proteins RPS2 and RPM1 [192]. Consequently, the pathogen-induced defensive response is attenuated and disease resistance conferred by these proteins is impaired [192-194]. Similarly, in

Nicotiana benthamiana, virus-induced gene silencing of Hsp90 results in the loss of resistance mediated by R proteins PRF (against *P. syringae)*, RX (against potato virus X), and N (against tobacco mosaic virus) [195,196]. Thus, Hsp90 is critical for disease resistance in plants as this chaperone regulates both the stability and function of multiple R proteins.

Similar to these R proteins, in this report we demonstrate that, in addition to regulating NLRP12 stability, Hsp90 also controls NLRP12 function. Although NLRP12 still bound NIK, it no longer induced NIK degradation in the presence of an Hsp90 inhibitor. Thus, while Hsp90 is required for the negative regulatory function of NLRP12, it is not required for NLRP12 to form molecular complexes with NIK. A similar observation has been made for the Hsp90 client protein, Raf. In this report, inhibition of Hsp90 reduced cytoplasmic Raf levels but did not prevent Raf from binding downstream signaling proteins [197]. These results suggest that Hsp90 activity is required for the processing steps that function downstream of NLRP12-NIK complex formation to promote degradation of the kinase. Future studies will elucidate if Hsp90 regulates the association if NLRP12-NIK complexes with ubiquitin conjugating enzymes and/or the proteasome degradation complex.

In addition to our findings regarding NLRP12, two recent reports have demonstrated a role for Hsp90 in the regulation of other NLR family members. During the review of this manuscript, Hsp90 was shown to be critical for the pro-inflammatory activity of NOD1, NOD2, IPAF and NALP3 [198]. Furthermore, inhibition of Hsp90 resulted in proteasome mediated degradation of NOD1 and NALP3 [198,199], demonstrating that similar to NLRP12, Hsp90 also serves to stabilize these NLR proteins. Together with our results, these findings suggest that a role for Hsp90 in regulating NLR stability and function represents a common feature of this family.

In summary, in this report we utilized an unbiased proteomic approach to show that NLRP12 associates with Hsp70, and a further investigation shows that it also associates with Hsp90. These two heat shock proteins regulate protein stability, and Hsp90 in particular is required for the stability of numerous inflammatory signaling molecules [177,191]. The effect of Hsp90 on NLRP12 was studied using specific Hsp90 inhibitors, and was observed not only in experiments investigating NLRP12 protein derived from transfected expression plasmids, but also with endogenous NLRP12 protein in both a monocytic cell line and primary human monocytes. Our results indicate that Hsp90 protects NLRP12 from proteasome-mediated degradation, and further regulates the function of NLRP12. These findings suggest an evolutionary conserved regulation of mammalian NLR proteins by Hsp90 that is strikingly similar to the regulation of plant R proteins.

Table 2.1 Summary of Hsp70 proteins identified by MALDI-TOF MS

Spot Number	Accession	Protein Identity	Matched/ Total Peptides	% Coverage
5	AAA52697	Hsp70	7/16	12
7	AAD21816	Hsp70.1	4/9	7
8	BAD96505	Hsp70 protein 8 isoform 1 variant	7/20	10
10	NP_006588	Hsp70 protein 8 isoform 1	11/16	20

[1] Protein spots shown above achieved a Mowse score above the significance threshold ($p<0.05$)

Figure 2.1

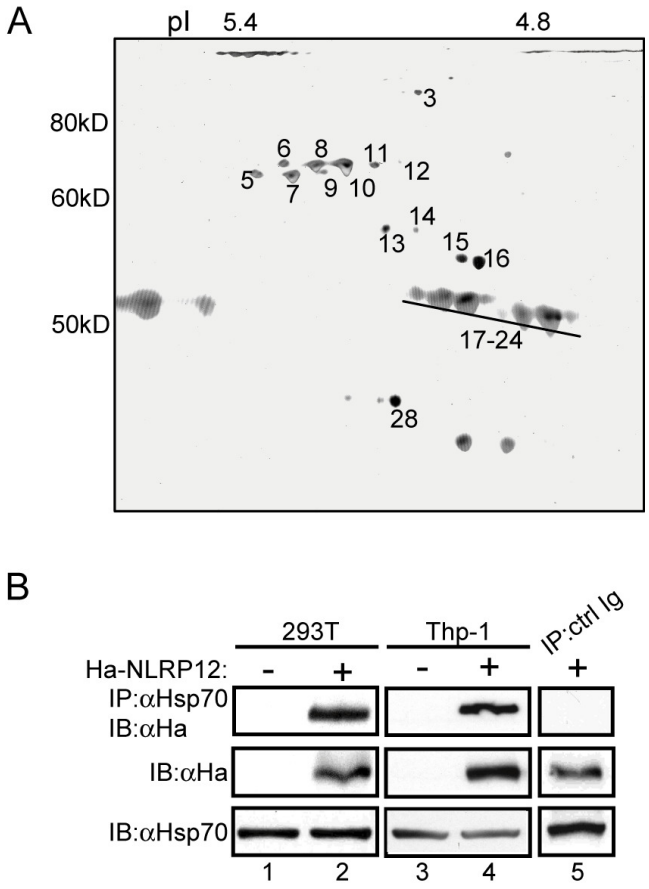

Figure 2.1: NLRP12 interacting proteins include Hsp70. (**A**) HEK293T cells were transfected with empty vector or Flag-NLRP12. Cell lysates were immunoprecipitated with anti-Flag antibodies and protein complexes were fractionated by 2D gel electrophoresis. Individual proteins were visualized by silver stain and those unique to NLRP12 transfected cells were excised and analyzed by MALDI mass spectrometry. Protein identities are described in Table 2.1. (**B**) HEK293T cells transfected with an empty control vector (lane 1) or Ha-NLRP12 (lane 2), or THP-1 monocytic cells stably transfected with an empty control vector (lane 3) or Ha-NLRP12 (lane 4) were lysed and protein complexes were immunoprecipitated with an anti-Hsp70 antibody. Following fractionation by SDS-PAGE, Western blots were probed with an anti-Ha antibody to detect NLRP12. Lysate controls show the presence of Hsp70 in all lanes and Ha-NLRP12 in the expected lanes (2 and 4). In lane 5, lysates from THP-1 cells stably expressing Ha-NLRP12 were immunoprecipitated with an isotype control antibody, mouse anti-I-A^d, and Western blots do not show non-specific binding of NLRP12. These data are representative of at least three independent experiments.

Figure 2.2

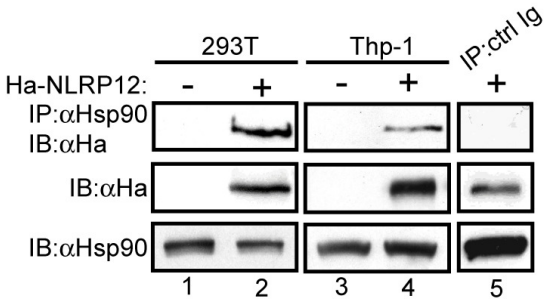

Figure 2.2: NLRP12 interacts with Hsp90. HEK293T cells transfected with an empty control vector (lane 1) or Ha-NLRP12 (lane 2), or THP-1 cells stably transfected with an empty vector (lane 3) or Ha-NLRP12 (lane 4) were lysed and protein complexes were immunoprecipitated with an anti-Hsp90 antibody. Following fractionation by SDS-PAGE, Western blots were probed with an anti-Ha antibody to detect NLRP12. Lysate controls show the presence of Hsp90 in all lanes and Ha-NLRP12 in the expected lanes (2 and 4). In lane 5, lysates from THP-1 cells stably expressing Ha-NLRP12 were immunoprecipitated with an isotype control antibody, rabbit anti-CagA, and Western blots do not show any non-specific binding of NLRP12. These data are representative of at least three independent experiments.

Figure 2.3

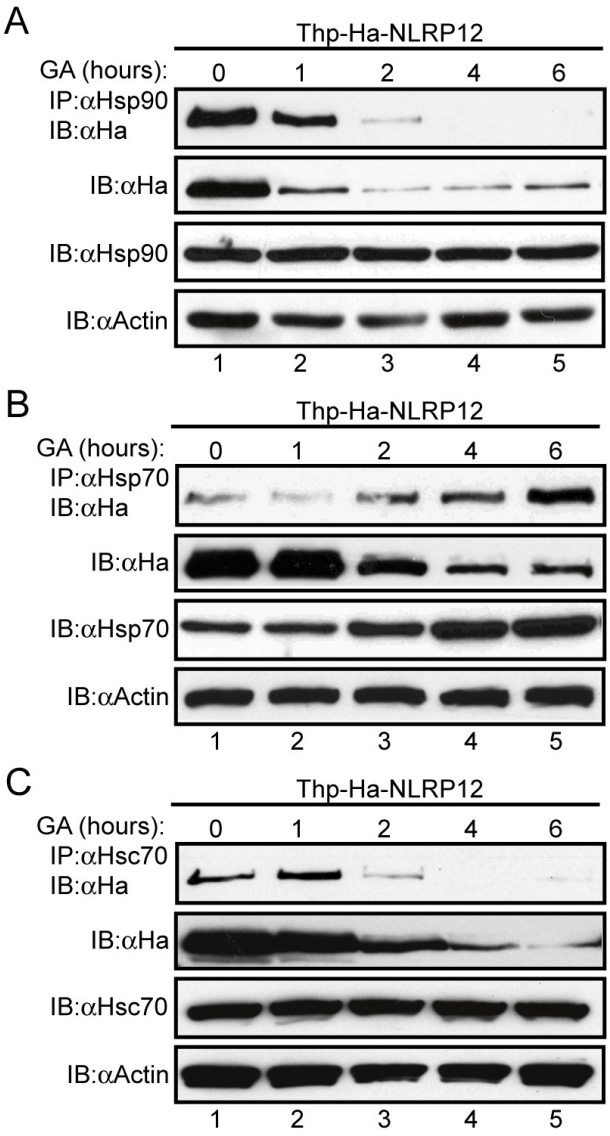

Figure 2.3: Hsp90 inhibition alters the association of NLRP12 and heat shock proteins. THP-Ha-NLRP12 monocytes were treated over the course of six hours with 0.2μM of the Hsp90 inhibitor, geldanamycin (GA). Lysates were immunoprecipitated with (**A**) anti-Hsp90 antibodies, (**B**) anti-Hsp70 antibodies, and (**C**) anti-Hsc70 antibodies, and fractionated by SDS-PAGE. Western blots were probed with an anti-Ha antibody to detect NLRP12. Immunoblots were performed with the indicated antibodies to monitor protein expression. All panels are representative of at least three independent experiments.

Figure 2.4

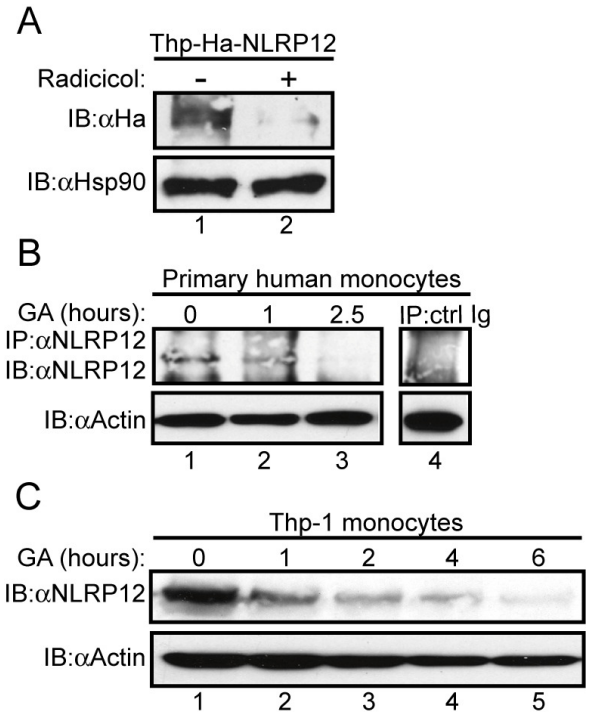

Figure 2.4: Endogenous NLRP12 stability is dependent on Hsp90 activity. (**A**) THP-Ha-NLRP12 monocytes were treated with 0.1μM of the Hsp90 inhibitor, Radicicol, for 1 hour. Lysates were fractionated by SDS-PAGE. Western blots were probed with an anti-Ha antibody to detect NLRP12. Control immunoblots were probed with an anti-Hsp90 antibody to ensure equal loading. (**B**) Human THP-1 monocytes were treated over the course of six hours with 0.2μM GA and lysates were fractionated by SDS-PAGE. Western blots were probed with a mouse anti-NLRP12 antibody to detect endogenous NLRP12. Control immunoblots were probed with an anti-actin antibody to ensure equal loading. (**C**) Human primary adherent monocytes were enriched from PBMC by adherence and treated with 0.2μM GA for the indicated times. To detect endogenous NLRP12, lysates were immunoprecipitated with a rabbit anti-NLRP12 antibody, fractionated by SDS-PAGE, and Western blots were probed with a mouse anti-NLRP12 antibody. Control immunoprecipitations were performed with normal rabbit serum and Western blots were probed with a mouse anti-NLRP12 antibody. Control immunoblots were probed with an anti-actin antibody to ensure equal loading. Each panel is representative of at least three independent experiments.

Figure 2.5

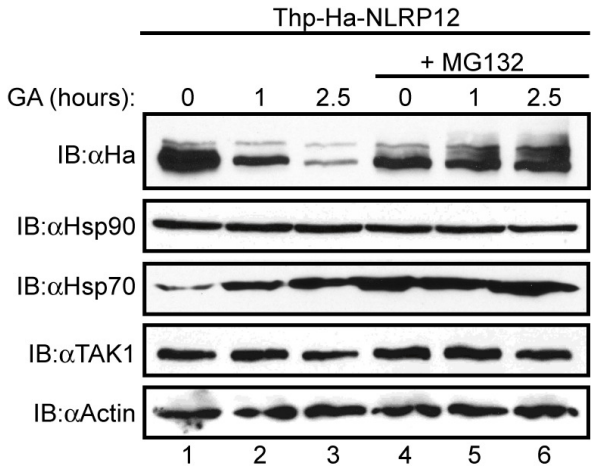

Fig. 2.5: Inhibition of Hsp90 induces proteasome-mediated degradation of NLRP12. THP-Ha-NLRP12 human monocytes were treated with 0.2μM GA for the indicated times in the absence (lanes 1-3) or presence (lanes 4-6) of the proteasome inhibitor, MG-132 (10μM). Lysates were fractionated by SDS-PAGE and immunoblots were probed with an anti-Ha antibody to detect NLRP12. Lysate controls show protein levels of Hsp90, Hsp70, TAK1, and Actin. These data are representative of at least three independent experiments.

Figure 2.6

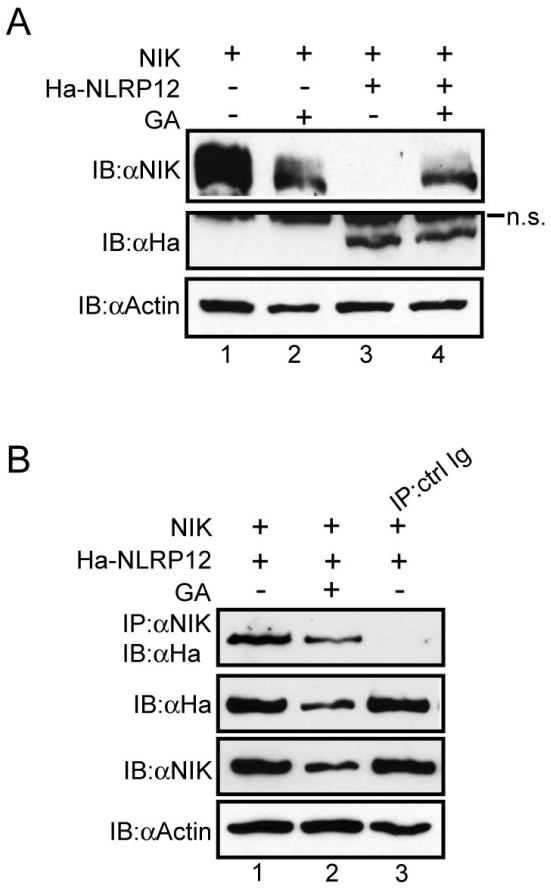

Fig. 2.6: Hsp90 is required for NLRP12-induced NIK degradation. (**A**) HEK293T cells were transfected with NIK and in the indicated lanes, Ha-NLRP12. Cells were treated for six hours with 0.1μM GA. Lysates were fractionated by SDS-PAGE and Western blots were probed with an anti-NIK antibody. NLRP12 was detected in the expected lanes using an anti-Ha antibody; "n.s." denotes non-specific band. Control immunoblots were probed with an anti-actin antibody. (**B**) HEK293T cells were transfected with NIK and Ha-NLRP12 and treated the following day for six hours with 0.2μM GA, where indicated. Protein complexes were immunoprecipitated from cellular lysates with an anti-NIK antibody (lanes 1-2), fractionated by SDS-PAGE, and Western blots were probed with an anti-Ha antibody to detect NLRP12 in NIK-containing complexes. In lane 3, lysates were immunoprecipitated with an isotype control antibody, rabbit anti-CagA and Western blots do not show any non-specific binding of NLRP12. NLRP12 was detected in cellular lysates using anti-Ha, NIK was detected using an anti-NIK antibody, and control immunoblots were probed with an anti-actin antibody. Each panel is representative of at least three independent experiments.

CHAPTER 3: NLRP12 CONTROLS ADAPTIVE IMMUNE RESPONSES BY REGULATING DENDRITIC CELL MIGRATION

This research was originally submitted to *Nature* in November of 2008.

It was submitted to *Science* in a revised form in April of 2008 and is currently under review.

Arthur JC, Lich JD, Ye Z, Allen IC, Gris D, Schneider M, O'Connor BP, Moore CB, Morrison A, Sutterwala FS, Bertin J, Liu Z, and Ting JP. NLRP12 controls adaptive immune responses by regulating dendritic cell migration. *Submitted to Nature November 2008*.

A. ABSTRACT

Long-term immuno-reactivity is a salient feature of protective immunity as well as destructive autoimmune and hyperinflammatory responses. The establishment of this reactivity relies largely on the ability of dendritic cells (DCs) to orchestrate innate and adaptive immune pathways [200,201]. Under homeostatic conditions, these cells survey peripheral tissues, collect local antigens, and then migrate to secondary lymphoid organs. Under inflammatory conditions the migration to draining lymph nodes intensifies and these cells become highly potent activators of antigen specific T cells [200, 202]. In this study, we described an unexpected role for a nucleotide-binding domain leucine rich repeat (NLR) protein, NLRP12 (formerly Monarch-1[154]), an NLR linked to atopic dermatitis[58] and hereditary periodic fever[57]. We show that *Nlrp12* is expressed in DCs and is required for the adaptive immune response to hapten-induced contact hypersensitivity, a model of atopic dermatitis, and experimental autoimmune encephalitis (EAE), a model of multiple sclerosis. The inability of *Nlrp12*$^{-/-}$ cells to induce adaptive immunity is not due to failure in antigen processing and presentation nor to defects in cytokine production including IL-1β. Rather, *Nlrp12*$^{-/-}$ DCs display a significantly reduced capacity to migrate to draining lymph nodes, associated with a perturbation of intracellular signaling pathways that govern DC activation and migration. These findings reveal a novel role for NLRP12 in regulating the ability of DCs to influence adaptive immunity.

B. INTRODUCTION

NLRs constitute a large gene family of 20-30 members that are preserved from plants to humans. NLR proteins have rapidly emerged as important components of inflammation and immunity. To date, at least five NLR proteins are predicted to form a large protein complex termed the inflammasome [95]. This complex functions in caspase-1 activation and subsequent release of bioactive IL-1β and related cytokines. Other NLR proteins have been shown to function as regulators of inflammatory signals such as NF-κB [118,147,203]. We previously showed that human *NLRP12*/*Monarch-1* is expressed by monocytes/macrophages and granulocytes and it inhibits the activation of noncanonical NF-κB [152,154]. This activity requires ATP binding by NLRP12 [35,154]. More recently, a mutation that results in a truncated form of NLRP12 has been linked to periodic fever syndrome, a recurrent hyperinflammatory disease of unknown etiology [57].

C. MATERIALS AND METHODS

Mice: An *Nlrp12* targeting vector was electroporated into 129SvEvBrd Lex-1 embryonic stem (ES) cells and homologous recombinant ES cells were microinjected into C57BL/6 blastocysts. Chimeras were backcrossed to C57BL/6 mice (Jackson Laboratories) for 9 generations and PCR of genomic tail DNA confirmed germline transmission. *Nlrp3*$^{-/-}$ mice[204] were also backcrossed onto C57BL/6 for 9 generations. OT-II mice, which express the $OVA_{323–339}$-specific TCR transgene on the C57BL/6 background, were obtained from M. Croft (La Jolla Institute of Allergy and Immunology). All mice were maintained in specific pathogen free housing at the University of North Carolina (UNC) at Chapel Hill. All experiments were performed with age- and sex- matched mice between 6-12 weeks old. All studies were conducted in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* as well as the Institutional Animal Care and Use Committee guidelines of UNC Chapel Hill.

Genotyping: Genomic tail DNA was isolated from *Nlrp12*$^{+/-}$ crosses and amplified by PCR with the following primers: F1 5'-CCCACAAAGTGATGTTGGACTG-3', F2 5'-GCAGCGCATCGCCTTCTATC-3', R1 5'-GAAGCAACCTCCGAATCAGAC-3'.

Generation of bone marrow derived dendritic cells (BMDC): Dendritic cells were generated from bone marrow precursors as previously described[205]. Surface marker phenotype was determined by single-color flow cytometry as described[205,206]. Staining was quantified with a CyAn ADP flow cytometer (Beckman Coulter), and analyzed using FlowJo software (Tree Star Inc.).

Contact hypersensitivity (CHS)[207]: Mice were sensitized by topical application of hapten to the depilated abdomen and footpads: either 200µl of 3% oxazolone (Sigma-

Aldrich) in ethanol, or 200µl of 0.5% FITC (Sigma-Aldrich) in 1:1 acetone:dibutyl phthalate (Sigma-Aldrich). Five days later, 20µl of 1% oxazolone in ethanol or 20µl of 0.5% FITC in acetone:dibutyl phthalate was topically applied to one ear. The contra-lateral ear was mock-treated with carrier, and control mice were treated with carrier on both ears. After 24 hours, mice were euthanized and 8mm circular samples of ear tissue were excised and weighed. CHS response was assessed by subtracting the weight of the carrier-treated ear from that of the hapten-treated ear. Ear tissue was then fixed in formalin, paraffin embedded, sectioned, and stained with H&E. Immune cell infiltration was quantified as average pixel density (x 10^4) using ImageJ software[208] from 4 fields per ear. Ear thickness was measured at 20 µm intervals under 40x magnification.

Ear tissue homogenates: Ear tissue harvested during CHS experiments was homogenized and sonicated in T-PER reagent (Thermo Scientific) as previously described[209]. Total protein concentration was determined by Bradford assay (Bio-Rad) and IL-1β was measured by ELISA (BD Biosciences).

EAE: EAE was induced as previously described[210]. Mice were immunized s.c. to the flanks with MOG_{35-55}(250µg; Genemed Synthesis) in CFA containing 500µg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco laboratories); 200 ng pertussis toxin (List Biological Labs) was administered i.p. at the time of immunization and 2 days later. Animals were scored daily by two independent observers: 0 absence of tail weakness, 0.5 tail touching ground frequently, 1 tail dragging on ground constantly, 1.5 weakness of hind limbs, 2 severe paralysis of at least one hind limb, 2.5 severe paralysis of both hind limbs, 3 complete hind limb paralysis, 3.5 front limb weakness, 4 severe paralysis of front limbs, 4.5 complete front limb paralysis, 5 moribund state.

FITC-induced in vivo migration[211]*:* Twenty µl of 0.5% FITC in 1:1 acetone:dibutyl phthalate was applied topically to both ears. After 24 or 48 hours, draining and non-draining lymph nodes were removed and single-cell suspensions were stained with anti-CD11c-APC (HL3, BD Biosciences) and analyzed by flow cytometry. Ear epidermal sheets were prepared 24 hours after FITC application,[212] stained with biotin-labeled anti-I-A^b (AF6-120.1; BD Biosciences) followed by streptavidin-Alexa Fluor 595 (Invitrogen), then mounted with Vectashield (Vector Laboratories) and visualized by fluorescent microscopy. DCs per 400x field are represented as the mean of 4 fields per sample, counted by a blinded reader. Percent of DCs remaining after FITC treatment was calculated as DCs per 400x field in treated ear divided by DCs per 400x field in contra-lateral untreated ear x 100.

Ova-induced in vivo migration[213]*:* Mice were injected s.c. into either flank with 50µl of Alexa Fluor 647 – labeled Ova (4 mg/ml in PBS; Invitrogen) emulsified in CFA. After 24 hours, draining and non-draining lymph nodes were removed and single-cell suspensions were stained with anti-CD11c-PeCy7 (HL3, BD Biosciences) and analyzed by flow cytometry.

In vitro migration: BMDC were seeded at $2x10^5$ per upper well of 96-well transwell plates with 5µm pores (ChemoTx System; NeuroProbe) and chemokines (Peprotech) in serum-free RPMI were added to lower wells. Plates were incubated at 37°C for 3 hours. Cells were removed from upper wells and plates were centrifuged to recover migrated cells. Migrated cells were incubated for 4 hrs with Tetrazolium hydroxide XTT (Sigma) then quantified by measuring the reduction of XTT to formazan at 450nm-650nm.

Immunoblots: BMDC were serum starved, stimulated as indicated, and lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM NaF, 2 mM plus protease

inhibitor cocktail (Roche). Protein concentrations were determined by Bradford assay (Bio-Rad), separated by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), probed with the indicated antibody, and visualized by chemiluminescence (Pierce). TNFα and CCL19 were obtained from Peprotech. Antibodies to pAKT and pERK were from Cell Signaling Technologies, antibodies to AKT, ERK and HSP90 from Santa Cruz Biotechnology, and antibody to histone H3 from Upstate Biotechnology.

Statistics: Central tendencies are presented as mean ± s.e.m. Pairwise comparisons were evaluated for statistical significance using two-tailed Mann Whitney *U* test, $\alpha = 0.05$. Statistics were computed using Prism 4 (GraphPad).

D. RESULTS AND DISCUSSION

To assess the *in vivo* role of NLRP12, mice deficient in *Nlrp12* (*Nlrp12*$^{-/-}$) were generated by homologous recombination, replacing a region of exon 3 containing the Walker A and Walker B sequences with the neomycin resistance gene (Fig. 3.1 a-b). Animals were backcrossed to the C57BL/6 strain for nine generations. *Nlrp12*$^{-/-}$ mice displayed no gross abnormalities, and there was no difference from wild type C57BL/6 (WT) mice in cellularity of the peripheral blood, bone marrow, spleen or lymph nodes (Fig. 3.2 and Table 3.1). In line with myeloid expression of human *NLRP12* [151,179], mouse *Nlrp12* was expressed in bone marrow and spleen tissues but not other non-immune organs (not shown) and, at the cellular level, in granulocytes and dendritic cells (Fig. 3.3). Unlike human *NLRP12,* mouse *Nlrp12* was not detected in bone-marrow derived or residential peritoneal macrophages.

Previous studies have demonstrated that deletion of other NLR genes expressed in innate immune cells, such as *Nlrp3*, results in a profound protection from endotoxic shock [102,103]. To determine if *Nlrp12*$^{-/-}$ mice share a similar phenotype, mice were administered LPS via intraperitoneal (i.p.) injection. No statistically significant difference in mortality was observed, although *Nlrp12*$^{-/-}$ displayed a trend of decreased survival at two difference doses of LPS (Fig. 3.4). Liver and kidney functions were similar between WT and *Nlrp12*$^{-/-}$ mice (Table 3.2). The resistance to endotoxic shock observed in *Nlrp3*$^{-/-}$ mice has been attributed to its role in IL-1β secretion [102]. *Nlrp12*$^{-/-}$ mice displayed no significant defect in IL-1β production from bone marrow cells or bone marrow derived dendritic cells (BMDCs) stimulated with different TLR agonists while *Nlrp3*$^{-/-}$ did (Fig 3.5). Similarly, no differences were observed in the production of IL-12p40 in BMDCs or IL-6 and TNFα in TLR stimulated bone marrow cells (Fig. 3.5). To determine if NLRP12 functions in the innate

immune response to live bacteria, *Klebsiella pneumoniae* was administered intra-tracheally in a model of bacterial pneumonia [214]. This bacteria causes a Nlrp3-dependent inflammation (S. Willingham, personal communications). Similar to LPS, no statistically significant differences were detected in body temperature, weight loss, BALF cellularity or mortality (Fig. 3.6). These results demonstrate that unlike Nlrp3, NLRP12 does not play a detectable role in these models of innate immune activation.

An emerging role for NLR genes is the regulation of the adaptive immune response [102,118]. *Nlrp12* expression in dendritic cells suggests that NLRP12 may affect these pathways. To evaluate the role of NLRP12 in adaptive immunity and due to a previous genetic association of *NLRP12* with atopic dermatitis[58], we performed a model of contact hypersensitivity (CHS) [207]. WT and *Nlrp12*$^{-/-}$ mice were sensitized epicutaneously to the abdomen with hapten, oxazolone or fluoroscein isothiocyanate (FITC). Five days later, the same hapten was epicutaneously applied to the ear to elicit a hapten-specific immune response. Twenty-four hours post-elicitation, *Nlrp12*$^{-/-}$ mice displayed a significantly weaker response to both haptens, as indicated by reduced swelling and reduced inflammatory cell infiltration to the site of elicitation (Fig. 1 c-g). In *Nlrp3*$^{-/-}$ mice, CHS is attenuated due to decreased production of IL-1β [215]. In *Nlrp12*$^{-/-}$ mice, however, equivalent levels of IL-1β were detected in ear tissue homogenates from hapten-treated WT and *Nlrp12*$^{-/-}$ mice (Fig. 1h). This suggests that the role of NLRP12 in CHS is independent of inflammasome-mediated cytokine production.

To further investigate the role of NLRP12 in adaptive immunity, we induced EAE by subcutaneous (s.c.) immunization with MOG peptide in complete Freund's adjuvant (CFA). Severity of EAE was evaluated by daily monitoring and scoring of mean clinical score as

described by others[210]. WT mice developed measurable EAE within 8 days of immunization (mean ± s.e.m., 7.25 ± 0.750) and progressed steadily (Fig. 1i). In sharp contrast, *Nlrp12*$^{-/-}$ mice displayed no measurable abnormalities for up to 15 days following immunization (mean ± s.e.m., 15.43 ± 2.08). Overall, the onset was delayed (p=0.0037) and the disease less severe in *Nlrp12*$^{-/-}$mice.

These results indicate that NLRP12 plays a key role in adaptive immunity. Based upon the expression pattern of *Nlrp12*, we next focused on other DC functions. Analysis of cell surface levels of MHC class II and co-stimulatory factors indicated no difference in BMDC maturation between WT and *Nlrp12*$^{-/-}$ mice (Fig 3.7). To determine if *Nlrp12*$^{-/-}$ DCs have a defect in antigen processing and presentation, WT and *Nlrp12*$^{-/-}$ BMDCs were cultured in the presence of ovalbumin (Ova) and then incubated with CFSE-labeled Ova-specific T cells (Fig. 3.8). WT and *Nlrp12*$^{-/-}$ BMDCs induced antigen dependent T cell proliferation to similar levels, indicating that NLRP12 is not required for antigen processing and MHC class II restricted presentation.

A key function of DCs in CHS and EAE models is to collect peripheral antigens and migrate to draining lymph nodes. To evaluate DC migration *in vivo*, FITC was applied epicutaneously to the ears of WT and *Nlrp12*$^{-/-}$ mice[211]. Draining lymph nodes were removed 24 and 48 hours later, and migration of FITC^{+}CD11c^{+} dendritic cells was assessed by flow cytometry. In agreement with the CHS results, the amount of FITC^{+} DCs in the draining lymph nodes of *Nlrp12*$^{-/-}$ mice was significantly reduced compared to WT mice at both 24 and 48 hours after FITC application (Fig. 3.9 a-b). These DCs did not traffic to other immune organs, as no FITC^{+}CD11c^{+} DCs were detected in the bone marrow or spleen (data not shown). In contrast to *Nlrp12*$^{-/-}$ mice, *Nlrp3*$^{-/-}$ DCs migrated to draining lymph nodes to

levels comparable to WT DCs. (Fig. 3.9c). Quantification of DCs in untreated skin revealed no difference in overall resting DC numbers (Fig. 3.9d). However, following FITC treatment the number of WT DCs decreased by approximately 40% while the number of *Nlrp12*$^{-/-}$ skin DCs did not change, indicating that more WT DCs egressed from the skin than *Nlrp12*$^{-/-}$ DCs (Fig. 3.9e). These data suggest that DC migration from the periphery to the draining lymph node is significantly impaired in *Nlrp12*$^{-/-}$ mice.

During CHS, antigen is administered epicutaneously; whereas in the EAE model, antigen is administered subcutaneously. Thus we next determined if upon s.c. antigen delivery, *Nlrp12*$^{-/-}$ DCs fail to migrate to the lymph nodes. Fluorescent-labeled Ova in CFA was injected s.c. into the flanks of WT or *Nlrp12*$^{-/-}$ mice and draining lymph nodes were removed after 24 hours[213]. Ova^{+}CD11c^{+} DCs were quantified by flow cytometry (Fig 3.9f). Analogous to epicutaneous application of FITC, *Nlrp12*$^{-/-}$ DCs displayed a significant decreased in migration to draining lymph nodes following s.c. administration of Ova antigen. Together, these results demonstrate that *Nlrp12*$^{-/-}$ mice have a defect in the ability of DCs to migrate from the periphery to the draining lymph node, and this provides a mechanism by which CHS and EAE are attenuated in *Nlrp12*$^{-/-}$ mice.

Peripheral DCs migrate to draining lymph nodes in response to lymph node homing chemokines that act through CCR7 and CXCR4 on DCs [216-218]. Analysis of CCR7 and CXCR4 surface expression demonstrated that *Nlrp12*$^{-/-}$ BMDCs expressed these chemokine receptors at levels similar to WT cells (Fig. 3.10), suggesting that reduced *in vivo* migration was not due to differences in CCR7 or CXCR4 expression. To determine if *Nlrp12*$^{-/-}$ DCs were deficient in their ability to respond to CCR7 and CXCR4 ligands, BMDC migration was evaluated in an *in vitro* transwell migration assay. Compared to WT cells, *Nlrp12*$^{-/-}$

BMDCs demonstrated significantly reduced migration toward CCL19, CCL21, and CXCL12 (Fig. 3.11 a-c and Table 3.3). In contrast, *Nlrp3*$^{-/-}$ BMDCs migrated toward these chemokines at levels nearly identical to WT cells (Fig. 3.11 e-g). BMDC from all genotypes failed to migrate toward CCL5, a chemoattractant to immature dendritic cells[219], supporting the earlier observation that NLRP12 does not affect DC maturation (Fig. 3.11 d, h). These data suggest that NLRP12 plays a role in DC migration and this is not dependent on Nlrp3. Furthermore, based upon the restricted expression of *Nlrp12* to DCs, these results suggest that the inability of *Nlrp12*$^{-/-}$ cells to migrate is DC intrinsic.

In the periphery, DC migration toward draining lymph nodes is triggered by local production of inflammatory cytokines, such as TNFα, that induce the expression of CCR7 [216]. To model peripheral activation of DCs, WT and *Nlrp12*$^{-/-}$ BMDCs were stimulated with TNFα and then treated with CCL19, a CCR7 agonist. Key signal transduction pathways downstream of chemokine receptor activation include the PI3K, MAPK and NF-κB pathways[216]. The activation of PI3K leads to AKT phosphorylation. Levels of phosphorylated AKT (pAKT) were not reduced in *Nlrp12*$^{-/-}$ cells, indicating that AKT activation proceeded normally in both WT and *Nlrp12*$^{-/-}$ cells following treatment with CCL19 (Fig. 3.12a). In contrast, notable differences were found in ERK phosphorylation following CCR7 stimulation (Fig. 3.12b). In WT BMDCs, ERK phosphorylation was detected within minutes of CCL19 treatment. However, ERK activation was profoundly delayed in *Nlrp12*$^{-/-}$ cells, with ERK phosphorylation not detectable until 3 hours after CCR7 activation. Finally, our previous work in human monocytic/macrophage cell lines showed that NLRP12 suppresses activation of the noncanonical NF-κB pathway by inducing degradation of NF-κB inducing kinase (NIK)[154]. NIK is required for noncanonical NF-κB

p100 cleavage to its active form p52[167], thus to determine if NLRP12 has a similar function in mouse cells, we assessed p52 production in BMDC. Treatment of *Nlrp12*$^{-/-}$ BMDCs with TNFα alone induced the activation of noncanonical NF-κB, as evidenced by the presence of p52 (Fig. 3.12c). In contrast, TNFα treatment alone did not activate noncanonical NF-κB in WT BMDCs. Rather, these cells required secondary stimulation via CCR7 to induce detectable levels of p52. Thus *Nlrp12*$^{-/-}$ DCs displayed decreased activation of ERK but increased activation of p52 downstream of CCR7. These results demonstrate that the molecular signaling pathways in response to a combination of TNFα and CCL19 are perturbed in *Nlrp12*$^{-/-}$ mice and provide a basis for NLRP12 regulation of adaptive immunity.

The reduction in ERK activation is congruent with the reduction of migratory properties of *Nlrp12*$^{-/-}$ cells[220]. However, although it confirmed our previous findings, the enhanced activation of noncanonical NF-κB is more difficult to link to reduced DC migration. In *aly/aly* mice, which do not activate noncanonical NF-κB, DCs migrate normally[221]. This suggests that noncanonical NF-κB activation is not required for DC mobilization. Our findings support these results and correlate inappropriate activation of noncanonical NF-κB with suppressed DC migration, although the direct link between these two events remains to be elucidated.

In summary, this work describes an NLR protein with a novel regulatory role in dendritic cell migration that affects chemokine signal transduction and impacts the outcome of CHS and EAE, respectively animal models of atopic dermatitis and multiple sclerosis. It is of interest that NLRP12 polymorphisms have been associated with atopic dermatitis[58] and skin urticaria[57]. This is the first description of an NLR protein expressed by dendritic cells that directly affects the function of these cells to alter adaptive immunity and associated

disease models. While NLRP3, NOD1 and NOD2 also affect adaptive immune response, NLRP3 and NOD2 likely mediate this through the indirect effects of cytokines such as IL-1β, IL-18 or IL-12 on T cell function, and the mechanism of NOD1 is not well understood[102,118,119]. This expands the biologic importance of this family of novel proteins.

Table 3.1

Table 3.1: Cellularity of peripheral blood

	WBC (10^3/mm^3)	RBC (10^6/mm^3)	HGB (g/dl)	HCT (%)	MON (10^3/mm^3)	GRA (10^3/mm^3)	LYM (10^3/mm^3)
WT	8.5 ± 1.0	9.5 ± 0.2	15.3 ± 0.5	42.5 ± 1.4	0.8 ± 0.1	0.9 ± 0.0	6.8 ± 0.9
Nlrp12 $^{-/-}$	7.2 ± 2.5	9.2 ± 0.4	14.5 ± 0.6	41.3 ± 1.7	0.7 ± 0.2	1.1 ± 0.3	5.4 ± 2.0

All values fall within normal reference ranges and depict mean ± s.e.m. WT n=3, *Nlrp12* $^{-/-}$ n=3.
WBC, white blood cells; HGB, hemoglobin; HCT, hematocrit; MON, monocytes; GRA, granulocytes; LYM, lymphocytes.
Mice were euthanized and cardiac blood was collected into tubes containing EDTA.
Analysis was performed by the Animal Clinical Chemistry and Gene Expression Laboratory at UNC Chapel Hill with the HESKA CBC Veterinary Hematology System.

Table 3.2

Table 3.2: Liver and Kidney function

	Albumin (g/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	BUN (mg/dL)	Creatine (mg/dL)	Na+ (mmol/L)	Cl- (mmol/L)
WT	2.7± 0.2	69.4 ± 13.5	46.9 ± 1.6	32.1 ± 8.4	19.0 ± 0.9	0.1 ± 0.0	151.0 ± 1.9	114.8 ± 1.8
Nlrp12 $^{-/-}$	2.4 ± 0.2	71.4 ± 17.2	47.8 ± 0.9	35.2 ± 12.0	17.8 ± 1.6	0.1 ± 0.0	149.0 ± 1.4	120.4 ± 3.9

All values depict mean ± s.e.m. WT n=7, *Nlrp12* $^{-/-}$ n=5.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.
Serum from survivors of the LPS endotoxic shock experiment (5 mg/kg) were tested for liver and kidney function
Analysis was performed by the Animal Clinical Chemistry and Gene Expression Laboratory at UNC Chapel Hill

Table 3.3

Table 3.3: *In vitro* DC migration

Chemokine	Migrated cells		*n*	P value
CCL19 (ng/ml)	WT	*Nlrp12* -/-		
1000	19,813 ± 1,225	12,454 ± 1,082	5	0.0004 ***
100	16,527 ± 1,017	9,736 ± 982	5	0.0002 ***
10	6,569 ± 777	4,180 ± 416	5	0.0465 *
1	1,908 ± 520	2,120 ± 239	4	0.4025
CCL21 (ng/ml)				
1000	9,900 ± 1,633	3,814 ± 784	3	0.0244 *
100	2,941 ± 869	1,166 ± 404	3	0.1304
10	433 ± 194	352 ± 150	3	0.7962
1	272 ± 175	647 ± 309	3	0.6048
CXCL12 (ng/ml)				
1000	22,666 ± 2,527	15,530 ± 1,823	4	0.0338 *
100	14,270 ± 1,907	8,482 ± 1,568	3	0.0360 *
10	3,862 ± 841	2,766 ± 562	3	0.3865
1	1,127 ± 279	1,807 ± 298	2	0.2403
CCL5 (ng/ml)				
1000	915 ± 520	436 ± 174	3	0.8148
100	364 ± 152	553 ± 150	3	0.3213
10	420 ± 206	325 ± 153	3	0.7430
1	292 ± 119	916 ± 376	3	0.1996
Media				
	1,342 ± 324	1,071 ± 195	4	0.6446

Pairwise comparisons were made using a two-tailed Mann Whitney *U* test, $\alpha = 0.05$
The number of migrated cells is indicated as mean ± s.e.m.
XTT was used to calculate the number of migrated cells; the lowest standard was 1,563 cells.
The number of experiments are indicated as *n;* replicate wells were seeded in each experiment.

Figure 3.1

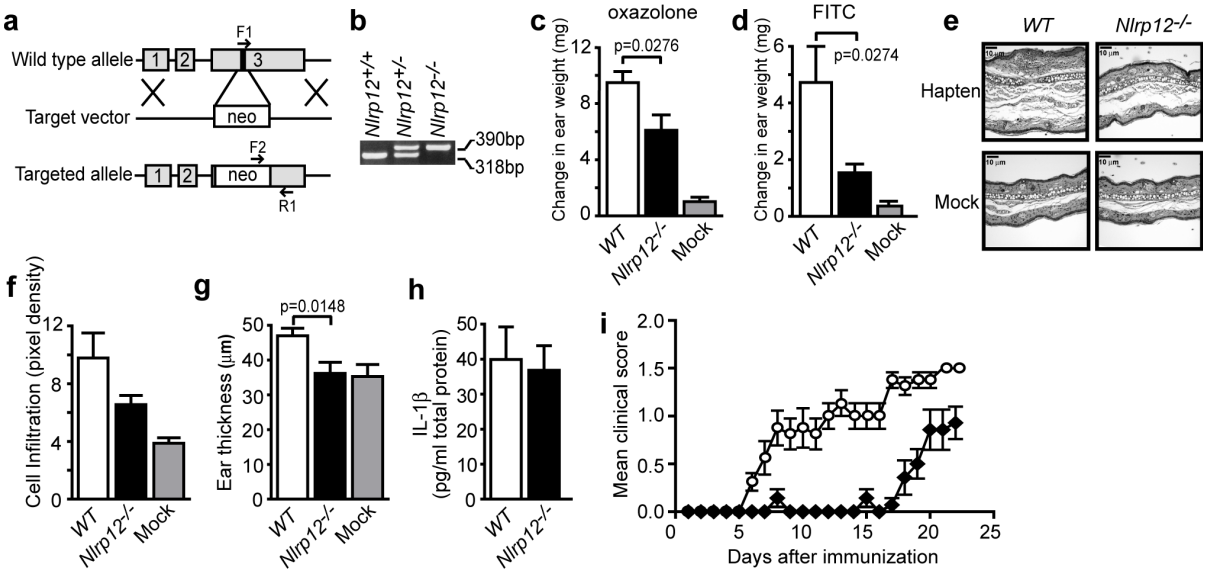

Figure 3.1: ***Nlrp12*$^{-/-}$ mice fail to mount robust adaptive immune responses.** (**a-b)**, Targeted disruption of the *Nlrp12 gene*. A 352bp region of Exon 3 containing the Walker A/B motifs was replaced with an IRES-LacZ/MC1 neo cassette. **b**, PCR genotyping of tail DNA from *Nlrp12*$^{+/-}$ crosses amplified a 318bp wild-type (primers F1 and R1) and a 390bp targeted band (primers F2 and R1). **c-g**, *Nlrp12*$^{-/-}$ mice exhibit reduced CHS response to (**c, e-g**) oxazalone (WT n=12, *Nlrp12*$^{-/-}$ n=13, Mock n=6) and (**d**) FITC (WT n=7, *Nlrp12*$^{-/-}$ n=8, Mock n=3). Results depict mean ± s.e.m and comparisons between WT and *Nlrp12*$^{-/-}$ were made using two-tailed Mann Whitney *U* test, $\alpha = 0.05$. Data are each comprised of two independent experiments. **i**, Development of EAE in WT (○ n=8) and *Nlrp12*$^{-/-}$ (♦ n=7). EAE was induced by s.c. injection of MOG_{35-55} in CFA. Animals were scored daily by two independent observers, one blinded. Data are presented as mean clinical score ± s.e.m. and are representative of 2 experiments totaling at least 10 mice per group.

Figure 3.2

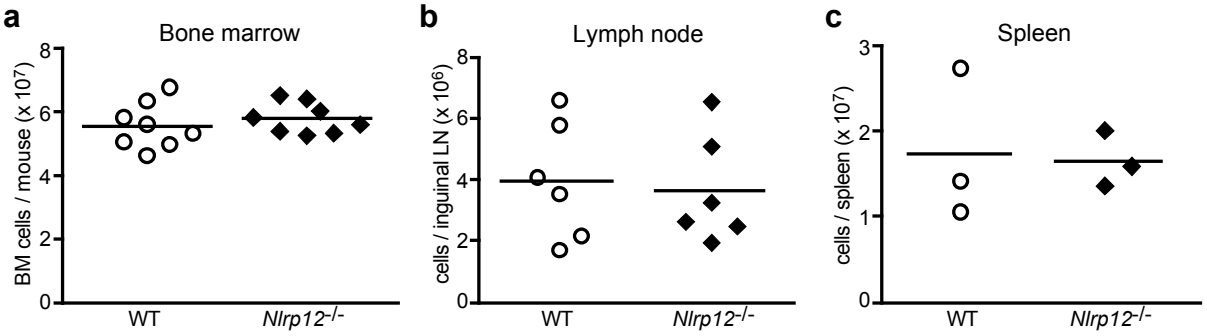

Figure 3.2: Quantification of total cells in WT (○) or *Nlrp12*$^{-/-}$ (♦) a, bone marrow **b,** lymph nodes and **c,** spleen. Total live cell number in bone marrow, spleen and lymph nodes were enumerated after red blood cell lysis using Trypan blue exclusion and hemacytometer.

Figure 3.3

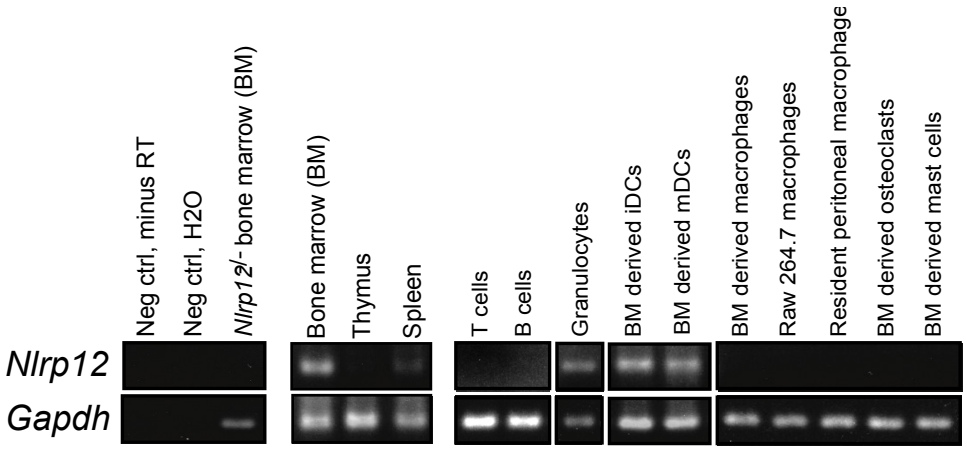

Figure 3.3: Expression analysis of *Nlrp12*. cDNA was synthesized from total RNA using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol. *Nlrp12* expression was assessed by PCR using intron-spanning primers: forward 5'-GTCCAGACTCAGTCCACATA, reverse 5'-GTATAAGGCCAGCTCGATCA. *GAPDH* was amplified with: forward 5'-TGAAGCAGGCATCTGAGGG, reverse 5'-CGAAGGTGGAAGAGTGGGAG. Cell populations were isolated from C57BL/6 mice by methods previously described: Splenic T cells[222] and B cells[223] were isolated by negative selection; Granulocytes were isolated from bone marrow[224]; Dendritic cells[205], macrophages[225], mast cells[226] and osteoclasts[227] were generated from bone marrow precursors; Raw264.7 macrophages were purchased from the American Type Culture Collection (ATCC); Resident peritoneal macrophages were obtained by lavage with PBS and enriched by adherence overnight.

Figure 3.4

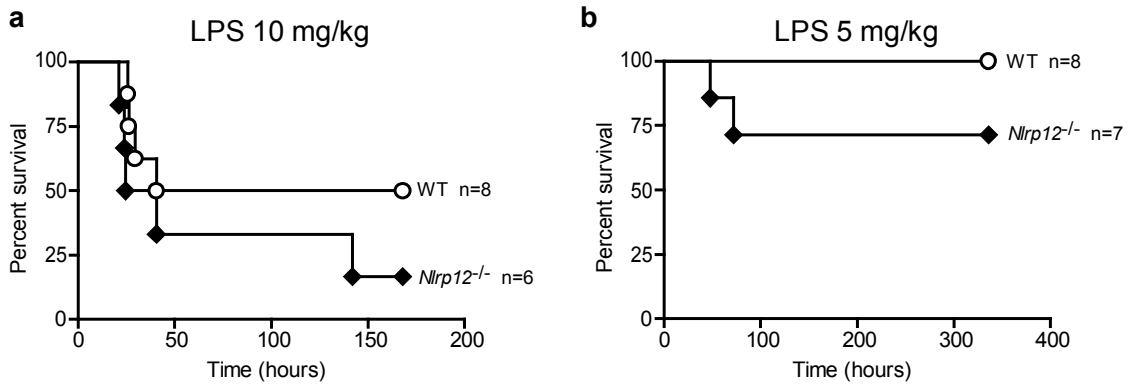

Figure 3.4: LPS-induced endotoxic shock in *Nlrp12*$^{-/-}$ mice. Mice were injected i.p. with **a,** 10 mg/kg or **b,** 5 mg/kg of *E. coli* LPS (serotype 0111:B4; Invivogen). Mice were monitored for lethality twice daily for up to 14 days. Percent survival was compared between WT (○) and *Nlrp12*$^{-/-}$ (♦) mice.

Figure 3.5

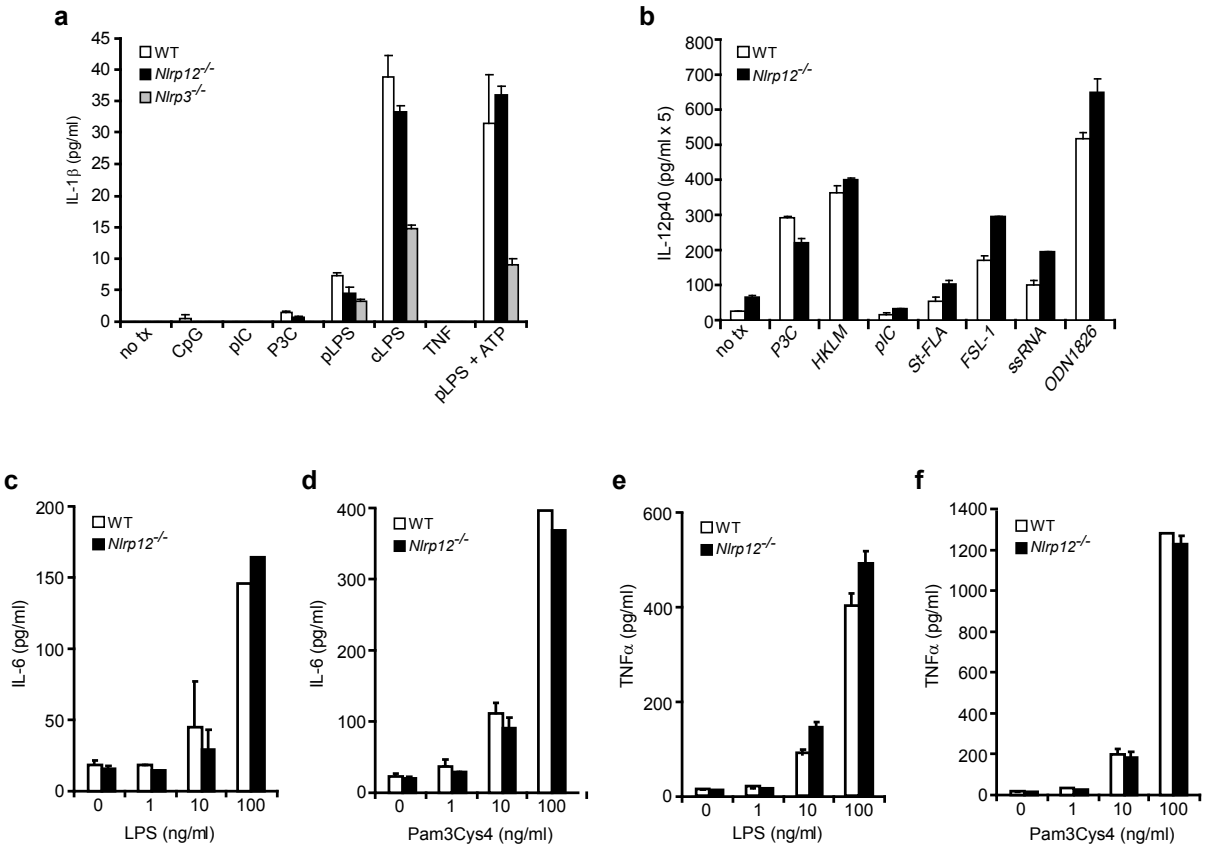

Figure 3.5: **Cytokine production in *Nlrp12*$^{-/-}$ cells**. **a-b**, BMDCs were stimulated for 24 hours with the indicated agonists (Invivogen). IL-1β and IL-12p40 were measure in supernatants by ELISA (R&D Systems and BD Bioscience). **c-f**, Freshly isolated bone marrow cells were stimulated with LPS or Pam3Cys4 (Invivogen) at the indicate concentrations. IL-6 and TNFα were measured in supernatants by ELISA (BD Bioscience).

Figure 3.6

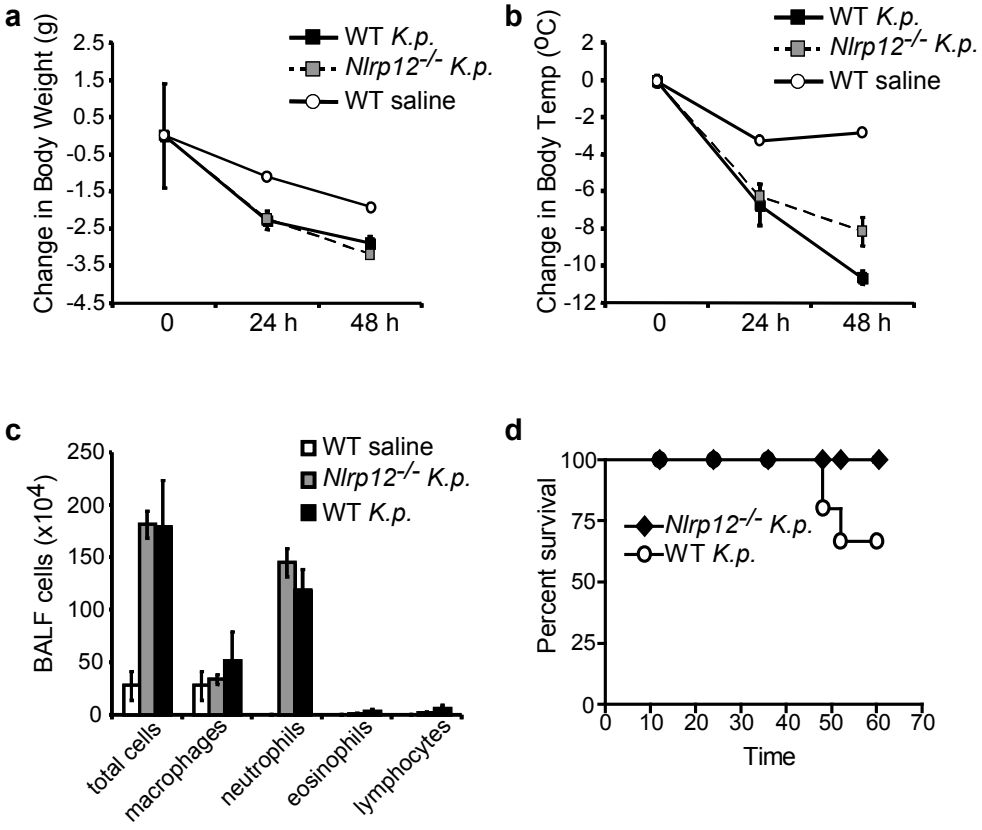

Figure 3.6: *In vivo* challenge of *Nlrp12*$^{-/-}$ mice with *Klebsiella pneumoniae* (*K.p.*). *K. p.* were grown in Luria broth (LB) for 1 h at 37°C. Bacteria numbers were estimated by measuring the absorbance at 600 nm[214]. The exact number used in each experiment was measured by plating aliquots on LB agar plates and counting CFUs. 4 x 10^4 CFU of *K.p.* in 50 µl Hank's balanced salt solution (HBSS) was delivered onto the trachea of anesthetized mice. **a**, Body weight and **b**, temperature were monitored to assess morbidity (WT n=7, *Nlrp12*$^{-/-}$ n=7, saline n=3). Forty-eight hours post infection the lungs of surviving mice were lavaged with HBSS. **c,** Cytospin slides were prepared, stained with Diff-Quick (Dade-Behring) and differential cell counts determined**.** Cellularity of bronchial alveolar lavage fluid from saline treated and inoculated mice (WT n=3, *Nlrp12*$^{-/-}$ n=5, saline n=4). Data (**a-c**) are represented as mean ± s.e.m. **d,** Percent survival was compared between WT (○ n=15) and *Nlrp12*$^{-/-}$ (♦ n=13) mice up to 48 hours. Data are representative of three independent experiments.

Figure 3.7

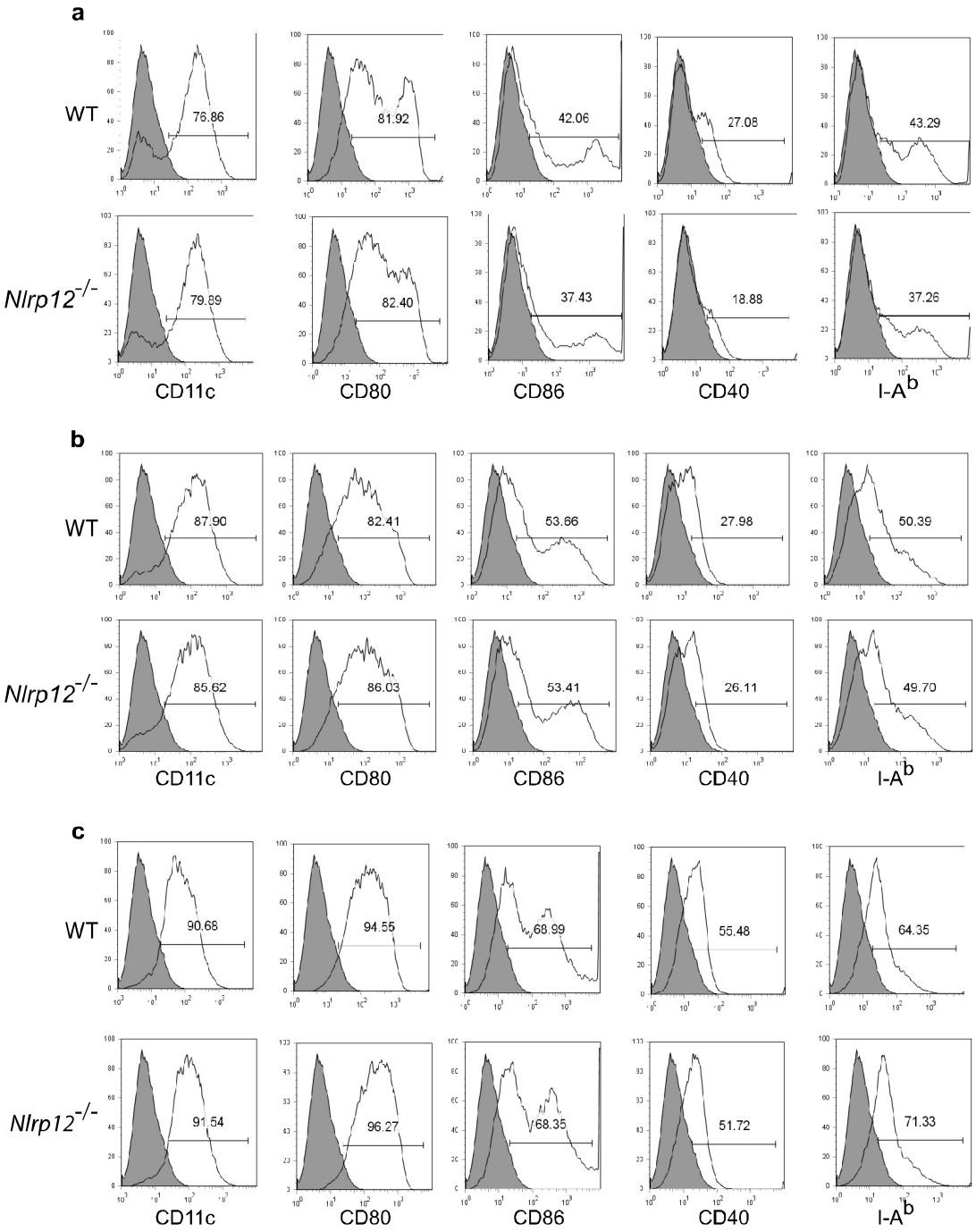

Figure 3.7: Analysis of BMDC cell surface markers on WT and *Nlrp12*$^{-/-}$ BMDC. BMDC were collected on **a,** days 8, **b,**10, and **c,** day 10 + 48hr TNFα and surface marker phenotype was determined by single-color flow cytometry after gating on live cells. The following PE-conjugated antibodies were used: CD11c (N418; eBioscience), I-A^b (AF6-120.1; BD Biosciences), CD80 (53-6.7; BD Biosciences), CD86 (PO3.1; eBioscience), CD40 (1C10; eBioscience). Data are representative of three independent experiments.

Figure 3.8

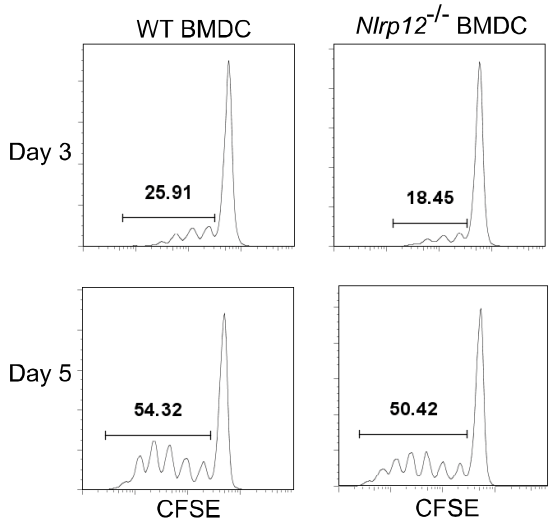

Figure 3.8: Antigen presentation assays comparing WT and *Nlrp12*$^{-/-}$ BMDC. BMDC from WT or *Nlrp12*$^{-/-}$ mice were pulsed with Ova whole protein (Worthington Biochemical Corp.) or mock-pulsed for 18-20 hours. Splenocytes were isolated from OT-II mice, labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen), and cultured 5:1 with BMDC (1×10^6) in 6-well plates. After 3-5 days in culture, cells were stained with biotin-labeled anti-Vβ5 (MR9-4 BD Biosciences), specific for the OTII transgenic TCR, followed by streptavidin-APC (eBioscience). Dilution of CFSE on Vβ5-positive cells was detected using flow cytometry. The data presented show the greatest observed difference between WT and *Nlrp12*$^{-/-}$ BMDC out of three independent experiments.

Figure 3.9

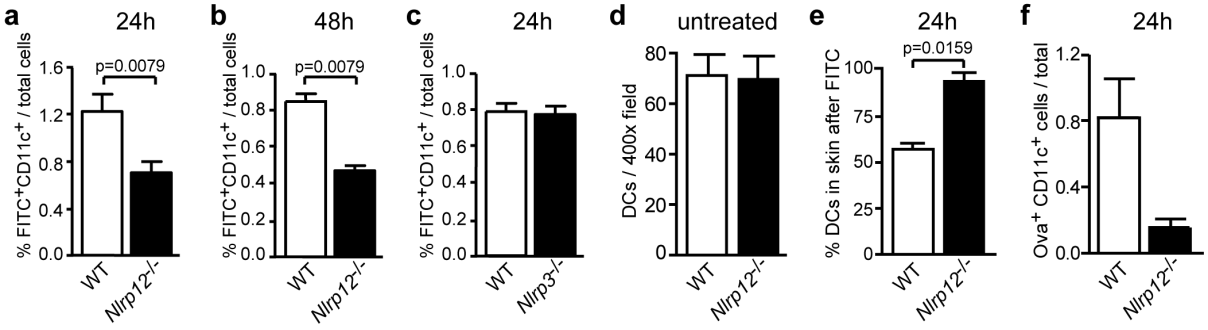

Figure 3.9: *Nlrp12*$^{-/-}$ mice exhibit attenuated migration of peripheral dendritic cells to draining lymph nodes. (a-b), Migration of CD11c^{+} WT (n=5) and *Nlrp12*$^{-/-}$ (n=5) DCs (**a)** 24 h and (**b)** 48 h following FITC application. **c**, Migration of CD11c^{+} WT (n=5) and *Nlrp3*$^{-/-}$ (n=5) DCs 24 h following FITC application. **d**, Quantitation of I-A^{b+} skin DCs in untreated WT (n=6) and *Nlrp12*$^{-/-}$ (n=6) ear epidermal sheets. **e**, Percent, relative to untreated, of I-A^{b+} DCs remaining in the skin 24 h following FITC application (WT n=4, *Nlrp12*$^{-/-}$ n=5). **e**, Migration of WT (n=3) and *Nlrp12*$^{-/-}$ DCs (n=3) 24 hours following s.c. injection of fluorescent Ova in CFA. All experiments were repeated 2-3 times. Data in all panels are presented as mean ± s.e.m. and pairwise comparisons were made using two-tailed Mann Whitney *U* test, $\alpha = 0.05$.

Figure 3.10

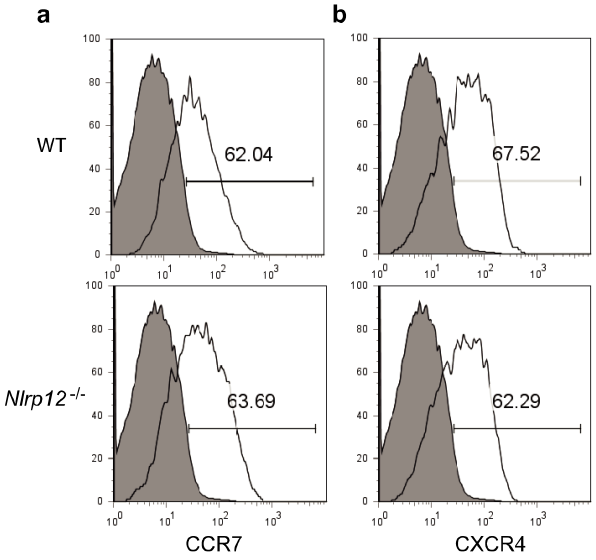

Figure 3.10: Cell surface expression of CCR7 and CXCR4 on WT and *Nlrp12*$^{-/-}$ BMDC. BMDC were stained with anti-CD11c-PeCy7 (HL3; BD Biosciences), anti-I-A^{b}-PE (AF6-120.1; BD Biosciences), and either anti-CCR7-APC (4B12; eBioscience) or anti-CXCR4-Alexa Fluor 647 (2B11; eBioscience). Staining was quantified by flow cytometry and analyzed using FlowJo software (Tree Star Inc.). BMDCs were gated on CD11c^{+}I-A^{b+} cells, and the percentages of (**a**) CCR7^{+} and (**b**) CXCR4^{+} DCs are shown.

Figure 3.11

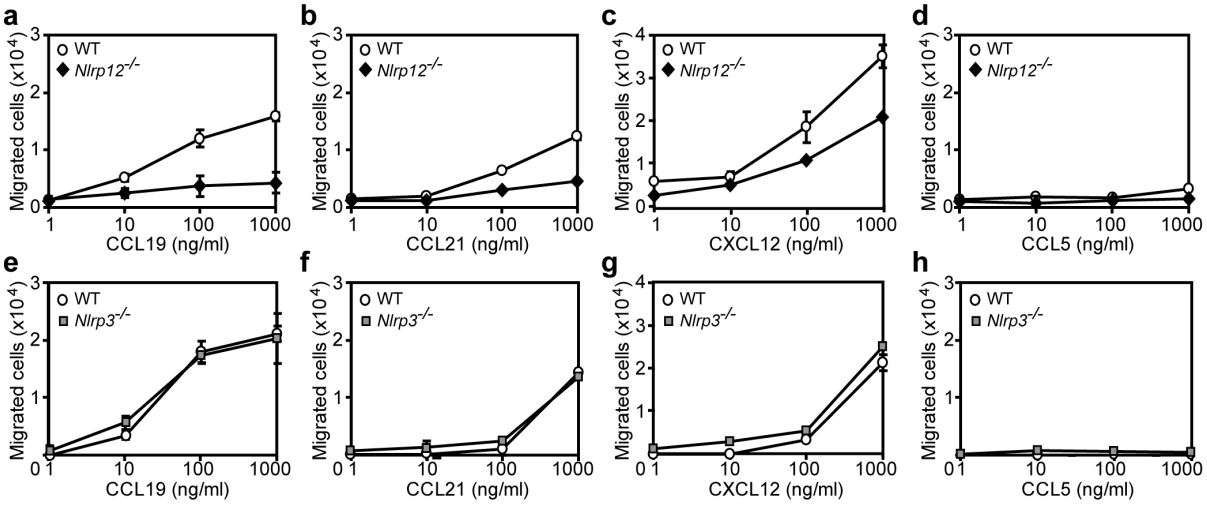

Figure 3.11: *Nlrp12*$^{-/-}$ BMDCs exhibit attenuated migration toward lymph node homing chemokines. Migration of (**a-d**) WT (○) and *Nlrp12*$^{-/-}$ (♦) BMDCs, and of (**e-g**) WT (○) and *Nlrp3*$^{-/-}$ (■) BMDCs to the indicated chemokine. Data are representative of 3-5 experiments and are presented as mean ± s.e.m. of one experiment. Data from all experiments with associated pairwise comparison statistics are presented in Supplemental Table A2.3.

Figure 3.12

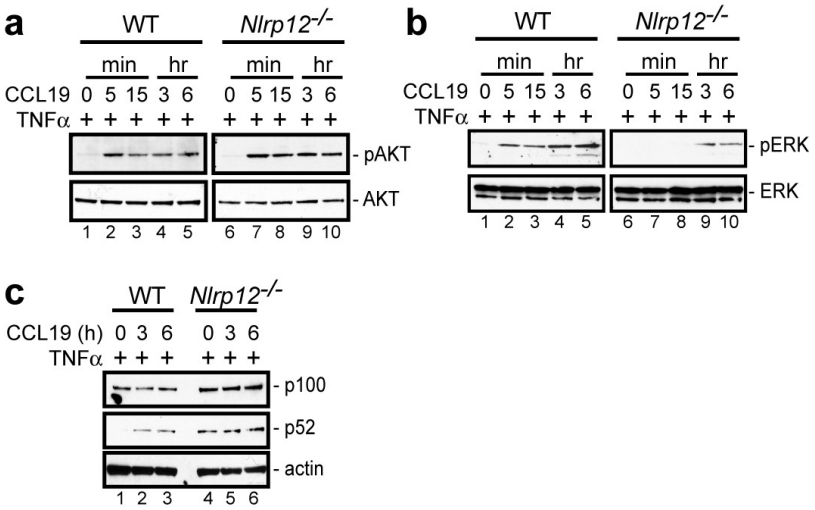

Figure 3.12: *Nlrp12*$^{-/-}$ BMDCs display altered activation of ERK and noncanonical NF-κB following CCR7 activation. a-c, BMDCs were pretreated with TNFα for 8 h and then stimulated with CCL19 for the indicated times. Immunoblots were probed with the indicated antibodies. Each panel is representative of at least three independent experiments.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation describes an unexpected role for the NLR family member *NLRP12*. Mutations in *NLRP12* have recently been linked to atopic dermatitis[58] and hereditary periodic fever with skin urticaria [57]. The etiology of these human disorders is not clear and mounting literature suggests that NLR mediated pathways may play a prominent role. Using rodent models, we demonstrate that NLRP12 functionally links innate and adaptive immune responses. In mice, *Nlrp12* is expressed almost exclusively in DCs and is required for DC migration from the periphery to secondary lymphoid tissues. Consequently, *Nlrp12*$^{-/-}$ mice fail to mount a robust immune response in two models of T cell mediated immunity, contact hypersensitivity (CHS) that models atopic and allergic dermatitis, and experimental autoimmune encephalitis (EAE) that models multiple sclerosis. The inability of *Nlrp12*$^{-/-}$ DCs to induce adaptive immunity is not due to failure in antigen processing and presentation. Rather, *Nlrp12*$^{-/-}$ DCs display a significantly reduced capacity to migrate to draining lymph nodes and respond to lymph node homing chemokines. This attenuated migratory capacity of *Nlrp12*$^{-/-}$ DCs is due to dysregulated NF-κB and MAPK signaling pathways. These results suggest that NLRP12 mediated pathways regulate the trafficking of antigen presenting cells. Disrupted migration of these cells may cause the advancement of atopic dermatitis and periodic fevers in individuals with mutations in NLRP12.

The discovery of a physiologic role for NLRP12 represents a significant advance in the study of this NLR protein. It is remarkable that an NLR with limited expression to innate immune cells can directly influence adaptive immune responses. In fact, with the exception of CIITA, NLRP12 is the first NLR found to directly affect the function of antigen presenting cells. Consequently, NLRP12 affects adaptive immunity and associated disease models.

While NLRP3, NOD1 and NOD2 also affect adaptive immune response, NLRP3 and NOD2 likely mediate this through the indirect effects of cytokines such as IL-1β, IL-18 or IL-12 on T cell function, and the mechanism of NOD1 is not well understood[102,118,119]. Of course, the role of NLRP12 in dendritic cell function and associated disease models is far from fully characterized.

Accordingly, four key questions remain:

1). How does NLRP12 control dendritic cell migration?

2). In the absence of NLRP12, are other peripheral dendritic cell subsets impaired in migration? What is the physiologic consequence?

3). Does deletion of NLRP12 affect neutrophil migration?

4). What stimulus/stimuli activates NLRP12?

The following discussion will address these issues.

1. How does NLRP12 control dendritic cell migration?

In this dissertation we show that NLRP12 regulates pro-inflammatory signaling pathways important for DC activation and migration. Previously our laboratory demonstrated that in human monocytes, NLRP12 induces proteasome-mediated degradation of NIK [154], the kinase responsible for activating the noncanonical NF-κB pathway [166]. In agreement with these findings, NIK levels are also increased in *Nlrp12*$^{-/-}$ DCs. Noncanonical NF-κB is inappropriately activated in these cells downstream of inflammatory stimuli, such as those encountered in the skin during CHS. The role of NIK and noncanonical NF-κB (NF-κB2) in dendritic cell migration is not well-studied. Studies of migration *in vivo* have been hampered by the phenotype of the NIK-deficient and NF-κB2-deficient mice, neither of

which have secondary lymphoid organs or normal lymphatic structure [228,229]. To determine if NLRP12 affects DC migration by controlling NIK and noncanonical NF-κB, *Nlrp12*$^{-/-}$ mice can be crossed to the NIK$^{-/-}$ mice. DC migration can be assessed using our *in vitro* migration assay. If NLRP12 affects DC migration through NIK, the phenotype of NLRP12 / NIK double-deficient mice should revert the migration phenotype back to wild type levels.

Our results suggest that in *Nlrp12*$^{-/-}$ DCs, excess NIK leads to defective DC migration. Known noncanonical NF-κB target genes include chemokines, chemokine receptors and adhesion molecules – all of which influence DC trafficking to draining lymph nodes. In *Nlrp12*$^{-/-}$ DCs, however, we have not detected a difference in expression, secretion, or cell surface levels of most noncanonical NF-κB targets. One NIK-regulated target we are currently evaluating is cyclooxygenase-2 (COX-2) [230,231]. COX-2 is an enzyme that metabolizes arachidonic acid to an intermediate prostaglandin, PGH_2. PGH_2 is then further metabolized by individual prostaglandin synthases to yield prostanoids including prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostacyclin (PGI_2) and thromboxane (TXA_2)[232]. It is well established that prostaglandins affect migration of many cell types, including dendritic cells. In fact, others have found that PGD2 is made by dendritic cells and suppresses dendritic cell migration [233-237]. We are currently assessing COX-2 expression in and prostaglandin production from *Nlrp12*$^{-/-}$ DCs. Preliminary data suggest that *Nlrp12*$^{-/-}$ DCs express higher levels of COX-2 compared to wildtype DCs. To determine if excess COX-2 affects migration, we are currently utilizing COX-2 inhibitors in our *in vitro* and *in vivo* DC migration assays. If COX-2 inhibitors restore migration, we will conclude that *Nlrp12*$^{-/-}$ DCs exhibit impaired migration due to increased expression of COX-2.

In contrast to noncanonical NF-κB, *Nlrp12*$^{-/-}$ DCs exhibit profoundly delayed ERK activation downstream of inflammatory stimuli and chemokine receptor activation. ERK activation is important for rear-end detachment and efficient cellular migration [220,238], thus a defect in ERK activation could contribute to impaired migration seen in *Nlrp12*$^{-/-}$ DCs. However, the mechanism by which NLRP12 affects ERK activation remains unclear. To determine if the effect of NLRP12 on ERK is mediated through NIK, we can assess ERK activation in NIK-deficient DCs. If NLRP12 promotes ERK activation by suppressing NIK and noncanonical NF-κB activation, NIK-deficient DCs will display robust ERK activation downstream of inflammatory stimuli and chemokine receptor activation. If the opposite occurs, NLRP12 likely is affecting ERK activation through another mechanism that is yet to be identified.

2. In the absence of NLRP12, are other peripheral dendritic cell subsets impaired in migration? What is the physiologic consequence?

Dendritic cells are constantly sampling antigens from the outside environment; consequently they reside in tissues that serve as surveillance sites. These include the skin, lungs, and gastric mucosa. In this dissertation we have shown that NLRP12 is required for efficient DC migration from the skin to the draining lymph nodes. It will be of interest to determine if *Nlrp12*$^{-/-}$ DCs residing in other peripheral tissues are also impaired in migration. In the lungs, DC activation and migration to draining lymph nodes are important in clearing viral lung infections like influenza [239]. An influenza mouse model is currently employed in our lab. Using this model, we can assess DC migration and determine what physiologic consequences arise from NLRP12 deletion during influenza lung infection. We are also interested in assessing the role of NLRP12 in DC function in the gastric mucosa. The role of

DCs in inflammatory bowel disease and colitis is not completely understood. However, their importance in resolving gut inflammation is highlighted by the finding that DC-depletion worsens colitis in mice [240]. The dextran sodium sulfate (DSS) colitis model is currently employed in our lab and we will utilize this model to assess the role of NLRP12 in gut inflammation.

3. Does deletion of NLRP12 affect neutrophil migration?

In addition to dendritic cells, *Nlrp12* is expressed in granulocytes, including eosinophils and neutrophils. Preliminary results suggest that neutrophil recruitment to the site of CHS elicitation is impaired in *Nlrp12*$^{-/-}$ mice. Impaired neutrophil recruitment during CHS may contribute to the reduced CHS response in *Nlrp12*$^{-/-}$ mice. We are currently evaluating *Nlrp12*$^{-/-}$ neutrophil migration *in vitro* toward the neutrophil-recruiting chemokine KC / IL-8. Migration of *Nlrp12*$^{-/-}$ neutrophils to KC is reduced to approximately half that of wildtype cells. Thus our data suggest that deletion of *Nlrp12* affects the migratory capacity of both dendritic cells and neutrophils. This affects CHS, a model dependent on both dendritic cells and neutrophils, and EAE, a model dependent on dendritic cells but not neutrophils. It will be interesting to utilize the *Nlrp12*$^{-/-}$ mice to assess the role of NLRP12 in a neutrophil-dependent model. For example, is NLRP12 important for neutrophil activation and clearance of bacterial skin infections, such as *Staphylococcus aureus*? Further studies will allow us to explore the role of NLRP12 in neutrophil-dependent infections and skin conditions.

4. What stimulus / stimuli activates NLRP12

One of the most pressing questions in NLR biology remains, what stimulus activates a particular NLR? Biochemical studies in human cell lines have shown that an activating stimulus is required for NLRP12 to associate with and induce the degradation of NIK [154]. These data suggest that NLRP12 responds to some molecular cue. One possibility is that NLRP12 responds to pathogen product(s), akin to NOD1 and NOD2 that respond to components of peptidoglycan in bacterial cell walls. However even in the case of NOD1 and NOD2, direct binding of these NLR proteins by peptidoglycan has not been shown despite many attempts. Thus to date the concept of NLR proteins as "pathogen receptors" has not been backed by experimental data. Another possibility is that NLRP12 responds to ion fluxes or endogenous danger signals, similar to that proposed for NLRP3.

A different possibility is that NLRP12 does not directly recognize pathogen products or danger signals, but instead recognizes modified self, as described by the "guard hypothesis." The guard hypothesis has been proposed as a mechanism by which plant *R* proteins indirectly recognize their cognate pathogen avirulence factors. Simply put, this hypothesis proposes that the plant *R* protein or mammalian NLR "guards" a host protein that is targeted by one or more microbial effectors / avirulence proteins. Upon recognition of this "pathogen-induced modified-self," [241] the plant *R* protein or mammalian NLR protein becomes activated and elicits a protective response. Because many pathogens activate the same signaling pathways and modify the same molecules, NLR proteins could respond to multiple effectors by guarding one host target.

Support for the guard hypothesis in NLRP12 activation lies in the following observations. In human THP-1 monocytes, NLRP12 resides in a molecular complex with

transforming growth factor beta-activated kinase 1 (TAK1), a kinase that is activated downstream of IL-1 receptor, TNF receptor, and TLRs. Within an hour of stimulation, NLRP12 leaves the TAK1-containing complex (Arthur, J.C., unpublished observations). In cells expressing a dominant negative TAK1, NLRP12 cannot associate with or induce NIK degradation (Lich, J.D. unpublished data). Perhaps NLRP12 guards TAK1 and in response to TAK1-activating stimuli, becomes activated and induces NIK degradation to suppress noncanonical NF-κB. Thus further investigation into host signaling components required for NLRP12 function may illuminate the specific NLRP12 activating stimulus.

Final Conclusions

This dissertation describes, for the fist time, the physiologic role of the NLR protein NLRP12. Earlier work in human monocytic cell lines suggested an anti-inflammatory role for NLRP12. Thus we hypothesized that *Nlrp12*-deficient mice would exhibit a pro-inflammatory phenotype. Our data have demonstrated this is not the case. NLRP12 does not play a detectable role in cytokine secretion or mouse models of innate immune activation. Instead, *Nlrp12*$^{-/-}$ mice fail to mount a robust immune response during CHS and EAE, two models of T cell mediated immunity. NLRP12 affects the outcome of these models by controlling dendritic cell migration. We find that *Nlrp12*$^{-/-}$ dendritic cells exhibit a reduced capacity to migrate to draining lymph nodes and respond to lymph node homing chemokines.

How does deletion of *Nlrp12*, a negative regulator of inflammation in human monocytic cell lines, lead to an anti-inflammatory phenotype in the mouse? There are a number of possibilities: a difference between primary cells and cell lines; a difference between monocytes and dendritic cells; a difference between human and mouse. Our data

suggests that the molecular mechanism by which NLRP12 exerts its effect is the same in both mouse dendritic cells and human monocytes. However, the resulting cellular phenotype is different. NLRP12 associates with NIK and induces its degradation via the proteasome. NIK is the sole kinase responsible for activating the noncanonical NF-κB pathway. By controlling the availability of NIK, NLRP12 controls activation of noncanonical NF-κB. Silencing of *Nlrp12* in human monocytic cell lines leads to NIK accumulation and inappropriate activation of noncanonical NF-κB. This results in increased expression of proinflammatory cytokines and chemokines including IL-6, TNFα, CXCL13, CXCL12, and CXCR4. In *Nlrp12*-deficient mouse dendritic cells, NIK accumulates and noncanonical NF-κB is inappropriately activated. In contrast to human monocytes, however, these cells do not express increased levels of cytokines or chemokines. They do display a reduced capacity to migrate both *in vivo* and *in vitro*. We are currently testing the hypothesis that an increased level of NIK-regulated genes affects the migratory capacity of these cells. One NIK-regulated gene whose expression is increased in *Nlrp12*$^{-/-}$ dendritic cells is COX-2. COX-2 is an inducible enzyme that metabolizes arachidonic acid to prostanoids. Prostanoids are well known to affect migration. Thus increased COX-2 expression seems a plausible mechanism by which NLRP12 affects migration. We are currently evaluating if COX-2 inhibition restores the migratory capacity of *Nlrp12*-deficient dendritic cells. It will be interesting to determine if *Nlrp12*-silenced human monocytes also express increased levels of COX-2 and exhibit a reduced migration capacity.

In conclusion, our findings demonstrate that NLRP12 provides a functional link between innate and adaptive immune responses. A central component of both protective and harmful immune responses is the ability of dendritic cells to collect peripheral antigens and

transport them to draining lymph nodes. Thus the ability to manipulate dendritic cell migration would open the door to more effective vaccines and better treatments for allergic and autoimmune diseases. The work presented in this dissertation furthers our knowledge of the role NLRP12 in such diseases and expands the biologic importance of the NLR family at the interface of innate and adaptive immunity.

APPENDIX 1. MONARCH-1/NLRP12 SUPPRESSES NONCANONICAL NF-KB ACTIVATION AND P52 DEPENDENT CHEMOKINE EXPRESSION IN MONOCYTES

This research was originally published in the *Journal of Immunology*.

Lich JD, Williams KL, Moore CB, Arthur JC, Davis BK, Taxman DJ, and Ting JP. Cutting Edge: Monarch-1 suppresses noncanonical NF-κB activation and p52-dependent chemokine expression in monocytes. *J Immunol* **178,**1256-60 (2007).

A. ABSTRACT

NLR (CATERPILLER, NOD, NBD-LRR) proteins are rapidly emerging as important mediators of innate and adaptive immunity. Among these, NLRP12 operates as a novel attenuating factor of inflammation by suppressing inflammatory responses in activated monocytes. However, the molecular mechanisms by which NLRP12 performs this important function are not well understood. In this report, we show that NLRP12 inhibits CD40-mediated activation of NF-κB via the noncanonical pathway in human monocytes. This inhibition stems from the ability of NLRP12 to associate with and induce proteasome-mediated degradation of NF-κB inducing kinase (NIK). Congruently, silencing NLRP12 with shRNA enhances the expression of p52-dependent chemokines.

B. INTRODUCTION

NLRP12, also known as Monarch-1 or Pypaf7, harbors an N-terminal pyrin domain and is expressed exclusively in cells of myeloid lineage [151]. We recently demonstrated that NLRP12 suppresses pro-inflammatory cytokine production in monocytes stimulated with TLR ligands, TNF-α, and *Mycobacterium tuberculosis* [242]. The mechanisms by which NLRP12 performs this anti-inflammatory function are not clear; however, a role for NLRP12 in the inhibition of NF-κB was suggested by these studies.

NF-κB activation occurs through two distinct mechanisms referred to as the canonical and noncanonical pathways. The canonical pathway proceeds very rapidly and can be activated by a number of upstream kinases that signal through the IKKα/β/γ complex. This results in nuclear accumulation of primarily RelA/p50 heterodimers that induce early immune response genes. In contrast, the noncanonical pathway displays slower kinetics and is dependent upon NIK [166]. In this alternative pathway, NIK activates IKKα leading to the nuclear accumulation of p52-containing NF-κB complexes that induce a different set of inflammatory genes to support the ongoing immune response [168]. The present study was initiated to elucidate the mechanisms by which NLRP12 suppresses NF-κB in monocytes. We found that NLRP12 suppresses activation of the noncanonical pathway by associating with NIK and inducing its proteasome-mediated degradation.

C. MATERIALS AND METHODS

Cell lines, Antibodies, and Reagents: HEK293T and Cos-7 cells were maintained in DMEM (Gibco) with 10% fetal calf serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. THP-1 derived cell lines stably expressing Ha-NLRP12 or shRNA targeting NLRP12 have been described [242]. The antibodies used were: anti-NIK (H-248), anti-p52 (C-5), anti-p50 (H-119) and anti-CagA (b-300; control Ab) from Santa Cruz Biotechnology; anti-HA antibodies (12CA4 and 13F10) from Roche; and anti-V5 from Invitrogen. CD40L was obtained from Peprotech, MG132 from Calbiochem. Ha-NLRP12 has been described [242]. NIK (MGC:45335) was obtained from the ATCC Mammalian Genome Collection. The luciferase reporter plasmids and p53 were obtained from Dr. Albert Baldwin. NLRP12 truncation mutants were PCR amplified and cloned into pcDNA3.1 V5/HIS TOPO cloning vector.

Luciferase Assays: HEK293T cells were transfected with 50 ng NF-κB or p53 reporter plasmid and 500 ng of NIK or p53. NLRP12 was co-transfected at the indicated concentrations and pcDNA3.1 was used to equalize the plasmid concentration among samples. Luciferase assays were performed in triplicate [242].

RNA Preparation and Real-time PCR: Total RNA was isolated with RNeasy (Qiagen). Real-time PCR was performed using SYBER Green as described [243]. Primer sequences are available upon request. Results were normalized to 18S ribosomal RNA internal controls and expressed in relative numbers.

Immunoprecipitations and Western Blot Analysis: HEK293T cells were transfected using Fugene 6 (Roche). The cells were lysed as described [242]. Nuclear and cytoplasmic fractions were generated using the NE-PER kit (Pierce). Protein concentrations were

determined by Bradford assay (BioRad) and equilibrated samples were immunoprecipitated with 2 ug of the indicated antibody for 18 h with rotation. Antibody complexes were captured with protein A/G agarose beads (Pierce). The beads were washed, eluted into sample buffer, boiled and separated by SDS-PAGE [242]. Unless indicated, all plasmids were used at equal concentrations.

Pulse-Chase analysis: Cos-7 cells were transfected with 3 ug of the indicated plasmids using Fugene 6 (Roche). The cells were incubated for 18 h, starved for 30 min in methionine/cysteine free DMEM with 5% FBS, pulsed with 0.4 mCi/ml (35)S methionine for 30 min, washed with warm PBS and incubated in methionine fortified DMEM containing 10% FBS. At the indicated time points, cells were washed in ice cold PBS then lysed in 1% Tx-100, 0.1% SDS, 0.5% DOC, 150 mM NaCl, 50 mM Tris pH 8, 50 mM NaF, 2 mM EDTA supplemented with protease inhibitors (Roche). NIK was immunoprecipitated and eluted into reducing sample buffer. Proteins were fractionated by SDS-PAGE and gels were dried. Control samples consisting of protein A/G beads alone confirmed the specificity of protein bands visualized in autoradiographs. Autoradiographs were scanned and analyzed by densitometry.

D. RESULTS AND DISCUSSION

NLRP12 inhibits noncanonical NF-κB in monocytes.

Previously, we reported that NLRP12 inhibits TLR-induced NF-κB driven luciferase. However, this reporter assay could not determine which NF-κB pathway was inhibited [242]. Activation of canonical NF-κB requires the degradation of IκBα to release RelA/p50 heterodimers. RelA is then phosphorylated allowing the expression of NF-κB responsive genes such as *IκBα* and *NF-κB2/p100*. To determine the role of NLRP12 in canonical NF-κB activation, THP-1 monocytes stably expressing Ha-NLRP12 (THP-Ha-NLRP12) or an empty vector control (THP-EV) were stimulated with the TLR2 ligand Pam3Cys for the indicated times (Figure 1A). Activation was monitored by western blot analysis of IκBα degradation, RelA phosphorylation, and the induction of NF-κB responsive genes. No difference in IκBα degradation or RelA phosphorylation was detected between THP-EV and THP-Ha-NLRP12 cells at the time points assayed. In addition, the expression of *NF-κB2/p100* and *IκBα* was equally up-regulated in both cells. Although a more detailed kinetic analysis indicated that NLRP12 did decrease RelA phosphorylation at 60 min (Figure 1B), these results indicate that initial activation of canonical NF-κB is not affected by NLRP12.

We next analyzed the role of NLRP12 in noncanonical NF-κB activation. This alternative pathway can be induced by TNF receptor family members such as CD40 and requires the processing of NF-κB2/p100 to its active form p52, which then rapidly translocates to the nucleus [158]. THP-Ha-NLRP12 cells or empty vector controls were pre-treated with Pam3Cys to induce p100 expression and then stimulated with CD40L to promote p100 processing (Figure 1C). Such prior activation of the canonical pathway followed by the subsequent activation of the noncanonical pathway has been documented [169].

Cytoplasmic and nuclear extracts were prepared and noncanonical NF-κB activation was determined by monitoring p100 processing to p52 by western blot. Pam3Cys treatment induced p100 expression in both THP-Ha-NLRP12 and THP-EV cells, confirming that the canonical pathway remained intact (Figure 1C). Subsequent CD40L treatment of THP-EV cells resulted in the accumulation of nuclear p52 within 1 h of treatment. The level of p52 peaked by 3 h and was maintained in the nucleus throughout the 6 h time course. In contrast, p52 was significantly reduced in THP-Ha-NLRP12 cells. A weak p52 band was detected within 1 h of treatment; however, this effect was only transient as p52 was not detected at later time points. Importantly, no difference in nuclear p50 levels was detected between the two cell lines, indicating that nuclear translocation of NF-κB proteins was not globally inhibited by the presence of NLRP12. These results indicate that NLRP12 suppresses noncanonical NF-κB activation.

NLRP12 associates with NIK.

While many kinases can stimulate the canonical pathway, the noncanonical pathway is uniquely dependent upon NIK [244]. To determine if NLRP12 intersects the noncanonical pathway by associating with this kinase, we performed co-immunoprecipitation experiments. HEK293T cells were transfected with Ha-NLRP12 and NIK, and NIK complexes were immunopurified and analyzed by western blot. NLRP12 co-precipitated with NIK but not a control isotype Ig (Figure 2A). As additional controls, no association was found between NLRP12 and IKKα when both proteins were overexpressed, nor did two other NLR proteins, CIITA and NOD2, interact with NIK (data not shown).

NLRP12 failed to co-precipitate with endogenous NIK when expressed alone in unstimulated cells (Figure 2A, lane 2). Instead, these complexes only formed when both proteins were co-expressed. It is known that ectopically expressed NIK displays a high level of functional activity, while endogenous NIK does not [245]. Thus an explanation for this result is that NLRP12 preferentially associates with active forms of the kinase. To explore this possibility in monocytic cells, THP-Ha-NLRP12 cells were stimulated with CD40L to activate endogenous NIK (Figure 2B) [158]. NLRP12 co-precipitated with endogenous NIK only in stimulated cells, thus supporting our hypothesis that complex formation depends on the activation status of NIK.

Structural domains of NLRP12 required for NIK binding.

NLRP12 possesses a tripartite domain architecture conserved in most NLR proteins [15]. To determine which structural elements of NLRP12 are required for NIK binding, truncation mutants were constructed and tested for the ability to bind NIK in immunoprecipitation assays. The N-terminal pyrin domain of NLRP12 failed to co-precipitate with NIK (Figure 3). However, the pyrin-NBD truncation mutant did co-precipitate with NIK, indicating a role for the NBD domain in NIK binding. NIK also co-precipitated with truncation mutants comprised of the NBD-LRR and the LRR alone. Thus, both the NBD and LRR domain of NLRP12 encode elements that mediate NIK binding. In contrast, the pyrin domain is not required for this interaction.

NLRP12 inhibits NIK-induced NF-κB activation.

To directly test the effect of NLRP12 on NIK-induced NF-κB activation, luciferase reporter assays were performed. As expected, ectopic expression of NIK led to strong activation of an NF-κB reporter plasmid (Figure 4A). Co-expression of NLRP12 resulted in a dose dependent inhibition of NIK-induced NF-κB activity, confirming a negative regulatory role for NLRP12. In contrast, NLRP12 did not inhibit activation of a p53-inducible reporter plasmid indicating specificity of its function.

We next sought to determine the biologic consequences of NLRP12-mediated suppression of NIK in monocytes using RNA silencing. THP-Ha-NLRP12 cells or THP-1 cells in which NLRP12 expression was silenced by shRNA (THP-shNLRP12) were stimulated with Pam3Cys and CD40L to induce activation of noncanonical NF-κB. Next, the expression of the p52-dependent genes *CXCR4*, *CXCL12*, and *CXCL13* was analyzed and compared to control THP-EV cells [246,247]. All three genes were strongly up-regulated in THP-shNLRP12 cells, demonstrating enhanced p52 activity in these cells in the absence of NLRP12. In contrast, gene expression was inhibited in THP-Ha-NLRP12 cells, indicating reduced activity of noncanonical NF-κB in the presence of NLRP12. No difference was detected in the expression of *CXCL8* or *CXCL9*, which are not known to be p52 dependent (data not shown). Together, these results suggest a mechanism whereby NLRP12 associates with NIK and suppresses its ability to activate noncanonical NF-κB in monocytic cells.

NLRP12 induces NIK degradation through a proteasome-dependent pathway.

Throughout the course of this study we consistently noticed reduced levels of NIK in cells co-expressing NIK and NLRP12, compared to cells in which NIK was expressed alone

(Figure 2A). Furthermore, a significant reduction in endogenous NIK was also observed upon stimulation of THP-Ha-NLRP12 cells compared to THP-EV cells (Figure 5A). These observations led us to question if NLRP12 suppresses NIK activity by regulating the stability of the kinase. To test this hypothesis, Cos-7 cells were transfected with NIK in the presence or absence of NLRP12 and pulse-chase assays were performed. NIK protein levels declined sharply in the presence of NLRP12 and densitometry quantified an approximate 75% decrease over the course of the experiment (Figure 5B). In contrast, in the absence of NLRP12 NIK remained stable throughout the 6 h chase period.

To determine which domains of NLRP12 regulate NIK stability, truncated forms of NLRP12 were co-expressed with NIK, and NIK levels were determined by western blot. These experiments revealed that the NBD is required to reduce NIK stability (Figure 5C). Interestingly, although the LRR domain associated with NIK, it had only a subtle effect on NIK stability (Figure 5C, lane 5). The LRR domains of the NLR proteins NOD1 and NOD2 sense breakdown products of peptidoglycan to trigger downstream signaling pathways, although there is no evidence that NOD1/2 directly bind to these products [19]. A specific ligand for NLRP12 also has not been identified; however, we predict that in the presence of ligand, the LRR domain would function to regulate NLRP12 activity. Nevertheless, our results indicate that the NBD is required for reducing NIK stability. The pyrin domain, in contrast, stabilizes NIK and may play an auto-inhibitory role in NLRP12 function (Figure 5C, lane 2).

The stability of many cellular proteins is regulated by the proteasome. To determine if NLRP12 regulates NIK stability through a proteasome dependent mechanism, cells were transfected with NIK in the presence or absence of NLRP12 (Figure 5D). Proteasome

inhibitor was added at increasing concentrations and NIK levels were determined by western blot analysis. As expected, co-expression of NIK and NLRP12 resulted in greatly reduced levels of NIK protein. This reduction was blocked in a dose dependent manner upon treatment of cells with proteasome inhibitor, demonstrating a role for the proteasome in NLRP12-mediated NIK degradation.

This report reveals a second mechanism whereby NLRP12 associates with and negatively regulates a signaling molecule in activated monocytes. In a previous report, we demonstrated that NLRP12 associates with IRAK-1 following TLR stimulation and blocks its hyperphosphorylation [242]. Since this correlated with decreased production of pro-inflammatory cytokines, we were initially surprised to find that immediate early activation of canonical NF-κB occurred normally in THP-Ha-NLRP12 cells. However, it has been shown that IRAK-1 can activate downstream signaling pathways in the absence of phosphorylation [248]. Therefore, it is likely that NLRP12 regulates other functions that are associated with IRAK-1 phosphorylation such as the ability to interact with other signaling molecules [155]. This may result in NLRP12-mediated suppression of canonical NF-κB activity at later time points. Indeed, we did observe decreased RelA phosphorylation 60 min after TLR2 stimulation in the presence of NLRP12.

It is not clear if the suppression of IRAK-1 phosphorylation and NIK degradation occur through a common pathway. Nevertheless, the results presented here demonstrate that NLRP12 operates at multiple points to attenuate inflammatory signaling._Given the preponderance of NLRP12 expression in monocytes, neutrophils and eosinophils, our results suggest NLRP12 may be critical for controlling inflammatory responses such as those that occur during allergy, asthma and infection.

Figure A1.1

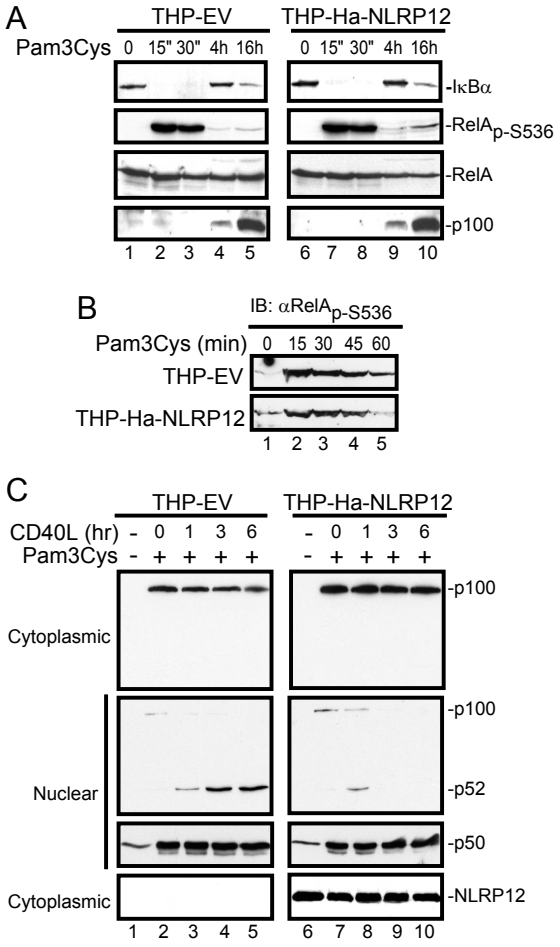

Figure A1.1: **NLRP12 suppresses noncanonical NF-κB activation.** (*A, B*) THP-EV and THP-Ha-NLRP12 cells were treated with 200 ng/ml Pam3Cys for the indicated times. Western blots were probed with Ab to detect IκBα, RelA, or p100. (C) THP-EV and THP-Ha-NLRP12 cells were stimulated with 200 ng/ml Pam3Cys to induce p100 expression and then treated with 250 ng/ml CD40L to activate noncanonical NF-κB. Nuclear and cytoplasmic fractions were analyzed by western blot.

Figure A1.2

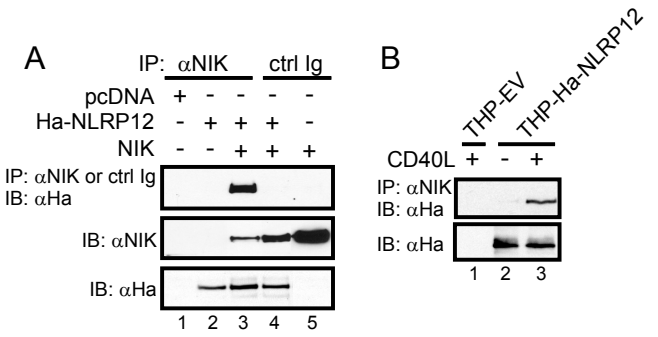

Figure A1.2: NLRP12 associates with NIK. (*A*) HEK293T cells were co-transfected with Ha-NLRP12 and NIK or pcDNA. Cell lysates were immunoprecipitated with anti-NIK and fractionated by SDS-PAGE. Western blots were probed with anti-Ha to detect NLRP12. Control samples (lanes 4 and 5) were immunoprecipitated with a control polyclonal Ab to monitor specificity. Lysate controls show the presence of NIK and NLRP12 in the expected lanes. (*B*) THP-EV or THP-Ha-NLRP12 cells were treated with 250 ng/ml CD40L. Cells were lysed and immunoprecipitated with anti-NIK. Western analysis of the precipitates was performed using anti-Ha to detect NLRP12.

Figure A1.3

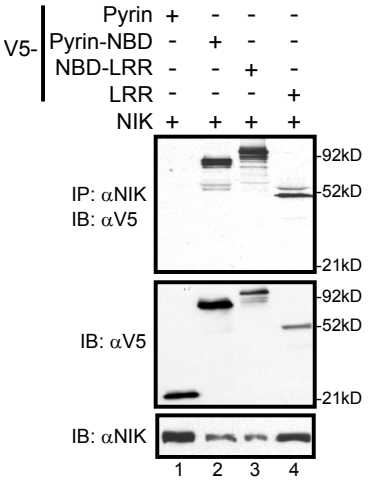

Figure A1.3: The NBD and LRR domains of NLRP12 mediate NIK binding. HEK293T cells were transfected with NIK and the indicated NLRP12 truncation mutant. Cell lysates were immunoprecipitated with anti-NIK and western blots probed with anti-V5 to detect NLRP12. The bottom panels show the presence of NLRP12 and NIK in lysates.

Figure A1.4

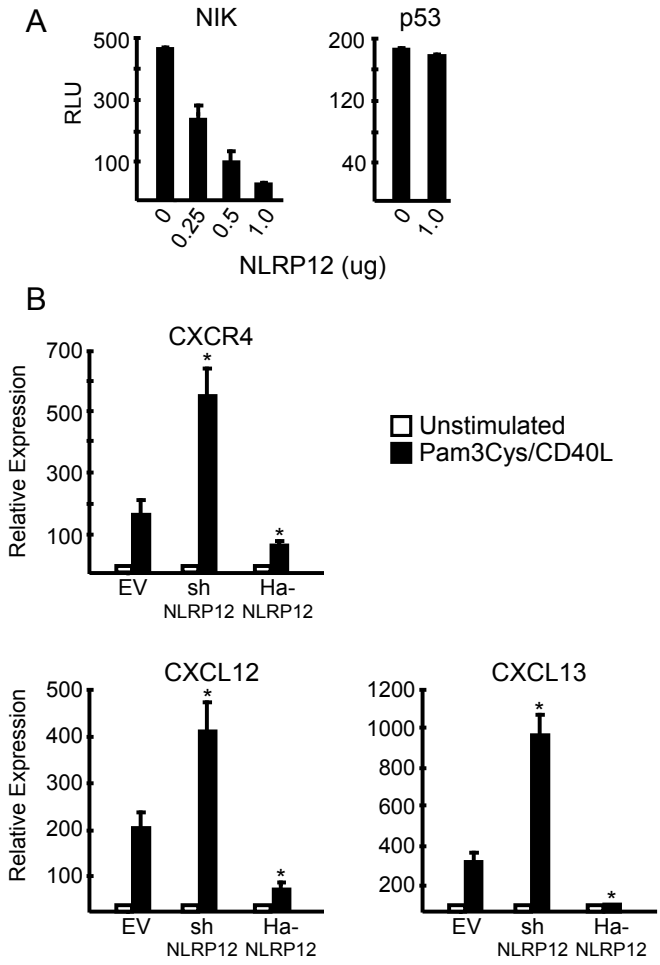

Figure A1.4: NLRP12 suppresses NIK-induced NF-κB activation. (*A*) HEK293T cells were transfected with NF-κB or p53 luciferase reporter plasmids in the presence of NIK or p53. NLRP12 was transfected at the indicated concentrations and luciferase activity was assessed. (*B*) THP-EV, THP-shNLRP12, and THP-Ha-NLRP12 cells were treated as described in Figure 1B. The expression of the indicated genes was measure by real-time PCR. The values presented are the average of three experiments measured in triplicate. The student's *t*-test was used to determine statistical significance in gene expression compared to control THP-EV cells, $p<0.05$.

Figure A1.5

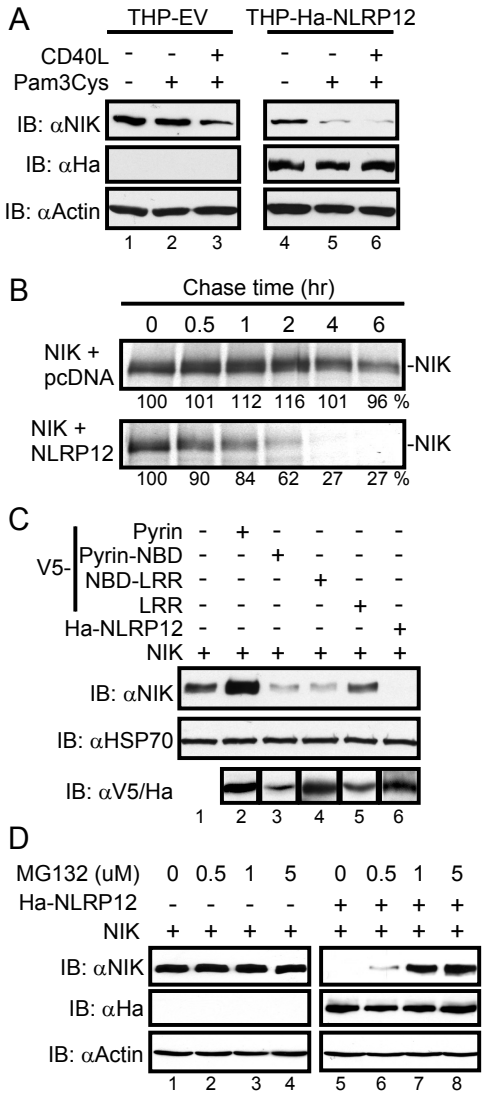

Figure A1.5: NLRP12 induces proteasome-mediated degradation of NIK. (*A*) THP-EV or THP-Ha-NLRP12 cells were stimulated as indicated and NIK levels were assessed by western blot. (*B*) Cos-7 cells were transfected with NIK in the presence or absence of NLRP12 and pulse-chase assays were performed. Gels were visualized by autoradiography and NIK bands were analyzed by densitometry. The percent NIK remaining, as compared to the 0 time point, is shown below each panel. (*C*) HEK293T cells were transfected with NIK plus full length or truncated NLRP12. NIK levels were determined by western blot. HSP70 was monitored to ensure equal loading. Immunoblots showing NLRP12 expression were cropped for space considerations. (*D*) HEK293T cells were transfected with NIK in the presence or absence of NLRP12. Six hours post-transfection the indicated samples were treated with increasing concentrations of MG132. Western blots were probed with anti-NIK. Actin levels were assessed to ensure equivalent protein loading.

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