Nucleotide-binding Oligomerization Domain Containing 2 Characterization and Function during *Mycobacterium tuberculosis* Infection of Human Macrophages

DISSERTATION

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Abstract

Macrophages are important innate immune regulators in the recognition and clearance of invading pathogens. *Mycobacterium tuberculosis* (*M.tb*), which causes tuberculosis, is a host-adapted intracellular pathogen of macrophages. Intracellular pattern recognition receptors in macrophages such as nucleotide-binding oligomerization domain (NOD) proteins regulate pro-inflammatory cytokine production. NOD2-mediated signaling pathways in response to *M.tb* have been studied primarily in mouse models and cell lines but not in primary human macrophages. Work in this dissertation examined whether NOD2 is expressed in human macrophages, controls the growth of *M.tb*, and functions in inducible nitric oxide synthase (iNOS) and novel protein kinase c (PKC) signaling pathways.

We examined NOD2 expression during monocyte differentiation and observed a marked increase in NOD2 transcript and protein following 2-3 days in culture. Pre-treatment of human monocyte-derived and alveolar macrophages with the NOD2 agonist muramyl dipeptide enhanced production of TNF-α and IL-1β in response to *M.tb* and *M. bovis* BCG (BCG) in a receptor interacting protein 2 (RIP2)-dependent fashion. The NOD2-mediated cytokine response was significantly reduced following knockdown of NOD2 expression by using small interfering RNA (siRNA) in human macrophages. Finally, NOD2 controlled the growth of both *M.tb* and BCG in human macrophages, whereas
controlling only BCG growth in murine macrophages. Together, our results provide
evidence that NOD2 is an important intracellular receptor in regulating the host response
to *M.tbc* and BCG infection in human macrophages.

Our observation of enhanced growth in NOD2 deficient cells at 24 hours (h) is of interest
because during this time *M.tbc* is becoming acclimated to the macrophage. This led us to
examine the involvement of NOD2 in early innate immune responses. In order to study
new biological functions and molecular mechanisms of NOD2 during early *M.tbc*
infection of human macrophages, we performed expression profiling of *M.tbc*-infected
NOD2 deficient macrophages using a microarray. Notably, we identified that inducible
nitric oxide synthase (iNOS) expression is regulated by NOD2 during *M.tbc* infection.
iNOS expression is enhanced at 24 and 48 h after *M.tbc* and BCG incubation in human
macrophages. Our data show iNOS expression is decreased in NOD2 deficient cells
stimulated with *M.tbc* and the *M.tbc*-specific NOD2 agonist N-glycolylated muramyl
dipeptide (GMDP), indicating that NOD2 is involved in iNOS protein expression. These
data provide evidence for a novel pathway involving NOD2 that controls *M.tbc* growth in
human macrophages through the regulation of iNOS expression.

We examined other potential signaling partners for NOD2 in human macrophages. Data
show that the NOD2 agonist, GMDP, stimulates phosphorylation and activation of PKCδ.
Corroborating these data, NOD2 deficient cells stimulated with PMA are significantly
reduced in PKCδ phosphorylation. PMA is a known stimulus for classical and novel
PKC isoforms. Additionally, the kinase known to interact with NOD2, RIP2, is activated by PMA. This activation is deficient upon knockdown of NOD2 in human macrophages. Thus, our findings strongly support a role for NOD2 in human macrophages during *M.tb* infection as well as in early innate immune responses that include iNOS and PKC pathways.
Dedication

This document is dedicated to my family and friends who encouraged me throughout life and molded me into the person I am today. I would like to specifically dedicate this to my mother, Katherine L. Brooks, who struggled in life so that I may succeed. My father, Michael S. Brooks, who taught me happiness and perseverance are the keys to life. My fiancé, Samuel E. Landes, who I am eternally grateful to for providing a shoulder to lean on during difficult times, listening to endless scientific presentations, and making my life during graduate school filled with fun and laughter. Thank you!
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Table of Contents

Abstract ....................................................................................................................................................... ii

Dedication .................................................................................................................................................. v

Acknowledgments ..................................................................................................................................... vi

Vita .............................................................................................................................................................. viii

List of Figures ........................................................................................................................................... xvii

CHAPTER 1 : Introduction ............................................................................................................................. 1

1.1 The immune system ............................................................................................................................ 1

1.2 Innate immunity ................................................................................................................................... 1

1.2.1 PKC isoforms .................................................................................................................................. 3

1.2.2 iNOS and NO production .............................................................................................................. 5

1.3 Adaptive immunity ............................................................................................................................ 6

1.4 Pulmonary innate immunity ............................................................................................................. 8

1.5 Alveolar macrophage ....................................................................................................................... 9

1.6 Macrophage activation ..................................................................................................................... 9

1.7 Macrophage receptors ..................................................................................................................... 11
1.8 Pattern recognition receptors ......................................................... 12

1.9 NOD-like receptors ........................................................................ 14
   1.9.1 Discovery .................................................................................. 14
   1.9.2 NLR structure .......................................................................... 15
   1.9.3 LRR domain ........................................................................... 15
   1.9.4 NOD domain ........................................................................... 16
   1.9.5 CARD domain ......................................................................... 16
   1.9.6 NOD agonists ......................................................................... 18
   1.9.7 Generation of NOD agonists .................................................. 18

1.10 NLRs and innate immunity ............................................................ 19

1.11 NOD2 signaling cascade ............................................................... 19

1.12 NOD2 and TLR crosstalk ............................................................. 22

1.13 NOD2 and microbial immunity .................................................... 23

1.14 NOD2 and inflammatory diseases .............................................. 24

1.15 NOD2: human versus mouse ....................................................... 25

1.16 Tuberculosis ................................................................................ 26
   1.16.1 Drug therapy and vaccines .................................................. 27
   1.16.2 Infection with M. tb ............................................................. 28
   1.16.3 M. tb and alveolus interaction ............................................. 29
2.2.8 Confocal microscopy........................................................................................ 49

2.2.9 Transfection of MDMs ..................................................................................... 49

2.2.10 Macrophage stimulation / infection, cell lysis, immunoprecipitation, and western blotting ......................................................................................................... 50

2.2.11 ELISAs ........................................................................................................... 52

2.2.12 M.\( tb \) and BCG intracellular growth assays in macrophages............... 52

2.2.13 M.\( tb \) and BCG cell association assay within macrophages ...................... 53

2.2.14 Cell enumeration of NOD2 siRNA transfected MDMs in monolayer culture 53

2.2.15 NOD2 antibody peptide competition assay ............................................... 54

2.2.16 Statistics.......................................................................................................... 54

2.3 Results ..................................................................................................................... 55

2.3.1 NOD2 mRNA and protein expression increase as monocytes differentiate into macrophages .............................................................................................................. 55

2.3.2 Activation of NOD2 enhances the M.\( tb \)- and BCG-mediated pro-inflammatory cytokine response through the NF-\( \kappa B \) pathway ................................................................. 58

2.3.3 siRNA-mediated knockdown of NOD2 activity in human macrophages leads to loss of its activity ................................................................................................. 61

2.3.4 NOD2 regulates the macrophage TNF-\( \alpha \) and IL-1\( \beta \) cytokine response to M.\( tb \) and BCG .................................................................................................................... 63
2.3.5 Expression of NOD2 in human AMs and its role in regulating the production of TNF-\(\alpha\) and IL-1\(\beta\) in response to \textit{M.tb} ................................................................. 66

2.3.6 NOD2 controls the intracellular growth of \textit{M.tb} and BCG in human macrophages .............................................................................................................. 68

2.3.7 Effects of NOD2 on \textit{M.tb} and BCG growth in mouse macrophages and on the cytokine response to infection................................................................. 70

2.4 Discussion ............................................................................................................... 72

CHAPTER 3 : Critical role for NOD2 in \textit{Mycobacterium tuberculosis} induced iNOS expression in human macrophages ................................................................. 81

3.1 Introduction ............................................................................................................. 81

3.2 Materials and Methods ............................................................................................ 84

3.2.1 Buffers and reagents ......................................................................................... 84

3.2.2 Antibodies ......................................................................................................... 85

3.2.3 Bacterial strains and culture ............................................................................. 85

3.2.4 Isolation and culture of human macrophages................................................... 86

3.2.5 RNA isolation ................................................................................................... 86

3.2.6 Gene expression studies by qRT-PCR ............................................................. 86

3.2.7 Transfection of MDMs ..................................................................................... 87

3.2.8 Microarray analysis .......................................................................................... 87
3.2.9 Macrophage stimulation / infection, cell lysis, and Western blotting ............... 88
3.2.10 DAF-FM diacetate staining ............................................................................. 89
3.2.11 Statistics .......................................................................................................... 90
3.3 Results ..................................................................................................................... 90
3.3.1 NOD2 regulates many genes during M.tb infection of human macrophages... 90
3.3.2 NOD2 agonists regulate iNOS expression and NO production in human macrophages .............................................................................................................. 93
3.3.3 M.tb-mediated iNOS expression is NOD2-dependent ..................................... 96
3.4 Discussion ............................................................................................................... 99

CHAPTER 4 : The role of PKCδ in the NOD2 signaling pathway in human macrophages ......................................................................................................................... 106

4.1 Introduction ........................................................................................................... 106
4.2 Materials and Methods .......................................................................................... 109
4.2.1 Buffers and reagents ....................................................................................... 109
4.2.2 Antibodies ....................................................................................................... 110
4.2.3 Isolation and culture of human macrophages............................................... 110
4.2.4 Transfection of MDMs ................................................................................... 111
4.2.5 Macrophage stimulation and preparation for ELISAs and Western blotting ..... 111
4.2.6 Kinase assays .................................................................................................. 112
4.2.7 ELISA............................................................................................................. 113
4.2.8 Statistics.......................................................................................................... 113
4.3 Results ................................................................................................................... 114
  4.3.1 Activation of NOD2 enhances the PMA-mediated pro-inflammatory cytokine response through its signaling cascade intermediates ............................................. 114
  4.3.2 Phosphorylated PKCδ expression and activity is NOD2-dependent............. 117
  4.3.3 RIP2 activation is PKCδ-dependent ............................................................... 121
4.4 Discussion ............................................................................................................. 123
CHAPTER 5 : Synthesis................................................................................................ 128
Bibliography ................................................................................................................... 140
List of Figures

Figure 1.1: NOD1 and NOD2 structure and domain functions ........................................ 17
Figure 1.2: NOD2 signaling cascades.................................................................................. 21
Figure 2.1: NOD2 expression increases during monocyte differentiation to macrophages. ........................................................................................................................................... 57
Figure 2.2: The NOD2 ligand MDP enhances \textit{M.\textit{tb}}- and BCG-mediated cytokine production in human macrophages, a process involving activation of RIP2 and \textit{IkB}α... 60
Figure 2.3: Loss of NOD2 activity in human macrophages by siRNA knockdown....... 62
Figure 2.4: NOD2 controls \textit{M.\textit{tb}}- and BCG-mediated cytokine production and RIP2 activation in human macrophages.................................................................................................................... 65
Figure 2.5: \textit{M.\textit{tb}}-mediated cytokine production in human AMs treated with MDP........ 67
Figure 2.6: NOD2 controls the intracellular growth of \textit{M.\textit{tb}} and BCG in human macrophages. ..................................................................................................................................................... 69
Figure 2.7: Effect of NOD2 on \textit{M.\textit{tb}} and BCG growth and cytokine response in mouse WT and NOD2\textsuperscript{−/−} BMMs. ........................................................................................................... 71
Figure 3.1: NOD2 regulates gene expression in human macrophages during \textit{M.\textit{tb}} infection. ........................................................................................................................... 92
Figure 3.2: NOD2 agonists regulate the mRNA and protein expression of iNOS and NO production in human macrophages. ................................................................. 95

Figure 3.3: *M.tb*-mediated induction of iNOS expression and NO production is NOD2-dependent in human macrophages. ............................................................... 98

Figure 4.1: The NOD2 signaling pathway is involved in PMA-stimulated cytokine production. ........................................................................................................ 116

Figure 4.2: NOD2 and PKCδ agonists stimulate PKCδ phosphorylation and activity in a NOD2-dependent manner. ................................................................. 120

Figure 4.3: RIP2 phosphorylation mediated by PMA and GMDP is PKCδ-dependent. 122

Figure 4.4: PKCδ is necessary for optimal NOD2 activity ........................................ 127
Abbreviations

ABC = ATP-binding cassette
AM = alveolar macrophage
APC = antigen presenting cell
BAL = bronchoalveolar lavage
BCG = *Mycobacterium bovis* BCG
BMM = bone marrow derived macrophage
BSA = bovine serum albumin
CARD = caspase activation and recruitment domain
CFP10 = culture filtrate proteins of 10 kDa
CR = complement receptor
CRD = carbohydrate recognition domain
DAF-FM = 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
DAG = diacylglycerol
DAN = diaminonaphthalene
DAP = diaminopimelic acid
DC-SIGN = dendritic cell-specific intercellular adhesion molecule-2 grabbing non-integrin
DUOX2 = dual oxidase 2
EBD = effector binding domain
ELISA = enzyme-linked immunosorbent assay
ESAT6 = early secreted antigen target of 6 kDa
ESX-1 = early secretory antigenic target 6 system 1
GlcNAc = N-acetylglucosamine
GMDP = N-glycolyl-MDP
HIV = human immunodeficiency virus
IFN = interferon
Ig = immunoglobulin
IL = interleukin
iNOS = inducible nitric oxide synthase
IRG = immunity-related guanosine triphosphatase
JAK = janus kinases
LM = lipomannan
LPS = lipopolysaccharide
LRD = ligand recognition domain
LRR = leucine rich repeats
ManLAM = mannose capped lipoarabinomannan
MAP = mitogen-activated protein
MDP = muramyl dipeptide
MDM = monocyte derived macrophage
MDR-TB = multi-drug resistant tuberculosis
MHC = major histocompatibility complex
MOI = multiplicity of infection
MR = mannose receptor
M. tb = Mycobacterium tuberculosis
MurNAc = N-acetylmuramic acid
NADPH = nicotinamide adenine dinucleotide phosphate hydrogen
NamH = N-acetyl muramic acid hydroxylase
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NK = natural killer
NLR = nod-like receptor
NO = nitric oxide
NOD = nucleotide-binding oligomerization domain
PAMPs = pathogen associated molecular patterns
PBMC = peripheral blood mononuclear cell
PKC = protein kinase c
PPARγ = peroxisome proliferator activated receptor
PRK = PKC related kinases
PRR = pattern recognition receptor
PMA = phorbol 12-myristate 13-acetate
qRT-PCR = quantitative real-time PCR
R = resistance
RCN = relative copy number
RD1 = region of difference 1
RIP2 = receptor interacting protein 2
RNI = reactive nitrogen intermediates
ROI = reactive oxygen intermediates
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA = small interfering RNA
SP-A = surfactant protein A
SP-D = surfactant protein D
SR = scavenger receptor
STAT = signal transducers and activators of transcription
TAK1 = TGF activated kinase 1
Tat = twin-arginase transport
TB = tuberculosis
Th1 = type 1 helper T cells
Th2 = type 2 helper T cells
TIR = toll IL-1 receptor
TLR = toll like receptor
TNF-α = Tumor necrosis factor alpha
XDR-TB = extensively drug resistant
WHO = World Health Organization
CHAPTER 1 : Introduction

1.1 The immune system

The immune system is highly complex, involving soluble components, cells, tissues, and organs that are responsible for keeping the host healthy from disease. There are many different cell types that make up host immunity including T cells, neutrophils, eosinophils, basophils, mast cells, and antigen presenting cells (1). These cell types are each unique in their function and work together to keep balance and order in the host. The immune system is made of two arms, adaptive and innate, that work together in order to keep the host healthy (2).

1.2 Innate immunity

The innate immune response is the first line of defense against invading pathogens, and is also known as native immunity. This response occurs rapidly upon encountering an infectious agent. There are four main principle components of innate immunity: physical and chemical barriers, phagocytic cells and natural killer (NK) cells, blood proteins, and cytokines (1). The major components that work in concert in order to protect the host include: soluble and membrane bound molecules, cells, and processes. The soluble
components include complement, collectins, and acute phase proteins that are known to decorate the surface of infectious agents and elicit a response. Membrane bound components are those found on innate immune cells such as pattern recognition receptors (PRR) (3). The cells of the innate immune response play a critical role in defending against pathogens. These cells include mast cells, eosinophils, basophils, macrophages, neutrophils, dendritic cells, and NK cells. The processes that occur include phagocytosis, reactive oxygen/nitrogen intermediate killing, phagosome acidification, and antimicrobial protein and peptide production (4). Macrophages are an important cell type in innate immunity. These cells use three mechanisms in order to uptake small particles or infectious agents. Pinocytosis is the uptake of substances that are < 1 μm, and is also known as fluid phase endocytosis. Receptor-mediated endocytosis is the uptake of specific substances that bind to surface receptors and this is mediated by clathrin. Phagocytosis is the uptake of microbes that are 1 μm or larger, and this mechanism is mediated by actin. Endocytosis uses clathrin-coated pits while phagocytosis uses cytoskeletal components such as paxillin, talin, and vinculin (5).

Once engulfed inside the macrophage, the pathogen is in a phagosome. This is known as an early phagosome characterized by Rab5 and the presence of cathepsin H. The phagosome matures and acquires Rab7 and cathepsin S. This is known as the late phagosome. Finally, the phagosome fuses with a lysosome that contains antimicrobial proteins and peptides accompanied by a lower pH (6).
Upon phagocytosis the macrophage uses a mechanism called the respiratory burst as its first line of defense, which causes the production of superoxide and hydrogen peroxide. Upon ligation of phagocytic receptor(s), phospholipase C releases diacylglycerol (DAG) and IP3. This is followed by calcium release from the endoplasmic reticulum (ER), which in conjunction with DAG activates protein kinase C (PKC) isoforms. PKC then phosphorylates the cytosolic component p47phox. This then activates the formation of the NADPH oxidase, allowing its components to translocate to the phagosome or plasma membrane, where the complex binds flavocytochrome subunits gp91 phox and p22 phox (7). One of the end products of the respiratory burst, superoxide anion, can dismutate to hydrogen peroxide, and can generate hydroxyl radicals and singlet oxygen. In neutrophils, hydrogen peroxide can also be converted by myeloperoxidase into hypochlorous acid and chloramines (4).

1.2.1 PKC isoforms

PKCs are a family of enzymes and were one of the first kinases identified. The major function of these kinases is to phosphorylate hydroxyl groups on the serine and threonine residues of other proteins in order for them to function properly. PKCs are most notably known for their function in the respiratory burst but are also important in cytokine signaling (8,9). PKCs are activated by phosphotidylserine or DAG in a calcium-dependent manner as well as by tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (10). There are 12 identified PKC isotypes in the mammalian PKC family, and this contributes to the complexity of understanding PKC
function. There are four subfamilies of isoforms that are classified based on the secondary messenger that is required for activation. Classical (conventional) PKCs have been the most thoroughly studied and require DAG, calcium, and phospholipid for activation. They include the isoforms PKCα, PKCβI, PKCβII, and PKCγ (11). PKCs that require DAG but not calcium for activation are known as novel PKCs and include the isoforms PKCd, PKCθ, PKCε, and PKCη (12). Atypical PKCs require neither calcium nor DAG for activation and include PKCi, PKCζ, PK-N1, PK-N2 (13). Both classical and novel PKC subfamilies can be activated by phorbol esters in the presence of phosphatidylinerine. PMA is able to activate the enzymes by abolishing the requirement of DAG and decreasing the concentration of calcium needed for activation (14). A final group, PKC-related kinases (PRK), are similar to atypical PKCs; however, they were discovered to bind and activate RhoA GTPase (15-18).

The novel PKCs are of interest here, specifically PKCd. PKCd is involved in B-cell signaling, and the regulation of growth, apoptosis, and differentiation of a variety of cell types (19-21). Phosphorylation of various tyrosine residues is necessary for activation of the enzyme; one specific residue is Tyrosine 311 (22). Interactions between this kinase include, but are not limited to, phosphoinositide-dependent kinase-1, STAT1, and the insulin receptor (23-26). PKCθ functions to activate T-cells, and is necessary in the activation of NF-κB and AP-1 through the T-cell receptor signaling complex (27,28). This kinase has been shown to interact with a few proteins including, RAC-alpha serine/threonine-protein kinase (AKT1) and Proto-oncogene tyrosine-protein kinase
(FYN) (29,30). Interestingly, PKCθ has been shown to phosphorylate CARD11 (CARMA1) and activation of NF-κB is mediated by these two proteins and B-cell lymphoma/leukemia 10 (BCL10) in T cells (31).

1.2.2 iNOS and NO production

Reactive nitrogen species (RNS) are also prominent in macrophages. Inducible nitric oxide synthase (iNOS) is regulated at the transcriptional level and is necessary in order for nitric oxide (NO) to be produced (4). NO is produced on the cytoplasmic side of the phagosome and can diffuse across membranes to reach intraphagosomal targets (32). Upon encountering ROS in the luminal environment NO can undergo spontaneous or catalytic conversion to RNS products that are important mediators in bacterial killing such as nitric dioxide, peroxynitrite, dinitrogen trioxide, and dinitrosyl iron complexes. The products can cause protein inactivation and lipid conversion by oxidative damage, causing impaired bacterial metabolism and inhibition of replication (33). Signal transduction is one fundamental role RNS plays in biology and has been shown experimentally during infections (34,35). It is now widely accepted that NO signaling is important in the cardiovascular system as well as in vascular homeostasis (36). Another role for NO is in controlling inflammation. While the mechanism is still unclear as to how exogenous NO puts forth an anti-inflammatory effect, it is thought that it inhibits NF-κB (37).
iNOS and NO production are dependent on many factors. iNOS mRNA is regulated at multiple steps, including transcription, stability, and translation (38). The regulation of iNOS protein may occur by calmodulin binding, dimer formation, substrate depletion (L-arginine), substrate recycling, tetrahydrobiopterin availability, end product inhibition, phosphorylation, and subcellular localization. Tetrahydrobiopterin plays a major role in iNOS enzymatic activity; however, production can be influenced by cytokines and LPS (38,39). Upon NO binding to the iron in heme, it acts as a feedback inhibitor of iNOS (40,41). iNOS mRNA can be induced by Interferon-gamma (IFNγ) through the binding of Interferon receptor 1 (IFNR1) and IFNR2 complex, which activates janus kinases (JAK) and signal transducers and activators of transcription (STAT) pathways. The transcription factor interferon response factor-1 (IRF1) is activated and iNOS mRNA is produced (42). Production of iNOS mRNA is also induced by toll-like receptor (TLR) ligands and use NF-κB transcription factors (43). While initial studies showed lower levels of iNOS expression and NO production in human cells, the availability of better techniques to study NO production has led to more convincing data for its presence in human cells (44).

### 1.3 Adaptive immunity

When the innate immune response cannot control an infection, it also serves to give warning that an infection is present and in turn activates the adaptive immune response. While the innate immune system is notorious for its immediate response to an infectious agent, the adaptive immune response is valued for its specificity and ability to recognize
a re-occurring previous antigen. Activation of this system is dependent on co-stimulatory molecules on antigen presenting cells (APCs) and T lymphocytes (T cells). There are two major types of adaptive immune responses, humoral and cell-mediated immunity. Humoral immunity is the production of antibodies by B lymphocytes (B cells) and cell-mediated is the interaction of T cells with macrophages. Extracellular microbes are neutralized and targeted for elimination by antibodies, using mechanisms such as phagocytosis or releasing inflammatory mediators from immune cells to remove them. T cells are responsible for infectious agents that are able to subvert the innate immune response and reside inside the macrophage where antibodies cannot reach. A major function of T cells is to mediate the killing of infected cells in order to control the infection. Activation of the adaptive immune response can elicit the production of several cytokines including, IL-2, IL-4, and IFN\(\gamma\). The T cell response to an infection is dependent on the cytokine produced, e.g. T\(_{\text{H}1}\) (IL-12) or T\(_{\text{H}2}\) (IL-4) (1).

Other characteristics of adaptive immunity include specificity and diversity, memory, specialization, self-limitation, and non-reactivity to self. Specificity is the ability of individual lymphocytes to recognize specific epitopes of antigens. The lymphocyte repertoire of an individual is great and this is due to the diversity of the structure of the antigen-binding site. Memory is another key characteristic of adaptive immunity. The second time an individual is exposed to an antigen, the response is quicker and greater compared to the primary interaction. Specialization refers to the variety of responses elicited, either humoral or cell-mediated, which allows the system to be more effective at
clearing an infection. The ability of the adaptive immune system to return to basal conditions and only functioning when an antigen is present is called self-limitation. Finally, nonreactivity to self may be one of the most important features because it controls the system from reacting to an individual’s own antigenic substances (45).

1.4 Pulmonary innate immunity

While the lung is known for its role in respiration, it is also a major player in innate immunity. The lung is constantly bombarded with particulates and microbes and serves as a major interface between the host and the external environment. An intricate pulmonary immune system is in place in order to protect the host, and this system encompasses innate and adaptive responses (46).

Upon inhalation, a particulate or microbe (< 5 μm) is able to avoid ciliary beat, the cough reflex, and mucus clearance to travel down the trachea and through the bronchi where it eventually settles in the alveolus. The alveolus is where the protection of the host occurs. Pulmonary innate immunity is controlled by cellular and soluble components. Airway and alveolar epithelial cells, as well as leukocytes are cellular components, while the antimicrobial products (e.g. collectins, defensins, lactoferrin, and cathelicidins) secreted into the epithelial lining fluid compose the soluble components of pulmonary innate immunity (47).
1.5 Alveolar macrophage

An important mediator of pulmonary immunity and one of the first lines of cellular defense in the alveolus is the alveolar macrophage (AM). There are only one to two AMs per alveolus which range in size from 9-40 μm in diameter (48). There are two major ways AMs are generated. The majority of AMs are thought to originate from monocytes circulating in the blood that eventually find their place in the alveolus, where they differentiate into macrophages. The second way, considered more controversial, is that the mononuclear phagocytes present in the lung replicate in the alveolus (48). In the alveolus, AMs are continuously bathed in surfactant, which is an important immune modulator that is produced by type II epithelial cells (49).

Two important functions of the AM are phagocytosis and inhibiting excessive pro-inflammatory signaling. It is necessary for these cells to have enhanced phagocytosis in order to clear the particles and microbes that are encountered by the alveolus. It is important to maintain homeostasis within the alveolus, and this is achieved by a balanced production of pro- and anti-inflammatory cytokines. AMs are unique in this ability to regulate cytokine production and are labeled alternatively activated for this purpose (48,50,51).

1.6 Macrophage activation

There are currently three types of activation states for macrophages based on the stimulus encountered: type I, type II, and alternative activation (52,53). Type I, also known as
classically activated macrophages or M1 macrophages, are those that encounter IFNγ and a stimulus that induces TNF-α, such as a microbe. Upon stimulation these cells produce pro-inflammatory cytokines such as TNF-α, IL-12, and IL-6, along with oxidants, increased MHCII expression, and a decrease in mannose receptor (MR) expression. Macrophages that encounter immune complexes are known as type II macrophages. These are similar to type I macrophages in that they produce pro-inflammatory cytokines, oxidants, and increased MHCII expression; however, they also produce the anti-inflammatory cytokine IL-10, and have less IL-12 production when compared to type I macrophages (53).

Alternatively activated, also known as M2 macrophages, are the third type of activation state. In vitro TH2 cytokines, IL-4 and IL-13, induce this macrophage type, which is consistent with the phenotype of AMs. These cells are characterized by having enhanced PRR expression, such as the MR, and decreased CD14 expression. They also produce fewer amounts of pro-inflammatory cytokines and oxidants, but instead secrete anti-inflammatory cytokines IL-10 and TGFβ more readily. Other markers of alternative activation found through mouse studies include receptors (CD23 and CD163), metabolic factors (L-arginase and lipoxygenase), and soluble proteins (FIZZI and Ym1/2) (52). Our laboratory has identified several features of alternatively activated human macrophages such as increased MR and TLR2 expression (54), decreased NADPH oxidase (55) and TLR activities (54), and increased activity of the nuclear receptor PPARγ which is linked to the MR (56).
1.7 Macrophage receptors

Phagocytosis is mediated by several different receptors. Opsonic phagocytosis occurs through the complement receptors (CR), CR1 and CR3, and Fcγ receptors. CR1 can bind mannose binding lectin (MBL), C1q, C3b, C4b, and requires additional signals to mediate phagocytosis. CR3 (Mac 1 or CD11b/CD18) binds C3bi-opsonized bacteria (57). The Fcγ receptors recognize particles opsonized with IgG antibodies (58). Other receptors are involved in non-opsonic phagocytosis. The MR is a C-type lectin, membrane bound Pattern Recognition Receptor (PRRs, see below) found on the cell membrane as well as on endosomes within the cell. Its function is normally to bind terminal mannose and fucose residues of host glycoproteins and glycolipids, which are also common components of bacterial cell walls. The tail of the receptor is necessary in order to induce phagocytosis (59). The scavenger receptor (SR) is known to phagocytose low-density lipoproteins (LDL) circulating in the body that can no longer interact with the LDL receptor, but has been shown to bind microbes as well (60).

There are several other membrane receptors that are not directly involved in phagocytosis, but instead signal in order to activate the phagocyte and in some cases regulate phagocytic receptors. These include dectin-1, TLRs, and G protein-coupled receptors. Dectin-1 is involved in signaling after recognition of non-opsonic beta glucan particles found in fungi (61). TLRs are trans-membrane PRRs that recognize various pathogen-associated molecular patterns (PAMPS) and induce a signaling cascade that
typically results in the production of inflammatory cytokines (62). G protein-coupled receptors recognize short peptides containing N-formylmethionyl residues and function to stimulate the recruitment of lymphocytes to the site of infection. The macrophage also has a secondary line of foreign molecule recognition with receptors located in the cytosol. These PRRs are known as the Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLRs) and function to induce an inflammatory response upon recognition of their PAMP intracellularly (63).

1.8 Pattern recognition receptors

As noted above, PRRs are unique in that they recognize specific patterns or PAMPS. These can range from bacterial peptidoglycan fragments, carbohydrates, lipids or flagella to intracellular DNA or single stranded RNA. They play a major role in innate immunity through functions such as phagocytosis and signaling an inflammatory response. PRRs can have a transmembrane domain and be located on the cell surface or endosomes, the cytosol, or secreted.

Two major membrane-bound PRRs are the MR and TLRs. The MR is 180 kDa protein that, along with its transmembrane domain, has four other domains: an extracellular amino terminal cysteine rich domain, a fibronectin type II repeat domain, eight tandem cysteine rich lectin-like carbohydrate recognition domains (CRD), and a cytoplasmic carboxy-terminal domain (64). TLRs are an important class of PRRs. There are 10 identified mammalian TLRs that are distinctly membrane-associated type I receptors (65-12
The exterior amino terminus contains variable arrangements of leucine-rich repeats (LRRs) which serve to recognize PAMPs. The cytosolic carboxy terminus of TLRs is highly homologous to the IL-1 receptor and contains a Toll-IL-1R (TIR) domain that forms the scaffold for the assembly of signaling intermediates such as Myd88, IRAK1, IRAK4, IRAKM and Mal/Tiram. TLR4 and TLR2 are the most thoroughly studied TLRs. TLR4 has been found to recognize the endotoxin (lipopolysaccharide, LPS) of Gram-negative organisms, while TLR2 recognizes Gram-positive PAMPS, such as lipoteichoic acid. In general, TLR signaling drives NF-κB activation via phosphorylation of IκBα (67-69), although several negative regulators such as IRAKM have been identified (70).

Cytoplasmic PRRs do not contain a transmembrane domain and are associated with cytoplasmic networks. NLRs are involved in processes such as apoptosis and pro-inflammatory signaling. RNA helicases recognize single stranded or double stranded RNA. The two recognized in mammalian cells are RIG-1 and MDA5, which activate antiviral signaling (71). Secreted PRRs include MBL, serum amyloid protein, and C-reactive protein (1,2).
1.9 NOD-like receptors

1.9.1 Discovery

NLRs were initially discovered in plants. Plants are able to recognize pathogens by disease resistance (R) genes, and in turn induce a response that allows the infected area to be isolated through cell wall remodeling, production of reactive oxygen species, cell death, and production of anti-pathogenic molecules at the site of infection (72). There are two classes of R genes, one that produces transmembrane proteins similar to TLRs and another that produces cytosolic NOD proteins. These cytosolic NOD proteins generally contain amino-terminal α-helix-rich or TIR domains, a central NOD, and carboxyl-terminal LRRs. Since there are known similarities between plants and animals, genome wide searches for homology between these plant R proteins and the mammalian system were embarked upon by investigators. The first searches yielded apoptosis regulator, Apaf-1 and its nematode homolog, CED-4, which revealed two proteins NOD1 (CARD4) and NOD2 (CARD15) (73). Further studies have described 23 mammalian NLRs in the innate immune recognition of intracellular pathogens (74). The current literature is heavily focused on NOD1 and NOD2. They are mainly expressed in APCs and epithelial cells. NOD1 protein is abundantly found in intestinal epithelial cells, while NOD2 protein is low or undetectable in these cells. NOD2 protein expression has been found in Paneth cells found at the base of intestinal crypts (75). NOD2 mRNA is present in monocytes but protein expression is low; however, NOD2 protein expression is abundant in human macrophages (76,77).
1.9.2 NLR structure

The structure of NOD1 and NOD2 are very similar to Apaf-1 and CED-4. They both contain amino terminal caspase activating and recruitment domains (CARDs) linked to a NOD domain; however, the mammalian NOD proteins contain LRRs in their carboxy termini (73). Most mammalian NOD family members contain 3 distinct functional domains, an amino terminal effector binding domain (EBD), a centrally located NOD, and a carboxy-terminal ligand recognition domain (LRD). Depending on the downstream signaling partner, the EBD is involved in homophilic and heterophilic interactions. The NLR family can be divided into four subfamilies depending on the composition of their N-terminal EBD. The differing N-terminal domains are as follows with subfamily name: acidic transactivation domains (NLRAs), CARD (NLRCs), pyrin domains (NLRPs), and baculovirus IPA repeat domains (NLRBs). NOD1 and NOD2 are part of the NLRC subfamily and contain a LRR domain, NOD domain, and CARD domains (78).

1.9.3 LRR domain

The LRR domain is made up of leucine rich repeats that are needed for the sensing of agonists. There are approximately 11 LRRs in a horseshoe shape made up of beta strands that constitute the concave face of the LRRs. Functional analysis of NOD2 revealed that the most C-terminal LRRs, 6-11, are critical for bacterial recognition (79). These residues are confined to the β-strand/β-turn (xxLxLxx) motif (L = leucine, x = amino
acid) (80). There are six non-conserved amino acids in the corresponding regions of the LRRs for each protein. These include G879, W907, V935, E959, K989, and S991. These amino acids face outward and are thought to be important in recognition of the specific agonist for each NOD protein (79). Currently, no one has been able to show direct binding of NOD proteins with their specific agonist. Some speculate that there may be an intermediate that has not been discovered that causes activation.

1.9.4 NOD domain

This region contains an ATP-binding cassette (ABC) that includes catalytic residues with canonical binding site motifs for phosphate residues (Walker’s A box) and magnesium ions (Walker’s B box). Similarities have been found between the AAA+ family of ATPases oligomerization module and ABC region of NOD-LRR proteins (81). In this region, there are also motifs involved in hydrolysis of nucleosides, ATP, deoxyATP and/or GTP. Nucleotide hydrolysis is necessary for protein activation of NOD1 and NOD2. Mutational analysis identified 21 critical residues in the NOD domain important for signaling (79).

1.9.5 CARD domain

The CARD domain is necessary for interactions with the CARD domain of the downstream kinase receptor interacting protein 2 (RIP2), which is necessary for the signaling cascade to occur and NF-κB activation. The CARD effector domain is
involved in homophilic interactions. There are 6 helices and a hydrophobic core. On helices 2 and 3 there is an acidic patch formed by residues E53, E54, and E56. This acidic patch has been found to be involved in interactions between NOD1 and RIP2 (82). The crystal structure of NOD1 revealed a dimer of two monomers where the sixth helix is swapped between two monomers (83). NOD1 contains one CARD domain, while NOD2 contains two CARD domains (Figure 1.1) (84).

Figure 1.1: NOD1 and NOD2 structure and domain functions

(A) NOD1 contains one CARD domain that is necessary for signaling, a NOD domain involved in activation of the protein, and a LRR domain essential for agonist recognition. (B) NOD2 contains all of the same domains as NOD1, but contains an additional CARD domain.
1.9.6 NOD agonists

The NLRC proteins, NOD1 and NOD2, recognize specific muropeptides found in the peptidoglycan layer of Gram-positive and Gram-negative bacteria. The repeating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) are arranged in parallel by stem peptides made up of amino acids. Cleavage of these chains leads to the agonists for NOD1 and NOD2. NOD2 recognizes muramyl dipeptide (MDP), which is found in Gram-positive and Gram-negative bacteria, while NOD1 recognizes meso-diaminopimelic acid (meso-DAP) only found in Gram-negative bacteria (85). Recently, it was found that NOD2 recognizes an N-glycolylated form of MDP (GMDP) that can be found in *Mycobacterium tuberculosis* (*M.tb*). The chemically synthesized GMDP is a more potent stimulator of the immune response (86). Likewise the synthetic truncated form of DAP, γ-D-glutamyl-meso-DAP, is sufficient to activate NOD1 (87,88).

1.9.7 Generation of NOD agonists

Even though the agonists are positioned in the peptidoglycan layer there are several ways these muropeptides can be generated by the bacteria and host. As bacteria replicate, cell division occurs, peptidoglycan remodeling occurs, and small fragments are released (89). During peptidoglycan biosynthesis bacteria are also able to produce small molecules such as MDP and iE-DAP (89-91). Lysozyme is secreted from the host in tears, saliva, mucous, and is also found inside phagocytic lysosomes in order to breakdown the cell wall of bacteria. This glycoside hydrolase catalyzes the hydrolysis of MurNAc and GlcNAc thereby generates muropeptides, which include MDP and iE-DAP (92). N-
acetylmuramoyl-L-alanine amidases are found in bacteria and host cells. This amidase cleaves the bond between MurNAc and the stem peptide in order to generate MDP. There are proposed models for the agonists to get into the cytosol from inside the phagosome in order to be recognized by NLRs. One mechanism is bacteria can divide inside the phagosome and cause it to burst, in turn releasing the bacteria as well as any peptidoglycan fragments that may have been generated by bacterial replication or host response factors. The other method is through bacterial secretion systems (93).

1.10 NLRs and innate immunity

NLRs play a major role in innate immunity. They are a second line of recognition and are responsible for inducing a pro-inflammatory response to bacteria that get inside the macrophage. While there are 23 NLRs with a wide range of known functions including cell signaling and cell death/survival pathways, the NLRC family’s established function is in inducing an inflammatory response through NF-κB upon recognition of specific mucopeptides from bacteria.

1.11 NOD2 signaling cascade

The determining region of whether a NOD protein is active or inactive has been revealed by mutational analysis to be the most carboxy-terminal region of the NOD domain also known as the “switching region” (79). Upon recognition of MDP, the NOD domain of NOD2 undergoes oligomerization, which allows the CARD domain to be exposed
leading to an electrostatic interaction with the CARD domain of the adapter protein RIP2 (85,88). This in turn causes autophosphorylation of RIP2 at serine 176 in cell lines and murine macrophages (94). The NOD2-RIP2 complex then binds TGF activated kinase 1 (TAK1), which can then either directly activate by phosphorylation of the inhibitor of NF-κB, IκBα, and or activate the MAPKs and thus indirectly allow for NF-κB and AP-1 activation leading to the production of pro-inflammatory cytokines (95,96). More recently researchers discovered that NOD2 can directly bind and activate caspase-1 and interact with the NALP1/NALP3 inflammasome, which causes the activation of caspase-1, a necessary enzyme needed to cleave pro-IL-1β into its active secreted form (97) (Figure 1.2). Also, a truncated form of NOD2 exists that is able to negatively regulate NOD2 activity (98).
Upon bacterial phagocytosis, MDP is released into the cytosol by various mechanisms and is sensed by the NOD2 LRR domain. (A) The NOD2 protein oligomerizes and recruits RIP2 and this NOD2/RIP2 complex interacts with TAK1. After TAK1 activation, either MAPKs are activated to induce transcription of pro-inflammatory cytokines or the IKK complex is activated, which in turn removes the inhibitor or NF-κB, IκBα, and allows for transcription of pro-inflammatory cytokines to occur. (B) The NOD2 protein interacts with the NALP1 or NALP3 inflammasome or directly binds pro-caspase1. Pro-caspase-1 is then cleaved into its active form which enables cleavage of pro-IL-1β into the released form, IL-1β.
1.12 NOD2 and TLR crosstalk

There are many reports on the synergy between TLR agonists and NOD2 agonists in pro- and anti-inflammatory cytokine release (99-106). Proper signaling through NF-κB by NOD2 and TLRs involves the same mechanism of K63-linked ubiquitination of NEMO (107). A biochemical mechanism for the synergy between TLRs and NOD2 has been reported. Both signaling cascades utilize TAK1/TAB/Ubc13 to activate NF-κB, which allows TLR and NOD2 signaling to synergistically induce cytokine release. However the primary source of this activation is different, NOD2 uses RIP2 while TLRs utilize TRAF6 (108).

On the level of transcription it has been shown that TLR activation and cytokines induce NOD2 mRNA expression (109,110). MDP enhances MyD88 expression, which is a necessary adapter protein in TLR signaling and may explain the high sensitivity of TLR responses after peptidoglycan priming (111). The location of the NOD proteins may also be a factor in crosstalk between TLRs and NOD2. While NOD2 is found in the cytosol, studies have shown that it can be found localized at the cell-membrane and this localization may be important in signaling (112-114). The proximity of NOD2 at the membrane where TLRs can be found also suggests a mechanism for crosstalk.
1.13 NOD2 and microbial immunity

The biological function of NOD2 in innate and adaptive immune responses is poorly understood; however, studies over the past decades on purified or synthetic MDP have given some insight. In animal models the administration of MDP induces activity against bacteria, fungi, viruses, protozoa, and tumor growth (115). The microbicidal activity of MDP can be explained by the induction of TNF-α and IL-1β. This was thoroughly tested by antibody neutralization studies (116-121). Further studies proved that MDP activates NF-κB and that all MDP-inducible genes require NOD2 activity (122). MDP has been described to be involved in the secretion of multiple immune mediators that are important in pathogen clearance: pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β), chemokines (IL-8, CCL3, CCL4), and hematopoietic growth/survival factors (IL-6, G-CSF, GM-CSF) (116-119,121-123). Adhesion molecules (ICAM1, CD44, TNFAIP6) known to be important in the recruitment of inflammatory cells are also induced upon MDP stimulation. NOD2 has also been shown to play a role in linking the innate and adaptive immune response due to its adjuvant activity. MDP generates antigen specific T- and B-cell responses, delayed-type hypersensitivity, and antibody production (115). This activity is due to the ability of MDP to induce the expression of costimulatory molecules (CD40, CD80, CD86) in monocytes and dendritic cells therefore differentiating naive T cells into effector T cells (123-125).
Several microbial models have substantiated the importance of NLRs in the recognition of pathogens including *Shigella flexneri, Chlamydia pneumonia, Pseudomonas aeruginosa, Helicobacter pylori, Streptococcus pneumoniae, Mycobacterium tuberculosis, Bacillus antracis, Listeria monocytogenes, Legionella pneumophila,* and *Staphylococcus aureus* (77,97,126-137).

1.14 NOD2 and inflammatory diseases

Mutations in the NOD2 gene, *CARD15*, have been linked to many inflammatory diseases including: Crohn’s disease, Blau syndrome, and early-onset sarcoidosis (138-141). Mutations are found in the NOD domain for Blau syndrome and early-onset sarcoidosis, while mutations in the LRR domain are linked to Crohn’s disease. Crohn’s disease presents as inflammation of the gut along with granulomas, and is mediated by over production of IL-1β, IL-6, IL-12, and TNF-α. There are two effective therapies; one is neutralizing antibodies against TNF-α, IL-6, and IL-12 or NF-κB inhibitors and the second is antibiotics (142). While debated, a correlative study reported that patients with Crohn’s disease are also likely to be infected with *Mycobacterium avium subspecies paratuberculosis* (143,144).

Studies have described mutations in the LRR domain as resulting in a ‘loss-of-function’ and ‘gain-of-function’. A ‘loss-of-function’ mutation results in a NOD2 protein that cannot activate the immune response. One hypothesis for the ‘loss of function’ phenotype is that this leads to excessive inflammation to the bacterial burden due to the
lack of NOD2-induced defense mechanisms. Another hypothesis is that NOD2 is involved in inhibiting the TLR2-mediated macrophage response and the lack of inhibition leads to excessive activation of NF-κB and IL-12 expression (104). A ‘gain-of-function’ mutation results in a new or abnormal function. The ‘gain-of-function’ mutation hypothesis is derived from ‘knockout-knockin’ CARD15 mouse studies which have a homologous mutation found in human Crohn’s disease. Stimulation of these mouse macrophages with MDP resulted in increased activation of NF-κB and caspase-1 mediated IL-1β processing and release (145). The excessive IL-1β production could lead to enhanced inflammation in the intestines. This study is debated as recent studies using human peripheral blood mononuclear cells (PBMCs) from patients with the homozygous NOD2 mutation displayed decreased IL-1β production in response to MDP (146). These differences reported are likely due to the difference in cell type, i.e. from mouse and human.

1.15 NOD2: human versus mouse

Researchers discovered that the promoter regions of NOD2 in humans and mice are different. Sequence alignment analysis revealed the canonical NF-κB binding site (-16 to -25 bp) is not found in the mouse or rat NOD2 core promoter (147). Furthermore, the NOD2 promoter (-500 to +300 bp) sequence alignment showed that there is only 49.7% similarity to mouse. Hu et al found that the NOD2 proximal promoter between human and chimpanzees is 98% identical (147). This indicates that human and chimpanzee NOD2 genes are regulated by a highly similar transcriptional mechanism, while the lack
of similarity between human and mouse is due to divergent mechanisms in NOD2 regulation. These differences are important to remember when studying the function of NOD2 in the mouse model.

1.16 Tuberculosis

*Mycobacterium tuberculosis* (*M.tb*) is the infectious agent that causes tuberculosis (TB). One third of the world’s population is currently infected with this bacillus, however only 5-10% of the people will develop clinical disease during their lifetime. In 2008, the World Health Organization (WHO) revealed that sub-Saharan Africa and the South-East Asia region are where TB cases are most heavily reported (148). In 2009, 1.7 million people died from TB with most deaths in the African region. This high incidence of TB deaths is due to the enhanced number of human immunodeficiency virus (HIV) positive people in Africa. HIV causes patients to be severely immuno-compromised (particularly in CD4 T cell number and function), and thus the probability of developing TB is greater. The WHO reported that TB is the leading cause of death among people who are HIV positive in Africa, and since 1990 HIV has been the single largest contributor to the increase in TB cases (148). The prevalence of TB combined with the increasing lack of effective drug therapy give reason to study the interaction of *M.tb* with the host in more depth. Understanding the molecular interactions of *M.tb* with host immune cells will potentially yield greater insight into the development of new targets for drug therapy as well as vaccine design.
1.16.1 Drug therapy and vaccines

The first TB vaccine was attempted by Robert Koch, who identified *M. tb* as the causative agent of TB. His vaccine used sterile filtrates from *M. tb* culture but it was not protective against TB; however it did open the door to further studies. Two French scientists, Albert Calmette and Camille Guerin, used a strain of mycobacteria that infects cattle, *M. bovis* made attenuated, by making serial passages for 13 years. This strain, *M. bovis* BCG (BCG), became avirulent in mice and vaccination of infants resulted in 90% reduction of mortality. Vaccination by BCG is effective against *M. tb* in children; however, its efficacy in adults varies from 80% to 0% (149,150). While the vaccine is not as effective in adults, it protects children, who are a highly susceptible group to disseminated and meningeal TB (151). While the exact reason is unknown, host and environmental factors are attributed to the lack of efficacy (152). The search continues for a cost effective vaccine that is protective for adults in order to one day eradicate TB.

Researchers are attempting to synthesize different types of vaccines including DNA, sub-unit, and living-attenuated vaccines. While there is currently no new TB vaccine in phase III clinical trials, a non-profit organization, the Aeras Global TB Vaccine Foundation, has a portfolio of promising vaccines. With its location in high incidence areas (South Africa and India), the company is capable of pursuing phase III clinical trials (153).

Although there are drugs to treat TB, incomplete or erratic usage has led to the development of drug resistant strains. Currently there are two categories of drug resistance, multi-drug resistant (MDR-TB) and extensively drug resistant tuberculosis
(XDR-TB) strains. MDR-TB strains are resistant to the two major first-line drugs, Isoniazid and Rifampin, while XDR-TB strains are resistant to the same drugs as well as fluoroquinolones, and any second-line injectible drugs (154). With 68 countries reporting in 2010 at least one case of XDR-TB, and the cost and long treatment regimen of current available drugs, it is clear that TB is a major public health concern and new drugs need to be identified to control the spread of this disease (155). As of 2010, there are ten new compounds for the treatment of active TB progressing through the clinical development pipeline. These drugs focus on novel regimens with 2-4 months of treatment (156).

1.16.2 Infection with *M. tb*

Individuals with active pulmonary TB can expel bacilli on minute aerosol droplets by coughing. A droplet containing as few as 1-10 bacilli is enough to cause an infection in a healthy individual (157). These droplets are small enough to subvert upper airway immune mechanisms such as ciliary beat and mucus clearance in order to reach the alveolus. In the alveolus the bacilli encounter antimicrobial products as well as the major cellular component of pulmonary innate immunity, the AM. The AM engulfs the bacteria, but *M. tb* is able to subvert the immune response through many different mechanisms (158).

The unique cell wall of *M. tb*, consisting of glycoproteins, glycolipids, lipoglycans and long-chain fatty acids, enables the bacteria to survive in the AM, while at the same time
these factors can be processed for presentation to T-cells. Over 2-12 weeks following primary infection the bacteria spread through the lymphatics and become systemic while the immune system recruits cells, notably T-cells, to the infected macrophages forming a granuloma. This cellular mass allows the healthy individual to contain the bacteria and remain asymptomatic; the individual is termed as having a latent infection. Active disease occurs when an individual becomes immunocompromised such as by old age or acquiring another infection, particularly HIV. At this time, the bacteria are able to replicate within the granuloma and induce a clinically evident disease state that the host cannot control. Reactivation occurs in the lung 80% of the time [most notably seen in the apex of the upper lobes of the lung (159)]; the remainder of cases are called extrapulmonary TB and occur in the lymph nodes (scrofula), spine (Potts disease), or systemic (miliary TB) among others.

1.16.3 *M.tb* and alveolus interaction

Upon entry into the alveolus, *M.tb* encounters surfactant, and two surfactant-associated components that regulate the early interaction of *M.tb* in the lung, SP-A and SP-D. SPA has been shown *in vitro* to enhance PRR activity, increase phagocytosis, alter production of pro-inflammatory cytokines, and decrease RNI and ROIs in response to stimuli (55,160). SPA enhances phagocytosis of *M.tb* in mouse and human macrophages, and also has been shown to inhibit *M.tb*-mediated NO production from IFNγ stimulated mouse macrophages (161-163). In contrast, SPD decreases phagocytosis of *M.tb* by binding to the mannose caps of ManLAM and inhibiting the normal interaction with the
MR (164). *In vitro* studies have shown that SPD-opsonized *M. tb* that do enter the macrophage have reduced intracellular growth that is due in part to the increased phagosome-lysosome fusion (164,165).

1.16.4 *M. tb* and AM receptor interaction

*M. tb* interacts with a number of receptors on the AM surface as well as in the cytosol. *In vitro* studies have shown that non-opsonic and serum opsonized bacteria coated with complement C3 are taken up by the CR3 on macrophages (166). While CR3 plays a clear role in phagocytosis, its role in pathogenesis is unclear. No affect was seen on the production of ROIs, NO or bacterial survival in CR3 deficient murine BMMs infected with *M. tb* (167) or in vivo in the mouse (168). Whether CR3 functions differently in the mouse compared to man with regards to TB pathogenesis is unknown. The importance of the MR during *M. tb* infection is better understood. *M. tb* is recognized by the MR through its mannosylated lipoarabinomannan (ManLAM) found on the cell wall. Upon phagocytosis by the MR, *M. tb* resides in a unique phagosome that does not fuse with the lysosome. Scavenger receptor A (SR-A) and CD-14 have been shown to mediate phagocytosis of unopsonized *M. tb* in different cell types (169,170). Avirulent and attenuated mycobacterial strains have been shown to induce dectin-1/TLR2 TNF-α production in murine macrophages (171). FcγRs mediate the phagocytosis of *M. tb* opsonized with immunoglobulin in the immune host (172,173). The 19 kDa lipoprotein, lipomannan (LM), and lower order PIMs found on the surface of the mycobacterial cell wall have all been shown to interact with TLR2 (174-176).
Recent studies have provided data to support *M. tb* activating intracellular receptors, i.e. NLRs. Masters *et al* published that *M. tb* inhibits inflammasome activation and IL-1β production in primary murine macrophages; however, using a human monocytic cell line researchers were able to show *M. tb* does activate the NLRP3/ASC inflammasome (177,178). Although the ligand has not been determined, several reports indicate that NOD1 and NOD2 recognize *M. tb* (127,135,179-181). Our recent data presented in this dissertation provide evidence that NOD2 is important in controlling the growth of *M. tb* in human macrophages and this is may be due to the role of NOD2 in inflammatory cytokine production (77).

1.16.5 *M. tb* and phagosome environment

Once inside the macrophage *M. tb* resides in a unique phagosome. The environment in the phagosome has a pH of 6.4, due to limited fusion of pre-formed lysosomes (182,183). Phagosomes containing *M. tb* resemble early endosomes. The *M. tb* cell wall component, ManLAM, is able to inhibit calcium increase in the cytosol that occurs normally during phagocytosis and without this calcium PI3K does not become activated (184). *M. tb* also inhibits sphingosine kinase, which inhibits calcium efflux (185). Due to the ability of *M. tb* to inhibit calcium- and Rab5-dependent recruitment of PI3K, PI3P is not formed on the membrane and there is a lack of Rab5 activity. This lack of activity inhibits full maturation of the phagosome by the lack of recruitment of Rab5 effector proteins EEA1 and syntaxin-6 (186). Entry through the MR leads to an anti-inflammatory program
along with a unique phagosome that has reduced fusion with the lysosome (187,188). This phagosome does not acidify normally in part because it does not acquire all subunits of the vacuolar proton ATPase (189,190).

1.16.6 *M.tb* secretion systems

The complex, thick and relatively impermeable cell wall of mycobacteria suggests the need for a secretion system, and in fact there are many secretion systems identified in mycobacteria including early secretory antigenic target 6 system 1 (ESX-1) through ESX-5. Many reports provide evidence for the release of proteins by *M.tb* into the supernatants of *in vitro* broth cultures (191). Specifically, culture filtrate proteins of 10 kDa (CFP10) and early secreted antigen target of 6 kDa (ESAT6), which are products of the ESX-1 secretion system, are particularly important. These two proteins are encoded by genes found in the region of difference 1 (RD1) and are attributed to *M.tb* virulence (192,193). During serial passage, the attenuated BCG lost 38 open reading frames, including the RD1 (192,193).

Putative analysis of the clustering of genes that encode membrane associated proteins with the genes that encode CFP10 and ESAT6 led to the idea and eventual confirmation of the ESX-1 secretion system (194-196). Addition of the RD1 region back into BCG restored the production of ESAT6. Also, disruption of genes in the ESX-1 locus, *Rv3870, Rv3871*, and *Rv3877* inhibited secretion of CFP10 and ESAT6. Further studies indicate that there are 14 possible genes in the RD1 necessary for optimal function of
ESX-1, and a number of these proteins have been associated with known functional domains. These include Rv3868, a putative cytoplasmic chaperone with an AAA+ ATPase domain, Rv3883c (MycP1), a subtilisin-like serine protease, and Rv3870/Rv3871, which together form an FtsK/SpoIIIE-like ATPase. Rv3860, Rv3877, Rv3881c, and Rv3882 are involved in ESAT6 secretion but have no known functions due to lack of homology with described proteins. These proteins are thought to be located in the cytoplasmic membrane. Rv3877 is predicted to be a multi-trans-membrane-spanning protein and hypothetically could be the translocation pore in the cytosolic membrane (191). The discovery of a second gene cluster, the Rv3614c-Rv3616c locus, involved in ESAT6 secretion has given further complexity to the ESX-1 system (197,198). The lack of similarity to type I- type VI secretion systems and the identification of additional ESX-1 systems within mycobacteria and other Gram-positive species has led to the characterization of a type VII secretion system (191).

While there is no structure determined yet for the type VII secretion system there are protein-protein interaction studies that indicate the mechanism of secretion. In order for secretion to occur, ESAT6 and CFP10 form a tight dimer held together by hydrophobic interactions. These two proteins are dependent on each other for secretion (199-201). Yeast two-hybrid screening showed Rv3871 interacts with Rv3870 along with CFP10. One hypothesis is that Rv3871 is involved in recognition of the CFP10-ESAT6 substrate pair and delivers it in an ATP-dependent manner to Rv3870 and thereby to the cell membrane secretion machinery. This is mediated by an interaction of Rv3871 with the
carboxy-terminus of CFP10 (199). This carboxy-terminus is similar to the secretion signal found in the type IV secretion system (202-204). EspA or Rv3616c and Rv3615c are also substrates that are secreted similarly to CFP10 and ESAT6 (198). All of the substrates of the ESX-1 system are dependent on each other for secretion. While the mechanism is not yet understood, in the absence of EspA or Rv3615c, the CFP10/ESAT6 dimer is not secreted (197,198).

Although their roles in virulence are not as clear as the type VII system, mycobacteria contain two other types of secretion systems, general secretion pathways (Sec) and the twin-arginase transport (Tat) pathway. Mycobacteria contain a general secretion pathway, like all bacteria, in order to secrete unfolded proteins across the cytosolic membrane. SecA2 was identified in mycobacteria as the non-essential homologue of SecA and its role in pathogenesis is unclear. However, SecA2 functions in L. monocytogenes as an accessory protein to secrete enzymes that remodel the peptidoglycan layer and these broken down products are shown to modify the innate immune response to favor bacterial colonization (205). The mycobacterial SecA2 mutant induces a more robust inflammatory immune response in infected macrophages indicating a role for it in suppressing the immune response (206). The Tat pathway is a Sec-independent pathway that has the ability to transport folded protein substrates across the plasma membrane (207,208). In the mouse model, four M.tb phospholipase C enzymes are secreted by Tat and are required for full M.tb virulence (207,209).
Secretion systems may play a key role in the ability of intracellular NLRs to sense mycobacteria, since the bacteria reside in a distinctive phagosome separate from the cytosol. Given that mycobacteria secrete proteins, the potential exists for these proteins to get across the phagosomal membrane and into the cytosol of infected macrophages to interact with NLRs. Secretion systems are reported to be important in enabling NLRs to sense *H. pylori* and *Legionella pneumophila* (93,137,210,211).

1.16.7 *M.tb* and entry into the cytosol

It is widely accepted that *M.tb* resides in a unique phagosome, which allows for its survival in antigen presenting cells along with the recruitment of CD4 T cells through MHCII-based antigen presentation. However, the mechanisms underlying the ability of virulent *M.tb* to use MHC-I-based antigen presentation in order to recruit CD8 T cells remains less clear.

While still debated, there is evidence that *M.tb* can escape from the phagosome and reside in the cytosol. The first report, using electron microscopy (EM), found that at 18-24 h 60 – 100% of endocytosed *M.tb* H37Rv by rabbit alveolar macrophages resulted in bacteria in the cytosol. While the less virulent strain, *M.tb* H37Rv a was located in intact phagosomes 99% of the time (212). Another report found, also using EM, that *M.tb* H37Rv was located in the cytosol or vesicles 50% of the time four days post infection. The authors found the *M.tb* H37Rv a was intermediate, while BCG was never found in the cytosol (213). These results are debated due to the cell types as well as the harsh fixation techniques.
used to prepare samples for EM. The cells in this study were J744.16, a mouse macrophage cell line, and also human monocytes. It is important to note that monocytes do not express the MR, and *M.tbc* entry through this receptor causes a decrease in phagosome-lysosome fusion (188,214).

Recently, another group reported visualizing *M.tbc* in the cytosol using cryogenic-EM, a more credible method for the preparation of samples when using EM. Cryogenic-EM is the use of low temperatures in order to keep the sample in its native state. They also used immunogold labeling, which allowed visualization of phagolysosomes. Using monocyte-derived dendritic cells, the authors report visualizing *M.tbc* and *M. leprae* in the cytosol 48 to 96 hours post infection. While they did visualize BCG in a phagolysosome 7 days post infection, no bacteria were found in the cytosol. The data also indicate the genes encoding CFP-10 and ESAT6 are necessary for mycobacterial entry into the cytosol (215). The presence of *M.tbc* in the cystosol has implication for recognition by intracellular NLRs.

1.17 *M.tbc* and NOD2

Recent studies provide evidence that NOD2 is involved in the pathogenesis of mycobacterial infections. For example, a genome analysis of patients with leprosy found a single nucleotide polymorphism in the NOD2 gene (216) which is linked to *Mycobacterium leprae* susceptibility. Furthermore, African American patients with TB have variants in their NOD2 gene rendering them more susceptible to *M.tbc* infection.
In addition, polymorphisms in \textit{CARD15}, which encodes NOD2, have been linked to inflammatory diseases, including Crohn’s disease \cite{138, 218}, whose cause is speculated to be of bacterial origin \cite{219}. The association of Crohn’s disease with \textit{Mycobacterium avium subspecies paratuberculosis} continues to be reported \cite{144}.

The majority of studies to examine the role of NOD2 in the host response to \textit{M.tb} infection have been done in mice, murine macrophages, or in transfected cell lines \cite{127, 135, 179-181}. There are limited reports using either normal PBMCs, PBMCs from patients with the NOD2 mutation associated with Crohn’s disease, mixed bronchoalveolar lavage (BAL) leukocytes, and none prior to our work using human monocyte derived macrophages (MDMs) \cite{127, 220}. A recent report indicates that NOD2 and TLR pathways are non-redundant in the recognition of \textit{M.tb}, but can synergize to induce a robust pro-inflammatory response \cite{127}. In addition, one study shows that RIP2 polyubiquitination occurs during \textit{M.tb} incubation in a NOD2-dependent manner in BMMs \cite{180}. Other studies in mouse macrophages have shown that NOD2 does not have a significant role in controlling \textit{M.tb} growth during early infection \cite{179} but may have during late infection \cite{135}. A decrease in pro-inflammatory cytokine production was observed in mouse NOD2 knockout BMMs and naive murine AMs in response to \textit{M.tb} without affecting intracellular bacterial growth \cite{135, 179}. Studies using human macrophages report the same role for NOD2 in cytokine production in response to \textit{M.tb}; however in contrast, data indicate an important role for NOD2 in controlling the growth...
of *M.tb* (77). This finding is yet another example of the difference between human and mouse.

1.18 Mouse and human differences in the immune system

While the human and mouse genomes only differ in approximately 300 genes, they differ greatly on development, activation, and response to challenge in the innate and adaptive immune system (221). For example, there are differences in the bronchus-associated lymphoid tissue that is present in the mouse but not human (222). Though it is not clear the consequence, mice have more lymphocyte rich blood compared to humans who have more neutrophils present (223). In the area of microbial defense, defensins are produced by neutrophils in humans and by Paneth cells in mice. Humans produce 2 defensins from Paneth cells, compared to mice, which produce over 20 defensins from this cell type (224). NO production is easily detected in mouse cells whereas it is more difficult to measure in human macrophages. It is debated whether or not the assays used to detect NO are sensitive enough to measure the NO produced in human macrophages, but it is clear that mouse macrophages produce a great deal of this antimicrobial mediator compared to human macrophages (225). There are five structurally distinct homologues of the receptor Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing (DC-SIGN) in mice and only two found in humans (226). DC-SIGN is an adhesion, signaling, and uptake receptor that functions to recognize mannose type carbohydrates found on bacteria and fungi (227). Non-integrin FeR receptors are important links between the innate and adaptive immune response and there are numerous differences between mice
and humans. Mice lack the FcαRI receptor, which is an important IgA receptor found in humans, but they likely use other receptors for this function. FcγRIIA and FcγRIIC are two IgG receptors expressed in humans and not mice (228,229). It has also been found that depending on the mouse strain there are differences in the expression of IgG isotypes as well as class switching. For example IL-4 in humans induces class switching of IgG4 and IgE, whereas in mice it induces switching to IgG1 and IgE (230). A few other significant differences to be noted between mice and humans are in B cell development, development and regulation of T cells, and IFN-α signaling (231-233). Finally, mice and humans differ in immunity-related guanosine triphosphatases (IRGs), which are important in innate immunity against pathogens and play a role in autophagy. There are 23 identified IRG genes in mice and only 3 in humans (234,235). The enhanced number of IRG genes in mice could potentially lead to different functional outcomes in mice when compared to humans.

1.19 Summary

The mammalian immune system, adaptive and innate, is uniquely designed to recognize and respond to invading pathogens. The innate immune system plays an important role in this function through the deployment of phagocytes that have extracellular and intracellular PRRs.

One bacterial pathogen in particular that has found mechanisms to evade the innate immune system is *M.tb*. The necessity to find better methods to control this pathogen is
at an all time high due to the emergence of MDR- and XDR-TB strains. In order to do this, the mechanisms for how this bacterium is recognized inside of the macrophage is important to understand because it will allow for the development of new ideas for the generation of novel microbe- and host-directed therapeutic approaches. The intracellular PRR, NOD2, has emerged as an important innate immune player in the recognition and control of pathogens in the host and data suggest that NOD2 may be important in the pathogenesis of \( M.tuberculosis \).

\[1.20\text{ Specific aims}\]

It is important to study the interaction between \( M.tb \) and the immune system in order to understand its pathogenesis and find new potential drug targets. Studying \( M.tb \)’s interaction with the first major innate immune cell it comes in contact with in the alveolus, the macrophage, is of particular relevance. The interaction of \( M.tb \) and membrane receptors has been well studied; however, \( M.tb \)’s interaction with cytosolic receptors has yet to be thoroughly studied in human macrophages. We hypothesize that NOD2 is important in controlling the growth of \( M.tb \) in human macrophages by regulating the nature of the inflammatory response in order to contain infection. We also hypothesize that NOD2 plays a role in several novel early innate immune responses in human macrophages and focus on its role in iNOS expression and PKC\( \delta \) activation. The specific aims of this dissertation are as follows:

1. Characterize NOD2 expression in human macrophages and determine the role of NOD2 during \( M.tb \) infection of human macrophages.
(2) Determine if NOD2 is necessary for iNOS expression in human macrophages in response to agonists as well as *M.tb*

(3) Determine NOD2 signaling partners in the innate immune response, specifically the function of NOD2 in the novel PKC signaling cascade
CHAPTER 2: NOD2 controls the nature of the inflammatory response and subsequent fate of *Mycobacterium tuberculosis* and *M. bovis* BCG in human macrophages

### 2.1 Introduction

Tuberculosis (TB) is currently the leading cause of death in HIV-infected persons (236). Multi-drug resistant *Mycobacterium tuberculosis* (*M.tb*) strains have limited our ability to use the current antibiotic supply, and the development of new antibiotics requires a better understanding of the biological mechanisms underlying *M.tb* growth and survival within the host. Humans are the only known natural host for *M.tb* and alveolar macrophages (AMs) in the lung microenvironment are the first immune cells that encounter inhaled mycobacteria (237). Thus, knowledge about pathogenic events for *M.tb* in primary human macrophages is particularly important.

Macrophages are equipped with an array of defense mechanisms to recognize and clear invading pathogens. Several studies demonstrate that *M.tb* interacts with macrophage membrane receptors such as the mannose receptor (MR), complement receptors, Toll-like receptors (TLR) and scavenger receptors (237). Engagement of specific receptor
pathways during phagocytosis of mycobacteria regulates the early host response through defined signaling pathways (158,175,238). Among these are the group of cytosolic receptors called Nucleotide-binding Oligomerization Domain (NOD)-like receptors (NLRs) (93) which function as intracellular pattern recognition receptors (PRRs) that recognize pathogens or pathogen secreted molecules (239).

There are 23 NLRs which can be divided into four subfamilies depending on the composition of their N-terminal effector domain. For example, those containing a caspase recruitment domain (CARD) as their effector domain are known as the NLRC subfamily and consist of proteins that initiate a pro-inflammatory response by signaling through their CARD domain. NOD2, a member of this subfamily, is found in epithelial cells and antigen presenting cells such as macrophages (76,240,241). NOD2 regulates the production of inflammatory mediators in response to muramyl dipeptide (MDP) which is found in Gram-negative and Gram-positive bacteria, and mycobacteria (86,88). Many studies have found that NOD2 is able to recognize and generate a response to a variety of microorganisms (97,126,127,136,242-248). NOD2 senses bacteria through its leucine rich repeats (LRR), then oligomerizes through its NOD domain and is able to recruit receptor interacting protein 2 (RIP2) through an electrostatic interaction of the CARD domain in each protein. This leads to a signaling cascade in which activation and translocation of nuclear factor-κB (NF-κB) into the nucleus occurs in order to transcribe pro-inflammatory cytokines (108,147,241,249,250).
As reported earlier in the introduction section (See: 1.17 M.tb and NOD2), the literature indicates that during mycobacterial infections NOD2 is an important player in the host response. Two genomic analysis studies revealed an association of NOD2 with susceptibility to mycobacterial infections. Patients with *Mycobacterium leprae* or African American patients with TB had polymorphisms or variants in their NOD2 gene (216,217). Patients with Crohn’s disease, an inflammatory granulomatous disease, are reported to have polymorphisms in their NOD2 gene (*CARD15*) (138,218). While debated, it is reported that the cause of Crohn’s disease is *Mycobacterium avium subspecies paratuberculosis* (144).

The role of NOD2 in the host response to *M.tb* infection has mainly been studied in mice, murine macrophages, or transfected cell lines, with limited reports using PBMCs from healthy donors, PBMCs from patients with mutations in their NOD2 gene, and mixed BAL leukocytes (127,127,135,179-181,220). NOD2 and TLR pathways are non-redundant in the recognition of *M.tb*, but can synergize to induce a robust pro-inflammatory response (127). In addition, RIP2 polyubiquitination occurs during *M.tb* incubation with BMMs in a NOD2 dependent manner (180). While a decrease in pro-inflammatory cytokine production in NOD2 knockout BMMs and naïve murine AMs in response to *M.tb* was found, NOD2 did not control intracellular bacterial growth during early in vivo infection in mice (179) but did during late infection (135).
Interest in the potential interactions between *M.tb* and cytosolic PRRs such as NOD2 continues to grow based on recent reports that *M.tb* secretes virulence factors and immune activators or suppressors through a type VII secretion system (191,251,252). Other reports provide evidence that *M.tb* may be able to escape the phagosome to the cytosol under specific conditions (213,215).

Based on the known differences between human and mouse macrophages in receptor expression, signaling pathways, and innate immune function (253-256), we decided to study NOD2 in human macrophages and in response to *M.tb* by optimizing an antibody to NOD2 and by accomplishing effective small interfering RNA (siRNA)-mediated knockdown of NOD2 in these cells. We show that NOD2 expression increases during monocyte differentiation. In contrast to previously published work using mouse macrophages, we report that NOD2 is important in controlling both pro-inflammatory cytokine production and the growth of *M.tb* in human macrophages; whereas NOD2 controls the growth of the related attenuated BCG strain in both human and mouse macrophages.

### 2.2 Materials and Methods

#### 2.2.1 Buffers and reagents

Dulbecco’s PBS with and without Ca\(^{2+}\) and Mg\(^{2+}\) ions, Iscoves, FBS, TRIzol, and RPMI 1640 medium with L-glutamine were purchased from Invitrogen (Invitrogen Life
Technologies). RHH medium [RPMI 1640 plus 10 mM HEPES (Invitrogen) plus 0.4% human serum albumin (CSL Behring LLC)] was used for cell culture experiments. N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP; Sigma-Aldrich) and H-Ala-D-γ-Glu-diaminopimelic acid (iE-DAP; AnaSpec) were used for functional assays involving ELISAs. 7H11 agar was prepared with Bacto Middlebrook 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol (Difco Laboratories).

2.2.2 Antibodies

Unconjugated rabbit anti-human NOD2 was purchased from ProSci Inc. and another anti-human NOD2 antibody was obtained from Dr. Nunez, University of Michigan, and available by ebioscience. Syk (N-19), TLR2, β actin, goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology. The aforementioned antibodies were used for Western blot. The NOD2 antibody from ProSci Inc., Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes and Invitrogen) and normal rabbit IgG (Santa Cruz Biotechnology) were used for confocal microscopy experiments. Monoclonal anti-human NOD2 (Cayman Chemical) and anti-mouse IgG1 kappa (Abcam) were used for IP experiments. Antibodies specific for phospho-RIP2 (Ser176) and phospho-IκB (Ser32) were purchased from Cell Signaling Technology. The caspase-1 antibody was a kind gift from Dr. Mark Wewers, Ohio State University.
2.2.3 Bacterial strains and culture

Lyophilized M.tb H37Rv (ATCC #25618) and BCG (ATCC #35734) were obtained from American Tissue Culture Collection, reconstituted, and used as described (172). The concentration of bacteria (1–2 x 10^8 bacteria /mL) and the degree of clumping (≥10%) were determined by counting in a Petroff-Hausser chamber. Bacteria prepared in this fashion are ≥90% viable by CFU assay.

2.2.4 Isolation and culture of human monocytes and macrophages

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using an approved protocol by The Ohio State University Institutional Review Board (IRB) was processed as described (257). Briefly, PBMCs were isolated from heparinized blood on a Ficoll-Paque cushion (Amersham Biosciences/GE Healthcare) and cultured in Teflon wells (Savillex) for 1 (monocytes) through 5 (MDMs) days in the presence of RPMI 1640 medium containing 20% autologous serum (2.0 x10^6 PBMCs/mL) at 37ºC/5% CO₂ (258). Human AMs were isolated from BALs of healthy human donors as previously described (161) using an approved protocol by The Ohio State University IRB. Briefly, the BAL was centrifuged (200 x g, 4°C, 10 min), supernatant was removed, and the pellet re-suspended in RPMI and washed two more times with RPMI. AMs were plated in tissue culture plates with 10% human serum supplemented with penicillin (1000U/mL), incubated in CO₂ incubator at 37°C for 2 h, washed with warm RPMI to remove non-adherent cells and penicillin, and used for experiments.
2.2.5 Mice and mouse macrophages

Wild-type C57BL/6 and NOD2−/− mice were backcrossed to C57BL/6 background for 10 generations (242). BMMs were prepared from femurs of five to eight week-old mice as previously described (137,259).

2.2.6 RNA isolation

One to 5 day-old PBMCs in Teflon wells were harvested and mononuclear phagocytes adhered to a 6-well tissue culture plate with 10% autologous serum (8 x 10^6 PBMCs/mL). After washing away lymphocytes, the monocytes or MDMs (8 x 10^5 cells) were lysed in TRIzol and total RNA was isolated by using the Qiagen RNAeasy column method. The Experion (Bio-Rad) was used to determine the quality and quantity of RNA samples.

2.2.7 Gene expression studies by qRT-PCR

RNA (100ng) was reverse transcribed to cDNA by reverse transcriptase enzyme, (SuperScript II, Invitrogen) and qRT-PCR was performed using a human NOD2 or IL-1β TaqMan gene expression kit (Applied Biosystems). NOD2 and IL-1β amplifications were normalized to β actin as a housekeeping gene (ΔCt). Relative copy number (RCN) and fold change were determined. RCN was calculated as follows: \( \text{RCN} = E^{-\Delta Ct} \times 100 \), where \( E \) is the efficiency (2 = 100% efficiency) and \( C_t = C_t \text{ (target)} - C_t \text{ (reference)} \) (260). Fold change was calculated from the RCN values compared to day 1 for NOD2

48
mRNA experiments, and compared to the siRNA resting group for IL-1β mRNA experiments. Triplicate samples were analyzed in duplicate wells in each experiment.

2.2.8 Confocal microscopy

Untransfected MDMs or transfected (scramble siRNA or NOD2 siRNA) MDMs (2 x 10^5) were adhered to a glass coverslip in a 24-well tissue culture plate for 2 h at 37°C and washed to remove lymphocytes. The cells were washed, fixed with 2% paraformaldehyde (10 min at room temperature), permeabilized by treatment with 100% methanol for 4 min, washed and then incubated overnight in 10% non-fat milk in PBS as blocking reagent. The cells were then incubated with NOD2 (2.5µg/mL) or the appropriate isotype (2.5µg/mL) control antibody for 1 h at room temperature, washed with blocking reagent, and counterstained with an Alexa Fluor 488-conjugated secondary antibody (1:500 dilution) for 1 h at room temperature. Nuclei were labeled with 0.1µg/mL of the DNA stain 4,6-diamidino-2-phenylindole (DAPI; Invitrogen/Molecular Probes) in PBS for 5 min at room temperature. The cells were next washed with PBS and coverslips were mounted on glass slides. Slides were viewed using an Olympus Flowview 1000 Laser Scanning Confocal microscope.

2.2.9 Transfection of MDMs

PBMCs were transfected with Accell scramble siRNA or NOD2 siRNA purchased from Thermo Scientific Dharnacon RNAi technologies that targeted the sequence
CUUUAGGAUGUACAGUUA (368nM) using the Amaxa Nucleofector (Lonza Group Ltd). Briefly, PBMCs (1× 10⁷) were resuspended in 100 μl of nucleofector solution followed by the addition of 5ug/mL of scramble siRNA or NOD2 siRNA, incubated at room temperature for 5 min, and nucleofected according to the manufacturer’s instructions. PBMCs were then seeded on 12-well plates containing 1.0 ml of RPMI supplemented with 10% autologous serum and incubated for 2 h at 37°C and 5% CO₂. After 2 h, adhered transfected cells (MDMs) were washed and repleted with warm RPMI containing 20% autologous serum for 72 h. Transfected cells were used for subsequent experiments, such as CFU assays, cytokine assays, confocal microscopy, cell enumeration assays, and Western blotting. Assessment of monolayer density in experiments was performed as described below.

2.2.10 Macrophage stimulation / infection, cell lysis, immunoprecipitation, and western blotting

Day 5 MDMs or MDMs transfected with scramble siRNA or NOD2 siRNA (2.0 x 10⁵) were incubated with M.tb or BCG (MOI 5:1; triplicate wells) in RHH for 30 min at 37°C in 5% CO₂ on a platform shaker for equal dispersion of bacteria followed by an additional incubation for 90 min without shaking. The cells were then washed three times with warm RPMI, repleted with RPMI containing 1% human autologous serum and incubated for 24 h at which time supernatants were collected for ELISAs (see below). For experiments using the NOD2 ligand MDP, 2.0 x 10⁵ day 5 MDMs were adhered to a 24-well plate for 2 h at 37°C in 5% CO₂, washed to remove lymphocytes, and then incubated
with MDP (5µg/mL) for 90 min. Cells were then incubated with bacteria as described above (2 h total), washed and repleted with MDP in 1% autologous serum for 24 h. Cell culture supernatants were harvested and used for ELISAs. The monolayers were washed once with PBS and lysed in TN-1 lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM Na₄PO₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, 10 µg/ml aprotinin, and 10 µg/ml leupeptin], incubated on ice for 10 min and then centrifuged at 17,949 x g for 10 min at 4°C to remove cell debris (261). For infections, MDM monolayers were incubated with M.tb or BCG in RHH for 15 min, 30 min, 1 h, 1.5 h, 2 h, and 3 h (MOI 5:1). At each time point, cells were washed with PBS and lysed as above. For NOD2 knockdown assessment, lysates were collected as above at 72 h and 96 h post-knockdown. Protein concentrations of the cleared lysates were measured using the BCA-protein assay kit (Pierce). Proteins were separated by SDS-PAGE and analyzed by Western blot by probing with the primary and secondary antibody of interest and development using ECL (Amersham Biosciences). For the IP experiment whole cell lysates were collected from HEK293T (empty vector) and NOD2-expressing HEK293T cells. Equal amounts of protein were incubated overnight with NOD2 antibody (Cayman Chemicals) or control IgG1, kappa. Then cells were incubated for 2 h with Protein G agarose beads (Invitrogen), washed, pellets boiled in Laemmli buffer and loaded on a 7.5% SDS-PAGE gel, and Western blot was performed using a NOD2 antibody (ProSci Inc.). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then
normalized to β-4actin. Fold change was determined as follows: (treated sample band intensity) / (untreated sample band intensity).

2.2.11 ELISAs

For MDM experiments, cell free culture supernatants were collected at 24 h and used to measure TNF-α and IL-1β by ELISA (R&D Systems). For mouse macrophage studies, WT and NOD2−/− macrophage monolayers were incubated with serum-opsonized mycobacteria (M. tb MOI 1:1 and BCG MOI 2.5:1) in the same manner as described for MDMs. Cell free culture supernatants were collected at 24 h, 72 h, and 168 h and measured for IL-1β by ELISA (BD Biosciences).

2.2.12 M. tb and BCG intracellular growth assays in macrophages

A CFU assay using infected macrophage lysates was performed as described (262). Briefly, scramble siRNA or NOD2 siRNA transfected MDM monolayers were incubated with M. tb or BCG (MOI 5:1; triplicate wells) in RHH for 30 min at 37°C in 5% CO₂ on a platform shaker for equal dispersion of bacteria and for an additional 90 min stationary. The cells were then washed three times with warm RPMI and either lysed or repleted with RPMI containing 1% human autologous serum and incubated for 2 h or 24 h. The supernatants and MDM monolayers were then processed for ELISA or enumeration of CFUs, respectively (triplicate samples in each test group). The number of colonies was counted after 4 weeks and data analyzed. For mouse macrophage experiments, WT and
NOD2−/− macrophages were incubated with serum-opsonized mycobacteria (M.tb MOI 1:1 and BCG MOI 2.5:1) in Iscoves plus 10% FBS using the same incubation protocol as for MDMs and then lysed at 2 h, 24 h, 72 h, and 168 h.

2.2.13 M.tb and BCG cell association assay within macrophages

Scramble siRNA or NOD2 siRNA transfected MDM monolayers (2.0 x 10⁵), or WT or NOD2−/− mouse macrophages monolayers (2.5 x 10⁵) were incubated with M.tb or BCG (MOI 10:1; triplicate wells) in RHH for 30 min at 37°C in 5% CO₂ on a platform shaker for equal dispersion of bacteria and then for an additional 90 min without shaking. The cells were then washed three times with warm RPMI and fixed with 10% formalin for 10 min at room temperature. Coverslips were then washed three times with PBS, dried and cell-associated M.tb or BCG was stained with auramine-rhodamine (BD Biosciences). Three hundred consecutive macrophages/coverslip/test group were counted using phase-contrast and fluorescence microscopy (166).

2.2.14 Cell enumeration of NOD2 siRNA transfected MDMs in monolayer culture

Scramble siRNA and NOD2 siRNA transfected MDMs were plated in 24 well plates and after 2 h, washed and repleted with 20% autologous serum for 72 h. Cells were then washed one time with PBS and treated with 1% Cetavlon in 0.1M citric Acid with 0.05% Napthol blue black (Sigma-Aldrich), pH 2.2, for 15 min at room temperature. Cell
lysates were then loaded on a hemacytometer and stained nuclei enumerated using phase contrast microscopy (263).

2.2.15 NOD2 antibody peptide competition assay

The NOD2 antibody and the NOD2 peptide (ProSci Inc.) used to produce the antibody were incubated in a ratio of 2:1 (peptide:antibody) at 37°C for 30 min in a 5% milk solution in low retention tubes. Day 5 MDM lysates were run in parallel on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and the membrane cut so that each lane could be probed with either the antibody-peptide mixture or antibody alone overnight at 4°C. Western blotting was performed as described above. Membranes were then developed using ECL.

2.2.16 Statistics

A paired one tailed student’s t test was used to analyze the differences between two groups in figures showing one representative figure of three independent experiments. An unpaired one tailed student’s t test was used to analyze differences between two groups in figures showing cumulative data from three independent experiments. Significance was P < 0.05.
2.3 Results

2.3.1 NOD2 mRNA and protein expression increase as monocytes differentiate into macrophages

The expression of NOD2 in human macrophages has not been studied and there are few reports on NOD2 mRNA expression in human monocytes (76,220). We began our studies by determining the expression of NOD2 during monocyte differentiation to MDMs in an established model by quantitative real-time PCR (qRT-PCR) and Western blot (54). Results from qRT-PCR provide evidence that NOD2 transcript levels are increased during the differentiation of monocytes to macrophages. While we saw production of mRNA in day 1 monocytes, the results in Fig. 2.1A show a significant increase in mRNA at day 3 of differentiation which remains elevated as the cells differentiate further into mature macrophages. Next, we compared the mRNA levels with the amount of protein produced during cell differentiation by Western blotting. Due to the sensitivity and specificity issues in using antibodies specific for human NOD2, we optimized an available commercial antibody (ProSci Inc.) with human macrophage lysates. The specificity of this antibody was confirmed by a peptide competition assay (Figure 2.1B), an immunoprecipitation (IP) experiment (Figure 2.1C), and in siRNA experiments shown later. For IP experiments, proteins in cell lysates from HEK293T cells expressing human NOD2 or HEK293T cells containing the empty vector were immunoprecipitated with NOD2 antibody (Cayman Chemical) and then probed with NOD2 antibody from ProSci Inc. As shown in Figure 2.1C, NOD2 antibody specifically binds to the human NOD2 protein expressed in the HEK293 cell line. We were also able
to detect NOD2 in human lysates using the antibody available from eBioscience, although with more background (data not shown). Thus, in all subsequent experiments we used the antibody from ProSci Inc. In accordance with the mRNA studies, NOD2 protein levels in cell lysates increased markedly at day 3 of differentiation and remained high on day 5 MDMs (Figure 2.1D upper panel). The densitometric analysis of Western blots from three independent experiments shows that NOD2 protein expression levels increased during cell differentiation (Figure 2.1D lower panel). Finally, our results were confirmed using confocal microscopy where NOD2 protein was easily detected throughout MDMs in a punctuate appearance (Figure 2.1E).
PBMCs were harvested from Teflon wells each day from day 1 through day 5 and monocytes/MDMs plated on tissue culture plates, and incubated at 37°C / 5% CO2 for 2 h. The resultant monocyte/MDM monolayers were either lysed with TRIZol for extraction of total RNA or TN-1 lysis buffer for cell lysate preparation. (A) NOD2 steady-state mRNA levels were measured during monocyte to macrophage differentiation by real time PCR. Data were normalized to the β actin gene and RCN was determined. Shown are cumulative data obtained from three independent experiments each performed in triplicate (mean ± SEM, *P < 0.05). (B) NOD2 antibody specificity was confirmed by peptide blocking using 2-fold excess NOD2 peptide (used to generate the NOD2 antibody) and Western blotting using anti-NOD2 antibody. Shown is a representative blot from two independent experiments. (C) HEK293T vector (lane 1) and HEK293T cells expressing NOD2 (lane 2) were lysed and 100 µg of total protein in each case was immunoprecipitated with NOD2 antibody (Cayman Chemicals) or isotype control (IgG1 kappa) and subsequent Western blots of the immunoprecipitants probed.
with NOD2 antibody (ProSci Inc.). Shown is a representative Western blot from two independent experiments. (D) Monocyte/macrophage cell lysates were analyzed for NOD2 protein expression by Western blot using NOD2 antibody. The same membrane was re-probed with β actin to verify equal loading of lysate proteins. Shown is a representative Western blot and the bar graph shows the cumulative densitometric analysis of Western blots from three different donors (mean ± SEM, *P < 0.05 ** P < 0.005). (E) Day 5 MDM monolayers on coverslips were fixed, permeabilized, and stained using either NOD2 antibody or isotype control antibody followed by Alexa flour 488 conjugated secondary antibody. Cells were then examined by confocal microscopy (Olympus FV1000). Shown is a representative section (63x mag) of a Z series image from one of three independent experiments performed on duplicate coverslips.

2.3.2 Activation of NOD2 enhances the *M.tb*- and BCG-mediated pro-inflammatory cytokine response through the NF-κB pathway

We next investigated whether activation of NOD2 leads to an increase in the *M.tb*- and BCG-mediated pro-inflammatory cytokine response in macrophages. Incubation of macrophages with MDP, a NOD2 agonist, is known to induce the production of pro-inflammatory cytokines such as IL-1β (122). A recent report indicates that in some TB patients, NOD2 mRNA is enhanced in PBMCs; furthermore, patients on anti-tuberculosis treatment have enhanced NOD2 levels in leukocytes (220). MDMs were pre-incubated with the NOD2 ligand MDP for 90 min followed by incubation with *M.tb* or BCG as described in the methods in the presence of MDP. Cell free culture supernatants were analyzed for TNF-α and IL-1β secretion at 24 h. The data show an additive (TNF-α) or synergistic (IL-1β) effect on cytokine production in cells pre-treated with MDP compared to cells incubated with mycobacteria alone (Figure 2.2A and B). Thus, our results indicate that pre-engagement of NOD2 by its ligand significantly enhances *M.tb*- or
BCG-mediated pro-inflammatory cytokine production. This effect is likely due to the activation of NF-κB through the phosphorylation of IκBα and its subsequent degradation (180,264).

To further explore the signaling pathway(s) involved, we examined whether \textit{M.tb}- or BCG-mediated signaling events include those known for the NOD2 pathway. NOD2 engagement leads to recruitment of a serine threonine kinase adapter protein called RIP2 by an electrostatic interaction through their CARD domains, which in turn causes autophosphorylation of RIP2 at serine 176 (94) in cell lines and murine macrophages. Activated RIP2 phosphorylates inhibitor of kB (IκBα) resulting in the activation and translocation of NF-κB. MDMs were incubated with \textit{M.tb} or BCG for different time points and the cell lysates were analyzed for activation of RIP2 and IκBα by Western blotting of the phosphorylated proteins. The data show that both \textit{M.tb} and BCG phosphorylate RIP2 during incubation but with different kinetics and pattern. BCG- and \textit{M.tb}-mediated phosphorylation of RIP2 peaks at 30 min and 1 h, respectively, and the phosphorylation decreased at 3 h during \textit{M.tb} incubation (Figure 2.2C). \textit{M.tb} and BCG infection also leads to phosphorylation of serine 32 of IκBα (Figure 2.2D) which is known to result in its degradation and subsequent activation of NF-κB. We found that phosphorylation occurs as early as 15 min for both mycobacteria and continues through 2 h for BCG but not \textit{M.tb}. In addition, there is a decrease at 30 min which can be explained by the degradation of IκBα and recruitment of newly re-synthesized pools of IκBα from the cytoplasm, as previously described (264).
Figure 2.2: The NOD2 ligand MDP enhances *M. tb*- and BCG-mediated cytokine production in human macrophages, a process involving activation of RIP2 and IkBα.

MDM monolayers in a 24 well tissue culture plates were pre-incubated with or without MDP (5μg/mL) for 90 min and then incubated with *M. tb* or BCG (MOI 5:1) for 2 h, washed 3 times and repleted with MDP (5μg/mL) in RPMI with 1% autologous serum for 24 h. Supernatants were collected and the amounts of TNF-α (A) and IL-1β (B) were measured by ELISA. Shown is the mean ± SD of a representative experiment performed in triplicate (n = 5, * P < 0.05). In parallel, MDMs were incubated with *M. tb* or BCG (MOI 5:1) for different time points and cells were lysed with TN-1 lysis buffer for RIP2 (C) and IkBα (D) phosphorylation, and re-probed with β actin as a loading control. Shown is a representative Western blot from three independent experiments.
2.3.3 siRNA-mediated knockdown of NOD2 activity in human macrophages leads to loss of its activity

In order to more definitively examine the causal role of NOD2 in controlling the nature of the inflammatory response and growth of mycobacteria in human macrophages, we developed an effective and reliable protocol for knockdown of NOD2 in human macrophages by using NOD2-specific siRNA and Amaxa nucleofector reagent. MDMs were transfected with scramble siRNA or NOD2-specific siRNA under optimized conditions, plated in tissue culture plates, and lysed after 72 h and 96 h. The cell lysates were analyzed for NOD2 protein levels by Western blotting. Results shown in Figure 2.3A demonstrate the loss of NOD2 protein in MDMs transfected with NOD2-specific siRNA at both time points. These results were confirmed by confocal microscopy (Figure 2.3B). Cell enumeration of scramble siRNA- and NOD2 siRNA-treated MDMs evaluated at 72 h post nucleofection by naphthol blue black nuclear staining of cells demonstrated no loss of cells on the monolayer in either experimental group (Figure 2.3C). There was also no difference in monolayer cell viability as seen by trypan blue exclusion staining (95% viable in both groups, data not shown). An assay was performed in order to verify that NOD2 protein knockdown led to loss of its function. Scramble siRNA and NOD2 siRNA transfected macrophages were treated with MDP and the cell free culture supernatants were analyzed for TNF-α production (Figure 2.3D). NOD2 knockdown led to a significant reduction in TNF-α production in response to MDP. We also tested the specificity of NOD2 knockdown by stimulating the NOD2 siRNA transfected MDMs with the NOD1 ligand H-Ala-D-γ-Glu-diaminopimelic acid (iE-DAP)
and measured TNF-α levels in the supernatant. There was no significant change in NOD1 mediated TNF-α production between scramble siRNA and NOD2 siRNA transfected cells providing evidence that NOD2 knockdown by siRNA did not affect the activity of NOD1, another NLR family member (Figure 2.3D). We also found that expression of the effector proteins TLR2, Syk and total RIP2 is unaffected in scramble siRNA and NOD2 siRNA transfected MDMs during *M.tb* and BCG infection (Figure 2.3E).

Figure 2.3: Loss of NOD2 activity in human macrophages by siRNA knockdown.

Continued on next page
MDMs were transfected with either 368nM of scramble siRNA or NOD2 siRNA using the Amaxa nucleofector. (A) The transfected macrophages were plated in RPMI containing 20% autologous serum and incubated for the indicated time points (72 h and 96 h). At each time point macrophages were lysed and NOD2 or the loading control β-actin protein levels were examined by Western blot (representative blot from n = 3). (B) NOD2 or scramble siRNA transfected MDM monolayers on coverslips were incubated for 72 h, washed, fixed, permeabilized, and stained using either NOD2 antibody or isotype control antibody followed by Alexa flour 488 conjugated secondary antibody. Cells were then examined by confocal microscopy. Shown is a representative micrograph from the Z series (63x mag for isotype; 126x mag for scramble/NOD2 siRNA, n = 3). (C) Cell numbers were assessed 72 h post transfection to ensure equal numbers of cells between scramble siRNA and NOD2 siRNA transfected cells. Nuclei in cell lysates were counted on a hemacytometer after nuclei were stained with Naphthol blue black. Shown are cumulative data obtained from three independent experiments each performed in triplicate (mean ± SEM) (D) Scramble siRNA or NOD2 siRNA transfected MDM monolayers in culture for 72 h were treated with MDP (5μg/mL) or DAP (10μg/mL) for 24 h. Cell supernatants were analyzed for TNF-α by ELISA. Shown is a representative experiment performed in triplicate (mean ± SD, n =2, ** P < 0.005; N.S. P = 0.1519). (E) Scramble siRNA or NOD2 siRNA transfected MDM monolayers were incubated with M.tb or BCG (MOI 5:1) for the indicated time points (15 min and 2 h) and cells were lysed with TN-1 buffer. TLR2, Syk, and RIP2 were analyzed by Western blot. Shown is a representative experiment of two independent experiments.

2.3.4 NOD2 regulates the macrophage TNF-α and IL-1β cytokine response to M.tb and BCG

We next investigated whether NOD2 regulates the production of pro-inflammatory cytokines during mycobacterial infection which can control the host defense response through autocrine and paracrine functions (265). Results shown in Figure 2 had already indicated a potential link between NOD2 and the cytokine response to mycobacteria. Scramble and NOD2 siRNA transfected MDM monolayers were incubated for 72 h to achieve the maximum NOD2 knockdown and subsequently incubated with M.tb or BCG
(MOI 5:1) for 24 h. The cell free culture supernatants were analyzed for cytokine levels by ELISA. We found that NOD2 deficiency leads to a significant decrease in the production of TNF-α and IL-1β in response to \textit{M.tb} and BCG (Figure 2.4 A and B). Next, we examined RIP2 activation in NOD2 knockdown MDMs in response to \textit{M.tb} and BCG infection. NOD2 deficient MDM monolayers were incubated with \textit{M.tb} or BCG for short time periods and cell lysates were analyzed for RIP2 phosphorylation by Western blot. Our results show that NOD2 siRNA transfected MDMs have a partial but reproducible decrease in RIP2 phosphorylation (Figure 2.4C: upper panel, Western blot; lower panel, band densitometry), particularly at 2 h, compared to scramble siRNA transfected MDMs in the case of both \textit{M.tb} and BCG. The partial reduction of RIP2 phosphorylation may be explained by the fact that the mycobacteria also produce NOD1 agonists that activate RIP2 independently of NOD2. The smaller reduction of RIP2 phosphorylation in NOD2 knockdown cells incubated with \textit{M.tb} compared to BCG may relate to the amount of the NOD1 agonist DAP in the former (266).

Next we elected to study whether \textit{M.tb} and BCG regulate IL-1β at the transcriptional level or through caspase-1, the enzyme necessary for cleavage of pro IL-1β to the secreted form. During BCG and \textit{M.tb} infection, NOD2 knockdown macrophages demonstrate either no effect or an increase in pro IL-1β transcription (Figure 2.4D). On the other hand, since NOD2 knockdown leads to a significant reduction in active (cleaved) caspase-1, NOD2 does appear to play a role in caspase-1 activation during \textit{M.tb} infection (Figure 2.4E). Similar results were found for caspase-1 using TCA.
precipitation of infected supernatants (data not shown). Interestingly, there was a lack of caspase-1 activation during BCG infection. However, this represents the results from only one experiment and thus further conclusions cannot be reached.

Figure 2.4: NOD2 controls *M. tb*- and BCG-mediated cytokine production and RIP2 activation in human macrophages.

Continued on next page
Scramble or NOD2 siRNA transfected MDM monolayers were incubated with *M.tb* or BCG (MOI 5:1) for 2 h, washed 3 times with warm RPMI, and repleted in 1% autologous serum for 24 h. Cell culture supernatants were analyzed for TNF-α (A) and IL-1β (B) production by ELISA. The graphs shown are from a representative experiment of *n* = 3. *P < 0.05 or **P < 0.005. (C) Scramble or NOD2 transfected MDM monolayers were incubated with *M.tb* or BCG (MOI 5:1) for the indicated time points (15 min and 2 h) and cells were lysed with TN-1 buffer. NOD2-dependent RIP2 phosphorylation was analyzed by Western blot of cell lysates from scramble and NOD2 deficient MDMs (upper panel). Shown is a representative experiment of three independent experiments. Densitometry of the band intensities in this experiment is shown as a bar graph (lower panel). (D) IL-1β steady-state mRNA levels were measured during BCG and *M.tb* infection by real time PCR. Data were normalized to the β actin gene and RCN was determined. Shown is a representative experiment from two independent experiments each performed in triplicate. (E) Macrophage cell lysates were analyzed for caspase-1 protein expression by Western blot using caspase-1 antibody. The same membrane was re-probed with β actin to verify equal loading of lysate proteins. Shown is a representative Western blot, and the bar graph below shows the densitometric analysis during *M.tb* infection (*M.tb* n = 2; BCG n = 1).

2.3.5 Expression of NOD2 in human AMs and its role in regulating the production of TNF-α and IL-1β in response to *M.tb*

A recent report showed the presence of NOD2 transcripts in human alveolar lavage cells, but to date there is no evidence for the presence of NOD2 protein in AMs (220). Therefore we examined whether NOD2 protein is expressed in human AMs. Cell lysates were prepared from MDMs and AMs, the latter were isolated from healthy donors and both cell types were analyzed for NOD2 expression by Western blot. Results shown in Figure 2.5A demonstrate that AMs express a detectable amount of NOD2 protein but the level is less than that in an equivalent number of MDMs. To further examine the potential involvement of NOD2 in the production of inflammatory cytokines in response
to *M. tb*, we incubated AMs with or without MDP and then with *M. tb* (MOI 1:1) for 24 h. The cell culture supernatants were analyzed for TNF-α and IL-1β production. We found that MDP or *M. tb* treatment induces the production of TNF-α whereas only *M. tb* induces the production of IL-1β, and that the levels of both cytokines were further enhanced by the addition of both MDP and *M. tb* (Figure 2.5 B and C). The magnitude of the responses was relatively low when compared with the response seen with MDMs (Figure 2.2). Together, these results provide evidence that AMs express functional NOD2 protein in the lung microenvironment, albeit with lower activity than in MDMs.

![Figure 2.5: *M. tb*-mediated cytokine production in human AMs treated with MDP.](image)

(A) The NOD2 expression level in healthy donor AMs was compared with day 5 MDMs using protein-matched cell lysates by Western blot with a NOD2-specific antibody and β actin as a loading control. Shown is a representative blot of two independent experiments. AMs were pre-incubated with MDP (5μg/mL) or media alone for 90 min and then incubated with *M. tb* (MOI 1:1) for 2 h, washed 3 times and repleted with MDP in 1% autologous serum for 24 h. Culture supernatants were measured for TNF-α (B) and IL-1β (C) production by ELISA. Shown is a representative experiment of the mean ± SD of triplicate wells in each test group (*n* = 2 * P < 0.05).
2.3.6 NOD2 controls the intracellular growth of *M.tb* and BCG in human macrophages

We next focused our studies on determining the role of NOD2 in controlling the growth of mycobacteria in human macrophages. MDMs were transfected with scramble or NOD2 siRNA and after 72 h of transfection, macrophages were incubated with *M.tb* or BCG (MOI 5:1) for 24 h. Experiments were performed for 24 h due to the fact that NOD2 protein levels in macrophages began to reappear after 120 h of knockdown. Intracellular growth of *M.tb* and BCG was determined by a colony forming unit (CFU) assay (262). The results in Figure 2.6A show that silencing of NOD2 in macrophages significantly enhances the growth of *M.tb* (84.6 ± 8.0%, *n* = 3). A similar result was observed for BCG growth in Figure 2.6B (108.4 ± 30.4%, *n* = 5). Western blotting confirmed NOD2 knockdown in each experiment (Figure 2.6C). We next determined whether the increase in mycobacterial growth at 24 h was the result of increased cell association (both bacterial attachment and phagocytosis). We compared the association of bacteria with scramble and NOD2 siRNA transfected MDMs by phase contrast and fluorescence microscopy after 2 h infection. Our results show that bacterial association is not altered by NOD2 siRNA transfection (Figure 2.6D and E). Thus, these results indicate that NOD2 plays a role in regulating the early host defense response during infection of both *M.tb* and BCG.
Figure 2.6: NOD2 controls the intracellular growth of *M. tb* and BCG in human macrophages.

(A) Scramble and NOD2 siRNA transfected MDMs in monolayer culture for 72 h were incubated with *M. tb* or BCG (MOI 5:1) for 2 h, washed 3 times with warm RPMI, repleted in 1% autologous serum for 24 h and lysed to analyze CFUs for (A) *M. tb* growth and (B) BCG growth. Shown is a representative experiment (mean ± SD of triplicate wells in each test group) for each bacterium (for *M. tb* *n* = 3 and for BCG *n* = 5; *P* < 0.05). (C) Western blot in the experiment shown confirming NOD2 knockdown. Mycobacterial association with macrophages was assessed by phase contrast and fluorescent microscopy. Scramble and NOD2 siRNA transfected MDMs were plated on coverslips for 72 h and then incubated with *M. tb* (D) or BCG (E) for 2 h (MOI 10:1). Cells were then washed, fixed in 10% formalin, mycobacteria were stained with auramine-rhodamine and enumerated as bacteria per macrophage. Cumulative data from 3 independent experiments, each performed in triplicate, are shown as fold change relative to the scramble siRNA condition (mean ± SEM).
2.3.7 Effects of NOD2 on *M. tb* and BCG growth in mouse macrophages and on the cytokine response to infection

Previous studies have reported that NOD2 is not critical for controlling the growth of *M. tb* and BCG in mouse macrophages (135,179). Based on our results in human macrophages, we elected to examine the host response to these mycobacteria in mouse macrophage experiments. We reproduced the published finding that NOD2 does not control the growth of *M. tb* in mouse macrophages using WT and NOD2<sup>−/−</sup> BMMs (Figure 2.7A). In contrast to the published work with BCG that analyzed growth in naive murine AMs at 48 h (135), our data show that NOD2 is important in controlling the growth of BCG at the later time points of 72 h and 168 h in WT and NOD2<sup>−/−</sup> BMMs (Figure 2.7B). We also examined the cell association of bacteria with WT and NOD2<sup>−/−</sup> BMMs by microscopy and found no difference with *M. tb* and only a small difference with BCG (Figure 2.7C and D). Finally, consistent with the literature, we found a reduction in the pro-inflammatory cytokine IL-1β in response to *M. tb* and BCG at 24 h, 72 h, and 168 h in NOD2<sup>−/−</sup> BMMs (Figure 2.7E).
Figure 2.7: Effect of NOD2 on *M. tb* and BCG growth and cytokine response in mouse WT and NOD2−/− BMMs.

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BMMs from WT and NOD2\textsuperscript{\textminus/\textminus} mice were plated in 24 well tissue culture plates and incubated with \textit{M.tb} (1:1) or BCG (2.5:1) for 2 h, washed 3 times, and immediately lysed or incubated for different time points (2 h, 24 h, 72 h and 168 h) prior to lysis for assessment of CFUs for \textit{M.tb} (A) and BCG (B). Shown is a representative experiment for each bacterium (mean ± variance of duplicate wells in each test group, \( n = 2 \)). Cell association of \textit{M.tb} (C) and BCG (D) with WT and NOD2\textsuperscript{\textminus/\textminus} BMMs was analyzed at 2 h post-infection by microscopy. Cumulative data from 3 independent experiments, each performed in triplicate, are shown as fold change relative to WT condition (mean ± SEM, *P<0.05). The cytokine response IL-1\textbeta (E) was analyzed by ELISA in culture supernatants harvested from \textit{M.tb} and BCG infected WT and NOD2\textsuperscript{\textminus/\textminus} BMMs. The data shown are from a representative experiment (mean ± SD, \( n = 2 \), with four test results in each group * P < 0.05; ** P < 0.005, ***P < 0.0005).

2.4 Discussion

Macrophages play a critical role in the recognition and removal of pathogens through various PRRs, including intracellular receptors such as NLR family CARD-containing domain 4 (NLRC4), Baculoviral IAP repeat-containing protein 1 (NAIP), NOD1, and NOD2. Studies on host recognition of pathogens by human macrophages have demonstrated the importance of transmembrane receptors; however the field is relatively lacking in defining the role of intracellular receptors in these cells. NOD2 is an intracellular PRR that senses bacteria through its LRR domain and induces a pro-inflammatory response. While this inflammatory response can activate the macrophage, it also allows for the recruitment and activation of other immune cells to the site of infection. NOD2 has been shown to serve as a microbial sensor and activator of the pro-inflammatory response to several pathogens, including \textit{M.tb} (126,127,135,179,242-244). In the case of \textit{H. pylori}, it is thought that the bacterium is able to use a secretion system to secrete peptidoglycan fragments that are then recognized by NOD2 in the cytosol (93).
Since *M. tb* is an intracellular pathogen, and known to secrete virulence factors, it is necessary to understand the role of intracellular receptors and immune functions in human macrophages with regards to this pathogen. Demonstrating a role for NOD2 can lead to the development of new methods for targeting this host defense molecule/pathway for therapy in the case of *M. tb*.

The presence of NOD2 mRNA has only been reported in monocytes (76). Thus, we initiated studies of NOD2 mRNA transcript levels as monocytes differentiate into macrophages using an established *in vitro* model. Our results indicate that NOD2 steady state mRNA transcripts increase as monocytes differentiate into macrophages, specifically on day three of differentiation. This result is similar to our previously published data on a membrane PRR that recognizes *M. tb*, the MR, whose mRNA transcript levels are also enhanced in macrophages when compared to monocytes. On the other hand, TLR2 and TLR4 expression follows a different pattern on these cells (54).

Since mRNA levels do not always correlate with protein expression, we elected to explore NOD2 protein expression in human macrophages compared to monocytes. Studies in the field have been limited to assaying for mRNA levels in primary human cells; whereas protein expression has been studied in cell lines by flag tagging NOD2 (114,267). Thus, a major contribution to the field by the current work is the optimization of available antibodies for characterization of NOD2 protein expression in primary human monocytes/macrophages by Western blot and confocal microscopy. Our findings
demonstrate that NOD2 protein is expressed in human macrophages and levels increase during monocyte to macrophage differentiation, suggesting a particularly important role for NOD2 in human macrophages. Our microscopy results show NOD2 protein in a punctuate pattern in cells, suggesting the possibility that it resides within organelles or in complexes present in the cytoplasm.

We also detected NOD2 protein in human AMs from healthy donors. An interesting finding in our work is that AMs appear to express less of NOD2 protein than MDMs. It is possible that reduced NOD2 levels in AMs represent another phenotypic marker of alternatively activated macrophages of which AMs are a prototype and consequently leads to less effective killing of *M. tb* on first contact during the innate immune response. On the other hand, later during TB disease, NOD2 may play an important role in host defense since NOD2 mRNA levels are increased in patients with TB disease (220).

NOD2 is engaged in response to pathogens which leads to the production of pro-inflammatory cytokines. In cell lines expressing NOD2, incubation with *M. tb* enhances NF-κB activation (86,127). We found that pre-stimulation of human macrophages (both MDMs and AMs) with the NOD2 ligand MDP followed by *M. tb* or BCG incubation caused a significant increase in TNF-α and IL-1β production. Although the addition of MDP is not the paradigm for human infection, the result provided evidence for the potential involvement of NOD2 in augmenting the cytokine response to *M. tb* and BCG. This result raised the question as to what are the major downstream signaling kinases
activated in response to *M.tb* or BCG. RIP2 is a kinase that autophosphorylates at serine 176, and its kinase activity is necessary for NOD2-mediated pro-inflammatory cytokine production (94). We found that *M.tb* and BCG both activate RIP2 by phosphorylation at serine 176 in a NOD2-dependent manner; however the kinetics and pattern of activation were different for these two bacteria. RIP2 phosphorylation peaked earlier for BCG (30 min) and continued throughout the time course; whereas RIP2 phosphorylation peaked at 1 h for *M.tb* and the level of phosphorylation decreased by 3 h. Consistent with this we observed that BCG continues to activate downstream *IκBα* [thereby enabling the transcriptional activity of NF-κB and the resultant up-regulation of expression of macrophage pro-inflammatory response genes (264)] through 2 h whereas activation of *IκBα* decreases for *M.tb* at this time point. Thus, it appears that *M.tb* but not BCG can down-regulate RIP2 and *IκBα* activation perhaps as a mechanism to dampen the innate immune response. One possibility for this down-regulation is that *M.tb* possesses virulence factors that affect this pathway that are not present in BCG such as those present in the Region of Difference-1 (RD1) which is responsible for the production of several virulence-associated proteins in *M.tb* (191). For example, culture filtrate protein-10 kDa (CFP-10) and early secreted antigenic target-6 kDa (ESAT-6) in *M.tb* but not in BCG have been shown to inhibit lipopolysaccharide-induced NF-κB transactivation by inhibiting reactive oxygen species production (251). Apart from RIP2, recent work from our laboratory has identified a PPARγ-mediated pathway for *M.tb* that is not operative for BCG in human macrophages (56).
To more directly assess a causal role for NOD2 in regulating the inflammatory response to mycobacteria in primary human macrophages, we developed a reliable knockdown protocol for NOD2 that is specific and does not appreciably affect the viability of the cells on the monolayer. To date, studies on the role of NOD2 in response to different pathogens in primary human cells have relied on either expressing the NOD2 allele with the Crohn’s disease mutation in cell lines (139) or by obtaining cells from individuals homozygous for the NOD2 mutation, which was shown to render this protein reduced in activity or non-functional (85). Obtaining cells from Crohn’s disease patients with this mutation is difficult and the results may not be consistent among patients. Also, since these patients have a mutation in this protein, their cells may be primed in other ways that may alter the immunological response. The accomplished knockdown of NOD2 in human macrophages is an important advance enabling the study of NOD2 more comprehensively in primary monocytes/macrophages.

Our results are consistent with those found with mouse macrophages with regards to the role of NOD2 in controlling the inflammatory response to mycobacteria. Gandotra et al found that TNF-α and IL-12 production is decreased in NOD2 knockout BMMs in response to \textit{M.tb} (179) and Divingahi et al found that these cytokines are decreased in NOD2 knockout naive alveolar mouse macrophages in response to BCG (135). We observed that both TNF-α and IL-1β production are significantly reduced in NOD2-deficient human macrophages and found a decrease in RIP2 phosphorylation in response to \textit{M.tb} and BCG infection. It is noteworthy that RIP2 activity is only partially reduced
rather than abolished by the knockdown of NOD2, which may be due to the activation of RIP2 by other intracellular receptors. For example, NOD1 is involved in the activation of RIP2 through the CARD-CARD interaction (94).

The role of NOD2 in IL-1β production during M.tb and BCG infection is of interest. A recent publication reports that PBMCs from Crohn’s disease patients that are homozygous for the 3020insC NOD2 mutation exhibit decreased production of IL-1β in response to M.tb (268). The mechanism for the production of mature IL-1β in response to M.tb involved the activation of ERK, p38 and RIP2 following recognition by TLR2/TLR6 and NOD2 in PBMCs; however, the mechanism was not explored in human macrophages. In PBMCs, NOD2 is reported to have a dual effect on pro IL-1β mRNA transcription and secretion of mature IL-1β (269). Our preliminary observations propose that during M.tb infection, NOD2 may play a small role in down-regulating pro IL-1β transcription, suggesting that NOD2 may play a negative regulatory role (Figure 2.4D). However, NOD2 is responsible for enhancing caspase-1 activation during M.tb infection, since we observed a decrease in active caspase-1 expression in NOD2 deficient macrophages (Figure 2.4E). In our experiments in which we added MDP to un-transfected macrophages followed by M.tb infection, we observed an additive effect on TNF-α secretion, but a synergistic effect on IL-1β secretion (Figure. 2.2 and 2.5). We speculate that the differential response of TNF-α and IL-1β may be explained by NOD2 being relatively more important in the release of mature IL-1β due to a role in processing
pro IL-1β through caspase-1. The relationship between NOD2 and caspase-1 has been reported (97,270).

Previous studies have reported that NOD2 knockout in mouse macrophages has no effect on controlling *M.tb* growth (135,179). In contrast, we demonstrate here for the first time that NOD2 plays a role in controlling intra-macrophage growth of *M.tb* and BCG in human macrophages. The difference in the role of NOD2 in controlling mycobacterial growth is validated by doing a direct side by side comparison of mycobacterial growth in NOD2-deficient human macrophages and macrophages from NOD2 knockout mice. This difference can be generally explained by the growing list of differences seen in the innate immune response of human versus mouse macrophages (225,254,271-275). It is relevant that the NOD2 knockout mouse does not show any signs of abnormalities in the intestinal tract, which is inconsistent with the NOD2 mutations leading to Crohn’s disease and inflammatory bowel disease in humans (276). In addition, differences in promoter structure between humans and mice indicate divergent mechanisms in NOD2 regulation (147).

There have been reports over time providing evidence that *M.tb* can translocate into the cytosol of macrophages under defined conditions (212,213,215,277) and also that mycobacterial phagosomes are porous (278). Nonetheless, the weight of the evidence to date is that mycobacteria replicate in phagosomes in human and mouse macrophages. In the context of the current work, we speculate that *M.tb* interacts with NOD2 by either
secreting virulence factors from the phagosome into the cytosol via a secretion system(s) (181,191), or possibly translocates proteins across the phagosome through the action of a yet to be defined flippase. Alternatively, a NOD2-RIP2 complex has been reported to signal from the cell membrane along with the need for NOD2 to be present at the membrane of intestinal epithelial cells for signaling to occur (114,279). These reports raise the possibility that NOD2 may localize with the phagosome of M.tb leading to engagement of this cytosolic PRR during residence of the bacterium within the phagosome.

The mechanism(s) involved in the recognition of NOD2 by M.tb and BCG may differ since BCG lacks the ESX-1 secretion system and is reported to be unable to stimulate NOD2 dependent RIP2 polyubiquitination in murine macrophages (181). Nonetheless, BCG does generate TNF-α in a NOD2-dependent manner [this report and (86)]. Of relevance, mycobacteria contain the N-acetyl muramic acid hydroxylase, NamH, which changes the acetyl group on MDP to a glycolyl group, causing it to be a more potent stimulator of the immune response via NOD2 suggesting that NOD2 may be more tuned at sensing mycobacterial infections in generating an immune response (86).

In summary, here we report that NOD2 is present and functional in human macrophages, including AMs. We provide evidence that NOD2 plays a role in controlling the growth of M.tb and BCG in human macrophages along with regulating the nature of the inflammatory response. The role for NOD2 differs with respect to growth of M.tb and
BCG in human and mouse macrophages. Finally, the assays established and described in this report now enable a more thorough evaluation of the role of NOD2 in regulating the innate immune response of human macrophages to mycobacteria and other infectious agents.
CHAPTER 3: Critical role for NOD2 in *Mycobacterium tuberculosis* induced iNOS expression in human macrophages

3.1 Introduction

*Mycobacterium tuberculosis* (*M. tb*) is the causative agent of tuberculosis (TB). TB infects approximately one-third of the world's populations, and was responsible for 1.7 million deaths in 2009. The high incidence of death is attributed to the increase in HIV infections, drug resistant strain development, and length/cost of drug treatment (155). It is imperative to understand the mechanism that underlies the survival of *M. tb* in humans in order to effectively treat patients for this disease as well as create useful vaccines.

An important immune cell that is involved in recognizing and aiding in the clearance of pathogens is the macrophage. Macrophages use a myriad of defense mechanisms in order to protect the host including the production of reactive oxygen/nitrogen intermediates (ROI/RNI), pro-inflammatory cytokines, and proteases, as well as acidification of the phagosome. The type of response induced during the interaction of *M. tb* with the macrophage is dependent on the receptor activated (186). For example,
entry through the mannose receptor leads to limited phagosome-lysosome fusion and an anti-inflammatory response (238).

A group of intracellular receptors, Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), have been described to recognize and induce a pro-inflammatory response to specific portions of the peptidoglycan layer of Gram-negative and Gram-positive bacteria cell walls (85). NOD2 is a NLR that was originally found to recognize N-acetyl muramyl dipeptide (MDP) found in Gram- negative and positive bacteria. *M.tb* produces N-glycolyl-MDP (GMDP), and this change in functional groups (acetyl to glycolyl) enhances the NOD2 stimulating activity (86). As discussed in chapter 2, our laboratory has recently provided evidence that in primary human macrophages NOD2 plays an important role in controlling the inflammatory response as well as the growth of *M.tb* (77).

The role of NOD2 during early innate immune responses is of interest to our laboratory, specifically the role of NOD2 in RNI production. Inducible nitric oxide synthase (iNOS or NOS2) is responsible for the production of the RNI, nitric oxide (NO). NO is known to play a role in signaling during inflammation, which suggests a potential role for it in the NOD2 biochemical pathway (37,280). NOD1 is a close family member of NOD2, and has been found to be involved in iNOS induction in rat vascular smooth muscle cells and a murine macrophage cell line (281). Furthermore, interferon gamma (IFNγ) activated mouse macrophages stimulated with MDP induces NO production (282). It has
also been shown that in mouse macrophages MDP is able to induce iNOS expression (283). Little is known about the relationship of NOD2 and iNOS during *M. tb* infection. A study using IFNγ-activated NOD2 deficient mouse macrophages showed a decrease in NO production in response to *M. tb* (179). In the mouse model for TB NO plays an important roles in controlling *M. tb* growth (284). However, it has been difficult to duplicate these data using human macrophages, which may be due to differences in the amount of NO produced by mouse macrophages and human macrophages as well as a lack in sensitivity of assays (285). There are also differences in upstream pathways of NO production that exist between human and mouse macrophages. L-arginine is required for the production of NO in mouse macrophages, but not in human macrophages (286,287). This is possibly due to the lack of tetrahydrobiopterin in human macrophages, which is a necessary cofactor in the conversion of L-arginine to NO; however it has been shown that addition of this cofactor does not induce NO production (288). Recent studies indicate that, while low in comparison to mouse macrophages, human monocytes produce NO in response to *M. tb* (289). Furthermore, clinical studies indicate that patients with TB have increased iNOS expression in the inflammatory zone of granulomas (290). More specifically, alveolar macrophages of patients with TB have enhanced iNOS protein expression (291).

Our recent finding of the ability of NOD2 to control *M. tb* growth at 24 h is of interest, and led us to believe that NOD2 is involved in early innate immune mechanisms. We performed a microarray at this time point using NOD2-deficient human macrophages and
found inducible nitric oxide synthase (iNOS) to be down-regulated. Based on reports of
*M.tb* inducing iNOS and NO using in vitro cell models as well as clinical specimens, the
recent evidence that NOD2 plays a role in NO production during *M.tb* infection of IFNγ
activated mouse macrophages, and our microarray data, we decided to study the role of
NOD2 in the iNOS pathway during *M.tb* infection of human macrophages. Here we
show that NOD2 agonists increase the expression of iNOS mRNA and protein expression
as well as NO production. In coordination with previous reports we show that *M.tb*
induces iNOS expression and NO production, however, we show for the first time in
human macrophages that this phenomena is NOD2-dependent. Together these findings
provide evidence for a novel role for NOD2 and its importance in the macrophage
induction of NO in response to pathogens.

3.2 Materials and Methods

3.2.1 Buffers and reagents

Dulbecco’s PBS with and without Ca²⁺ and Mg²⁺ ions, Iscoves, FBS, TRIzol, and RPMI
1640 medium with L-glutamine containing phenol red or phenol red deficient were
purchased from Invitrogen (Invitrogen Life Technologies). RHH medium [RPMI 1640
plus 10 mM HEPES (Invitrogen) plus 0.4% human serum albumin (CSL Behring LLC)]
was used for cell culture experiments. N-Acetylmuramyl-L-alanyl-D-isoglutamine
hydrate (MDP; Sigma-Aldrich), N-Glycolyl-muramyldipeptide (GMDP; Invivogen), and
Interferon gamma (IFNγ; BD Biosciences) were used for assays involving iNOS
expression and NO production. 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-FM diacetate) was purchased from invitrogen for NO production assays. 7H11 agar was prepared with Bacto Middlebrook 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol (Difco Laboratories).

3.2.2 Antibodies

Unconjugated rabbit anti-human NOD2 was purchased from ProSci and optimization for human macrophages can be found in the previous report (77). β actin, goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology. Antibodies specific for iNOS were purchased from Cell Signaling Technology. The aforementioned antibodies were used for Western blot.

3.2.3 Bacterial strains and culture

Lyophilized M.tb H37Rv (ATCC #25618) and BCG (ATCC #35734) was obtained from the American Tissue Culture Collection, reconstituted, and used as described (172). The concentration of bacteria (1–2 x 10^8 bacteria /mL) and the degree of clumping (≤10%) were determined by counting in a Petroff-Hausser chamber. Bacteria prepared in this fashion are ≥90% viable by CFU assay.
3.2.4 Isolation and culture of human macrophages

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using an approved protocol by The Ohio State University Institutional Review Board (IRB) was processed as described (292). Briefly, PBMCs were isolated from heparinized blood on a Ficoll-Paque cushion (Amersham Biosciences/GE Healthcare) and cultured in Teflon wells (Savillex) for 1 (monocytes) through 5 (MDMs) days in the presence of RPMI 1640 medium containing 20% autologous serum (2.0 x10^6 PBMCs/mL) at 37°C/5% CO₂ (258).

3.2.5 RNA isolation

Day 5 PBMCs in Teflon wells were harvested and mononuclear phagocytes adhered to a 12-well tissue culture plate with 10% autologous serum (4 x 10^6 PBMCs/mL). After washing away lymphocytes, the MDMs (4 x 10^5 cells) were lysed in TRIzol and total RNA was isolated by using the Qiagen RNAeasy column method. For microarray studies RNA was not processed through the Qiagen RNAeasy column. The Experion (Bio-Rad) was used to determine the quality and quantity of all RNA samples.

3.2.6 Gene expression studies by qRT-PCR

RNA (100ng) was reverse transcribed to cDNA by reverse transcriptase enzyme, (SuperScript II, Invitrogen) and qRT-PCR was performed using a human NOS2 TaqMan gene expression kit (Applied Biosystems). NOS2 amplification was normalized to β
actin as a housekeeping gene (ΔCt). Relative copy number (RCN) and fold change were determined. RCN was calculated as follows: \( \text{RCN} = E^{-\Delta Ct} \times 100 \), where \( E \) is the efficiency (2 = 100% efficiency) and \( Ct = Ct \text{ (target)} - Ct \text{ (reference)} \) (260). Fold change was calculated from RCN compared to resting group for NOS2 mRNA experiments. Triplicate samples were analyzed in duplicate wells in each experiment.

3.2.7 Transfection of MDMs

PBMCs were transfected with Accell scramble siRNA or NOD2 siRNA purchased from Thermo Scientific Dharmacon RNAi technologies that targeted the sequence CUUUAGGAUGUACAGUUA (368nM) using the Amaxa Nucleofector (Lonza Group Ltd). Transfected cells were used for subsequent experiments, such as the DAF-FM diacetate assay and Western blotting. See section 2.2.9 for more details of the transfection protocol. Assessment of the NOD2 siRNA specificity and effects on monolayer density/viability were performed and can be found in chapter 2 (77).

3.2.8 Microarray analysis

Scramble and NOD2 siRNA-transfected cells were incubated with \( M.\text{tb} \) for 24 h in 1% autologous serum. Cells were then lysed with TRIzol and RNA was isolated as described above. Subsequent labeling and hybridization to Affymetrix hgu133plus2 chips was performed at The Ohio State University (OSU) Comprehensive Cancer Center microarray facility. Analysis was performed at the OSU Comprehensive Cancer Center Biomedical
Informatics core laboratory. The Venn diagram was generated using Bioinformatics Organization’s Gene List Venn Diagram program (http://www.bioinformatics.org/gvenn/).

3.2.9 Macrophage stimulation / infection, cell lysis, and Western blotting

Day 12 MDMs or day 5 MDMs transfected with scramble or NOD2 siRNA (2.0 x 10^5) for 72 h were incubated with *M.tb* or BCG (MOI 5:1; triplicate wells) in RHH for 30 min at 37°C in 5% CO₂ on a platform shaker for equal dispersion of bacteria followed by an additional incubation for 90 min without shaking. The cells were then washed three times with warm RPMI, repleted with RPMI containing 1-2% human autologous serum and incubated for 24 h. For experiments using the NOD2 agonists MDP, GMDP, or the cytokine IFNγ, 2.0 x 10^5 day 5 MDMs were adhered to a 24-well plate for 2 h at 37°C in 5% CO₂, washed to remove lymphocytes, and then incubated with MDP (5ug/mL), GMDP (5ug/mL), or IFNγ (10ng/mL), for 24 h in RPMI containing 1% autologous serum. The monolayers were washed once with PBS and lysed in TN-1 lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM Na₃PO₄, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, 10 μg/ml aprotinin, and 10 μg/ml leupeptin)], incubated on ice for 10 min and then centrifuged at 17,949 x g for 10 min at 4°C to remove cell debris (261). For NOD2 knockdown assessment, lysates were collected as above at 72 h or 96 h post-knockdown. Protein concentrations of the cleared lysates were measured using the BCA-protein assay kit (Pierce). Proteins were separated by SDS-PAGE and analyzed by Western blot by probing with the primary and secondary antibody of interest and
development using ECL (Amersham Biosciences). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then normalized to β-actin. Fold change was determined as follows: (treated sample band intensity) / (untreated sample band intensity).

3.2.10 DAF-FM diacetate staining

Untransfected MDMs or transfected (scramble or NOD2 siRNA) MDMs (2 x 10^5) were adhered to glass coverslips in a 24-well tissue culture plate for 2 h at 37°C and washed to remove lymphocytes. The cells were treated with GMDP, IFNγ, or M.tb for 24 h in no phenol red RPMI containing 1% autologous serum. At 24 h cells were washed one time with warm no phenol red RPMI and repleted with 5 μM/mL DAF-FM diacetate for 30 min at 37°C. Next cells were washed with warm no phenol red RPMI 3 times and left in the third wash for 15 min at 37°C to allow for deesterification of the dye. Finally, cells were fixed with 10% formalin (10 min at room temperature), washed with PBS, and mounted on glass slides. Slides were viewed using an Olympus Flowview 1000 Laser Scanning Confocal microscope at an excitation/emissions maxima of 495/515 nm. One hundred and fifty to three hundred consecutive macrophages/test group were counted and Mean fluorescence intensity (MFI) was calculated using Olympus FluoView FV1000 software.
3.2.11 Statistics

A paired one tailed student’s $t$ test was used to analyze the differences between two groups in figures showing one representative figure of three independent experiments. An unpaired one tailed student’s $t$ test was used to analyze differences between two groups in figures showing cumulative data from three independent experiments. Significance was $P < 0.05$.

3.3 Results

3.3.1 NOD2 regulates many genes during *M. tb* infection of human macrophages

As discussed in chapter 2, our laboratory has published that NOD2 plays an important role in controlling the growth of *M. tb* in human macrophages at 24 h post infection. Between initial infection and 24 h there is limited bacterial replication; the bacteria are becoming acclimated to the phagosome of the macrophages. Thus, enhanced bacterial viability at 24 h in NOD2-deficient macrophages suggests an alteration in primary host defense mechanisms of the cell. In order to study this enhanced viability at 24 h, we used our established method to knock-down NOD2 in human macrophages and performed a microarray of scramble and NOD2 siRNA-transfected MDMs 24 h post infection. In total, 1,583 transcripts were found to be significantly different with a fold difference of 2 or more, which is depicted by scatterplot (Figure 3.1A).

In order to quantify the overlap in the number of genes NOD2 regulates under resting conditions and during *M. tb* infection, a Venn diagram was created from the 1,583
regulated transcripts (Figure 3.1B). Of these, 22% were regulated by NOD2 under resting conditions, (scramble versus NOD2 siRNA-transfected MDMs) indicating that NOD2 plays a role in regulating genes involved in homeostasis of macrophages. 54% of the genes were regulated by NOD2 during *M.tb* incubation for 24 h (scramble versus NOD2 siRNA-transfected MDMs incubated with *M.tb*) and 24% of the genes were regulated under both conditions (resting and *M.tb* incubation combined). While it is of interest that NOD2 regulates genes involved in normal macrophage homeostasis, we focused on the role of NOD2 during *M.tb* infection due to the fact that approximately half of the genes obtained in the microarray were regulated by NOD2 under this condition.

We first analyzed the data by assessing genes regulated in major macrophage signaling pathways. We found genes affected by NOD2 deficiency during *M.tb* infection in the iNOS, JAK/STAT, TLR, autophagy, TGF-β, and apoptosis signaling pathways (Figure 3.1C). While the percentage of genes in each pathway affected in the microarray may differ depending on the number of genes involved, it is of interest to note that a large number of genes were identified to be affected in the apoptosis signaling cascade. When looking further at the fold change of up- or down-regulated genes in each cascade, we found that iNOS was down-regulated 3.07-fold. We were able to validate that NOD2 deficiency in human macrophages led to a decrease in iNOS mRNA expression during *M.tb* infection using qRT-PCR (Figure 3.1D). This was of interest to us because of previous reports indicating that patients with TB have enhanced iNOS expression in macrophages when compared to non-TB patients (291).
Figure 3.1: NOD2 regulates gene expression in human macrophages during *M. tb* infection.
Scramble or NOD2 siRNA-transfected MDM monolayers were incubated with \textit{M.tb} (MOI 5:1) for 2 h, washed 3 times with warm RPMI, and repleted in 1\% autologous serum for 24 h. Cells were lysed with TRIzol to obtain total RNA. An Affymetrix Human Genome U133 plus 2.0 array was performed and processed. (A) Shown is a scatter plot (log base 2 scale) with NOD2 siRNA treated cells incubated with \textit{M.tb} (y-axis) plotted against scramble siRNA treated cells incubated with \textit{M.tb} (x-axis). (B) A Venn Diagram showing all significantly different genes that were up- or down-regulated twofold or more, with 380 genes common to both conditions (Resting = Scramble vs NOD2 siRNA treated cells and \textit{M.tb} = Scramble vs NOD2 siRNA treated cells incubated with \textit{M.tb}). (C) A pie chart showing the major signaling pathways analyzed, and the proportion of genes in each pathway that are regulated by NOD2 during \textit{M.tb} infection of human macrophages. (D) Scramble or NOD2 siRNA transfected MDM monolayers were incubated with \textit{M.tb} (MOI 5:1) for 24 h, and then monolayers were lysed with TRIzol for extraction of total RNA. Quantitative real time RT-PCR was used to determine iNOS mRNA levels. Shown is the mean ± SD of a representative graph of two independent experiments.

3.3.2 NOD2 agonists regulate iNOS expression and NO production in human macrophages

We next determined whether NOD2 agonists were able to induce iNOS mRNA in human macrophages by using quantitative real-time PCR (qRT-PCR). MDM monolayers were incubated with MDP or GMDP for 24 h and then lysed in order to isolate RNA for qRT-PCR analysis or cell lysates were collected for Western blotting analysis of iNOS. Results from qRT-PCR provide evidence that MDP, the NOD2 agonist found in Gram negative and Gram positive bacteria, induces iNOS mRNA expression (Figure 3.2A). However the agonist for NOD2 generated from the cell wall of mycobacteria, GMDP, induces iNOS mRNA at a significantly higher level. This is not surprising because it has
been shown that GMDP is a more potent stimulator of the immune response (86). Next, we compared the iNOS mRNA levels with the amount of protein expressed during MDM stimulation with NOD2 agonists by Western blotting. While there were no differences in the amount of iNOS protein expression between agonists, we did observe that expression is induced by NOD2 agonists (Figure 3.2B). IFNγ was used as a positive control for the ability of MDMs to induce iNOS protein expression (294). We next investigated whether the NOD2 agonist GMDP and IFNγ activation of iNOS is NOD2-dependent. Scramble and NOD2 siRNA-transfected MDM monolayers were incubated for 72 h in order to achieve the maximum NOD2 knockdown and then incubated with GMDP or IFNγ for 24 h. At this time cell lysates were collected and analyzed for iNOS expression. We found that NOD2 deficiency leads to the loss of iNOS expression by GMDP and IFNγ (Figure 3.2C). This result indicates that iNOS expression is NOD2-dependent. Finally, we sought to confirm whether the iNOS expression we observed in response to these agonists in MDMs resulted in NO production. It was previously observed that IFNγ activated mouse macrophages stimulated with MDP led to the production of NO, and this was NOD2-dependent (179). In our MDM model, we used a membrane permeable, NO-sensitive fluorescent dye, DAF-FM diacetate, in order to measure the production of NO (293). Figure 2D shows that exposure of MDMs to GMDP for 24 h led to a 45% increase in NO production. IFNγ stimulation for 24 h resulted in a 57% increase in NO production, similar to previously reported data using primary human alveolar macrophages (294). Two representative images from each test group are shown in figure 3.2E.
Figure 3.2: NOD2 agonists regulate the mRNA and protein expression of iNOS and NO production in human macrophages.

(A) MDM monolayers were treated with MDP (5 μg/mL), GMDP (5 μg/mL) or IFNγ (10ng/mL) for 24 h, then monolayers were lysed with TRizol for extraction of total RNA. Quantitative real time RT-PCR was used to determine iNOS mRNA levels. Data were normalized to the β actin gene and RCN was determined. Shown are cumulative data from five independent experiments (mean +/- SEM; *P < 0.05, **P < 0.005). (B) Cell lysates were analyzed for iNOS expression or loading control β actin by Western blot at 24 h. Shown is a representative experiment of three independent experiments. (C) Scramble or NOD2 siRNA-transfected MDM monolayers were incubated with GMDP (5 μg/mL) or IFNγ (10 ng/mL) in 1% autologous serum for 24 h. Cells were lysed with TN-1 buffer and iNOS or the loading control β actin protein levels were examined by Western blot. Shown is a representative experiment of three independent experiments. (D) Day 5 MDM monolayers on coverslips were treated with GMDP or IFNγ for 24 h, and then cells were incubated with DAF-FM diacetate in RPMI containing no phenol red for 30 min. Cells were washed 3 times with warm RPMI containing no phenol red and then allowed to sit for 15 min in order for

Continued on next page
deesterification to occur. Next, cells were fixed and mounted on slides. Cells were then examined by confocal microscopy with excitation/emission maxima of 495/515 nm (Olympus FV1000). Shown are cumulative data of the mean fluorescence intensities for each test group obtained from three independent experiments (mean +/- SEM; **P < 0.005, ***P < 0.0005). (E) Two representative micrographs (40x) are shown of DAF-FM diacetate staining for each test group of three independent experiments performed on duplicate coverslips.

3.3.3 M.tb-mediated iNOS expression is NOD2-dependent

It is known that the attenuated M.tb strain, H37Rv, enhances iNOS mRNA and protein expression as well as NO production in human alveolar macrophages (295). The virulent strain, Erdman, was shown to induce NO production in human monocytes and monocytic cell lines (289). Corroborating these data, it was discovered that patients with TB have higher levels of iNOS protein expression in alveolar macrophages (295). We next examined whether the virulent strain of M.tb, H37Rv, and the attenuated vaccine strain, M. bovis BCG (BCG), caused increased iNOS expression in MDMs. Day 12 MDM monolayers were incubated with M.tb or BCG (MOI 5:1) for 24, 48, 72, and 96 h at which time cell lysates were analyzed for iNOS expression by Western blotting (Figure 3.3A). We found iNOS protein expression is enhanced at 24 and 48 h for both bacteria and decreased at 72 h. A notable difference between the virulent and attenuated strain is seen at 24 h, M.tb increases iNOS expression to a > level than BCG. We next confirmed that M.tb-mediated induction of iNOS resulted in NO production in MDMs. MDM monolayers were incubated with M.tb for 24 h and then exposed to DAF-FM diacetate in order to quantify NO production. Our results show a 71% increase of NO production in
MDMs infected with *M. tb* (Figure 3.3B). Two representative images of each test group are shown in figure 3.3C. The previous observation of a decrease in iNOS expression upon stimulation of NOD2 deficient MDMs with the component of peptidoglycan that can be found in *M. tb*, GMDP, led us to study whether NOD2 is responsible for the *M. tb*-induced iNOS expression. MDMs were transfected with scramble or NOD2 siRNA, and after 72 h of transfection MDMs were incubated with *M. tb*. At 24 h cells were lysed and analyzed for iNOS expression by Western blotting (Figure 3.3D). Silencing of NOD2 in MDMs and incubation with *M. tb* resulted in decreased iNOS protein expression. Thus, our results indicate that in human macrophages, NOD2 is an important mediator of iNOS expression during *M. tb* infection.
Figure 3.3: *M. tb*-mediated induction of iNOS expression and NO production is NOD2-dependent in human macrophages.

(A) Day 12 MDMs were incubated with *M. tb* or BCG (MOI 5:1) for 2 h, washed 3 times and repleted with 2% autologous serum for 24, 48, 72, and 96 h. Cells were lysed with TN-1 buffer at each time point and iNOS or β actin protein levels were examined by Western blot. Shown is a representative experiment of three independent experiments. (B) Day 12 MDM monolayers on coverslips were incubated with *M. tb* as previously described for 24 h, and then cells were incubated with DAF-FM diacetate as described in the methods, fixed and examined by confocal microscopy with excitation/emission maxima of 495/515 nm (Olympus FV1000). Shown are cumulative data of the mean fluorescence intensities for each test group obtained from three independent experiments (mean +/- SEM; ***P < 0.0005). (C) Two representative micrographs (40x) are shown of DAF-FM diacetate staining for each test group of three independent experiments performed on duplicate coverslips. (D) Scramble and NOD2 siRNA-transfected monolayers were incubated with *M. tb* (MOI 5:1) for 2 h, washed 3 times and repleted with 1% autologous serum for 24 h. Cells were lysed with TN-1 buffer and iNOS or β actin protein levels were examined by Western blot. Shown is a representative experiment of three independent experiments.
3.4 Discussion

While NOD2 is one of the most well characterized members of the NLR family, there are limited reports of the functions of NOD2 in other innate immune responses besides cytokine production. These recent reports indicate a role for NOD2 in autophagy and MHC class I and class II presentation (296-298). Our own findings of the growth of *M. tb* being controlled at an early time point, led us to question whether other early innate immune responses were being affected by NOD2 (77). Since NOD2 plays such a major role in bacterial recognition and clearance, we sought to study other roles of NOD2 through a microarray (299).

Our microarray results show that NOD2 regulates 1,583 transcripts in human macrophages. While we did not pursue this avenue, our microarray results indicate that NOD2 is important for normal macrophage homeostasis (independent of *M. tb*). The role of NOD2 in homeostatic macrophage functions has not been studied and may be an important aspect of the receptor to study. Over half of the genes are up- or down-regulated during *M. tb* infection of NOD2 deficient macrophages, which suggests that NOD2 plays a role in many more macrophage responses during infection than previously reported. Our array analysis revealed that during *M. tb* infection of human macrophages NOD2 plays a role in many signaling cascades including: iNOS, JAK/STAT, TLR, autophagy, TGFβ, and apoptosis. Thus a major contribution to the field by this current work is the identification of important genes involved in inflammation/immunity affected by NOD2 deficiency in human macrophages.
We chose to study the role of NOD2 in the iNOS signaling pathway due to its relevance to *M. tb* infection. In the mouse model, *NOS2* is identified as a protective locus against TB. Mice deficient in the *NOS2* gene succumb to infection within 33-45 days compared to WT mice which survive an average of 160 days. Large, granulomatous lesions were found in the lung, liver, and spleens at 30 days post infection, while few were found in WT mice at this time point (284). Similar results of mortality and tissue damage were found using iNOS inhibitors, aminoguanidine and N^G^-monomethyl-L-arginine, in the in vivo mouse model (300). The role of NO during *M. tb* pathogenesis in humans has been a controversial topic in the past; however, there is now more evidence in the literature that supports its impact. Initial reports testing human cells for the production of NO were negative, due to the low amounts of NO produced by these cells and the usage of insensitive assays. The discovery of a more sensitive assay using the fluorescent probe diaminonapthalene (DAN), which detects as little as one pmol of nitrite, allowed researchers to detect nitrite in human monocytes incubated with *M. tb* (301). Jagannath *et al* reported using the DAN assay that *M. tb* is able to induce NO production in human monocytes and this NO is bacteriostatic (289). Whether or not NO is bacteriostatic or bactericidal is dependent on the specificity of the RNI and the strain of mycobacteria (302-304). Although the mechanism has not been identified, the ability of *M. tb* to resist the effects of RNI is due to a novel gene, *noxR1* (305). It is interesting to note that iNOS deficient mice treated with the same drugs that cure *M. tb* infected immunocompetent
mice are ineffective. This suggests that the effectiveness in vivo of tuberculocidal drugs may be dependent on iNOS-derived NO (306).

The precise mechanism(s) underlying how NO antagonizes *M. tb* is unknown; however, it may include disruption of bacterial DNA, proteins, signaling, or induction of apoptosis. Since we recently found that NOD2 is an important modulator of the immune response to *M. tb* and is necessary to control its growth in primary human macrophages, we decided to study whether NOD2 plays a role in the expression of the necessary mediator of NO production, iNOS. Our microarray results indicated that iNOS mRNA expression is down-regulated during *M. tb* infection of NOD2 deficient human macrophages. We verified this result using quantitative RT-PCR. Recently, it was found in mouse macrophages that MDP induces iNOS protein expression in a RIP2-dependent manner (283). Thus, we initiated our studies by investigating whether or not NOD2 agonists induced iNOS mRNA expression in human macrophages. Our results indicate that the agonist found in Gram negative and positive bacteria, MDP, is a relatively poor inducer of iNOS mRNA at 24 h. However, the agonist produced by *M. tb*, GMDP, induces robust mRNA expression of iNOS. This is not surprising because comparative studies of MDP and GMDP indicate that GMDP is a more robust stimulator of the immune response (86).

mRNA expression does not always translate to protein expression in eukaryotic cells, so we explored the ability of NOD2 agonists to induce iNOS protein expression in human macrophages. Our findings demonstrate that both NOD2 agonists are effective at
inducing iNOS protein expression and this is at the same level as a known inducer of iNOS in human alveolar macrophages, IFNγ (294). These NOD2 agonists are known to induce the production of pro-inflammatory cytokines, TNF-α and IL-1β. As discussed in chapter 2, our laboratory has shown that the M.tb-induced cytokine production is NOD2-dependent in human macrophages (77). Alveolar macrophages from TB patients produce TNF-α and IL-1β at a higher level than healthy patients and the usage of an inhibitor of NO led to a reduction in TNF-α and IL-1β (307). It is known that iNOS expression can be induced by pro-inflammatory cytokines (308,309). The lack of these cytokines being produced is a possible reason why iNOS is not expressed when NOD2 is deficient.

In order to directly evaluate the role of NOD2 in regulating iNOS expression, we used a well established method in our laboratory to knockdown NOD2 in human macrophages (77). To date the role of NOD2 in iNOS and NO production has relied solely on the use of primary mouse macrophages or the iNOS knockout mouse model (179,282,283). Our results show that NOD2 is necessary to induce the protein expression of iNOS in human macrophages. Since iNOS is a critical enzyme that catalyzes the production of NO, this suggests that NOD2 plays a role in iNOS-induced NO production. NO is an effective host-defense molecule against microbial pathogens. NO can damage DNA as well as interact with accessory protein targets and result in enzymatic inactivation (310). Using a compound that is non-fluorescent until it binds NO to form a fluorescent benzotrizole, we found that the GMDP-induced iNOS expression consequently results in NO production.
Since GMDP is a portion of the cell wall of \textit{M. tb}, it is possible that it mediates \textit{M. tb}-induced iNOS expression and NO production.

Our results are consistent with previous findings that \textit{M. tb} enhances iNOS expression. However, here we compare virulent \textit{M. tb} to the attenuated vaccine strain, BCG. We observed that \textit{M. tb} and BCG induce iNOS at 24 h, but \textit{M. tb} enhances iNOS expression at a $\geq$ level when compared to BCG. However, the levels decrease at 72 h for both strains. In this study, we were able to confirm that the \textit{M. tb}-induced iNOS expression results in NO production using a different technique than previously published (DAF-FM diacetate). This NO could potentially be bacteriostatic or bactericidal as previously mentioned. Human alveolar macrophages isolated from patients with fibrosis and challenged with BCG for 24 h displayed enhanced iNOS expression. This led to peroxynitrate production and the subsequent killing of BCG (311).

For the first time we show that the induction of iNOS by \textit{M. tb} in human macrophages is NOD2-dependent. This cytosolic PRR is an important protein in the iNOS signaling pathway, which leads to the production of NO. The role of a PRR in the induction of an inflammatory mediator such as iNOS is not reported in human macrophages. In mouse macrophages, iNOS expression is mediated by TLR4 in MyD88-dependent and independent manners (312); however its expression in human macrophages has not been reported. For example in mouse macrophages bacterial lipoprotein activation of TLR2 leads to NO-dependent killing of \textit{M. tb}, but in human alveolar macrophages and
monocytes, the TLR2 activated antimicrobial pathway was found to be NO-independent (313). This finding is important because it identifies a cytosolic PRR potentially involved in the antimicrobial killing effects of NO. NOD2 is thought to be a secondary line of defense in macrophages to fight bacteria that are able to avoid the initial host defense mechanisms at the surface, but now these data suggest an even more important role for this receptor in inducing a pathway that results in bacterial killing. This is of great importance during M. tb infection, where the bacteria are able to modulate the host immune response and reside in a unique phagosome. Clinical samples have high levels of iNOS expression in the inflammatory zone surrounding tuberculous granulomas and NOD2 could be the mechanism for this expression (290).

Patients with Crohn’s disease have inflamed mucosa. They are homozygous for the 3020insC NOD2 mutation (loss-of-function) and are reported to have enhanced levels of iNOS expression and NO production in the active mucosa (314). This is somewhat contradictory to the results in this manuscript, since NOD2 knockdown led to decreased iNOS expression. However, the enhanced iNOS expression and NO production in Crohn’s disease patients is likely due to other infiltrating cells such as neutrophils or epithelial cells found in the area. It has been shown that elicited but not circulating neutrophils produce larger amounts of NO (315,316). Furthermore, we show here that NOD2 is important in iNOS expression but we did not study endothelial NOS, which is expressed in cultured human bronchiolar epithelium and is also responsible for NO
production (317). These reasons could explain why patients with Crohn’s disease have increased iNOS and NO levels.

In summary, we report results of a microarray indicating that NOD2 plays a role in many signaling pathways important in inflammation/immunity. We confirm published work in that human macrophages are able to produce NO, dependent on NOD2 agonists as well as \textit{M. tb}. Finally, we provide evidence that the cytosolic PRR, NOD2, is a mediator in the production of iNOS in response to \textit{M. tb}. These findings uncover a new mechanism for how NOD2 plays a role during \textit{M.tb} pathogenesis and point towards GMDP as being clinically useful for controlling \textit{M.tb} infection in an NO-dependent manner.
CHAPTER 4: The role of PKCδ in the NOD2 signaling pathway in human macrophages

4.1 Introduction

Inflammation is a process that involves the production of cytokines that are important to control infections and involves various regulators. The most notable transcription factor that is involved in pro-inflammatory cytokine production is NF-κB. There are several signaling cascades responsible for NF-κB activation, including Protein kinase C (PKC) signaling (318). The PKC family of enzymes consists of several isoforms that are important kinases in ensuring that other proteins function properly through the phosphorylation of hydroxyl groups on serine and threonine residues. The PKC subfamilies of isoforms are classified based on their activation stimulus and are called classical, atypical, and novel. Classical PKCs require DAG, calcium, and phospholipid for activation and include the isoforms PKCα, PKCβI, PKCβII, and PKCγ (11). Atypical PKCs require neither calcium nor DAG for activation. They include the isoforms PKCε, PKCζ, PK-N1, PK-N2 (13). The novel PKCs are activated by diacylglycerol and do not require calcium. They include the isoforms PKCδ, PKCθ, PKCe, and PKCη (12). PKCδ is of interest because it is expressed in many cell types including macrophages and is
thought to be important in microbial infections (319,320). During *Listeria monocytogenes* infection PKCδ is necessary for the confinement of bacteria to the phagosome (320). PKCδ is also necessary for the control of Raf and MAPK signaling in response to the bacterium *H. pylori* (321). The role of PKCδ in intracellular events during infection points to the possibility that it may be involved in intracellular signaling pathways that involve cytosolic PRRs.

A more recently discovered intracellular group of receptors are the Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). These receptors recognize specific muropeptides from the peptidoglycan of Gram-negative and Gram-positive bacteria and induce a pro-inflammatory cytokine response through activation of NF-κB and MAPK (85). Of the 23 NLRs, NOD2 is one of the best characterized. It recognizes the muropeptide, muramyl dipeptide (MDP), which is found in both Gram-negative and Gram-positive bacteria, as well as a glycolyl-MDP (GMDP) found in mycobacteria (86). While it is a well characterized NLR, there are still several fundamental questions that need to be answered regarding the signaling pathways that it is involved in. For example, it is established that MDP is an agonist of NOD2 and that NOD2 “senses” the muropeptide, but the underlying biochemistry for this recognition is still not understood. It is hypothesized that another molecule may be involved in the recognition of MDP which ultimately binds to NOD2; however it is yet to be discovered (74,322).
It is important to study the signaling cascades involving NOD2 in order to have a clearer understanding of how this PRR functions during bacterial infection. The current model of NOD2 signaling includes recruitment of a key kinase, receptor interacting protein 2 (RIP2). This kinase has a CARD domain that interacts with the CARD domain of NOD2 by a homotypic interaction and induced proximity signaling (76). The interaction only occurs upon activation and oligomerization of the NOD2 protein (85,88). The next interaction that occurs is the binding of TGF activated kinase 1 (TAK1) by the NOD2-RIP2 complex, which leads to subsequent NF-κB and AP-1 mediated activation and pro-inflammatory cytokine production (95,96). It was previously thought using cell lines that RIP2 kinase activity was not necessary for proper NOD2 signaling (107). However a recent study using RIP2 knockout mice, RIP2 kinase-dead knockin mice, as well as RIP2 deficient primary mouse cells proved that RIP2 kinase activity is necessary for protein stability and downstream signaling of NOD1 and NOD2 (94). However, the exact role of RIP2 kinase activity in the activation of signaling molecules is still unknown.

A short isoform of NOD2 has been shown to inhibit the NOD2-RIP2 assembly complex by interacting with these two proteins and interfering with the oligomerization of NOD2 (98). The protein Erbin, a member of the leucine rich repeat and PDZ domain (LAP) family, also binds NOD2 through the CARD domain and inhibits NOD2-mediated NF-κB activation (112,113). Another protein, CARD8, has also been shown to inhibit NOD2 signaling through a physical interaction with NOD2 (323). While these studies give insight into possible mechanisms of proteins that interfere with NOD2 signaling, they are
all done in transfected cell lines and thus provide only indirect evidence for the signaling pathways that may be occurring in primary cells.

Studies indicate that NOD2 may play a role in PKC signaling. In epithelial cells, NOD2 interacts with dual oxidase 2 (DUOX2) and co-localizes at the plasma membrane. DUOX2 is involved in NOD2-dependent ROS production (324). PKC activation with low concentrations of PMA causes DUOX2 to be highly induced (325). A more recent study using mouse macrophages indicates that MDP stimulates PKCβII phosphorylation in a RIP2-dependent manner (283). These studies along with the lack of full understanding of NOD2 signaling in primary cells led us to study the role of PKCδ in the NOD2 signaling cascade. PKCδ has been shown to play an important role in signal transduction pathways active during infection. We show that NOD2 agonists activate PKCδ kinase activity and PKCδ-induced cytokine production through a NOD2-dependent mechanism. We also provide evidence to support that PKCδ is needed for RIP2 phosphorylation. Together these findings indicate that PKCδ is a newly identified important mediator in the NOD2 signaling cascade.

4.2 Materials and Methods

4.2.1 Buffers and reagents

Dulbecco’s PBS with and without Ca²⁺ and Mg²⁺ ions, and RPMI 1640 medium with L-glutamine containing phenol red were purchased from Invitrogen (Invitrogen Life
Technologies). RHH medium [RPMI 1640 plus 10 mM HEPES (Invitrogen) plus 0.4% human serum albumin (CSL Behring LLC)] was used for cell culture experiments. Phorbol myristate acetate (PMA; Sigma-Aldrich), N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP; Sigma-Aldrich), and N-Glycolyl-muramyldipeptide (GMDP; Invivogen) were used for assays involving PKCδ expression and kinase activity.

4.2.2 Antibodies

Unconjugated rabbit anti-human NOD2 was purchased from ProSci and optimization for human macrophages can be found in chapter 2 and our recently published work (77). Total PKCδ, β actin, goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology. Phospho-RIP2, Phospho-PKCδ, Phospho-PKCθ, and Phospho-PKCα/βII antibodies were purchased from Cell Signaling Technology. The aforementioned antibodies were used for Western blot.

4.2.3 Isolation and culture of human macrophages

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using an approved protocol by The Ohio State University Institutional Review Board (IRB) was processed as described (292). Briefly, PBMCs were isolated from heparinized blood on a Ficoll-Paque cushion (Amersham Biosciences/GE Healthcare) and cultured in Teflon wells (Savillex) for 1 (monocytes) through 5 (MDMs) days in the presence of RPMI 1640 medium containing 20% autologous serum (2.0 x10⁶ PBMCs/mL) at 37°C/5% CO₂ (258).
4.2.4 Transfection of MDMs

PBMCs were transfected with Accell scramble siRNA, NOD2 siRNA, or PKCδ siRNA purchased from Thermo Scientific Dharmacon RNAi technologies that targeted for NOD2 the sequence CUUUAGGAUGUACAGUUA (368nM) and for PKCδ the sequence GGCUGAGUUCUGGCUGGACTT (250nM) using the Amaxa Nucleofector (Lonza Group Ltd). Transfected cells were used for subsequent experiments, such as ELISAs and Western blotting. See section 2.2.9 for more details on the transfection protocol. Assessment of the NOD2 siRNA specificity and effects on monolayer density/viability were performed and can be found in chapter 2 and our recently published work (77). Assessment of the PKCδ siRNA specificity can be found in previous reports (326). PKCδ siRNA did not affect monolayer density/viability.

4.2.5 Macrophage stimulation and preparation for ELISAs and Western blotting

Day 5 MDMs or day 5 MDMs transfected with scramble, NOD2 siRNA, or PKCδ siRNA (supernatants, 2.0 x 10⁵; lysates 4.0 x 10⁵) for 72 h were incubated with NOD2 agonists MDP (5ug/mL), GMDP (5ug/mL), or the phorbol diester PMA (0.123ug/mL in RHH for various time points between 0.5 and 4 h at 37°C in 5% CO₂. For day 5 MDM ELISA experiments using the NOD2 agonist MDP for pre-stimulation, 2.0 x 10⁵ day 5 MDMs were adhered to a 24-well plate for 2 h at 37°C in 5% CO₂, washed to remove lymphocytes, and then incubated with MDP (5ug/mL) for 90 min. Cells were then
washed and incubated with PMA for 1 h, 2 h, 4 h, 6 h, or 24 h. Cell culture supernatants were harvested and used for ELISAs (see below). For Western blotting experiments, the monolayers were washed once with PBS at the time points indicated and lysed in TN-1 lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM Na₄PO₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, 10 µg/ml aprotinin, and 10 µg/ml leupeptin], incubated on ice for 10 min and then centrifuged at 17,949 x g for 10 min at 4°C to remove cell debris (261). For NOD2 knockdown assessment, lysates were collected as above at 72 h post-knockdown. Protein concentrations of the cleared lysates were measured using the BCA-protein assay kit (Pierce). Proteins were separated by SDS-PAGE and analyzed by Western blot by probing with the primary and secondary antibody of interest and development using ECL (Amersham Biosciences). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then normalized to β-actin. Fold change was determined as follows: (treated sample band intensity) / (untreated sample band intensity).

4.2.6 Kinase assays

MDMs were lysed in TN-1 lysis buffer and 250 µg of cell lysates were immunoprecipitated overnight at 4°C, with 200 ng of anti-PKCδ or PKCα (SC-937) or IgG isotype control (SC-2027) antibodies followed by 1 h incubation with 25 µl of protein G-agarose beads (Amersham Biosciences). Immunoprecipitates were rinsed four times with 700 ul of TN-1 buffer followed by one rinse with PKCδ-kinase buffer (25 mM HEPES pH 7.4, 10 mM MnCl2, 1 mM MgCl2, 1 mM DTT, 0.1 mM PMSF) and
subjected to in vitro kinase assays by incubating the immunoprecipitates for 1 h at 37°C in the presence of 20 μl of kinase assay buffer containing 2 μCi of [γ-32P]-ATP (Perkin Elmer, Boston, MA), 0.5 mM ATP, 200 μg/ml phosphatidylinerse, 20 μg/ml diacylglycerol, and 1 μg histone 2B (H2B) (Roche Applied Science, Mannheim, Germany), the latter as exogenous substrate.

4.2.7 ELISA

For MDM experiments, cell free culture supernatants were collected at 1 h, 2 h, 4h, 6h, and 24 h and used to measure TNF-α by ELISA (R&D Systems).

4.2.8 Statistics

A paired one tailed student’s t test was used to analyze the differences between two groups in figures showing one representative figure of three independent experiments. An unpaired one tailed student’s t test was used to analyze differences between two groups in figures showing cumulative data from three independent experiments. Significance was P < 0.05.
4.3 Results

4.3.1 Activation of NOD2 enhances the PMA-mediated pro-inflammatory cytokine response through its signaling cascade intermediates

We began our studies by investigating whether activation of NOD2 leads to an increase in the phorbol-myristate acetate (PMA)-mediated pro-inflammatory cytokine response in macrophages. PMA is a phorbol ester that activates various PKC isoforms by mimicking the structure of diacylglycerol (DAG). Incubation of macrophages with MDP, a NOD2 agonist, is known to induce the production of pro-inflammatory cytokines such as TNF-α (85). MDMs were pre-incubated with the NOD2 agonist MDP for 90 min followed by incubation with PMA. Cell free culture supernatants were analyzed for TNF-α secretion at 1 h, 2 h, and 4 h. The data show a synergistic effect on cytokine production in cells pre-treated with MDP compared to cells incubated with PMA alone (Figure 4.1A). Since our agonist studies indicated a potential link between NOD2 and the cytokine response to PMA, we next wanted to explore if the PMA-induced TNF-α production was dependent on NOD2. Scramble and NOD2 siRNA transfected MDM monolayers were incubated for 72 h to achieve the maximum NOD2 knockdown and subsequently incubated with PMA for 3 h. The cell free culture supernatants were analyzed for cytokine levels by ELISA. We found that NOD2 deficiency leads to a significant decrease in the production of TNF-α in response to PMA (Figure 4.1B). Similar results were found at 6 h and 24 h (data not shown). Thus, our results indicate that pre-engagement and hence activation of NOD2 by its agonist significantly enhances PMA-mediated pro-inflammatory cytokine
production. Furthermore, we show that PMA-mediated cytokine production is NOD2-dependent.

The role of NOD2 in PMA-mediated TNF-α production led us to study the downstream signaling molecules during PMA stimulation of macrophages. RIP2 has been identified as a signaling partner for NOD2 that is autophosphorylated upon interaction with the CARD domain of NOD2 which leads to NF-κB-mediated pro-inflammatory cytokine production (94). MDMs were incubated with PMA or MDP for 0.5 h, 1.5 h, and 3 h. Cell lysates were analyzed for phosphorylation of RIP2 by Western blotting of the phosphorylated protein. The data show that both PMA and MDP phosphorylate RIP2 during incubation but with different intensities. The NOD2 agonist MDP has a distinct kinetic pattern in the activation of RIP2 peaking at 0.5 h and decreasing over time, while PMA more robustly stimulates the phosphorylation of RIP2 and does not cause a wane in expression (Figure 4.1C). In order to understand whether the enhanced expression of RIP2 phosphorylation was NOD2-dependent or mediated by a different mechanism, we used siRNA to knockdown NOD2 in macrophages. Scramble and NOD2 siRNA transfected MDM monolayers were incubated with PMA for 3 h and cell lysates were analyzed for phosphorylation of RIP2 by Western blotting. We observed that PMA-mediated phosphorylation of RIP2 in day 5 MDMs was greatly enhanced when compared to scramble siRNA transfected cells incubated with PMA (Figure 4.1C and D). Even though the level of PMA-mediated RIP2 phosphorylation of scramble siRNA treated cells was lower than day 5 MDMs, we did find that NOD2 deficiency led to a decrease in
RIP2 phosphorylation (Figure 4.1D). Taken together, these results provide evidence for an important role for NOD2 and RIP2 in PMA-mediated signaling pathways such as those involving PKC.

Figure 4.1: The NOD2 signaling pathway is involved in PMA-stimulated cytokine production.

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4.3.2 Phosphorylated PKCδ expression and activity is NOD2-dependent

The ability of PMA to induce pro-inflammatory cytokine production and robustly activate RIP2 in a NOD2-dependent manner led us to formally investigate whether PKC isoforms were involved in this pathway. PMA is a known activator of several PKC isoforms including PKCα, PKCβ, PKCδ, and PKCθ (14). We elected to examine whether the NOD2 agonist, MDP, led to increased phosphorylation of PKC isoforms by using a PKC phospho-array of isoforms, δ, θ, and, α / βII (Figure 4.2A, B, C). MDP caused enhanced phosphorylation of PKC δ, PKCθ, and PKCα / βII. PKCθ is not well characterized in macrophages (327,328). So, we focused our studies on PKCδ and PKCα / βII due to their presence in macrophages and roles in signaling during microbial entry of host cells (320,329). We first compared the NOD2 agonists MDP and GMDP, the latter
is a NOD2 agonist from the cell wall of mycobacteria (see chapter 2), for their abilities to induce PKCδ phosphorylation. MDMs were incubated with MDP or GMDP for 0.5 h, 1.5 h, and 3 h. Cell lysates were analyzed for phosphorylation of PKCδ by Western blotting of the phosphorylated protein. The data show that GMDP leads to phosphorylation of PKCδ at early time points (0.5 h and 1.5 h) and is lost at the later time point (3 h), while MDP only slightly increased the phosphorylation of PKCδ at 0.5 h (Figure 4.2D). Since GMDP was a more potent stimulator of PKCδ phosphorylation, we proceeded in our studies using this agonist.

Detection of phosphorylation of PKC isoforms by Western blotting is not an accurate indicator of the magnitude of its kinase activity because in order to be active the kinase must be phosphorylated at multiple sites. In order to test for PKCδ kinase activity, we performed a PKCδ kinase assay in immunoprecipitates using GMDP and PMA. The results indicate that PKCδ activity is non-existent at 0.5 h and peaks at 1.5 h when stimulated with GMDP (Figure 4.2E). This difference in kinetics of phosphorylation and kinase activity of PKCδ is not surprising, as it may take time for all of the relevant sites to be phosphorylated and the kinase to be fully functional. We also elected to study the kinase activity of PKCα specifically during PMA and GMDP stimulation; however the results were less consistent when compared to delta. Despite phosphorylation of PKCα/βII at 1.5 h and 3 h (Figure 4.2C), the kinase assay shows PKCα is active only at 0.5 h (Figure 4.2F). Also, the kinetics of activation does not match RIP2. PKCα is activated by GMDP at 0.5 h, which is at the same time that RIP2 is phosphorylated,
indicating that they would be working simultaneously. While this may occur it suggests that other mechanisms are involved, making it more complicated to understand the general role of PKCs in NOD2 signaling. Thus, we elected to move forward with studying only PKCδ.

The ability of a NOD2 agonist to fully activate PKCδ led us to study whether NOD2 is important in mediating PKCδ phosphorylation. Scramble and NOD2 siRNA transfected MDM monolayers were incubated with PMA for 3 h and cell lysates were analyzed for phosphorylation of PKCδ by Western blotting. Our results show decreased expression of phosphorylated PKCδ in response to PMA in NOD2 deficient MDMs (Figure 4.2G). We next wanted to determine whether the NOD2-dependent PKCδ phosphorylation was only through the PMA-mediated pathway or also through the NOD2-mediated pathway. Scramble and NOD2 siRNA transfected MDM monolayers were incubated with GMDP for 3 h and cell lysates were analyzed for phosphorylation of PKCδ by Western blotting. Our results show a marked decrease in phosphorylated PKCδ in NOD2 deficient MDMs (Figure 4.2H). These results indicate that NOD2 is important in the classical PKCδ signaling cascade (PMA) and the NOD2-signaling cascade (GMDP).
Figure 4.2: NOD2 and PKCδ agonists stimulate PKCδ phosphorylation and activity in a NOD2-dependent manner.

Day 5 MDM monolayers were incubated with PMA or MDP in RHH for the indicated time points, and cells were lysed with TN-1 lysis buffer and probed for either PKCδ (A), PKCθ (B), or PKCα/βII (C) phosphorylation, and then re-probed for the total proteins, and/or β actin as a loading control. Shown is a representative Western blot from two independent experiments. (D) Day 5 MDM monolayers were incubated with MDP or GMDP in RHH for the indicated time points, and cells were lysed with TN-1 lysis buffer and probed for PKCδ phosphorylation, and then re-probed for total PKCδ. Shown is a representative experiment of two independent experiments. Cell lysates were collected and 250 μg of total protein were immunoprecipitated with

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PKCδ (E) or PKCα (F) antibody or isotype control. Immunoprecipitates were subjected to in vitro kinase assays. Shown are representative kinase assays of phosphorylation of recombinant H2B visualized by autoradiography from two independent experiments. Scramble or NOD2 siRNA-transfected MDM monolayers were incubated with PMA (G) or GMDP (H) in RHH for 3 h and cells were lysed with TN-1 lysis buffer and probed for PKCδ phosphorylation, and then re-probed for total PKCδ and β actin as a loading control. Shown are representative Western blots from three independent experiments.

4.3.3 RIP2 activation is PKCδ-dependent

Our results indicate that NOD2 is necessary for the activation of PKCδ and this result points to it being a potential player in the NOD2 signaling cascade. Therefore we next sought to determine where PKCδ activation fits into the known NOD2 signaling cascade. RIP2 is felt to be the next identified kinase to interact with NOD2, so we studied whether PKCδ is necessary for RIP2 phosphorylation. Scramble and PKCδ siRNA transfected MDM monolayers were incubated with PMA for 3 h and cell lysates were analyzed for phosphorylation of RIP2 by Western blotting. Our results show decreased amounts of phosphorylated RIP2 in PKCδ deficient MDMs (Figure 4.3A). We next wanted know if this PKCδ dependent RIP2 phosphorylation was only through the PMA-induced pathway or also through NOD2-induced pathway. Scramble and PKCδ siRNA transfected MDM monolayers were incubated with GMDP for 1.5 h and cell lysates were analyzed for phosphorylation of RIP2 by Western blotting. Our results show a small but consistent decrease in amounts of phosphorylated RIP2 in PKCδ deficient MDMs (Figure 4.3B).
These results indicate that PKCδ is necessary for RIP2 phosphorylation and suggests that it may be activated upstream of RIP2 during NOD2-mediated signaling.

Figure 4.3: RIP2 phosphorylation mediated by PMA and GMDP is PKCδ-dependent.

Scramble or PKCδ siRNA-transfected MDM monolayers were incubated with PMA for 3 h (A) or GMDP for 1.5 h (B) in RHH and cells were lysed with TN-1 lysis buffer and probed for RIP2 phosphorylation, and then re-probed for β actin as a loading control. Shown are representative Western blots from three independent experiments.
4.4 Discussion

NOD2 is an important intracellular receptor in controlling bacterial infections (77,299). There are many reports on the function of this receptor during bacterial infection, but much less is known about the biochemical mechanisms (i.e. signaling pathways) underlying its activation and ability to mediate its functions. Increased knowledge in this area will not only expand our understanding of the role of NOD2 in host defense but may also provide new targets for host-directed therapies that alter the activity of this receptor. Currently there are reports of several proteins that potentially interact with NOD2 in *in vitro* cell systems, and these reports point towards the ability of NOD2 to interact with proteins other than its main regulator RIP2 (98,112,113,323).

The PKC enzyme family plays a role in pro-inflammatory cytokine production through the transcription factor NF-κB (318). They also play a major role in signal transduction cascades through their ability to phosphorylate hydroxyl groups of serine and threonine residues. A member of the novel PKC family, PKCδ, is of interest in this chapter. Diacylglycerol is necessary in order to activate this novel PKC, however, PMA is used instead of diacylglycerol *in vitro* to mimic its actions (12). Our interest lies in PKCδ because it is found in macrophages and recent findings provide evidence that PKCδ is involved in microbial infections, including those due to *Listeria monocytogenes* and *Helicobacter pylori* (319-321). PKCδ’s established role in signal transduction pathways (including those that regulate ROS production) along with the discovery of its role during microbial infection makes it plausible that it may intersect with pathways involving...
intracellular receptors involved in microbial recognition such as NOD2. Thus, we sought to study the role of PKCδ in the NOD2 signaling cascade in human macrophages (283,325).

We initially found that using the NOD2 agonist MDP as an activator of NOD2 followed by PMA stimulation caused a significant increase in TNF-α production at early time points. We also found that PMA-induced cytokine production was NOD2-dependent. PMA is a phorbol ester that mimics DAG, a lipid involved in second messenger signaling. A major function of DAG is activation of PKCs. Upon activation, PKCs translocate to the plasma membrane and perform their function of phosphorylating other proteins that mediate the induction of cytokines. The substrates that PKCs activate are dependent on the cell type. The recent finding that NOD2 co-localizes with an activator of PKCs, DUOX2, at the plasma membrane indicates a role for PKCs in NOD2 signaling (324).

In our model of human macrophages, we found that PMA led to the phosphorylation of PKCs as expected, but were surprised to find that PMA also led to the phosphorylation of the downstream signaling partner of NOD2, RIP2. The level of RIP2 phosphorylation was in fact much higher than the known inducer of RIP2 phosphorylation, MDP. We also found that this phosphorylation is partially NOD2-dependent. RIP2 is a serine threonine kinase that is felt to act as a scaffolding protein more than a kinase during NF-κB activation; however in NOD2 signaling the kinase activity is important for protein
stability (330). RIP2 has been shown to associate with TNFR1, TRAF1, TRAF5, TRAF6, CIAP1, Cflip, TAK1, as well as caspase-1 (330-333). It is interesting to note that PKCδ interacting protein kinase (DIK), also known as RIP4, shares 45% similarity to the RIP2 kinase domain (334). RIP4 is a membrane-associated kinase with ankyrin repeats that is known to bind PKCβ and contributes to activation of NF-κB (334,335). This activation is dependent on the catalytic activity of RIP4. The ability of RIP2 to interact with other proteins as well as the capability of a RIP family member to interact with PKCs suggested to us that RIP2 may interact with PKCδ. Our findings along with the literature provide evidence for the convergence of the NOD2 and PKC signaling pathways. This discovery raises new possibilities for the role of NOD2 in a number of macrophage host defense functions.

The ability of a NOD2 agonist to induce novel PKC isoforms is of interest. The enhancement of PKCδ activity by NOD2 agonists indicates that NOD2 and PKCδ signaling may be dependent on one another. Our studies show that PKCδ activity is NOD2-dependent when stimulated with its classical agonist, PMA, or the NOD2 agonist GMDP. The discovery that NOD2 is necessary for optimal PKCδ kinase activity is a novel finding and may be an important mechanism for PKC-dependent cytokine production. In order to evaluate the location of PKCδ in the NOD2 pathway, we used a well-established method of knocking down PKCδ (326). We also show that the downstream kinase of NOD2 is dependent on PKCδ, which is of interest. Our results indicate that NOD2 is necessary for PKCδ activity and PKCδ is needed for RIP2
phosphorylation. We speculate in Figure 4.4 that NOD2, PKCδ, and RIP2 may be in complex at the membrane that in turn binds TAK1 for proper NF-κB induced cytokine production to occur. Upon PKC activation, RACK proteins translocate the kinase to the membrane. It has been previously shown that NOD2 and RIP2 are at the plasma membrane and signaling can occur here as well (112,114,279). In addition, we found that PKCα is activated at an earlier time point which implies that this isoform and RIP2 are activated simultaneously. While we did not pursue this area of interest, the data suggest other possible signaling pathways may be involved. For example, NOD2 and PKCα may be involved in earlier events, like p47phox activation for reactive oxygen species production through the NADPH oxidase.

In summary, here we report a new addition to the NOD2 signaling pathway and provide new insight into PKC-induced cytokine production. We show that NOD2 is important for PKCδ activity as well as PMA-induced cytokine production. In addition, we show that RIP2 phosphorylation is PKCδ-dependent. These findings divulge a new mechanism for the NOD2 signaling cascade and for PKC-mediated cytokine production (Figure 4.4). It will be interesting to further define the importance of PKCδ to the NOD2 signaling pathway. These data provide useful knowledge for targeting NOD2 in host-directed therapies to control bacterial infections.
Figure 4.4: PKCδ is necessary for optimal NOD2 activity.

GMDP is able to stimulate PKCδ activity. PMA and GMDP induce PKCδ activation and the release of cytokines, both of which rely on NOD2 for maximal effectiveness. PKCδ is necessary for RIP2 phosphorylation during PMA stimulation and to a lesser extent during GMDP stimulation. It is plausible that upon stimulation, NOD2, RIP2, and PKCδ form a complex that is dependent on all members. This complex may then bind TAK1 and allow for the production of pro-inflammatory cytokines.
The human immune system is a well defined entity that humans depend on to protect them against pathogens. Humans are equipped with a complex immune system that encompasses an adaptive and innate immune response. While both components are necessary in the clearance and eventual immunity against pathogens, the innate immune system is the first line of defense against invading organisms. Epithelial cells, neutrophils, monocytes, and macrophages are cell types that play key roles in protecting the host. Important components of these cells involved in the recognition and clearance of microorganisms are PRRs (1). The innate immune system is not always enough to eliminate pathogens, wherein lies the role of the adaptive immune response. The adaptive immune response protects the host by remembering previous invaders and producing an enhanced immune response in order to control the infection (1,336).

Our laboratory is interested in the innate immune response to the pathogen \(M.\text{tb}\). More specifically we study the interaction of \(M.\text{tb}\) with human macrophages. \(M.\text{tb}\) infects one-third of the world’s population and is a great public health concern. It is easily transmissible by aerosol droplets with a low infectious dose of ten bacilli causing
infection (157). One of the first immune cells that *M. tb* comes in contact with in the alveolus is the alveolar macrophage. While treatments are available, their expense and long regimens make it difficult for under developed countries to control the spread of disease. The recent increase in multi- and extensive-drug resistant strains makes it difficult for even developed countries to control. It is important to gain more understanding of the pathogenesis of *M. tb* in order to be successful in the development of more effective drug therapies and possible vaccines. This study focuses on one of the recently described PRRs found in macrophages, NOD2, and its role during *M. tb* pathogenesis.

In order to study the interaction of NOD2 with *M. tb*, our laboratory uses the most clinically relevant model for *M. tb*, human MDMs and alveolar macrophages. These two cell types are critical during *M. tb* infection and are relevant since this bacterium only naturally infects humans. While most of our studies use MDMs, this work and previous work from our laboratory show that they have similarities to alveolar macrophages. For example, they both express common PRRs such as the MR, TLRs, CRs, and NOD2 (54,77,258). Therefore MDMs are an excellent model to study the first interaction of *M. tb* with the host and understand the early innate immune response.

Prior to our NOD2 studies, data were lacking regarding basal mRNA and protein expression on mononuclear phagocytes during monocyte differentiation into macrophages. Previous NOD2 studies were limited to cell lines, murine macrophages,
and some human monocyte and mixed BAL studies (127,127,135,179-181,220).
Importantly, there are significant differences between the mouse and human promoter of NOD2 (147). Furthermore, the field was only able to identify NOD2 mRNA expression in human PBMCs and not protein. Therefore, we began our research by examining NOD2 expression during monocyte differentiation into macrophages using our MDM model [Chapter 2]. Interestingly, we observed NOD2 mRNA and protein expression is low during day one and two of differentiation and significantly increases at day three of differentiation after which it remains relatively unchanged (Figure 2.1). These data suggest that NOD2 plays a relatively more important role during an effective innate immune response in macrophages, while monocytes more readily rely on other PRRs to control infection. We also observed that NOD2 protein has a punctate rather than diffuse appearance in the cytosol (Figure 2.1, 2.3). This appearance suggests that NOD2 may be located either in organelles or as complexes in the cytoplasm. There also appeared to be less NOD2 protein in human alveolar macrophages than in MDMs (Figure 2.5). Since NOD2 is an important receptor in controlling early infection, this finding provides one explanation for why alveolar macrophages are less effective at controlling initial M.tb infection. However, we were able to show that pre-treatment of alveolar macrophages with the agonist for NOD2, MDP, followed by infection leads to enhanced pro-inflammatory cytokine production (Figure 2.5). Even though NOD2 expression is less in HAMs when compared to MDMs (Figure 2.5), this result implies that this receptor can be up-regulated, at least to some degree, in alveolar macrophages to potentially control infection.
After determining NOD2 expression during the differentiation into macrophages, we examined whether MDP pre-treatment followed by *M. tb* or BCG incubation leads to enhanced cytokine production. We found that stimulating NOD2 with its agonist followed by *M. tb* or BCG leads to increased pro-inflammatory cytokine production. Additionally, these two bacteria are able to activate downstream mediators (RIP2 and IkBα) in the NOD2 signaling cascade, albeit with different expression patterns (Figure 2.2). The differences in expression indicate that *M. tb*, which has a shorter and less robust activation compared to BCG, is able to dampen the immune response. One possibility is that *M. tb* utilizes secreted virulence proteins found in the RD1 to dampen NOD2-mediated signaling. These proteins are absent in the attenuated BCG strain (192,193).

The enhanced pro-inflammatory cytokine production after NOD2 pre-engagement, suggests that NOD2 is involved during mycobacterial infection of macrophages. However, use of an agonist is not the most direct method to study a receptor’s activity. Thus, we next used siRNA knockdown of NOD2 in human macrophages and observed decreased RIP2 phosphorylation and pro-inflammatory cytokine production in response to the two mycobacteria (Figure 2.4). In addition, we found enhanced *M. tb* and BCG growth in NOD2 deficient macrophages (Fig. 2.6). This increase in growth is different from previously published data (135,179), as well as our own data, using NOD2 knockout BMMs incubated with *M. tb* (Figure 2.7). In contrast, BCG showed enhanced growth in NOD2 knockout BMMs suggesting that *M. tb* has acquired a mechanism for
evading the immune system of the mouse, while BCG has not. One possibility is that under normal conditions *M. tb* utilizes virulence proteins, that BCG is lacking (such as those in the RD1 in the genome), in order to inhibit proper NOD2 functioning. These virulence proteins may be more effective in inhibiting NOD2 function in mice because they target the promoter of NOD2, which has been found to be structurally different in mice (147).

It is of interest that NOD2 plays a greater role in IL-1β production during *M. tb* infection, with slight enhancement of pro-IL-1β transcription and a decrease in caspase-1 expression in NOD2 deficient MDMs (Figure 2.4). As one explanation, NOD2 may play a relatively more important role in enabling the release of mature IL-1β through caspase-1 activation in response to *M. tb* than in increasing TNF-α production, since NOD2 has been shown to interact with inflammasomes (97,270).

Our studies of NOD2 involvement during *M. tb* infection lead to unanswered questions, such as how does NOD2 recognize *M. tb* and what molecule(s) does NOD2 regulate in order to affect viability of mycobacteria at 24 h? Several potential mechanisms exist for NOD2 recognition of *M. tb*, including the ability of *M. tb* to escape the phagosome, the bacteria’s ability to secrete virulence proteins, and the possibility that NOD2 signals from the phagosomal membrane. Since our data show that NOD2 regulates cytokine production and *M. tb* growth at 24 h, we reason that *M. tb* is not escaping the phagosome, which has been shown to begin to occur in human dendritic cells at 48 h post infection.
In addition, our results with the attenuated vaccine strain, BCG, argue that secreted virulence proteins are not the sole mechanism for NOD2-mediated recognition of *M.tb*; however, we cannot rule out the possibility that *M.tb* down-regulates downstream signaling molecules important to NOD2 signaling when compared to BCG. Mycobacteria express a hydroxylase, NamH, which glycolylates MDP (GMDP) causing it to induce a strong immune response (86). Our NOD2 confocal data support the possibility that NOD2 localizes at the *M.tb* phagosome membrane. NOD2 signaling in epithelial cells occurs at the plasma membrane and NOD2 must be localized there for this signaling to occur (114,324). Therefore, we hypothesize that NOD2 localizes at the mycobacterium-containing phagosome and is engaged by bacterial cell wall products such as GMDP released during replication of the bacteria inside the phagosome. Such a hypothesis would require that bacterial cell wall products be locally transported to the cytosolic face of the phagosome, possibly through a secretion or transport system. In order to confirm or refute this hypothesis, we would need to either isolate mycobacterium-containing phagosomes to look for the presence of NOD2 by Western blotting or perform immunofluorescence microscopy studies to see if NOD2 is located on the *M.tb*-containing phagosome membrane.

While we observed that NOD2 regulates the viability of *M.tb* and BCG in human macrophages, the mechanism(s) behind this role remains unknown. One possible mechanism that our data support is through the production of pro-inflammatory cytokines which can activate the macrophages through autocrine and paracrine means. We
performed experiments using MDP to “prime” the macrophage to induce early innate immune responses such as ROIs / RNIs in a NOD2-dependent manner. We found a consistent decrease in the growth of BCG and \textit{M.\textit{tb}} under these conditions, but the results were variable (10-26\% decrease for BCG and 4-29\% for \textit{M.\textit{tb}}). Thus, the results are generally consistent with NOD2 being involved in controlling the viability of these mycobacteria, however it is possible that the experimental conditions were not optimized, \textit{e.g.} other parameters such as time and dose should be further tested. In order to determine the role of NOD2 on other early innate immune responses during \textit{M.\textit{tb}} infection, we would need to examine which processes are involved. Such studies would broaden the roles of NOD2 in innate immunity beyond those that involve enhancement of pro-inflammatory cytokine production.

Related to the discussion above, we became interested in what processes NOD2 regulates during infection [Chapter 3]. We began these studies by performing a microarray of NOD2 deficient macrophages infected with \textit{M.\textit{tb}} for 24 h. Interestingly, we observed many of the genes in signaling pathways involved in host defense were regulated by NOD2 in human macrophages including iNOS, autophagy, and apoptosis (Figure 3.1). Although somewhat controversial, studies in the literature support involvement of iNOS, and thus its product NO, during \textit{M.\textit{tb}} infection of human cells (290,291). Thus, we decided to study this enzyme in the context of NOD2. NOD2 agonists have previously been shown to induce iNOS protein expression in mouse macrophages (283), but no one has studied the role of NOD2 in iNOS activity in human macrophages. We observed that
iNOS mRNA and protein are expressed in response to NOD2 agonists (Figure 3.2). It is of interest that higher mRNA expression was observed in response to the mycobacterial NOD2 agonist, GMDP, when compared to the Gram negative / Gram positive NOD2 agonist, MDP, but no differences were observed in iNOS protein expression. One potential explanation for this result is that GMDP is able to induce iNOS protein expression for a longer time period as a result of increased stability of transcript levels. This idea was not studied in the context of the current work but if this was found to be the case, the result would add to our understanding of iNOS transcriptional regulation.

After determining that NOD2 may be involved in iNOS expression through the usage of NOD2 agonists, we more directly assessed the role of NOD2 using siRNA knockdown. NOD2 deficient macrophages treated with GMDP or IFNγ resulted in decreased iNOS expression (Figure 3.2). Thus, these data indicate that NOD2 is involved in iNOS expression in human macrophages. In addition, we observed that NO was enhanced in response to the NOD2 agonist GMDP (Figure 3.2). These results raise the possibility that NOD2 is necessary for NO production in response to invading organisms. Ongoing studies involve assessing NO production in NOD2 deficient cells stimulated with these agonists.

We next explored whether \textit{M.tb} induces iNOS expression and NO production in our MDM model, since the literature is not in agreement on this subject matter (337). We observed enhanced iNOS expression and NO production in response to \textit{M.tb} (Figure 3.3).
In addition we found that *M.tb*-induced iNOS expression is NOD2-dependent (Figure 3.3). It is of interest that NOD2 regulates *M.tb*-induced iNOS expression and potentially NO production. This links a cytosolic PRR with the induction of iNOS and the antimicrobial killing effects of NO. It would be of interest to study which molecules in the iNOS signaling pathway are regulated by NOD2 during *M.tb* infection. This experiment would add to our understanding of the relationship between NOD2 and an antimicrobial mediator, iNOS. It would also be interesting to identify if GMDP is the main component of *M.tb* responsible for iNOS expression. To study this we are currently using *M.tb* deficient in NamH, the gene necessary for the production of GMDP (86), to analyze iNOS expression and NO production.

Since NOD2 is a relatively new PRR and its ability to recognize its agonist is still unknown, we decided to study the signaling cascade to get a better understanding of how NOD2 signals in response to pathogens [Chapter 4]. We began these studies by stimulating NOD2 deficient cells with PMA and observed a decrease in cytokine production. In addition, we found that PMA induces the NOD2 downstream signaling kinase RIP2 in a NOD2-dependent manner (Figure 4.1). This is of interest because PMA induces many PKC isoforms by mimicking DAG, and suggests that NOD2 and PKC signal in concert to produce pro-inflammatory cytokines.

A PKC phospho-array identified PKCδ as a kinase phosphorylated by the NOD2 agonists, MDP and GMDP, and kinase assays confirmed that PKCδ is active (Figure 4.2).
In addition, we observed that PMA and GMDP-induced PKC\(\delta\) phosphorylation is NOD2-dependent (Figure 4.2). These data indicate that NOD2 is a necessary molecule in optimally activating PKC\(\delta\).

To delineate PKC\(\delta\)’s involvement in PMA-induced RIP2 phosphorylation and where PKC\(\delta\) belongs in the NOD2 signaling cascade, we incubated PKC\(\delta\) deficient macrophages with PMA or GMDP. We observed a decrease in RIP2 phosphorylation, indicating that PKC\(\delta\) must be activated prior to RIP2 (Figure 4.3). These data provide new evidence for PKC\(\delta\) being a necessary kinase in the NOD2 signaling pathway. We hypothesize that PKC\(\delta\) is in the NOD2-RIP2 complex, and therefore it would be of interest to perform immunoprecipitations with NOD2 and PKC\(\delta\) or RIP2 and PKC\(\delta\) in order to confirm. Additionally, we would like to know whether this complex binds to the downstream molecule TAK1, which the NOD2-RIP2 complex is known to bind (95,96). Finally we would like to understand the importance to PKC\(\delta\) in the NOD2 signaling cascade. Since PKC\(\delta\) is known to phosphorylate hydroxyl groups on serine and threonine residues, we hypothesize that it does this to RIP2 in order for RIP2 to be active. Currently the literature suggests that RIP2 is autophosphorylated when recruited by its CARD domain to NOD2 (338). More experiments in this area would add to our understanding of the NOD2 signaling cascade that contributes to the innate immune response to invading organisms.
New approaches to the treatment and prevention of inflammatory or infectious diseases could involve the identification of novel activators or inhibitors of NOD2. Since NOD2 mutations (gain and loss of function) are associated with Blau syndrome and Crohn’s disease, future treatment for these diseases could include NOD2 replacement or modifying therapy (depending on the disease) (339). NOD2 polymorphisms in certain populations are associated with susceptibility to TB and leprosy, so NOD2 replacement or modifying therapy could also be given to high risk individuals as a preventative. Our findings that NOD2 is important in controlling early *M.tb* infection of macrophages suggest that activators such as MDP, could be given to individuals with primary infection or potentially to those with reactivation disease. MDP has been used for decades as a key component in complete Freund’s adjuvant to elicit adjuvant activity to injected antigens, and the use of MDP in immunotherapy of infections and cancer is currently being studied (340,341). However, since NOD2 gain of function mutations result in Blau syndrome, MDP therapy should be used in moderation and studies should be done to test for the correct dosage and length of administration.

In summary, work in this dissertation adds to our understanding of the complex role of the cytosolic receptor, NOD2, in innate immunity. We focused on the role of NOD2 during *M.tb* infection. This PRR has been shown to regulate NF-κB and subsequent pro-inflammatory cytokine secretion in response to specific bacterial products. In this dissertation, we observed in human macrophages the role of NOD2 in cytokine secretion and viability of *M.tb*, its importance in *M.tb*-induced iNOS expression, and its
relationship to a new molecule in the NOD2 signaling cascade. In addition, we
optimized antibodies for the detection of NOD2 in human cells, which led to the first
report of NOD2 protein expression in human macrophages. Furthermore, the
contribution of a new model, siRNA mediated knockdown of NOD2, to study NOD2 in
human cells is of great importance to the field. We would argue that previous studies
using mouse models have given misleading results on the role of NOD2 in TB. As a
result of work presented in this dissertation, we have elucidated the role of NOD2 using a
more clinically relevant model, which has the potential of identifying new drug targets to
treat or prevent the disease. Our studies on the relationship between NOD2 and iNOS
bring us a step closer to understanding the role of NOD2 in controlling the viability of
M. tb in human macrophages. Moreover, our reports on the involvement of PKCδ in the
NOD2 signaling cascade will be beneficial in future drug development studies. In all, our
data provide strong evidence for the role of NOD2 during M. tb infection of human
macrophages. Further studies will identify how NOD2 recognizes M. tb, the molecules
involved in controlling M. tb viability, whether NOD2 is directly involved in NO
production, and the importance of PKCδ in the NOD2 signaling cascade, thus further
increasing our knowledge of the role of NOD2 in innate immunity.


148


149


153


156


157


168


