The Role of NOD-Mediated Innate Immune Activation in Lung Epithelia

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Abstract

The ability to recognize and remove pathogens that invade the lower airway, while maintaining homeostasis in the lung microenvironment, is an essential function of the innate immune system. An evolving hypothesis in this relatively new area of research is that in addition to maintaining normal host function, the molecular machinery responsible for pathogen recognition and activation of the innate immune response, may also contribute to chronic inflammatory diseases if a break down in normal function occurs [9].

This thesis focuses on the investigation of NOD-like receptor proteins (NLRs) which are involved in intracellular pathogen recognition and activation of the innate immune response. Recent work by others has identified that NOD1 and NOD2, of the NLR family, are critical components in intestinal inflammatory diseases [1]. An association between NOD1 and NOD2 with autoimmune diseases of the lung, primarily asthma and sarcoidosis respectively, has also been described [3,11]. The main goal of this thesis is to investigate the intracellular NLRs present in lung epithelium and their role in directing innate immune activation. The specific hypothesis is that NOD1 and NOD2 activate the innate protein complex called the "signalosome" leading to NF-κB activation in human lung epithelia.

Data presented show that NOD1 and NOD2 along with RIP2, a key signaling protein of the NLR pathway, are constitutively present and associate in the lung epithelia. Upon recognition of the PAMP, iE-DAP, NOD1 facilitates physical recruitment of RIP2 activating the "signalosome". Furthermore exposure to the NOD1 specific agonist, iE-DAP, but not NOD2 specific agonist, MDP, result in increased release of Interleukin 6 and Interleukin 8 mediated through the activation of the transcription factor NF-κB. These findings provide a framework to explain the recognition of gram negative bacterial invasion by lung epithelium and provide a valid model for future studies that will investigate the proximal entry of PAMPs into the lung epithelium thereby activating the "signalosome" upon NOD recognition and signal transduction.

In conclusion our findings indicate that, NOD1 activates the "signalosome" machinery resulting in activation of the NF-κB pathway thereby inducing proinflammatory cytokine and chemokine release. This highlights the importance of NOD1 function in bacterial clearance and epithelial homeostasis, as well as, a potential for aberrant immune responses potentiated by dysregualtion in lung pathogenesis and inflammatory diseases.

Introduction

All organisms must protect themselves from attack by foreign invaders. The vast biodiversity among microorganisms requires that the host immune system can function effectively in terms of detection and elimination. Therefore it is no surprise that multiple mechanisms of defense have evolved in humans. The interaction and cooperation, of such a network, provides for an accurate and precise defense system; precise by distinguishing self from non-self and accurate by eliminating foreign invaders and not host tissue.

Pulmonary Host Defense

The lumen of the lung is kept sterile by a system of very effective defenses that protect the interior of the body. The first consists of physical and chemical barriers that prevent the entrance of infectious agents into tissues of the lung. The second line of defense consists of cellular and chemical defenses that exist to remove and destroy infectious agents that have gained entrance to these tissues [10]. These pulmonary defense mechanisms are therefore essential in recognizing and removing pathogens, while maintaining barrier function and tissue homeostasis in the lung microenvironment.

Innate Immune Defense in the Lung

When microorganisms breech the anatomical barrier, additional defense is required. First, the system must have the ability to recognize common pathogenic structures that distinguish it as foreign[10]. Indeed, the innate immune defense system consists of germ-line encoded receptors and effecter molecules that recognize highly conserved pathogen motifs [11]. These pathogen recognition receptors (PRRs) "sense" the presence of invading microbes and recruit effecter molecules that in turn initiate the host defense response thereby protecting and ultimately maintaining normal tissue function [9]. A well recognized effector cell in this process is the macrophage that upon recognition engulfs and digests pathogens thereby effectively killing it.

The Lung Epithelium

The epithelium of the human airway is responsible for forming a barrier that divides the airway from the interstitial space [12]. It is a diverse population of ciliated cell types that form tight junctions and secrete mucosa and antimicrobial peptides. The mucociliary clearance system removes infectious agents from the airway of the lung by propelling mucus containing pathogenic particles up the airway were it is eliminated [8]. Also present in the mucosal layer are the antimicrobial peptides lysozymes, cathelicidin, and beta defensins that inhibit and kill microorganisms [6, 8]. Upon bacterial invasion these enzymes liberate bacterial motifs that play a crucial roll in innate sensory recognition and immune activation in the lung [9, 11]. Impairment of these functions results in longer residence time leading to pathogen colonization, propagation and infection. The dynamic interplay between barrier defense and innate defense is central to lung epithelial function and host homeostasis.

Molecular Aspects of NOD-mediated Immune Activation

Pathogen-associated molecular patterns (PAMPs) are components of microorganisms that are recognized by specific receptors of the innate immune system. Categorically, three pathogen recognition receptor types (PRRs) are known: extracellular complement, transmembrane toll-like receptors (TLRs), and intracellular nucleotide-binding oligermerisation domain (NOD) proteins. We will focus our attention on investigation of NOD1 and NOD2 and evaluate their role in pathogen recognition (of PAMPs) in the lung epithelium leading to activation of the innate immune system. PAMP recognition is known to occur by NOD proteins via interaction with a luceine rich repeat region (LRR) located at their C-terminus. The amino terminus of NODs harbors a caspase-recruitment domain (CARD) that promotes protein interaction with caspase-1 or Receptor Interacting Protein 2 (RIP2) [5,9]. RIP2 is a serine-threonine kinase adapter molecule that is recruited into the signalosome following PAMP recognition by NODs, leading to NF-κB activation and cytokine production [5].

Innate Immunity in Health and Disease

Innate immunity is essential for protection of the lung and functions to help maintain barrier function and host homeostasis in the lung microenvironment. However, as shown in other disease states, disorders of the innate immune response can lead to disease. Immunodeficiency diseases occur when the immune response is less then sufficient resulting in life-threatening infection. Alternatively, autoimmune diseases are the result of a hyperactive immune response. The pathogenesis of such disease is the result of dysregulation of the molecular machinery responsible for regulating innate activity.

Scope of this Thesis

The general aim of this thesis has been to investigate how the innate immune system senses intracellular pathogen invaders and relays that information through an effector pathway in the lung epithelia. In particular, the involvement of intracellular recognition of bacterial dipeptides by NOD receptors triggering “signalosome” recruitment and subsequent activation of NF-κB –mediated transcription was the subject of our investigation. Based on preliminary studies, our goal became more focused on NOD1.

1 Molecular Biology

1.1 Plasmids

-NOD1

The human full length NOD1 cDNA was a generous gift of the Nunez laboratory. The 2862 bp cDNA sequence (accession: NM_006092) was inserted into a pcDNA3 vector with KpnI and XhoI restriction sites with a CMV promoter region and N-terminal flag tag.

-*Firefly* Luciferase

Dr. Anasuya Sarkar (Wewers laboratory) graciously provided the firefly plasmid. It was unknown to what construct vector the firefly luciferase was inserted.

- *Renilla* Luciferase

Sudarshan Seshadri (Wewers Laboratory) graciously provided the Renilla plasmid.

1.2 Transformation and Propagation of Plasmids

Note: All plasmids followed the same protocol.

MAX Efficiency® DH5α™ competent cells (Invitrogen) were thawed on wet ice. 100 ng of was mixed with 50 µl of competent cells. Cells were incubated on ice for 30 minutes followed by a 45 second heat-shock in a 42°C water bath. Cells were returned to ice for 2 minutes upon which 900 µl Super optimal catabolite repression (S.O.C.) medium (Invitrogen) was added and then shaken on an orbital shaker at 225 rpm (37°C) for 1 hour. Then on a Luria-Bertani (LB) plus ampicillin agar 100 µl of competent cell mixture was spread an incubated at 37°C overnight. 2 ml LB medium and 100 µg/ml Ampicillin was inoculated with a single colony and incubated for 6 hours at 37°C with vigorous shaking. Upon which time a 1:500 dilution of starter culture in LB medium was incubated overnight at 37°C with vigorous shaking (500 µl : 250 ml) . 2 ml of cultured bacteria plus 20% glycerol was stored at -20°C.

1.3 Isolation and Purification of Plasmids

The protocol below is for the Promega Wizard® Plus Midiprep DNA Purification System.

100 ml of bacterial culture was centrifuged at 6,000g for 15 minutes at 4°C to pellet the cells. Using 3 ml of Cell Resuspension Solution to suspend the cells an additional 3 ml of Cell Lysis Solution was added then added. Once suspension became clear (no greater than 5 minutes) 3 ml of neutralization Solution was added to inhibit further cell lysis. Another centrifugation at 14,000g for 15 minutes at 4°C was performed. Supernatant containing DNA was decanted and saved in a 50 ml falcon tube; avoiding white precipitant. 10 ml of resin was added to DNA and transferred to Midicolumn. Vacuum was applied; releasing when liquid had passed through the column. 15 ml of wash solution containing ethanol was added and vacuum applied once more; again releasing vacuum when liquid had passed through the column. A wash was repeated once more, however, this time vacuum was continued for approximately 30 seconds after liquid had passed through the column. The Midicolum was separated from the reservoir and placed in a 1.5 ml microcentrifuge tube and centrifuged at 10,000g for 2 minutes.

1.4 Elution and Quantitation of Plasmids

The midicolumn was placed in a new 1.5 ml tube and 300 µl of (70°C) RNAse/DNAse free water added. After 1 minute elapsed the tube was centrifuged at 10,000g for 20 seconds to elute the DNA. Elute was then centrifuged an additional 5 minutes at 10,000g to pellet resin fines. Supernatant was then transferred to a new centrifuge tube and stored at -20°C. Additionally, a sample 1:49 dilution was performed and using a spectrophotometer concentration was determined.

2 Cellular Biology

Experiments were performed using sterile technique and a laminar flow hood. Care has been taken to ensure that all solutions were sterilized by autoclaving and/or filtration through a 0.22 µm filter unit (Nalgene), and that the DNAs used for tranfection were not contaminated.

2.1 Biochemicals

All chemicals and reagents used for the studies were of the highest chemical grade and were available commercially. Ala-γ-D-Glu-Diaminopimelic acid (γ-iE-DAP) and Ala-D-Glu-DAP (iE-DAP) were purchased from Anaspec (San Jose, CA). N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) and Glycyl-sarcosine (Gly-Sar) was purchased from Sigma (St. Louis, MO).

2.2 Primary Cell Culture

Primary human lung upper airway epithelial cells (hUAECs) were isolated by enzymatic dissociation of trachea, bronchi, and bronchioles of adult lungs. Cells were seeded on a collagen coated, semi-permeable matrix (0.6 cm2; Millicell-HA, Millipore, Bedford, Massachusetts) and grown at an air-liquid interface. The donor lung was held constant for all experiments (TBD 016). The hUAECs were maintained with a 1:1 mixture of Dulbecco's modified Eagle's media andHam's F12 media (DMEM/F12), supplemented with 2% Ultroser G (BioSepra; Villeneuve, La Garenne, France) and antibiotics. 24 hours pre-experiment medium was replaced with DMEM plus 10% fetal bovine serum no antibiotics unless otherwise noted.

2.2.1 Immune Activation Studies

Culture inserts were washed twice and conditioned in phosphate buffer saline 1X (PBS) for 20 minutes followed by washing with sterile apical transport buffer (AP) (DMEM/F-12 w/o phenol red, 50:50 mix, pH 6.5). 300 µl of basal transport buffer (BL) (DMEM/F-12 w/o phenol red, 50:50 mix, pH 7.9; Gibco, Carlsbad, CA) was placed on the basolateral side and 100 µl of AP buffer on the apical side. hLEC's were treated apically with 5 µg/mL of the bacterial dipeptide diaminopimelic acid (γ-ieDAP) or muramyl dipeptide (MDP). For competitive inhibition studies, the apical chamber received a 10,000-fold excess of GlySar (0.1mM) prior to the bacterial dipeptide challenge. At the end of a 4 hour incubation the hLEC's were washed three times and placed normal culture medium (DMEM plus 10% FBS; Cellgro-Mediatech, Inc.). The cell supernatants from the apical and basolateral chambers was collected and pooled at the end of 24 hours, snap frozen, and stored at -80˚C for cytokine analysis.

2.2.2 Cytokine Determination

Measurement of human IL-6 and IL-8 concentrations in cell culture supernatants was performed using the *Immulite* automated chemiluminometer (Diagnostic Products Corporation, Los Angeles, CA) by Dr. Mark Hall. Manufacturer-supplied standards were used for calibration prior to sample analysis. Samples were measured over a range of dilutions to validate accuracy.

2.2.3 Western Analysis of hUAECs

Cell lysis buffer (Cell Signaling, Beverly, MA) with 1 mM PMSF and 1X Complete Mini^{TM} protease inhibitor cocktail (Roche) was directly added to the apical surface of the transwell chamber after removal of the culture medium from the basolateral chamber. Cells were scraped and collected with lysis buffer and then centrifuged at 13,200 rpm for 10 min at 4°C. Supernatants were quantified by protein assay (Bio-Rad, Hercules, CA) and then mixed with Laemmli buffer (Bio-Rad) containing 5% (vol/vol) 2-mercaptoethanol, boiled for 5 min, separated on 10% SDS-PAGE gel (Bio-Rad), and transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% (wt/vol) nonfat milk in phosphate-buffered saline (PBS)-0.1% Tween 20 (PBS/T) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C. After washing, the membranes were incubated with the secondary antibody for 1 h at room temperature. Signal was detected with an ECL kit (Amersham Biosciences) and a Fluor-S Multi-Imager Max/Bio-quantity one (Bio-Rad). The following antibodies were used in our experiments: anti-NOD1 (1:1,000; IMGENEX, San Diego, CA); anti-NOD1 (1:1,000; Dr. Philpott, Paris, France); anti-RIP2 (1:1,000; Cell Signaling, Beverly, MA); and goat anti-rabbit IgG-HRP (1:3,000; 1:10,000; Zymed, San Francisco, CA).

2.2.4 Immunoprecipitation Experiments of hUAECs

Cultured hUAECs were stimulated with 250 units/mL of IFN-γ, or 100 ng/mL of TNF-α. After 24 hours cells were recovered and lysed in Cell lysis buffer (Cell Signaling, Beverly, MA) with 1 mM PMSF and 1X Complete Mini^{TM} protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation (10 min at 13,200rpm), protein quantified (as described above), and aliquoted in 200 μg/200 μL samples. Samples were pre-cleared by incubation of Recombinant Protein G (rProtein G) Agarose (Invitrogen Corporation, Carlsbad, California) for 30 minutes with cell lysates. Immunoprecipitation of NOD1 was performed by incubating overnight at 4 °C with commercially available anti-NOD1 (2 μL) antibody or pre-immune rabbit serum (2 μL) followed by a 1-hr incubation with rProtein G. Immune complexes were washed extensively with lysis buffer, boiled in Laemmli buffer and separated by SDS/PAGE using a 10% gel. Proteins were then transferred on to nitrocellulose membranes and detected by immunoblotting with the anti-NOD1 antibody from Dr. Philpott.

3 HEK 293 Cell Line Culture

HEK-293T cells (Human embryonic kidney) were used for some *in vitro* experiments; as they do not constitutivley express NOD1. They were maintained in Dulbecco's modified Eagle medium low glucose 1X (Gibco) supplemented with 10% FBS. Cells were grown in an incubator at 37°C in presence of 5 % of CO2.

3.1 NOD1 Expression

3.1.1 Cell Transfection

Cells were plated on a six well plate a day before transfection using DMEM plus 10% FBS and no antibiotics. On the day of transfection one sterile 1.5 ml centrifuge tube was designated *Tube 1* and DMEM medium and DNA was added. Another was designated *Tube 2* and DMEM medium and Lipfectamine 2000 (Invitrogen) was added; let this sit for 5 minutes. The tubes were then combined into one tube and sat for 30 minutes at room temperature. This allows for the DNA/Lipo to complex. While waiting for complexes to form, cell medium was removed and washed once with DMEM (No FBS). After the 30 minutes transpired the DNA/Lipo complexes were gently added to the cells. Once added the plate was gently rocked back and forth. Then additional DMEM was added to bring the final transfection volume up to the desired volume. The tranfections were placed inside the 37°C incubator for 2 hours. After 2 hours the transfection medium was washed replaced with DMEM plus 10% FBS and stored overnight in a 37°C incubator.

Note: The amounts of DNA and Lipofectamine are based on the chart below:

plate	# cells	DNA	DNA media vol	Lipo 2000	Lipo media vol	total transf. vol
96 well	2 x 104	0.1 ug	10 ul	1 ul	10 ul	50 ul
8 w chamber	1 x 105	0.25 ug	25 ul	1 ul	25 ul	100 ul
24 well	2 x 105	0.5 ug	50 ul	1.75 ul	50 ul	200 ul
12 well	1 x 106	0.7 ug	100 ul	2.5 ul	100 ul	400 ul
6 well	2 x 106	1.0 ug	150 ul	5 ul	150 ul	800 ul
60 mm dish	2-5 x 106	2.0-5.0 ug	300 ul	20 ul	300 ul	3 mL

3.1.2 Western Analysis

Cell lysis buffer (Cell Signaling, Beverly, MA) with 1 mM PMSF and 1X Complete Mini^{TM} protease inhibitor cocktail (Roche) was directly added to the surface of the cells. Cells were scraped and collected with lysis buffer and then centrifuged at 13,200 rpm for 10 min at 4°C. Supernatants were quantified by protein assay (Bio-Rad, Hercules, CA) and then mixed with Laemmli buffer (Bio-Rad) containing 5% (vol/vol) 2-mercaptoethanol, boiled for 5 min, separated on 10% SDS-PAGE gel (Bio-Rad), and transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% (wt/vol) nonfat milk in phosphate-buffered saline (PBS)-0.1% Tween 20 (PBS/T) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C. After washing, the membranes were incubated with the secondary antibody for 1 h at room temperature. Signal was detected with an ECL kit (Amersham Biosciences) and a Fluor-S Multi-Imager Max/Bio-quantity one (Bio-Rad). The following antibodies were used in our experiments: anti-NOD1 (1:1,000; IMGENEX, San Diego, CA); anti-NOD1 (1:1,000; Dr. Philpott, Paris, France); anti-flag (1:1,000; Sigma-Aldrich, St. Louis, MI); and goat anti-rabbit IgG-HRP (1:3,000; 1:10,000; Zymed, San Francisco, CA).

4 BEAS-2B Cell Line Culture

The BEAS-2B cell line was derived by transforming human bronchial cells with an adenovirus 12-simian virus 40 construct. They were maintained in DMEM supplemented with 10% FBS. BEAS-2B cells were used because of the difficulty in the transfection of primary cells and are a good model cell.

4.1 Co-Immunoprecipitation of RIP2

The BEAS-2B cell line was transfected with 1 μg flag tagged NOD1. 24 hours post cells were stimulated with 5 μg/ml iE-DAP for 0, 5, 15, and 30 minutes. Cells were recovered and lysed in Cell lysis buffer (Cell Signaling, Beverly, MA) with 1 mM PMSF and 1X Complete Mini^{TM} protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation (10 min at 13,200rpm), protein quantified (as described above), and aliquoted in 200 μg/1000 μL samples. Immunoprecipitation of flag tagged NOD1 was performed by incubating overnight at 4 °C with commercially available monoclonal anti-flag (1 μL) (Sigma) antibody or mouse IgG (1 μL) followed by a 1-hr incubation with anti-flag M2 beads (Sigma). Immune complexes were washed extensively with lysis buffer, boiled in Laemmli buffer and separated by SDS/PAGE using a 10% gel. Proteins were then transferred on to nitrocellulose membranes and detected by immunoblotting with the anti-RIP2 antibody from Dr. Wewers lab.

4.2 NF-κB Activation Study

The protocol is for the Promega® Luciferase Assay System. It is important to remember that all reagents and samples equilibrate to room temperature prior to performing an assay.

Cells were transfected, as previously mentioned with HEK 293 cells, this time with firefly luciferase. However, after the two hours of transfection cells were washed once and then submersed in 1 ml of AP buffer (pH= 6.5). A dose of 5 μg/ml of iE-DAP was then administered. The plate was then placed in a 37°C incubator for 0, 2, 6, 12, and 24 hours. 1X PBS was used to wash the cells and then 100 μl of Passive Lysis Buffer (Promega) was added to the surface of the cells. Cells were scrapped on ice, collected, vortexed for 15 seconds, and then centrifuged at 13,200 rpm for 10 minutes at 4°C. 100 μl of Luciferase Assay Reagent was dispensed into each luminometer tube (two tubes per sample). 20 μl of cell lysate was added to a luminometer tube and mixed by pipetting 3 times. Then the tube was placed in the luminometer and read. Luciferase values were normalized for transfection efficiency by protein concentration. NK-κB assays were performed once in triplicate.

4.2 Small Interfering RNA (siRNA) Assay

The following NOD1 siRNA used in this study was purchased from Dharmacon (Lafayette, CO). BEAS-2B cells were transfected with 5-nM ON-TARGETplus™ SMARTpool® siRNA by using HiPerfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. 48 hours after transfection, cells were subjected to western analysis.

4.3 Real-Time PCR

RNA was isolated with TRIzol reagent followed by reverse transcrition of 2 μg of total RNA with ThermoScript RNase H^- reverse transcriptase (Invitrogen Corporation, Carlsbad, California) then diluted to 100 μl. Between 20 to 60 ng of cDNA was used for quantitative PCR with SYBR green I PCR master mixture and the PRISM 7700 apparatus (Applied Biosystems). The total volume of the PCR reaction was set at 20 μl and included 2 μl of cDNA template and 0.25 μM of each primer. The NOD1/CARD4 primer was designed with the following criteria: to give an 80- to 200-nt amplicon, GC range of 30–70%, melting temperature of 59–60°C, free of secondary structure, and a free-energy change (ΔG) of less than or equal to –7 kcal/mol. Primer pairs intentionally spanned introns to avoid false negatives by amplification of genomic DNA. Relative copy numbers (RCN) and expression ratios of NOD1 was normalized to two housekeeping genes [GAPDH and CAP-1 (cAMP-accessory protein)] and calculated with the following equation: $RCN = E^{-Ct} \times 100$ (refer to Gavrilin *et al*).

Results

1 Constitutive Presence of NLRs in Lung Epithelium

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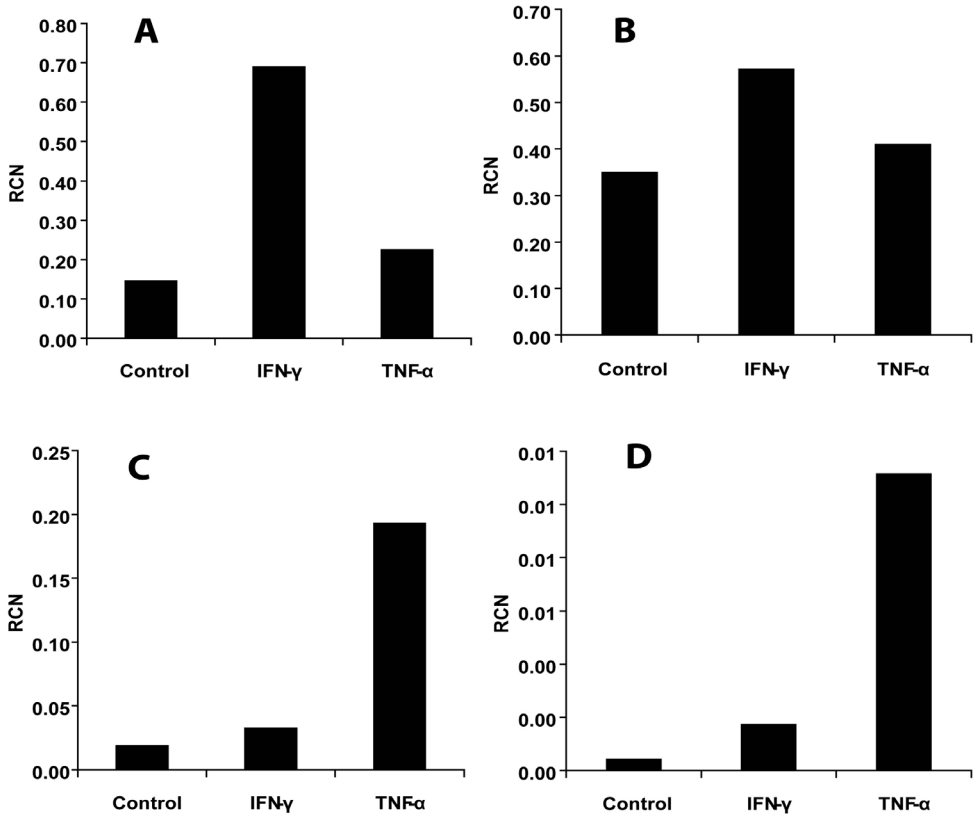
1.1 RT-PCR Analysis

To determine whether hUAECs and BEAS-2B cells in fact had the ability to express NOD protein, we examined steady state message RNA levels under baseline and cytokine challenged conditions. RT-PCR analysis was used to quantify NOD expression. Our results show that both hUAECs and BEAS-2B cells express endogenous levels of messenger RNA for NOD1 (Fig 1.1 A,B) and NOD2 (Fig 1.1 C,D). The data also reveals NOD1 inducibility by IFN-γ (Fig 1.1 A,B) and NOD2 inducibility by TNF-α (Fig 1.1 C,D) and was anticipated based upon the previously published work of others (Ref). This was observed in a parallel fashion across epithelial cultures.



***Figue 1.1*: Quantitative characterization of transcriptional message expression of CARD4/NOD1 in hUAECs**. (A) IFN-γ induces NOD1 mRNA in primary lung epithelial cells. (B) TNF-α induces NOD2 mRNA in primary lung epithelial cells. RT-PCR analysis for NOD mRNA following the addition of 250 units/mL IFN-γ, and 100 ng/mL TNF-α stimulation for 24hrs. GAPDH and CAP were used as the internal control to standardized and quantify mRNA expression.

1.2 Immunoprecipitation (IP) Assay

To confirm protein expression we examined NOD1 by Western analysis. In order to concentrate NOD protein in our samples an IP was performed on both hUAECs and BEAS-2B cells. From this we were able to effectively probe NOD1 at 107 Kd under baseline, IFN-γ and TNF-α conditions. Consistent with RT-PCR data, our results show both a constitutive and inducible expression of NOD1 protein (Fig 1.2).

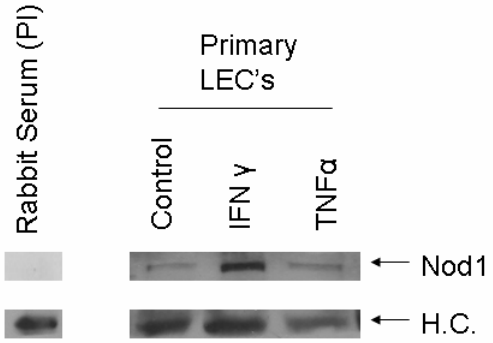


Fig 1. **Effect of IFNγ on Nod1/Card4 Protein Expression**. Protein obtained from primary lung epithelial cells . Pre-immune rabbit serum was used as a negative control. Whole cell lysates obtained from LEC's cultured with 250 units/mL of IFNγ, or 100 ng/mL of TNFα, for 24 hours were immunoprecipitated with affinity-purified anti-Nod1/Card4 commercially available antibody (IMGENEX), and then immuno-blotted with borrowed anti-Nod1/Card4 antibody.

1.3 Western Analysis

To determine the presence of key molecules involved in the NLR-mediated signalosome response in the lung epithelium, cells were analyzed under baseline conditions. Standard Western analysis was performed on different cell types and immuno-blotted for RIP2 and NOD1, both involved in innate immune activation. Our results catalog the presence of RIP2, ASC, ICE, and other essential players in the NLR pathway in the lung epithelium (Fig 1.3).

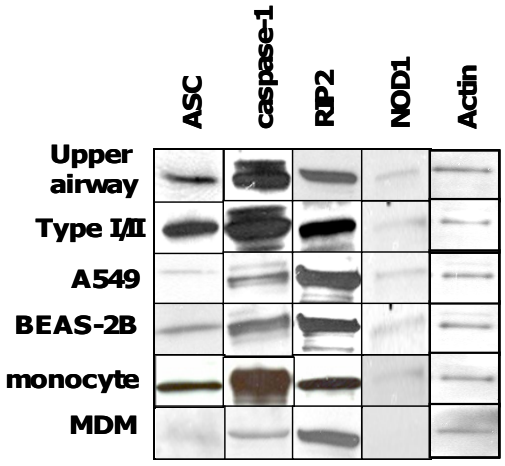


Figure 1.3: **Constitutive Presence of NLR proteins.** Western analysis was performed on different cell types that included the human lung epithelial cell lines A549 and BEAS2B as well as primary human upper airway epithelia.

2 Immune Activation Upon NOD1 Specific Agonist

2.2 Cytokine Assay

After characterizing the presence of NOD1, NOD2 and RIP2 we set out to determine if the lung epithelium was sensitive to NOD specific agonists. To measure this we ran a cytokine profile for Interleukin-6 and Interleukin-8 release in the supernatant of hUAECs and BEAS-2B cells following exposure to NOD specific agonists. Our results show increased secretion of IL-6 and IL-8 in primary (Figure 2.3) and BEAS-2B cells (Figure 2.2) upon iE-DAP, but not MDP treatment. Based on these findings and knowing that iE-DAP is specific for NOD1, we turned our attention to NOD1 in subsequent studies.

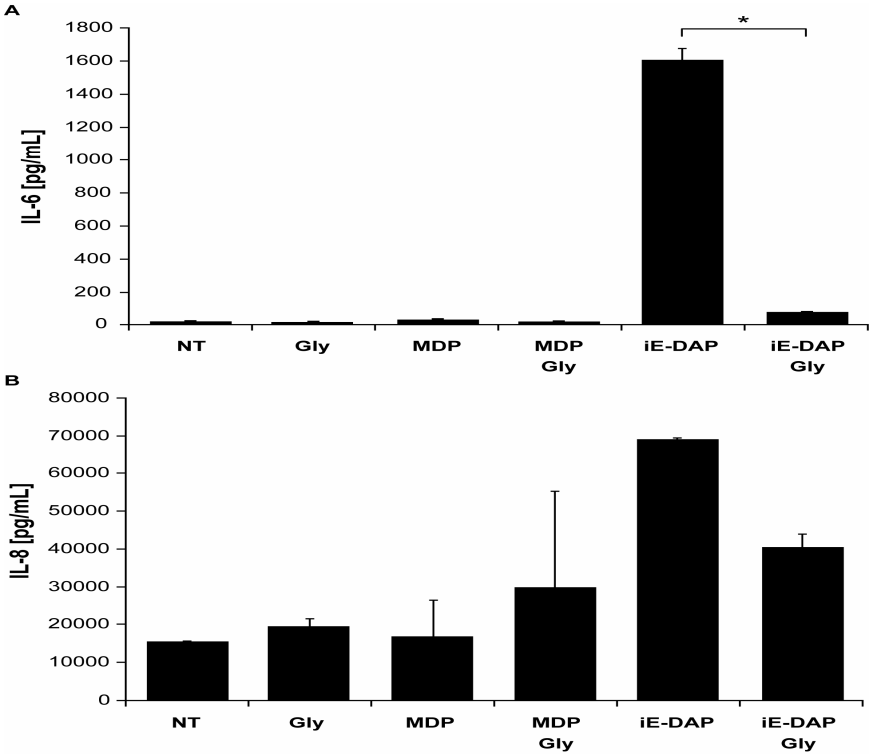


Figure 2.3: **Immune activation upon NOD1 specific agonist, but not NOD2 specific agonist.** hUAECs were treated apically with 5 μg/mL of the bacterial dipeptide diaminopimelic acid (γ-ieDAP) or muramyl dipeptide (MDP). The cell supernatants from the apical and basolateral chambers was collected and pooled at the end of 24 hours for cytokine analysis.

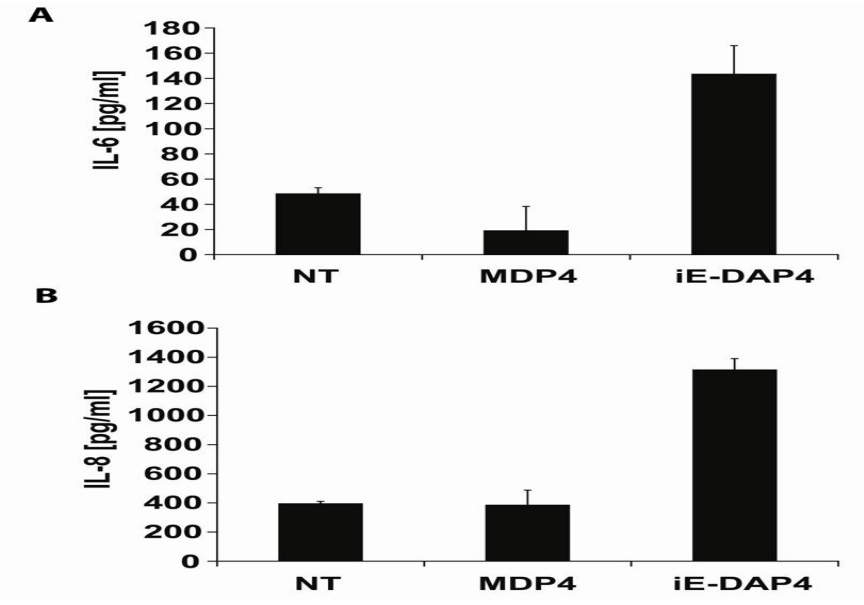


Figure 2.2: **Immune activation upon NOD1 specific agonist, but not NOD2 specific agonist**. BEAS-2B cells were treated apically with 5 μg/mL of the bacterial dipeptide diaminopimelic acid (γ-ieDAP) or muramyl dipeptide (MDP). The cell supernatants were collected at the end of 24 hours for cytokine analysis.

3 Nuclear factor-kappa B (NF-κB) Activation

3.1 NF-κB Luciferase Assay

To further confirm the role of NOD1 protein in mediating the effects of iE-DAP, downstream activation of the transcription factor NF-κB was measured to elucidate transactivation of IL-6 and IL-8. A firefly luciferase reporter was overexpressed in the BEAS-2B cell line. Cells were treated with iE-DAP following transfection of the luciferase plasmid. Results demonstrate a peak in NF-κB activity as early as 2 hours that persisted out to 24 hours (Fig 3.1).

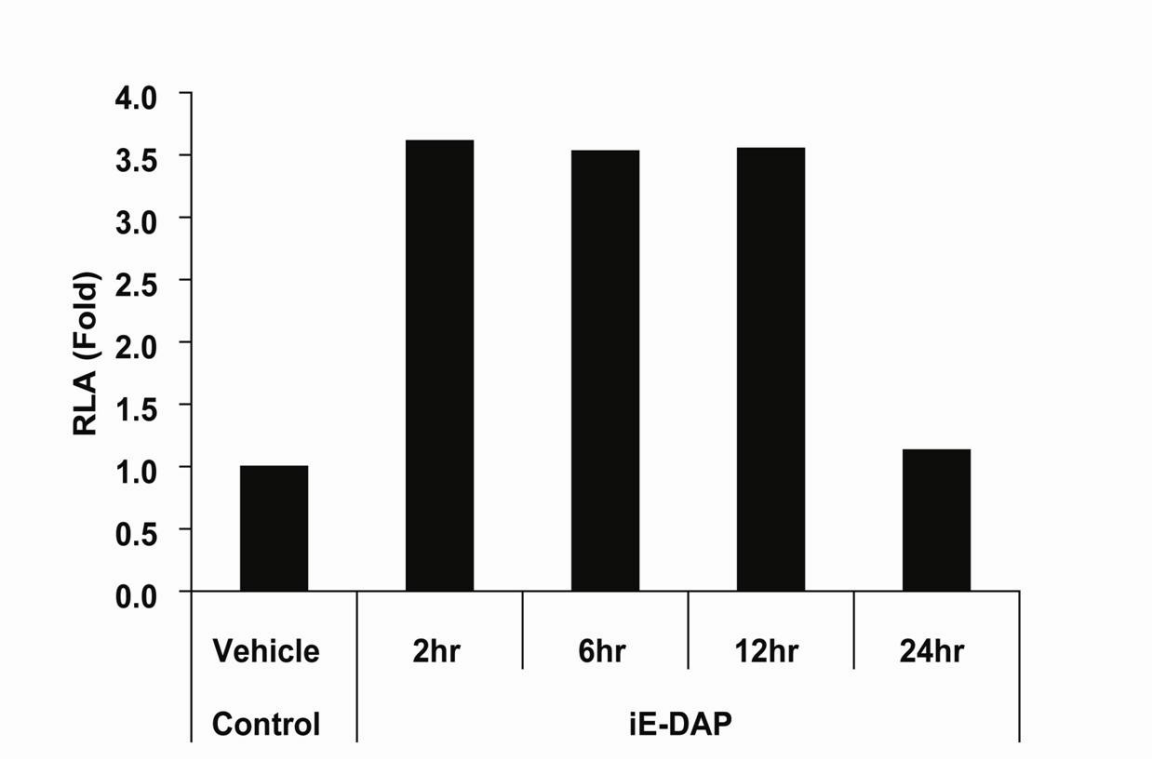


***Figure 3.1*: Activation of the transcription factor NF-κB upon NOD1 specific agonist challenge.** BEAS-2B cells were transfected with 1 µg of the firefly reporter. Two hours post transfection cells were administered 5 µg/ml iE-DAP for 0, 2, 6, 12, and 24 hours. NF-κB-mediated luciferase was expressed as early as 2 hours post treatment. Luciferase units were normalized to total protein concentrations

3.2 Dual NF-κB Luciferase Assay

In order to validate specificity of the firefly reporter assay an internal control was incorporated to normalize the relative luciferase units measured upon iE-DAP treatment. A pilot study to optimize the conditions of a dual luciferase assay was performed. Our results show that 1) Firefly reporter activity increases with a decrease in renilla reporter concentration (Figure 3.2A) and that renilla reporter shows decreased sensitivity in a dose dependent manner (Fig 3.2B). Thus, a 1:10,000 fold dilution of renilla plasmid will be used in future studies involving the activation of the transcriptional factor NF-κB.

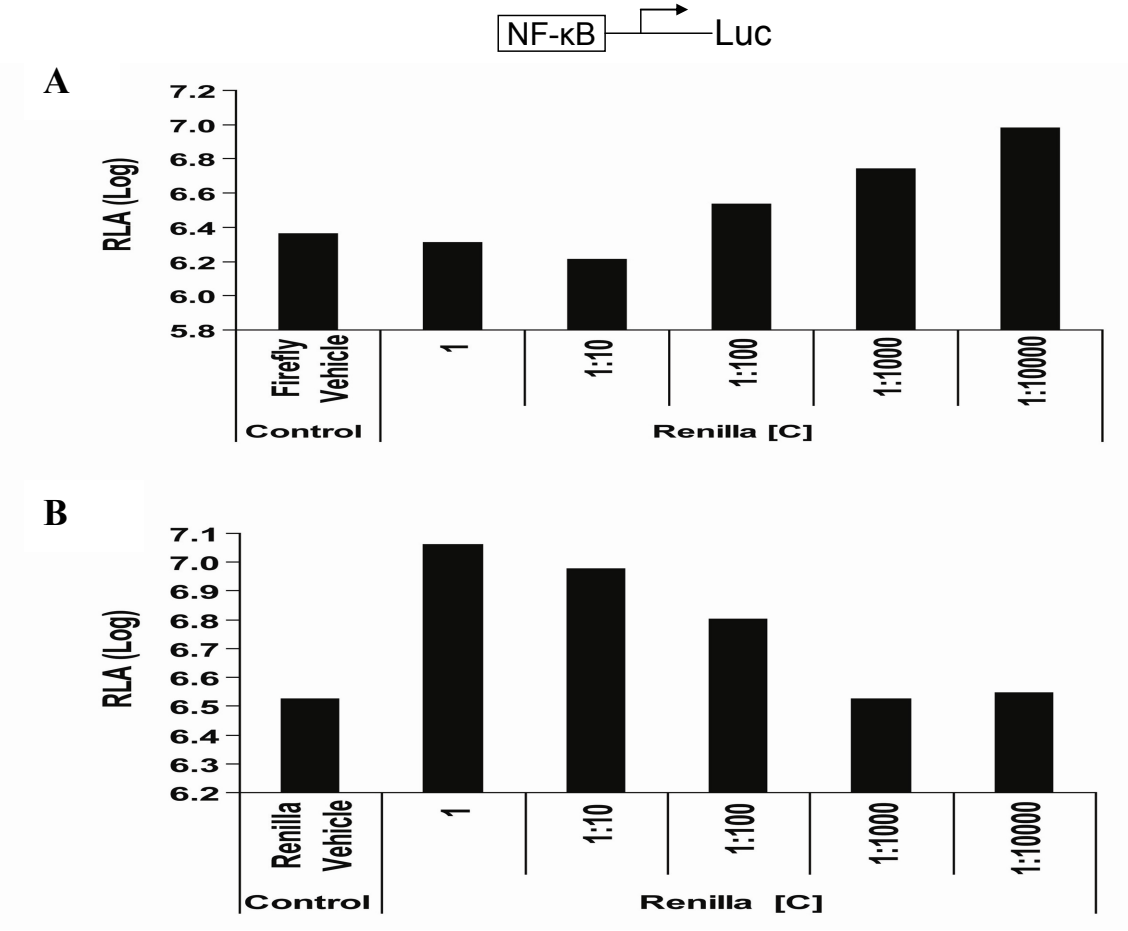


***Figure 3.2*: Optimization parameters of the dual luciferase assay.** (A) Firefly reporter activity increases with a decrease in renilla reporter concentration. (B) renilla reporter shows decreased sensitivity in a dose dependent manner

4 NOD1 Activates the Signalosome

4.1 Transfection of Flag Tagged NOD1

HEK-293 cells, a cell line that does not naturally express NOD1, was transfected with 1μg of a Flag-tagged NOD1 plasmid construct. Western analysis was performed to evaluate NOD1 protein expression. Figure 4.1A confirms successful transfection and overexpression of NOD1 in HEK-293 cells. Transfection efficiencies over 70% are routinely achievable (data not shown).

4.2 Co-Immunoprecipitation Study

Transfection of Flag-tagged NOD1 was performed in BEAS-2B cells. At 24 hours post-transfection, cells were exposed to increasing concentrations of iE-DAP followed by IP pull-down of cell lysates with Flag antibodies and the indicated time points. Immunoblots were then conducted with an anti-human-RPI2 antibody. The results show physical recruitment of RIP2 (to NOD1) at 15 and 30 minutes (Fig 4.1B). From this we conclude that NOD1 and RIP2 interaction, a key step in initiating formation of the signalosome, occurs following recognition of the gram-negative bacterial dipeptide iE-DAP.

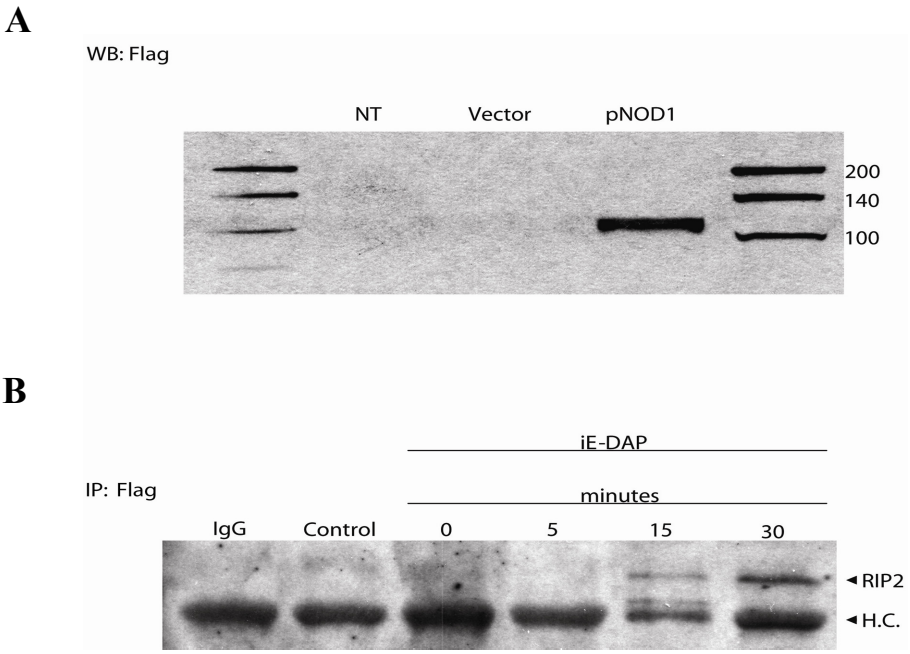


***Figure 4.1*: Transfection studies using NOD1-flag** (A) Expression of NOD1-flag protein after transfection into HEK-293 cells. Western analysis was performed on HEK-293 cells by loading 40 μg whole cell lysate on a 10% gel and immuno-blotted with monoclonal anti-flag antibody (Sigma). (B) Interaction between NOD1 and RIP2 upon NOD1 agonist in BEAS-2B cells. Co-Immunoprecipitation of RIP2 was performed in BEAS-2B whole cell extracts following treatment with 5μg/mL iE-DAP in a time dependent manner. Mouse IgG was used as a negative isotype control. Lysates were immunoprecipitated with monoclonal anti-Flag antibody (Sigma) and then immuno-blotted with anti-RIP2 antibody (rabbit).

5 NOD1 Silencing

5.1 siNOD1 Experiment

A pilot study was performed using four small interfering RNA's that are complimentary to NOD1 mRNA. BEAS-2B cells were treated with siNOD1 and then treated with IFN-γ and blotted for NOD1. Densitometry was used to quantify protein expression. Results show 98% inhibition of NOD1 protein expression in the BEAS-2B cell (Figure 5.1). This will allow us to further address the essential requirement and specificity of NOD1 in future studies.

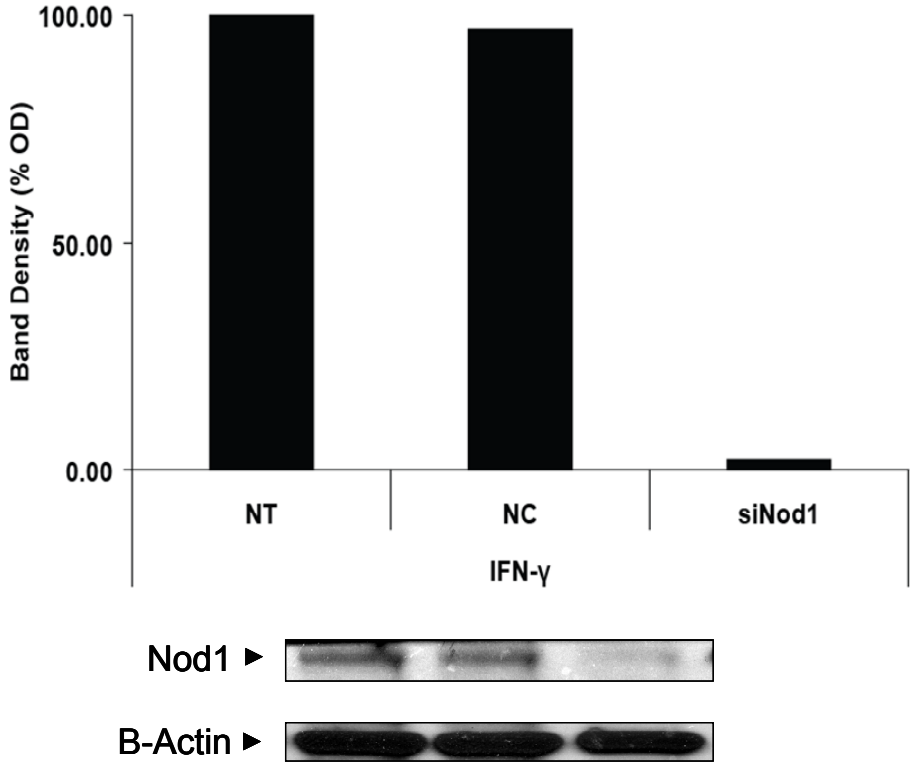


***Figure 5.1*: Suppression of NOD1 protein expression.** BEAS-2B cells were transfected with siNOD1. 24 hours later cells were stimulated with 250 U/ml IFN-γ for 24 hours. Western analysis was performed on whole cell lysates with 80 μg of total protein loaded on a 10% gel and immuno-bloted with anti-NOD1/Card4 antibody (IMGENEX)

Discussion

To date, studies involving NOD-mediated immune activation has largely focused on the phagocytes and gut epithelium. Based on literature review, there are no studies involving lung epithelia. Our first experiments set out to characterize the presence of NOD1 and NOD2 and at the same time, catalog the presence of other NLR-related effector molecules. Our results demonstrate the endogenous expression of NOD1 and NOD2 mRNA and NOD1, RIP2, ICE, and ASC protein in the lung epithelium. Work by others had previously reported that the pro-inflammatory cytokines IFN-γ and TNF-α induce NOD1 and NOD2 expression, respectively, in gut epithlia (1). Our data shows an identical response in the lung epithelium proving that NOD protein exists constitutively and can also be induced following cytokine exposure (acute inflammation).

The next phase of investigation was to determine NOD-mediated immune activation by NOD specific agonists. It has been reported that iE-DAP and MDP are the minimal di-peptides required for NOD1 and NOD2 recognition, respectively, resulting in signal transduction and immune activation. Our results demonstrate that iE-DAP specifically induces IL-6 and IL-8; key markers of the innate immune response. Alternatively, MDP did not lead to immune activation as measured by IL-6 and IL-8 release, suggesting that NOD1, but not NOD2, plays a critical role in NLR signaling and activation of the innate immune response. Since both NOD proteins are present in the lung epithelium it may be that proximal events determine NOD recognition although this remains to be studied. The mechanism by which these di-peptides gain entrance into the cell has also not been elucidated. This is an important question that will be addressed in future studies.

The promotor regions of both IL-6 and IL-8 become activated by the transcription factor NF-κB. Results from the firefly luciferase assay demonstrate that iE-DAP induces activation of the NF- κB pathway. This suggests that NOD1-mediated events may result following NF-κB activation thereby resulting in cytokine and chemokine production and release. NOD1-mediated participation of NF-κB-mediated expression of other innate immune effector molecules is an important question to be addressed in future studies. Our results, however, do not rule out the possibility that iE-DAP may be activating additional signaling pathways. In future studies we will utilize the NF-κB-mediated promotor dual luciferase assay and NOD1-specific small interfering RNA knockdown to determine if NOD1 is an essential requirement for the lung epithelial response to iE-DAP.

How NODs mediate the activation of the NF-κB pathway is still poorly understood. RIP2 an adaptor protein with a serine threonine kinase and a caspase recruitment domain interacts with NOD1 through a homophilic -CARDCARD interaction. Our preliminary results show supportive evidence that physical recruitment of RIP2 to NOD1 occur upon iE-DAP exposure. This supports the concept that binding of NOD1 and RIP2 activates a multimeric protein complex of other signal-transducing molecules coined the "signalosome" thereby activating the transcription factor NF-κB. These intermediary signaling molecules leading to NF-κB activation by NOD1 will be also be evaluated in future studies.

In summary, our results demonstrate that the innate immune recognition of bacteria involves the lung epithelium, in addition to other immune type cells. Mounting evidence suggests a vital role for NOD1 in bacterial recognition and activation of the signalosome complex thereby activating transcription factor NF-κB releasing IL-6 and IL-8. Thus, lung epithelial cells which largely constitute the physical front line defense against microbes, also play an important role through sensory perception and the expression of proinflammatory cytokines and chemokines that can recruite additional inflammatory cells into the lung microenvironment to enhance host defense.

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