NOD2 AND THE INNATE IMMUNE DEFENSE

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The innate immune response is the first barrier against external stimuli arising from disease-causing pathogens. The primary membrane associated Toll-Like receptors (TLRs) play an important role in the innate immune response by recognizing pathogen-associated molecular patterns (PAMPs). Recently nucleotide-binding oligomerization domain (NOD) proteins have been shown to serve as intracellular receptors that are also involved in the innate immune response. Caveolae are small plasma membrane invaginations that exist in a wide range of cell types. The overall goals of this proposal are to examine the molecular determinants of the interaction of the intracellular pathway (NOD2) with plasma membrane (TLR2) mediated events in the response of epithelial cells to bacterial pathogens and to identify the role of caveolae in the signaling events that may underlie this association. Revealing the mechanisms of interaction between NOD2 and pathogens has significant importance to control or prevent Crohn's disease and benefits the public health issues. Accordingly, the specific aims are:

Aim 1: To reveal the molecular mechanism for the interaction between NOD2 and MDP. We hypothesize that a cognitive sequence in the LRR of NOD2 recognizes MDP and the cognitive sequence accounts for the interaction of PGN from Gram-positive microorganism in polarized epithelial cells.

Aim 2: to determine the requisite role of NOD2/MDP pathway in response to Gram-positive infection. We hypothesize that constitutive levels of NOD2 are low in polarized epithelial cells. Gram-positive infection results in synthesis of TNFα that increases NOD2

expression and TNFα in an autocrine fashion and enables the cells to participate in host defense via synthesis of IL-8.

Aim 3: to identify an interaction between NOD2 and TLR2 pathways in mediating Gram-positive bacterium *Staphylococcus aureus* activation (IL-8 synthesis) in polarized MDCK cells. IL-8 synthesis in response to gram-positive infection will be contrasted in wildtype MDCK cells vs. cells in which NOD2, TLR2 or both have been silenced by siRNA. We also hypothesize that cross talk between NOD2 and TLR2 pathways occurs at a MAP kinase step and accordingly, experiments will be repeated in the presence of pharmacological (or genetic, e.g. dominant negative) inihibitors of p38, JNK and MAPK.

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1.   Background and Significance

Crohn's disease (CD) is an inflammatory disorder that is characterized by transmural inflammation, epithelial ulceration, fissure formation, and stenosis of segments of the entire gastrointestinal tract[1, 2]. There is a significant environmental influence on the development of Crohn's disease, but a genetic element also plays a role; at least five separate genetic loci have been linked to Crohn's disease. Nucleotide-binding oligomerization domain protein NOD2(Card15), the first susceptibility gene for CD, has also been found to be an intracellular recognition receptor of the muramyl dipeptide component of peptidoglycan (PGN)[3]. However, it's not yet known in detail how the mutation of NOD2 is connected to the pathogenesis of CD. Since the NOD2 gene product is thought to be an intracellular receptor for bacterial wall products, microbial factors have been the recent focus of attention. Through a better understanding of the mechanisms of interaction between NOD2 and pathogens, we may uncover novel means to utilize the anti-bacterial function of NOD2 to develop strategies to control or prevent CD.

1.1.   Innate immunity:

Organisms depend on innate and adaptive immune responses to defend themselves against invaded pathogens. Innate immunity relies on specific pattern-recognition receptors (PRRs) to recognize the pathogen-associated molecular patterns (PAMPs) thus creating the immune system first line of defense. It is well known that Toll-like receptors (TLRs) play a key role in mediating responsiveness to bacteria and bacterial products in cells of the myeloid

lineage. TLRs set the tone and pace of the inflammatory response that follows initial contact with

pathogens within different periods of time. To date, more than 10 TLRs have been identified as

well as their potential ligands. For example, TLR4 recognizes lipopolysaccharide (LPS), TLR3

recognizes double-stranded RNA, and TLR9 recognizes bacterial DNA. All TLRs are

transmembrane molecules and express on either typical immune cells such as macrophages,

neutrophils, dendritic cells, or epithelial cells. The stimulation of TLRs by their agonists requires

the recruitment of specific adaptor molecule including MyD88. An overview of TLR signaling

pathways is shown in figure 1[4].
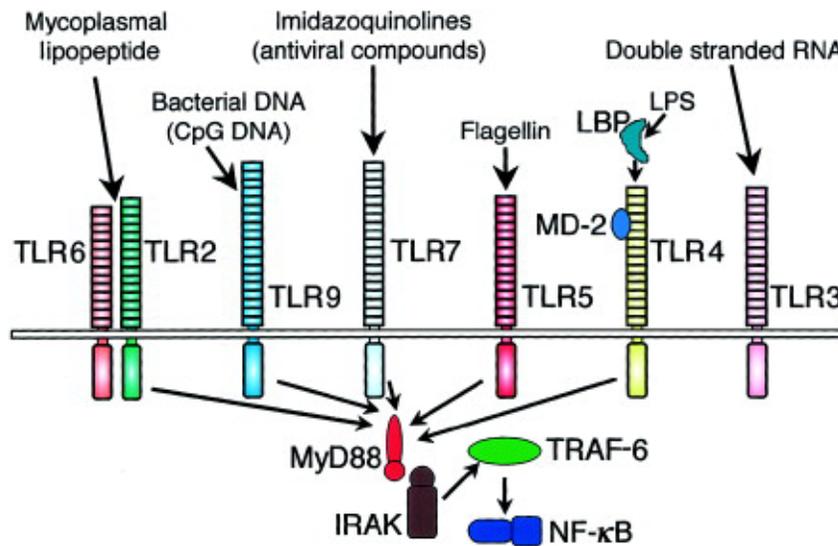
Figure 1: Ligands recognized by Toll-like receptor (TLR) family.

Among TLRs, the study of function of TLR2 has been addressed in relatively more

detail. Ligand recognition and signaling of TLR2 is a little more complicated than other TLRs.

The characters and function of TLR2 have been noted as: a) TLR2 is a cell-activating receptor

for gram-positive bacteria (For example, *Staphylococcus* and *Streptococcus*), mycobacteria, and

for the cell wall components (lipoteichoic acid lipopeptides, lipoproteins et al); b) TLR2 mainly

recognizes its ligands as a heterodimer with either TLR1 or TLR6 (some other TLRs or non-TLR molecules will also form the heterodimerization with TLR2) [5]; c) CD14 is a major co-receptor required for signaling induced by TLR2 ligands. For example, upon binding to ligand, CD14 can efficiently transfer as well as enhance the recognition of TLR2 to pathogen components [6]; d) Like other TLRs, the TLR2 signaling is significant, because it induces antigen presenting cell activation, pro-inflammatory cytokine production, and increases expression of co-stimulatory ligand expression. Alteration or lack of TLR2 function *in vivo* may lead to decreased protection response required by TLR2. Evidence strongly supports the critical role of TLR2 in the induction of innate immune responses and improved acquired immunity.

TLRs are mainly expressed at the surface of innate immune-responsive cells and detect microbes in the extracellular compartment. In recent reports, it was found that two nucleotide-binding oligomerization domain (NOD) family member proteins, Nod1 (Card4) and NOD2 (Card15), can detect intracellular pathogens that avoid TLR-mediated detection[7]. The responsiveness conferred by NODs is MyD88-independent. Since both Nod1 and NOD2 are cytoplasm proteins, these findings highlight that Nod1 and NOD2 are intracellular PRRs for peptidoglycan.

1.2. NOD2 gene and protein scheme:

NOD proteins belong to a growing family of cytosolic factors[8]. NOD proteins have three functional domains— an amino-terminal effector-binding domain (EBD) (which mediates binding to effector molecules, controlling the downstream events), a centrally located NOD (Which mediates self-oligomerization for downstream effector molecules activation), and carboxy-terminal leucine-rich repeats (LRRs) (Fig.2.)[1]. The caspase-recruitment domain (CARD) and the pyrin domain (PYD) are two EBDs. The LRRs are important for ligand

recognition. Mutagenesis in the LRRs abolishes the NF-κB activation of NOD2 upon MDP stimulation. Although functional properties have been demonstrated, limited data has been published to address the question of weather or not NOD2 will interact with MDP[1] directly or indirectly. **In this proposal, a series of studies will be conducted to examine the mechanism of the interaction of NOD2 with MDP. We will test the influence of LRR domain mutation on the interaction of NOD2 with MDP in an *in vitro* assay system as well as in a cell culture system.**
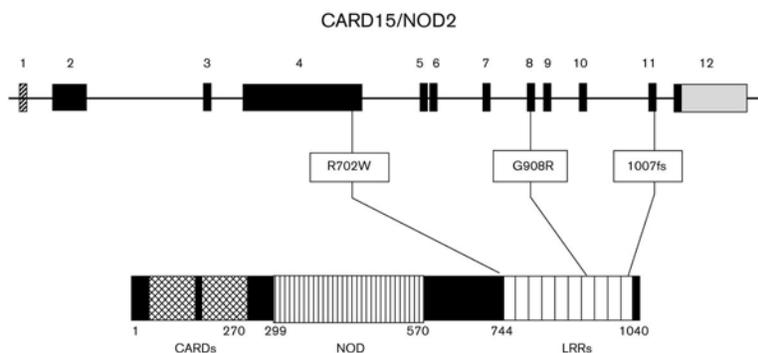
Figure 2:   The NOD2/CARD15 gene contains 12 exons. The encoded protein contains two caspase recruitment domains (CARDs), a nucleotide binding domain (NBD) and 10 leucine-rich repeats (LRRs)[1].

1.3.   NOD2 ligand specificity and signal transduction:

In contrast to Nod1 which is broadly expressed in tissues[9], the expression of NOD2 seems to be more restricted to monocytes. Recent reports showed that NOD2 was also expressed in granulocytes, dendritic cells, and to a lesser extent in T lymphocytes[10]. In inflammatory conditions, NOD2 expression levels are significantly up-regulated in myelomonocytic and epithelial cells upon stimulation with TNFα and bacterial degradation products. Results from Aldhous et al.[11] showed that the expression of NOD2 in the Paneth cell could be critical in the pathogenesis of Crohn's disease.

Inohara et al. and Bertin et al.[9, 12] first reported that Nod1 could activate the NF-κB pathway when overexpressed in the human embryonic kidney cell-line 293T(HEK 293). Very recently, Girardin and Chamaillard et al. [13-15] identified NOD1 as an intracellular sensor for specific peptidoglycan (PGN) muropeptides. By excluding the possibility of contamination of bacterial cell-wall components, further results confirmed that NOD2 is a PRR for PGN[15]. So besides the extracellular guarding system of TLRs, NOD proteins have been discovered for intracellular sensing of bacteria in mammals. NOD2 recognizes both Gram-negative and Gram-positive bacteria through the N-acetylmuramyl dipeptide (MDP) component in their PGN.

Signaling through NOD2 is mediated through the kinase RICK, which interacts with NOD2 through homophilic CARD-CARD interactions. Experiments on mouse embryo fibroblasts lacking in RICK showed that Nod1 and NOD2 do not activate NF-κB[16], but as long as exogenous RICK is added in, NF-κB activity can be restored. An intermediate region exists between the CARD and the kinase domain of RICK that interacts with IKK complex regulatory subunit IKKγ[9, 12], thus linking Nod1 and presumably NOD2 to the IKK. In addition to NF-κB, Nod1 has been reported to activate c-JUN N-terminal kinase (JNK) in response to bacteria but the concrete mechanism is unclear. It is also unclear how TLR2 and NOD2 collaborate with each other. Both TLR2 and NOD2 are important PRRs for Gram-positive bacterial and are involved in the recognition of same bacterial product, either outside or inside cells. **In this proposal we will try to find out the detail overlap, or redundant functional relationship between NOD2 and TLR2**.

1.4.   MDP:

Peptidoglycan (PGN) and lipopolysaccharide (LPS) are the two most commonly studied components of Gram-positive and Gram-negative bacterial cell walls. Muramyldipeptide (MDP) is the minimum structural moiety of bacterial peptidoglycan (PGN) for adjuvant and related activities[17]. MDP is ubiquitously distributed in bacterial cell walls and expresses especially high levels in Gram-positive bacteria. The receptor and signaling system for MDP were shown to be different from that for PGN, suggesting the presence of a recognition system other than CD14/TLR. MDP activated human monocytic cells in a CD14-, TLR2- and TLR4-independent manner to increase MyD88 expression[17]. MDP not only synergizes with LPS but also acts similarly with PGN to induce the synthesis of TNFα in the human monocytic cell line Mono Mac 6[18]. In quiescent epithelial cells, the expression of NOD2 is low or null, but the up-regulation of NOD2 is significant after TNFα stimulation.

1.5.   Caveolae localization and function:

Caveolae have gained attention in recent years because of the discoveries that caveolae play a role in the cellular uptake and intracellular delivery of certain bacterial toxins, viruses, and bacteria[19-21]. Caveolae are rich in cholesterol, sphingolipids, caveolin-1 and are considered to be a subset of lipid rafts[22]. They are flask-shaped invaginations 50-100 nanometers in diameter and are highly ordered microdomains residing within the plasma membrane. The intracellular trafficking of bacteria via caveolae is the pathway different from the classical route of ligands internalized by clathrin-medited endocytosis. Caveolins are the major protein component of caveolae. The caveolin gene family has three members in vertebrates: caveolin-1, caveolin-2, and caveolin-3. Caveolins distribute predominantly at the plasma membrane but also in the Golgi, the endoplasmic reticulum, in vesicles, and at cytosolic locations. Caveolae are expressed

ubiquitously in mammals and certain continuous endothelial and epithelial cells[23, 24]. The finding that silencing the expression of caveolin protein decreased the uptake of pathogen reagents confirmed the important role of caveolae in the vesicle docking and transport into the cell. The high concentration of signaling molecules (such as heterotrimeric G proteins, non-receptor tyrosine kinases, et al.) determines the versatile functions of caveolae. Caveolins participate in many important cellular processes, including vesicular transport, cholesterol homeostasis, signal transduction, and tumor suppression. Recent documents states that caveolae constitute an alternative endocytic pathway to clathrin-coated pits and transport their select cargo for ultimate delivery to the Golgi[25]endoplasmic reticulum(ER)[26], or lysosome [27]. Caveolae have participated in Gram-negative bacteria endocytosis. We predict a similar function of caveolae for internalization of Gram-positive bacteria. MDP is one known ligand for NOD2, but how epithelial cells take up MDP is poorly understood. The mechanisms by which Gram-positive bacteria enter the cell, and by which MDP is recognized by NOD2 have not been addressed. **We propose to examine the role of caveolae in the NOD2/MDP pathway.**

Although there have been years of intensive research devoted to Crohn disease (CD), the etiology of the disease is still unknown. In 2001, NOD2, the first susceptibility gene for CD was discovered. Although mutational assays and epidemiological data have shown that patients with mutations on the NOD2 gene are susceptible to CD disease, it remains difficult to establish the impact of these discoveries on the clinical management of patients. Revealing the molecular mechanism of Nod2 function may provide a better understanding the pathogenesis of Crohn's disease [28].

2.   Preliminary Studies:

2.1.   To reveal the molecular mechanism for the interaction between NOD2 and MDP.

2.1.1.   NOD2 is a general sensor of bacterial PGN but not of LPS:

Commercial and endotoxin-free purified bacterial PGN triggered NF-κB in NOD2-transfected cells instead of LPS. This kind of PGN-induced activation was NOD2-dose dependent and required the LRR domain. In figure 3, HEK293 cells (transfected with or without NOD2) were treated with different PAMPs. The results suggested that NOD2 confers responsiveness to PGN, but not to LPS. When NOD2 was mutated at the LRR domain, PGN could not trigger NF-κB in NOD2-transfected cells. (Fig.3)[29, 30]. Study also revealed that Nod2 was a general sensor of peptidoglycan through MDP detection[15].
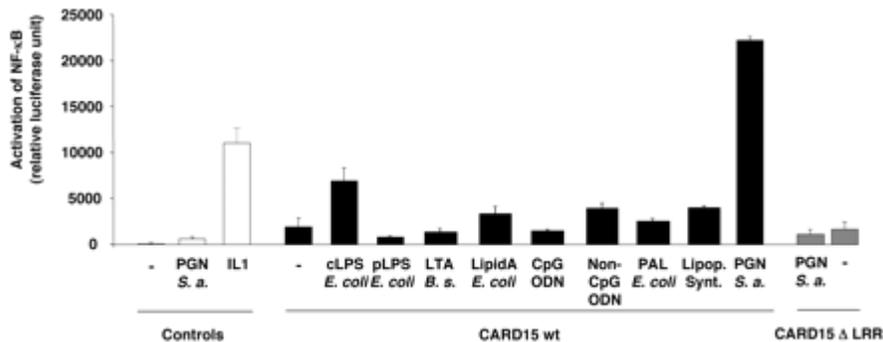
Figure 3: Differential sensing of cytosolic PAMP by NOD2.

The delivery of MDP to the cytosol varies in different pathogens and cells. Invasive pathogens penetrate the cells freely. Phagocytic cells containing intracellular hydrolases can digest bacterial peptidoglycan, resulting in the release of muropeptides. Lysozyme presenting in the serum might also process extracellular peptidoglycan to release muropeptides. Some intracellular bacteria like Listeria could produce muropeptides during replication. Nonetheless, it's still unclear how muropeptides enter the cytosol from extracellular compartments or from the

phagosome across phagosome membranes. Whether MDP directly binds to NOD2 or interacts with this protein via other intracellular factors has to be determined. The mechanism concerning how NOD2 recognizes MDP is waiting for further investigation. **In this proposal we will try to investigate the interaction between NOD2 with MDP as well as the role of caveolae in the MDP internalization and NOD2/MDP interaction**.

2.1.2.   Caveolae involve in Gram-negative bacterial invasion:

It's well known that Nod2 is a cytosolic protein and recognizes the minimal structure of bacterial cell wall component peptidoglycan — MDP. Studies have shown that degradation of peptidoglycan into MDP may be caused by enzymes like ceradase. It's not known how the bacteria and their components come inside the cell and how the cytosolic protein NOD2 recognizes the MDP. Clathrin-coated vesicles are the general pathway for bacterial endocytosis. However, pathogens may skip to the alternative pathway such as caveolae to enter into the cells to avoid the entry of lysosome. Caveolae have also been found in immune cells like macrophage, monocyte, or dendritic cells[31], which suggesting that caveolae may participate in the cell surveillance for pathogen invasion.

Indeed, caveolae have been found as portals of entry for microbes, at least for Gram-negative bacterial invasion. Various concentrations of either filipin or cyclodextrin were incubated with confluent monolayers of Human Brain Microvascular Endothelial Cells (HBMEC) before performing the *E. coli* invasion assays. Caveolae disrupters Filipin as well as cyclodextrin were found to be effective in blocking the invasion of OmpA$^+$ *E. coli* (E44) in a dose-dependent manner (Figure 4) [32]. In contrast, the binding activity was not influenced. Caveolae may also play a role in the entry of bacteria into other nonphagocytic cells like epithelial cells[33]. Since

caveolae help Gram-negative bacterial invasion, we predict that caveolae also participate in the Gram-positive bacterial internalization. **In this study we will examine if caveolae is involved in 1) the binding of MDP to NOD2, and 2) the MDP-induced IL-8 synthesis.**
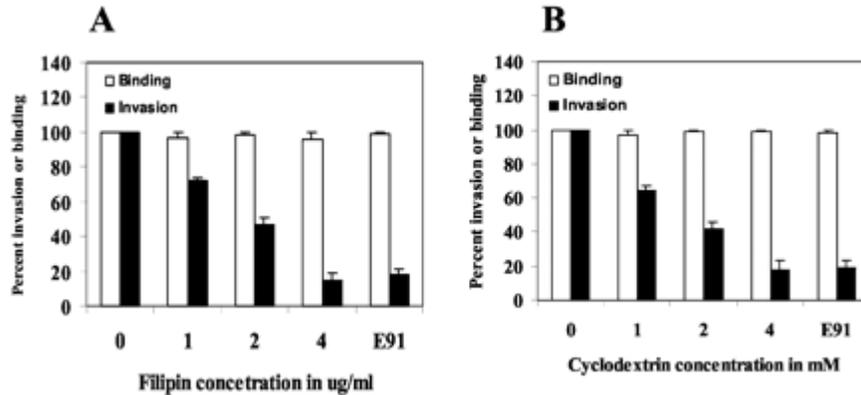
Figure 4: Inhibition of *E. coli* invasion of HBMEC by filipin and cyclodextrin in a dose-response manner.

2.1.3.   NOD2 but not NOD1 could recognize Gram-positive internalized bacterial Streptococcus pneumoniae:

Nod1 and Nod2 are both intracellular receptors for pathogen recognition. However, Nod1 can not recognize Gram-positive bacteria while Nod2 can act on both Gram-positive and negative bacteria. The broad recognition properties of Nod2 enable it to be important for Gram-positive bacterial detection. As shown in Figure 5, HEK293 cells were transiently transfected with or without Nod1/Nod2 expression plasmids, an NF-κB-dependent luciferase reporter, and an RSV β-galactosidase plasmid. Nod2 conferred a dose-dependent responsiveness to inactivated pneumococci and MDP [34], but Nod1 does not. Obviously, the result suggested that both as an intracellular receptor, NOD2 is more important for Gram-positive bacterial sensing.
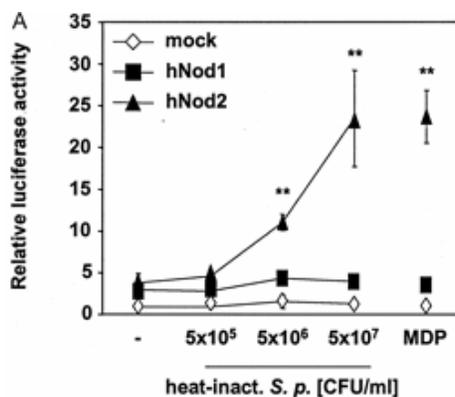
Figure 5: Nod2 mediates NF-κB activation by the inactivated pneumococci when intracellularly challenged.

2.2. To determine the requisite role of NOD2/MDP pathway in response to Gram-positive infection.

2.2.1. Nod2 gene could be regulated by TNFα in intestinal epithelial cells:

Although Nod2 is expressed at low levels in quiescent stage, NOD2 mRNA would be increased greatly in a monocyte cell line in response to lipopolysaccharide, lipoteichoic acid, interferon-γ and TNFα. The expression of NOD2 in cloned at epithelial cells is more sensitive to TNFα stimulation[10]. In this experiment, cells were stimulated by TNFα for 24 h and evaluated for the elevation of NOD2 mRNA. The levels of NOD2 mRNA in the SUM159 and MCF-7 were increased about 1.5- and 6-fold respectively (Fig.6, A and B). Up-regulation of NOD2 mRNA levels was more prominent (23-fold) in the colon epithelial cell line FHC (Fig. 6B) [10]. The fact that the NOD2 gene can be regulated by proinflammatory cytokines suggests an important role for NOD2 in innate immunity. Up-regulation of NOD2 genes as a consequence of proinflammatory cytokines stimulation could be accomplished via NF-κB activation:
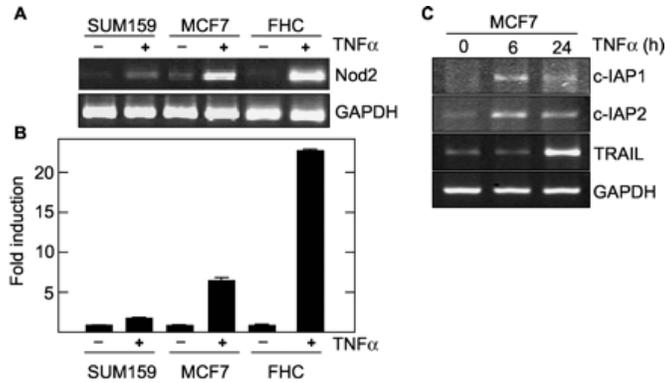
Figure 6: Induction of NOD2 mRNA in epithelial cells treated with TNFα.

The increased expression of NOD2 in intestinal epithelial cells has been confirmed, what not been known to us is that how the NOD2 expression been regulated by cytokines. Improper regulation of NOD2 expression will either lead to inactive reaction for pathogen invasion or induce overwhelming inflammatory response.

2.3.   To identify an interaction between NOD2 and TLR2 pathways in mediating Gram-positive bacterium Staphylococcus aureus activation (IL-8 synthesis) in polarized MDCK cells

2.3.1.   Role of TLR2 in the innate immunity:

The function role of TLR2 for recognizing Gram-positive bacteria has been described in quantities of studies. After transfection with TLR1 or TLR2 plasmids, HEK293 cells were treated with Gram-positive bacteria (Micrococci) and Peptidoglycan (PGN) to observe the IL-8 level as reflected by CAT activity. Micrococci and PGN induced transcription of IL-8 in HEK293 cells expressing Toll-like receptor 2 (TLR2) and CD14 but not in those expressing TLR1 or TLR4 (Figure 7)[35]. It has also been shown that *S. aureus* cell wall-mediated TNFα production was fully TLR2 dependent [36]. Study on various doses of bacterial challenge on

HEK293T cell showed that TLR2-dependent NF-κB activation is bacterial concentration dependent, rather than PGN content dependent (Figure 8) [37].
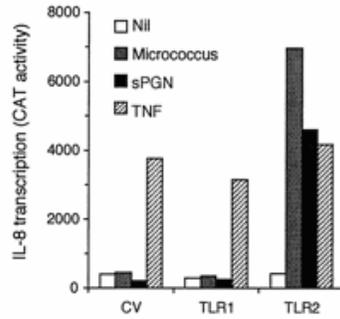
Figure 7: Micrococci (Micro) and sPGN induce transcription of IL-8 in HEK293 cells expressing TLR2, and this induction is NF-κB dependent.
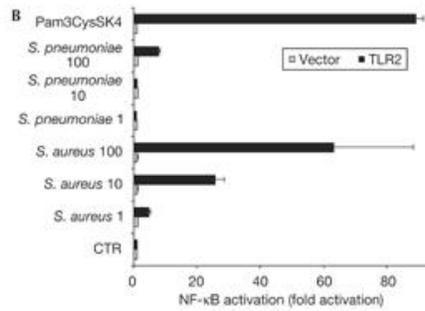
Figure 8: Dose-dependent HEK293T cells stimulation with tenfold increasing amounts of HK S. aureus and S. pneumoniae.

The critical role for TLR2 in host defense against Gram-positive bacteria was strongly supported by *in vivo* TLR2 gene disruption experiment. In these studies, TLR2-/- mice were significantly more susceptible to lethal infection after a high-dose challenge (1×10^7 CFU *S. aureus*). As shown in Figure 9, all wild-type mice survived for 8 days after *S. aureus* inoculation and 60% survived on day 14. In contrast, about 80% of TLR2-deficient mice succumbed to *S. aureus* and died within 8 days, and only 10% survived on day 14 [38].
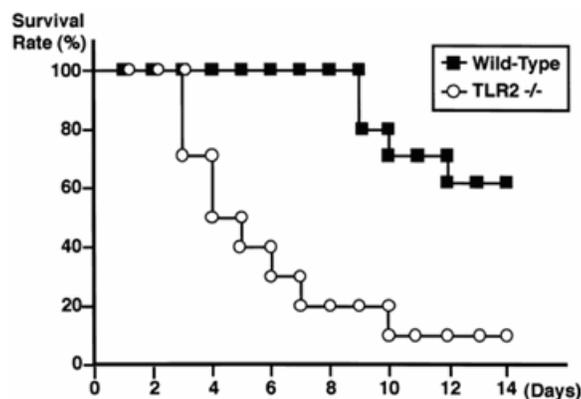
Figure 9: Survival of TLR2-deficient mice (n = 10, ■) and wild-type mice (n = 10, ○) after i.v. infection of 1 x 10^7 CFU of *S. aureus.* p < 0.03 in log rank test.

2.3.2.   Potential relationship between NOD2 and TLR2:

The potential cross talk between NOD2 and TLR2 has been implicated in several studies[3, 39, 40]. It is conceivable that the TLR and NOD pathways cooperate to enhance the immunological response. Experiments in Nod2 (-/-) mice showed that intact NOD2 signaling inhibited TLR-2 driven activation of NF-κB [40]. As shown in Figure 10, total splenocytes obtained from wild-type and NOD2-/- mice were stimulated with pure synthetic TLR2 ligands (Pam3Cys or Pam3CSK4) and MDP, Culture supernatants were collected and analyzed for cytokine production by ELISA. Results showed that TH1 cytokines (IL-12, IFN-ɣ and IL-18) secretion increased in NOD2 -/- mice. The fact that NOD2 stimulation initiates both pro-inflammatory and anti-biotic response suggests the complicated role of NOD2 in the innate immunity.
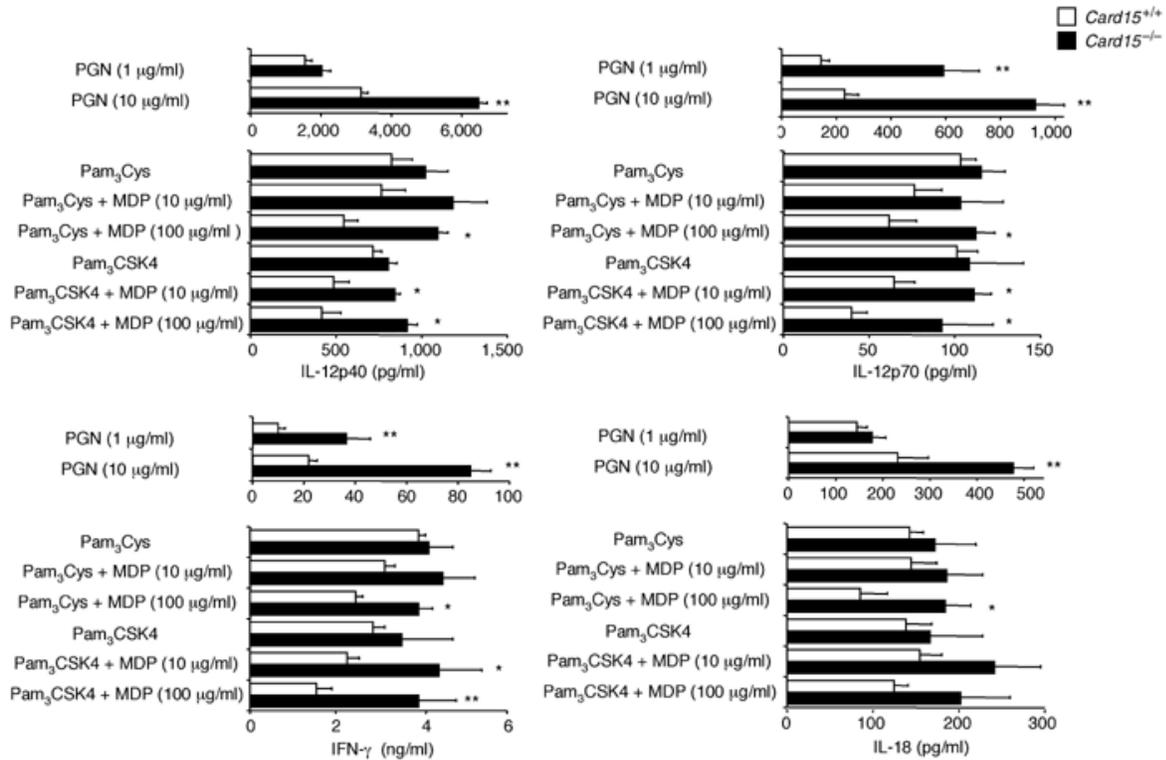
Figure 10: Splenocytes from NOD2 -/- mice show enhanced TH1 cytokines production after stimulation with MDP together with the pure (synthetic) TLR2 agonists Pam3Cys and Pam3CSK4.

Both TLR2 and NOD2 are used for sensing Gram-positive bacterial, TLR2 acts as an extracellular receptor while NOD2 acts as an intracellular sensor. **In this study, we will examine if there is any cross talk between TLR2 and NOD2 in bacteria-induced IL-8 synthesis in epithelial cells. Particularly, we would like to examine MAP kinase signal pathway to see any relationships between TLR2 and NOD2 on Gram-positive bacterial stimulation.**

3.   Research Design and Methods:

In this part, experimental design and specific protocols will be listed first and then followed by necessary experimental techniques.

3.1.   Experimental design and specific protocols

3.1.1.   To reveal the molecular mechanism for the interaction between NOD2 and MDP.

3.1.1.1.   <u>Objective</u>:

To test the hypothesis that a cognitive sequence recognizes MDP in the LRR of NOD2 and accounts for the interaction with PGN from Gram-positive microorganism in polarized epithelial cells. Particularly, we will try to examine 1) How mutagenesis of the LRR domain of NOD2 will affect the interaction of NOD2 with MDP, and 2) How caveolae are involved in the internalization of MDP in the cells and the subsequent interaction with NOD2. To address the first question, we will mutate the various sequences within both the N-terminal and C-terminal of the LRR domain of NOD2, and then we will examine whether this modification will affect the interaction of MDP with NOD2 in an i*n vitro* assay as well as in intact cells.

To figure out the role of caveolae in MDP internalization and its subsequent interaction with NOD2, we will first determine the subcellular co-localization of NOD2 and MDP with caveolae. To further investigate if biochemical disruption of caveolae by detergent will influence the MDP internalization, or its interaction with NOD2 and IL-8 synthesis as a consequence. We will use optical imaging to trace the co-localization of transfected GFP-caeoline-1 and Alexa 588 labeled MDP via laser scanning confocal microscopy. We also want to find out whether disruption of caveolae with detergent will affect IL-8 systhesis.

3.1.1.2.   <u>Protocol</u>:

To address the question of pathway selection by Gram-positive bacteria, we will investigate whether the bacteria are internalized via caveolae in MDCK cells.

1) Mutagenesis:

We will generate the mutagenesis of the various sequences within both the N-terminal and C-terminal of LRR domain of NOD2. The strategy to perform random mutagenesis of NOD2 has been described before [41, 42]. Each cassette containing the coding region of NOD2 will be mutated using the GeneMorph PCR kit. Each primer will be designed to contain restriction sites so that each PCR product will be digested with appropriate restriction enzymes and inserted in-frame into the remainder of the coding region of NOD2. The NOD2 3020insC mutation that renders truncation of the NOD2 at position 1007 was determined using the allele-specific multiplex PCR assay described by Ogura et al. Deletion and site-directed mutants as well as wide-type NOD2 will be cloned into pcDNA3 vector. Expression plasmids will be amplified and purified for *in vitro* binding assay.

a) *In vitro* binding assay: The microplate will be pre-coated with Avidin. We will label wild type and mutated NOD2 with biotin and incubate with Avidin. Two hours later fluorescently labeled MDP will be added and incubated for another 2 hours before measurement of fluorescence intensity.

b) Interaction of NOD2 with MDP in intact cells: Wild type and mutated NOD2 cDNA will be cloned into pcDNA3-HA. Expression plasmids will be transient transfected into MDCK cells. Twenty four hours post-transfection, 10ng/ml biotin labeled MDP will be transfected in the presence of calcium phosphate to allow their entry into the cells as reported[39, 43]. Proteins from cell lysate will be coimmunoprecipitated with an anti-HA antibody and subsequently detected with bioin-Avidin interaction to track the existence of MDP. For colocalization assay, we will construct various NOD2-EGFP fusion proteins, transfect express plasmids into MDCK cells and add rhodamine-labeled MDP. At different time points, we will fix cells and observe

NOD2/MDP co-localization using laser scanning confocal microscopy to detect the binding of MDP with NOD2.

2) Interaction of caveolae with NOD2/MDP:

Construction of GFP-caveolin-1: GFP will be attached to the C-terminal end of caveolin-1. *Hin*dIII restriction sites will be added to both 5' and 3' ends of the caveolin-1 cDNA by PCR using the Expand High Fidelity PCR system. The PCR product will be digested with *Hin*dIII and inserted into the *Hin*dIII site of the EGFP-N1 eukaryotic expression vector. The proper orientation and sequence of caveolin-1-GFP will be verified by sequencing. The polybrene method will be used to transfect MDCK cells with the caveolin-1-GFP construct. Transfected cells will be selected by growing in 0.8 mg/ml G418 for 14 days. Individual clones will be isolated and screened for expression of the caveolin-1-GFP by fluorescent microscopy.

Methyl-b-cyclodextrin and filipin pretreatment: MDCK cells with be preincubated with different amounts of methyl-b-cyclodextrin (1, 2, 5, 10 mM) and filipin (1,5,10 and 20 mM) in 25 mM Hepes buffered DMEM for 1 h at 37°C, wash thoroughly with Hepes-DMEM, and then incubate with 0.5 mg ml-1 Alexa 488 coupled with MDP for 15 min. Infection will be stopped by fixation with 1% formaldehyde. Or, we will do time- and concentration-dependent MDP uptake experiment under which caveolin will either over-expressed or be downregulated by siRNA.

Immunofluorescence and confocal microscopy: MDCK cells expressing the GFP-caveolin-1 construct will be grown on coverslips and pretreated with and without detergent. They will then be treated with Alexa Fluor 588 labeled MDP and incubated for 8 h. We will wash the cells five times in PBS and fix with 2% paraformaldehyde for 15 minutes at room temperature and continue incubation with 5% normal serum blocking solution for 20 minutes. Cells will then

be immunostained to detect NOD2 by incubation with primary and secondary antibodies at room temperature for 1 h and for 45 min, respectively. Coverslips will be mounted using Fluoromount-G and viewed with epifluorescence microscopy. For colocalization of NOD2 and caveolin, MDP, confocal microscopic analysis will be carried out using the TCS SP1 confocal imaging system.

IL-8 assays: We will expose confluent monolayers of MDCK cells to the medium without serum for 24 hours then to *Staphylococcus aureus* for 60 minutes with and without pretreatment with Methyl-b-cyclodextrin or filipin for 30 minutes. Cells will be washed, sterilized with gentamicin (100 αg/ml), and the supernatants harvested at the different time points for IL-8 ELISA assay.

3.1.1.3.   Expected Results, Problems, and Solutions:

In this protocol, we try to figure out any potential role of caveolae in the interaction of NOD2 and MDP. Because almost all the cells share the same IL-8 promoter, the functional analysis indicated by IL-8 expression has the cell type significance. The expression pattern of fluorescently labeled NOD2 will be compared for the similarity in several different clones and different cell lines. Our results will show the recruitment migration of the NOD2 toward internalized MDP. Within the subcellular level, we will reveal if NOD2 will be expressed in the ER or Golgi complex and how and where the NOD2-MDP will be processed. We expect that fluorescent microscopy will reveal a diffuse cytoplasmic staining of NOD2 in untreated cells. Subsequent stimulation with MDP will induce rapid redistribution of NOD2 and perinuclear condensation after interval exposure. MDP will colocalize with intracellular NOD2 staining as

well as the cellular distribution of Golgi marker C5 ceramide. We expect that the LRR will be critical for MDP binding. Alternatively, we will consider CARD region of NOD2.

Based on the special functional role of caveolae in the internalization of pathogens and previous results, we expect that MDP/NOD2 will be relevant to caveolae. In the event that neither filipin nor caveolin-1 overexpression will inhibit the internalization and transportation of MDP in confluent MDCK cells, we would like to do the following things: use siRNA to specifically silence the expression of caveolin-1 or other caveolin isoforms caveolin-2 or 3. Considering the alternative pathway, which is named clathrin-dependent endocytosis, we may pick up other inhibitors such as chlorpromazine and potassium depletion to destroy clathrin and evaluate the possible pathway via clathrin. Others such as cholesterol-disruption agents, dynamin function inhibitors may be served in multiple studies with different cell strains to argue the critical role of caveolae.

3.1.2.   To determine the requisite role of the NOD2/MDP pathway in response to Gram-positive infection.

3.1.2.1.   Objective:

We hypothesize that constituitive levels of NOD2 are low in polarized epithlelial cells. Gram-positive infection results in the synthesis of TNFα that increases NOD2 expression in an atuocrine fashion and enables the cells to participate in host defense via synthesis of IL-8. We will measure IL-8 synthesis after Gram-positive infection in wild type MDCK cells and compare this response to cells in which TNFα or NOD2 is downregulated by siRNA. Complementation of the deleted genes with direct gene transfer will be used to confirm their critical roles.

3.1.2.2.   Protocol:

The methods for the NOD2 and TNFα siRNA design will be used as follows: A double-stranded oligonucleotide siRNA will be designed to contain a sequence derived from the NOD2 open reading frame (ORF). For the target purpose, siRNA design will be focused on NOD2 ORF promoter region, both in forward and reverse orientation. The TNFα siRNA sequence will be generated via a similar way. For the siRNA transfection, MDCK cells will be seeded at a density of 10^5/ml in 24-well plates. Graded concentrations of siRNAs for NOD2/ TNFα siRNA and a control siRNA will be transfected into cells using Oligofectamine according to the manufacturer's instruction.

Role of Nod2/MDP pathway in the direct TNFα stimulation: We will transfect MDCK cells with or without Nod1/Nod2 siRNA (different dose with different time points). Forty eight hours later, MDCK cells will be treated with TNFα. To observe the functional consequences of Nod2, at the different time points, MDCK cell supernatant will be collected and the IL-8 level will be assayed.

Role of Nod2/MDP pathway in the endogenous TNFα stimulation: MDCK cells will be treated with Gram-positive bacteria *S. aureus*. siRNA experiment will be designed as follows: a) siRNA for NOD2; b) siRNA for TNFα; c) siRNA for both NOD2 and TNFα. At various time points, we will assay IL-8 level from the cell supernatant. The production of TNFα will be analyzed using an ELISA kit. We will blot total cell lysates with specific antibodies for NOD2 or TNFα.

Reinstatement assay: We will redirect the NOD2 or TNFα gene into the above cells already being treated with siRNA to see if the functional synthesis of IL-8 will be restored.

Transfect NOD2/TNFα containing plasmids into the siRNA pretreated cells, and then we will stimulate them with *S. aureus*. Finally we will re-evaluate the synthesis of the IL-8 level.

3.1.2.3.   Expected Results, Problems, and Solutions:

We predict that TNFα increases NOD2 mRNA in a manner that is dependent on dose and time. The NOD2/MDP pathway has an important role in proinflammatory stimulation (sensitive to siRNA to NOD2). The down-regulation effect of siRNA could be recovered after restoring the NOD2 and/or TNFα genes. To overcome the possibility that the siRNA fragment is not the critical one for the gene silencing, we will try to pick up multiple sites. Alternatively, we will co-transfect multiple siRNA fragments in order to silence the functional sites of NOD2. These siRNA target sites could either be located at the LRR domain (interfere MDP recognition) or at the Card domain (disrupt NF-κB activation). To further confirm the hypothesis, in vivo experiments will be needed. Specifically, an animal model with conditional knockout NOD2 or TNFα genes will be used. Alternatively, considering the external sources of TNFα, we may add in the TNFα antibody to block the entry of exogenous TNFα to observe the autocrine effect. To exclude the possible role of the TLR2 effect, we may choose other cell types, or using HEK293 cells (TLR2 exclusive).

3.1.3.   To identify an interaction between NOD2 and TLR2 pathways in mediating Gram-positive bacterium Staphylococcus aureus activation (IL-8 synthesis) in polarized MDCK cells.

3.1.3.1.   Objective:

We will try to find out if there is any co-operation between TLR2 and NOD2 in the immune response against Gram-positive bacterial infection. We will also determine whether

MAP kinase activation plays any role in the TLR2/NOD2 synergistic effect. To address this issue, we will silence the expression of either TLR2 or NOD2 by siRNA and examine whether this will affect MDP-induced IL-8 synthesis. To address the second question, we will treat the MDCK cells with inhibitors that are specific for ERK, p38 and JNK and then examine the IL-8 synthesis.

3.1.3.2.   Protocol:

Studies on the synergistic effect between Nod2 and TLR2 pathway: To investigate if TLR2 interacts with NOD2, we try to see if TLR2 signaling upregulates Nod2 expression in MDCK cells: a) We stimulate MDCK cells with TLR2 specific agonists Pam3Cys and then detected NOD2 using Western blot and RT-PCR in MDCK cells. RNAse protection assay will be used to demonstrate the significant induction of IL-8 mRNA after TLR2 stimulation in MDCK cells in vitro. The induction of TL-8 will be demonstrated by ELISA. As we see from the preliminary studies, we predict that NOD2 expression will be elevated in MDCK cells; b) We then want to delineate the pathways that regulate NOD2 function. We will investigate the effect of TLR2 on NOD2-induced NF-κB activation. HEK293T cells will be transfected with pcDNA-NOD2 (3 ng) with reporter constructs RSV-KB-Luc and RSV-β-galactosidase plus the dominant negative form of TLR2. TLR2DN doses will be used from 0 to 150 ng. 24 hours after transfection, luciferase activity will be determined and normalized on the basis of β-galactosidase activity[39]; c) Further, we will try to see if NOD2 regulates MDP-induced NF-κB activation in NOD2-expressing cells. HEK293T cells will be transfected with NOD2 and TLR2DN, and 8 h later, stimulated with MDP (100 ng/ml). The inhibition effect of TLR2DN on MDP-induced NF-κB activation in NOD2-transfected HEK293T cells will be observed

compared with the control vector; d) If TLR2 indeed regulates NOD2-mediated NF-κB activation, we will further investigate the possibility that TLR2 interacts with NOD2. FLAG-NOD2 and HA-TLR2 will be coexpressed in HEK293T cells and immunoprecipitated with anti-HA antibody. Subsequently, we will resolve immune complexes by SDS-PAGE. Then, we will do the Western blot analysis with an anti-FLAG antibody to show if the TLR2-precipitated complexes contain NOD2.

Studies on the role of MAP kinase pathway on the cross talk between NOD2 and TLR2: MAPKs can also play an important role in regulating proinflammatory gene expression other than NF-κB. To determine whether MAP kinase is involved in Nod2 function, we generate MDCK cells that stably express Nod2 or TLR2 and compare their p38 activation to the control MDCK cells that have been transformed with the antibiotic resistance gene only. We will then seek to use Nod2DN or TLR2DN constructs to investigate whether functional Nod2/TLR2 signaling is necessary for *S. aureus*-induced p38 MAPK activation. Both control MDCK cells and MDCK cells stably transfected with Nod2 will be stimulated with *S. aureus* for the indicated time and lysed, and p38 MAPK activation will be measured via immunoblotting. Induced p38MAP kinase phosphorylation in the MDCK cells will be assayed by performing Western blotting using the phospho-p38 MAP kinase antibody. Parallel blots will be done using the antibody recognizing total p38 MAP kinase[42]. Total cell lysates will also be blotted with a phosphospecific antibody (*Ab*) for ERK and JNK. To verify the amount of loaded protein, the lysates will be also probed with anti-ERK1/2.

JNK activity will be analyzed by a quantitative JNK kinase assay[43, 44]. Briefly, MDCK cells will be transfected with 500 ng of Flag-JNK1 expression vector plus 2 μg of empty vector, siRNA for TLR2 or NOD2 or control RNA. Forty eight hours post-transfection, we will

infect cells with *S. aureus* for 20 min. Flag-JNK1 will be immunoprecipitated using antibodies to the Flag epitope. JNK1 activity of the immunoprecipitates will be determined by dividing the level of c-Jun phosphorylation by the amount of overexpressed Flag-JNK1 levels for each sample as described [44]. The level of c-Jun phosphorylation and Flag-JNK1 expression will be determined.

To see if p38 regulates IL-8 expression via a posttranscriptional mechanism, we will consider the consequences of MAPK activation on the epithelial expression of the proinflammatory chemokine IL-8. We will stimulate cells with *S. aureus* or TNFα in the presence of the indicated concentration of p38 inhibitor SB-203580(2－20 μM), ERK inhibitor (U-0126) or JNK inhibitor (JNK Inhibitor 1) (0.01% DMSO). At 5 h after the stimulation, we will remove the media for IL-8 assay via ELISA.

NOD2/TLR2 siRNA experiment: The siRNA design will refer to the method mentioned before. In three separate experiments, TLR2 and NOD2 will be silenced by siRNA separately or at the same time. Two days later, we will infect cells with Gram-positive bacterium *S. aureus* and compare the IL-8 levels. To further confirm the results, reinstatement assay will be done as mentioned. We will redirect the NOD2 or TLR2 gene into the above cells already being treated with siRNA to see if the functional synthesis of IL-8 will be restored.

To see whether *S. aureus* will induce a NOD2/TLR2-dependent transcription of the gene for IL-8, two different assays will be tested: a) reverse transcription-PCR of IL-8 mRNA in MDCK cells stably transfected with TLR2 and/or NOD2 siRNA; b) transactivation of an IL-8 promoter-CAT construct in MDCK cells transiently transfected with pIL8(wt)CAT and with plasmids expressing NOD2 and TLR2.

3.1.3.3. Expected Results, Problems, and Solutions:

Based on the different characters of NOD2/TLR2 sensing, we expect that Gram-positive bacterial sensing specificities and functions between TLR2 and NOD2 are distinct and non-overlapping. There exists a synergetic effect between them. We will observe TLR2DN will exert its effect in a dose-dependent manner to inhibit NOD2-induced NF-κB activation. Cells will be stimulated by either inactivated or alive Gram-positive bacterial *S. aureus* and we will observe that TLR2DN will exert its effect in a dose-dependent manner to inhibit NOD2-induced NF-κB activation. Further direction could determine the role of NOD2/TLR2 in innate immunity *in vivo*. Alternatively, overexpression of TLR2 and NOD2 could be done to observe the synergistic effect of NOD2/TLR2. Considering the expression model of NOD2, low level expressions in quiescent state but up-regulated after proinflammatory stimulation in epithelial cells, we would like to check the dichotomous role of NOD2. The upregulation of NOD2 in epithelial cells has two implications: initiate innate immunity against pathogen invasion or inhibit abnormal inflammation at the certain circumstance. To observe the inflammatory regulation mechanism, we utilize both a polarized model of intestinal epithelia expressing endogenous TLR2 as well as MDCK cells engineered to express TLR2, allowing both physiologic and mechanistic assessments of this signaling pathway. Cells may be directly stimulated with TNFα. Twenty four hours later, we will remove TNFα and treat the cells with Gram-positive bacterial, and compare the IL-8 level. In the parallel experiment, NOD2 will be up-regulated by TNFα first, after 24 h, we will treat cells with Gram-positive bacterial without depleting TNFα, and compare the IL-8 level among different groups.

3.2. Experimental details

3.2.1. Cell culture:

The Madin–Darby canine kidney (MDCK) cell lines will be cultured in Eagle's minimum essential medium containing 10% FBS and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Polarized monolayers will be generated from cells cultured in complete medium in 12-mm and 24-mm Transwell® filter chambers (0.4 μm pore size). The medium will be changed every 2 days and cell monolayers will be considered polarized when the trans-epithelial electrical resistance is $\geq 200 \ \Omega \ cm^2$ as measured with the Epithelial Volt Ohmmeter [45].

3.2.2. Bacteria and infection:

S. aureus 834 strain will be prepared as described [46]. In brief, bacteria will be cultured on trypticase soy agar, inoculated with trypticase soy broth, and incubated for 15 h at 37°C. The bacteria will be collected and resuspended by PBS. The concentration of resuspended cells will be adjusted spectrophotometrically at 550 nm. *S. aureus* suspension (1×10^9 CFU/ml in PBS) will be boiled for 30 min and used as heat-killed *S. aureus*.

siRNA: Transfections for targeting endogenous TLR2, NOD1, NOD2 will be carried out using oligofectamine and dsRNA (final concentration, 200 nM) for 24 h at 37°C. The sequences of target mRNAs will be used in this study are TLR2, 5'-AATCCGGAGGCTGCATATTCC-3'; NOD1, 5'-AAGA GCCTCTTTGTCTTCACC-3'; and NOD2, 5'-AAGACATCTTCC AGTTACTCC-3'[47].

3.2.3. NF-κB Activation Assays:

Cells will be cotransfected with RSV-KBLuc (100 ng), a reporter construct encoding the luciferase reporter gene κB-luc under the control of a minimal promoter with an NF-κB-binding

site; RSV-β-galactosidase (50 ng), an expression construct containing β-galactosidase; and an indicated amount of each expression construct, by FuGENE 6, in triplicate. Cell lysates will be prepared 24-36 h after transfection, and the relative luciferase activity will be determined according to the manufacturer's instructions (Promega). Results will be normalized for transfection efficiency on the basis of β-galactosidase activity[39].

RNAse protection assay: RNAse protection assay will be used to measure the pro-inflammatory chemokines IL-8 induction on mRNA level following Tlr2 stimulation. In short, ^{32}P UTP radioactive labeled RNA probes will be generated using manufacturers' hCK-2 or hCK-5 cDNA multi-templates including 15 cytokines and chemokines and 6 house keeping genes and T7 RNA polymerase. Radioactive probes will be used to hybridize 2 μg total RNA from cultured cells. Protected RNA fragments will be quantified after film exposure using Quantity One software [48].

3.2.4.   MAPK assessments:

Cells will be stimulated and rinsed in cold HBSS, lysed (in PBS with 1% Triton X-100, 1 mM EDTA, 1 mM NaVO$_4$,1mM NaF) cleared by centrifugation (10 min at 5,000 g at 4°C), and assayed for phospho-p38, phospho-ERK, or total P38 by SDS-PAGE immunoblotting. Densitometry will be performed via scanning with Scion Image densitometry software [49].

3.2.5.   Statistical analysis:

For statistical analysis, ELISA samples with IL-8 levels will be performed in triplicate. Results will be expressed as means and standard deviations. Statistical significance will be determined using a one-way analysis of variance with Dunnett's post test. Differences will be considered significant if P values are <0.05.

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