Nod1-Mediated Endothelial Cell Activation by Chlamyphila Pneumoniae

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Abstract—Seroepidemiological and animal studies, as well as demonstration of viable bacteria in atherosclerotic plaques, have linked Chlamyphila pneumoniae infection to development of chronic vascular lesions and coronary heart disease. Inflammation and immune responses are dependent on host recognition of invading pathogens. The recently identified cytosolic Nod proteins are candidates for intracellular recognition of bacteria, such as the obligate intracellular chlamydia. In the present study, mechanisms of endothelial cell activation by C. pneumoniae via Nod proteins were examined. Viable, but not heat-inactivated, chlamydia activated human endothelial cells, suggesting that invasion of these cells is necessary for their profound activation. Endothelial cells express Nod1. Nod1 gene silencing by small interfering RNA reduced C pneumoniae–induced IL-8 release markedly. Moreover, in HEK293 cells, overexpressed Nod1 or Nod2 amplified the capacity of C pneumoniae to induce nuclear factor κB (NF-κB) activation. Interestingly, heat-inactivated bacteria were still able to induced a NF-κB reporter gene activity via Nod proteins when transfected intracellularly, but not when provided from the extracellular side. In contrast, TLR2 sensed extracellular heat-inactivated chlamydia. In conclusion, we demonstrated that C pneumoniae induced a Nod1-mediated and Nod2-mediated NF-κB activation in HEK293 cells. In endothelial cells, Nod1 played a dominant role in triggering a chlamydia-mediated inflammatory process. (Circ Res. 2005;96:000-000.)

Key Words: endothelial cells ■ Nod proteins ■ Toll-like receptors ■ chlamydia ■ siRNA

Chlamydia are Gram-negative bacteria that are obligate intracellular parasites of eukaryotic cells. Chronic or recurrent infections with Chlamyphila (Chlamydia) pneumoniae have been associated with the development of vascular lesions and atherosclerosis.1–4 Chronic infection of susceptible target cells (monocytes, macrophages, endothelial cells, fibroblasts, and smooth muscle cells), as well as direct activation of endothelial cells, may initiate and perpetuate a local inflammation by inducing cytokine release (eg, IL-6, IL-8, etc), and increased expression of adhesion molecules (P-selectin/E-selectin, ICAM [intercellular adhesion molecule]-1, VCAM [vascular cell adhesion molecule]-1), which subsequently results in increased adhesion of leukocytes or platelets to the endothelium.5,6 Although chlamydiae may reside and replicate in different cell types and induce a chronic immune activation, little is known about the mechanisms of C pneumoniae–induced target cell alteration. The field is troubled by the “hen versus egg” problem and causative proof is difficult because there are no good markers to differentiate among new versus old (IgM versus IgG), as well as acute versus chronic persistent (IgM versus IgA), C pneumoniae infection. Chlamydia are able to persist in infected target cells. These persistent bacteria are resistant against almost all of the known chlamydia-targeting antibiotics (macrolides, tetracycline).7 Therefore, all studies (like WIZARD or PROVE-IT) to evaluate the importance of chlamydial infections for the development of vascular diseases via the use of antibiotics are unlikely to obtain useful results.

The innate immune system relies on surveillance proteins to recognize pathogens by sensing pathogen-associated molecular patterns. A well-studied group of pattern recognition receptors are the Toll-like receptors (TLRs), which are mainly expressed on the surface of a broad diversity of cells. TLR2 and TLR4 have been demonstrated to mediate host defense against C pneumoniae or chlamydial components.8–11 The recently identified nucleotide-binding oligomerization domain (Nod) proteins, also called caspase recruitment domain–containing proteins, are molecules that have been implicated in intracellular pattern recognition.12,13 More than 20 proteins that are homologues to Nod1 have been identified in the human genome, but only a few members of this growing family are functionally characterized.13 So far, Nod1 has been described to mediate the activation of nuclear factor κB (NF-κB) induced by peptidoglycans containing meso-diaminopimelate acid found mainly in Gram-negative bacte-
ria,14,15 whereas Nod2 (caspase recruitment domain 15) mediates responsiveness to the muramyldipeptide MurNAc-l-Ala-d-isogln conserved in peptidoglycans of basically all bacteria.16,17 Little is known about the Nod-dependent signaling cascade activated by ligand-binding. There is evidence that oligomerization of Nod1 (and Nod2) induces the recruitment of its interacting partner Rip2 kinase (RICK or CARDIAK). Subsequent activation of NF-κB therefore relies on activation of downstream effectors of RICK, like the inhibitor of NF-κB kinase complex.18–20

Because persistent intracellular infection of endothelial cells with C pneumoniae has been associated with development of cardiovascular diseases, we examined the importance of cytosolic immune receptors in this context. We demonstrate that Nod proteins mediate NF-κB activation by C pneumoniae and suggest that Nod1 plays a major role in endothelial cell immunity.

**Materials and Methods**

**Chlamydia Strains**

C pneumoniae strain TW-183 (VR2282; ATCC, Rockville, Md) was cultured and purified as described by Maass et al.2 Briefly, TW-183 was grown to high titers in cyclohexamide-treated HEp-2 cells. Infected monolayers were harvested from culture flasks and sonicated for 30 seconds. Cellular debris was removed by centrifugation at 500g for 10 minutes at 4°C. Aliquots of purified elementary bodies were stored at −75°C until use. For some experiments, C pneumoniae were inactivated by heat treatment (90°C, 30 minutes).

**Infection of Human Umbilical Vein Endothelial Cells, Human Aortic Endothelial Cells, and HEK293 Cells**

Human aortic endothelial cells (HAECs) were obtained from Clonetix (San Diego, Calif), and human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veils. Both cell types were cultured as described previously.4 HUVECs, HAECs, or HEK293 cells were inoculated with C pneumoniae using a multiplicity of infection (MOI) of 0.5 to 5 in MCDB131, EGM-2, or DMEM, respectively. Plates were centrifuged at 800g for 30 minutes at 37°C for 1 hour and subsequently incubated for 7 hours (luciferase assay) or overnight (IL-8 enzyme-linked immunosorbent assay [ELISA]). For some experiments, chlamydia or lipopolysaccharide (Sigma, Deisenhofen, Germany) were preincubated with polymyxin B (10 μg/mL; Sigma) to inactivate lipopolysaccharide. To investigate importance of TLR2 and Nod1 or Nod2 for intracellular activation of target cells, heat-inactivated C pneumoniae (MOI=5) were transfected intracellularly using the calcium phosphate method (Clonetech, Palo Alto, Calif) before transfection with different plasmids as has been described for different stimuli.15,17 Briefly, immediately before DNA/calcium phosphate suspensions were added, cells were fed with culture medium containing bacterial components.

**RNA Interference**

Control nonsilencing small interfering RNA (siRNA) (sense, UUCUGGACACGUUCAGCUT; antisense, AGGUGACAGGUUCGGAGGAAUtt) and different siRNAs targeting Nod1 (sequence 1 sense, GGUGAGACCAUCUCAUCUt; antisense, GAUGAACGAUGGUUCACCCCCtt; sequence 2 sense, GGCAGAUCUAUGAGAAt; antisense, AUCUCAUGACUUGGCCCct) were purchased from Ambion (Huntingdon, UK). HUVECs and HAECs were transfected using an Amaxa Nucleofector (Amaxa, Cologne, Germany) according to the manufacturer’s protocol with 1.5 or 2 μg siRNA, respectively.

**Expression Plasmids and HEK293 Cell Overexpression Experiments**

Expression plasmids for Nod1 and Nod2 were kindly provided by G. Nunez, (Ann Arbor, Mich). Dominant-negative Rip2-2 were a generous gift from P. Dempsey (Los Angeles, Calif). The constructs encoding TLR2 and dominant-negative MyD88 were kindly donated by K. Kirschning (Munich, Germany) and Tularik Inc (San Francisco, Calif). Mal was generously provided by K. Fitzgerald (Worchester, Mass). HEK293 cells were cultured in 24-well plates with DMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum. Subconfluent HEK293 cells were cotransfected using the calcium phosphate method (Clonetech, Palo Alto, Calif) with 0.05 μg NF-κB–dependent luciferase reporter and 0.3 ng Nod expression vectors or 0.05 μg TLR2. For some experiments, cells were cotransfected with dominant-negative mutants of signaling molecules Rip2-2 or MyD88.

**Luciferase Assay**

Luciferase activity was measured using the Luciferase Reporter-Gene Assay (Promega, Mannheim, Germany) as described previously.5 The luminescence readings demonstrated were normalized to total protein and expressed as fold activation relative to unstimulated, mock-transfected cells±SE (Figures 2 and 3). In Figure 4, mock-transfected cells were not demonstrated; therefore, the relative luminescence readings were expressed as fold activation relative to unstimulated, Nod1-transfected or Nod2-transfected cells.

**Confocal Laser Scanning Microscopy Analysis of HUVECs and HEK293 Cell Infection**

HUVECs or HEK293 cells grown on Thermostax slides (Falcon Culture Slide; Becton Dickinson, Rutherford, NJ) were infected with C pneumoniae (MOI=5) for 24 hours, and cells were fixed with 3% PFA for 20 minutes and permeabilized with 1% Triton for 15 minutes. For chlamydia staining, the primary Ab (a genus-specific monoclonal antibody, “chlamydia cell conformation system”; Sanofi-Diagnostics Pasteur, Freiburg, Germany) was incubated overnight at 4°C. Bound antibodies were detected with ALEXA-488–conjugated goat anti-rabbit mAb (4°C; overnight: Molecular Probes, Eugene, Ore). Endothelial F-actin was counterstained using ALEXA-546–conjugated phalloidin (Molecular Probes). Cells were analyzed using a Pascal 5 confocal laser scanning microscope (Zeiss, Jena Germany).

**IL-8 ELISA**

IL-8 concentrations in the supernatants of infected HUVECs or HAECs were quantified using a commercially available sandwich ELISA Kit (R&D Systems, Wiesbaden, Germany).

**Reverse-Transcription Polymerase Chain Reaction Analysis**

Total RNA from different cell lines were isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse-transcribed using AMV reverse-transcriptase (Promega). The generated cDNA was amplified by polymerase chain reaction (PCR) using specific primers (Nod1-sense 5ˊ-AAGGAGACGGTGCGAACCATA-3ˊ; Nod1-antisense 5ˊ-TTCTGATCTTGGCCTCCTC-3ˊ; Nod2-sense, 5ˊ-AGCCATTTGTCAGGGGTCT-3ˊ; Nod2-antisense, 5ˊ-CGTCG- TCTGCTCATCATAGG-3ˊ; Tib MolBiol, Berlin, Germany). After 30 amplification cycles, the PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide, and subsequently visualized. To confirm equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression.

**Statistics**

Stimulatory effects of C pneumoniae and inhibitory effects of dominant-negative signaling molecule mutants or siRNAs were statistically evaluated using Student t test. Throughout the figure, P<0.05 is indicated by one asterisk; P<0.01 is indicated by double asterisks.
Results

Viable but Not Heat-Inactivated C pneumoniae Infect Human Endothelial Cells and Induce IL-8 Expression

Infection of endothelial cells by C pneumoniae was demonstrated by confocal laser scanning microscopy ofHUVECs 24 hours after addition of bacteria. ALEXA-546 phalloidin was used to counterstain endothelial F-actin; Figure 1A represents uninfected endothelial cells. C pneumoniae (Figure 1B) were inoculated onto HUVEC monolayer using an MOI of 5. This resulted in bacterial adhesion after the first minutes of cell contact and subsequent internalization. After 24 hours of exposure, only few bacteria were adherent to the cell surface; instead, a perinuclear formation of internalized bacteria was observed.

C pneumoniae infection of human endothelial cells induces a NF-κB–dependent pro-inflammatory phenotype as indicated by enhanced expression of cytokines/chemokines and adhesion molecules.6,21,22 To further characterize the chlamydial stimulation of endothelial cells, we incubated HUVECs or HAECs with viable or heat-inactivated C pneumoniae and assessed IL-8 secretion in the supernatant. Viable, but not heat-inactivated, chlamydia were able to induce IL-8 expression in HUVECs (Figure 1C) or HAECs (Figure 1D) 24 hours after infection. Preincubation of C pneumoniae with polymyxin B to inactivate lipopolysaccharides did not significantly reduce IL-8 expression (Figure 1E). The fact that intracellular infection of endothelial cells appeared...
and Nod1 or Nod2, respectively. Cells were infected with increasing concentrations of C. pneumoniae as described in Materials and Methods. The bacteria caused a slight induction of reporter gene activity if a control vector (“mock”) was transfected instead of Nod1/2 (Figure 2B). In cells overexpressing Nod1 or Nod2, C. pneumoniae induced a marked concentration-dependent NF-κB activation, and maximal effects were seen using a MOI of 5.

**Nod1 and Nod2 Recognize Only Intracellular, Whereas TLR2 Detects Extracellular, Viable or Heat-Inactivated Chlamydia**

Nod proteins are receptors for peptidoglycans. Because these molecules are heat-stable, we next assessed the ability of viable or heat-inactivated C. pneumoniae to activate Nod1, Nod2, and TLR2 while applied extracellularly or intracellularly.

To allow intracellular stimulation with heated bacteria, heat-inactivated chlamydia were transfected into target cells as described in Materials and Methods. Activation of NF-κB was used as “read out.” The Nod1/2-mediated NF-κB activation was only initiated when C. pneumoniae (viable or heat-killed) were provided intracellularly, either by infection with viable chlamydia or by intracellular transfection of heat-inactivated chlamydia (Figure 3). When applied extracellularly, the heat-inactivated chlamydia failed to initiate Nod-mediated signal transduction in HEK293 cells. Note that Nod1/2-overexpressing cells have a high constitutive activity of NF-κB. This observation is currently unexplained but clearly in line with several recently published studies.16,18 Viable and heat-inactivated C. pneumoniae were very well able to activate NF-κB in a TLR2-dependent manner (Figure 3) when chlamydia were provided from the extracellular side. When performing intracellular stimulation with inactivated bacteria, some TLR2-related reporter gene activity could be observed. This phenomenon is most likely attributable to the fact that during transfection of inactivated chlamydia, some bacteria nevertheless stay outside of the cells and possibly signal via TLR2.

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**Figure 2.** Nod1 and Nod2 mediated NF-κB activation in HEK293 cells by chlamydia. A, HEK293 cells were grown on Thermanox coverslips and stimulated with C. pneumoniae (MOI=5) for 24 hours. Cells were then fixed, permeabilized, and stained with FITC-conjugated genus-specific monoclonal antibody to demonstrate the intracellular presence of chlamydia. Cells were analyzed by confocal laser scanning microscopy (magnification ×630). Z-stack analysis confirmed that in infected HEK293 cells, all visualized chlamydia were intracellular. B, HEK293 cells were transiently transfected with a control vector, Nod1 or Nod2 expression plasmids, and a NF-κB-dependent luciferase reporter. The next day, cells were infected with the indicated doses of C. pneumoniae for 7 hours, and luciferase activity was measured. Data are shown as means±SE for one representative experiment of three, with each transfection performed in triplicate (**P<0.01).
Importance of Rip2 but Not of MyD88 for Chlamydia-Mediated NF-κB Activation via Nod Proteins

Next, we investigated the involvement of the signaling molecules Rip2 or MyD88, which participate during NF-κB activation by Nod proteins or TLRs, respectively. Therefore, we cotransfected HEK293 cells with a NF-κB luciferase reporter, Nod1 or Nod2, as well as different amounts of dominant-negative mutants of Rip2 or MyD88, and stimulated these cells with viable C pneumoniae. TLR2-dependent NF-κB activation served as a positive control for dominant-negative MyD88, and overexpressed Mal (an adapter molecule of TLR2 and TLR4) was used as a negative control for dominant-negative Rip2. Dominant-negative MyD88 failed to exhibit inhibitory effects on Nod protein-mediated NF-κB activation by chlamydia but clearly reduced the TLR2-dependent cell activation in HEK293 cells (Figure 4A and 4C). Dominant-negative Rip2, however, inhibited the Nod protein-associated NF-κB activation and overexpressed Mal (an adapter molecule of TLR2 and TLR4) was used as a negative control for dominant-negative Rip2.

Nod1 Is Fundamental for Chlamydia-Induced IL-8 Production in Endothelial Cells

To confirm the critical involvement of Nod1 in endothelial cell activation by C pneumoniae, we used siRNA technology to inhibit the expression of endogenous Nod1. First, we evaluated three different siRNA duplexes targeting Nod1 for their ability to reduce Nod1 mRNA expression in endothelial cells. Nonsilencing siRNA served as a control. Only the Nod1-specific oligonucleotides inhibited Nod1 mRNA levels in HUVECs and HAECs (Figure 5A and data not shown). Importantly, these Nod1-specific siRNAs also blocked the IL-8 production induced by C pneumoniae in HUVECs and HAECs (Figure 5B and 5C). In contrast, the tumor necrosis factor-α–induced (10 ng/mL) cytokine production in HUVECs was not altered by any siRNA used (Figure 5B), thus demonstrating specific effects of the siRNAs in our experiment. These observations confirmed our results from the HEK293 cell studies and demonstrated that Nod1 is important for endothelial cell activation by C pneumoniae.

Discussion

The study presented demonstrates that Nod1 is indispensable for intracellular sensing of C pneumoniae in endothelial cells. Nod1 gene silencing by siRNA blocked the IL-8 production induced by C pneumoniae in HUVECs and HAECs. In addition, we demonstrated that C pneumoniae activated a Nod1-mediated and Nod2-mediated signal transduction pathway in HEK293 cells involving Rip2, but not MyD88. Heat-stable chlamydial components are responsible for the demonstrated effects. TLR2 acted as an extracellular receptor for initial chlamydia-induced target cell stimulation. These observations can improve our understanding how C pneumoniae infection may contribute to chronic inflammatory events associated with atherosclerosis.

After initial attachment, chlamydial elementary bodies are internalized and dissociate from the endocytic pathway by actively modifying the vacuole to become fusogenic with exocytic vesicles. Interaction with this secretory pathway appears to provide a pathogenic mechanism that allows chlamydia to establish themselves in a site that is not destined to fuse with lysosomes. Almost nothing is known about the
relationship between distinct steps of this chlamydial development cycle and initiation of host cell signaling pathways.

Nod proteins have so far been associated with recognition of different types of peptidoglycans. Although recent studies suggest a functional peptidoglycan pathway in chlamydia, clear-cut biochemical evidence for the synthesis of peptidoglycans in chlamydia is missing. Chlamydia, however, are sensitive to antibiotics that inhibit peptidoglycan synthesis. This phenomenon has been referred to the “chlamydial anomaly.” Our finding that C pneumoniae induced a Nod-mediated endothelial cell activation via heat-stable components does not prove, but may lend support to, the existence of peptidoglycan-like structures in chlamydia.

Recognition of chlamydia by Nod proteins could thus be interpreted in at least two different ways. First, the chlamydial cell wall contains peptidoglycan or peptidoglycan-like structures. This hypothesis is in accordance with several studies suggesting the expression of peptidoglycan-like structures not on the surface of elementary bodies but—invasion of the target cells—on the subsequently developed reticular bodies. Second, Nod proteins act as receptors for molecules other than peptidoglycans. Proteins such as GroEL-1 or MOMP could be involved in Nod activation because the minimal motif recognized by Nod1 is a dipeptide containing diaminopimelic acid, and chlamydia could possibly synthesize this dipeptide in a peptidoglycan-independent way. In line with this hypothesis are preliminary data suggesting the recognition of recombinant GroEL-1 by Nod proteins in HEK293 cells (data not shown). However, additional studies using purified components from C pneumoniae are necessary to further analyze the importance of different chlamydial virulence factors for Nod-mediated signal transduction.

Nod1 has been found in multiple tissues, whereas the expression of Nod2 is mainly restricted to leukocytes, dendritic cells, and epithelial cells. Analyzing endothelial cells and different epithelial, monocytic, and lymphocytic cell lines, we found that human endothelial cells seemed to express the highest levels of Nod1 mRNA, suggesting that Nod1 might play a key role in endothelial cells.

Little is known about the different functions of Nod1 and Nod2 proteins as receptors for intracellular pathogens like chlamydia. Because no differences in the downstream signaling of Nod2 was observed so far, both receptors might substitute for each other in some cases for intracellular recognition of bacteria in varying tissues.

Several recent studies demonstrated the involvement of TLR2 and TLR4 in initiation of target cell activation by C pneumoniae or chlamydial components. TLR2 was suggested to be more important than TLR4 for recognition by and activation of innate immune cells. In our system of TLR2-overexpressing HEK293 cells, we could demonstrate that viable and heat-inactivated C pneumoniae were able to induce NF-κB-activation on cell contact when “applied” from the extracellular side. Heat-killed chlamydia, however, failed to activate NF-κB in Nod1 or Nod2-overexpressing HEK293 cells on extracellular challenge, indicating that Nod proteins serve as intracellular receptors. These considerations are in line with our observation that viable, but not heat-inactivated, chlamydia were able to induce a marked release of IL-8 from infected endothelial cells, because HUVECs express Nod1 but barely express TLR2. Thus, Nod proteins rather than TLR2 appear to contribute to C pneumoniae–mediated endothelial cell activation. Recently, a report was published...
showing a MyD88-dependent and TLR4-dependent interferon-α/β induction by C pneumoniae in macrophages. In contrast, NF-κB activation and tumor necrosis factor-α, IL-1, and IL-6 secretion were MyD88-independent and TLR4-independent. These data support the hypothesis of a TLR-independent but Nod-dependent mechanism of target cell activation by C pneumoniae.

The association between chronic/persistent chlamydial infection and development of atherosclerosis is still controversially discussed. The idea was supported by: (1) experimental studies in animal models demonstrating enhanced development of atherosclerotic lesions after infection with C pneumoniae; (2) detection of C pneumoniae in atherosclerotic plaques (electron microscopy, immunocytochemistry, PCR); and (3) serological association between C pneumoniae infection and coronary heart disease. However, many epidemiological studies failed to consider the various confounding risks of atherosclerosis, such as cigarette smoking and hyperlipidemia. In addition, it was reported that mice that were held in a germ-free environment revealed no differences concerning atherosclerotic disease when compared with mice that were kept in normal environment. Finally, most of the clinical trials trying to demonstrate a direct link between antichlamydial antibiotic therapy and improved outcome of atherosclerosis failed. However, intracellularly persistently chlamydia are antibiotic-resistant, and therefore the interventional studies are not helpful to prove the concept. Thus, it still remains uncertain whether C pneumoniae represents a causative role in atherosclerosis or is just a concomitant phenomenon. In contrast, the role of the TLRs in atherosclerosis is firmly established. It is thus conceivable that other innate immune receptors such as Nod proteins might be involved in this inflammatory process. The recently established Nod1 and Nod2 knockout mice will be of outstanding value for future studies.

Taken together we provide evidence that Nod1 is a potent innate immune receptor for C pneumoniae in endothelial cells. Whereas TLR2 might be important for initial cell activation on chlamydial contact in some cell types, Nod proteins are crucial for an intracellularly triggered prolonged activation on chlamydial contact in some cell types, Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB, and Nod2 detects a unique muramyl dipeptide from gram-negative bacterial peptidoglycan. Thus, Nod1 and Nod2 might be important for initial cell activation on chlamydial contact in some cell types, and the role of the TLRs in atherosclerosis is firmly established. Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB, and Nod2 detects a unique muramyl dipeptide from gram-negative bacterial peptidoglycan.

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