**Nucleotide-binding Oligomerization Domain Proteins Are Innate Immune Receptors for Internalized *Streptococcus pneumoniae***

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*Streptococcus pneumoniae*, the major cause of community-acquired pneumonia and bacterial meningitis, has been shown to transiently invade epithelial and endothelial cells. Innate immune receptors including Toll-like receptors recognize various pathogens, such as *S. pneumoniae*, by identifying conserved pathogen-associated molecular patterns. Recently, two members of a novel class of pattern recognition receptors, the cytosolic proteins nucleotide-binding oligomerization domain 1 (Nod1)/CARD4 and Nod2/CARD15, have been found to detect cell wall peptidoglycans. Here we tested the hypothesis that Nod proteins are involved in the intracellular recognition of pneumococci. Data indicate that pneumococci invade HEK293 cells. Genetic complementation studies in these cells demonstrate that NF-κB activation induced by *S. pneumoniae* depends on Nod2. Moreover, intracellular transfection of inactivated pneumococci yielded similar effects, confirming the Nod2 dependence of NF-κB activation by pneumococci in HEK293 cells. By dominant negative overexpression and small interfering RNA experiments, we show for the first time that interleukin-1 receptor-associated kinase participates in Nod2-dependent NF-κB activation. Additionally, dominant negative interleukin-1 receptor-associated kinase 2, tumor necrosis factor receptor-associated factor 6, NF-κB-inducing kinase, transforming growth factor-β-activated kinase-binding protein 2, and transforming growth factor-β-activated kinase 1 also inhibited Nod2-dependent NF-κB activation. We finally demonstrate that in C57BL/6 mouse lung tissue in vivo as well as in the bronchial epithelial cell line BEAS-2B, Nod1 and Nod2 mRNA expressions were up-regulated after pneumococcal infection. Data presented suggest that Nod proteins contribute to innate immune recognition of *S. pneumoniae*. Furthermore, Rip-2 and members of the Toll-like receptor-signaling cascade are involved in the Nod2-dependent activation of NF-κB induced by pneumococci.

3 The abbreviations used are: PAF, platelet-activating factor; CARD, caspase recruitment domain; dn, dominant negative; IRAK, interleukin-1 receptor-associated kinase; MyD88, myeloid differentiation protein 88; Mal, MyD88 adapter-like; MDP, muramyl dipeptide; NIK, NF-κB-inducing kinase; nod, nucleotide-binding oligomerization domain; Rip2, receptor-interacting protein 2; TAB, transforming growth factor-β-activated kinase-binding protein; TAK, transforming growth factor-β-activated kinase; TLR, Toll-like receptors; TRAF, tumor necrosis factor receptor-associated factor; TRIF, TIR domain-containing adapter-inducing interferon-β; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; cDNA, colony-forming units; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; RT, reverse transcription.

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actions and eventually leads to nuclear translocation of the transcription factor NF-κB via IκB release by the IκB kinase complex (16–18). However, there might be intermediate signaling molecules leading to NF-κB activation by Nod proteins that are still unknown.

Because pneumococci penetrate epithelial as well as endothelial cells and Nod proteins mediate intracellular detection of bacterial flagellins, we tested the hypothesis that Nod proteins are involved in host recognition of *S. pneumoniae*. Overexpression experiments indicate that Nod2 recognizes *S. pneumoniae*. We furthermore show that Nod protein expression is up-regulated in *vivo* and *in vitro* by pneumococcal infection. Moreover, this study suggests that in addition to Rip2, the signal-transducing molecules IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 are involved in NF-κB activation by Nod proteins in pneumococci-stimulated cells.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Capsulated *S. pneumoniae* serotype 3 (NCTC 7978) were used for the murine pneumonia model. Bacteria were incubated at 37°C with 5% CO2 on Columbia agar with 5% sheep blood at 37°C. Single colonies were expanded by resuspension in Todd-Hewitt broth supplemented with 0.5% yeast extract and incubation at 37°C for 3–4 h in midlogarithmic phase (*Amp*: 0.2–0.4). Bacteria were harvested by centrifugation at 1500 g for 15 min at 4°C and were washed twice in sterile PBS. Bacteria were then resuspended in sterile PBS, at ~2.5 × 108 CFU/ml, as determined by plating serial 10-fold dilutions onto Columbia-agar plates.

The *S. pneumoniae* R6x is the unencapsulated derivative of serotype 2 strain D39 (22). A pneumolysin-negative mutant of R6x ply− was generated by insertion-duplication mutagenesis using the pJDc9p plasmid. After transformation of the plasmid into *S. pneumoniae* strain R6x, erythromycin-resistant transformants were selected with 0.025% Triton X-100, and 100 μg/ml of Rifamycin S (Rifamycin S, Mannheim, Germany) was subcloned into the human expression vector cDNA3.1 (Invitrogen). Human embryonic kidney HEK293 cells (Shimizu, Tokyo, Japan) was subcloned into the human expression vector pCMV-CAT (8). For cell culture stimulation studies, R6x or R6x ply− pneumococci were expanded as described above, harvested by centrifugation, and resuspended in suitable cell culture medium or inactivated by heating at 56°C for 30 min and sonication. The ethanol inactivation was performed as described before (7).

**HEK293 Cell Invasion Assay and PAF Receptor Overexpression**—The cDNA encoding the human PAF receptor (kindly provided by T. Shimizu, Tokyo, Japan) was subcloned into the human expression vector cDNA3.1 (Invitrogen). Human embryonic kidney HEK293 cells (ATCC) were cultured in 12-well plates with Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. In some experiments, subconfluent cells were transfected with 0.3 μg of PAF receptor plasmid using Effectene transfection reagent (Qiagen, Hilden, Germany). Pneumococcal invasion of HEK293 was assessed by antibiotic protection assay as described previously for endothelial and epithelial cells (4, 5). Briefly, HEK293 cells were infected with 106 CFU/ml *S. pneumoniae* strain R6x ply−. After the indicated time intervals, cells were washed three times with PBS and incubated for 1 h with culture medium supplemented with penicillin G (10 μg/ml) and gentamicin (200 μg/ml) to kill extracellular bacteria. Subsequently, HEK293 cells were washed three times again with PBS and lysed by using ice-cold 0.025% Triton X-100, and 100-μl aliquots were plated on Columbia agar with 5% sheep blood at 37°C. Colonies were counted after 24 h of incubation at 37°C. In some experiments, subconfluent cells were transfected with 0.3 μg of PAF receptor plasmid using Effectene transfection reagent (Qiagen, Hilden, Germany).

**Expression Plasmids, HEK293 Cell Transfection, and Luciferase Assay**—Expression plasmids for Nod1 and Nod2 were kindly provided by G. Nuñez (An Arbor, MI). Expression vectors encoding wild-type Myc-Rip2 and dominantly negative (dn) Rip-2 were a generous gift from T. Shimizu (8). Expression plasmids for Nod1 and Nod2 were kindly provided by T. Shimizu (9). Expression plasmids for Nod1 and Nod2 were kindly provided by T. Shimizu (10). The constructs encoding dnMyD88, dnIRAK, dnIRAK2, dnTRAF6, and dnNIK were from C. Kirschning (Munich, Germany) and Tulark Inc. (San Francisco, CA); dnTAK1 and dnTAK2 were a kind gift from Thohru Ishitani (Nagoya, Japan); dnMal and dnTRIF were from K. Fitzgerald (Golenbock Laboratory, University of Massachusetts, Amherst, MA). Subconfluent HEK293 cells were cotransfected the following day using the calcium phosphate method according to the manufacturer’s instructions (Clontech) with 0.1 μg of NF-κB-dependent luciferase reporter (23), 0.1 μg of RSV β-galactosidase plasmid, 0.1 μg of PAF receptor, and 0.3 ng of Nod or 0.5 μg of Rip2 expression vectors. In some experiments, 0.5 μg/ml MDP (Calbiochem) or 104 CFU/ml inactivated S. pneumoniae were added to the cells prior to transfection to allow their entry into the cells. When indicated, cells were additionally transfected with 0.1 μg of dominant negative mutants of several signaling molecules. The experiment shown in Fig. 4B was performed using 5, 25, and 125 ng of dnIRAK or dnIRAK2. In some experiments, cells were incubated with S. pneumoniae strain R6x ply− for 5 h on the following day. Luciferase activity was measured by using the Luciferase Reporter-Genie Assay (Promega, Mannheim, Germany), and results were normalized with values obtained by RSV-β-galactosidase or protein concentrations (Fig. 1A).

**RNA Interference**—Control nonsilencing siRNA (sense, UUUUCGCGAGCUGUGACUUCUU; antisense, ACUGUACGCUUGUCGAGGAGAAA) and different siRNAs targeting IRAK were purchased from Dharmacon (Huntingdon, UK). Different siRNAs were checked for their ability to silence IRAK protein expression in HEK293 cells by Western blots using anti-human IRAK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 72 h after siRNA transfection (RNAiFect; Qiagen). Sequence 5 (sense, GGGUGCUUCUAGUGAUAAUAtt; antisense, UUAUCAACGGAACACGCG) was most effective in inhibition of protein expression and was therefore used for further studies. 72 h after cDNA transfections, siRNA duplexes (final concentration 100 μM) were transfected by using RNAiFect (Qiagen).

**Murine Model of Pneumococcal Pneumonia**—Pathogen-free, female C57BL6 mice were obtained from Charles River (Sulzfeld, Germany). Animals were lighted with dark/white 12-h light/dark cycles and were given free access to food and water. The study was approved by the Institutional Review Board for the care of animal subjects. Mice were challenged at 10 weeks of age and 19–20 g of weight. Mice were lightly anesthetized by intraperitoneal injection of ketamine and xylazine and were inoculated intranasally with 20 μl of bacterial suspension (5 × 106 CFU of *S. pneumoniae* serotype 3). Control mice were challenged with 20 μl of sterile PBS. Groups of three mice were sacrificed 6, 12, 24, or 48 h postinfection, and lungs were removed aseptically and immediately frozen in liquid nitrogen. Control mice were sacrificed 6 h postinfection (n = 3). Frozen lung tissue was crushed using a sterile, nitrogen-cooled high grade steel homogenizer. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. For cDNA synthesis, 2 μg of pooled RNA from each group was reversed transcribed using a Pro Star First Strand RT-PCR kit (Strategene, Europe, Amsterdam) following the manufacturer’s protocol by using oligo(dT) primers. Murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as housekeeping gene. After 35 (Nod1) or 40 (Nod2) PCR amplification cycles, samples were resolved on a 1.2% agarose gel. The PCR primers for Nod1 (expected size 469 bp) were 5′-CTTGGATCCTAACGGTCACTCTTC-3′ and 5′-ACATCCTGGTGTCACGTGTTGAGG-3′; primers for Nod2 (expected size 515 bp) were 5′-AGCGAATTTCTTGTGCTCTGGA-3′ and 5′-TCACAAAGAATGTGCGGTCTCTCT-3′; and primers for GAPDH were 5′-TATCGTGGTTGGAACACGAG-3′ and 5′-TCACGTGTAGCCCAAGATGCC-3′. Total RNA from lipopolysaccharide-stimulated murine macrophages (RAW 264.7; murine macrophage cell line) served as positive control.

**Stimulation of BEAS-2B Cells, mRNA Isolation, and Reverse Transcription-PCR**—The human bronchial epithelial cell line BEAS-2B (a generous gift of Dr. Curtis Harris, Bethesda, MD) was cultured in keratinocyte medium supplemented according to the manufacturer’s instructions (ATCC, Manassas, VA). Cells were grown to confluence in 3.5-cm well plates, subsequently infected with 106 CFU/ml *S. pneumoniae* strain R6x for different durations, stimulated with 5 × 103 CFU/ml heat-inactivated pneumococci or 20 ng/ml TNF-α (R&D Systems, Minneapolis, MN). Total RNA was isolated using a RNeasy minikit (Qiagen) and reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega, Heidelberg, Germany). The generated cDNA was amplified by PCR using specific primers (Nod1-sense, 5′-AAGCGAAGAGCTCAGCAAAAT-3′; Nod1-antisense, 5′-TTTCTAGAATTTGCTCCCTC-3′; Nod2-sense, 5′-CGTCTCTCTCTGCTCCTCATGAT-3′; Nod2-antisense, 5′-CGTCTCTCTCTGCTCCTCATGAT-3′). All primers were purchased from TIB MOLBIOL (Berlin, Germany). After 35 (Nod1) or 40 (Nod2) 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 GADPH mRNA expression. The effect of the Nod2-dependent inflammatory effects of active or inactive *S. pneumoniae* as well as inhibitory effects of dominant negative signaling molecule mutants were statistically evaluated using Student’s t test. Throughout the figures, p values <0.05 are indicated by one asterisk, and p values <0.01 are shown by double asterisks.
RESULTS

*S. pneumoniae Invade HEK293 Cells and Activate NF-κB in a Nod2-dependent Manner*—To explore a possible function of Nod proteins in host recognition of *S. pneumoniae*, we performed overexpression assays in HEK293 cells. This cell line has been described as not expressing TLR2, a well known receptor for Gram-positive patterns (24). Moreover, we could not detect any Nod2 mRNA and detected only very low levels of Nod1 mRNAs in these cells by RT-PCR analysis (data not shown). We transiently transfected the cells with an NF-κB-dependent luciferase reporter construct and Nod1 or Nod2, respectively. Additionally, since the PAF receptor has been shown to be involved in internalization of pneumococci (4, 5), we cotransfected a plasmid encoding the PAF receptor. On the next day, cells were exposed to different concentrations of viable *S. pneumoniae* strain R6x ply⁺. We used this pneumolysin-negative mutant for HEK cell experiments in order to avoid pneumolysin-induced damage of HEK293 cells. The bacteria failed to cause induction of reporter gene activity if only a control vector was transfected (Fig. 1A). After overexpressing Nod1 or Nod2, we observed a constitutive reporter activity, which is in line with previously published results (14). Pneumococcal infection further enhanced the reporter gene activity in Nod2-transfected cells in a dose-dependent manner, thus indicating a role of Nod2 in NF-κB activation by pneumococci. Nod2-dependent NF-κB activation by pneumococci was less pronounced in cells that were transfected with an empty control vector instead of the PAF receptor plasmid, indicating the importance of cell invasion of pneumococci for Nod signaling (data not shown). Taken together, the data indicate that Nod2 is involved in recognition of internalized *S. pneumoniae*.

To further confirm internalization of pneumococci into HEK293 cells, we performed an antibiotic protection assay as described previously (4, 5). As seen in Fig. 1B, *S. pneumoniae* strain R6x ply⁺ was internalized into HEK293 cells overexpressing PAF receptor in a time-dependent manner.

**Nod2 Mediates NF-κB Activation by Inactivated S. pneumoniae**—To further characterize the role of Nod proteins in the recognition of pneumococci, we used heat-inactivated, sonicated, or ethanol-inactivated pneumococci; MDP served as a positive control. In contrast to viable bacteria, inactivated pneumococci as well as MDP failed to induce NF-κB activation in Nod-transfected cells when extracellular stimulation was performed (data not shown), an observation compatible with the notion that Nod2 detects only intracellularly located pathogen-derived material. We therefore assessed Nod-dependent NF-κB activity after allowing the internalization of bacterial components into the cells (as described under “Experimental Procedures”). Under these conditions, Nod2 conferred a dose-dependent responsiveness to inactivated pneumococci and MDP (Fig. 2, A and B). Hereby, the ethanol-inactivated pneumococci were more potent than the heat-inactivated pneumococci, displaying Nod2 activity already at a concentration of 5 × 10⁵ CFU/ml. Again, we observed a certain constitutive activity caused by overexpression of Nod1 and Nod2. The different activities in these background activities may be caused by slightly variable transfection conditions.

**Involvement of Rip2, but Not of the TLR Adapter Proteins, in the NF-κB Activation by Inactivated *S. pneumoniae* and MDP**—Following our observation that Nod2, and not the TLRs, mediates NF-κB induction by *S. pneumoniae* in our assay, we tried to further exclude any TLR involvement by using dominant negative mutants of TLR adapter proteins (25). Therefore, we cotransfected HEK293 cells with an NF-κB-dependent reporter gene, Nod2, as well as dominant negative mutants of different signaling molecules and stimulated these cells with heat-inactivated pneumococci or MDP. Vectors containing dominant negative mutants of the TLR adapter molecules MyD88, Mal, and TRIF failed to exhibit significant inhibitory effects on Nod2-dependent NF-κB activation (Fig. 3). As expected and shown earlier, dnMyD88, dMal, and dnTRIF clearly inhibited TLR-dependent NF-κB activity brought about by respective TLR ligands (24, 26) (data not shown). In contrast, dominant negative mutants of Rip2, a kinase known to associate with Nod proteins, suppressed Nod2-dependent
NF-κB activation induced by inactivated pneumococci and MDP. We thus conclude that Nod proteins and Rip2, but not the TLRs or their adapter molecules, mediate NF-κB activation in our assay.

The Signaling Cascade Downstream of Nod2—Little is known about the further downstream signaling cascade of Nod2. In order to identify potential molecules involved, experiments using dominant negative mutants of different signaling molecules were carried out. As shown in Fig. 4A, dnIRAK, dnIRAK2, dnTRAF6, dnNIK, dnTAB2, and dnTAK1 suppressed the Nod2-dependent NF-κB activity induced by pneumococcal components and MDP. Moreover, dominant negative mutants of IRAK and IRAK2 inhibited NF-κB activation in a dose-dependent manner (Fig. 4B).

To further confirm the unexpected role of IRAK in Nod2 signaling, gene silencing experiments using siRNA were performed. To assess gene silencing activities, HEK293 cells were transfected with different siRNAs targeting IRAK as well as nonsilencing siRNA for 24 h, and Western blot analyses using anti-IRAK antibodies were performed 72 h after transfection. Sequence 3 was most effective in gene silencing (Fig. 4C) and was therefore used in subsequent reporter gene assays. As can be seen in Fig. 4D, siRNA targeting IRAK inhibited the NF-κB activation brought about by overexpression of Nod2. In contrast, IRAK siRNA duplexes had no inhibitory effect on TNF-α response.

IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 Are Located Downstream of Nod2 and Rip2—To examine whether IRAK, IRAK2, NIK, TAB2 and TAK1 lie upstream or downstream of Rip2, plasmids encoding wild-type Rip2, an NF-κB reporter, and the dominant negative mutants of signaling molecules examined were cotransfected in HEK293 cells. Overexpression of Rip2 leads to a strong NF-κB activation (Fig. 5). In line with the above mentioned studies, the reporter gene activation could not be suppressed by dnMyD88, dnMal, and dnTRIF. Furthermore, dnIRAK, dnIRAK2, dnTRAF6, dnNIK, dnTAB2, and dnTAK1 inhibited Rip2-induced NF-κB activation. Thus, IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 seem to be located downstream of Rip2 and play essential roles in transducing information from Nod2 to NF-κB activation.

Nod1 and Nod2 mRNA Levels Were Up-regulated in Vivo and in Vitro after Pneumococcal Infection—Finally, we analyzed the expression of Nod1 and Nod2 in mouse lung tissue and examined whether their mRNA levels changed during pneumococcal infection in vivo. C57BL/6 mice were infected with capsulated S. pneumoniae serotype 3 for different durations, and subsequently total RNA of mouse lung tissue was isolated and RT-PCR was performed. Our analyses detected Nod1 transcripts in lung tissue, whereas the expression of Nod2 was lower (Fig. 6A). After pneumococcal infection, a marked increase of Nod1 and Nod2 mRNA levels could be observed.

To further confirm up-regulation of Nod1 and Nod2 expression during infection with S. pneumoniae, we used the human bronchial epithelial cell line BEAS-2B. Cells were infected with S. pneumoniae strain R6x or stimulated with heat-inactivated pneumococci or TNF-α. The levels of Nod1 and Nod2 transcripts were low in untreated epithelial cells examined (Fig. 6B). Increased Nod1 and Nod2 mRNA levels could be detected after infection. Furthermore, Nod1 and Nod2 mRNA up-regulation was also observed after stimulation with inactivated pneumococci for 24 h and when TNF-α was used as a control.
FIG. 4. Inhibition of Nod2-dependent activation of NF-κB by dominant negative mutants or siRNA targeting IRAK as well as dominant negative IRAK2, TRAF6, NIK, TAB2, and TAK1. HEK293 cells were cotransfected with Nod2 expression plasmid, NF-κB-dependent luciferase reporter, and RSV β-galactosidase plasmid in the presence of $5 \times 10^7$ CFU/ml heat-inactivated pneumococci (S.p.) or 0.5 μg/ml MDP. Additionally, 0.1 μg of dn mutants of IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 (A) or different amounts as indicated of dnIRAK and dnIRAK2 (B) were cotransfected. Luciferase activity was obtained by luciferase assay and normalized with β-galactosidase activity. Relative luciferase activities are shown as means ± S.E. for one representative experiment of two with transfection performed in triplicate (A), and titration of dominant negative constructs was performed in duplicate (B). C, HEK293 cells were transfected with nonsilencing siRNA (c) or different sequences of siRNA targeting IRAK (S1–S3) for 24 h. After 72 h, cells were lysed, and Western blots (WB) using antibodies recognizing IRAK or p38 MAP kinase (as a control) were performed. D, HEK293 cells were transfected with nonsilencing siRNA or siRNA targeting IRAK (final concentration 100 nM) for 24 h. After further 24 h, cells were transfected with an NF-κB-dependent luciferase reporter, RSV β-galactosidase plasmid, and Nod2 expression plasmid where indicated. Stimulation with 10 ng/ml TNF-α was used as a control. siRNA experiments were performed three times with similar results, each transfection performed in duplicate (D).
this hypothesis, the penetration of epithelial and endothelial cells by \textit{S. pneumoniae} is initiated during the first hours after infection, and it is most pronounced after 4–6 h (5). mRNA levels of Nod1 and Nod2 increased within a similar time frame, suggesting that Nod-mediated NF-\(\kappa\)B activation might play an important role in this subsequent phase of host responses against \textit{S. pneumoniae}.

The downstream signaling cascade induced by Nod activation is currently poorly understood. By two different approaches, such as dominant negative overexpression and siRNA experiments, we demonstrated that IRAK participates in Nod2 signaling. Moreover, data presented suggest that molecules like IRAK2, TRAF6, NIK, TAB2, and TAK1 are additionally involved in NF-\(\kappa\)B activation and seemed to be located downstream of Nod2 and Rip2. Thus, Nod-mediated signaling might be partly homologous to TLR/MyD88 downstream signaling (25). Importantly, the TLR adapter proteins MyD88, Mal, and TRIF did not participate in NF-\(\kappa\)B activation by Nod2, indicating that Nod and TLR pathways merge downstream of MyD88, Mal, and TRIF. In the Rip2 knockout mice, even TLR-dependent signal transduction was attenuated, showing that Rip2 is not exclusively involved in Nod-regulated cell activation (27, 28), and suggesting a model in which Rip2 could be a junction. Nevertheless, analyses using knockout mice and further siRNA experiments should be initiated to clarify differences and homologies of Nod and TLR signaling pathways.

The Nod-dependent NF-\(\kappa\)B activation by intact or inactive \textit{S. pneumoniae} is most likely due to cell wall peptidoglycan. Nod2 has been found to mediate cell activation by a muramyldipeptide conserved in basically all kinds of peptidoglycans (14, 15), whereas meso-diaminopimelate acid is responsible for Nod1 utilization (12, 13). Our finding that pneumococci showed a clear Nod2 but no Nod1 activity is thus in line with the fact that lysine, and not meso-diaminopimelate acid, has been found as the third amino acid in peptidoglycans of various pneumococcal strains (29). The high constitutive activity of overexpressed Nod1 and Nod2 might be responsible for the limited effect of viable pneumococci on Nod-dependent NF-\(\kappa\)B activation (in contrast to the more distinct effects of intracellularly transfected pneumococci).

A recently published paper describes macrophages from Nod2 knockout mice as responding normally to different pathogen-associated molecules, leading these authors to challenge the role of Nod2 in host defenses (30). In this study, cells were stimulated with known TLR ligands from the extracellular side, and no viable pathogens were used. It remains to be determined how these Nod2-deficient cells would respond to living invasive bacteria. Thus, the Nod2-deficient mice as well as the recently generated Nod1 knockout mice will be of valu-
able help to further elucidate the precise role of these proteins in host defense (12, 13, 30).

Overall, besides *S. pneumoniae* recognition by TLRs, Nod2 activation seems to play an important role in host cell activation by internalized pneumococci. Downstream of Nod2 and Rlp2, signal-transducing molecules like IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 might mediate NF-κB-dependent cell activation. Knowledge about the molecular interaction of pneumococci with target cells may pave the way to innovative therapeutic strategies.

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REFERENCES

1. Bartlett, J. G., and Mundy, L. M. (1995) *N. Engl. J. Med.* 333, 1618–1624
2. Schuchat, A., Robinson, K., Wenger, J. D., Harrison, L. H., Farley, M., Reingold, A. L., Lefkovitz, L., and Perkins, B. A. (1997) *N. Engl. J. Med.* 337, 970–976
3. Centers for Disease Control and Prevention (1997) *MMWR Recomm. Rep.* 46, 1–24
4. Cundell, D. R., Gerard, N. P., Gerard, C., Idanpaan-Heikkila, I., and Tuo- manen, E. I. (1995) *Nature* 377, 1–5
5. Ring, A., Weiser, J. N., and Tuomanen, E. I. (1998) *J. Clin. Invest.* 102, 347–360
6. Koedel, U., Angele, B., Rupprecht, T., Wagner, H., Roggenkamp, A., Pfister, H. W., and Kirschning, C. J. (2003) *J. Immunol.* 170, 438–444
7. Malley, R., Hennke, P., Morse, S. C., Cieslewicz, M. J., Lipsitch, M., Thompson, C. M., Kurt-Jones, E., Paton, J. C., Wessels, M. R., and Golen bock, D. T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 1966–1971
8. Schroder, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gebel, U. B., Weber, J. R., and Schumann, R. R. (2003) *J. Biol. Chem.* 278, 15587–15594
9. Yoshimura, A., Lien, E., Ingalls, R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999) *J. Immunol.* 163, 1–5
10. Weber, J. R., Freyer, D., Alexander, C., Schroder, N. W., Reiss, A., Kuster, C., Pfeil, D., Tuomanen, E. I., and Schumann, R. R. (2003) *Immunity* 19, 269–279
11. Girardin, S. E., Tournebize, R., Mavris, M., Page, A. L., Li, X., Stark, G. R., Bertin, J., DiStefano, P. S., Yaniv, M., Sansonetti, P. J., and Pulppot, D. J. (2001) *EMBO Rep.* 2, 736–742
12. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Su, Q., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nunez, G., and Inohara, N. (2003) *Nat. Immunol.* 4, 702–707
13. Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zahringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, P. J., and Pulppot, D. J. (2003) *Science* 300, 1584–1587
14. Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Pulppot, D. J., and Sansonetti, P. J. (2003) *J. Biol. Chem.* 278, 8869–8872
15. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crepas, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nunez, G. (2003) *J. Biol. Chem.* 278, 5509–5512
16. Bertin, J., Nir, W. J., Fischer, C. M., Tayber, O. V., Errada, P. R., Grant, J. R., Keilty, J. J., Gosselin, M. L., Robison, K. E., Wong, G. H., Glucksman, M. A., and DiStefano, P. S. (1999) *J. Biol. Chem.* 274, 12955–12958
17. Inohara, N., Koedel, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nunez, G. (1999) *J. Biol. Chem.* 274, 14560–14567
18. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nunez, G. (2001) *J. Biol. Chem.* 276, 4812–4818
19. Gutierrez, O., Piacon, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, N., Nunez, G., and Fernandez-Luna, J. L. (2002) *J. Biol. Chem.* 277, 41701–41705
20. Hisamatsu, T., Suzuki, M., and Podolsky, D. K. (2003) *J. Biol. Chem.* 278, 32962–32968
21. Rosenstiel, P., Fantini, M., Brautigam, K., Kuhbacher, T., Waetzig, G. H., Seegert, D., and Schreiber, S. (2003) *Gastroenterology* 124, 1001–1009
22. Tiraby, J. G., and Fox, M. S. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 3541–3545
23. Krull, M., Klucken, A. C., Wuppermann, F. N., Fuhrmann, O., Mayerl, C., Saybold, J., Huppenstiel, S., Hegemann, J. H., Jantos, C. A., and Suttorn, N. (1999) *J. Immunol.* 162, 4834–4841
24. Kirschning, C. J., Wesche, H., Merrill Ayres, T., and Rothe, M. (1998) *J. Exp. Med.* 188, 2091–2097
25. O'Neill, L. A. (2002) *Curr. Top. Microbiol. Immunol.* 270, 47–61
26. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) *J. Immunol.* 169, 6666–6672
27. Chin, A. I., Dempsey, P. W., Bruhn, K., Miller, J. F., Xu, Y., and Cheng, G. (2002) *Nature* 416, 190–194
28. Kobayashi, K., Inohara, N., Hernandez, L. D., Elson, O. C., Akira, S., Nunez, G., and Janez, C. A., Medzhitov, R., and Flavell, R. A. (2002) *Nature* 416, 194–199
29. Severin, A., and Tomasz, A. (1996) *J. Infect. Dis.* 174, 168–174
30. Paulaus, A. L., and Murray, P. J. (2003) *Mol. Cell. Biol.* 23, 7531–7539
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