The mammalian innate immune system recognizes pathogen-associated molecular patterns through pathogen recognition receptors. Nod1 has been described recently as a cytosolic receptor that detects specifically diaminopimelate-containing muropeptides from Gram-negative bacteria peptidoglycan. In the present study, we investigated the potential role of Nod1 in the innate immune response against the opportunistic pathogen Pseudomonas aeruginosa. We demonstrate that Nod1 detects the P. aeruginosa peptidoglycan leading to NF-κB activation and that this activity is diminished in epithelial cells expressing a dominant-negative Nod1 construct or in mouse embryonic fibroblasts from Nod1 knock-out mice infected with P. aeruginosa. Finally, we demonstrate that the cytokine secretion kinetics and bacterial killing are altered in Nod1-deficient cells infected with P. aeruginosa in the early stages of infection.

Pseudomonas aeruginosa is a typical opportunistic pathogen that can cause a variety of systemic infections, particularly in immunocompromised patients such as those with cancer, AIDS, cystic fibrosis, and burns (1, 2). The virulence of P. aeruginosa is multifactorial including several cell-associated and secreted proteins such as elastase A (3), phospholipase C (4), and those translocated through the type III secretion system (5).

The mammalian innate immune system recognizes pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs). PAMPs are conserved structures present in all or large groups of microorganisms but are absent in mammalian cells. PRRs are located either at the cell membrane, where they respond to extracellular PAMPs, or in the cytosol of host cells, where they respond to PAMPs that are present in the cytosol (6). Human Toll-like receptors interact mainly with extracellular PAMPs for example, TLR5 interacts with bacterial flagellin (7), and TLR4 interacts with bacterial lipopolysaccharide (LPS) (8). Signaling through TLRs leads to the activation of NF-κB and, subsequently, of NF-κB target genes.

Recent studies have highlighted the importance of Nod1 and Nod2 in innate immunity. It is thought that they act as cytoplasmic sensors of bacteria and bacterial products (5). Nod1 structurally contains a CARD domain, a centrally located nucleotide-binding oligomerization domain, and multiple C-terminal leucine-rich repeats. Nod1 engages in a homophilic CARD–CARD interaction with Rip2, a CARD-containing protein kinase that in turn interacts with IKK-γ and results in NF-κB activation (9). Nod1 and Nod2 have been recognize muropeptides from bacterial peptidoglycan (PG), although they require distinct molecular motifs within PG to achieve this sensing (10).

Although traditionally considered as an extracellular pathogen, P. aeruginosa has been shown to be internalized by and to survive within different types of mammalian cells, in particular epithelial respiratory cells from nonjunctional epithelia (1). P. aeruginosa infections are usually associated with a marked host inflammatory response, and previous studies have shown that bacterial products such as LPS, flagellin, and CpG DNA from P. aeruginosa are strong stimuli for TLR4 (11), TLR5 (12), and TLR9 (13), respectively.

In this study, we investigated the ability of P. aeruginosa to induce NF-κB activation through Nod1. Our results demonstrate that in addition to data from literature that show the role of TLRs in the detection of products from P. aeruginosa, signaling through Nod1 constitutes a potential important back-up system against P. aeruginosa.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human embryonic kidney epithelial cell line HEK293T were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were maintained in 95% air, 5% CO2 at 37 °C.

**Isolation of Mouse Embryonic Fibroblasts (MEFs)**—Wild-type and Nod1 knock-out mice were used to isolate MEFs as follows. The livers were removed from newborn pups and transferred to a Petri dish containing fresh PBS, cut into small pieces with a sterile blade, and resuspended in trypsin-EDTA in Hanks’ balanced salt solution (Invitrogen). The suspensions were incubated twice...
for 20 min at 37 °C under magnetic stirring and then filtered and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in 20 ml of complete medium (DMEM, 10% fetal calf serum, 2 mM l-glutamine, 50 units/ml penicillin) and cultured to 90% confluence. Each culture was expanded to five new dishes and frozen in liquid nitrogen. Before use, the cells were thawed in complete medium.

**Bacteria and Other Reagents**—The following *P. aeruginosa* strains were used in this study. PAO-1, PAK, and a PAK mutant lacking flagellin (PAKΔflic) (the last two strains were kindly provided by Dr. Steve Lory, Harvard Medical School, Boston, MA). tri-DAP was obtained by treatment of the corresponding MurNAc peptide with partially purified *Escherichia coli* N-acetylmuramyl-l-alanine amidase according to the method of van Heijenoort et al. (14). Muramyl dipeptide (15) was obtained from Calbiochem (San Diego, CA). PG from *P. aeruginosa* PAO-1 was prepared as previously described (16). PG samples were lyophilized in a SpeedVac to estimate the amount of PG and determine the yield per colony-forming unit. Peptidoglycan samples were resuspended in pyrogen-free ultrapure water (Biochrom AG, Berlin, Germany). Amino acid and amino-sugar compositions were determined with a Hitachi L8800 analyzer (ScienceTec, Les Ulis, France) after hydrolysis of samples in 6 M HCl at 95 °C for 16 h. Recombinant TNF-α was from Pharmingen (San Diego, CA).

**Infection Protocol**—Bacteria were grown in Trypticase soy broth at 37 °C overnight under agitation, harvested by centrifugation, and resuspended in DMEM culture medium to an OD600 of 0.1 (10^8 colony-forming units/ml). HEK293T cells or MEFs grown to 80% confluence in 24-well plates in DMEM supplemented with 10% fetal calf serum were infected with bacteria at a multiplicity of infection of 100 or stimulated with flagellin-enriched supernatants from *P. aeruginosa* cultures unless otherwise stated. After incubation for 1 h at 37 °C, extracellular bacteria were removed by washing and by incubation for different periods with DMEM containing 300 μg/ml gentamicin to kill the remaining extracellular bacteria. Flagellin-enriched supernatants were prepared as follows. Bacterial cells from 96-h-old cultures were harvested by centrifugation, and the supernatants were filter-sterilized with 0.22-μm membranes and boiled at 100 °C for 30 min to destroy proteins other than flagellin.

**Plasmids, Transfection, and Luciferase Assays**—Studies on the synergistic activation of NF-κB by PGS were carried out as described previously (17). Studies on NF-κB activation following *P. aeruginosa* infection were carried out as described (18). Briefly, cells were transfected with 75 ng of the reporter plasmid Iκg-luc alone or in combination with 3 ng of Nod1-HA or a dominant-negative form of Nod1 (DN-Nod1-HA/ΔCARD4-HA). The pcDNA3.1 vector was used to balance the transfected DNA concentration. The PG preparations were used at 1 μg/ml, and tri-DAP or muramyl dipeptide (MurNAc-l-Ala-d-isoGln) (10 pmol) were used as the positive and negative control for Nod1, respectively.

**Western Blots**—HEK293T cells were transfected as described above. Briefly, cells were harvested from 6-well plates to detect Nod1-HA expression. Cells were lysed and kept on ice until separation in SDS-8% PAGE. After separation, proteins were transferred to nitrocellulose membranes and probed with an anti-HA (Upstate Biotechnology). After three washes, membranes were incubated with an anti-rabbit IgG conjugated to horseradish-peroxidase. Proteins were visualized by Western Lightening ECL labeling (PerkinElmer Life Sciences). Blots were also probed with a control antibody against human β-actin.

**Cytokine Measurements**—Murine cytokines (TNF-α, MIP-2, and KC) released into the medium were measured using BD Pharmingen opt enzyme immunoassay kits or R&D Systems’ Duoset enzyme-linked immunosorbent assay kits.

**Intracellular Bacterial Survival**—MEFs obtained from wild-type and Nod1 knock-out mice were infected with *P. aeruginosa* for 1 h, washed, and incubated with gentamicin-containing culture medium. After different periods of incubation, the cells were washed with sterile PBS and lysed with 0.1% Triton X-100 in PBS. Viable counts of cell-associated bacteria were determined by serial plating of cell lysates.

**Cell Viability Assays**—The cell membrane integrity was analyzed by flow cytometry after propidium iodide labeling as described previously (2). Briefly, control non-infected or infected cells were detached from the microplate wells with a 0.05% trypsin, 0.02% EDTA solution and pooled with spontaneously detached cells present in the cell culture supernatants. After rinsing, cells were resuspended in PBS (pH 7.2, containing 1% bovine serum albumin) and incubated with propidium iodide at 5 μg/ml for 5 min at room temperature. Samples were kept on ice and analyzed with a FACScalibur flow cytometer (BD Biosciences) equipped with a standard argon ion laser.

**Statistics**—Data were analyzed with the Student’s t-test and Mann-Whitney test, as appropriate. Differences in data values were considered significant at a p value of less than 0.05.

**RESULTS**

*P. aeruginosa Induces NF-κB Activation in Epithelial Cells*—We first investigated whether *P. aeruginosa* was able to induce NF-κB activation in HEK293T cells transiently transfected with a Iκg-luc reporter plasmid. Cells were exposed to different concentrations of a laboratory strain (PAO-1) for 1 h and treated for an additional 3-h period with gentamicin-containing culture medium to eliminate extracellular bacteria. The increase in luciferase activity of infected cells was shown to be up to 5.2 ± 0.5-fold higher than the increase in non-infected control HEK293T cells. Additionally, the increase in luciferase activity was shown to be dependent on the number of bacteria added to the cells (Fig. 1A).

**Flagella Do Not Account for NF-κB Activation of P. aeruginosa-infected Cells**—HEK293T cells, although not expressing TLR2 or TLR4, do express TLR5 (20), which recognize bacterial flagellin. Because in our assay HEK293T cells were exposed to a *P. aeruginosa* flagellated strain for 1 h before the gentamicin treatment, and flagellin monomers may be released from the bacterial cell, we could not exclude the participation of TLR5 in the response to *P. aeruginosa* infection detected in our assays. To determine whether flagella accounted for the NF-κB activation, we compared the levels of luciferase activity following infection with the parental laboratory strain PAK and the nonflagellated mutant PAKΔflic. As shown in Fig. 1B there was no significant difference in the luciferase activity of cells infected with PAK or PAKΔflic. Gentamicin protection assays with PAK and PAKΔflic showed no difference in the invasive ability between these two strains (data not shown). Interestingly, the flagellin-enriched supernatant from the PAK strain was still capable of inducing high levels of NF-κB activation, whereas the supernatant of the PAKΔflic strain had no activity, demonstrating that the flagellin monomers of *P. aeruginosa* are a potent proinflammatory stimulus (Fig. 1B). The results suggest that flagellin monomers are a better stimulus for TLR5 than the assembled molecule, as described previously (21).

**Nod1 Participates in the Innate Immune Response to P. aeruginosa Infection**—The observation that PAKΔflic strain could still induce NF-κB activation, apparently in a TLR-independent manner, led us to hypothesize that the recently described cytosolic receptor Nod1 could be involved in the innate immune recognition of *P. aeruginosa*. It has
been demonstrated that Nod1 detects DAP-containing muropeptides from the PG of Gram-negative bacteria. To determine whether Nod1 is involved in \textit{P. aeruginosa} detection, we first tested whether the highly purified PG of \textit{P. aeruginosa} is recognized by Nod1. To this end, we co-transfected HEK293T cells with a Nod1 expression vector and the purified PG from \textit{P. aeruginosa}. The PG induced a 7.0-fold increase in the luciferase activity when compared with the nonstimulated control (Fig. 2A). In contrast, the transient transfection of a dominant-negative form of Nod1 (DN-Nod1) expression vector inhibited the activation of the co-transfected Igx-luc reporter gene in response to \textit{P. aeruginosa} PG by ~3-fold (Fig. 2C). After the observation that \textit{P. aeruginosa} PG was detected by Nod1, we questioned whether Nod1 would participate \textit{in vitro} in the induction of NF-κB during \textit{P. aeruginosa} infection. To this end HEK293T cells transfected with increasing amounts of DN-Nod1 were infected with PAK and PAK\textsubscript{ΔfliC} strains. The results showed that luciferase activity was inhibited in response to infection PAK and PAK\textsubscript{ΔfliC} strains in a dose-dependent manner (Fig. 3A). Accordingly, MEFs from Nod1-deficient mice infected with PAO-1 strains showed significant lower activation of NF-κB (Fig. 3B).

\textbf{Nod1 Deficiency Leads to Slower Cytokine Production Kinetics}—We next investigated the consequences of Nod1 signaling in the production of proinflammatory cytokines and/or chemokines in response to \textit{P. aeruginosa} infection. To this end MEFs from wild-type and Nod1 knock-out mice were infected with PAO-1 strain for different periods of time, and the supernatants were assayed for the presence of TNF-α, MIP-2, and KC. \textit{P. aeruginosa} infection did not lead to TNF-α or MIP-2 secretion either in the knock-out cells or in the control wild-type cells (data not shown). In contrast, it induced the secretion of high amounts of KC. At 3 h post-infection the wild-type cells secreted significantly more KC than the knock-out cells. However, at the end of 20 h post-infection both wild-type and knock-out cells secreted similar amounts of KC (Fig. 4A).

\textbf{DISCUSSION}—The innate immune system recognizes and responds to an array of bacterial products, such as LPS, lipoproteins, and flagellin, through TLRs (7, 8, 16). These products are highly conserved among both pathogenic and commensal microorganisms. The recognition of PG by PRRs has been shown to be a important feature in the innate immunity of flies and mammals (23). In mammals TLR2 was the first PRR implicated in PG recognition. However, it has been recently demonstrated that TLR2
There is no clear question or statement in the provided text. The text seems to be discussing the role of Nod1 in the immune response against bacterial infection, particularly focusing on the detection of Gram-negative bacteria. However, without a specific question or statement, it is difficult to provide a coherent response.
secretion in Nod1 knock-out mice leads to decreased neutrophil challenge (36). It will be interesting to look at whether this delayed nas...}

Specifically, in a murine model of pneumonia, these chemokines appear crucial to the innate host defense. Indeed, wild-type MEFs were able to eliminate intracellular bacteria more efficiently than Nod1 knock-out MEFs after 2 h of infection in g...}

We have tested and that was not detected in periods of infection of up to 24 h) peaks on day 2. Complex temporal patterns of chemokine expres...}

Inflammation, consistent with its role in regulating the early infiltration of neutrophils and the production of various cytokines and chemokines in the local tissue. KC is a CXC chemokine that recruits and activates neutrophils and the production of various cytokines and chemokines in a number of settings; however, the regulation of such expression has not been explored in detail. In murine models of acute Gram-negative bacterial and fungal pneumonia, these chemokines appear crucial to the innate host defense. Specifically, in a murine model of Pseudomonas pneumonia, KC levels are associated with the presence of neutrophils in the lung (19). Furthermore, studies have demonstrated that site-specific transgenic expression of KC resulted in enhanced clearance of bacteria after Pseudomonas challenge (36). It will be interesting to know at which this delayed secretion in Nod1 knock-out mice leads to decreased neutrophil recruitment and exacerbation of disease.

In summary, we conclude that Nod1 expressed by epithelial cells takes part in the activation of NF-κB and the up-regulated production of an important epithelial cell chemotactic response to P. aeruginosa. Murine models of infection will help to further elucidate the role of Nod1 in P. aeruginosa infection.

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