Toll-Like Receptor 2 Represses Nonpilus Adhesin-Induced Signaling in Acute Infections with the Pseudomonas aeruginosa pilA Mutant

Eva Lorenz,1* Diana C. Chemotti,1 Karen Vandal,2 and Philippe A. Tessier2

Department of Internal Medicine, Section of Molecular Medicine, Wake Forest University Health Sciences, Winston-Salem, North Carolina,1 and Centre de Recherche en Infectiologie, Centre de Recherche du Centre Hospitalier Universitaire de Québec, Université Laval, Sainte-Foy, Quebec, Canada2

Received 18 November 2003/Returned for modification 25 February 2004/Accepted 9 April 2004

Expression of pili and associated proteins is an important means of host invasion by bacterial pathogens. Recent evidence has suggested that the binding of Pseudomonas aeruginosa through nonpilus adhesins may also be important in respiratory diseases, since adhesins bind mucins. Using wild-type C57BL/6 and TLR2KO mice, we compared the induction levels of the host response to P. aeruginosa that either expressed pili or lacked pilus expression due to a mutation in the structural gene pilA. In C57BL/6 mice, deletion of pili led to a decreased immune response, evidenced by a lower secretion of cytokines and a lack of neutrophil chemotaxis. By contrast, the P. aeruginosa pilA mutant induced a hyperresponsive phenotype in TLR2KO mice. TLR2KO mice showed an increased number of neutrophils in lavage fluid compared to the levels seen when either mouse strain was exposed to wild-type P. aeruginosa. Further analysis indicated that the increased neutrophil influx was associated with an increased expression of calgranulins, possibly through an induction of Toll-like receptor 4 (TLR4) expression. The hyperresponsive phenotype of TLR2KO mice exposed to the P. aeruginosa pilA mutant was associated with TLR4 induction and indicated that nonpilus adhesin-induced signaling was repressed by TLR2 function and, if not blocked by the host, could induce airway hyperresponsiveness.

Pseudomonas aeruginosa is an opportunistic human pathogen that routinely infects the host through invasion of the epithelial cells and can be easily cleared by healthy individuals. In susceptible individuals, P. aeruginosa can cause chronic infections, such as the lung infections commonly seen in cystic fibrosis patients, which eventually lead to a severe impairment of lung function (31). In immunocompromised patients, the presence of P. aeruginosa can also cause more severe infections that may lead to the development of sepsis-like disease (36).

P. aeruginosa expresses a variety of surface structures, such as flagella, pili, and endotoxin. With the discovery of the Toll-like receptors (TLRs), experimental evidence has identified several members of this protein family as receptors for bacterial surface components (2). TLRs are known to signal in response to a variety of bacterial and viral components, leading to the initiation of a signal transduction cascade that results in the activation of transcription factors NF-κB and AP-1 (27). More recently, mutations in TLR4 and TLR2, which lead to a decreased function of the receptors, were shown to be associated with an increased risk for severe bacterial infections (18, 19). P. aeruginosa, a gram-negative bacterial species, is known to signal through TLR4 with its lipopolysaccharide (LPS) moiety (11) and through TLR2, as well as TLR1, by way of lipoproteins on its surface (4, 39). In addition, flagella on the surface of P. aeruginosa were shown to signal through TLR5 (12), while the bacterial DNA with its CpG motifs has been shown to signal through activation of TLR9 (3). Since additional polymorphisms in the TLRs have been identified (16) and heterodimer formation of the TLRs has been described (24, 29), it has become important to define the role of the various TLRs in the cumulative host response following exposure to P. aeruginosa. The interaction of bacterial surface components with the host receptors is especially important in our understanding of chronic infections, since bacterial isolates from cystic fibrosis patients, who have a chronic presence of P. aeruginosa in their airways, indicate that the long-term presence of bacteria in the airways leads to changes in the bacterial phenotype (11, 21). P. aeruginosa isolates from cystic fibrosis patients generally lack flagella and express modified endotoxin moieties, which cause a differential activation of TLR4 and TLR5 and may enhance the survival of the bacteria in the host airways (11, 21).

In addition to flagella and LPS, pili, another surface component of P. aeruginosa, are required for the bacteria to attach to and invade host cells (15). Interestingly, similar to the expression of flagella and LPS, pilus expression by P. aeruginosa is modified in patients with chronic infections. Bacterial isolates from cystic fibrosis patients lack pilus expression, suggesting that additional adhesins may play a role in host attachment during chronic infections. In contrast to findings for flagella and LPS, no specific TLR that initiates a host response following pilus adhesion has been identified. The interaction between pili and epithelial cells is thought to be an important first step in the infection process (13). Moreover, the surface of epithelial cells derived from cystic fibrosis patients may favor expression of certain glycolipid structures, including asialoGM1 residues, which contain attachment sequences for P. aeruginosa (35). Pili bind to these structures, indicating that changes in the epithelial cells of cystic fibrosis patients may favor an interaction between pili and epithelial cells during the initial colonization. The interaction of bacterial pili with the host epithelial

* Corresponding author. Mailing address: Department of Internal Medicine, Section of Molecular Medicine, Wake Forest University Health Sciences, Medical Center Blvd., Winston-Salem, NC 27157-1042. Phone: (336) 716-4322. Fax: (336) 716-1214. E-mail: elorenz@wfubmc.edu.
cells induces an inflammatory cascade that results in interleukin 8 (IL-8; macrophage inflammatory protein 2 [MIP-2]) secretion (7). In addition, in epithelial cells from cystic fibrosis patients, increased IL-8 production can also be achieved in the absence of bacterial adhesion, suggesting that IL-8 secretion may be a stress response to the accumulation of mutant cystic fibrosis transmembrane regulator protein in the endoplasmic reticulum.

Pili are complex bacterial appendages made up of more than 40 proteins expressed from several unlinked gene clusters throughout the bacterial genome (14). The environmental triggers that lead to pilus production are unknown, but pili are important in the twitching motility that allows the bacteria to move across cellular surfaces (22, 41). In addition, twitching motility through type IV pili is important in biofilm formation, an important means of bacterial survival in the host (26).

Mutations within each of the pilus-contributing genes can have a wide range of effects, from a total lack of pilus formation to a hyperpiliated phenotype. Inactivation of the pilA or pilB gene results in a lack of pilus formation, while mutations in pilT or pilU lead to overproduction of pili and a lack of motility on solid surfaces (6). Interestingly, the pilA mutant bacteria show reduced cytotoxicity but are better able to adhere to mammalian cells than P. aeruginosa expressing a pilT or pilU mutation (6). The difference in adherence was thought to indicate the expression of alternate adhesins by the pilA mutant bacteria or the possible role of the nonfunctional pili expressed by the P. aeruginosa pilT or pilU mutant bacteria as dominant negative repressors of such alternate adhesins. In addition to pili, nonpilus adhesions have gained importance, since they bind mucins (5). Mucus hypersecretion is a prominent feature in airway diseases, such as asthma, cystic fibrosis, and chronic bronchitis (20). While upregulation of mucin genes during acute respiratory infections is beneficial to the host, persistent upregulation of mucins leads to airway obstruction. In addition, mucus hypersecretion is thought to decrease the effectiveness of antibiotic treatments by generating protective compartments within the mucus, in which bacteria can survive (23). Since the P. aeruginosa pilA mutant is significantly less virulent than wild-type P. aeruginosa but maintains the ability to adhere to epithelial cells and mucin (6), we used this bacterial strain as a model to determine the interaction of nonpilus adhesions with the host response. In a mouse model of acute pneumonia, acute respiratory infections with the P. aeruginosa pilA mutant indicated that TLR2 expression is required to block host signaling induced by these adhesions. In our mouse model, the pilA mutation caused a significant reduction in the host response in C57BL/6 mice, while TLR2KO mice showed a hyperresponsive phenotype. The hyperresponsive phenotype involves elevated neutrophil chemotaxis, calgranulin synthesis, and TLR4 expression. These results suggest that the deletion of wild-type pili in P. aeruginosa leads to the uncovering of alternative adhesions. The signaling by these adhesins is blocked by TLR2. Only in the absence of TLR2 can these adhesins induce a host response, leading to an increased expression of calgranulins and elevated neutrophil chemotaxis.

**MATERIALS AND METHODS**

**Mice.** Six- to eight-week-old C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). TLR2KO mice were obtained from S. Akira (Osaka University) (38). Mice were given food and water ad libitum. The animal investigative protocol was approved by the Wake Forest University School of Medicine Animal Care and Use Committee.

**Inhalation exposure system.** The exposure setup for the saline and bacterial challenges used a previously described nose-only aerosol system (10). Briefly, the exposure system included a jet nebulizer (Malinckrodt Medical, St. Louis, Mo.) and a small Lucite chamber. The chamber had holes to accommodate the intake and outlet valves for the delivery of the aerosol cloud to the chamber. Additional holes were sized to hold 50-ml Falcon-type tubes for restraining the mice during challenges. The tips of the tubes were trimmed off to permit the rostrum of the mouse to protrude into the aerosol cloud entering the chamber. A driving pressure of 25 l/min was used to provide airflow to the chamber. Previously, it was determined that the aerosol delivered to the chamber at room temperature and humidity is polydisperse, with a mean aerodynamic droplet diameter of 0.65 μm, a size well suited for efficient deposition in the mouse lung. For each aerosol challenge, 4.0 ml of the appropriate bacterial dilution (see below) was added to the nebulizer, and during the 24-min nebulization period, approximately 2.8 ml of the bacterial suspension was consumed.

In cases in which a comparison of multiple mouse strains was performed, mice from the appropriate mouse strains were exposed in parallel to minimize experimental variations.

**Bacterial culture and generation of the bacterial aerosol.** The P. aeruginosa pilA mutant and the PA01 (wild-type) strain (gift of C. Whitchurch, Institute for Molecular Bioscience, Brisbane, Australia) (6) were grown in rich Luria-Bertani (LB) medium as described previously (6) to ensure maximum expression of alginate. Cells were pelleted and resuspended in 0.9% NaCl. The concentration of bacteria in the culture was established by dilution plating on LB agar plates without antibiotics to ensure the purity of the stock and by measurement of the optical density to establish the precise concentration of the bacteria in the stock solution. All bacterial stock was diluted to a concentration of 1011 CFU/ml in 0.9% NaCl and stored for a maximum of 12 h at 4°C to maintain viability. On the day of the experiment, the bacterial stock was diluted to a final concentration of 1011 CFU/ml in Hanks buffered saline (Invitrogen, Carlsbad, Calif.). The identical saline was used for the saline control exposures to establish baseline values.

**Measurement of bacterial concentration in the aerosol.** To ensure the delivery of a consistent number of bacteria to the mouse airways, we employed bacterial counts from prewetted filters in the setup phase of the bacterial aerosol model. To measure the numbers of bacteria delivered in the aerosol during a given time frame, prewetted nitrocellulose filters were placed in the outlet tube of the exposure chamber prior to the start of the exposure. Following completion of the exposure, the filters were placed in 15-ml Falcon tubes filled with 5 ml of sterile deionized water. Following incubation at 37°C and shaking at 250 rpm for 5 h, 200 μl of the bacterial eluate was plated on LB agar and Pseudomonas isolation agar plates without antibiotics to determine the numbers of Pseudomonas bacteria in the aerosol and of any additional bacteria as part of the normal lung flora. The plates were incubated overnight, and the bacteria were counted the next morning. The numbers of bacteria on the LB plates ranged from 35 to 45.

**Whole-lung lavage.** At specific time points after the completion of the inhalation exposure, the mice were sacrificed, their chests were opened, and the lungs were lavaged in situ via PE-90 tubing inserted into the exposed tracheas. Following the lavages, the samples were processed as described previously (17). The lavage liquid was centrifuged for 5 min at 200 x g, and the supernatant was decanted and frozen at −70°C for subsequent use. The residual pellet of cells was resuspended and washed twice in Hanks balanced salt solution (without Ca2+ or Mg2+). After the second wash, a small aliquot of the sample was taken for a cell count with a hemocytometer. The cells were then washed once more and resuspended in RPMI medium so that the final concentration had a cell count of 105 cells/ml. The cells that were present in 10 to 12 μl of the 106-cell/ml suspension were spun for 5 min onto a glass slide with a cytocentrifuge (CytoSpin-2; Shandon Southern, Sewickley, Pa.). Duplicate slides were obtained for each lavage sample. Staining was carried out with a Diff-Quick stain set (Harleco, Gibbstown, N.J.). Cells counts were determined in a blinded fashion with respect to mouse strain and exposure condition.

**Assay for bacterial clearance from lungs.** To determine the amount of bacterial infiltrate present in the lungs at 4 or 24 h postexposure, we plated 50 or 100 μl of the original lung lavage fluid, respectively, on LB agar plates to measure the total bacterial loads and on Pseudomonas isolation agar plates to count the P. aeruginosa bacteria that were present in the lungs. Following an overnight incubation at 37°C, the bacterial isolates on each plate were counted.

**Cytokine assays.** Tumor necrosis factor alpha (TNF-α) and MIP-2 cytokine assays were done in duplicate based on 50 μl of lavage fluid and included a standard curve for normalization between different experiments. The lowest limit
of detection for the cytokines for the commercially available kit used (R&D Systems, Minneapolis, Minn.) is 15.2 pg/ml for TNF-α. Calgranulin expression in the lung lavage fluid was measured by enzyme-linked immunosorbent assays specific for either monomer or the calgranulin heterodimer, as previously described (40).

RNA isolation and gene expression analysis. Total RNA was isolated from mouse lung tissue by using RNA-sta station (Tel-Test Inc., Friendswood, Tex.) according to the manufacturer's recommendations. First-strand cDNA synthesis was performed with 1 μg of total RNA by using a First Strand cDNA synthesis kit (Amersham Biotech, Piscataway, N.J.). The subsequent PCR was performed with an Advantage cDNA kit (Clontech, Palo Alto, Calif.) with gene-specific primers for mouse β-actin (forward, 5′- TGT TAC CAA CTG GGA CGA CAT-3′; reverse, 5′- CCG TCA GAT TGG AAA AAT ATG 3′) and TLR4 (forward, 5′- GCA TGG ATC ATA AAC ATG ACA TCA G 3′; reverse, 5′- TCG TCA GAT TGG AAA AAT ATG 3′). PCR products were run on agarose gels containing ethidium bromide to visualize amplification.

Real-time PCR. Real-time PCR was performed with a SYBR green detection kit and an ABI Prisma 7000 sequence detection system (Applied Biosystems, Foster City, Calif.) cDNA was generated as described above for reverse transcription (RT)-PCR. Each 50-μl reaction mixture contained 10 to 80 ng of cDNA (to establish linearity of response), SYBR green universal PCR mix with hot-start AmpliTaq Gold enzyme (Applied Biosystems), and 100 nM concentrations of gene-specific primers. For each unknown sample, the relative amounts of TLR4 mRNA were calculated by the comparative cycle threshold (Ct) method described in the user bulletin supplied by the manufacturer (ABI Prisma 7700 sequence detection system update, 2001; Applied Biosystems) (Table 1). The ΔCt value was calculated by the subtraction of the unstimulated, saline-exposed control ΔCt value from the stimulated ΔCt value. Results are the average percentages of induction relative to those of the saline-treated control mice and ± standard deviations. Real-time PCRs were done in duplicate and repeated twice.

Statistical analysis. A minimum of five mice were used per condition and time point. Readouts for inflammatory markers for each set of mice as well as the numbers of live bacteria recovered in the lavage fluid were averaged, and standard deviations were determined. Statistical significance of differences between sets was analyzed by using the SPSS 9.0 software package. A P value of 0.05 by a two-sided t test with equal variances assumed was used to determine statistical significance.

RESULTS

Deletion of pili reduces the bacterial load in the mouse lung. Pili are an essential component in the process of bacterial adherence and invasion. Infection of C57BL/6 and TLR2KO mice with either the wild-type or pilA mutant bacteria did not indicate any differences in clearance between the pilA mutant bacteria and the wild-type P. aeruginosa in the initial stages of the infection. At 4 h postexposure, the two strains of mice showed almost identical numbers of wild-type bacteria in their lungs. In addition, a comparison of the numbers of the pilA mutant and wild-type bacteria did not indicate any statistically significant numbers. By 24 h postexposure, differences in results between the mouse strains and between the bacteria used became evident. The numbers of wild-type bacteria increased similarly in C57BL/6 and TLR2KO mice by 24 h postexposure (P < 0.05), indicating that TLR2 does not play an essential role in bacterial clearance of wild-type bacteria. In contrast, pilA mutant bacterial numbers did not increase in C57BL/6 mice, and the bacteria were cleared by 24 h postinfection in TLR2KO mice (Fig. 1). This accelerated clearance of pilA mutant bacteria in TLR2KO mice compared to that in C57BL/6 mice exposed to the P. aeruginosa pilA mutant (P = 0.004) suggests that the presence of functional TLR2, even though not affecting the survival of wild-type bacteria, delays the clearance of pilA mutant bacteria from C57BL/6 mice.

Deletion of pil has differential effects on cytokine and chemokine secretion in C57BL/6 and TLR2KO mice. To determine whether the accelerated clearance of pilA mutant bacteria translated into changes in other aspects of the host response to the P. aeruginosa pilA mutant in TLR2KO mice, we measured the expression levels of TNF-α, a proinflammatory cytokine, and of MIP-2, a chemokine in the lung lavage fluids of exposed mice (17). The two proteins yielded similar expression profiles in the mice tested. Exposure to wild-type P. aeruginosa yielded elevated levels of MIP-2 and TNF-α in TLR2KO and C57BL/6 mice (Fig. 2) compared to the levels seen in C57BL/6 and TLR2KO mice exposed to saline. Since the

TABLE 1. Changes in TLR4 mRNA expression in C57BL/6 and TLR2KO mice exposed to pilA mutant bacteria

<table>
<thead>
<tr>
<th>Mouse strain and time of bacterial exposure</th>
<th>TLR4</th>
<th>GAPDH</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>33.47 ± 1.117</td>
<td>21.02 ± 0.806</td>
<td>12.45 ± 0.311</td>
<td>0 ± 0.311</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>32.44 ± 1.06</td>
<td>19.66 ± 1.089</td>
<td>12.78 ± 0.028</td>
<td>0.33 ± 0.028</td>
<td>-4.067 ± 2.07</td>
</tr>
<tr>
<td>4 h</td>
<td>35.30 ± 0.615</td>
<td>21.125 ± 1.124</td>
<td>14.18 ± 0.509</td>
<td>1.75 ± 0.51</td>
<td>-6.59 ± 1.09</td>
</tr>
<tr>
<td>TLR2KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>34.3 ± 1.37</td>
<td>21.725 ± 0.898</td>
<td>12.575 ± 0.434</td>
<td>0 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>32.295 ± 0.969</td>
<td>23.045 ± 0.884</td>
<td>9.25 ± 0.085</td>
<td>-3.325 ± 0.085</td>
<td>92.3 ± 25.33</td>
</tr>
</tbody>
</table>

* None, saline control exposure.

Values for TLR4 and GAPDH are averages ± standard deviations. See Materials and Methods for details.

FIG. 1. Determination of the bacterial load. C57BL/6 and TLR2KO mice were infected with P. aeruginosa wild-type and pilA mutant bacteria and euthanized at 4 and 24 h postexposure as described in Materials and Methods. Aliquots of the lung lavage fluid were plated on Pseudomonas isolation agar plates, and colonies were counted to determine the bacterial load. Data indicate the average results for a minimum of five mice for each data point.
TNF-α and MIP-2 levels were almost identical in C57BL/6 and TLR2KO mice, TLR2 expression is not required for secretion of these inflammatory markers during the acute infection phase.

In C57BL/6 mice exposed to the pilA mutant strain of *P. aeruginosa*, TNF-α and MIP-2 levels were significantly decreased (*P* = 0.0001), indicating that expression of pil is required for an induction of the host response. In TLR2KO mice exposed to pilA mutant bacteria, TNF-α and MIP-2 levels were significantly higher than those seen in C57BL/6 mice (*P* = 0.0002) but were not different from those in TLR2KO mice exposed to wild-type bacteria (*P* = 0.06). This result indicates that in TLR2KO mice, deletion of pil has a minimal effect on the host response, as measured by the secretion of TNF-α and MIP-2. In wild-type mice, on the other hand, the presence of TLR2 function is associated with significantly lower levels of TNF-α and MIP-2 in lung lavage fluid, indicating a weakened immune response in C57BL/6 mice to the *P. aeruginosa pilA* mutant bacteria.

Deletion of pili decreases the influx of neutrophils in C57BL/6 mice but not in TLR2KO mice. In addition to cytokines and chemokines, neutrophil chemotaxis is another defense mechanism against bacterial infection. The accumulation of neutrophils in the airways, such as that seen in cystic fibrosis patients, is commonly associated with progressive airway damage. To determine whether neutrophils were differentially activated in TLR2KO mice, we measured the absolute number of neutrophils in the lung lavage fluid. The absolute number of neutrophils in 1 ml of lung lavage fluid was determined as described in Materials and Methods. Data are averages of results for a minimum of five mice per experimental set. Counting was done by an investigator who was blinded to the experimental condition.

**FIG. 2.** Cytokine and chemokine secretion following infection with wild-type and pilA mutant *P. aeruginosa* bacteria. C57BL/6 and TLR2KO mice were infected as described in Materials and Methods. By using a commercially available kit (R&D Systems), levels of TNF-α (A) and MIP-2 (B) were measured in the lung lavage fluid from mice euthanized at 4 and 24 h postexposure. Results for saline-exposed C57BL/6 and TLR2KO mice are given as a comparison of baseline TNF-α and MIP-2 secretion levels. Results for a minimum of five mice were averaged for each experimental condition.

**FIG. 3.** Neutrophil infiltration in C57BL/6 and TLR2KO mice infected with *P. aeruginosa pilA* mutant and wild-type bacteria. (A) Percentage of neutrophils (polymorphonuclear leukocytes [PMNs]) in the lung lavage fluid. The relative frequency of neutrophils compared to macrophages was determined as described in Materials and Methods. Only neutrophils and macrophages were visible in the lung lavage fluid. Two slides from each lavage sample were stained, and the relative frequencies of macrophages and neutrophils were determined by counts done in a blinded fashion. The percentage of neutrophils indicated is the average result for all mice undergoing the identical exposure protocol. Standard deviations are indicated by bars. Data are averages of results for at least five mice per condition. (B) Total number of neutrophils in the lung lavage fluid. The absolute number of neutrophils in 1 ml of lung lavage fluid was determined as described in Materials and Methods. Data are averages of results for a minimum of five mice per experimental set. Counting was done by an investigator who was blinded to the experimental condition.
neutrophils from well below 60% at 4 h to an almost exclusive presence of neutrophils at 24 h postexposure, TLR2KO mice showed levels of about 60% neutrophils at both time points. Differences between the mouse strains were also observed when the percentages of neutrophils in the lavage fluids were measured following acute exposures to P. aeruginosa pilA mutant bacteria. C57BL/6 mice still showed an increase in lavage fluid neutrophils from 4 to 24 h postinfection with the P. aeruginosa pilA mutant. The levels of neutrophils observed were lower than those observed for infections with wild-type bacteria, consistent with the reduction in the host response to pilA mutant bacteria. By contrast, in TLR2KO mice, the percentage of neutrophils in the lung lavage fluids increased, suggesting that lack of pilus expression led to an increased host response in mice lacking TLR2 function.

Differences in neutrophil chemotaxis between C57BL/6 and TLR2KO mice were maintained when the total numbers of neutrophils were determined. Exposure to wild-type P. aeruginosa resulted in similar numbers of neutrophils in both mouse strains (Fig. 3B). Both strains also showed increases in the total numbers of neutrophils from 4 to 24 h, suggesting a peak in the host response at 24 h, concomitant with the maximum number of bacteria (Fig. 1) seen at the later time point. Differences between the mouse strains became obvious when the P. aeruginosa pilA mutant bacteria were used in the aerosol challenges. In C57BL/6 mice, the total number of neutrophils was not altered compared to that resulting from exposures to wild-type bacteria. Combined with the previously observed decreases in the percentages of neutrophils (Fig. 3A), this result indicates that macrophages are present at elevated numbers in the lung lavage fluids. In TLR2KO mice, neutrophil levels in the lung lavage fluids were altered following exposures to the P. aeruginosa pilA mutant. As early as 4 h postinfection, the total number of neutrophils in the lavage fluids exceeded the number of neutrophils seen in TLR2KO mice following acute exposure to wild-type P. aeruginosa or in C57BL/6 mice exposed to the P. aeruginosa pilA mutant (P = 0.0006). By 24 h postexposure, the total numbers of neutrophils in TLR2KO mice exposed to the pilus-deficient P. aeruginosa bacteria were almost 10-fold higher than the number of neutrophils seen in either mouse strain exposed to wild-type P. aeruginosa (P < 0.0001). This result confirms the hyperresponsive phenotype of TLR2KO mice when they are exposed to nonpiliated P. aeruginosa. The increased levels of lavage fluid neutrophils in TLR2KO mice exposed to wild-type bacteria tie in with a previously published report that indicates cross talk between TLRs (9) and support for the expression of the S100A8/S100A9 complex in lung lavage fluid neutrophils to determine whether an increase in this protein complex would correlate with the changes in the numbers of lavage fluid neutrophils. In humans, elevated levels of the heterodimer were previously associated with chronic respiratory infections, such as those seen in cystic fibrosis patients in which the heterodimer shows increased expression (32). We measured S100A8 and S100A9 both separately and together as a heterodimer in lung lavage fluids (Fig. 4). Wild-type bacteria induced significant levels of S100A8, S100A9, and the heterodimer in C57BL/6 mice, while the P. aeruginosa pilA mutant induced only minimal levels of calgranulins in wild-type mice. TLR2KO mice showed lower levels of calgranulins than C57BL/6 mice following exposure to wild-type bacteria at 4 h postexposure, suggesting that TLR2 function may be required for the maximum induction of calgranulins. By 24 h postinfection, the differences between TLR2KO and C57BL/6 mice in lavage fluid calgranulins had decreased, with the heterodimer and S100A9 present at almost identical levels in the two mouse strains.

Exposure to the P. aeruginosa pilA mutant induced only minimal levels of calgranulins in C57BL/6 mice, supporting the association between calgranulin expression and neutrophil influx. Based on the elevated neutrophil influx seen in TLR2KO mice exposed to pilA mutant bacteria, we expected an increased expression of lavage fluid calgranulins. Analysis of calgranulin expression confirmed that TLR2KO mice secrete elevated levels of calgranulins when exposed to the P. aeruginosa pilA mutant. The expression of lavage fluid calgranulins in TLR2KO mice exposed to the P. aeruginosa pilA mutant was significantly increased compared to the levels seen in C57BL/6 mice (P value of <0.007 for the heterodimer) and reached levels that were similar to those seen in C57BL/6 mice exposed to wild-type bacteria at 4 h postexposure (P value of 0.065 for the heterodimer). At 24 h postexposure, the expression of calgranulins was higher in TLR2KO mice exposed to the P. aeruginosa pilA mutant than in C57BL/6 mice exposed to nonpiliated bacteria and comparable to the calgranulin levels in C57BL/6 mice exposed to wild-type P. aeruginosa (P value of 0.14 for the heterodimer). These results support the strong association between neutrophil influx and calgranulin expression. The results further suggest that the hyperresponsive phenotype of TLR2KO mice is mediated in part by host factors regulating calgranulin expression.

TLR4 expression is higher in TLR2KO mice than in C57BL/6 mice. TLR4 was previously shown to be crucial in mediating the LPS response in airway epithelial cells (28), and TLR4 signaling was able to induce TLR2 expression (9) in endothelial cells. We therefore hypothesized that the increased expression of calgranulins in the absence of functional TLR2 and the resulting hyperresponsive phenotype of TLR2KO mice may be associated with an altered expression of TLR4. Measurements of the TLR4 expression levels in the lungs of TLR2KO and C57BL/6 mice exposed to mutant and wild-type P. aeruginosa bacteria confirmed a differential expression of TLR4 in the two mouse strains following exposure to the P. aeruginosa pilA mutant. Exposure to wild-type bacteria induced powerful neutrophil chemotaxtants. Since the calgranulin heterodimer was shown to be required for the activation of neutrophils following acute LPS stimulation, we measured the expression of the S100A8/S100A9 complex in lung lavage fluid neutrophils to determine whether an increase in this protein complex would correlate with the changes in the numbers of lavage fluid neutrophils. In humans, elevated levels of the heterodimer were previously associated with chronic respiratory infections, such as those seen in cystic fibrosis patients in which the heterodimer shows increased expression (32). We measured S100A8 and S100A9 both separately and together as a heterodimer in lung lavage fluids (Fig. 4). Wild-type bacteria induced significant levels of S100A8, S100A9, and the heterodimer in C57BL/6 mice, while the P. aeruginosa pilA mutant induced only minimal levels of calgranulins in wild-type mice. TLR2KO mice showed lower levels of calgranulins than C57BL/6 mice following exposure to wild-type bacteria at 4 h postexposure, suggesting that TLR2 function may be required for the maximum induction of calgranulins. By 24 h postinfection, the differences between TLR2KO and C57BL/6 mice in lavage fluid calgranulins had decreased, with the heterodimer and S100A9 present at almost identical levels in the two mouse strains.

Exposure to the P. aeruginosa pilA mutant induced only minimal levels of calgranulins in C57BL/6 mice, supporting the association between calgranulin expression and neutrophil influx. Based on the elevated neutrophil influx seen in TLR2KO mice exposed to pilA mutant bacteria, we expected an increased expression of lavage fluid calgranulins. Analysis of calgranulin expression confirmed that TLR2KO mice secrete elevated levels of calgranulins when exposed to the P. aeruginosa pilA mutant. The expression of lavage fluid calgranulins in TLR2KO mice exposed to the P. aeruginosa pilA mutant was significantly increased compared to the levels seen in C57BL/6 mice (P value of <0.007 for the heterodimer) and reached levels that were similar to those seen in C57BL/6 mice exposed to wild-type bacteria at 4 h postexposure (P value of 0.065 for the heterodimer). At 24 h postexposure, the expression of calgranulins was higher in TLR2KO mice exposed to the P. aeruginosa pilA mutant than in C57BL/6 mice exposed to nonpiliated bacteria and comparable to the calgranulin levels in C57BL/6 mice exposed to wild-type P. aeruginosa (P value of 0.14 for the heterodimer). These results support the strong association between neutrophil influx and calgranulin expression. The results further suggest that the hyperresponsive phenotype of TLR2KO mice is mediated in part by host factors regulating calgranulin expression.

Calgranulin expression is elevated in TLR2KO mice exposed to the P. aeruginosa pilA mutant. Calgranulins are pow-
robust levels of TLR4 mRNA in C57BL/6 mice (Fig. 5A). By contrast, significantly lower levels of TLR4 mRNA were seen when C57BL/6 mice were exposed to the P. aeruginosa pilA mutant, suggesting a weakened immune response following an acute infection with gram-negative bacteria. TLR4 mRNA also decreased in abundance from 4 to 24 h postinfection following exposure to the P. aeruginosa pilA mutant, possibly indicating the establishment of a tolerance phenotype associated with a downregulation TLR4 surface expression (28). By contrast, TLR2KO mice showed robust levels of TLR4 mRNA in re-
response to either bacterial strain. The expression levels of TLR4 mRNA in TLR2KO mice following exposure to pilA mutant bacteria suggest that increased TLR4 signaling may cause the hyperresponsive phenotype of TLR2KO mice in response to infections with nonpiliated bacteria. Previous evidence has linked increases in TLR4 mRNA to increased sensitization of epithelial cells to LPS, which is a component of the P. aeruginosa cell wall, and subsequently increased mitogen-activated protein kinase production, which also led to higher levels in TNF-α and MIP-2 expression (25).

Using quantitative real-time PCR, we measured the relative expression levels of TLR4 in C57BL/6 and TLR2KO mice exposed to the P. aeruginosa pilA mutant to verify the increased expression of TLR4 in TLR2KO mice. Our analysis of the TLR4 mRNA induction levels relative to baseline expression levels in saline-exposed TLR2KO and C57BL/6 mice indicated that TLR4 mRNA was actually decreased in C57BL/6 mice following an acute exposure to the P. aeruginosa pilA mutant. By contrast, TLR2KO mice exposed to the mutant bacteria showed significant inductions of TLR4 mRNA at both 4 and 24 h postexposure (Fig. 5B). There is no statistically significant difference between the induction levels of TLR4 at 4 and 24 h postexposure, but the induction of TLR4 in TLR2KO mice exposed to the P. aeruginosa pilA mutant suggests that the increased host response is mediated in part through TLR4, which is down-regulated in exposed C57BL/6 mice. These results indicate that TLR2 expression and function are preventing the induction of TLR4 during acute infections with the P. aeruginosa pilA mutant.

DISCUSSION

P. aeruginosa is a common respiratory pathogen that can cause both acute and chronic airway infections in humans. During the course of chronic infections, P. aeruginosa undergoes specific phenotypic changes, such as loss of flagella and the development of a mucoid phenotype (30). Both modifications are thought to contribute to the enhanced survival of the bacteria in human airways. In addition to these changes, P. aeruginosa expresses other surface components and proteins that are important in the initial infection as well as in the ability of the bacteria to survive over the long term in the host. Previous evidence has implicated the presence of quorum-sensing mechanisms with the enhanced ability of P. aeruginosa to evade detection by the host through modulation of the timing at which virulence factors are secreted (33). We have focused in this report on nonpilus adhesins and their interaction with the host. Through the use of a pilA mutant strain of P. aeruginosa, which lacks functional pili (6), we were able to determine how the lack of pili affects the induction of the initial host response in a mouse model of acute infection. In addition, we used wild-type and knockout mice lacking TLR2 expression (37) to study how modification of the host genotype may affect the interaction with adhesins.

Comparing bacterial clearances of the wild-type and pilA mutant bacteria in C57BL/6 mice did show a decreased survival of the pilA mutant in mouse lungs at 24 h postexposure. At an earlier time point, 4 h postexposure, the two bacterial strains had shown equal survival rates. As seen in wild-type C57BL/6 mice, the wild-type bacteria also increased in abundance in the lavage fluid of TLR2KO mice by 24 h postexposure. By 24 h postinfection, no live pilA mutant bacteria were present in the lavage fluid of TLR2KO mice, while they could be recovered from C57BL/6 mice. This result suggests that TLR2 function does not affect the clearance of wild-type bacteria, while TLR2 expression delays the clearance of pilA mutant bacteria.

Since the analysis of bacterial survival suggested an interaction between TLR2 and nonpilus adhesins, we further analyzed the induction of the host response by measuring cytokine secretion and neutrophil numbers in lavage fluid. Analysis of the TNF-α and MIP-2 levels in the lung lavage fluid confirmed the requirement of pili for the induction of a full host response in C57BL/6 mice but not in TLR2KO mice. Moreover, TLR2KO mice showed a hyperresponsive phenotype to the P. aeruginosa pilA mutant, as indicated by the high levels of MIP-2 and TNF-α in the lavage fluid. This result indicates that TLR2 expression reduces the host response to bacterial signaling through nonpilus adhesins.

The potential role of TLR2 expression in reducing the host response to the pilA mutant bacteria was supported by an analysis of the lavage fluid neutrophils. Analysis of the percentage of neutrophils in the lung lavage fluid showed a much stronger influx of neutrophils in TLR2KO mice than in C57BL/6 mice when the P. aeruginosa pilA mutant was used in the aerosol challenges. Analysis of total lavage fluid neutrophils further supported the hyperresponsive phenotype of TLR2KO mice in response to the P. aeruginosa pilA mutant. While TLR2KO and C57BL/6 mice showed similar numbers of neutrophils in response to wild-type bacteria, exposure to the P. aeruginosa pilA mutant caused a significantly elevated neutrophil influx in TLR2KO mice. C57BL/6 mice showed only a minor influx of neutrophils, consistent with a reduction in the host response. This increase in neutrophils in TLR2KO mice is not merely due to a dysregulation of neutrophil apoptosis, which is regulated by TLR2 expression (34), but suggests a direct role of TLR2 in reducing host signaling during acute infections with pilA mutant bacteria, since the difference in neutrophil chemotaxis between TLR2KO and C57BL/6 mice is increased when nonpiliated instead of wild-type P. aeruginosa bacteria are used in aerosol challenges.

To define the molecular basis for the altered neutrophil influx in TLR2KO and C57BL/6 mice in response to P. aeruginosa pilA mutant infection, we measured the levels of calgranulins in lavage fluid. Calgranulins are a family of powerful neutrophil chemoattractants that are overexpressed in respiratory infections. The original name for the S100A8/S100A9 heterodimer, cystic fibrosis antigen, was due to the high levels of calgranulins found in cystic fibrosis patients (32). The analysis of the lavage fluid calgranulins indicated that their levels closely mimic the numbers of neutrophils. Exposure to wild-type bacteria induces higher levels of calgranulins in C57BL/6 mice than in TLR2KO mice, indicating a requirement for functional TLR2 expression in the host response to wild-type P. aeruginosa. By contrast, exposure to the P. aeruginosa pilA mutant induced significantly reduced calgranulin levels in C57BL/6 mice, supporting the requirement for pili for an efficient infection of wild-type mice. TLR2KO mice showed an upregulation of S100A8 and S100A9 as well as the heterodimer following exposure to the P. aeruginosa pilA mutant. The host response to adhesins is mediated by TLR4 signaling.
in addition to calgranulins, since the hyperresponsive phenotype in TLR2KO mice exposed to the P. aeruginosa pilA mutant is associated with an induction of TLR4 expression that is specific for TLR2KO mice and is not seen in C57BL/6 mice. The results of this study support a direct interaction of TLR2 with nonpilus adhesins. TLR2 expression reduces host-signaling in response to adhesins during acute infections. In relation to cystic fibrosis pathogenesis, this result suggests an interesting scenario. Mucins are persistently induced in the airways of cystic fibrosis patients, leading to mucus hypersecretion. Adhesins are thought to be important in binding to mucins, and the interaction of adhesins with the host, if not blocked by TLR2 expression, leads to an overexpression of calgranulins. Calgranulins are overexpressed in the airways of cystic fibrosis patients. Since adhesin-induced host signaling and the subsequent overexpression of calgranulins can be blocked by TLR2 function, therapeutic measures aimed at the overexpression of TLR2 may be beneficial for cystic fibrosis patients. The finding also suggests that the modulation of TLR2 expression during chronic infections may have important implications for cystic fibrosis pathogenesis. Rather than lead to a decreased host response, a reduction in TLR2 expression and signaling would augment the effect of phenotypic adaptation in bacteria during chronic infection and lead to an exacerbation of the host response. Further research is needed to determine how the loss of pil correlates with expression changes in the host. Since pil are essential for the initial binding of P. aeruginosa to epithelial cells, it will be important to determine whether decreases in the expression level of TLR2 coincide with the loss of pili as the infections in the airways of cystic fibrosis patients progress to reach a chronic stage. In addition, phenotypic changes such as loss of flagella, a modification associated with the chronic presence of P. aeruginosa in human airways, may also increase the host response rather than decrease it. While the pilA mutant P. aeruginosa shows a TLR2-specific effect, additional phenotypic changes, such as the loss of flagella and the conversion to a mucoid phenotype, may target additional TLRs in a similar way. Research into the role of flagella and the effect their loss has on the host response may be of particular interest, since flagellin was recently shown to signal through both TLR2 and TLR5 (1). A loss of flagella could therefore induce similar effects in TLR2KO mice as did acute infections with the P. aeruginosa pilA mutant. Further research will therefore have to address changes in the host response to these additional bacterial modifications associated with chronic infections, when factors such as tolerance may significantly affect the function of the TLRs (8). If several TLRs are associated with enhanced signaling due to changes in the bacterial phenotype during chronic infections, anti-inflammatory therapies aimed at one single receptor would remain ineffective. The change from a requirement for pili during the initial infection to a loss of pili during chronic infections, both of which are associated with increases in the host response, indicates that potential therapies may also have to differentiate between the acute and chronic stages of a respiratory infection.

ACKNOWLEDGMENTS

We thank Letitia D. McDougal and Rebecca W. Todd for excellent technical assistance.

This work was supported by a grant-in-aid from the American Heart Association. E.L. is the recipient of the C. Scott Venable Research Award from the American Lung Association.

REFERENCES


