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Importance of TLR2 in Early Innate Immune Response to Acute Pulmonary Infection with Porphyromonas gingivalis in Mice

George Hajishengallis, Min Wang, Gregory J. Bagby, and Steve Nelson

The periodontal pathogen Porphyromonas gingivalis is implicated in certain systemic diseases including atherosclerosis and aspiration pneumonia. This organism induces innate responses predominantly through TLR2, which also mediates its ability to induce experimental periodontitis and accelerate atherosclerosis. Using a validated mouse model of intratracheal challenge, we investigated the role of TLR2 in the control of P. gingivalis acute pulmonary infection. TLR2-deficient mice elicited reduced proinflammatory or antimicrobial responses (KC, MIP-1α, TNF-α, IL-6, IL-12p70, and NO) in the lung and exhibited impaired clearance of P. gingivalis compared with normal controls. However, the influx of polymorphonuclear leukocytes into the lung and the numbers of resident alveolar macrophages (AM) were comparable between the two groups. TLR2 signaling was important for in vitro killing of P. gingivalis by polymorphonuclear leukocytes or AM and, moreover, the AM bactericidal activity required NO production. Strikingly, AM were more potent than peritoneal or splenic macrophages in P. gingivalis killing, attributed to diminished AM expression of complement receptor-3 (CR3), which is exploited by P. gingivalis to promote its survival. The selective expression of CR3 by tissue macrophages and the requirement of TLR2 inside-out signaling for CR3 exploitation by P. gingivalis suggest that the role of TLR2 in host protection may be contextual. Thus, although TLR2 may mediate destructive effects, as seen in models of experimental periodontitis and atherosclerosis, we have now shown that the same receptor confers protection against P. gingivalis in acute lung infection. The Journal of Immunology, 2008, 181: 4141–4149.

Porphyromonas gingivalis is a Gram-negative oral anaerobic bacterium that is strongly associated with human periodontitis (1, 2) and is implicated in several systemic diseases including aspiration pneumonia (3–5), which is responsible for significant morbidity and mortality in the elderly (6). In this regard, it is thought that the dental plaque biofilm serves as a persistent reservoir for respiratory infections and that oral pathogens can be aspirated into the lung to cause aspiration pneumonia (7–9). Indeed, certain oral pathogens and especially P. gingivalis are common isolates from aspiration pneumonia and lung abscesses (5, 10, 11). Recently, periodontitis was epidemiologically implicated as a mortality risk factor for aspiration pneumonia in the elderly (12).

To facilitate the study of host pulmonary-bacterial interactions and the pathogenesis of anaerobic pulmonary infections, a well-defined mouse model was established by one of the coauthors (13). In this model, intratracheal challenge with Bacteroides (Porphyromonas) gingivalis causes inflammation in the lung leading to severe bronchopneumonia and lung abscess (13). The inflammation is characterized by a marked recruitment of polymorphonuclear leukocytes (PMN) (3) and significant bacterial killing (13). Subsequent mouse studies by independent groups characterized the inflammatory pulmonary response against P. gingivalis, which includes induction of TNF-α, IL-1β, IL-6, and KC, a functional homolog of human IL-8 (14, 15). However, the host receptor(s) involved in inflammation and control of P. gingivalis lung infection have not been addressed.

In the absence of lung infection or inflammation, PMN are essentially absent and alveolar macrophages (AM) constitute >98% of the total leukocyte population in the alveolar space. AM form the first line of pulmonary defense at the air-tissue interface and their role lies in phagocytosis of airborne particles or invading microorganisms, killing, and coordination of the innate immune response (16). For instance, when the invading pathogen represents an overwhelming load or is too virulent to be contained by the AM alone, AM-released chemokines and other inflammatory mediators recruit and activate large numbers of PMN from the pulmonary vasculature into the alveolar space (17). Studies in humans and experimental animals have underscored the importance of certain cytokines/chemokines in pulmonary host defense, including TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, MIP-1α, and IL-17 (16–20). Also important for host defense of the lung are microbial products such as phagocyte-derived reactive oxygen and nitrogen intermediates (21). Recruited PMN and resident AM utilize these mechanisms to mediate early bacterial clearance (19, 21).

*Department of Periodontics/Oral Health & Systemic Disease and **Department of Microbiology and Immunology, University of Louisville Health Sciences Center, Louisville, KY 40292; and Section of Pulmonary and Critical Care Medicine, Alcohol Research Center, and Department of Physiology, Louisiana State University Health Science Center, New Orleans, LA 70112

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2 Address correspondence and reprint requests to Dr. George Hajishengallis, University of Louisville Health Sciences Center, 501 South Preston Street, Room 206, Louisville, KY 40292. E-mail address: ghajij01@louisville.edu

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These antimicrobial activities and the pulmonary innate response in general are mediated, in large part, by TLRs (21, 22). TLRs are expressed on a number of immune effector cells, including AM and PMN, and serve as sensors of infection for triggering cytokine and antimicrobial responses (16, 21). Studies with different lung pathogens suggest that the role of TLRs in the host response to lung infection ranges from resistance to susceptibility (22–26). In general, TLRs can mediate critical antimicrobial responses, although they may be exploited by certain pathogens for induction of IL-10-mediated immunosuppression (27, 28).

The objective of this study was to determine whether TLR2 plays a role in the control of P. gingivalis acute infection in the lung. TLR2 was selected for investigation owing to its importance in P. gingivalis-induced innate immune responses. Specifically, we have shown that TLR2-deficient cells exhibit diminished induction of NF-κB activation and cytokine production in response to P. gingivalis or purified components thereof (29, 30). The TLR2 dependence of the innate response to P. gingivalis was confirmed in vivo by an independent group (31). Indeed, s.c. infection with P. gingivalis elicited high levels of cytokines in wild-type or TLR4-deficient mice, although in TLR2-deficient mice cytokine responses were hardly detectable or delayed (31). In the same study, TLR2 was associated with exacerbation of P. gingivalis-induced periodontal bone loss (31). This finding is supported by another group, which attributed periodontal bone loss to TLR2-induced TNF-α-dependent osteoclastogenesis (32, 33). TLR2 has moreover been implicated in the acceleration of atherosclerotic heart disease by P. gingivalis (34).

In this study, we show for the first time a protective role for TLR2 in acute lung infection with P. gingivalis. Indeed, TLR2-deficient mice elicited reduced proinflammatory or antimicrobial responses and impaired clearance of P. gingivalis compared with wild-type controls. At an in vitro mechanistic level, both PMN and AM exhibited TLR2-dependent killing of P. gingivalis, and the bactericidal activity of the latter required production of reactive nitrogen species. Interestingly, AM were more potent in P. gingivalis killing than peritoneal or splenic macrophages. This is probably because most tissue macrophages, but not AM, express complement receptor-3 (CR3), which is exploited by P. gingivalis to promote its survival in host tissues and cause disease (35–37). The selective expression of CR3 by tissue macrophages (37, 38) and the requirement of TLR2 inside-out signaling for CR3 exploitation by P. gingivalis (36, 39, 40) suggest that TLR2 deficiency may not always predispose to increased host susceptibility to P. gingivalis. Indeed, our results in conjunction with those of others (31, 32, 34) suggest that TLR2 may play disparate roles in P. gingivalis infections, exacerbating certain chronic conditions (e.g., periodontitis, atherosclerosis) while providing protection against acute pulmonary infection.

Lung infection model

Mice were intratracheally infected with P. gingivalis essentially as previously described (13, 41). Briefly, following mouse anesthesia with intraperitoneal ketamine/xylazine injection, the trachea was aseptically exposed and a P. gingivalis inoculum (10^9 CFU in 40 μl PBS) was administered via a 28-gauge needle. Unchallenged naive mice were administered PBS only. Groups of animals were sacrificed immediately after infection (time 0) or at later time points, 5 or 24 h postinfection. To determine lung bacterial clearance, the lungs were aseptically removed, homogenized in a tissue homogenizer, and serial 10-fold dilutions were plated onto hemin/ menadione-supplemented blood agar plates and cultured anaerobically for enumerating recovered CFU. To determine induction of cytokine production and recruitment of inflammatory cells, each pair of administered lungs (or infected mice not used for assessing lung bacterial clearance) was surgically removed in toto and lavaged with 1.0-ml aliquots of PBS/0.5 mM EDTA up to 10 ml. The first milliliter of recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 500 × g and the supernatant was stored at −80°C for subsequent cytokine analysis. The cell pellet was combined with additional lavage fluid obtained from the same pair of lungs and analyzed for total cell count and differential cell counts performed in cytopsins stained with Wright-Giemsa (Diff-Quick, Baxter Healthcare).

Cytokine and antimicrobial molecule induction analysis

Cytokine levels in the collected BALF samples were assessed by means of a Bio-Plex cytokine bead array using a multiplex for eight cytokines: IL-1β, IL-6, IL-10, IL-12p70, IL-17, TNF-α, KC, and MIP-1α (Bio-Rad Laboratories). Induction of cytokine production in cell culture supernatants was determined using ELISA kits (eBioscience). Myeloperoxidase (MPO) levels in BALF were determined using an ELISA kit (Hycult Biotechnology). NO production was assessed by measuring the amount of nitrite (NO_2^-), a stable oxidative metabolite of NO, in lung tissue homogenates using an assay kit based on the Griess reaction (Cayman Chemical).

Cell isolation and killing assays

AM were obtained for cell culture from the BALF of naive mice. The BALF cell population contained >95% macrophages as determined by morphology upon cytologic examination. The cells were cultured at 37°C and 5% CO_2 atmosphere, in complete RPMI (RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin; Invitrogen). Mouse macrophages were also isolated from the peritoneal cavity via peritoneal lavage with ice-cold HBSS or from splenic cell suspensions by positive selection using anti-CD11b magnetic bead cell sorting (Miltenyi Biotec) (42). Cells were washed and incubated in complete RPMI. After 2 h, aspiration and three successive washes with HBSS were performed to remove any residual nonadherent cells, resulting in a population containing >95% macrophages. To determine intracellular killing of P. gingivalis, an antibiotic protected based intracellular survival assay was used, as we previously described (36). Briefly, following incubulation of P. gingivalis with macrophages (at a multiplicity of infection (MOI) of 10:1) for various time points, extracellular bacteria were eliminated by washing and antibiotic treatment. Viable internalized bacteria were released by macrophage lysis, and serial dilutions of the lysates were plated onto blood agar plates for anaerobic culture and CFU enumeration.

PMNs were purified from mouse blood through three-layer Percoll gradient centrifugation as previously described (43). This method yielded ∼200,000 cells per mouse containing >95% PMN. Killing of P. gingivalis by mouse PMN was determined according to the method of Mydel et al. (44) with minor modifications. PMN-P. gingivalis suspensions (MOI = 1:1) were incubated at 37°C and 5% CO_2 for 2 h without washing to remove extracellular bacteria, and enumeration of viable CFU on anaerobically cultured blood agar plates after PMN lysis was used to determine the killing of both extracellular and intracellular bacteria. The killing index was calculated according to the following formula: [CFU in the absence of PMN – CFU in the presence of PMN]/CFU in the absence of PMN × 100. The selected 2-h time point was based on a time-course study showing significant mouse PMN killing of P. gingivalis in the interval between 105 and 135 min (44).

Oxidative burst assay

The PMN oxidative burst (H_2O_2 production) was monitored as we described previously (45) following the method of Bass et al. (46). The assay is based on the ability of 2',7'-dichlorofluorescin diacetate (DCFH-DA) to diffuse into the cells where it is hydrolyzed to 2',7'-dichlorofluorescin (DCFH) and is thereby trapped within the cells. During the oxidative burst,
nonfluorescent intracellular DCFH is oxidized to highly fluorescent dichlo-rofluorescein (DCF), and the generated fluorescent signal has been quan-titatively correlated with the PMN oxidative burst (46). PMN in glucose-containing HBSS (Invitrogen) were preincubated at 37°C for 15 min with 100 μM DCFH-DA (Invitrogen/Molecular Probes) in microtiter culture plates (2 × 10^5 cells/well). The samples were then incubated with P. gingivalis (MOI = 1:1) at 37°C for various time points. The fluorescent signal resulting from the oxidation of DCFH into DCF was measured as relative fluorescence units on a microplate fluorescence reader (BioTek Instruments) with excitation/emission wavelength settings of 485/530 nm.

Flow cytometric uptake assay

Mouse macrophages were incubated at 37°C with FITC-labeled P. gingivalis at a MOI of 10:1 (36). Phagocytosis was stopped at various time points (5–60 min) by cooling the incubation tubes on ice. After cell washing to remove nonadherent bacteria, extracellular fluorescence (representing attached but not internalized bacteria) was quenched with 0.2% trypan blue. The cells were washed again, fixed with 1% paraformaldehyde, and analyzed by flow cytometry (percent positive cells for FITC-P. gingivalis) using the FACS Calibur and the CellQuest software (Becton Dickinson).

Statistical analysis

Data were evaluated by ANOVA using the InStat v3.06 program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed t tests were conducted. Statistical differences were considered signif-i-cant at the level of p < 0.05. The experiments were performed at least twice for verification.

Results

Impaired clearance of P. gingivalis acute lung infection and reduced cytokine responses in TLR2−/− mice

To determine the role of TLR2 in the acute response to P. gingivalis, a 5-h time point was carefully selected as being the most appropriate for the purpose. Indeed, a previous time-course study, which established the P. gingivalis-induced aspiration pneumonia model, has demonstrated pronounced recruitment of PMNs and significant killing of P. gingivalis in wild-type mice at 5 h postinfection (13). A comparable time point (6 h) was shown to be ideal for simultaneously assessing cytokine induction and inflammatory cell influx (47). Moreover, we included a 24-h time point, which represents the peak of the PMN influx upon intratracheal P. gingivalis challenge (13). Preliminary experiments using wild-type mice confirmed that intratracheal infection with 10^8 P. gingivalis bacteria causes a marked increase in PMN recruitment at 5 h postinfection. Although PMN were essentially undetectable in the BALF of naive mice, their numbers increased to 4.5 ± 1.1 × 10^6 in the BALF of infected mice (n = 5), accounting for >80% of the total cell count. On the other hand, the number of AM remained virtually unaltered 5 h postinfection compared with the levels seen in naive mice (0.9 ± 0.2 × 10^6). Comparison of wild-type and TLR2-deficient (TLR2−/−) mice at 5 or 24 h postinfection showed that TLR2 did not play a significant role in PMN recruitment since both groups showed comparable total cell counts and differential cell counts (Table 1). Consistent with this finding, no statistically significant differences were observed between wild-type and TLR2−/− mice in MPO levels (reflecting PMN activity) in the BALF (Table 1). Mock inoculation (intratracheal injection of plain PBS) resulted in relatively low levels of PMN recruitment to the lungs after 5 or 24 h (Table 1). Indeed, PMN comprised only ~19–27% of the total cell count in the BALF of mock-inoculated wild-type or TLR2−/− mice compared with 79–83% in P. gingivalis-infected wild-type or TLR2−/− mice (Table 1). As the BALF total cell count was ~4–6-fold higher in infected mice (Table 1), the estimated PMN numbers in mock-inoculated mice were consistently <8% of the numbers corresponding to infected mice. Consistent with this, the MPO levels in the BALF of mock-inoculated mice were <9% of the levels seen in infected mice (Table 1).

Although TLR2−/− mice exhibited quite normal recruitment of PMNs, they were defective in controlling P. gingivalis. Indeed, although wild-type mice cleared ~80% of the bacteria by 5 h postinfection, there was no sign of net killing activity in TLR2−/− mice (Fig. 1A). In this regard, no statistically significant differences were observed between P. gingivalis CFU recovered from the lungs of TLR2−/− mice and CFU counts from mice sacrificed immediately upon infection, representing the initial observed inoculum (Fig. 1A). In contrast, P. gingivalis CFU counts recovered from the lungs of wild-type mice were significantly lower compared with the initial inoculum or the CFU levels of TLR2−/− mice (p < 0.05; Fig. 1A). At 24 h postinfection, the viable P. gingivalis counts recovered from the lungs of wild-type mice were ~300 less than those seen in TLR2−/− mice and 500 times less than those in the initial observed inoculum (p < 0.05; Fig. 1B). In contrast, an apparent modest reduction in viable CFU counts in TLR2−/− mice, relative to the initial observed inoculum, did not reach statistical significance (Fig. 1B). In conclusion, no significant net growth or killing of P. gingivalis was observed in the lungs of TLR2−/− mice, whereas wild-type mice readily clear the organism, suggesting that TLR2 mediates effective control of P. gingivalis lung infection.

Cytokine responses in the BALF were generally significantly higher in wild-type mice compared with TLR2−/− mice at 5 h postinfection. Significant differences (p < 0.05) were seen for KC, MIP-1α, TNF-α, IL-6, and IL-12p70, but not for IL-1β, IL-10, or IL-17, with the latter being undetectable (Fig. 2A). At 24 h postinfection, TLR2−/− mice still exhibited significantly (p < 0.05) lower levels of KC, MIP-1α, and IL-12p70 compared with wild-type controls, but not of TNF-α or IL-6 (Fig. 2B). The relatively increased bacterial challenge in TLR2−/− mice (compared with wild-type controls, which effectively eliminated most of the bacteria; Fig. 1B), as well as the possibility for TLR2-independent induction of certain cytokines, may have somewhat blunted differences in cytokine production between the two groups at 24 h. Wild-type or TLR2−/− naive mice had no or negligible cytokine

### Table 1. Inflammatory cell recruitment in wild-type and TLR2−/− mice intratracheally infected with P. gingivalis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT Naive</th>
<th>WT Infected</th>
<th>TLR2−/− Naive</th>
<th>TLR2−/− Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC (×10^6)</td>
<td>1.18 ± 0.44</td>
<td>1.07 ± 0.28</td>
<td>5.16 ± 1.51</td>
<td>4.02 ± 1.34</td>
</tr>
<tr>
<td>% AM</td>
<td>78.5 ± 3.24</td>
<td>80.9 ± 5.72</td>
<td>16.8 ± 6.42</td>
<td>20.6 ± 5.27</td>
</tr>
<tr>
<td>% PMN</td>
<td>21.5 ± 3.24</td>
<td>19.1 ± 5.72</td>
<td>83.2 ± 6.42</td>
<td>79.4 ± 5.27</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td>329 ± 76</td>
<td>266 ± 98</td>
<td>4775 ± 1519</td>
<td>3136 ± 1040</td>
</tr>
</tbody>
</table>

*WT TLR2−/−: mice (n = 5 for each group) were challenged intratracheally with P. gingivalis (10^6 CFU) or PBS only (mock-inoculated naive mice) and sacrificed 5 or 24 h postinfection. BALF was used for total cell counts (TCC) and differential cell counts, and for determination of MPO levels by ELISA.*
fected TLR2-deficient mice. Wild-type or TLR2-deficient mice were infected intratracheally with P. gingivalis (10^8 CFU; expected inoculum). The actual infecting dose (observed inoculum) was determined in mice sacrificed immediately after intratracheal administration of the organism. The wild-type and TLR2-deficient experimental mice were sacrificed 5 (A) or 24 h (B) postinfection and their lungs were aseptically removed. Serial 10-fold dilutions of lung homogenates were plated for anaerobic growth and enumeration of recovered CFU. Data are shown for each mouse, and horizontal lines indicate the mean value. *, Significantly (p < 0.05) lower CFU values compared with those seen in TLR2-deficient mice and the observed inoculum. No statistically significant differences were found between TLR2-deficient CFU and observed inoculum.

Responses (Fig. 2). With regard to induction of antimicrobial molecules, wild-type mice elicited significantly higher levels of NO at 5 or 24 h postinfection (p < 0.05 vs TLR2-deficient mice), whereas NO production was undetectable in wild-type or TLR2-deficient naive mice (Fig. 3).

In summary, TLR2 contributes to induction of innate immune responses in the lung and, although not essential for inflammatory cell recruitment, is required for efficient clearance of P. gingivalis infection.

**FIGURE 1.** Impaired clearance of P. gingivalis from the lungs of infected TLR2-deficient mice. Wild-type or TLR2-deficient mice were infected intratracheally with P. gingivalis (10^8 CFU; expected inoculum). The actual infecting dose (observed inoculum) was determined in mice sacrificed immediately after intratracheal administration of the organism. The wild-type and TLR2-deficient experimental mice were sacrificed 5 (A) or 24 h (B) postinfection and their lungs were aseptically removed. Serial 10-fold dilutions of lung homogenates were plated for anaerobic growth and enumeration of recovered CFU. Data are shown for each mouse, and horizontal lines indicate the mean value. *, Significantly (p < 0.05) lower CFU values compared with those seen in TLR2-deficient mice and the observed inoculum. No statistically significant differences were found between TLR2-deficient CFU and observed inoculum.

**FIGURE 2.** Cytokine levels in the BALF of wild-type and TLR2-deficient mice in response to P. gingivalis. Wild-type or TLR2-deficient mice were infected intratracheally with P. gingivalis (10^8 CFU) and sacrificed 5 (A) or 24 h (B) postinfection. Cytokine responses in BALF samples were determined using a Bio-Plex cytokine bead array. Baseline levels were determined using uninfected (naive) wild-type or TLR2-deficient mice intratracheally administered PBS only. Data are means ± SD (n = 5). *, Statistically significant (p < 0.05) reduction of cytokine responses in TLR2-deficient mice compared with infected wild-type controls.

**FIGURE 3.** Induction of NO in the lungs of P. gingivalis-infected wild-type or TLR2-deficient mice. Wild-type or TLR2-deficient mice were infected intratracheally with P. gingivalis (10^8 CFU) and sacrificed 5 (A) or 24 h (B) postinfection. Wild-type or TLR2-deficient mice that were intratracheally given PBS only are denoted as “naive”. Levels of NO_2, a stable oxidative metabolite reflecting production of NO in lung homogenate supernatants were measured by the Griess assay. Data are means ± SD (n = 5). *, Statistically significant (p < 0.05) difference between wild-type and TLR2-deficient mice.

**TLR2-deficient PMN display reduced P. gingivalis killing**

The impaired clearance of P. gingivalis in the lungs of TLR2-deficient mice could be attributed to reduced bactericidal activities of their alveolar phagocytes (PMN and AM). We have thus used freshly explanted wild-type and TLR2-deficient phagocytes to address this hypothesis. We first evaluated the ability of TLR2-deficient PMN to kill P. gingivalis and found that their bactericidal capacity was reduced by 54% in comparison to wild-type controls (p < 0.05; Fig. 4A). Therefore, TLR2 signaling in PMN contributes significantly to P. gingivalis killing. An additional factor that could have affected the in vivo ability of TLR2-deficient PMN to clear P. gingivalis may be related to reduced priming due to decreased cytokine responses in the lungs of TLR2-deficient mice. Indeed, certain cytokines and especially TNF-α (which was reduced by 62% in TLR2-deficient mice; Fig. 2) can effectively prime the bactericidal capacity of PMN, even though they do not directly activate killing mechanisms (48). Priming of wild-type neutrophils with recombinant mouse TNF-α (10 ng/ml) resulted in modest enhancement of their killing activity (data not shown); however, priming of TLR2-deficient PMN with...
FIGURE 4. Role of TLR2 in PMN killing of *P. gingivalis*. A. Comparative killing by wild-type (WT) or TLR2<sup>−/−</sup> PMN (2 × 10<sup>6</sup> cells) of *P. gingivalis* (MOI = 1:1) after a 2-h incubation at 37°C. The killing index was calculated as described in Materials and Methods. B, TLR2<sup>−/−</sup> PMN were primed, or not, for 30 min with recombinant mouse TNF-α (10 ng/ml) or with culture supernatants from activated wild-type or TLR2<sup>−/−</sup> PMN, before incubation with *P. gingivalis* for performing a killing assay as above. The killing activities of the variously primed TLR2<sup>−/−</sup> PMN were normalized to that of unprimed wild-type PMN. C. Comparative induction of oxidative burst in WT or TLR2<sup>−/−</sup> PMN (2 × 10<sup>6</sup> cells) by *P. gingivalis* (MOI = 1:1) over time at 37°C. The oxidative burst was monitored using a quantitative DCFH assay, as explained in Materials and Methods. D, PMN oxidative burst and killing of *P. gingivalis* was determined as above in the presence or absence of 10 μM DPI. Data are means ± SD (A and D, n = 5; B and C, n = 3). *p < 0.05; Fig. 5A, B, C, D, respectively, compared with CFU recovery from wild-type controls (A and C) or to medium-only controls (B and D).

TNF-α or even with activated culture supernatants from wild-type PMN did not significantly enhance their killing capacity against *P. gingivalis*, which ranged within 37–47% of the killing capacity of wild-type PMN (Fig. 4B).

The oxidative burst is an important mechanism whereby PMN eliminate pathogens (49). However, TLR2<sup>−/−</sup> PMN did not display significantly reduced capacity for oxidative burst in response to *P. gingivalis*, when compared with their normal counterparts (Fig. 4C). Thus, the reduced bactericidal activity of TLR2<sup>−/−</sup> PMN may not be attributed to defective induction of the oxidative burst. Moreover, the killing of *P. gingivalis* by PMN was not significantly suppressed by the NADPH oxidase inhibitor DPI at 10 μM (Fig. 4D), a concentration demonstrated to abrogate the oxidative burst (Fig. 4D). Therefore, induction of reactive oxygen species may not be an effective mechanism to eliminate *P. gingivalis*. Finally, we could not detect production of NO in *P. gingivalis*-stimulated PMN (not shown), ruling out a possible role for inducible NO synthase (iNOS) in PMN killing of *P. gingivalis*.

In summary, the Fig. 4 data show that TLR2 signaling in PMN is required for maximal killing activity against *P. gingivalis*. Moreover, the differential killing capacity of wild-type and TLR2<sup>−/−</sup> PMN appears to involve NADPH oxidase- and iNOS-independent mechanisms.

TLR2 mediates killing of *P. gingivalis* in AM; comparison with other macrophage types

We have previously shown that TLR2 deficiency does not impair the ability of peritoneal macrophages for intracellular killing of *P. gingivalis* (36). In striking contrast, TLR2<sup>−/−</sup> AM exhibited defective intracellular killing of this pathogen compared with normal controls (*p < 0.05; Fig. 5A). Specifically, using an antibiotic protection-based intracellular survival assay, we found that the recovery of viable *P. gingivalis* CFU from TLR2<sup>−/−</sup> AM was significantly higher by ~0.5 or 1.3 log<sub>10</sub> units after a 5- or 24-h incubation, respectively, compared with CFU recovery from wild-type controls (*p < 0.05; Fig. 5A). These data, in conjunction with the Fig. 4A results, indicate that TLR2 contributes to the killing of *P. gingivalis* and provide a mechanistic basis for the observed impaired clearance of this pathogen from the lungs of TLR2<sup>−/−</sup> mice (Fig. 1).

However, it was puzzling that TLR2 appeared to play disparate roles in alveolar and peritoneal macrophages regarding the control of *P. gingivalis* intracellular infection. Side-by-side comparison conclusively showed that AM are more potent than peritoneal macrophages in *P. gingivalis* intracellular killing (Fig. 5B). Indeed, the recovery of viable *P. gingivalis* CFU from AM was significantly lower by 0.6 or 1.6 log<sub>10</sub> units after a 5- or 24-h incubation,

FIGURE 5. Intracellular killing of *P. gingivalis* by AM. A. Wild-type and TLR2<sup>−/−</sup> AM were incubated with *P. gingivalis* (MOI = 10:1) for the indicated times. The persistence of viable internalized bacteria was determined using an antibiotic protection-based survival assay. B. Similar procedures were followed to measure the intracellular killing of *P. gingivalis* by alveolar and peritoneal macrophages. Data are means ± SD (n = 5). *p < 0.05; Fig. 5B, respectively, compared with CFU levels (i.e., increased killing) in AM compared with their TLR2<sup>−/−</sup> counterparts (A) or to peritoneal macrophages (B).
respectively, compared with CFU recovery from peritoneal macrophages (p < 0.05; Fig. 5B). Moreover, comparison of wild-type and TLR2−/− peritoneal macrophages confirmed our previous findings that TLR2 actually promotes rather than controls the intracellular persistence of P. gingivalis (36) (data not shown). This is attributed to TLR2 inside-out signaling, which activates CR3-mediated uptake of P. gingivalis, resulting in increased intracellular survival of this pathogen (36). By contrast, this CR3-dependent immune evasion mechanism of P. gingivalis is unlikely to operate in AM, which express little or no CR3 (37, 38). Indeed, we found that CR3 does not play a significant role in the uptake of P. gingivalis by AM. Specifically, there were no significant differences between wild-type and CR3-deficient AM in P. gingivalis uptake, as opposed to significant (p < 0.05) differences between wild-type and CR3-deficient peritoneal macrophages (Fig. 6A). Moreover, although CR3 is associated with increased intracellular survival of P. gingivalis in peritoneal macrophages (36) (confirmed in Fig. 6B), as well as in splenic macrophages, which also express CR3 (Fig. 6B), this was not the case with AM. Indeed, no significant differences were observed between wild-type and CR3-deficient AM in terms of their ability to control the intracellular fate of P. gingivalis (Fig. 6B). Thus, the TLR2/CR3 inside-out pathway is not exploited by P. gingivalis in AM, and this may explain at least partly why AM TLR2 inhibits, rather than promotes, the intracellular survival of P. gingivalis.

**TLR2-mediated killing of P. gingivalis in AM involves reactive nitrogen intermediates**

NO is a key antimicrobial molecule produced by activated macrophages for pathogen killing (49). To determine whether AM utilize this mechanism for intracellular killing of P. gingivalis, we pretreated them with l-NAME, a specific inhibitor of NO synthesis, or with the inactive enantiomer d-NAME (50). The macrophages were then infected with P. gingivalis (MOI = 10:1) and its intracellular fate was monitored using the antibiotic-based intracellular survival assay. The pathogen was recovered at significantly higher CFU numbers from l-NAME-treated AM than from untreated cells (p < 0.05; Fig. 7A). In contrast, d-NAME had no effect in this regard (Fig. 7A). The increased recovery of P. gingivalis CFU from l-NAME-treated cells could not be attributed to increased uptake (relative to medium-only or d-NAME-treated cells) since l-NAME had no effect on the ability of the AM to take up P. gingivalis (data not shown). We also confirmed that l-NAME, but not d-NAME, dramatically diminished production of NO (measured as NO2−, its stable oxidative metabolite) by AM in response to P. gingivalis (p < 0.05; Fig. 8A). However, l-NAME did not affect P. gingivalis-induced cytokine production (Fig. 8A), thus ruling out potential toxic effects on AM.

In contrast to the findings from wild-type AM, the intracellular survival of P. gingivalis in TLR2−/− AM was not significantly affected by l-NAME (Fig. 7B). Therefore, NO does not significantly contribute to the intracellular control of P. gingivalis in the absence of TLR2 signaling. In fact, P. gingivalis-induced NO levels were significantly diminished in TLR2−/− AM compared with wild-type controls (p < 0.05; Fig. 8B). Therefore, NO production in this in vitro system is heavily dependent on TLR2, as seen in vivo (Fig. 3). The observed inhibition of NO production was ~75%, comparable to the inhibition of cytokine production (TNF-α and MIP-1α) in TLR2−/− AM (Fig. 8B).

In conclusion, the data from Figs. 7 and 8 suggest that induction of NO is at least one mechanism whereby AM control P. gingivalis lung infection. Moreover, this antimicrobial mechanism accounts,
responses in AM.

A

dose (10^8 CFU) may represent a realistic aspirated in -
gingivalis

tory cells. Indeed, despite reduced production of certain chemo-
kines (KC, MIP-1α) in the BALF of P. gingivalis-infected TLR2^−/− mice, and although TLR2 deficiency has occasionally been associated with decreased PMN influx (26, 54), we did not observe significant differences between TLR2^−/− and wild-type control mice regarding PMN recruitment to the lungs after 5 or 24 h postinfection. It is possible that TLR2-independent mecha-

nisms have compensated for this function, such as stimulation by bacterially derived formyl peptides of the formyl-peptide receptor, which plays a major role in PMN recruitment to infected alveoli (20).

In principle, the defective killing of P. gingivalis in the lungs of TLR2^−/− mice could result, at least partly, from decreased prim-
ing of their alveolar phagocytes (PMN and AM) owing to reduced proinflammatory cytokine responses. Alternatively, or in addition to the above, the lack of TLR2 signaling in the TLR2^−/− alveolar phagocytes may have directly affected their ability to kill P. gin-
givalis. Our in vitro mechanistic data are consistent with the latter interpretation. TLR2 was also shown to mediate AM killing of Mycobacterium tuberculosis (55) and PMN killing of Streptococcus pneumoniae (56).

In stark contrast, TLR2 promotes the sur-
vival of Staphylococcus aureus in peritoneal macrophages (57). It is unclear whether the outcome depends on the phagocytic cell type or the pathogen involved (or both), but note that the antimicro-

bial role of TLR2 may be contextual (discussed in greater detail below) and thus may not be predicted a priori in the absence of experimental evidence.

Our findings that the oxidative burst is not important for P. gingivalis killing are consistent with a recent study showing no difference in killing activity against this pathogen between wild-
type and NADPH oxidase-deficient (Cybb^−/−) PMN (44). It is th-
us possible that PMN use nonoxidative mechanisms to eliminate P. gingivalis, such as granule proteases or antimicrobial peptides, which can be delivered to the phagosome or released to the extra-
cellular space (58, 59). In contrast, NO may be an effective anti-
microbial mechanism for the control of P. gingivalis since inhibi-
tion of its production in normal AM resulted in impaired intracellular killing of this pathogen. Therefore, the diminished capacity TLR2^−/− AM to induce NO production may, at least partly, explain why TLR2^−/− AM failed to efficiently control P. gin-
givalis. An additional mechanism may involve impaired phago-

some maturation in the absence of TLR2 signaling (60), although a competing view supports that phagolysosomal maturation is a TLR-independent process (61). Our data are consistent with earlier findings that iNOS-deficient mice exhibit reduced killing of P. gin-
givalis in a s.c. chamber model (62). Our results are also in line with a recent study that demonstrated NO-dependent killing of P. gingivalis by mouse peritoneal macrophages (44).

Although we have previously demonstrated intracellular killing of P. gingivalis by peritoneal macrophages (36), we have now found that AM are even more potent than their peritoneal or splenic counterparts in this activity. This finding is at first sight surprising given that AM mediate critical immunosuppressive functions for maintaining respiratory tract homeostasis (16). This dif-

ference in P. gingivalis killing by distinct macrophage types may be attributed to selective expression of CR3 by tissue mac-

rophages. Specifically, the fact that resident AM express little or no CR3 (37, 38) renders them resistant to a CR3-dependent immune evasion strategy of P. gingivalis (35, 36). Note that the inhibi-

tory effect of CR3 deficiency on the intracellular survival of P. gingivalis is quite dramatic (viability is reduced by up to 2.5–3.0 log_{10} units) which cannot be attributed to a ~50% reduction in bacterial uptake (Ref. 36 and Fig. 6). Rather, the enhanced intra-
cellular survival of P. gingivalis in CR3-expressing macrophages could be attributed to the notion that CR3 phagocytosis does not

FIGURE 8. Regulation of P. gingivalis-induced NO and cytokine re-

ductions in AM. A, Wild-type AM were pretreated with t-NAME (inhibitor of NO production) or the inactive enantiomer d-NAME (both at 1 mM) and then stimulated for 5 h with P. gingivalis (MOI = 10:1). B, Wild-type or TLR2^−/− AM were stimulated for 5 h with P. gingivalis (MOI = 10:1). In both experiments, culture supernatants were assayed for production of NO (stable oxidative metabolite of NO), TNF-α, and MIP-1α. Data are means ± SD (n = 3). * Statistically significant (p < 0.05) inhibition of NO or cytokine responses due to t-NAME (A) or TLR2 deficiency (B).

Discussion

Our findings of impaired P. gingivalis clearance from the lungs of TLR2^−/− mice after 5 or 24 h postinfection support a protective role for TLR2 in the control of acute pulmonary infection with this pathogen. Given the mechanism of oral pathogen-induced pneu-

monia and the fact that 1 mm^3 of dental plaque biofilm contains >10^8 viable bacteria (7–9), it is likely that the selected P. gingivalis dose (10^8 CFU) may represent a realistic aspired in-
oculum in patients with severe periodontitis. TLR2 was also shown to confer protection against pulmonary infection with Francisella tularensis (24), Chlamydia pneumoniae (26), or Aspergillus fu-
migatus (51), although its role is relatively minor in infections with Cryptococcus neoformans (52) or Legionella pneumophila (53).

Interestingly, TLR2 signaling may, in contrast, exacerbate certain pulmonary infections. For example, TLR2^−/− mice are more re-
sistant to Acinetobacter baumanii-induced acute pneumonia than are normal controls, although TLR4^−/− mice are quite susceptible (23). In the same study, TLR2^−/− mice exhibited enhanced innate responses in the lung, and it was thus concluded that TLR2-mediated antimflammatory signaling (23). In a similar context, the virulence of Paracoccidioides brasiliensis in pulmonary infection of susceptible B10.A mice was attributed to TLR2-dependent induction of IL-10 (25). We did not observe significant differences in IL-10 production in the BALF of P. gingivalis-infected normal or TLR2^−/− mice, both of which had very low levels of this immu-

nosuppressive cytokine. In contrast, TLR2 signaling was associ-
ated with increased levels of chemokines, certain proinflammatory cytokines, and antimicrobial molecules (NO) in our P. gingivalis lung infection model.

The defective killing of P. gingivalis in the lungs of TLR2^−/− mice could not be attributed to impaired recruitment of inflamma-

tory cells. Indeed, despite reduced production of certain chemo-
promote phagolysosomal fusion (63). Wild-type AM behave closer to CR3-deficient rather than wild-type peritoneal or splenic macrophages in terms of their ability to clear \( P. \) gingivalis. Note that AM may not be inherently stronger than peritoneal macrophages in general microbialidal function, since alveolar and peritoneal macrophages display similar killing activities against \( Pneumocystis \) carinii (64).

In vitro, TLR2 inside-out signaling activates CR3-mediated uptake of \( P. \) gingivalis (39), leading to inhibition of IL-12p70 induction and enhanced intracellular persistence of the pathogen (36). In vivo, CR3 blockade leads to increased clearance of \( P. \) gingivalis from murine periodontal tissues and reduced periodontal disease and enhanced intracellular persistence of the pathogen (36). In the presence of these and other factors, remains to be established.

TLR2 deficiency has been associated with increased in vivo expression of CR3 by tissue macrophages (37, 38), can at least from murine periodontal tissues and reduced periodontal disease while providing protection against acute pulmonary infection (31, 32, 34 and this study), we suggest that TLR2 may take of \( P. \) gingivalis and resistance to \( P. \) gingivalis-induced periodontitis in the mouse model (31). On the basis of available evidence (Refs. 31, 32, 34 and this study), we suggest that TLR2 may play diverse and contextual roles in immunity (Refs. 31, 32, 34 and this study), we suggest that TLR2 may play diverse and contextual roles in immunity (Ref. 31, 32, 34 and this study), we suggest that TLR2 may take of \( P. \) gingivalis and resistance to \( P. \) gingivalis-induced periodontitis in the mouse model (31). On the basis of available evidence (Refs. 31, 32, 34 and this study), we suggest that TLR2 may play diverse and contextual roles in immunity (Ref. 31, 32, 34 and this study), we suggest that TLR2 may take of \( P. \) gingivalis and resistance to \( P. \) gingivalis-induced periodontitis in the mouse model (31). On the basis of available evidence (Refs. 31, 32, 34 and this study), we suggest that TLR2 may play diverse and contextual roles in immunity. For example, TLR2 deficiency has been associated with increased in vivo clearance of \( P. \) gingivalis and resistance to \( P. \) gingivalis-induced periodontitis in the mouse model (31). On the basis of available evidence (Refs. 31, 32, 34 and this study), we suggest that TLR2 may play diverse and contextual roles in immunity.


