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TLR2-Dependent Inflammatory Response to *Porphyromonas gingivalis* Is MyD88 Independent, whereas MyD88 Is Required To Clear Infection

Elia Burns,* Tal Eliyahu,* Satoshi Uematsu,† Shizuo Akira,† and Gabriel Nussbaum*

***Porphyromonas gingivalis* is a Gram-negative anaerobe considered to be a major periodontal pathogen. TLR2 plays a central role in the response to *P. gingivalis* infection in vivo. In its absence there is a weak inflammatory response; however, bacteria are cleared rapidly compared with wild-type mice. We examined the role of the TLR adaptor proteins MyD88 and TLR/IL-1R–domain-containing adaptor-inducing IFN-β in the inflammatory response to *P. gingivalis* in vivo and in the ability to clear the bacterial infection. Proinflammatory cytokine production in response to *P. gingivalis* infection depends on TLR2, but it does not require MyD88 or TLR/IL-1R–domain-containing adaptor-inducing IFN-β. In contrast, the generation of intracellular toxic oxygen species and the ultimate clearance of *P. gingivalis* infection depend critically on MyD88, independent of TLR2. Thus, robust cytokine production and bacterial clearance are independent events mediated by distinct signaling pathways following infection with *P. gingivalis*. *The Journal of Immunology*, 2010, 184: 1455–1462.**

Porphyromonas gingivalis is a Gram-negative anaerobe considered to be a major etiological factor in adult periodontitis (1), a chronic inflammatory disease in the oral cavity resulting in tissue destruction of the attachment apparatus of the teeth. Surface components of *P. gingivalis*, such as LPS, lipoproteins, and fimbriae, interact with host-expressed TLRs (2, 3), key control elements of the innate immune response to microbial challenge (4). TLRs are expressed on multiple cell types at the site of periodontal infection (5), and their activation leads to nuclear translocation of NF-κB and the induction of inflammation-related genes. Innate TLR-mediated host responses to pathogens are critical for host protection. For example, mice carrying TLR4 mutations that interfere with the response to enterobacterial LPS are highly sensitive to infection with Gram-negative organisms (6). Similarly, TLR2$^{-/-}$ mice are far more susceptible to infections with bacteria that activate host immunity through TLR2 (7), and TLR3 mutant mice succumb more easily to viral infections (8). However, according to prevailing concepts, TLR-mediated inflammation can behave as a double-edged sword: although contributing to control over bacteria, the inflammation is the ultimate culprit in the ensuing host damage that can lead to mortality. Thus, although TLR4-deficient mice are far more sensitive than wild-type (WT) mice to challenge with live Gram-negative bacteria, they are protected from lethal doses of purified LPS extracted from those bacteria (9).

In a previous report, we showed that TLR2 is specifically required for the host response to *P. gingivalis* challenge in vivo (10). The host cytokine response is greatly diminished in TLR2$^{-/-}$ mice; in fact, these mice are resistant to alveolar bone loss following oral infection with *P. gingivalis*. However, despite a diminished cytokine response, TLR2-deficient mice rapidly cleared *P. gingivalis* compared with WT mice, suggesting that TLR2-driven inflammation hinders, rather than helps, the host in overcoming bacterial challenge.

The TLR signaling pathways depend on cytoplasmic TLR/IL-1R (TIR) domain interactions, which are conserved among all TLRs. Homotypic interactions between the TLR TIR domain and TIR domains of intracellular adaptor proteins initiate the signaling cascade that ultimately leads to transcription factor activation (11). MyD88 is the main adaptor protein implicated in TLR signaling and is considered essential for the induction of inflammatory cytokines triggered by most TLRs. An additional adaptor protein TIR–domain-containing adaptor-inducing IFN-β (TRIF) is involved in TLR3- and TLR4-mediated MyD88-independent signaling (4). In general, the elements involved in TLR signaling have been deciphered using in vitro studies of isolated cell lineages (12). Thus, macrophages from MyD88$^{-/-}$ mice are unresponsive in vitro to a variety of microbial ligands. In contrast, studies showed that MyD88$^{-/-}$ mice can mount inflammatory responses in vivo (13) and develop mature adaptive immune responses (14, 15). This discrepancy may be explained by intercellular interactions in vivo that overcome the absence of MyD88 signaling, or it may reflect cytokine production through MyD88-independent signaling in cell types not usually studied in vitro, such as neutrophils.

Although additional signaling pathways may contribute to pathogen recognition via TLR2 (16, 17), proinflammatory cytokine production following TLR2 activation by microbial and synthetic ligands was shown to depend on MyD88 (18). In the current study, we analyzed the role of MyD88 signaling in the TLR2-driven response to in vivo infection with live *P. gingivalis*. Surprisingly, we found that TLR2-mediated proinflammatory cytokine production in response to *P. gingivalis* is independent of MyD88. Furthermore, in contrast to the proinflammatory cytokine response

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Abbreviations used in this paper: BL, baseline; DHR, dihydrorhodamine; MAL, MyD88-adapter-like; MOI, multiplicity of infection; TIR, TLR/IL-1R; TRIF, TLR–domain-containing adaptor-inducing IFN-β; TRAF6, TNF receptor-associated factor 6; WT, wild-type.

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in which MyD88 is not required, we show that MyD88 signaling is critical for controlling bacterial load.

Materials and Methods

Bacterial growth

P. gingivalis (strain 381) was cultured for 48 h in Wilkins broth (Oxoid, Basingstoke, U.K.) under anaerobic conditions at 37°C. An OD of 0.1 (650 nm) was determined to correlate to 10^{10} CFU/ml.

Mice

C57BL/6 mice were from Harlan (Jerusalem, Israel). TLR2$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice backcrossed to the C57BL/6 background were housed at the specific pathogen-free unit of the Hebrew University at the Hadassah Medical Center. TLR2$^{-/-}$/MyD88$^{-/-}$ double-knockout mice were generated by screening F_2 progeny of TLR2$^{-/-}$ females crossed with MyD88$^{-/-}$ males. The genotypes were verified using primer sets and PCR conditions developed for each knockout (19, 20) All experiments were approved by the institutional animal care and use committee of the Hebrew University of Jerusalem.

s.c. chamber model

Two titanium coil chambers were inserted s.c. into anesthetized 8–10-wk-old female mice ($n \geq 6$ per mouse strain), as previously described (21). Seven to 10 d later, live *P. gingivalis* (10^9 CFU in 100 μl PBS) or Pam3Cys as a known TLR2 ligand (10 μg in 100 μl PBS) was injected into each chamber. Chamber exudates were collected at baseline (BL; immediately before challenge) and at 2 and 24 h postinfection (each chamber was sampled only once). PBS was injected into the chambers of the control groups. Exudates were centrifuged, and supernatants were collected for bacterial counts and cytokine analysis.

Cytokine analysis

Chamber exudate cytokine levels were determined by ELISA using mouse OptEIA sets (BD Biosciences, San Jose, CA) for TNF-α and IL-10 and mouse DuoSet (R&D Systems, Minneapolis, MN) for IL-1β, according to the manufacturer's instructions.

Viable bacterial counts

Ten microliters of chamber exudates were serially diluted in PBS and plated in triplicate on tryptic soy agar containing sheep blood (HyLabs, Israel). Plates were incubated in anaerobic conditions for 5–7 d at 37°C. *P. gingivalis* colonies were identified by their black pigment and by phase-contrast microscopy.

In vivo phagocytosis assay

P. gingivalis 381 was labeled with 0.1 mg/ml FITC (Sigma-Aldrich, Rehovot, Israel) in carbonate buffer pH 9.5 for 20 min at room temperature. Chamber exudates from 24 h postinfection (10^9 CFU/chamber) were centrifuged, and cell pellets were washed three times in PBS to remove nonadherent bacteria. Cells (~7×10^6 cells per mouse are recovered) were resuspended in 0.2% trypan blue in PBS for quenching of any remaining extracellular bacteria. Trypan blue was removed by two additional washes, and cells were resuspended in 0.5% BSA/PBS. Phagocytosis was measured using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

Ex vivo phagocytosis assay

Chamber exudates from WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice obtained prior to *P. gingivalis* challenge (BL) and 24 h postchallenge were drawn and centrifuged at 14,000 rpm for 10 min to obtain supernatants clear of all host and bacterial (*P. gingivalis*) cells. In parallel, cells from chambers implanted in WT and TLR2$^{-/-}$ mice were recovered (1–3 $\times$ 10^6 cells per mouse) without exposure to *P. gingivalis* infection in vivo and pooled for each mouse strain before being divided equally for further treatment. Then cells were exposed to FITC-labeled *P. gingivalis* (multiplicity of infection [MOI] 10) in the presence of exudates elicited from the *P. gingivalis*-challenged mice. In some experiments, the cells were exposed to preboiled exudates (20 min at 100°C). Plates were incubated for 4 h at 37°C with 5% CO_2. Following incubation, cells were washed three times in PBS and resuspended in 0.2% trypan blue in PBS for quenching remaining extracellular bacteria. Trypan blue was removed by an additional two washes, and cells were resuspended in 0.5% BSA/PBS. Phagocytosis was measured using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

Intracellular oxidative burst

Chamber cells. Naive cells from s.c. chambers implanted in WT and MyD88$^{-/-}$ mice were plated in 24-well plates in PBS/1% BSA. Cells were exposed to *P. gingivalis* (MOI 10) for 45 min at 37°C with 5% CO_2. Following exposure to *P. gingivalis*, dihydrorhodamine (DHR) 123 (Calbiochem, San Diego, CA) was added for 15 min (final concentration 10 μM). Following incubation, cells were washed, quenched with trypan blue (as described above), and resuspended in 0.5% BSA/PBS. Peroxynitrate-induced reduction of DHR 123 was measured using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences). Positive cells exposed to *P. gingivalis* and DHR 123 were defined as those with florescence above the level of cells exposed to DHR 123 alone.

Peripheral blood cells. Heparinized blood drawn from the facial vein of WT versus MyD88$^{-/-}$ mice (n = 6/group) was pooled, diluted 1:10 with PBS, and centrifuged for 10 min at 1500 rpm. Erythrocytes were lysed using RBC lysis buffer (eBioscience, San Diego, CA), according to the manufacturer's instructions. Cells were washed, counted, and divided equally into Eppendorf tubes. For each tube, DHR 123 was added to a final concentration of 10 μM and incubated for 5 min on ice to allow DHR 123 to penetrate the cells (22). Next, *P. gingivalis* was added to each tube (MOI 10) and incubated at 37°C with 5% CO_2. Following incubation with the bacteria, cells were centrifuged, washed twice, and resuspended in 0.5% BSA/PBS for FACS analysis. Peroxynitrate-induced reduction of DHR 123 was measured using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences). Positive cells were defined as those with florescence above the level of cells exposed to DHR 123 alone.

Statistical analysis

Data are reported as mean ± SEM. Comparisons were made using the unpaired Student t test.

Results

MyD88-dependent and -independent responses to P. gingivalis *s.c. challenge*

The intracellular signaling cascade downstream of TLR2 that leads to production of proinflammatory cytokines depends upon the adaptor protein MyD88 (23). Therefore, we expected to find diminished cytokine production in MyD88$^{-/-}$ mice in response to *P. gingivalis*, similar to TLR2$^{-/-}$ mice. To test this, we implanted s.c. chambers in WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice and challenged them with live *P. gingivalis.* Chamber exudates were sampled 2 and 24 h following challenge, and cytokines were determined by ELISA. Injection of PBS into the chambers did not induce cytokine production compared with BL levels in any of the mouse strains (data not shown). Surprisingly, we observed a rapid increase in TNF-α and IL-1β in MyD88$^{-/-}$ mice 2 h following infection to levels similar to those seen in WT mice (Fig. 1*A*, 1*B*). In contrast, significantly less TNF-α and IL-1β were found in TLR2$^{-/-}$ mice at 2 h ($p < 0.01$), as we previously reported (10). Thus, early TNF-α and IL-1β production in response to live *P. gingivalis* seems to be mostly TLR2 dependent, but independent of MyD88. In contrast, IL-10 production was absent in MyD88$^{-/-}$ mice at both time points following challenge with *P. gingivalis*, consistent with the finding of little IL-10 at 2 h and none at 24 h in TLR2$^{-/-}$ mice (Fig. 1*C*). Therefore, IL-10 production in response to *P. gingivalis* in vivo requires MyD88 and is, in large part, dependent on recognition through TLR2.

Twenty-four hours following infection, TNF-α levels returned to BL in all strains tested (Fig. 1*A*). Although significantly less IL-1β was found in TLR2$^{-/-}$ mice at 2 h following infection, the response at 24 h was similar to that in WT mice (Fig. 1*B*, 1*C*), indicating that the proinflammatory cytokine response is independent of TLR2 at later time points. One possible explanation is that by 24 h of infection, other TLRs or non-TLRs are involved in *P. gingivalis* recognition and the initiation of an inflammatory response. For example, TLR4 and TLR7 were shown to play an important role in the recognition of surface molecules of *P. gingivalis* (2, 24); therefore, we anticipated that IL-1β at 24 h might

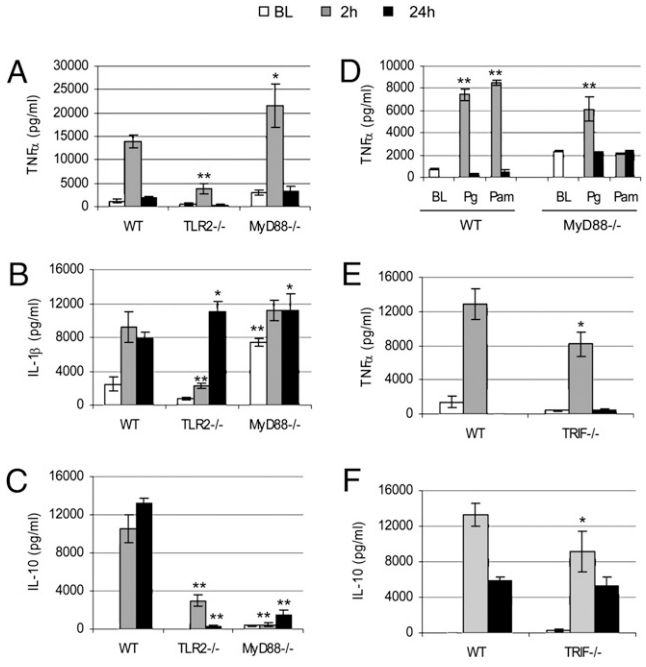

FIGURE 1. Cytokine levels following *P. gingivalis* and Pam3Cys challenge. TNF-α (*A*), IL-1β (*B*) and IL-10 (*C*) levels were examined in exudates at BL and 2 and 24 h after *P. gingivalis* challenge in WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice. One representative experiment of three is shown. Significance shown represents comparisons to WT at the same time point. *D*, TNF-α levels in chamber exudates of WT and MyD88$^{-/-}$ mice was measured following Pam3Cys challenge at BL and 2 and 24 h. One representative experiment of two is shown. Significance represents comparisons of each mouse strain to its own BL level. TNF-α (*E*) and IL-10 (*F*) levels were measured in chamber exudates of TRIF$^{-/-}$ mice after live *P. gingivalis* challenge. Significance shown represents comparisons to WT at the same time point. $*p < 0.05$; $**p < 0.01$.

require MyD88 signaling. However, high levels of IL-1β, similar to levels in WT mice, persisted in MyD88$^{-/-}$ mice at 24 h, suggesting that MyD88 signaling is not required for IL-1β production in response to live *P. gingivalis* infection.

To determine whether TLR2-dependent inflammatory cytokine production is, in general, MyD88 independent in the context of the challenge model we were using, we injected WT and MyD88$^{-/-}$ mice with Pam3cys, a synthetic lipopeptide shown to induce inflammatory cytokines through a TLR2–MyD88–dependent pathway (25). In contrast to infection with *P. gingivalis*, TNF-α secretion in response to the defined TLR2 ligand Pam3cys is MyD88 dependent, because no TNF-α was detected in MyD88$^{-/-}$ mice at 2 h, in contrast to the high level found in WT mice. WT and MyD88$^{-/-}$ mice challenged in parallel with live *P. gingivalis* responded by producing high levels of TNF-α (Fig. 1*D*). Thus, the nature of the ligand seems to determine whether TLR2 signaling involves MyD88-independent pathways. We next found, as other investigators have described (2), that isolated macrophages respond to live *P. gingivalis* in a MyD88-dependent manner (data not shown). Therefore, TLR2-dependent, MyD88-independent proinflammatory cytokine production depends on the nature of the ligand and the in vivo context of exposure.

TLR3 and TLR4 are known to signal through a MyD88-independent alternative pathway that uses the adaptor protein TRIF. In the case of TLR4, TRIF-dependent NF-κB activation leads to inflammatory cytokine production, although with delayed kinetics compared with MyD88-dependent signaling (23). Although TLR2 has not been shown to signal through TRIF, the TRIF TIR domain contains the conserved proline residue that is required for activation of TLR-mediated signal transduction (11), and epitope-tagged TRIF was shown to interact with TLR2 and TLR3 (26). Therefore, we examined the possible involvement of TRIF in mediating TLR2-dependent proinflammatory cytokine production in response to *P. gingivalis*. TRIF$^{-/-}$ mice produced high amounts of TNF-α 2 h post-s.c. challenge with *P. gingivalis* in a similar manner to WT mice (Fig. 1*E*), indicating that TLR2 signaling leading to TNF-α production in response to *P. gingivalis* is not MyD88 or TRIF dependent. The secretion of high levels of IL-10 in TRIF$^{-/-}$ mice (Fig. 1*F*), in contrast to MyD88$^{-/-}$ mice (Fig. 1*C*), is consistent with the observation that IL-10 production in vivo requires MyD88.

P. gingivalis *persists in WT and MyD88$^{-/-}$ mice*

In our previous report, we showed that *P. gingivalis* is rapidly cleared following s.c. infection in TLR2$^{-/-}$ mice, whereas the presence of TLR2 correlated with significantly prolonged bacterial persistence in WT and TLR4$^{-/-}$ mice (10). Despite proinflammatory levels similar to WT mice, and the absence of IL-10, *P. gingivalis* persisted in MyD88$^{-/-}$ mice; within 24 h after challenge, CFU counts significantly exceeded those measured at 2 h. Clearance of *P. gingivalis* in TLR2$^{-/-}$ mice proceeds rapidly, and within 24 h of infection *P. gingivalis* cannot be recovered from a portion of the mice (Fig. 2*A*). Bacterial CFUs recovered from TRIF$^{-/-}$ mice did not differ from those recovered from WT mice (Fig. 2*B*).

The significant increase in *P. gingivalis* CFU at 24 h compared with 2 h in MyD88$^{-/-}$ mice suggested that bacterial clearance is impaired compared with the slow, but consistent, clearance we previously observed in WT mice (10). Therefore, we followed the kinetics of bacterial clearance over a 10-d period in WT and MyD88$^{-/-}$ mice (Fig. 2*C*). Consistent with our previous findings (10), CFU counts gradually decreased in WT mice; however, even at 10 d postinfection, viable *P. gingivalis* could be cultivated. The kinetics of bacterial clearance in TLR4$^{-/-}$ mice was similar to that in WT mice (Fig. 2*D*). Consistent with the results at 24 h, bacterial clearance in MyD88$^{-/-}$ mice was significantly slower than that observed in WT mice. We previously found that *P. gingivalis* disseminates from the s.c. space to the systemic circulation in WT and TLR4$^{-/-}$ mice and that bacteremia persists for $>$ 1 wk following challenge (10). Nevertheless, bacteremic WT mice do not display physical signs of systemic illness, and other investigators reported that even repeated i.v. challenge with *P. gingivalis* did not cause systemic illness or death (27). MyD88$^{-/-}$ and TRIF$^{-/-}$ mice are also bacteremic for $>$ 1 wk (data not shown), similar to WT mice. However, in contrast to the other strains, MyD88$^{-/-}$ mice displayed signs of systemic infection starting at 48 h postchallenge, and s.c. infection led to 40% mortality by day 10 following challenge (data not shown). Therefore, a TLR2-dependent response significantly prolongs bacterial persistence in the host (compare TLR2$^{-/-}$ mice with WT mice; Fig. 2*A*); however, the ability to overcome lethal infection in healthy mice requires MyD88.

Impaired P. gingivalis *phagocytosis in My88$^{-/-}$ mice*

To examine the requirement for MyD88 in bacterial clearance, we followed *P. gingivalis* phagocytosis in WT, TLR2$^{-/-}$ and MyD88$^{-/-}$ mice. As we reported previously, phagocytosis is significantly more efficient in TLR2$^{-/-}$ mice compared with WT mice (Fig. 3*A*). In contrast, phagocytosis in MyD88$^{-/-}$ mice is less efficient than in their WT counterparts (Fig. 3*B*). Thus, TLR2-dependent inflammation leads to reduced phagocytosis; however, efficient phagocytosis in WT mice is dependent, in part, on MyD88.

Inflammatory exudates reduce P. gingivalis *phagocytosis*

We next tested whether the TLR2-driven inflammatory response contains a secreted factor that reduces phagocytosis. Because TLR2 is not known as a phagocytic receptor, we hypothesized that

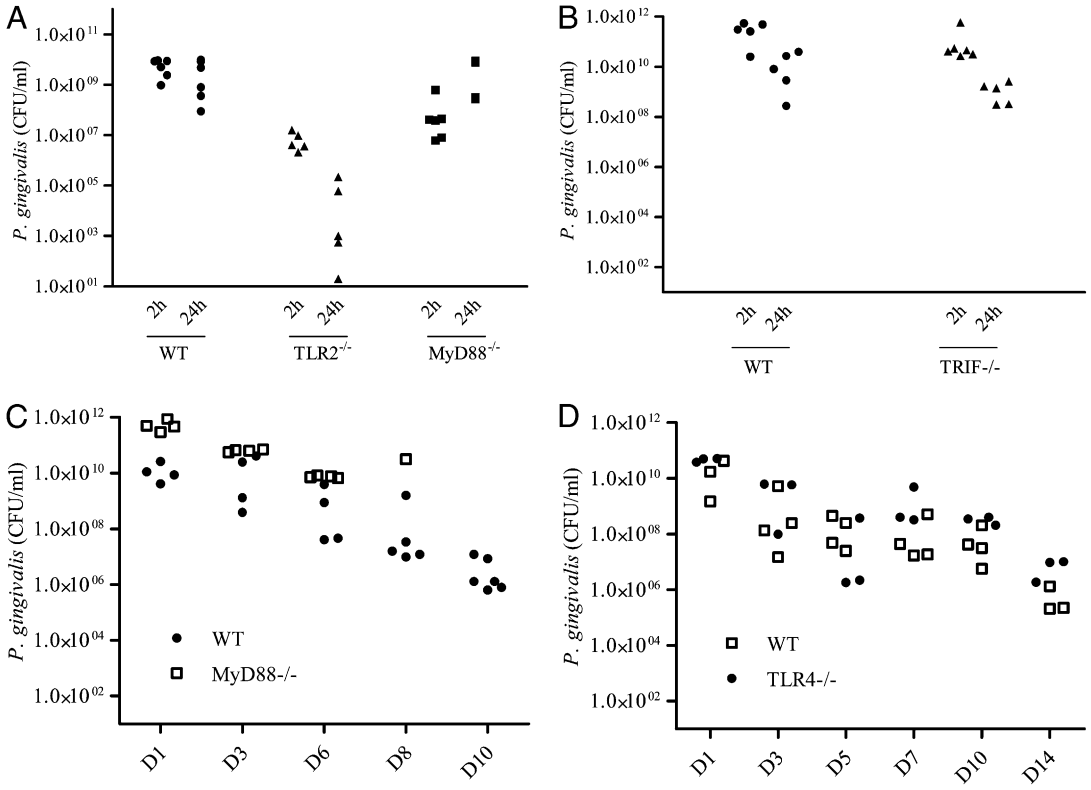

FIGURE 2. *P. gingivalis* survival in WT, TLR2$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice. *A*, Viable *P. gingivalis* counts from chamber exudates of WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were determined 2 and 24 h postintrachamber challenge. Each symbol represents an individual mouse ($n \geq 6$ per strain). One representative experiment of three is shown. CFU counts significantly decreased between 2 and 24 h in TLR2$^{-/-}$ mice ($p < 0.001$) and increased significantly in MyD88$^{-/-}$ mice ($p < 0.05$). There was no significant change between 2 and 24 h in WT mice. There was significantly fewer CFU in TLR2$^{-/-}$ mice than in WT mice at both time points ($p < 0.005$). *B*, Viable *P. gingivalis* counts from chamber exudates of WT and TRIF$^{-/-}$ mice were determined 2 and 24 h postintrachamber challenge. Each symbol represents an individual mouse ($n \geq 6$ per strain). One representative experiment of two is shown. No significant differences were found between WT and TRIF$^{-/-}$ mice at either time point. *C*, *P. gingivalis* survival postinfection was followed in WT and MyD88$^{-/-}$ mice ($n = 6$ per time point). Each symbol represents one mouse. By day 8, MyD88$^{-/-}$ mice had succumbed to infection, or chamber exudates were too thick to be plated. By day 10, no exudates could be recovered from surviving MyD88$^{-/-}$ mice. CFU counts at 1, 3, and 6 d postinfection were significantly higher in MyD88$^{-/-}$ mice compared with WT mice ($p < 0.005$ at 1 and 3 d; $p < 0.001$ at 6 d). One representative experiment of two is shown. *D*, *P. gingivalis* survival postinfection was followed in WT and TLR4$^{-/-}$ mice ($n = 3$–4 mice per time point). Each symbol represents one mouse. There were no significant differences between the groups at any time point. One representative experiment of two is shown.

such a factor would downregulate phagocytosis independently of TLR2 expression on the phagocytic cells. To test this, we incubated TLR2$^{-/-}$ cells with exudates from WT or TLR2$^{-/-}$ mice infected with *P. gingivalis* and determined the extent of phagocytosis of FITC-labeled *P. gingivalis* compared with exposure to BL exudates isolated prior to infection (Fig. 4*A*). TLR2$^{-/-}$ cells exposed to inflammatory exudates from WT mice showed greatly reduced phagocytosis compared with BL exudates from WT mice or the inflammatory exudate recovered from TLR2$^{-/-}$ mice following infection with *P. gingivalis*. In fact, exposure to the postinfection exudate recovered from TLR2$^{-/-}$ mice enhanced phagocytosis compared with the BL exudates. Similar results were obtained by incubating the exudates with WT cells (data not shown). These results confirmed that *P. gingivalis* activation of TLR2 induces a secreted factor that downregulates phagocytosis. Reduced phagocytosis was also obtained by incubating cells with exudates from MyD88$^{-/-}$ mice infected with *P. gingivalis* to a similar extent as exudates from infected WT mice (Fig. 4*B*). Preboiling of the exudates obtained 24 h postinfection completely reversed the inhibition of phagocytosis, suggesting that the secreted factor is proteinaceous (Fig. 4*C*). Interestingly, exudates obtained 48 h postinfection (solid line) were far less able to suppress phagocytosis compared with exudates from 24 h postinfection (dotted line). Boiling of exudates from 48 h postinfection also reversed the phagocytosis-suppressive effect (data not shown). Thus, a TLR2-dependent, but MyD88-independent, pathway is activated following infection with *P. gingivalis*, leading to the secretion of a heat-sensitive factor that downregulates phagocytosis independently of TLR2 expression on the phagocytic cells.

Intracellular toxic oxygen species formation in response to P. gingivalis *is impaired in the absence of MyD88*

Our results showed that *P. gingivalis* survival in MyD88$^{-/-}$ mice was enhanced relative to WT mice, and although WT and MyD88$^{-/-}$ mice are bacteremic, only the MyD88$^{-/-}$ mice displayed signs of systemic illness and succumbed to infection. Because the difference in phagocytosis between WT and MyD88$^{-/-}$ mice was small (Fig. 3*B*), we examined the role of MyD88 in bactericidal mechanisms in response to *P. gingivalis*. No differences were found in NO levels in response to *P. gingivalis* infection in any of the mouse strains at 2 and 24 h following challenge (data not shown). We next used the oxidation of DHR to measure the generation of intracellular toxic oxygen species, a representative bactericidal mechanism. Oxidation of DHR occurs mainly as a result of peroxynitrate formation and produces a fluorescent signal that can be measured by FACS (28). The induction of peroxynitrate by *P. gingivalis* was significantly impaired in cells recovered from s.c. implanted chambers of MyD88$^{-/-}$

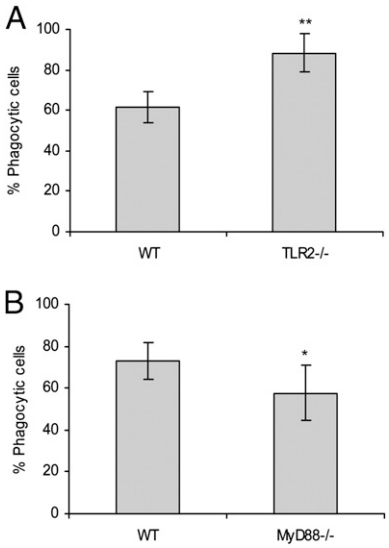

FIGURE 3. In vivo phagocytosis of *P. gingivalis* in WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice. Phagocytosis of FITC-labeled *P. gingivalis* was measured 24 h after s.c. infection (n = 5–6 mice per group). The percentage of phagocytic cells in each mouse was determined relative to the autofluorescence of cells alone. Significance was determined by comparing the percentage of phagocytic cells from TLR2$^{-/-}$ (*A*) and MyD88$^{-/-}$ mice (*B*) with those of cells from WT mice. One representative experiment of two is shown. $*p < 0.05$; $**p < 0.01$.

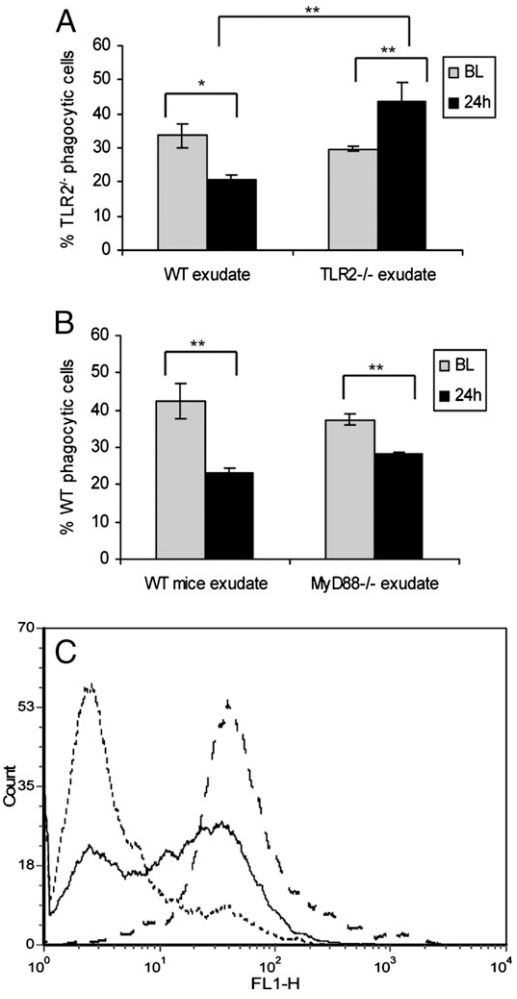

FIGURE 4. Ex vivo phagocytosis of *P. gingivalis* by WT, TLR2$^{-/-}$, and MyD88$^{-/-}$ cells in the presence of inflammatory exudates. *A*, Naive TLR2$^{-/-}$ cells from s.c. chambers were exposed to FITC-labeled *P. gingivalis* in the presence of exudates from WT (*left*) and TLR2$^{-/-}$ (*right*) mice (at BL or 24 h post *P. gingivalis* infection). One representative experiment of two is shown. *B*, Naive WT cells from s.c. chambers were exposed to FITC-labeled *P. gingivalis* in the presence of exudates from WT (*left*) and MyD88$^{-/-}$ (*right*) mice (at BL or 24 h post *P. gingivalis* infection). *C*, Naive WT cells from s.c. chambers were exposed to FITC-labeled *P. gingivalis* in the presence of untreated WT mice exudates drawn 24 h (dotted line) and 48 h (solid line) post *P. gingivalis* infection or in the presence of boiled WT mice exudates (dashed line) drawn 24 h following infection. One representative experiment of two is shown. $*p < 0.05$; $** p < 0.01$.

mice compared with WT mice (Fig. 5*A*). No differences were observed between WT and TLR2$^{-/-}$ cells (data not shown). We next examined the induction of peroxynitrate in peripheral blood cells (following erythrocyte lysis) by exposure to *P. gingivalis* for 15, 45, and 90 min. At all times tested, intracellular generation of toxic oxygen species was severely impaired in MyD88$^{-/-}$ peripheral cells compared with WT cells (Fig. 5*B*).

The response to P. gingivalis *in TLR2$^{-/-}$/MyD88$^{-/-}$ double-knockout mice*

After confirming the importance of MyD88 to bacterial clearance and killing in comparison with WT mice, the next question we addressed was whether the presence of MyD88 in TLR2$^{-/-}$ mice explains the rapid clearance that we observed in these mice. To test this, we generated TLR2$^{-/-}$/MyD88$^{-/-}$ mice. These mice could also be used to confirm that the proinflammatory cytokine response in MyD88$^{-/-}$ mice requires TLR2. S.c. challenge with *P. gingivalis* in TLR2$^{-/-}$/MyD88$^{-/-}$ mice yielded a cytokine profile almost identical to the one observed in TLR2$^{-/-}$ mice for TNF-α (Fig. 6*A*) and IL-1β (Fig. 6*B*), i.e., no TNF-α was detected 2 h postinfection, and IL-1β secretion was delayed and detectable only 24 h postinfection. Thus, proinflammatory cytokine secretion in vivo in response to *P. gingivalis* infection is TLR2 dependent and independent of MyD88 signaling. Consistent with the absence of IL-10 in MyD88$^{-/-}$ mice, no IL-10 was found in TLR2$^{-/-}$/MyD88$^{-/-}$ mice (Fig. 6*C*), confirming that IL-10 secretion in vivo in response to *P. gingivalis* is MyD88 dependent.

We next followed bacterial survival in TLR2$^{-/-}$/MyD88$^{-/-}$ mice and found a pattern of survival similar to that in MyD88$^{-/-}$ mice, best reflected in increased CFU at 24 h compared with 2 h following challenge (Fig. 7). The dramatic difference in bacterial clearance between TLR2$^{-/-}$ mice (most mice completely cleared the infection by 24 h) and TLR2$^{-/-}$/MyD88$^{-/-}$ mice supports the argument that a TLR2-independent, but MyD88-dependent, pathway governs rapid bacterial clearance in TLR2$^{-/-}$ mice.

Discussion

In this study, we described TLR2-dependent proinflammatory cytokine production in vivo, independent of the two main intracellular adaptor proteins involved in TLR signaling (MyD88 and TRIF) (11, 29). The TNF-α and IL-1β produced in MyD88$^{-/-}$ mice in rapid response to s.c. infection with *P. gingivalis* (Fig. 1*A*, 1*B*) was surprising, because inflammatory cytokine production in vitro and in vivo following TLR2 stimulation has consistently been shown to depend on MyD88 (18, 30, 31). The absence of proinflammatory cytokines in response to *P. gingivalis* infection in TLR2$^{-/-}$/MyD88$^{-/-}$ mice (Fig. 6) confirmed that the MyD88-independent response requires TLR2. In contrast, the production of IL-10 in response to *P. gingivalis* infection is mostly through TLR2 and is entirely dependent on signaling through MyD88 because no IL-10 is produced in response to *P. gingivalis* infection of MyD88$^{-/-}$ or

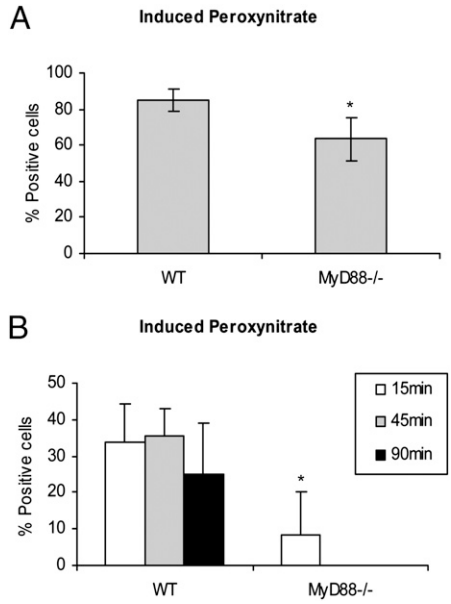

FIGURE 5. Generation of toxic oxygen species in response to *P. gingivalis* in WT and MyD88$^{-/-}$ mice. *A*, Naive chamber cells from WT ($n = 6$) and MyD88$^{-/-}$ mice ($n = 5$) were exposed ex vivo to *P. gingivalis* and then incubated with DHR 123. Positive cells were defined as those with fluorescence above the level of spontaneous DHR 123 reduction. One representative experiment of two is shown. *B*, WBCs of WT and MyD88$^{-/-}$ mice were incubated with *P. gingivalis* and DHR 123 for 15, 30, or 90 min, and the percentage of cells with induced DHR 123 reduction was determined at each time point. No induced DHR 123 reduction was observed at 45 and 90 min for cells from MyD88$^{-/-}$ mice. One representative experiment of two is shown. $*p < 0.05$.

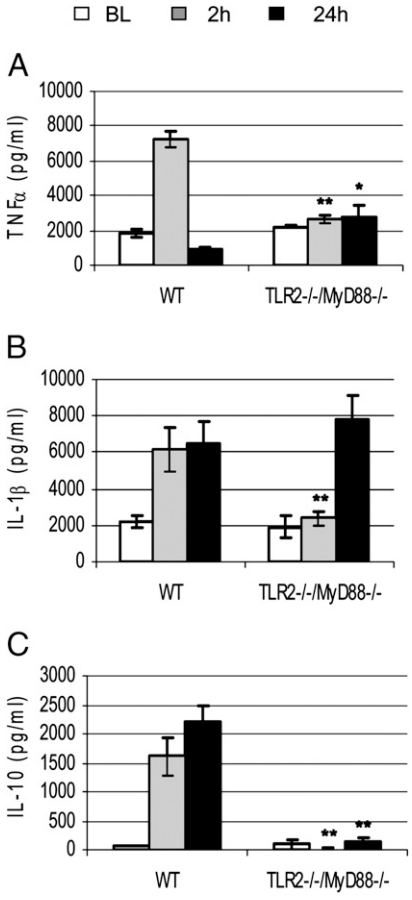

FIGURE 6. Cytokine levels following *P. gingivalis* challenge in TLR2$^{-/-}$/MyD88$^{-/-}$ mice. TNF-α (*A*), IL-1β (*B*), and IL-10 (*C*) levels were measured in chamber exudates at BL and 2 and 24 h after *P. gingivalis* challenge. One representative experiment of two is shown. Significance shown represents comparisons with WT mice at the same time point. $*p < 0.05$; $** p < 0.01$.

TLR2$^{-/-}$/MyD88$^{-/-}$ mice (Figs. 1*C*, 6*C*). Several nonmutually exclusive mechanisms may underlie TLR2-dependent, MyD88-independent inflammatory cytokine production in vivo. First, the nature of the Ag seems to be critical; Pam3Cys, a synthetic lipopeptide TLR2 ligand, does not induce cytokines in MyD88$^{-/-}$ mice (Fig. 1*D*), suggesting that particular TLR2 ligands available during *P. gingivalis* infection may recruit additional receptors or adaptor proteins whose signaling involves factors other than MyD88 (32). Second, TLR signaling pathways that may not require MyD88 have been described. For TLR2, signaling can proceed through Rac1, PI3K, and Akt, leading to NF-κB activation in macrophages (3, 16) and neutrophils (17). Rapid production of IL-6 involving Ca^{2+}, adenylyl cyclase 3-generated cAMP, and CREB has been reported for TLR4 in human bladder epithelial cells in response to uropathogenic *Escherichia coli* (33). Although not described for TLR2, this or a similar pathway may be involved in the MyD88-independent cytokine response to *P. gingivalis* infection. TLR2-driven responses to *P. gingivalis* were also shown to activate β2-integrin inside-out signaling (3), and this may initiate cell–cell interactions that lead to cytokine production through non-TLR pathways. Finally, the adaptor protein MyD88-adapter-like (MAL), which is necessary as a bridging protein to recruit MyD88 to TLR2 and TLR4, also contains a binding domain for the downstream kinase TNF receptor-associated factor 6 (TRAF6). In fact, we found that MAL coimmunoprecipitates with TRAF6 in RAW264.7 macrophages and in cells recovered from s.c. chambers (data not shown), similar to the findings of other investigators (34). Therefore, MAL may lead to TRAF6 phosphorylation and NF-κB activation, even in the absence of MyD88 (18, 34). Although we have yet to establish which, if any, of these pathways is active following *P. gingivalis* infection in vivo, the formal demonstration of in vivo proinflammatory cytokine production that is TLR2 dependent, but independent of MyD88 or TRIF, should inspire a new look at the requirements for TLR2-driven inflammation in vivo.

Despite levels of TNF-α and IL-1β in MyD88$^{-/-}$ mice equivalent to those found in WT mice following s.c. infection with

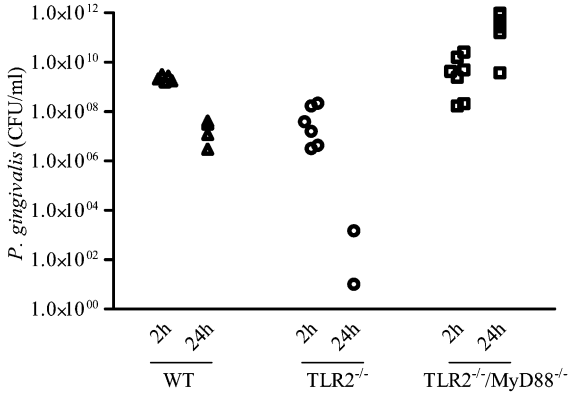

FIGURE 7. *P. gingivalis* survival in TLR2$^{-/-}$/MyD88$^{-/-}$ mice. Viable *P. gingivalis* CFU counts from chamber exudates of WT, TLR2$^{-/-}$, and TLR2$^{-/-}$/MyD88$^{-/-}$ mice were determined at 2 and 24 h postintrachamber challenge. Each symbol represents an individual mouse ($n \geq 6$ per strain). One representative experiment of two is shown. CFU counts in TLR2$^{-/-}$/MyD88$^{-/-}$ mice were significantly higher at 24 h compared with 2 h ($p < 0.05$), whereas there was a significant decrease in CFU between 2 and 24 h in the other strains ($p < 0.05$ for WT; $p < 0.00001$ for TLR2$^{-/-}$).

P. gingivalis, MyD88$^{-/-}$ mice were much less able to clear *P. gingivalis* (Fig. 2*A*, 2*C*) and eventually demonstrated signs of systemic infection, with 40% mortality at 10 d postinfection. Signaling through a TLR4-MyD88 pathway does not seem to explain bacterial survival in MyD88$^{-/-}$ mice, because bacterial clearance in TLR4$^{-/-}$ mice was similar to that in WT mice (Fig. 2*D*). In contrast to our finding that *P. gingivalis* is rapidly cleared in TLR2$^{-/-}$ mice (Fig. 2*A*) (10), Hajishengallis et al. (35) reported impaired *P. gingivalis* clearance in TLR2-deficient mice following intratracheal challenge. Following s.c. challenge, the majority of cells encountering *P. gingivalis* are neutrophils (10); however, alveolar macrophages are the major cell type involved in the response to *P. gingivalis* following intratracheal challenge. Neutrophils may promote *P. gingivalis* replication, as was shown for *Chlamydia pneumoniae* (36). Thus, the role of TLR2 in promoting or clearing infection may depend on the particular cell types present at the site of infection. In addition to its role in TLR signal transduction, MyD88 is critical to IL-1βR signaling; however, this pathway is unlikely to be responsible for rapid *P. gingivalis* clearance, because *P. gingivalis* load decreases dramatically at 2 h postinfection in TLR2$^{-/-}$ mice, whereas IL-1β production is observed only at later time points in these mice. IL-10 production might be expected to protect *P. gingivalis* from clearance, such as shown for *Mycobacterium tuberculosis* (37) and *Yersinia* spp. (38); however, this mechanism does not explain *P. gingivalis* survival, because IL-10 is absent from the response to *P. gingivalis* in MyD88$^{-/-}$ mice (Fig. 1*C*). Our findings are consistent with the observation that *M. tuberculosis* bacterial load in lungs of MyD88$^{-/-}$ mice far exceeds the load observed in WT mice, despite active production of TNF-α in vivo in MyD88$^{-/-}$ mice following *M. tuberculosis* infection (13). Similarly, pulmonary infection with *C. pneumoniae* is fatal in MyD88$^{-/-}$ mice compared with WT mice, despite similar levels of TNF-α and IFN-γ (39).

MyD88-dependent signaling seems to be much more important for phagocytosis and generating intracellular oxidative bactericidal activity in our system than for cytokine production. Although reactive oxygen species generation may not be effective against *P. gingivalis* (35, 40), our results suggest that intracellular bactericidal mechanisms follow a TLR2-independent, MyD88-dependent pathway that is distinct from the pathway leading to cytokine generation. Blander and Medzhitov (41) showed that phagocytosis of Gram-negative and -positive bacteria is impaired in MyD88$^{-/-}$ macrophages, and they and other investigators (42) showed that MyD88 signaling is critical to phagosome maturation. MyD88's role in phagocytosis and phagosome maturation may be dependent (41) or independent (42) of TLR2 and TLR4 activation. According to our results, MyD88-dependent clearance of *P. gingivalis* is independent of TLR2 activation; in fact, it is more efficient in TLR2$^{-/-}$ mice (Fig. 2*A*), suggesting that the TLR2-directed inflammatory response conceals *P. gingivalis* from MyD88-dependent clearance. The increased bacterial survival in TLR2$^{-/-}$/MyD88$^{-/-}$ mice compared with WT or TLR2$^{-/-}$ mice (Fig. 7), in the absence of proinflammatory cytokines, confirms that two independent events are triggered following *P. gingivalis* infection: TLR2-dependent, MyD88- and TRIF-independent inflammatory cytokine production and TLR2-independent, but MyD88-dependent, bacterial containment and ultimate clearance.

Our results demonstrate an uncoupling of the proinflammatory cytokine response from what would naturally be considered its purpose, bacterial clearance. In fact, activation of signaling cascades leading to inflammatory cytokine expression was shown to proceed independently of pathways leading to bacterial uptake and the generation of bactericidal molecules (43). Although NF-κB is involved in activating the expression of proinflammatory cytokines and bactericidal molecules (44), the differential requirement for additional factors can explain why inflammation does not predict bacterial clearance. For example, regulation of inducible NO synthase expression requires IFN regulatory factor-1 and phosphorylated STAT1, which are induced independently of MyD88, in addition to NF-κB and AP-1 (induced by MyD88-dependent signaling) (44). Furthermore, the absence of MyD88 would be expected to eliminate the effector function of IL-1β and -18; however, it may have a more general dampening effect on cytokine effector function. For example, MyD88$^{-/-}$ mice infected with *C. pneumoniae* succumb to infection, despite pulmonary levels of IFN-γ and TNF-α that are similar to WT mice (36).

The oral cavity is *P. gingivalis*' only known ecological niche. Thus, selective pressures may have led *P. gingivalis* to evolve strategies that enable persistence in the presence of inflammation. It is hoped that understanding how TLR2 is hijacked by *P. gingivalis* to mask rapid MyD88-dependent bacterial clearance will lead to new therapeutic strategies to control chronic infection-driven inflammation.

Disclosures

The authors have no financial conflicts of interest.

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