J Periodont Res 2006; 41; 447–454



JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00890.x

# Differences in innate immune responses upon stimulation with gram-positive and gram-negative bacteria

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*Konrad Tietze, Alexander Dalpke, Sigfried Morath, Reinier Mutters, Klaus Heeg, Claudia Nonnenmacher: Differences in innate immune responses upon stimulation with gram-positive and gram-negative bacteria. J Periodont Res 2006; 41: 447–454.*


*Background and Objectives:* Host recognition pathways for gram-negative and gram-positive bacteria comprise pattern recognition receptors among which Toll-like receptors (TLRs) play a pivotal role. TLRs share common signaling pathways yet exhibit specificity as well. Periodontal disease is initiated and maintained in the first line by gram-negative but also gram-positive bacterial infection of the gingival sulcus. To date only limited information is available on whether gram-positive and gram-negative bacteria induce different host responses (strength or quality).

*Materials and methods:* To elucidate these differential effects we focused on pro-inflammatory cytokine releases by assessing *ex vivo* stimulation of whole blood with heat-killed gram-negative and gram-positive bacteria and thereof derived microbial products associated with distinct TLRs. Tumor necrosis factor-α and interleukin-8 release were measured in the supernatants by enzyme-linked immunosorbent assay. In addition, innate immune responses of peritoneal macrophages from mice lacking TLR2 and TLR4 were tested.

*Results:* We observed that gram-negative and gram-positive species induced distinct patterns of cytokine production. Gram-negative species produced higher amounts of tumor necrosis factor-α while gram-positive species released higher amounts of the chemokine interleukin-8. Data from TLR knockout mice and TLR-transfected HEK cells revealed a somehow specific role of TLR4 and TLR2 for the recognition of gram-negative and gram-positive bacteria, respectively, an observation that goes along with the dominant recognition of the respective pathogen associated molecular patterns lipopolysaccharide and lipoteichoic acid.

*Conclusions:* The results show that gram-negative and gram-positive bacterial species induce different patterns of immunoregulatory activity, which might be the result of activation of different TLRs.

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Keywords: Toll-like receptors; cytokines; *Porphyromonas gingivalis* lipopolysaccharide; gram-negative; gram-positive

Accepted for publication January 17, 2006

Gram-negative and gram-positive microbial products are believed to evoke different immune responses in which Toll-like receptors play a decisive role. Periodontitis differs from many other types of infections because it is not caused by a single bacterium but by a group of bacteria. Significant doses of viable gram-negative bacteria may invade periodontal connective tissues and subsequently enter the circulation during various periodontal treatment procedures. Although more than 500 different types of bacteria have been isolated from the oral cavity (1), only a small fraction of these bacteria has the potential to cause destruction of periodontal tissues (2).

A variety of host cell receptors have been implicated in the recognition of and response to bacteria and their components. Initial host defense against bacterial infection is executed by innate immunity stimulated by pathogen-associated molecular patterns (PAMPs), conserved molecular structures common to different groups of pathogens that are recognized by host receptors. The innate immune system enables the host to mount an immediate response to the presence of pathogens (3). However, successful defense of bacteria might need different responses depending upon structure, life-style, and virulence of bacteria. At present it is not clear whether or to what extent the innate immune system is able to mount somehow specific responses to the different pathogens.

Recognition of gram-negative bacteria involves shed or membrane-associated lipopolysaccharide. In contrast, gram-positive species are recognized by the host through contact with membrane peptidoglycans, lipoteichoic acid, or soluble extracellular toxins (4). The products of different gram-negative and gram-positive microbes may induce distinct patterns of cytokine production (5,6). Examining the diversity in host response to different gram-negative and gram-positive species suggests that they exhibit distinct patterns of cytokine release (7) but this was not seen when examining different serotypes from the same bacteria (8).

In humans, 10 toll-like receptors (TLRs) have been identified to date, each conferring responsiveness to various infectious agents as well as some endogenous ligands (9). Bacteria express a broad array of structural and soluble cell wall components that play an important role in the pathogenesis of infection. Lipopolysaccharide is the major constituent of the outer cell wall of gram-negative bacteria and is associated with immediate cell activation and the release of proinflammatory cytokines. TLR4 was reported to function as a signaling receptor for lipopolysaccharide from enterobacterial species (10). TLR2 is known to be a signal-transducing molecule for lipoteichoic acid and bacterial lipoproteins (10). Furthermore, TLR2 was also considered the primary signal-transducing molecule for structurally different lipopolysaccharide species derived from the gram-negative bacterium *Porphyromonas gingivalis* as well as from *Prevotella intermedia*, *Leptospira interrogans* and *Helicobacter pylori* (11–13).

Considering that the immunobiology of TLR is based on a multifaceted cellular response that occurs during infection with whole microbial pathogens *in vivo* we examined the diversity in immune response to gram-negative and gram-positive bacteria that play a role in periodontal disease. Here we focused on the differential proinflammatory cytokine releases by assessing *ex vivo* stimulation of whole blood with gram-negative and gram-positive bacteria and microbial products associated with the response to TLRs.

## Materials and methods

### Bacterial species

The bacterial species used in this study are reported in Table 1. Columbia agar (Becton Dickinson) with 5% sheep blood was used for the cultivation of aerobic and microaerophilic microorganisms and Schaedler agar plates were used for the recovery of obligatory anaerobic gram-negative rods and gram-positive cocci. The bacteria were harvested by scraping and centrifugation (1000 *g*, 10 min) and washed in phosphate-buffered saline. McFarland was determined and bacteria were suspended at a concentration of $1 \times 10^9$ cell/ml. The strains were heat inactivated at 65°C for 10 min and then stored at − 70°C.

### Reagents

Lipoteichoic acid from *Staphylococcus aureus* was prepared as described previously (14). Highly purified lipopolysaccharide from *Salmonella minnesota* was kindly provided by U. Seydel (Borstel, Germany). Lipopolysaccharide from *P. gingivalis* was prepared as follows: bacteria were disrupted by sonification and were mixed with an equal volume of *n*-butanol (Merck, Darmstadt, Germany) under stirring for 30 min at room temperature. After centrifugation at 13,000 *g* for 20 min, the aquatic phase was lyophilized, resuspended in chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate, pH 4.7), and centrifuged at 45,000 *g* for 15 min. The supernatant was subjected to hydrophobic interaction chromatography on an octyl-Sepharose column (2.5 × 11 cm) with a linear gradient

*Table 1.* Species used in this study

| Species | Collection number |
|---|---|
| **Gram-positive bacteria** | |
| *Actinomyces meyeri* | CCUG 18285 |
| *Actinomyces odontolyticus* | CCUG 32402 |
| *Enterococcus faecalis* | ATCC 29212 |
| *Micromonas micros* | MCCM 03084 |
| *Peptostreptococcus asaccharolyticus* | ATCC 29743 |
| *Staphylococcus aureus* | ATCC 29213 |
| *Staphylococcus intermedius* | MCCM 03330 |
| *Streptococcus sanguis* | MCCM 00829 |
| **Gram-negative bacteria** | |
| *Actinobacillus actinomycetemcomitans* | MCCM 02810 |
| *Campylobacter rectus* | MCCM 00818 |
| *Dialister pneumosintes* | ATCC 33048 |
| *Eikenella corrodens* | MCCM 02491 |
| *Escherichia coli* | ATCC 25922 |
| *Fusobacterium nucleatum* | Clinical isolate |
| *Porphyromonas gingivalis* | MCCM 03199 |
| *Prevotella intermedia* | ATCC 25611 |
| *Pseudomonas aeruginosa* | ATCC 27853 |
| *Salmonella minnesota* | Clinical isolate |

CCUG = Culture Collection University of Göteborg; ATCC = American Type Culture Collection; MCCM = Medical Culture Collection Marburg.

from 15 to 60% *n*-propanol in 0.1 M ammonium acetate, pH 4.7. Fractions containing lipopolysaccharide were detected by UV absorption.

### Cell transfection

Human embryonic kidney (HEK) 293 cells ($2.5 \times 10^5$ cells/well/24-well plate) were transiently transfected using lipofectamin transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions with 0.5 μg of a human TLR2 expression construct fused to yellow fluorescence protein. Human TLR4, fused to green fluorescence protein, was transfected together with human MD2 (hMD2). TLR plasmids were a donation of T. Espevik, Trondheim, Norway. Additionally, cells were transfected with a nuclear factor-κB reporter gene using luciferase. Cells were stimulated with different stimuli for 6 h and chemiluminescense was measured using a LucLit® Kit (PerkinElmer, Boston, MA, USA) in a TopCount NXT.

### Whole blood assay

Heparinized blood was obtained from healthy volunteers and diluted five-fold with pyrogen-free culture medium Click/RPMI-1640 (Biochrom, Berlin, Germany) containing 100 IU/ml penicillin G and 100 μg/ml streptomycin. The blood samples were treated with different bacterial species or specific stimuli and were then incubated for 22-h intervals. Culture supernatants were collected after centrifugation and cytokine production was monitored by enzyme-linked immunosorbent assay (ELISA).

### Mice

Mice of the strains C3H/HeJ and C3H/HeN were purchased from Charles River (Deutschland GmbH, Sulzfeld, Germany) and $TLR2^{-/-}$ mice were a kind gift from C. Kirschning, Munich, Germany.

Thioglycollate-induced peritoneal macrophages were isolated by peritoneal lavage with ice-cold phosphate-buffered saline. Then, $1.5 \times 10^5$ cells/well were stimulated in 96-well flat-bottom tissue culture plates with different stimuli. The 22-h culture supernatant was analyzed for tumor necrosis factor-α (TNF-α) content using ELISA.

### Cytokine measurement

Cytokine levels (TNF-α and interleukin-8) were determined using commercially available ELISA kits (OptEIA, Becton Dickinson, Heidelberg, Germany). The assays were performed according to the manufacturer's protocol and each value shown represents the mean of duplicate values.

## Results

### *In vitro* cytokine production by whole blood stimulated with different bacteria species

During infection, humans are challenged by whole microorganisms possessing different components that activate the innate immune system. We analyzed the ability of whole bacteria from gram-positive and gram-negative species, which are involved in the progression of periodontal disease, to induce the secretion of cytokines in whole blood cells as a substitute for a local immune reaction in the tissue. *Ex vivo* stimulation of whole blood appears to represent a more physiological environment where the immune cells are present in natural ratios and can interact with each other. As no isolation procedure beyond the drawing of blood is required, the assay is characterized by few preparation artifacts and standardized performance. In addition, the cellular interactions are preserved and the presence of various plasma components, such as lipoproteins, sCD14, lipopolysaccharide-binding protein, bactericidal/permeability-increasing protein, albumin and transferrin, are maintained.

Here, freshly collected blood was incubated with different bacterial species and after 22 h of culture the production of TNF-α and interleukin-8 was quantified in the culture supernatants using ELISA. To better compare the interassay variability, stimulation with lipopolysaccharide was set as 100% and all other bacteria species and stimuli examined were normalized to this. The bacterial species tested showed different abilities to stimulate whole blood cells. Gram-negative species like *Pseudomonas aeruginosa*, *Escherichia coli*, *Campylobacter rectus*, *Dialister pneumosintes*, *Eikenella corrodens* and *S. minnesota* induced greater amounts of cytokines in comparison to other gram-negative species and especially gram-positive bacteria (Fig, 1A,B). Interestingly, gram-negative species seemed to provide a different cytokine pattern release than gram-positive species, in that they produced a higher ratio of TNF-α to interleukin-8. Some gram-positive species, in contrast, provoked relatively higher amounts of IL-8 while other gram-positive species were nearly devoid of activity. *P. gingivalis*, which is a gram-negative bacterium that presents a structurally and functionally different lipopolysaccharide than the Enterobacteriacea, gave a similar answer to that for gram-positive species inducing higher amounts of interleukin-8 in comparison to TNF-α. Differences were reflected by stimulation with typical PAMPs from gram-positive and gram-negative species namely lipoteichoic acid and lipopolysaccharide (Fig. 1C). To this, lipoteichoic acid induced far less TNF-α while it was able to activate interleukin-8. Lipopolysaccharide from *P. gingivalis* behaved like lipoteichoic acid but not like lipopolysaccharide confirming a different stimulatory activity.

### Cytokine production in macrophages from TLR2- and TLR4-deficient mice upon stimulation with whole bacteria

To analyze the effects of heat-killed whole cell preparations derived from gram-negative and gram-positive species on innate immune responses peritoneal macrophages from mice lacking $TLR2^{-/-}$ were stimulated with whole bacteria as well as bacterial PAMPs. Figure 2(A) shows that cells exposed to *P. gingivalis* lipopolysaccharide and lipoteichoic acid from *S. aureus* exhibited barely detectable TNF-α in mice lacking TLR2 in comparison to lipopolysaccharide derived from *S. minnesota*. Analyzing whole bacteria,

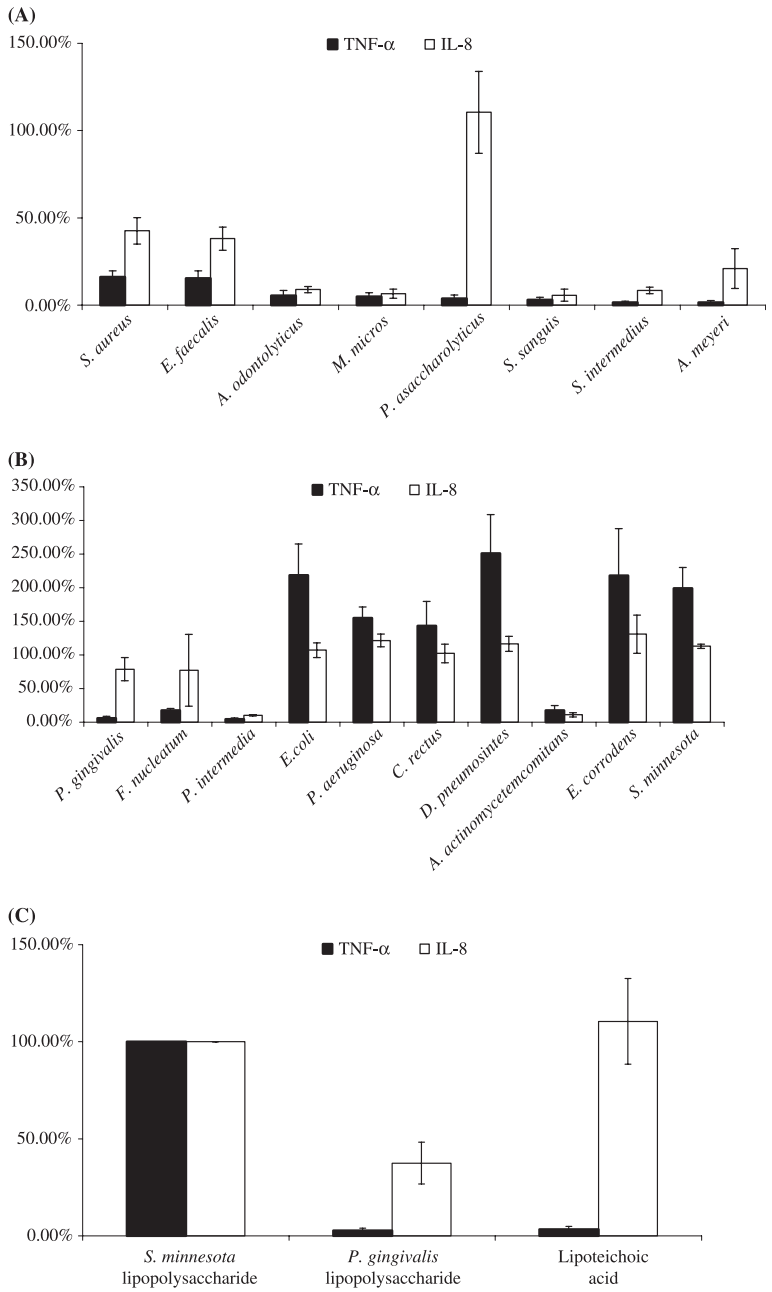


*Fig. 1.* Production of cytokines in human whole blood cells upon stimulation with whole bacteria, *Porphyromonas gingivalis* lipopolysaccharide (1 μg/ml), *Salmonella minnesota* lipopolysaccharide (100 ng/ml) and lipoteichoic acid (10 mg/ml) (C). For the production of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8), whole blood cells were stimulated with $1 \times 10^8$/ml heat-inactivated bacteria from gram-positive (A) and gram-neagtive (B) species for 22 h and the cell-free supernatants were evaluated by ELISA (mean ± SD). The data shown represent one of three independent experiments.

*S. aureus* induced high amounts of TNF-α release in wild-type control peritoneal macrophages while this was reduced in TLR2$^{-/-}$ cells. All other gram-positive species tested were only weakly active. Gram-negative species like *E. coli*, *E. corrodens and C. rectus* induced slightly greater amounts of TNF-α in peritoneal macrophages from wild-type mice while *D. pneumosintes* and *P. aeruginosa* were more active on macrophages from mice lacking TLR2. Interestingly, TNF-α production in response to the gram-negative bacteria *P. gingivalis* and *Fusobacterium nucleatum* was decreased in cells from TLR2$^{-/-}$ mice compared to wild-type mice. This is consistent with earlier observations that *P. gingivalis*-induced signaling pathways are mainly mediated through TLR2 in the same way as gram-positive bacteria.

TLR4 has been the principal TLR species involved in lipopolysaccharide signaling. As previously established, the C3H/HeJ mice with a mutant allele of the TLR4 gene are protected from endotoxic shock and do not manifest any of the symptoms which afflict the C3H/HeN mice upon TLR4 stimulation. To further determine the contribution of gram-negative bacterial species to the production of cytokines, the production of TNF-α induced by different stimuli was evaluated in peritoneal macrophages from lipopolysaccharide-non-responder (TLR4-mutant) C3H/HeJ mice stimulated with whole bacteria, *S. minnesota* lipopolysaccharide, *P. gingivalis* lipopolysaccharide and lipoteichoic acid as compared with wild-type C3H/HeN mice (Fig. 2D). *P. gingivalis* lipopolysaccharide and lipoteichoic acid were equally active to induce TNF-α secretion in C3H/HeJ mice compared to C3H/HeN mice. In contrast, TLR4 mutant C3H/HeJ mice showed dramatically impaired production of TNF-α in response to lipopolysaccharide from *S. minnesota*.

Next, we tested the response of peritoneal macrophages to gram-positive and gram-negative whole bacteria. We found that C3H/HeJ macrophages did not respond to most gram-positive bacteria with the exception of *Enterococcus faecalis* while peritoneal macrophages obtained from the control mice C3H/HeN did (Fig. 2E) although in a smaller proportion in comparison to gram-negative bacteria. C3H/HeJ cells stimulated with different gram-negative species like *F. nucleatum*, *E. coli*, and *P. aeruginosa* showed a decreased TNF-α production in comparison to control wild-type while macrophages stimulated with *P. intermedia*, *C. rectus*, *Actinobacillus actinomycetemcomitans*, and *D. pneumosintes* did not respond. In contrast, *P. gingivalis* induced a higher TNF-α response in the TLR4 mutant C3H/HeJ mice.

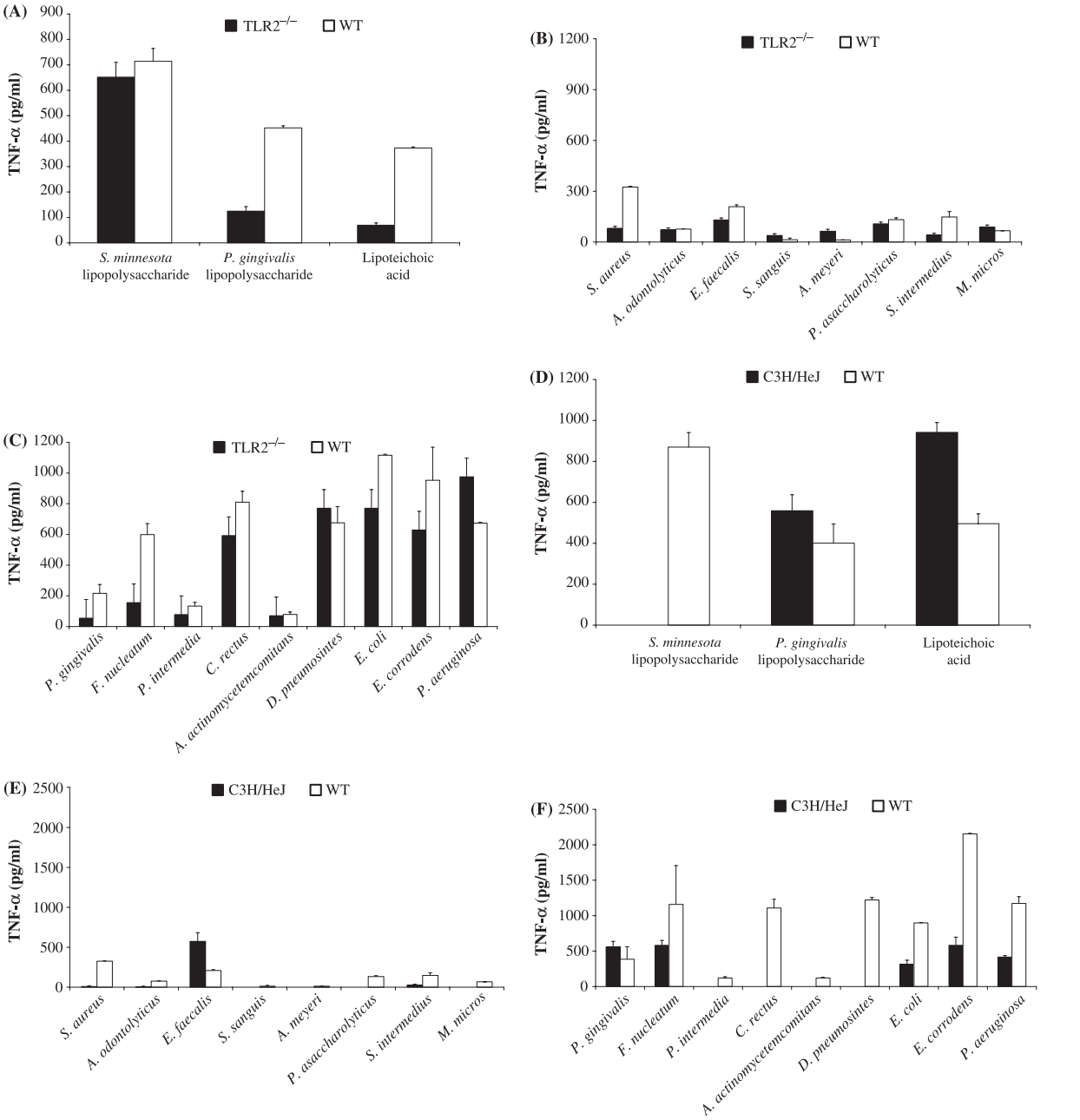


*Fig. 2.* Tumor necrosis factor-α production in peritoneal murine macrophages stimulated with whole bacteria. Thioglycollate-elicited peritoneal macrophages from TLR2-deficient ($TLR2^{-/-}$) and TLR4 mutated (C3H/HeJ) mice, were stimulated with purified bacterial stimuli (A,D), gram-positive species (B,E), and gram-negative species (C,F) for 22 h. C3H/HeN mice were employed as wild-type (WT).

### Requirement of TLRs for the recognition of whole bacteria

HEK293 cells were transiently transfected with human TLR2 and human TLR4. All cells were cotransfected with a nuclear factor-κB-dependent luciferase reporter construct and human TLR4 cells were additionally transfected with hMD2. The transfected cells were stimulated with whole bacteria from different species and with control stimuli *S. minnesota* lipopolysaccharide, *P. gingivalis* lipopolysaccharide and lipoteichoic acid. HEK293 cells transfected with human TLR2 showed a high luciferase induction while in TLR-4-transfected cells this response was markedly reduced (Fig. 3A). The *P. gingivalis* lipopolysaccharide-induced nuclear factor-κB activation was similar to that seen for lipoteichoic acid. These data support the concept that although *P. gingivalis* is a gram-negative bacterium, TLR-2 is involved in signaling events caused by these bacteria as well as by gram-positive species. Nuclear factor-κB activation followed activation with whole bacteria from gram-positive species showed an increased luciferase induction in cells transfected with hTLR2 in comparison to hTLR4-transfected cells (Fig. 3B).

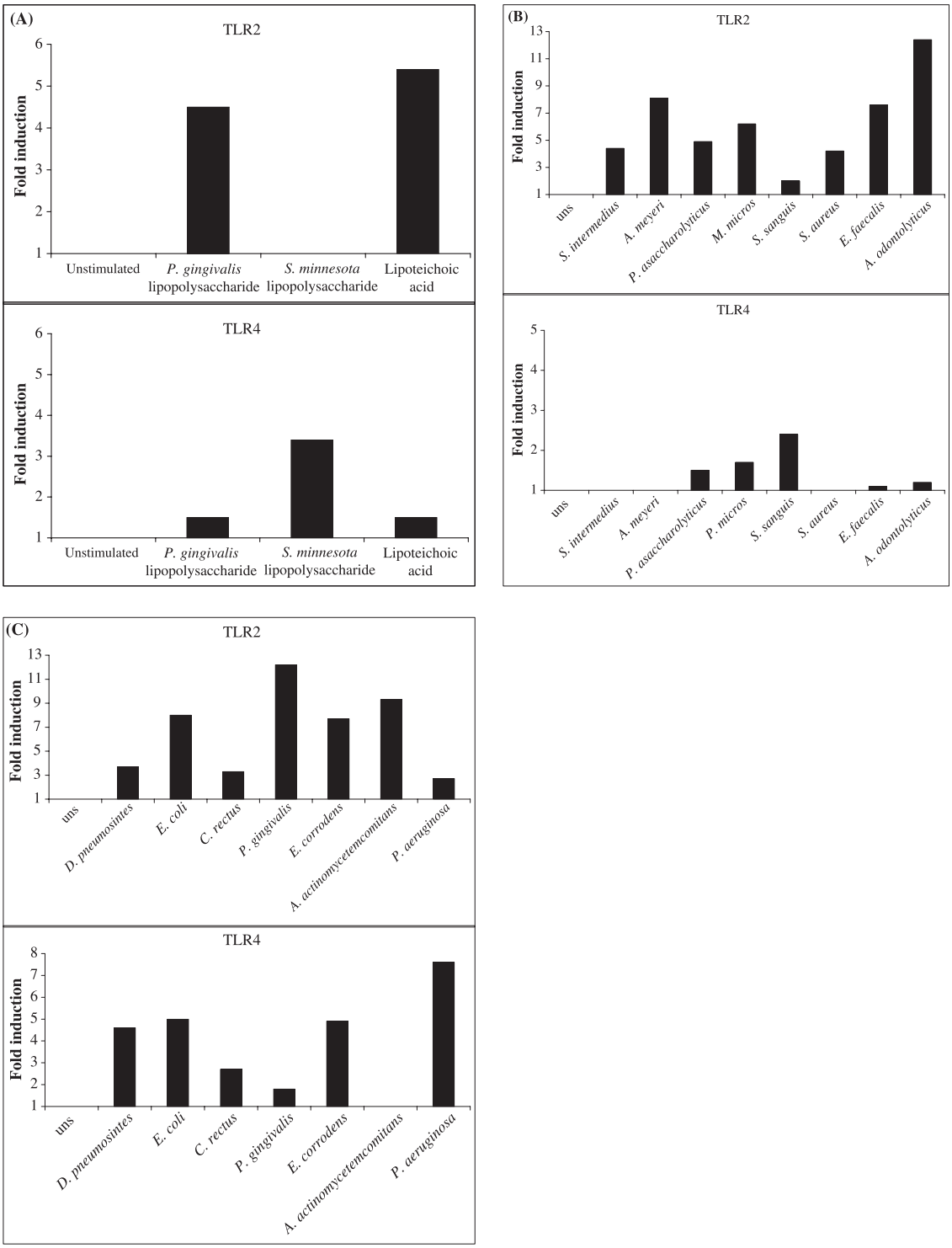


*Fig. 3.* Activation of nuclear factor-κB in HEK293 cells transiently transfected with expression plasmids encoding human TLR2 and TLR4. All cells were cotransfected with a nuclear factor-κB-dependent luciferase reporter construct and human TLR4 was addionally transfected with hMD2. Cells were stimulated with control stimuli (A) as well as whole bacteria from gram-positive (B) and gram-negative (C) species. Luciferase activity was measured after 6 h of stimulation. The data represent one of three independent experiments.

## Discussion

The presence of specific PAMPs during the periodontal infection stimulates an inflammatory cascade that finally results in periodontal tissue destruction (15). In this study we report that gram-negative and gram-positive bacterial species that are involved in the progression of periodontal disease induce different patterns of immunostimulatory activity in which gram-negative species

induced higher amounts of TNF-α release while gram-positive bacteria released higher amounts of interleukin-8.

Gram-negative and gram-positive bacteria have been evolutionarily separated and have developed in parallel for a long time (16); they present several differences. Gram-positive bacteria are bounded by a single cell membrane, and most of these contain a thick cell wall containing peptidoglycan layers approximately 50 times thicker than those of gram-negative bacteria. Peptidoglycan and lipoteichoic acid are two of the major cell wall components in gram-positive bacteria. Both peptidoglycan and lipoteichoic acid have been shown to stimulate inflammatory responses in a number of *in vivo* and *in vitro* experimental models (17–20). In contrast, all gram-negative bacteria contain only a thin peptidoglycan layer (i.e. cell wall) lying between two different cell membranes in addition to lipopolysaccharide, which presents a great compositional variation depending on the particular bacterial origin.

In the present study we chose to compare whole blood responses to whole bacteria from different species, rather than comparing individual elements of the gram-positive and gram-negative microbes. This decision was based on the assumption that the responses to purified gram-negative and gram-positive products are not necessarily comparable and may not mimic the response to whole pathogen. Interestingly, gram-positive species elicited significantly different levels of inflammatory responses *in vivo* where higher amounts of IL-8 were released. In contrast, stimulation of whole blood cells with gram-negative species led to the higher production of TNF-α. Furthermore, lipopolysaccharide from *P. gingivalis,* as well as whole *P. gingivalis*, which has been implicated as a major pathogen in the development and progression of periodontal diseases, and *F. nucleatum*, both gram-negative bacteria, behaved like gram-positive bacteria causing the release of higher amounts of interleukin-8 than TNF-α. One explanation could be that gingipains, the bacterial cysteine proteinases that are released in large amounts by *P. gingivalis*, have been shown to shed and degrade the lipopolysaccharide receptor, CD14, which may result in diminished inducibility of TNF-α (21,22).

Investigation of the precise role of the different PAMPs in interacting with cells during an infection with whole bacteria has been somewhat neglected in favor of studies with purified bacterial components that have allowed a comprehensive understanding of the biology of TLRs and signaling pathways in cellular responses (10). *In vitro*, TLR2 was shown to respond to a variety of microbial products, such as lipoteichoic acid specific for gram-positive bacteria, peptidoglycan particularly from gram-positive bacteria, lipoproteins from bacteria such as gram-negatives and particularly spirochetes, as well as from mycoplasma, and mycobacterial products such as lipoarbinomannan (23,24). Highly purified lipoteichoic acid from *S. aureus* was tested in whole blood cells as well as *in vitro* and is demonstrated to be TLR2 dependent, as already described (25). Making use of whole bacteria from different species in knock-out TLR models we observed that gram-positive species are severely diminished in their immunostimulation in $TLR2^{-/-}$ mice while gram-negative species are more affected in TLR4 mutant C3H/HeJ mice.

Most investigators assessing the effect of lipoproteins have used lipopolysaccharide derived from Enterobacteriaceae species (*E. coli* or *Salmonella typhimurium*). However, lipopolysaccharide structures are a heterogeneous group of molecules with interspecies differences in the length and position of the acyl chains in the lipid A portion of the lipopolysaccharide, the length and polarity of the polysaccharide tail, and the formation of supramolecular structures (26,27). This may explain the difference in TLR4 responses for the different gram-negative species tested in this study. For instance, lipopolysaccharide from the gram-negative bacterium *P. gingivalis* has been suggested to signal through both TLR2 and TLR4 (28–30). Further, lipopolysaccharides from other gram-negative organisms, including *P. intermedia*, *L. interrogans* and *H. pylori* were reported to activate TLR2-dependent signaling (11–13). These different results are thought to be related to the differences in the chemical structure of lipid A moieties (31).

Studies of HEK293 transfectants revealed that although the gram-positive species present a tendency for signaling by TLR2, signaling was also observed, although at lower levels, by TLR4. The same was observed for gram-negative bacteria, which stimulated mostly TLR4, but also TLR2. Clearly, the response to microbial pathogens is believed to vary depending upon the specific pathogen or microbial product, its concentration, and the duration of the exposure. In addition, results of experiments using single knockout animals might be misleading because of the potential ability of one TLR to compensate for the lack of the other. Previous results from our group have already highlighted the differences in stimulatory effects as a result of bacterial DNA from distinct bacteria strains from the oral cavity (32). Furthermore, it has been shown that differential activation of TLRs by whole gram-positive or gram-negative bacteria evokes distinct gene expression profiles *in vitro* (33).

One has to further assume that the actual situation *in vivo* is much more complicated because periodontal infections are polymicrobial in nature and thus infections comprise gram-positive and gram-negative bacteria simultaneously. Accordingly the actual pattern of immunostimulation might depend on the bacterial composition as well as on the interrelationship between distinct TLR ligands and subsequent signaling pathways. There is clinical evidence that some genera of bacteria might aggravate while others might suppress acute inflammatory processes. Hence, it seems to be tempting to analyze the interrelationship of bacterial stimulation in a defined system as described here.

In summary, our findings illustrate the potential differences in the response patterns of whole-blood cells in response to different bacterial species that are responsible for the initiation and progression of periodontal disease. In addition, whole bacteria from either gram-negative or gram-positive species

may contain agonists that activate multiple TLRs. Thus, a thorough understanding of the initial interactions between host and pathogen and the complexities in the host response to different pathogens is essential for understanding the differential activation of the innate immune system.

## Acknowledgements

The authors would like to thank Helene Bykow, Nadine Schelberg and Claudia Trier for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG): DFG He 1452/4 and/5 and Da 592/1.

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