Oral Microbiology Immunology 2008: 23: 353–359
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

The role of Toll-like receptors 2 and 4 on reactive oxygen species and nitric oxide production by macrophage cells stimulated with root canal pathogens

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Marcato LG, Ferlini AP, Freire Bonfim RC, Ramos-Jorge ML, Ropert C, Afonso LFC, Vieira LQ, Ribeiro Sobrinho AP. The role of Toll-like receptors 2 and 4 on reactive oxygen species and nitric oxide production by macrophage cells stimulated with root canal pathogens.
Oral Microbiol Immunol 2008: 23: 353–359. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: Periapical lesions arise as a result of the activation and interaction of the host immune responses against root canal infection. Recently identified Toll-like receptors (TLR) seem to be involved in the recognition and development of immune responses against a myriad of microorganisms. However, very little information is available on the role of TLR in the induction of periapical lesions.
Method: The role of TLR-2 and TLR-4 in the activation of murine macrophages stimulated using *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* was investigated. The production of nitric oxide (NO) and reactive oxygen species (ROS) was assessed.
Results: The results demonstrate that TLR-2 and TLR-4 are involved in the production of ROS by activated macrophages. The microorganisms induced similar levels of NO production by TLR-2-competent and TLR-2-deficient macrophages, regardless of the addition of interferon-γ (IFN-γ), ruling out a role for TLR-2 in the NO production induced by these bacteria. Only *P. anaerobius* induced NO production by TLR-4-competent macrophages without the addition of IFN-γ. However, after IFN-γ addition, *F. nucleatum* induced macrophage NO production. Therefore, NO production stimulated by IFN-γ and these microorganisms seems to be TLR-4-independent.
Conclusion: TLR-2 seems to be involved in the induction of ROS production by macrophages in response to prevalent root canal bacteria, while only *F. nucleatum* induced ROS production by TLR-4-competent macrophages. Both microorganisms significantly induced large amounts of NO independent of TLR-2 and TLR-4. We conclude that microorganisms may participate in the induction and progression of periapical lesions through NO and ROS production by activated macrophages.

Key words: Innate immunity; nitric oxide; reactive oxygen species; root canal infection; Toll-like receptors

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Accepted for publication December 3, 2007

A successful defense by an organism against infection depends on the recognition of bacterial products. Using receptors codified in the germinative lineage, which selectively recognize essential pathogen constituents, organisms immediately respond to infection and later build adptative immune response (3). Different microorganisms express particular molecular patterns, which are identified by different molecular receptors present in the host (1).

Transduction signal receptors, which start the inflammatory cascade, were recently identified in mammals and, because of their similarity to *Drosophila* Toll receptors, were attributed the name toll-like receptors (TLR) (4). Strong evidence has shown that one of these receptors, TLR-4, is directly involved in immunological responses against lipopolysaccharides (LPS) present in the cellular wall of gram-negative bacteria (9, 26, 43). After activation, TLR-4 induces proinflammatory cytokine expression associated with bone resorption and tissue breakdown in endodontic periapical lesions (10, 23). Another receptor, TLR-2, has been associated with the recognition and development of immunological responses against gram-positive bacteria (20).

Root canal infections lead to the recruitment of immune competent cells to the periapical region, producing a variety of inflammatory mediators (36). To kill microorganisms, activated macrophages and neutrophils ingest microorganisms and use microbicidal molecules present in phagolysosomes (4, 14). Among these molecules, nitric oxide (NO) and reactive oxygen species (ROS) play key roles in the development of periapical lesions (11, 13, 17, 32, 34, 39).

To date, information on the role of TLRs in the induction and progression of periapical lesions is scarce in the literature. Therefore, this study assessed the effector immune response that occurs in this pathology, investigating the ROS and NO production by macrophages from TRL-2-defective and TLR-4-defective (C57BL/6 TLR-$2^{-/-}$ and C3H/HeJ) knockout mice and their respective wild-type controls (C57BL/6 and C3H/HeN, respectively). Macrophages were stimulated with the prevalent endodontic bacteria, *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius*. In addition, the NO production when interferon-γ (IFN-γ) was added to the cell cultures was evaluated.

Materials and methods

Animals

Six- to eight-week-old, male and female mice were used: mice that were TLR-4-defective by spontaneous mutation (C3H/HeJ) and TLR-4-sufficient (C3H/HeN); mice that were rendered deficient in TLR-2 by homologous recombination, (TLR-$2^{-/-}$) (9, 25) and TLR-2-sufficient wild-type controls (C57BL/6). TLR-$2^{-/-}$, CEH/HeJ, and C3H/HeN mice were obtained from Centro de Pesquisas Rene Rachou (Fiocruz, Belo Horizonte, MG, Brazil). C57BL/6 mice were obtained from CEBIO (ICB, UFMG, Belo Horizonte, MG, Brazil). Experimental animals were kept in a conventional animal house with barriers and controlled temperature, and light. Food and water were offered *ad libitum*. This research project was approved by the local ethics committee for research on animals (CETEA – UFMG, protocol number 129/04).

Microorganisms

The selected microorganisms were *F. nucleatum* (15), a gram negative bacterium, and *P. anaerobius* (ATCC 27337), a gram-positive bacterium. Zymosan A from *Saccharomyces cerevisiae* was used as a positive control for the ROS assay. The microorganisms were grown in brain–heart infusion broth (Difco, Detroit, MI, USA), supplemented, and prereduced (BHI-PRAS) in an anaerobic chamber (Forma Scientific, Inc., Marieta, OH) containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 for 48 h at 37°C. The samples were adjusted in phosphate-buffered saline to a concentration of 8.0×10^7 colony-forming units (CFU)/ml. To kill the bacteria, the methodology proposed by Ribeiro Sobrinho et al. (30) was used. Zymosan A was diluted in phosphate-buffered saline (10^9 particles/ml).

Macrophage cultures

Inflammatory macrophages were obtained from the peritoneal cavity as previously described (29). Briefly, cells were isolated from the peritoneal cavity of mice 3 days after injection of 2 ml of 3% thioglycollate medium (Biobrás S.A., Montes Claros, MG, Brazil) into the peritoneum. Cells were resuspended in complete medium: RPMI-1640 without phenol red (Sigma Chemicals Co., St Louis, MO), supplemented with 10% of fetal calf serum (Nutricell, Campinas, SP, Brazil), 0.2% penicillin (100 U/ml)/streptomycin (0.1 mg/ml), and 200 mM L-glutamine. The cells were counted in a Neubauer chamber, and the final concentration was adjusted for each experiment.

ROS assay

ROS were assayed as described by Trush et al. (42), with modifications. Briefly, *F. nucleatum* and *P. anaerobius* (10^7 CFU/well), live or heat-killed (30), or Zymosan A (10^7 particles/well) preparations were added to C96 White Maxisorp (Nalgene, Rochester, NY) plates containing 1×10^6 cells in RPMI-1640 without phenol red (Sigma Chemical Co.). Next, 0.05 mmol/ml luminol (Sigma Chemical Co.) was added to each well and ROS readings were carried out for 2 h (Lumi-Count® Packard Instrument Company Inc., Downers Grove, IL). Results were expressed as the area under each of the curves obtained.

NO production

Macrophages (1×10^6 cells/ml) were cultured in 96-well culture plates (Nunclon, Nalgene) in an incubator with a humidified atmosphere containing 5% CO_2, at 37°C. Some cultures were activated with 10 U/ml recombinant IFN-γ (Pharmingen, San Diego, CA). Cells were cultured for 48 h and NO production was assayed as nitrites in the supernatants. Sodium nitrite (0.977–125 μM) was used as a standard (22). Sensitivity was 0.5 μM.

Statistical analysis

Data were analyzed using parametric tests (Student's *t*-test; $P < 0.05$). Analyses were carried out using the SPSS 12.0 Inc. (Statistical Package for Social Sciences, Chicago, IL) software.

Results

Production of ROS

The analysis of the area under each ROS production curve showed that macrophages from C3H/HeN and C3H/HeJ mice, stimulated by live or heat-killed *P. anaerobius*, produced ROS at levels similar to those produced by unstimulated macrophages (Fig. 1C). In contrast, cells from wild-type mice, C3H/HeN, when stimulated with live or heat-killed *F. nucleatum*, were able to produce statistically higher levels of ROS than untreated macrophages (Fig. 1A and C). These results suggest that heat treatment diminishes the capability of *F. nucleatum* to induce ROS. Levels of ROS production in C3H/HeN macrophages stimulated by live cells were higher than those observed in macrophages stimulated by heat-killed bacteria. In addition, only live *F. nucleatum* triggered an increased production of ROS in C3H/HeJ macrophages. The addition of Zymosan increased ROS production in macrophages from both strains, C3H/HeN and C3H/HeJ (Fig. 1C).

Figure 1(B and D) represent the kinetics of ROS production by macrophages from C57BL/6 and C57BL/6 TLR-$2^{-/-}$ mice. Significantly higher levels of ROS

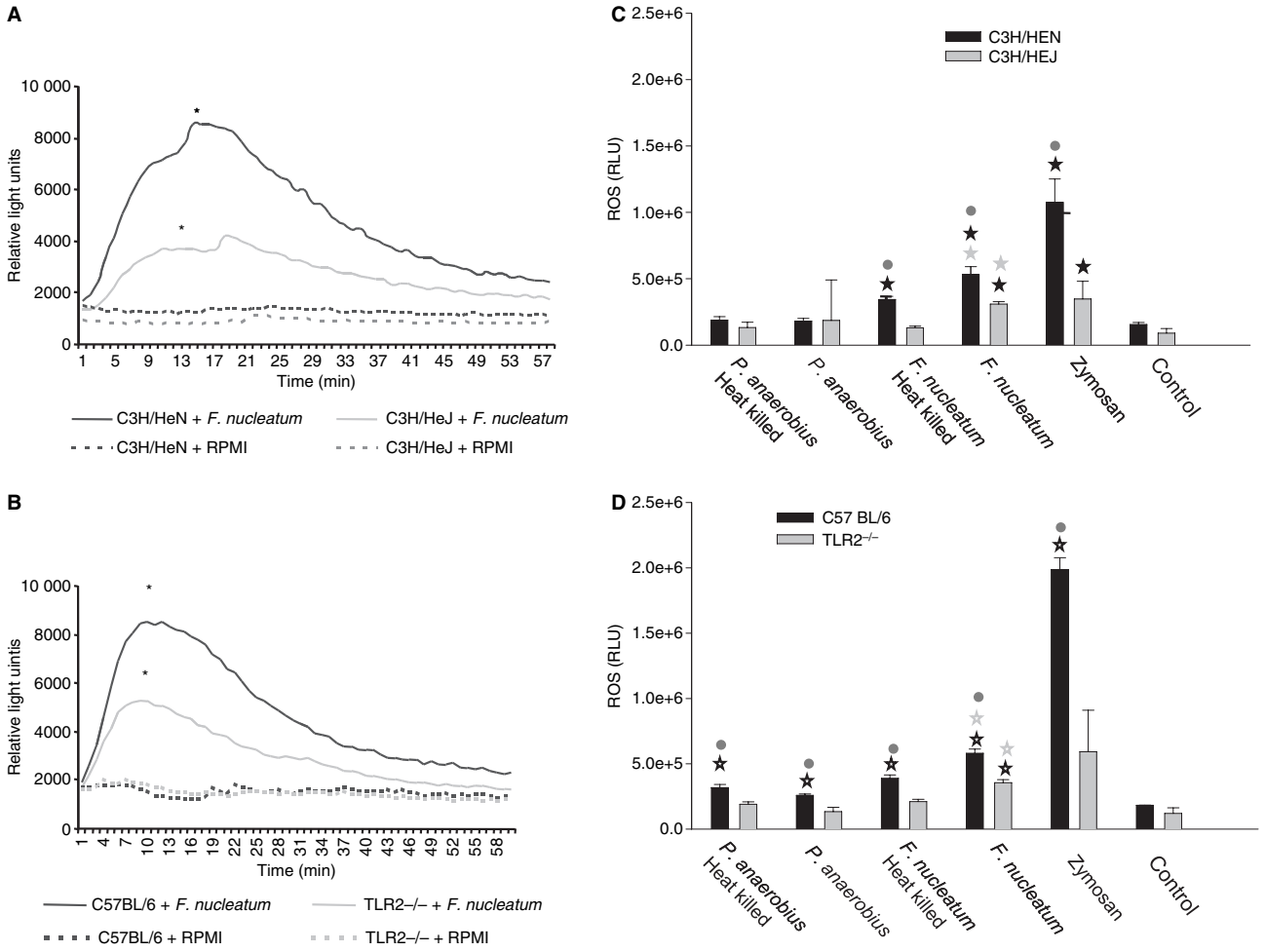

Fig. 1. Reactive oxygen species (ROS) production by macrophages from C3H/HeN and C3H/HeJ mice and from C57BL/6 and C57BL/6 TLR-2$^{-/-}$ mice. (A and C) Representative kinetics of ROS production by macrophages from C3H/HeN and C3H/HeJ (A) or C57BL/6 and C57BL/6 TLR-2$^{-/-}$ mice (B) stimulated with live *Fusobacterium nucleatum.* (C) Area under the curves obtained for macrophages from C3H/HeN and C3H/HeJ mice stimulated *in vitro* with live or heat-killed *Peptostreptococcus anaerobius* or *F. nucleatum.* Zymosan was used as a positive control and macrophages in medium were used as negative control. (D) Area under the curves obtained for macrophages from C57BL/6 and C57BL/6 TLR-2$^{-/-}$ mice stimulated *in vitro* with live or heat-killed *P. anaerobius* or *F. nucleatum.* Zymosan was used as a positive control and macrophages in medium were used as negative control. Bars represent the mean of three experiments performed in duplicate; lines stand for the standard error of the means. Black star indicates statistical difference for ROS production in response to presence or absence of stimuli; gray star indicates statistical difference between microbial stimuli type (live or heat-killed), and gray circle indicates statistical difference between macrophage sources ($P < 0.05$).

production by macrophages from C57BL/6 mice than those from C57BL/6 TLR-2$^{-/-}$ mice were observed when the stimuli were *P. anaerobius* and *F. nucleatum*, live or heat-killed. All stimuli, *P. anaerobius* and *F. nucleatum*, live or heat-killed, induced consistent increases in ROS production when compared to the negative control macrophages from C57BL/6 mice. Interestingly, heat treatment also seemed to interfere with the microorganism's capability to induce respiratory bursts because only live *F. nucleatum* stimulated ROS production by C57BL/6 TLR-2$^{-/-}$ macrophages. Live *F. nucleatum* induced higher ROS production by C57BL/6 than by TLR-2$^{-/-}$ macrophages (Fig. 1B and D). An increased ROS production was found in response to a Zymosan stimulus in C57BL/6 macrophage cultures (Fig. 1D).

NO production

Different levels of NO production were found in macrophage cultures obtained from TLR-4-defective C3H/HeJ and C57BL/6 TLR2$^{-/-}$ mice and their respective backgrounds (Figs 2 and 3). Figure 2 (A and B) demonstrate NO production by macrophages from C57BL/6 and C57BL/6 TLR-2$^{-/-}$ mice in the absence (Fig. 2A) or presence (Fig. 2B) of IFN-γ. All stimuli had significant effects on NO production, and statistical differences appeared upon comparing the test and control cultures (RPMI), in the presence or absence of IFN-γ. There were no statistical differences in NO production between cultures stimulated with live or heat-killed bacteria. The same was observed for the source of macrophages (wild-type or TLR2$^{-/-}$) (Fig. 2A and B). Taken together, these results suggest that the signal to induce NO production does not occur solely through the activation of TLR-2.

Figure 3 represents NO production by macrophages from C3H/HeN and C3H/HeJ mice in the absence (Fig. 3A) or presence (Fig. 3B) of IFN-γ. In the absence of IFN-γ, only live *P. anaerobius* triggered an increased production of NO in macrophage cultures from C3H/HeN mice (Fig. 3A). In contrast to these results, in the presence of this cytokine, live

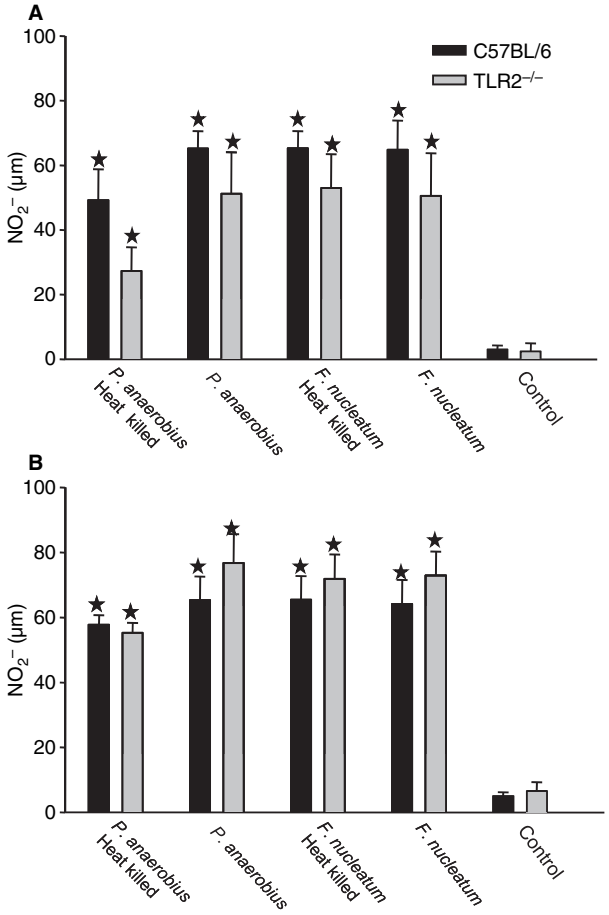

Fig. 2. Nitric oxide (measured as NO_2^-) production by macrophages from wild-type C57BL/6 and TLR-$2^{-/-}$ C57BL/6 mice. Cells were stimulated by live or heat-killed *Peptostreptococcus anaerobius*; live or heat-killed *Fusobacterium nucleatum*, and RPMI-1640 (negative control) in the absence (A) or presence (B) of interferon-γ (IFN-γ) as described in the Material and methods. Bars represent the mean of three experiments performed in duplicate; lines stand for the standard error of the means. Black stars indicate statistical difference for NO_2^- production in response to the presence or absence of stimuli, $P < 0.05$.

P. anaerobius induced not only a high level of NO production by C3H/HeN macrophages, but also an increased amount of NO in TLR-4-defective macrophage cultures (Fig. 3B). *F. nucleatum*, live or heat-killed, in the presence of IFN-γ, induced consistent increases in wild-type macrophage NO production while only live *F. nucleatum* stimulated this production in TLR-4-defective macrophage cultures (Fig. 3B).

Discussion

The periapical lesion development is the result of an inflammatory reaction induced by root canal infection. Bacteria and their byproducts promote the recruitment of a number of inflammatory cells, which play an important role in periapical health. However, in certain circumstances, this reaction induces cell and tissue breakdown, culminating in the installation and maintenance of a periapical disease (23). The specific function of each cell present in the inflammatory infiltrate has yet to be defined (35). Macrophages, along with lymphocytes, are the most prevalent cells in the periapical infiltrate (36, 39). In addition to interleukin-1 and tumor necrosis factor, macrophages produce several microbicidal substances, such as NO and ROS, which are involved in inflammatory reactions and bone resorption (1, 17, 18, 22, 33). During periapical lesion development, macrophages and other innate immune response cells use receptors that recognize bacterial constituents, transducing signals that start the inflammatory cascade. In endodontic infection, Hou et al. (10) demonstrated that TLR-4 function enhances inflammatory response and bone destruction.

In this study, *F. nucleatum* and *P. anaerobius*, well-known endodontic pathogens (8, 15), were selected to assess the role of TLR-2 and TLR-4 in inducing NO and ROS production. It was shown that both microorganisms affect ROS production in different ways. Macrophages from C3H/HeN and TLR-4-defective C3H/HeJ mice, when stimulated by *P. anaerobius*, did not induce ROS production. The low levels of ROS detected in the presence of this bacterium (Fig. 1A) are most likely related to its ability to evade phagocytosis through encapsulation or by superoxide dismutase production which consumes superoxide (13). On the other hand, *F. nucleatum* (both heat-killed and live cells) induced large amounts of ROS (Fig. 1A and C). Similar results for *F. nucleatum* have been demonstrated, in the presence or absence of plasma (13, 32, 33). Sheikhi et al. (33) suggested that opsonines present in the plasma are key participants in the phagocyte process and ROS production. However, *F. nucleatum* could be an exception because it may be directly bound to phagocytic cells (19). Another *F. nucleatum* morphological characteristic that might interfere in ROS production is its larger size (5–8 µm) compared to other bacteria (± 1 µm). Finally, the ROS production induced by *F. nucleatum* appears to be associated with a thermolabile component, because cells stimulated by heat-killed microorganisms produced lower levels of NO than those stimulated by live cells, as previously described by Kato et al. (11) (Fig. 1A and C).

TLR-4-defective macrophage cultures (C3H/HeJ) stimulated by *F. nucleatum* presented significantly lower ROS levels than C3H/HeN macrophages (Fig. 1A and C). This result is in agreement with the studies by Park et al. (24) and Ryan et al. (31); they demonstrated that ROS production, induced by LPS, is dependent on TLR-4. Conversely, Larouk et al. (16) noticed that macrophage ROS production, when activated by gram-negative bacteria, was MyD88 dependent but TLR-4 independent. Some researchers believe that these distinct results could be related to the methodology or to the different microorganisms used during cell stimulation (5, 26, 44).

Remer et al. (27) showed that murine neutrophils produced ROS in the presence of *Escherichia coli* LPS and serum, using TLR-4, while murine macrophages were not activated by LPS. TLR4-competent macrophage cultures stimulated by *F. nucleatum* produced ROS, while TLR-4-deficient macrophages produced no or

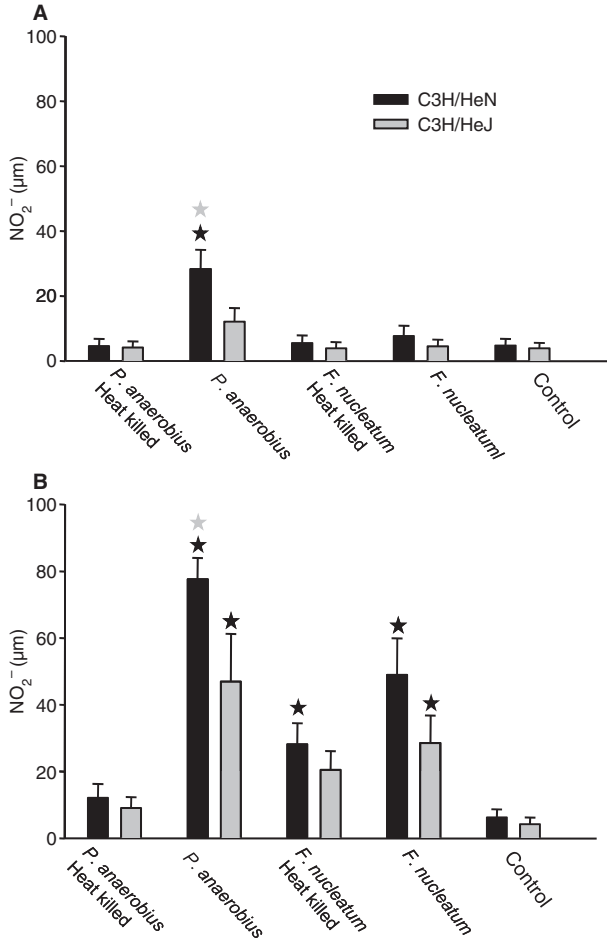

Fig. 3. Nitric oxide (measured as NO_2^-) production by macrophages from wild-type C3H/HeN and C3H/HeJ mice. Cells were stimulated by live or heat-killed *Peptostreptococcus anaerobius*; live or heat-killed *Fusobacterium nucleatum*, and RPMI-1640 (negative control) in the absence (A) or presence (B) of interferon-γ (IFN-γ) as described in the Material and methods. Bars represent the mean of three experiments performed in duplicate; lines stand for the standard error of the means. Black stars indicate statistical difference for NO_2^- production in response to presence or absence of stimuli, grey stars indicate statistical difference between microbial stimuli types (live or heat-killed), $P < 0.05$.

lower levels of ROS regardless of the presence of serum. As suggested by Flo et al. (5), the different results could be explained by the use of whole microorganism cells (either heat-killed or live cells) in our study as opposed to purified LPS.

In contrast to C3H/HeN macrophages, C57BL/6 cells produced ROS when stimulated by *P. anaerobius* (Fig. 1D). It is well known that macrophages from C57BL/6 mice present a proinflammatory profile, characterized by high NO and ROS production (21). We did not find, in the literature, a comparison between NO production by C56BL/6 and C3H macrophages; however, even between mouse strains that present a proinflammatory profile, small variations occur (21). In our study, *F. nucleatum* (heat-killed or live cells) greatly stimulated ROS production (Fig. 1B) by C57BL/6 mice, reinforcing its role in the induction of the tissue inflammatory response (13, 33). In addition, TLR-$2^{-/-}$ cells produced significantly lower levels of ROS than did wild-type macrophages when stimulated with either bacterium (Fig. 1D). Taken together, these results show that both microorganisms are recognized by TLR-2, which implies that this receptor plays a key role in the induction of the respiratory burst.

Several mechanisms may be involved in the inflammatory response to a microorganism. The peptidoglycan membrane component, present in *P. anaerobius* and recognized by TLR-2, might induce ROS production by macrophages (28, 41). In addition to LPS, *F. nucleatum* has other structures, such as lipoproteins, that might be recognized by TLR-2 and so play a role in macrophage activation (2). Yoshimura et al. (44) have demonstrated that both TLR-2 and TLR-4 may be involved in immune responses against *F. nucleatum*. In accordance with Hou et al. (10), TLRs may be promiscuous in terms of their ligand binding and activation properties, which could explain the use of different receptors by one microorganism.

Zymosan, used as a positive control, is thought to be recognized by TLR-2 (7); this was confirmed in this study (Fig. 1D). Somewhat surprisingly, TLR-4 was associated with Zymosan recognition (Fig. 1C), which could occur because mannose is present in the yeast cell wall (40). Incontestably, the results of this study demonstrated the role of TLRs in ROS production, suggesting their involvement in the ethiopathogeny of periapical diseases (6, 34).

NO synthesis, in several mammals, is the main cytostatic, cytotoxic, and proapoptotic mechanism of the innate immune response (14). In periapical lesions, NO production is associated not only with tissue breakdown (34, 38), but also with periapical lesion enlargement (17). Moreover, in biological assays performed *in vitro*, it is necessary to add IFN-γ to cell cultures when attempting to induce NO production (9, 41).

In this study, *P. anaerobius* and *F. nucleatum* significantly induced large amounts of NO in macrophage cultures from both wild-type and TLR-$2^{-/-}$ C57BL/6 mice, in the presence or absence of IFN-γ (Fig. 2). Similar results were reported by Kato et al. (12) who used *F. nucleatum in vivo*. Possibly the use of whole microbial cells (live or heat-killed) in this study could explain why IFN-γ did not increment the NO production in cells from this mouse strain, demonstrating, in turn, that both microorganisms are highly capable of inducing NO production.

NO production by macrophage cells taken from C3H/HeN and C3H/HeJ mice and stimulated by *P. anaerobius* and *F. nucleatum* was analyzed (Fig. 3). Live *P. anaerobius* cells were the only stimulus that induced NO production by C3H/HeN macrophages in the absence of IFN-γ (Fig. 3A). When this result is compared to that found in C57BL/6 macrophages (Fig. 2A and B), it becomes clear that heat treatment does indeed interfere with NO production, suggesting that cell wall heat-labile components may be associated with NO induction. In addition, NO production was observed in macrophages from C3H/HeJ mice, but only in those cultures treated with live microbial antigens in the presence of IFN-γ (Fig. 3B).

In summary, this study demonstrates that endodontic prevalent bacteria induce NO production through a redundant signaling pathway in the absence of a specific receptor. Similarly, some researchers have demonstrated that each microorganism may use different TLRs, such as immunological activation pathways (5, 37). Hence, the microorganisms used here may participate in the induction and progression of periapical lesions through NO and ROS production. However, the dissection of the precise roles of the TLRs, which directly impact periapical pathogenesis, requires further research to clarify the many gaps that remain in this area of study.

Acknowledgments

Thanks are due to the postgraduate program of the School of Dentistry, UFMG, as well as to the laboratories of Microbial Ecology and Oral Microbiology at UFMG for the use of equipment and useful discussions. We are indebted to Antonio Mesquita Vaz for his excellent animal care. This study has received financial support from CAPES, CNPq, and Fapemig.

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