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Mice Lacking Inducible Nitric Oxide Synthase Demonstrate Impaired Killing of *Porphyromonas gingivalis*

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Porphyromonas gingivalis **is a primary etiological agent of generalized severe periodontitis, and emerging data suggest the importance of reactive oxygen and nitrogen species in periodontal tissue damage, as well as in microbial killing. Since nitric oxide (NO) released from inducible NO synthase (iNOS) has been shown to possess immunomodulatory, cytotoxic, and antibacterial effects in experimental models, we challenged iNOS-deficient (iNOS$^{-/-}$) mice with *P. gingivalis* by using a subcutaneous chamber model to study the specific contribution of NO to host defense during *P. gingivalis* infection. iNOS$^{-/-}$ mice inoculated with *P. gingivalis* developed skin lesions and chamber rejection with higher frequency and to a greater degree than similarly challenged C57BL/6 wild-type (WT) mice. Chamber fluid from iNOS$^{-/-}$ mice possessed significantly more *P. gingivalis* than that of WT mice. The immunoglobulin G responses to *P. gingivalis* in serum was similar in WT and iNOS$^{-/-}$ mice, and the inductions of tumor necrosis factor alpha, interleukin-1β and interleukin-6, and prostaglandin E_2 were comparable between the two mouse strains. Although no differences in total leukocyte counts in chamber fluids were observed between iNOS$^{-/-}$ and WT mice, the percentage of dead polymorphonuclear leukocytes (PMNs) was significantly greater in iNOS$^{-/-}$ mouse chamber fluids than that of WT samples. Interestingly, casein-elicited PMNs from iNOS$^{-/-}$ mice released more superoxide than did WT PMNs when stimulated with *P. gingivalis*. These results indicate that modulation of superoxide levels is a mechanism by which NO influences PMN function and that NO is an important element of the host defense against *P. gingivalis*.**

Nitric oxide (NO) plays diverse roles in various physiological and pathological processes, including vasodilation in blood vessels, modulation of neurotransmission in the central and enteric nervous systems, and inhibition of microbial and tumor cell growth. Such widely different effects are possible in part because NO is released by three different NO synthase (NOS) isoforms, which are active in different cell types and release NO with different kinetics (41). One of these isoforms, the inducible NOS (iNOS), is activated by immunological and inflammatory stimuli, including cytokines such as gamma interferon (IFN-γ) and bacterial lipopolysaccharide (40). iNOS releases large amounts of NO in a prolonged fashion, and high local concentrations of NO and its oxidative derivatives such as peroxynitrite ($ONOO^-$) are shown to be toxic to eukaryotic cells, as well as to microbes (18, 39). In addition, NO also plays an immunomodulatory role by regulating leukocyte migration and inhibiting superoxide production (30, 33). Experiments with knockout mice lacking iNOS (iNOS$^{-/-}$ mice) indicated increased susceptibility to *Leishmania major*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, and *Salmonella* infections, suggesting a protective effect of iNOS-derived NO against these microorganisms (32, 35, 53). Similarly, pharmacological inhibition of NO synthesis in wild-type (WT) mice led to an increased bacterial burden during experimental tuberculosis and leishmaniasis, further supporting the idea that NO plays a role in a patent host response to infection (36, 50). On the other hand, in various sterile experimental inflammation models, including adjuvant arthritis (22), chronic experimental ileitis (37), and carrageenin-induced paw inflammation (21), NO appeared to be detrimental to host tissues. Moreover, NO is not always protective against infectious agents, since Karupiah et al. demonstrated that iNOS$^{-/-}$ mice were protected from influenza A virus-induced pneumonia at titers that were lethal in WT mice (26). NO is thus recognized as a molecule with dual effects in host defense, and it has been suggested that host tissue damage is the price to pay for the widespread cellular availability and broad spectrum of target organisms for NO (41).

Numerous microorganisms have been associated with periodontal disease, an infectious disease characterized by destruction of tooth-supporting tissues. Based on epidemiological and experimental data, *Porphyromonas gingivalis* has emerged as an important causative agent of advanced adult periodontal disease (1, 9, 15, 17). This organism possesses an array of virulence factors that allow for colonization and initiation of periodontal infection, including lipopolysaccharide, fimbriae, hemagglutinins, hemolysins, and proteolytic enzymes such as gingipains (14, 20, 38). *P. gingivalis* is sufficient to initiate alveolar bone loss in rodents and nonhuman primates (14, 19). *P.*

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gingivalis has been shown to induce iNOS expression in gingival fibroblasts, inflammatory cells, and basal keratinocytes (28); to stimulate NO release from macrophages (46); and to induce expression of IFN-γ, an important stimulant of NO release (42).

Although periodontal disease is initiated and maintained by a pathogenic oral flora, an overzealous host response is thought to contribute significantly to periodontal tissue destruction. For example, in localized aggressive periodontitis, polymorphonuclear leukocyte (PMN)-mediated tissue injury contributes significantly to periodontal destruction due to excessive superoxide (O_2^-) generation by these cells (25). Interestingly, patients with localized aggressive periodontitis also display elevated NOS activity, and inhibition of NO synthesis resulted in increased chemotaxis (48). NO has been demonstrated to mediate inflammation and bone loss in a ligature model of periodontal disease, where pharmacological inhibition of iNOS diminished plasma extravasation and bone destruction (34). However, no experimental data are available on NO's contribution to the host defense against any specific periodontal pathogen.

In the present study we investigated the role of NO in the defense against *P. gingivalis* by using a subcutaneous chamber model of local infection in WT and iNOS$^{-/-}$ mice (12). We report that NO generated by iNOS is required for control of *P. gingivalis* infection and that NO may be an important molecule in regulating oxidative killing and PMN survival.

MATERIALS AND METHODS

Mouse strains. All animal experiments were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of Boston University and in conformance to the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Five- to-six-week-old female iNOS$^{-/-}$ and WT (C57BL/6) mice were purchased from Taconic (Germantown, N.Y.). All animals received standard laboratory chow diet and water ad libitum.

***P. gingivalis* culture.** *P. gingivalis* strain A7436 was cultivated as described previously (12, 14). After 24 h of anaerobic growth in Schaedler broth (Difco, Detroit, Mich.), the bacteria were harvested by centrifugation, washed with sterile pyrogen-free saline (PFS) and adjusted to an optical density at 660 nm of 1.0 (ca. 10^9 CFU/ml). Bacterial cell counts were determined on all bacterial cultures to confirm *P. gingivalis* viability prior to mouse experiments.

Subcutaneous chamber inoculation with *P. gingivalis*. Sterile coil-shaped chambers prepared from stainless steel wire were inserted subcutaneously to the subscapular region of WT and iNOS$^{-/-}$ mice under isoflurane anesthesia as described previously (12). Both WT and iNOS$^{-/-}$ mice were divided into two groups each (*P. gingivalis*, or mock treated), at 10 animals/group (for a total of 40 mice), and baseline serum samples were collected from each mouse and stored at −80°C for enzyme-linked immunosorbent assays (ELISAs). After 10 days of rest, subcutaneous chambers from one group of WT mice and one group of iNOS$^{-/-}$ mice were injected with 0.1 ml of *P. gingivalis* suspended in PFS (10^9 CFU/ml), whereas the second groups of WT and iNOS$^{-/-}$ mice were injected with vehicle only.

For cachexia, skin abscess formation, and chamber rejection a score was given on a scale from 0 to 4, where 0 represents normal appearance, and 4 indicates the most severe lesion (e.g., complete chamber rejection). Scoring was performed by an examiner who was blinded to the identity of treatment groups. A minimum of 55 μl of chamber fluid was collected from each mouse at 1, 3, 7, and 11 days postchallenge. These fluids were separated as follows: 10 μl for determination of *P. gingivalis* viability (CFU/ml), 10 μl for total inflammatory cell counts, 5 μl for differential inflammatory cell counts, 10 μl for fluorescent microscopic analysis of chamber fluids, and 20 μl for determination of cytokines (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β] and IL-6) and prostaglandin E_2 [PGE_2] by ELISA. After 11 days, a final serum sample was obtained from all mice; each sample was stored frozen at −80°C, and then all mice were humanely sacrificed.

Determination of viable *P. gingivalis* from murine chamber fluids. Titers of viable *P. gingivalis* in chamber fluids were determined as described previously (12). Briefly, the 10-μl aliquot of chamber fluid from each mouse was serially 10-fold diluted with 1% peptone, the dilutions were plated onto anaerobic blood agar plates in duplicate and incubated anaerobically for 5 to 7 days, and the CFU counts of *P. gingivalis*/milliliter present in each chamber fluid were determined.

Determination of *P. gingivalis*-specific IgG by ELISA. *P. gingivalis*-specific immunoglobulin G (IgG) levels were determined by ELISA as follows. Broth-grown *P. gingivalis* was fixed overnight with 3% formaldehyde, washed, and adjusted to an optical density at 660 nm of 0.3 in carbonate-bicarbonate buffer (pH 9.6). A 50-μl aliquot of this suspension was added to each well of 96-well Immulon 4HBx ELISA plates (Dynatec, Chantilly, Va.). After overnight incubation, the plates were dried and blocked with bovine serum albumin (2%), and serial twofold dilutions of each chamber fluid sample in phosphate-buffered saline plus 0.05% Tween 20 were added to the wells, followed by incubation overnight. The plates were washed, incubated with a goat anti-mouse IgG-alkaline phosphatase conjugate (1:7,500 dilution; Sigma, St. Louis, Mo.), substrate was added, and after 1 h of incubation the absorbance was read at 405 nm. We also included one additional ELISA plate to perform quantitative assessments of murine IgG levels in serum. This plate was sensitized with 200 ng of rabbit anti-mouse IgG Fab fragment and blocked with bovine serum albumin, and quintuplicate wells were incubated with a serial twofold dilution of a mouse IgG standard (Sigma) ranging from 200 ng to 0.1 pg/ml. This plate was processed with the mouse chamber fluid samples, and the standard curve generated from this plate was used to calculate the concentration of *P. gingivalis*-specific IgG present in all chamber fluids.

Analysis of chamber fluid for *P. gingivalis*-host cell interactions. A fluorescence phagocytosis and killing assay, as modified by Cutler et al. (8), was used to assess *P. gingivalis* and host cell viability and interactions. In brief, 10-μl aliquots of chamber fluids from each mouse were diluted 1:10 in PFS, centrifuged to collect leukocytes and bacteria, and stained with propidium iodide (final concentration, 5 μg/ml), DAPI (4′,6′-diamidino-2-phenylindole; 15 μg/ml), and acridine orange (20 ng/ml). After being stained, the cells were attached to microscope slides by using a cytospin apparatus, a drop of cyanoacrylate was added to each slide, and a coverslip was attached. All samples were analyzed by epi-illumination UV microscopy. PMNs were differentiated by lobed nuclei and by a granular cytoplasm when stained with acridine orange. Dead PMNs were differentiated from live PMNs by their red staining with propidium iodide. A total of 100 cells were counted from each slide and the percentage of live and dead PMNs present in the chamber fluids were calculated for each mouse.

TNF-α, IL-1β, IL-6, and PGE_2 determinations. The 20-μl aliquots of chamber fluid samples obtained from experimental mice were diluted 1:50 with PFS, and the levels of TNF-α, IL-1β, IL-6, and PGE_2 were determined by using commercially available ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn.). Intra-assay standards were used to calculate the concentration of each molecule present in the undiluted chamber fluids.

Superoxide release by isolated neutrophils. Leukocytes were obtained by abdominal lavage from WT and iNOS$^{-/-}$ mice after casein stimulation (7), and PMNs were isolated by discontinuous density gradient centrifugation with Histopaque 1119 and 1077 (Sigma). Superoxide production was determined from purified mouse PMNs after *P. gingivalis* challenge by using the cytochrome *c* reduction assay (47). Briefly, WT and iNOS$^{-/-}$ PMNs were placed into microtiter plates and were either unstimulated or were stimulated with *P. gingivalis* (multiplicity of infection = 100) or fMLP (*N*-formyl-methionyl-leucyl-phenylalanine; Sigma) at 1 μM as a positive control stimulus in the presence or absence of superoxide dismutase (SOD). Superoxide production was monitored over a 10-min period at an absorbance of 550 nm with a microplate reader. The amount of SOD-inhibitable superoxide generated was calculated from raw optical density units and converted into nanomole production per minute.

Statistical analysis. Viable *P. gingivalis* cell counts, cytokine levels, and superoxide generation were compared by using analysis of variance (ANOVA) with the Fisher protected-least-significant-difference test for individual comparisons. Comparison of live PMN counts between mouse strains was performed by using a Student *t* test. For all statistical calculations, StatView 5.0.1 software was used (SAS, Inc., Cary, N.C.), and a P value of <0.05 was considered significant.

RESULTS

Detection of *P. gingivalis* in WT and iNOS$^{-/-}$ mouse chamber fluid samples. To determine the effect of iNOS-generated NO on the replication of *P. gingivalis*, 10 μl of fluid was drawn from the subcutaneous chambers of WT and iNOS$^{-/-}$ mice at

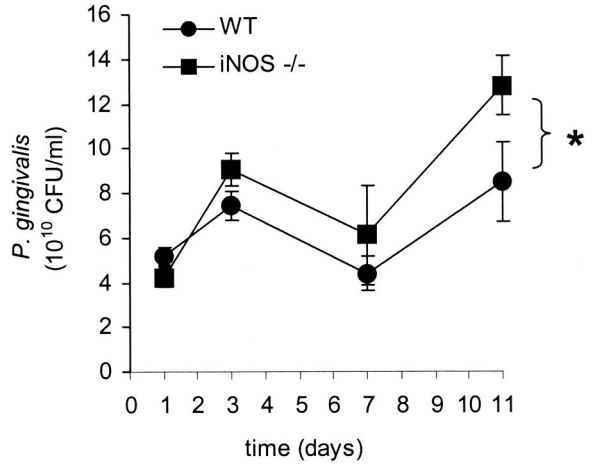

FIG. 1. Increased numbers of *P. gingivalis* were detected in chamber fluids of iNOS$^{-/-}$ mice compared to those of WT mice. Subcutaneous chambers were inoculated on day 0 with 10^8 CFU of *P. gingivalis* strain A7436, and aliquots of chamber fluid were collected on days 1, 3, 7, and 11. CFU/milliliter values were determined by bacterial growth on anaerobic blood agar plates ($n = 10$ mice for each group; ✻, $P < 0.01$ [ANOVA]).

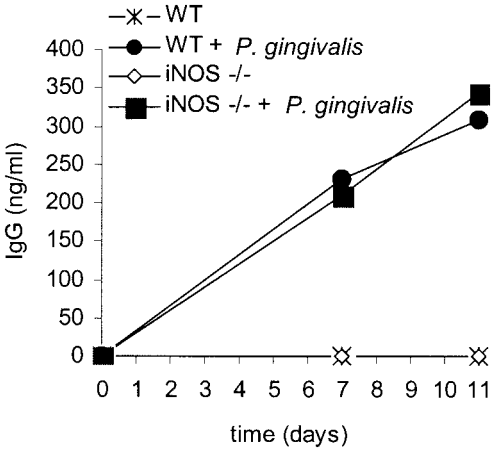

FIG. 2. IgG titers in serum against *P. gingivalis*. There was no detectable anti-*P. gingivalis* IgG before inoculation (day 0) or in the saline-injected mice of either genotype. After *P. gingivalis* inoculation, WT and iNOS$^{-/-}$ mice showed increased levels of anti *P. gingivalis* IgG in serum, as determined by quantitative ELISA on days 7 and 11 after inoculation. No statistical differences were observed in the levels of *P. gingivalis*-specific IgG in serum between WT and iNOS$^{-/-}$ mice.

days 1, 3, 7, and 11 postinoculation. At 3 days postinoculation, we observed a moderate elevation in the numbers of *P. gingivalis* organisms present in the chambers of iNOS$^{-/-}$ mice compared to those of WT mice, and this difference steadily increased by days 7 and 11 (Fig. 1). Bacteria were not recovered from chamber fluids of mice injected with saline only. These data demonstrate that mice deficient in the iNOS gene have impaired ability to clear *P. gingivalis* from the chambers compared to WT animals.

Host response after subcutaneous chamber inoculation with *P. gingivalis*. WT and iNOS$^{-/-}$ mice were monitored daily from days 1 to 11 for body weight changes and general health. No animals died during the experimental period, and there was no difference in body weight gain between the WT and iNOS$^{-/-}$ mice (Table 1). All mice were monitored for cachexia, the development of cutaneous abscesses, and chamber rejection. Signs of cachexia, such as ruffled fur, body hunching, and lethargy, were similar for both WT and iNOS$^{-/-}$ mice challenged with *P. gingivalis* during the course of these studies (data not shown). On the other hand, we found spreading skin lesions with greater frequency in iNOS$^{-/-}$ mice, and the sizes of the lesions were greater in iNOS$^{-/-}$ mice compared to those of WT mice, as determined by an unbiased examiner (Table 1). These skin lesions occurred predominantly on the ventral surface of mice. Moreover, chamber rejection was found more frequently in iNOS$^{-/-}$ mice than in WT mice, and the degree of rejection was more severe in iNOS$^{-/-}$ mice as well (Table 1). These data demonstrate an altered host response of the iNOS$^{-/-}$ mutant to *P. gingivalis* challenge compared to that of the WT mice.

TABLE 1. Host response in WT and iNOS$^{-/-}$ mice at 11 days postinjection of *P. gingivalis*

Mouse type[a]	Mean ± SD[b]		
	Wt gain (g)	Skin abscess (frequency)	Chamber rejection (frequency)
WT	1.96 ± 1.51	0.3 ± 0.48 (3/10)	0.90 ± 0.74 (7/10)
iNOS$^{-/-}$	2.48 ± 2.40	1.50 ± 1.08** (10/10)	1.70 ± 0.95* (10/10)

[a] There were 10 aminals per group.
[b] *, $P < 0.05$; **, $P < 0.005$ (as determined by the Student *t* test). Values for skin abscesses are on a scale of 0 to 4, where 0 represents no lesion, and 4 represents the most severe lesion. A similar scale was used to score chamber rejection, where 0 indicates that the chamber is in its original subcutaneous position, and 4 indicates that the chamber is completely exposed through the skin. The frequency of occurrence of any score greater than 0 is shown in parentheses.

Humoral immune response to *P. gingivalis*. The serum antibody response of WT and iNOS$^{-/-}$ mice after *P. gingivalis* challenge was monitored by quantitative ELISA. We did not detect *P. gingivalis*-specific IgG prior to inoculation or in mice injected with sterile saline solution in either WT or iNOS$^{-/-}$ mice. After *P. gingivalis* inoculation, the levels of *P. gingivalis*-specific IgG increased in the sera of both WT and iNOS$^{-/-}$ mice, and these increases were similar in the two genotypes (Fig. 2). These data show that there were no significant differences in the humoral host response between the WT and iNOS$^{-/-}$ mice to *P. gingivalis* infection.

Chamber fluid levels of proinflammatory cytokines and PGE_2. To assess the host inflammatory response to *P. gingivalis* challenge, we measured the levels of TNF-α, IL-1β, and IL-6 present in chamber fluid samples of WT and iNOS$^{-/-}$ mice by ELISA during the initial 3 days after challenge. TNF-α was detected in the chamber fluids of both WT and iNOS$^{-/-}$ mice 1 day after *P. gingivalis* challenge, and the level of TNF-α increased by day 3. However, there were no significant differences in the levels of TNF-α present in chamber fluid levels from WT or iNOS$^{-/-}$ mice at either day 1 or day 3 postinfection (Fig. 3A). IL-1β was also detected in chamber fluid samples of WT and iNOS$^{-/-}$ mice 1 day after *P. gingivalis* infection (Fig. 3B). By day 3, IL-1β levels markedly decreased in both mouse strains. No significant differences were observed in the levels of IL-1β between WT and iNOS$^{-/-}$ mice. The levels of

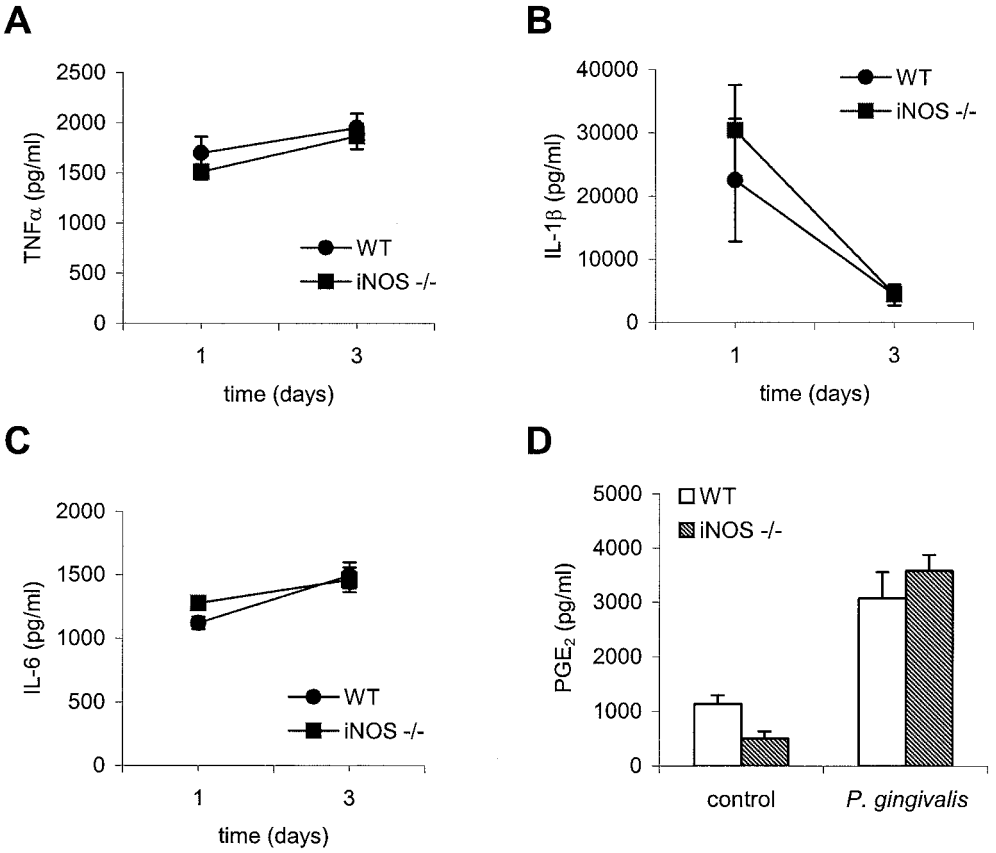

FIG. 3. Cytokine and PGE_2 levels in chamber fluids of WT and $iNOS^{-/-}$ mice after *P. gingivalis* challenge as measured by ELISA. Chambers were inoculated with 10^8 CFU of *P. gingivalis* or vehicle on day 0. (A) TNF-α levels measured 1 and 3 days postinoculation; (B) IL-1β levels measured 1 and 3 days postinoculation; (C) IL-6 expression measured 1 and 3 days postinoculation; (D) PGE_2 expression measured in vehicle or in *P. gingivalis*-inoculated chambers 3 days after inoculation. No statistically significant differences in cytokine levels were found between WT and $iNOS^{-/-}$ mice after *P. gingivalis* challenge.

IL-6 in chamber fluids were also found to be similar for both WT and $iNOS^{-/-}$ mice at 1 and 3 days after *P. gingivalis* challenge (Fig. 3C). Chamber fluids from mice not infected with *P. gingivalis* did not produce detectable amounts of TNF-α, IL-1β, or IL-6 (data not shown). We also assessed the level of the proinflammatory arachidonic acid metabolite PGE_2 by ELISA. We observed that the chamber fluid levels of PGE_2 were elevated in both WT and $iNOS^{-/-}$ mice challenged with *P. gingivalis*. However, the levels of PGE_2 detected in chamber fluids from WT and $iNOS^{-/-}$ mice were not significantly different at any time point after *P. gingivalis* stimulation (Fig. 3D).

Cellular inflammatory response to *P. gingivalis*. It is well established that subcutaneous challenge of mice with *P. gingivalis* elicits a host cellular inflammatory response (12). We observed that the total leukocyte counts recovered in chamber fluid samples from WT and $iNOS^{-/-}$ mice were similar throughout the course of the present study (Fig. 4A). Differential cell counts showed that the predominant inflammatory cell was the PMN (Fig. 4B). We used a fluorescence phagocytosis and killing assay to investigate the impact of iNOS mutation on the interaction between *P. gingivalis* and host cells (13). Aliquots of both WT and $iNOS^{-/-}$ chamber fluid samples were stained with DAPI to label bacteria, acridine orange to label PMNs, and propidium iodide to differentiate dead and live PMNs and bacteria. A total of 100 PMNs were examined for each sample. We found that the percentage of live PMNs was significantly lower in the chamber fluid of $iNOS^{-/-}$ mice (Fig. 4C). The average number of *P. gingivalis* found within a PMN was not different between the two mouse strains (WT mice, 3.13 ± 0.61; $iNOS^{-/-}$ mice, 3.30 ± 0.56), and the extent of *P. gingivalis* killing, expressed as the proportion of dead *P. gingivalis* found in PMNs, was also similar (WT mice, 45.3 ± 12.3%; $iNOS^{-/-}$ mice, 46.6 ± 4.4%).

Superoxide release in the absence of iNOS. To address the importance of NO in the oxidative response of PMNs to *P. gingivalis*, we harvested PMNs from peritoneal lavage fluids of casein-injected WT and $iNOS^{-/-}$ mice and stimulated these cells with either saline, fMLP (1 μM) or *P. gingivalis* at a multiplicity of infection of 100. We found no difference in the resting levels of O_2^- release between WT and $iNOS^{-/-}$ mice. PMNs from both mouse strains responded similarly to fMLP stimulation, indicating an intact G-protein-mediated activation pathway of O_2^- generation in the absence of NO. The peak response of WT and $iNOS^{-/-}$ PMNs to *P. gingivalis* occurred at 30 s poststimulation. Interestingly, stimulation of $iNOS^{-/-}$ PMNs with live *P. gingivalis* resulted in a higher peak O_2^- response compared to that of the WT PMNs (Fig. 5), indicat-

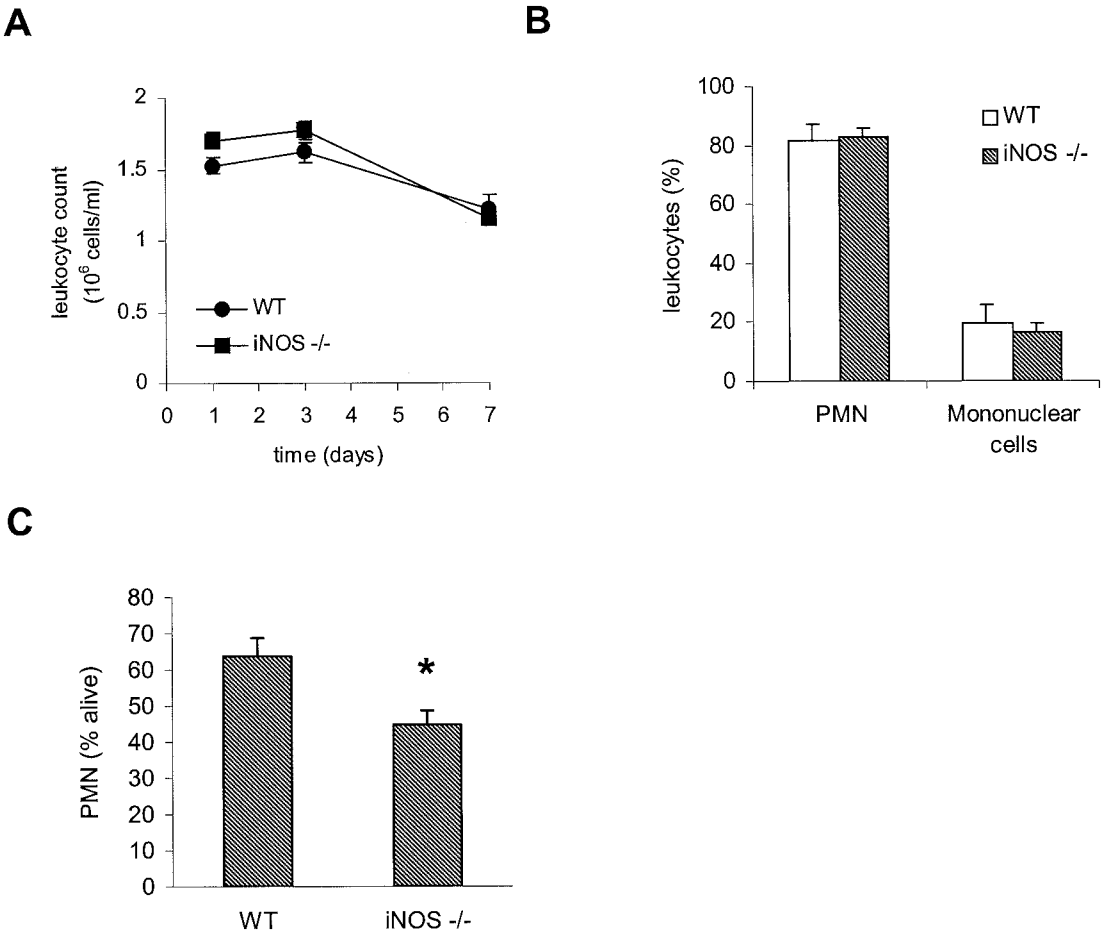

FIG. 4. Inflammatory cell infiltrate in the chambers of mice challenged with *P. gingivalis*. (A) Total white blood cell counts were similar between the two genotypes as determined by Trypan blue staining and cell counting. (B) Differential cell counts demonstrated that the dominant leukocyte type in the infected chambers is the PMN. There were no differences in the proportions of PMNs and mononuclear cells in the chamber fluids of WT and iNOS$^{-/-}$ mice. (C) The ratio of live and dead PMNs in the chamber fluid samples was decreased in iNOS$^{-/-}$ samples at day 3, as determined by counting the number of dead PMNs out of 100 PMNs after propidium iodine staining (n = nine mice for each group; ✻, $P < 0.05$ [Student t test]).

ing that either O_2^- release is increased or O_2^- scavenging is diminished in iNOS$^{-/-}$ PMNs after *P. gingivalis* challenge.

DISCUSSION

NO has been shown to be an effective agent against numerous pathogens, but its effect on specific periodontal bacteria has not been previously characterized. Our results indicate that NO is an important component of the host defense against *P. gingivalis*. In a subcutaneous chamber model of infection, the lack of NO leads to the development of a more severe form of infection characterized by impaired clearance of *P. gingivalis* and increased occurrence and severity of skin abscesses and chamber rejection. On the cellular and molecular level, we found that PMN survival is impaired in the absence of iNOS, whereas the amount of superoxide released by PMNs from iNOS-deficient mice is higher.

The humoral immune response to *P. gingivalis* appears to be intact in iNOS$^{-/-}$ mice, since anti-*P. gingivalis* IgG levels in serum were similar in WT and iNOS$^{-/-}$ mice. Cytokine activation also appears to be unchanged in the absence of iNOS,

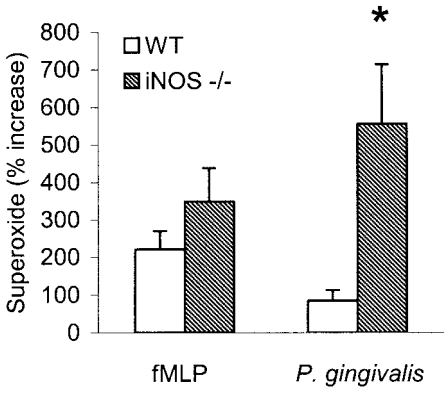

FIG. 5. Peak superoxide release by PMNs in response to fMLP (1 μM) or *P. gingivalis* (multiplicity of infection = 100). O_2^- was measured by using a cytochrome *c* reduction assay. Data are expressed as the percent increase over baseline O_2^- release. *P. gingivalis* induced significantly greater peak O_2^- responses in iNOS$^{-/-}$ PMNs compared to WT PMNs (n = five mice for each group; ✻, $P < 0.05$ [iNOS$^{-/-}$ mice versus WT mice, ANOVA]).

since TNF-α, IL-1β, IL-6, and PGE_2 were induced similarly in WT and $iNOS^{-/-}$ chamber fluids in response to *P. gingivalis*. NO of endothelial origin has been shown to modulate leukocyte attachment to vascular endothelium by downregulating E-selectin and ICAM-1 (30, 33). However, we observed no difference in leukocyte counts in the chambers of WT and $iNOS^{-/-}$ mice, which confirms earlier observations that iNOS-derived NO has no impact on leukocyte migration (35). On the other hand, our data indicate that once leukocytes migrate to the site of infection, iNOS becomes an indispensable part of the host defense, since PMN survival was impaired and O_2^- release was increased in the absence of iNOS after *P. gingivalis* challenge.

PMNs are among the first inflammatory cells to be mobilized against bacterial invasion, and phagocytosis coupled by oxidative killing by PMNs is a critical mechanism for the elimination of microorganisms. The role of NO in PMN survival is unclear. Although pharmacological NO donors can induce cell death and apoptosis in isolated PMNs (10, 52), and NO donors can also attenuate the antiapoptotic effect of lipopolysaccharide on PMNs (4), there is evidence that NO's effect on cell death is dose dependent, and sustained release of physiological doses of NO can be cytoprotecive (29, 51). Our finding that PMN cell death is increased in *P. gingivalis*-challenged $iNOS^{-/-}$ mice lends support to the latter theory by demonstrating that endogenously released NO can be protective to PMNs during bacterial challenge. An alternative explanation is that the increased loss of PMN viability in $iNOS^{-/-}$ mice is a direct consequence of increased bacterial burden and the subsequent increase in the levels of gingipains expressed by *P. gingivalis* in $iNOS^{-/-}$ chambers. However, the average numbers of bacteria found within a PMN were similar for WT and $iNOS^{-/-}$ samples, suggesting that an inappropriate host response rather than the increased bacterial numbers is responsible for the observed increase in $iNOS^{-/-}$ PMN cell death.

The macroscopic findings of more severe skin abscess and more advanced chamber rejection in $iNOS^{-/-}$ mice coincide with the microscopic observations of increased PMN death. Whether an overactive host response or the increased bacterial burden is the main cause for the skin tissue damage is not known. However, the increase in *P. gingivalis* titers in $iNOS^{-/-}$ chambers is relatively modest, which suggests that other factors, such as a self-damaging host response, might play a role.

The observed increase in O_2^- activity in isolated $iNOS^{-/-}$ PMNs might be an important component of the altered host response in the absence of NO. O_2^- is released by NADPH oxidase, an enzyme complex assembled in the plasma membrane of activated PMNs during respiratory burst. NO interacts with O_2^- metabolism on at least two levels: direct inhibition of the NADPH oxidase and scavenging free O_2^- (16, 44). Studies on the direct inhibition of O_2^- production by NO demonstrate that NO inhibits components of NADPH oxidase before or during their assembly (6, 11, 43). Our observation that there is increased peak release of O_2^- in $iNOS^{-/-}$ PMNs upon *P. gingivalis* stimulation thus might be due to the absence of NO-mediated inhibition of NADPH oxidase assembly. In addition, the lack of NO can further enhance O_2^- effects because NO is an important O_2^- scavenger (16, 23). It is likely that the available O_2^- is increased in $iNOS^{-/-}$ chambers, since the differential cell counts show that the dominant leukocyte in the chambers is the PMN, and PMNs isolated from $iNOS^{-/-}$ mice were found to release more O_2^- than those from WT mice. The increased O_2^- levels augment oxidative stress for host tissues and might be a key factor in the increases observed in skin lesions and chamber rejection in $iNOS^{-/-}$ mice. PMN-mediated host tissue damage has been demonstrated in numerous diseases, including glomerulonephritis, rheumatoid arthritis, and cystic fibrosis (54). A similar case has been made for localized aggressive periodontitis, where enhanced superoxide release from both stimulated and nonstimulated PMNs has been demonstrated (47). It is interesting that the product of the reaction by NO and O_2^-, $ONOO^-$, is a strong oxidant itself, capable of causing DNA strand breaks, oxidizing proteins, and nitrating tyrosine residues, resulting in altered protein function (3, 24). Moreover, $ONOO^-$ also has antimicrobial activity, e.g., against *Helicobacter pylori* (31), raising the possibility that diminished levels of $ONOO^-$ are contributing to the increase in *P. gingivalis* organisms in the subcutaneous chambers of $iNOS^{-/-}$ mice.

The primary source of NO in mice is the macrophage, the cell type for which iNOS gene expression was first described (55). In addition, mouse (2), rat (45), and human (5) PMNs have all been demonstrated to release NO upon stimulation. Although NO output from PMNs is generally lower (2 to 20 nmol/min/10^6 cells) (45), we found that PMNs represent the majority leukocyte cell type in *P. gingivalis*-inoculated chambers; thus, they are likely to contribute to the overall NO output. Regardless of the source, NO diffuses freely through cell membranes and can act as a paracrine molecule on neighboring cells (27). $iNOS^{-/-}$ cells, on the other hand, express no iNOS mRNA and fail to produce detectable NO upon stimulation with lipopolysaccharide and IFN-γ (35).

Our results demonstrate a functional interplay between the NO and O_2^- systems and indicate that upsetting the balance of these two effector molecules can result in cell and tissue damage. Complementary action between the NO and O_2^- systems has been elegantly demonstrated by Shiloh et al., who showed that whereas single knockouts deficient in either NO or O_2^- production are resistant to infections by commensal microorganisms such as *Escherichia coli*, iNOS/$gp91^{phox}$ double knockouts deficient in both NO and O_2^- production quickly succumb to infections by endogenous bacteria (49). Future studies with iNOS/$gp91^{phox}$ double-knockout mice will determine whether there is a causal relationship between the excessive O_2^- production and the increased host tissue damage in $iNOS^{-/-}$ mice.

In conclusion, our results indicate that iNOS-generated NO is an important element of the host defense against the periodontal pathogen *P. gingivalis*. Neutrophil PMNs require an intact iNOS enzyme for optimal survival, and modulation of O_2^- effects is likely to be a mechanism by which NO influences neutrophil function.

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