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Lethal outcome caused by *Porphyromonas gingivalis* A7436 in a mouse chamber model is associated with elevated titers of host serum interferon-γ

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Background/aims: Septic shock caused by gram-negative bacteria has been associated with cytokines produced by hosts. *Porphyromonas gingivalis* A7436, a disseminating strain, caused septic shock-like symptoms and even animal death in a mouse chamber model. However, *P. gingivalis* exhibits lower endotoxin activities in its lipopolysaccharide than other typical gram-negative bacteria. In this study, we examined the effects of *P. gingivalis* lethal infection on host pro-inflammatory cytokines production.
Methods: Nude and normal BALB/c mice were infected with a lethal dose of *P. gingivalis* A7436 using a mouse chamber model. Serum levels of tumor necrosis factor, interleukin (IL)-1β, IL-12 and interferon-γ were evaluated. The effects of tumor necrosis factor inhibitor (thalidomide) and anti-interferon-γ antibody on infection outcomes were examined.
Results: All nude mice survived infectious challenge, whereas 100% of normal mice died with abdominal lesions. Bacterial cultures indicated *P. gingivalis* dissemination to the circulation. Serum levels of tumor necrosis factor, IL-1β and IL-12 showed no significant differences between nude and normal mice. Thalidomide treatment did not protect normal mice from death but decreased remote lesion occurrence, with concurrent reduced bacterial counts recoverable from blood. There was a 3.5-fold elevation in normal mice serum interferon-γ titers compared to those of nude mice and anti-interferon-γ antibody treatment resulted in 100% protection from lethal outcome.
Conclusion: Lethal outcome following *P. gingivalis* A7436 infection is T-lymphocyte dependent and involves an increase in systemic interferon-γ levels. The data further indicate that *P. gingivalis* transvascular dissemination (bacteremia) alone is not sufficient for lethal outcome.

Key words: interferon-γ; mice; periodontitis/complications; *Porphyromonas gingivalis*; tumor necrosis factor

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Bacterial infection of the mammalian bloodstream can lead to overwhelming sepsis (2). The sequential release of pro-inflammatory mediators in response to bacterial infections is essential in the fight against the invading microorganisms (24). However, the overproduction of these mediators may be detrimental for the host leading to multi-organ failure, shock, and finally death (24, 35). Tumor necrosis factor is a monocyte-derived peptide released in response to lipopolysaccharide of gram-negative bacteria, and it has been implicated as a principal mediator of endotoxic shock (4, 5). In studies of mice (19) and primates (16) antibodies against tumor necrosis factor protected animals from shock, vital organ dysfunction, and

death. Anesthetized baboons developed hypotension followed by lethal renal and pulmonary failure when infused with *Escherichia coli*, whereas baboons passively immunized against endogenous tumor necrosis factor were protected against bacterial challenge. These results indicate that tumor necrosis factor is a major mediator of fatal bacteremic shock.

Other than tumor necrosis factor, the monocyte-derived proinflammatory cytokines interleukin (IL)-1β and IL-12 appear early in the course of endotoxic septic cascade, which also play key roles in the pathogenesis of endotoxic shock (1, 3, 34). These cytokines are central to the induction of interferon-γ production by T lymphocytes and natural killer cells (16, 36), which is another important intermediate in the development of endotoxin shock (9, 29).

Porphyromonas gingivalis, a gram-negative black-pigmented anaerobic rod, has been suggested to be strongly associated with the development and progression of periodontal diseases in humans (17). It has been reported that *P. gingivalis* lipopolysaccharide exhibited very low endotoxic activities *in vivo*, such as pyrogenicity, local Shwartzman reaction and activity in the Limulus test, compared with enterobacterial lipopolysaccharide (32). *P. gingivalis* lipid A induced very weak production of tumor necrosis factor and IL-1β in human peripheral blood monocyte culture as compared with lipid A from *E. coli* or *Salmonella* (25). *P. gingivalis* lipopolysaccharide appears less dependent on Toll-like receptor 4 signaling than *E. coli* lipopolysaccharide (26). This property is attributed mainly to the unique lipid A motif of *P. gingivalis* lipopolysaccharide, which contains unusually branched and relatively long fatty acids. Unlike enteric lipopolysaccharide, *P. gingivalis* lipopolysaccharide has been reported to induce the symptoms of endotoxic shock in C3H/HeJ mice, which have a point mutation in the gene that encodes Toll-like receptor 4 and are thus hyporesponsive to *E. coli* lipopolysaccharide (31).

To understand the alterations of these sepsis-associated cytokines during *P. gingivalis* infection, we investigated the endogenous productions of tumor necrosis factor, IL-1β, IL-12, and interferon-γ induced by lethal infections of *P. gingivalis.* The effects of *in vivo* administration of blockers against tumor necrosis factor and interferon-γ on the course of pathogenesis in mice were also examined. In the present study, we demonstrate the role of tumor necrosis factor in remote lesion formation and the role of interferon-γ in lethal outcome in host responses to *P. gingivalis* infection.

Material and methods

Bacterial strain and growth conditions

P. gingivalis A7436 was originally isolated from a refractory periodontitis patient and was characterized by Dr. V. R. Dowell (Anaerobic Microbiology Laboratory, Centers for Disease Control and Prevention, Atlanta, GA, USA). *P. gingivalis* strain A7436 was cultivated on Brucella blood agar plates with the addition of hemin and menadione in an atmosphere of 5% CO_2−10% H_2−85% N_2 at 37°C. Bacteria were passaged by picking colonies from plate surfaces at least five times on blood agar plates before challenging into mice. The bacterial cells were harvested from 24–30 h cultures and concentrated by centrifugation at $1000 \times g$ and resuspended in Brain-Heart Infusion broth.

Experimental animals and infection protocol

Normal female BALB/c mice and nude mice approximately 8 weeks of age with BALB/c background were purchased from the Experimental Animal Center, National Council of Science, Taiwan. The mice were surgically implanted with coils in the dorsolumbar region to create subcutaneous chambers (12). After allowing healing for at least 14 days, the animals were infection-challenged at day 0 by intra-chamber injection of *P. gingivalis* A7436 (1×10^9 colony-forming units (CFU) in 100 μl Brain-Heart Infusion).

Thalidomide purchased from Celgene (Summit, NJ) was injected intraperitoneally (100 μg per mouse) in 100 μl phosphate-buffered normal saline just before *P. gingivalis* infectious challenge and the injections repeated daily. Anti-interferon-γ XMG 1.2 purchased from Pierce (Rockford, IL) was injected mice at the dose of 50 μg in 100 μl phosphate-buffered saline using the same protocol. Blank phosphate-buffered saline (100 μl) was used for control of thalidomide and anti-interferon-γ treatments. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Chung Shan Medical University, Taiwan.

Sample fluids collection

Subcutaneous fluids of mouse abdomen were aspirated with 26 G Hamilton microliter syringes (Hamilton, NV) and diluted 1/50 in phosphate-buffered saline. Except for the experiments of bacterial recovery, which were immediately performed after collection, fluids were centrifuged at $300 \times g$ for 10 min, the pellets were removed, and the supernatants stored at − 80°C until further analysis. Blood was taken from the retro-orbital venous plexus with glass capillary tubes without heparin coating and placed in 4°C for 1 h before centrifugation to harvest serum.

Quantification of bacteria in fluid samples

Serial dilutions of serum and abdominal fluid samples were streaked on anaerobic blood agar plates and cultivated in an atmosphere of 5% CO_2−10% H_2−85% N_2 at 37°C for at least 7 days. This allowed the detection and quantification of *P. gingivalis* colonies. The presence of *P. gingivalis* was confirmed by their characteristic black colonies and foul odor on the blood agar plates.

Cytokine analysis

For cytokine analysis, aliquots of serum samples were diluted in phosphate-buffered saline. Commercial kits of enzyme-linked immunosorbent assay (ELISA) purchased from Endogen (Rockford, IL) were used for the detection of fluid tumor necrosis factor, IL-1β, interferon-γ, and IL-12, respectively. All determinations were carried out in duplicate. The average of both determinations was used for the calculation of the amount of each cytokine.

Data analysis

The results were expressed as the mean ± standard error. A statistical analysis software JMP (SAS Institute Inc., Cary, NC) was used in this study and a *P*-value of less than 0.05 was considered significant. One way analysis of variance (ANOVA) and Bonferroni t-tests were used to examine the differences between experimental groups and controls. Kaplan–Meier survival analyses with log-rank test were performed to examine animal survival rates and the presence of remote lesions between tested groups.

Results

Survival rates of *P. gingivalis* A7436 infection in wild and nude mice

All normal BALB/c animals ($n = 12$) infected with *P. gingivalis* A7436 developed remote lesions at the abdominal area

within 48 h of infection and died in a general debilitating condition with apparent body wasting, ruffled fur, and lethargy (Fig. 1). All the nude mice ($n = 6$) survived *P. gingivalis* infection, although they showed grave wasting in the first few days of bacterial infection and developed similar abdominal lesions before day 6 postinfection (Fig. 2). They lost an average 38% their body weight within 7 days of infection, but there were no apparent permanent effects, and they recovered their weight and health (data not shown).

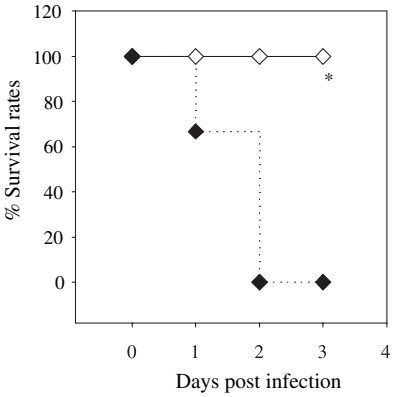

Fig. 1. Survival rates of normal (◆, $n = 12$) and nude (◇, $n = 6$) BALB/c mice after infection with *P. gingivalis* A7436. *****There was a significant difference in survival rate between normal and nude BALB/c mice (Kaplan–Meier Survival analysis, log-rank test).

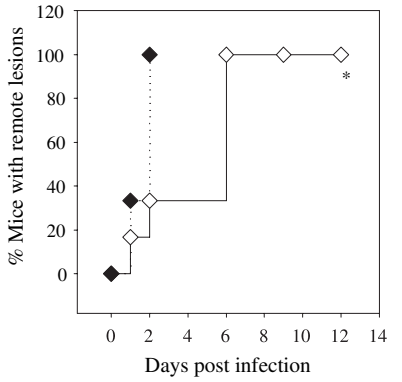

Fig. 2. Presence of remote lesions in normal (◆, $n = 12$) and nude (◇, $n = 6$) BALB/c mice after infection with *P. gingivalis* A7436. *****100% of normal mice had remote lesions by day 2, and 100% of the nude mice by day 6. The difference in the day of occurrence is significant according to Kaplan–Meier Survival analysis, log-rank test.

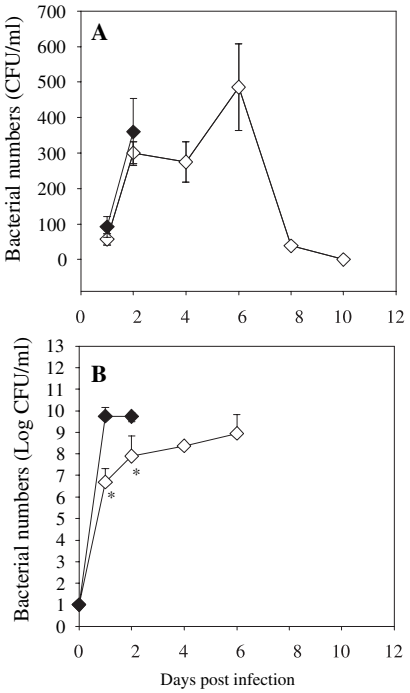

Fig. 3. Recoverable bacterial counts from (A) sera and (B) abdominal fluids after *P. gingivalis* A7436 infection in normal (◆, $n = 12$) and nude (◇, $n = 6$) BALB/c mice. *****Significantly fewer bacteria in day 1 and day 2 abdominal fluids in the nude mice compared to the normal mice according to One way analysis of variance and Bonferroni t-tests ($n = 6$ for both groups).

Recoverable bacteria from normal and the nude mice

As the animals demonstrated typical symptoms of septicemia after bacterial challenge, measurements of bacterial numbers in the bloodstream were performed to compare normal BALB/c and nude mice. The numbers of bacteria in both groups reached over 300 CFU/ml in serum in day 2 samples (the day all infected normal mice died) and the bacteria were still present in the circulation until at least day 6 postinfection in the nude mice, after which the mice gradually recovered from bacterial insult (Fig. 3A). Abdominal fluid samples have shown the presence of *P. gingivalis* in both normal BALB/c and the nude mice. The numbers of recoverable bacteria in normal BALB/c mice abdomens were $5.66 \pm 8.45 \times 10^9$ and $5.45 \pm 2.43 \times 10^9$ CFU/ml in day 1 and day 2 fluids, respectively (Fig. 3B), both statistically significantly larger than the bacterial numbers from the same day sampling in the nude mice. Recoverable bacteria in day 6 abdominal fluid in the nude mice was $8.89 \pm 5.54 \times 10^9$ CFU/ml, close to the amounts in day 2 fluid from normal mice (Fig. 3B).

IL-1β, tumor necrosis factor, IL-12, and interferon-γ titers in serum

Animal mortality differences between the normal and the nude mice indicated that T lymphocytes might play a vital role in the pathology of *P. gingivalis* infection. Four T-cell associated cytokines were examined in both groups – IL-1β, tumor necrosis factor, IL-12, and interferon-γ. IL-1β is an acute phase inflammatory cytokine, and both groups showed a spike of titers in day 1 serum samples (Fig. 4B). Tumor necrosis factor is also an acute phase inflammatory cytokine. Normal mice displayed an outburst of tumor necrosis factor in their serum tumor necrosis factor titers until animal death (Fig. 4A). In contrast, the titers of tumor necrosis factor in the nude mice gradually increased until day 6, declining thereafter (Fig. 4A). There were no significant differences between the normal and the nude mice in their IL-12 titers of day 1 and day 2 samples. The titers of IL-12 in the nude mice escalated from 113.67 ± 51.33 on day 1 to 508.67 ± 79.82 pg/ml on day 2 and remained at that level until at least day 12 (Fig. 4C). The responses of interferon-γ to *P. gingivalis* challenge in the nude mice were significantly lower than those of the normal animals. The titers of interferon-γ in normal animals was 42.67 ± 9.32 pg/ml in day 1 serum and 63.75 ± 1.58 pg/ml in day 2 serum, whereas those in the nude mice were well below 30 pg/ml during bacterial infection (Fig. 4D).

Blocking the effect of tumor necrosis factor or interferon-γ in normal BALB/c mice

The animals that died of *P. gingivalis* infection showed symptoms of septicemia, such as cachexia, ruffling, agitated, feverish first and then cold. Their urine outputs were decreased and ill-looking. Tumor necrosis factor may cause animal death. We administered thalidomide intraperitoneally 1 h before *P. gingivalis* bacterial challenge to curb the effects of host tumor necrosis factor. The results showed there was no improvement in animal mortality (Fig. 5A). The numbers of recoverable bacteria from day 2 serum samples was higher in the thalidomide-treated group than in the blank-treated group, although animals in both groups died within 2 days of *P. gingivalis* infection (Fig. 5B). There were significant decreases in the developments of remote lesions in thalidomide-treated group compared to blank controls. Only 33% of the thalidomide-treated group developed remote lesions in the abdominal area vs. 100% in control animals (Fig. 5C).

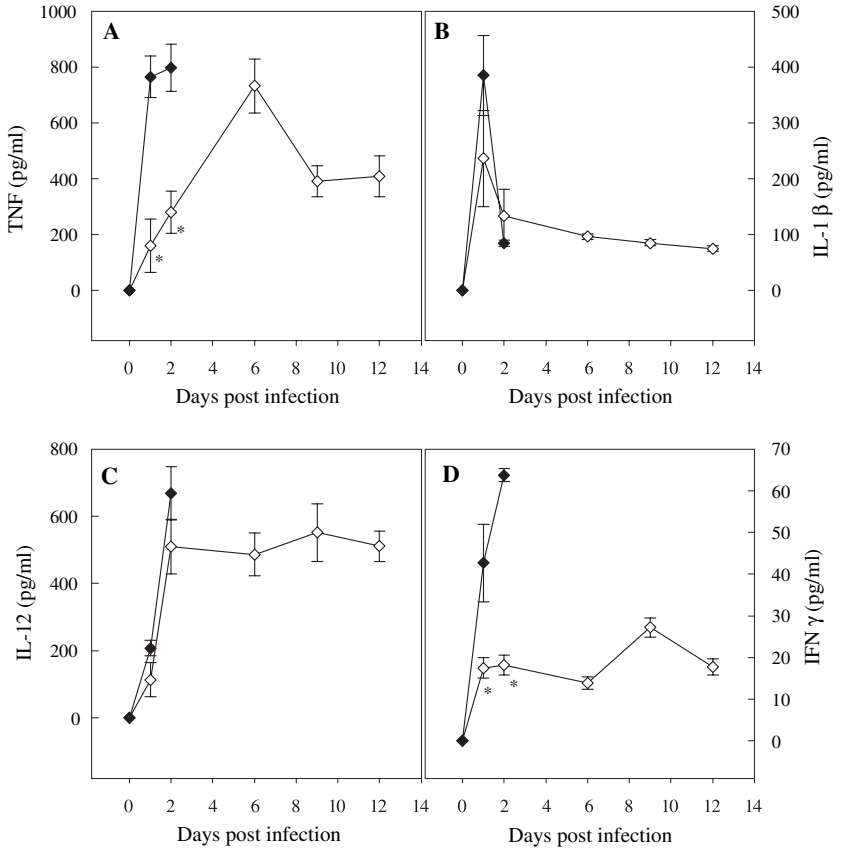

Fig. 4. Proinflammatory cytokine profiles in blood following *P. gingivalis* A7436 challenge in normal (◆, $n = 12$) and nude (◇, $n = 6$) BALB/c mice. A) Tumor necrosis factor. B) IL-1β. C) IL-12. D) Interferon-γ. *The levels of tumor necrosis factor and interferon-γ in the nude mice were significantly lower than those in normal BALB/c mice in day 1 and day 2 blood (One way analysis of variance, Bonferroni t-tests, $n = 6$).

Fewer numbers of *P. gingivalis* were recovered from the abdominal areas of thalidomide-treated than from blank controls on day 1 and day 2 (Fig. 5D). Interferon-γ titers in serum samples were greatly mobilized in normal animals infected with *P. gingivalis*. With daily treatment of anti-interferon-γ systemically, none of infected normal mice died (Fig. 5A), in spite of the fact of the recoverable bacterial number was higher in day 2 serum in anti-interferon-γ treated animals than that in blank control animals (Fig. 5B). The occurrence of remote lesions was not affected by anti-interferon-γ treatment, and all animals developed abdominal lesions within 2 days post bacterial infection (Fig. 5C).

Blocking the effects of tumor necrosis factor or interferon-γ in the nude mice

Thalidomide or anti-interferon-γ did not alter nude mice survival rates in that all animals survived *P. gingivalis* A7436 infectious challenge regardless of whether they were treated with thalidomide, anti-interferon-γ or blank (Fig. 6A). The curves of recoverable bacterial in sera in these three groups followed a similar trend (Fig. 6B), though there were marginally more in day 6 thalidomide-treated animals than in anti-interferon-γ or blank-treated animals ($P = 0.06$). Thalidomide-treated animals developed fewer remote lesions (67%) than the other two groups (both 100%; Fig. 6C). Accordingly, more *P. gingivalis* were found in day 1 or day 2 abdominal fluids in the anti-interferon-γ or blank-treated group than in thalidomide-treated animals. More bacteria were found in thalidomide-treated mice in day 9 and day 12 fluids when compared to corresponding anti-interferon-γ or blank-treated groups (Fig. 6D).

Discussion

The mouse chamber model used in this study creates a discrete niche inside hosts for bacterial colonization and was first introduced by Genco et al. (12) Those authors have shown that the bacterial loads needed to induce similar pathologies were much less in the mouse chamber model than in subcutaneous animal model without chamber (7). They characterized *P. gingivalis* A7436 along with W83 and W50 as virulent strains, causing septic shock and lethal outcomes, and strains ATCC 33277, HG405, and W50/BEI as avirulent, inducing only localized lesions. *P. gingivalis* A7436 was originally isolated from a refractory periodontitis patient and in this study demonstrates distinct pathologic outcomes progressing to a severe systemic response with remote abdominal lesions, cachexia and death consistent with endotoxic shock. The remote lesions in animal abdominal areas appeared between 24 h and 48 h with black exudates. All mice died by day 2 following *P. gingivalis* A7436 infection. There were anaerobic rod bacteria with *P. gingivalis* characteristic black colonies recoverable from animal circulation and abdominal subcutaneous tissue transudates at the time of animal death.

The thymus plays a central role in the maturation, differentiation, and conditioning of T lymphocytes, which are the principal intermediate cells of immune responses (11). To our surprise, the nude mice, which were deprived of the immune protection of mature functional T lymphocytes, all survived *P. gingivalis* A7436 insult even though the bacteria were present in their blood and bacterial numbers increased in the first 6 days post infection comparable to the levels in normal BALB/c mice (Fig. 3). Despite similar levels of bacterial replication, mice lacking functional T cells have a significantly lower mortality following *P. gingivalis* infection, suggesting that bacterial replication in the circulatory system is necessary, but not sufficient, to induce death. This result strongly suggests an immunopathologic contribution of T lymphocytes to fatal bacterial sepsis.

Although the cytokines tumor necrosis factor, interferon-γ, IL-12, and IL-1β all showed serum elevations in the first 2 days of bacterial infection in normal BALB/c mice, the most likely cytokine for such lethal involvement is interferon-γ. Nude mice showed significant lower interferon-γ titers, which coincided with complete protection against animal death. Also, mice treated with anti-interferon-γ were completely resistant to fatal *P. gingivalis* infection. Our data suggest that interferon-γ plays a role in fatal sepsis following *P. gingivalis* infection, apparently acting in the periphery as a regulator of the immune

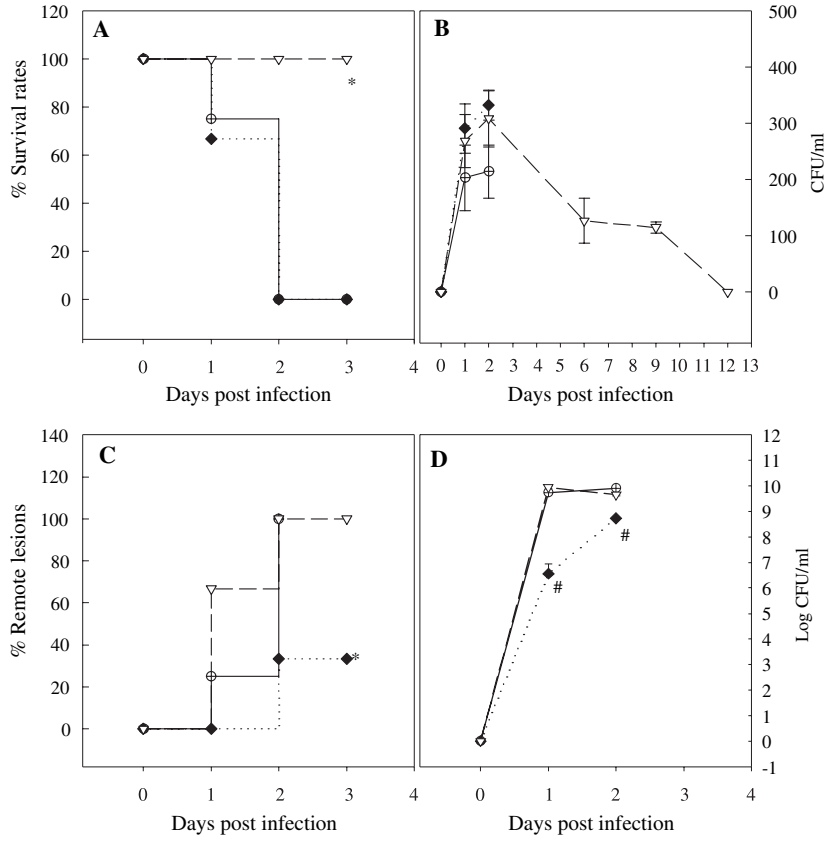

Fig. 5. Thalidomide (TH, ◆), anti-interferon-γ mAb (AI, ∇), and Blank (⊕) effects on pathologies in mouse chamber model (n = 6, 6, and 4 for TH, AI, and Blank, respectively). A) Animal survival rates. B) recoverable bacterial numbers from blood. C) Presence of remote lesions. D) Recoverable bacterial numbers from abdominal fluid. *****Anti-interferon-γ administration completely protected animals from death, and thalidomide administration significantly reduced the occurrence of remote lesions (Kaplan–Meier Survival analysis, log-rank tests). #Recoverable bacteria from abdominal fluids were reduced in thalidomide-treated mice on day 1 and day 2 (One way analysis of variance, Bonferroni t-tests).

response. Interferon-γ is a potent macrophage activator that can enhance a number of macrophage functions (10, 27) and modulate cytokine production (8, 28, 29). Some observations suggest that interferon-γ, in addition to tumor necrosis factor and IL-1β, might play a role in the pathophysiology of septic shock. Interferon-γ was found in the blood of septic shock patients (15) and in experimental models of lipopolysaccharide injections (13). Injections of murine interferon-γ enhanced cytokine production after lipopolysaccharide challenge (25). Finally, interferon-γ blockade protected mice against intravenous challenge of lipopolysaccharide (6, 14).

Lei & Morrison have demonstrated the presence of a lipopolysaccharide receptor on murine T cells (18). *In vitro* studies utilizing T cells in suspension have confirmed the ability of lipopolysaccharide to stimulate T-cell interferon-γ secretion (22). Human T cells become activated for interferon-γ secretion when co-cultured on an endothelial monolayer in the presence of endotoxin (33). Mattern et al. (21), utilizing T cells adherent to plastic, provided evidence that monocytes are indeed necessary and must be in direct contact with T cells for lipopolysaccharide-induced interferon-γ secretion. In a murine model of intra-abdominal polymicrobial sepsis, Miles et al. (23) injected mice with interferon-γ following cecal ligation and puncture, which led to increased mortality and earlier deaths in an interferon-γ dose-dependent fashion. Our study indicates that interferon-γ may contribute to immunopathology by direct effects on endothelial function and by promoting activation of resting monocytes, and that functional T cells are necessary for the lethal outcome of this animal model.

Tumor necrosis factor, a principal mediator responsive to endotoxic sepsis in previous studies (2, 4), was also markedly expressed during the course of infection in our animal model. Our data showed that the elevation of tumor necrosis factor titers was associated with the development of remote lesions. Both normal and nude BALB/c mice had comparably high concentrations of tumor necrosis factor in their systems when these lesions occurred. Blocking the effects of tumor necrosis factor by administering thalidomide reduced the occurrence of remote lesions in normal mice from 100% to 33% and in nude mice from 100% to 67%. The tissue destruction effects of *P. gingivalis* A7436-associated tumor necrosis factor expression were also observed in our laboratory with another strain of bacteria, *P. gingivalis* 381. *P. gingivalis* 381 is a tissue-contained strain and is incapable of disseminating *trans*-vascularly and thus incapable of causing animal death, but it did induce local tissue destruction and lesion formation. The local tissue destruction was shown to be associated with the host tumor necrosis factor production (20).

We cannot exclude the possibility of *P. gingivalis* A7436 could be unique in its pathogenesis in the infected animals. To validate our studies, other strains of *P. gingivalis* and other periodontopathic bacteria should be tested in this model in future research. Unlike most bacteria, which stay virtually outside the hosts in periodontal diseases, this chamber model allows the organism to colonize inside the host, directly exposed to antibodies, complement, phagocytes and other antimicrobial host mechanisms. The immune responses mounted by normal animals against *P. gingivalis* A7436 actually led to a lethal outcome in this study, whereas impaired functional T lymphocytes or hampered interferon-γ production protected infected animals. Our data imply that a clinical bacterial isolate from periodontitis patients which affects mainly periodontium in infected victims can become involved systemically and even threaten life once it gains internal access to the host.

It appears from data from our studies that the lethal outcome in mice following *P. gingivalis* A7536 infection is T-lymphocyte dependent and involves an increase in systemic interferon-γ levels. The data further indicate that dissemination of bacteria in the circulation is not sufficient for animal lethality. Our studies suggest that alterations in interferon-γ or in other T-cell effector responses will potentially result in perturbation of the complex network of host defense.

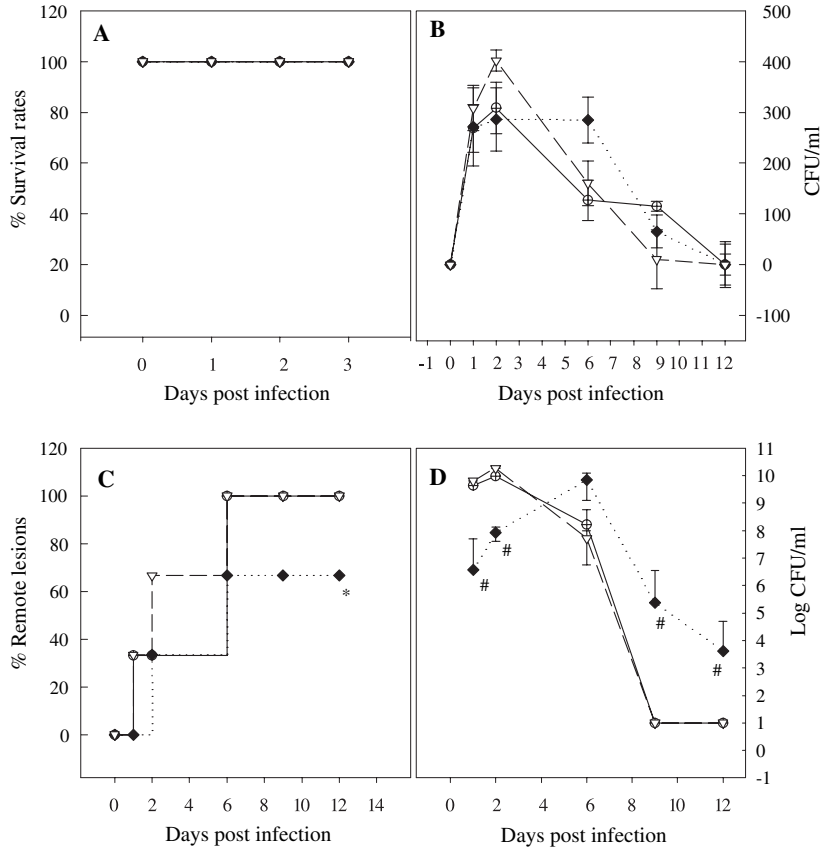

Fig. 6. Thalidomide (TH, ◆), anti-interferon-γ mAb (AI, ∇), and Blank (⊕) effects on pathologies of the nude mice ($n = 6$ for each group). A) animal survival rates. B) Recoverable bacterial numbers from blood. C) presence of remote lesions. D) Recoverable bacterial numbers from abdominal fluid. *****Thalidomide administration significantly reduced the occurrence of remote lesion compared to blank administration (Kaplan–Meier Survival analysis, log-rank tests). #The numbers of recoverable bacteria from abdominal fluids were reduced in thalidomide-treated mice in day 1 and day 2 but were significantly higher in day 9 and day 12 fluids compared to those in blank treatment group (One way analysis of variance, Bonferroni t-tests).

Acknowledgments

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