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Vaccination of mice with *Porphyromonas gingivalis* or *Fusobacterium nucleatum* modulates the inflammatory response, but fails to prevent experimental periodontitis*

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Abstract

Aim: To assess the potential of using vaccination with *Porphyromonas gingivalis* or *Fusobacterium nucleatum*, in modulating local subcutaneous inflammatory response and alveolar bone loss following coinfection with both bacteria.

Materials and Methods: Mice were immunized against either *P. gingivalis* or *F. nucleatum*. The cytokine response to mixed infection with *P. gingivalis* and *F. nucleatum* was evaluated using the subcutaneous chamber model. The alveolar bone loss induced by oral mixed infection was evaluated by micro-CT using the experimental periodontitis model. Serum levels of specific antibodies were determined by ELISA.

Results: Vaccination with either bacterium produced a specific humoral response before infection. Animals immunized against either bacteria following a mixed infection with *P. gingivalis* and *F. nucleatum*, showed decreased TNFα (but not IL-1β) levels as compared with non-immunized animals. However, the vaccination did not change the level of mixed infection-induced alveolar bone loss when compared with non-immunized animals. Six weeks following the oral mixed infection, specific antibody titres remained high. Furthermore, specific antibodies against the non-immunized bacterium were present at high levels.

Conclusions: While vaccination produced specific antibodies and suppressed the inflammatory response, it failed to prevent or reduce the progression of experimental periodontitis induced by mixed infection with *P. gingivalis* and *F. nucleatum*.

Key words: alveolar bone loss; *F. nucleatum*; mixed infection; *P. gingivalis*; vaccination

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Conflict of interest and source of funding statement

The authors declare that they have no conflict of interest.

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Vaccination is one of the preventive modalities suggested to control infectious diseases. The use of vaccination to prevent periodontal disease has been extensively investigated. Most of the studies have used animal models that utilized a single periodontal pathogen to induce experimental periodontitis. These studies often used *Porphyromonas gingivalis* as their single pathogen;

however, they produced variable and conflicting results (Page 2000, Persson 2005). Several studies using mice or primates as models demonstrated that vaccination with *P. gingivalis* results in protection against experimentally induced periodontal destruction (Rajapakse et al. 2002, Gonzalez et al. 2003, Gemmell et al. 2004), whereas other animal studies have been unable to demonstrate such protection (Ebersole et al. 1991, Moritz et al. 1998).

Despite the fact that periodontitis is considered to be an infection of more than one pathogen, few investigators have used models of experimental periodontits involving coinfection. In such experiments, where more than one bacteria were used, the experimental coinfection was shown to modulate the immune response and to affect the subsequent clinical outcome (Vitovec et al. 2001, Stoicov et al. 2004). More specifically, exposure to *Fusobacterium nucleatum* before *P. gingivalis* in a mouse model led to a Th2 subset response, whereas exposure to *P. gingivalis* alone, only evoked a Th1 subset response. In addition, all T-cell clones isolated from both groups were cross-reactive to both *P. gingivalis* and *F. nucleatum* (Choi et al. 2000). Gemmell et al. (2004) found that in animals vaccinated with *P. gingivalis* alone there is a propensity towards an IgG1 response, characterizing a Th2 subset response, whereas vaccination with *F. nucleatum* followed by *P. gingivalis* induces higher anti-*P. gingivalis* IgG2a levels, characteristic of a Th1 subset response. These studies indicate that prior vaccination may modulate the host response to a subsequent mixed infection with *P. gingivalis* and *F. nucleatum* and may shift the outcome of the inflammatory response.

A synergistic effect on soft tissue destruction was reported in experiments using an abscess model in mice infected with a mixture of *P. gingivalis* and *F. nucleatum* (Feuille et al. 1996). More recently, a study on rats using the oral infection model showed that oral infection with *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *F. nucleatum* together induced robust alveolar bone loss compared with infection with either bacteria alone (Kesavalu et al. 2007). Using the subcutaneous chamber model, we have recently shown that mixed infection with *P. gingivalis* and *F. nucleatum* induces a stronger inflammatory response, compared with that of mono-infection with either bacterium alone (Polak et al. 2009). As above, we also found that oral infection with the two bacteria resulted in augmented alveolar bone loss compared with that in mono-infected mice (Polak et al. 2009). However, to date there is no available information on the effect of vaccination mono or otherwise on the outcome of poly-microbial infections.

As such, the objectives of the present study were: (1) to investigate the effect of subcutaneous vaccination with either *P. gingivalis* or *F. nucleatum* on the immune response evoked by mixed infection with both bacteria. (2) To investigate the effect of the vaccination on subsequent experimental periodontitis induced by oral infection with a combination of the two bacteria.

Material and Methods

All experiments were performed in the Specific Pathogen-Free Unit of The Hebrew University-Hadassah Medical Center, and approved by the University's Animal Care and Use Committee.

Bacterial cultivation

P. gingivalis strain ATCC 33277 and *F. nucleatum* strain PK 1594 were separately grown in peptone yeast extract containing haemin and vitamin K (Wilkins Chalgren broth, Oxoid Ltd., Hampshire, UK), in an anaerobic chamber with 85% N_2, 5% H_2 and 10% CO_2. Following three washes in phosphate-buffered saline (PBS), the bacterial concentration was spectrophotometrically standardized to $OD_{650\,nm} = 0.1$ for *P. gingivalis*, corresponding to 10^{10} bacteria/ml (Genco et al. 1991), and $OD_{660\,nm} = 0.26$ for *F. nucleatum*, corresponding to 10^9 bacteria/ml (Kolenbrander & Andersen 1989).

Immunization

A subcutaneous injection (200μl) of heat-killed whole bacteria sonic extracts (sonicated 3×15 s each) was administered using *P. gingivalis* or *F. nucleatum* with an alum adjuvant (ratio, 1:1). Immunization was performed 14 and 7 days before the bacterial challenge in both the subcutaneous and the experimental periodontitis models (Fig. 1).

Subcutaneous chamber model (Fig. 1a)

This model was used to evaluate the local inflammatory response to bacterial challenge (Houri-Haddad et al. 2000). While under ketamine anaesthesia (20μl, intraperitoneal), 4–5-week-old experimental female BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA, $n = 6$ for each experimental group) were introduced with two chambers constructed from titanium wire that were surgically inserted into their dorsolumbar area. Ten days post-chamber-insertion, after collecting the baseline content of all chambers, the mice were challenged by an injection of 100 μl PBS containing a mixture of *P. gingivalis* and *F. nucleatum* (a total of 10^6 bacteria including 5×10^5 of each bacterium) into the chambers. A control group received PBS alone. Chamber exudates were harvested for cytokine analysis 2 and 24 h post-challenge (each chamber was sampled only once). Blood was collected by tail bleeding at baseline.

ELISA of cytokines

The secreted forms of mouse TNFα and IL-1β were quantified using the two site-ELISA, the method is based on commercially available antibody pairs (Pharmingen, San Diego, CA, USA) as described previously (Polak et al. 2009). The range of detection for each specific cytokine was 25–2000 pg/ml.

ELISA of antibodies

Plates containing 96 wells were coated with *P. gingivalis* or *F. nucleatum* sonic extracts, and relative quantities of IgG were determined using commercially available antibody pairs as described previously (Houri-Haddad et al. 2005).

Experimental periodontitis (oral infection) model (Baker et al. 1994) (Fig. 1b)

Female, 4–5-week-old BALB/c mice (Jackson Laboratories, $n = 8$ for each experimental group) were given sulphamethoxazole (10/500 ml water) ad libitum for 10 days followed by a 3-day wash-out (antibiotic-free period). The mice were then superinfected with a mixture of *P. gingivalis* and *F. nucleatum* ($\sim 400\,\mu$l of a 10^9 bacteria/ml concentration made up of equal volumes of each bacterium). The oral cavities of the animals were infected using 2%

carboxymethycellulose in PBS as vehicle, by three gavages at 2-day intervals. At 42 days after the final gavage (day 46), the maxillary jaws were harvested. Alveolar bone loss was evaluated by micro-CT as described previously (Wilensky et al. 2005). Blood was collected by tail bleeding at baseline and by heart puncture at 42 days.

Micro-CT analysis (Wilensky et al. 2005)

Maxillary hemi-jaws were analysed by compact fan-beam-type computerized tomography (μCT 40, Scanco Medical, Bassersdorf, Switzerland). The samples were placed in a cylindrical sample holder and ~ 200 micro-tomographic slices at increments of $12\,\mu$m were acquired, covering the entire bucco-palatal width of each hemi-jaw. Image segmentation of bone, dentin, enamel and pulp were obtained by applying a manually selected threshold for all the specimens. A reference line was set throughout the micro-tomographic slices at a set distance from the cemento-enamel junction and the residual coronal alveolar bone volume was measured. The measured area included the buccal alveolar bone around the mesio-buccal and disto-buccal roots of the middle molar tooth without the furcation area, so that $\sim 75\%$ of the buccal alveolar bone was measured. The results are presented as residual bone in mm^3 above the reference line.

Data analysis

The studies were carried out using at least six mice per treatment group. The data were analysed using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated measure analysis of variance was implemented to test the significance of the differences between the treated groups. If the results were significant, inter-group differences were tested for significance using Student's *t*-test and the Bonferroni correction for multiple testing.

Results

Specific antibody response to *P. gingivalis* or *F. nucleatum* was evaluated at baseline (before infection and 1 week after the last vaccination) in animals immunized against *P. gingivalis* or *F. nucleatum*, and compared with non-immunized animals. Anti-*P. gingivalis* antibodies were evident in the *P. gingivalis*-immunized animals, and absent in the *F. nucleatum*-immunized and non-immunized animals (Fig. 2a). The same pattern was found in the *F. nucleatum* antibodies, where only animals immunized against *F. nucleatum* produced specific antibodies with virtually none in the *P. gingivalis*-immunized and non-immunized animals (Fig. 2b). The difference between the groups for both antibodies was statistically significant ($p < 0.05$).

Using the subcutaneous chamber model (Fig. 1a), the local inflammatory response to mixed infection with *P. gingivalis* and *F. nucleatum* was evaluated in animals immunized against *P. gingivalis* or *F. nucleatum*, and compared with non-immunized animals. Non-immunized sham-infected animals served as a second control group. TNFα and IL-1β levels in the sham-infected group were not elevated at all the tested time intervals (Fig. 3a and b). Two hours post–infection, animals immunized with *P. gingivalis* or *F. nucleatum* showed significantly lower TNFα levels compared with non-immunized animals (Fig. 3a). The differences were statistically significant ($p < 0.05$). At 24 h post-infection, all groups showed reduced levels of TNFα, which was essentially the same as in the control group (Fig. 3a). IL-1β levels in all the groups peaked at 2 h, dropping somewhat at 24 h (Fig. 3b), yet no statistically significant differences were observed in the IL-1β levels between the immunized and non-immunized groups at any of the time intervals tested (Fig. 3b).

Alveolar bone loss in the experimental periodontitis model (Fig. 1b) was induced by oral mixed infection with *P. gingivalis* and *F. nucleatum* and evaluated in animals immunized with *F. nucleatum*, *P. gingivalis* or non-immunized animals. As above, non-immunized sham-infected animals served as a second control group. Oral mixed infection with *P. gingivalis* and *F. nucleatum* induced significant bone loss compared with that in the sham-infected group (Fig. 4). However, the alveolar bone loss induced by the mixed infection in the *P. gingivalis*-immunized animals and the *F. nucleatum*-immunized animals did not differ from the bone loss in the non-immunized animals (Fig. 4).

Because immunization with *P. gingivalis* or *F. nucleatum* did not appear to offer a protective effect on alveolar bone loss, a concern was raised that during the 42 days of the experiment the anti-

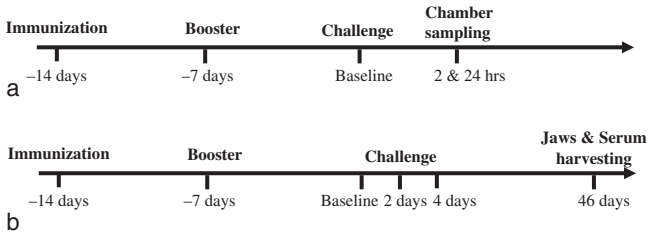

Fig. 1. Study design. (a) The subcutaneous chamber model design. (b) The experimental periodontitis model design.

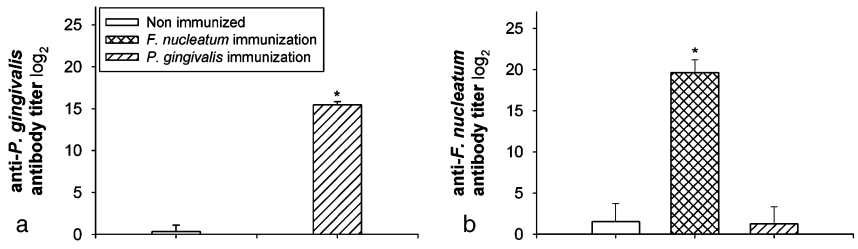

Fig. 2. Specific antibody response in animals immunized against *Porphyromonas gingivalis* or *Fusobacterium nucleatum* at baseline. Mice were immunized with *P. gingivalis* or *F. nucleatum* 14 and 7 days before baseline. Control non-immunized mice received saline. Sera were harvested at baseline from all groups and IgG titres were measured by ELISA. (a) Anti-*P. gingivalis* antibody levels. (b) Anti-*F. nucleatum* antibody levels. The results are expressed as the mean $\pm$ standard deviations. *Results significantly different from those obtained in the other groups. $p < 0.05$.

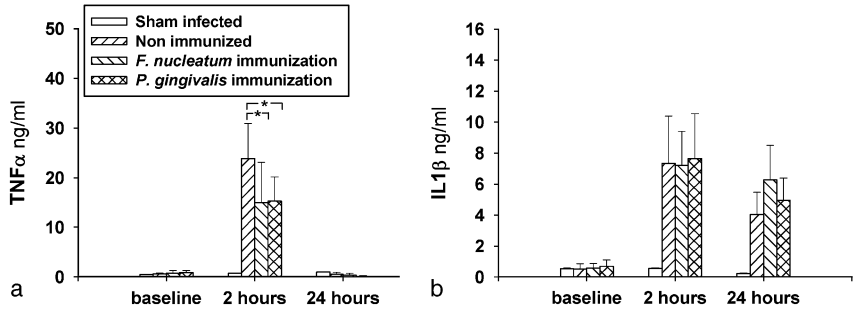

Fig. 3. Levels of inflammatory cytokines induced by mixed infection in animals immunized against *Porphyromonas gingivalis* or *Fusobacterium nucleatum*. Mice ($n = 6$ in each group) were immunized (14 and 7 days before challenge) with *P. gingivalis* or *F. nucleatum*. Control non-immunized mice received saline. The mice received an intra-chamber challenge of *P. gingivalis* and *F. nucleatum*. Chamber exudates were harvested at baseline (before challenge), 2 and 24 h post-challenge. The level of each cytokine in the chamber exudates was determined by ELISA. (a) TNFα levels. (b) IL-1β levels. The results are expressed as the mean ± standard deviations. *indicates results significantly different from those obtained in the other groups. $p < 0.05$.

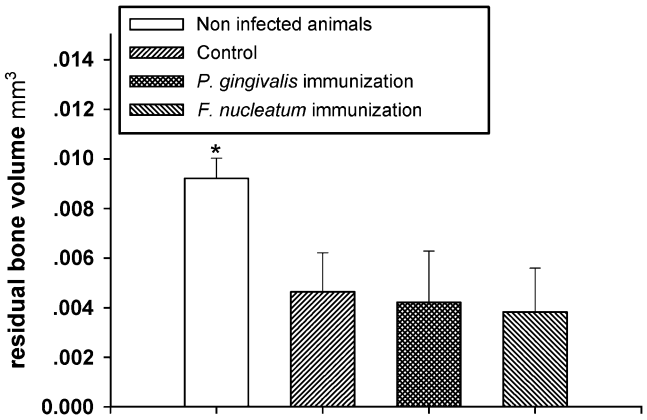

Fig. 4. Effect of immunization on residual bone levels following oral mixed infection. Mice ($n = 8$ in each group) were immunized (14 and 7 days before challenge) with *Porphyromonas gingivalis* or *Fusobacterium nucleatum*. Control non-immunized mice received saline. Thirteen days before challenge mice were treated with antibiotics for 10 days. Three days later, the mice were given an oral challenge of three gavages at 2-day intervals using an inoculum consisting of a mixture of *P. gingivalis* and *F. nucleatum*. Forty-two days later (at day 46), the jaws were harvested and the alveolar bone volume was measured using micro-CT. The results are expressed as the mean ± standard deviations.*Results significantly different from those obtained in the other groups. $p < 0.05$.

body levels dropped such that the protective effect of the vaccination faded. For this reason, we chose to examine the specific antibody titre at 42 days after oral infection (day 46, the time of jaw harvesting; Fig. 1b). Specific anti-*P. gingivalis* antibody levels were preserved during the 42 days of the experiment in animals immunized against *P. gingivalis* (Fig. 5a). Moreover, animals that were exposed to *P. gingivalis* only through the oral infection (not through the vaccination) also expressed significant anti-*P. gingivalis* antibodies (Fig. 5a). Still, the anti-*P. gingivalis* antibody levels expressed in the *P. gingivalis*-immunized animals were significantly higher than in the other groups. The same pattern was likewise observed in the anti-*F. nucleatum* antibody levels in *F. nucleatum* immunized mice as compared with the other groups. Specific anti-*F. nucleatum* antibodies levels were preserved during the 42 days of the experiment in animals immunized against *F. nucleatum* (Fig. 5b), and animals that were exposed to *F. nucleatum* only through the oral infection also expressed significant anti-*F. nucleatum* antibodies (Fig. 5b). The anti-*F. nucleatum* antibodies level in the *F. nucleatum*-immunized animals were significantly higher than in the other groups.

Discussion

The present experiments show that vaccination against *P. gingivalis* or *F. nucleatum* induced the expression of specific antibodies against the immunizing pathogen. Our results support the results of Gemmell et al. (2004), which showed that immunization with *P. gingivalis* or *F. nucleatum* induces the production of specific antibodies only against the immunizing pathogen. This induced humoral response expressed in specific antibody production could support a hypothesis that vaccination could provide protection against infection with periodontal pathogens. Considering the polymicrobial nature of periodontal disease and the fact that vaccination cannot be designed against all different bacteria present at the periodontal niche, a dual vaccination group (*P. gingivalis* and *F. nucleatum* together) was not included in the study design. Instead, we aimed to lucid if a vaccination with a single pathogen will be sufficient to create a protective effect against a mixed infection.

TNFα and IL-1β are well-recognized as tissue-destructive mediators (Bascones et al. 2005). We showed previously that mixed infection induces higher TNFα and IL-1β levels compared with mono-infection with either bacterium alone (Polak et al. 2009). Our present results demonstrate that vaccination with either *P. gingivalis* or *F. nucleatum* followed by mixed infection reduces the levels of TNFα as compared with non-immunized mice. This indicates that vaccination hinders the inflammatory response to the subsequent mixed infection. This decrease in the amplitude of the inflammatory response was similar in animals immunized against either pathogen, showing that the effect of vaccination on the host response need not be associated with a specific antigen, but is more general in nature. The above results imply that vaccination down-regulates the inflammatory response, and together with the humoral response may put forward a means for controlling tissue damage.

Because of a reduced inflammatory response and induced humoral response in immunized animals, we expected that the clinical outcome of experimental periodontitis, induced by oral mixed infection, would subside with preceded vaccination. To test this hypothesis, we investigated the effect of vaccination on alveolar bone loss induced by oral infec-

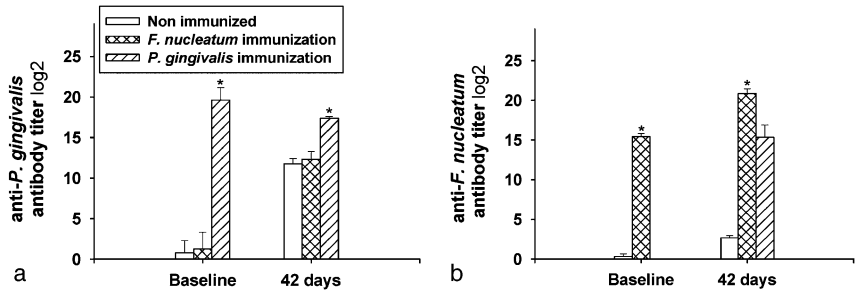

Fig. 5. Specific antibody response at 42 days post-infection in the experimental periodontitis model. Mice ($n = 8$ in each group) were immunized (14 and 7 days before challenge) with *Porphyromonas gingivalis* or *Fusobacterium nucleatum*. Control non-immunized mice received saline. Thirteen days before challenge mice were treated with antibiotics for 10 days. Three days later, the mice were given an oral challenge of three gavages at 2-day intervals using an inoculum consisting of a mixture of *P. gingivalis* and *F. nucleatum*. Forty-two days later (at day 46), serum was collected and specific IgG levels were measured by ELISA. (a) Anti-*P. gingivalis* antibody levels. (b) Anti-*F. nucleatum* antibody levels. The results are expressed as the mean ± standard deviations. *Results significantly different from those obtained in the other groups. $p < 0.05$.

tion with the two bacteria. We showed previously that oral mixed infection with *P. gingivalis* and *F. nucleatum* induced robust alveolar bone loss compared with infection with either bacterium alone (Polak et al. 2009), suggesting the two bacteria act synergistically. As in our previous study, the oral mixed infection in non-immunized animals resulted in significant alveolar bone loss when compared with non-infected animals. It was, however, surprising to find that neither vaccination with *P. gingivalis* nor with *F. nucleatum* was able to prevent or even reduce the alveolar bone loss induced by subsequent oral mixed infection. Several studies using a mono-infection model of *P. gingivalis* were able to demonstrate that *P. gingivalis* vaccination is able to partially prevent alveolar bone loss in experimentally induced periodontitis (Baker et al. 1994, Persson 2005). Other studies were not successful in demonstrating any protection through immunization (Ebersole et al. 1991, Moritz et al. 1998). Using a poly-infection model, our present study suggests that whole cell vaccination with one of the infecting microorganisms fails to prevent attachment loss, and supports the studies, which failed to find a protective effect in vaccination.

A possible explanation to the above results is that during the 42 days of subsequent poly-oral infection (the experimental periodontitis model), the specific antibody levels dropped and the protective effect of the vaccination was lost. To examine this notion, we measured the specific antibody levels at 42 days post-infection (at the termination of the experiment). We found that specific antibodies were preserved during the experimental period for both anti-*P. gingivalis* and anti-*F. nucleatum*. It was interesting to note that specific antibodies against the non-immunized bacteria, which were introduced only via the subsequent oral challenge, were also present at high levels at day 46. While many studies of vaccination as a preventive measure for periodontal disease measure antibody levels as an indication of effectiveness (Persson 2005), from our results it is clear that high levels of specific antibodies were not correlated with the actual effectiveness of the vaccination. This may be due to antibodies with low avidity or ineffective opsonization. Yet another explanation could be that the observed bone loss is not the result of bacterial invasion into the periodontal tissue, but perhaps it is rather that mere stimulation of the tissue, from the infection in the oral cavity, is sufficient to induce attachment loss. In addition, it is possible that antibody responses against whole-bacterial cell immunogens are directed against immunodominant antigens, not necessarily protective antigens. Furthermore, the indigenous flora is still present and may be altered by the mixed infection with *P. gingivalis* and *F. nucleatum*. This effect may cause a transformation in the flora to a more periopathogenic one, even if *P. gingivalis* or *F. nucleatum* presence may not persist in the flora.

The subcutaneous chamber and experimental periodontitis models used in the present study are complementary models. Each one is used to test different and specific hypotheses. The subcutaneous chamber model provides the means to investigate the inflammatory response to a specific infection at the cellular and molecular levels, whereas the oral infection model allows an investigation of the clinical outcome of the bacterial infection. However, there are differences in the two models, which must be addressed. A major difference is the nature of the inflammatory response: the subcutaneous chamber model represents an acute inflammatory response, whereas the inflammatory response to the oral infection is chronic in nature. In addition, in the subcutaneous chamber, the inflammatory response originates in the surrounding connective tissue, whereas the response to the oral infection is more complex and involves connective tissue, epithelium, periodontal ligament and alveolar bone. Perhaps it is the complexity of the experimental periodontitis model that holds the key towards a better understanding of the results of the alveolar bone loss.

In conclusion, in our experiments, vaccination with either *P. gingivalis* or *F. nucleatum* down-regulated the local inflammatory response to a subsequent mixed infection with *P. gingivalis* and *F. nucleatum*, and induced a specific humoral antibody response. However, both these vaccination protocols were unsuccessful in preventing or reducing the alveolar bone loss induced by the oral mixed infection. A protection against an infection like periodontitis with both mucosal and systemic aspects may require a mucosal immunization, which elicits both secretory and serum antibodies.

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Clinical Relevance

Scientific rationale for the study: Vaccination is considered to have the potential to serve as a preventive modality against periodontal disease. The effectiveness of vaccination with only one of the pathogens participating in an oral poly-microbial infection should advance our understanding of the feasibility of this treatment.

Principal findings: Although vaccination with *P. gingivalis* or *F. nucleatum* attenuated the local inflammatory response to mixed infection at the molecular level, it was unsuccessful in preventing the outcome of experimental periodontitis.

Practical implications: Although vaccination shows promise in its effectiveness to modulate the local inflammatory response, it was unsuccessful in preventing disease progression when examined using our poly-microbial model of periodontal disease, which more closely mimics the nature of the disease/infection. In conclusion, this treatment modality of vaccination may not offer a solution for the prevention of periodontal disease as originally hypothesized.