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Strain-dependent activation of the mouse immune response is correlated with *Porphyromonas gingivalis*-induced experimental periodontitis

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Abstract

Aims: To evaluate the effect of oral infection with three *Porphyromonas gingivalis* strains on alveolar bone loss (ABL) and its correlation with the mouse immune response.

Materials and Methods: Mice were orally infected with *P. gingivalis* strains 381, 33277 and 53977. After 42 days, maxillae were analysed for ABL using micro-computed tomography and the serum for anti-*P.gingivalis* IgG1 and IgG2a levels. The cytokine response to *P. gingivalis* was tested using the subcutaneous chamber model.

Results: The *P. gingivalis* 53977-infected group showed the highest ABL, which was significantly different from all other groups ($p<0.001$). In addition, the humoral response to *P. gingivalis* 53977 was significantly lower than the response to *P. gingivalis* 381 and 33277 ($p\leqslant0.01$). The IgG2a/IgG1 ratio was higher in the *P. gingivalis* 33277-infected group (1.6) compared with the *P. gingivalis* 381-infected group (0.51). Four days post-infection, interleukin (IL)-1β levels remained significantly higher in the *P. gingivalis* 53977-infected group only (1198.2 ± 260.0, $p<0.05$), while IL-4 levels remained significantly higher in the *P. gingivalis* 381-infected group (265.8 ± 131.6, $p<0.05$).

Conclusions: The high levels of ABL induced by *P. gingivalis* 53977 were inversely correlated with the humoral response to this bacterium. In addition, ABL was correlated with an elevated pro-inflammatory response.

Key words: alveolar bone loss; experimental periodontitis; interleukin-1β; interleukin-4; *Porphyromonas gingivalis*

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Conflict of interest and source of funding statement

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Periodontal disease is an infection accompanied by chronic inflammation. It is a multifactorial, polymicrobial disease characterized by the loss of connective tissue attachment, periodontal ligament and alveolar bone (Listgarten 1986). While human subgingival plaque harbours up to 500 bacterial species (Paster et al. 2001), it is likely that only a small percentage of these are, in fact, involved in the aetiology of periodontal disease (Moore & Moore 1994). Evidence suggests that *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium, is strongly associated with periodontal disease (Slots et al. 1988, Grossi et al. 1994). The nature and magnitude of the periodontium response to this periodontal infection is modulated not only by the bacteria itself but by the host immune response (Van Dyke et al. 1993) and the interactions between them.

The bacterial infection that exists in periodontal disease generates various

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host immune responses, resulting in the secretion of cytokines and immunoglobulins (Ig). The pro-inflammatory response is characterized by the dominant secretion of cytokines such as interleukin (IL)-1β, IL-6, IL-11, IL-17, interferon (IFN)-γ and tumour necrosis factor (TNF)-α (Cochran 2008). In contrast, an anti-inflammatory dominant response, which was thought to protect the periodontal tissues from destruction by bacterial infection (Eastcott et al. 1994), is characterized by IL-4, IL-12, IL-10, IL-13 and IL-18 secretion (Cochran 2008). Pro-inflammatory cytokines have been shown to support Ig-isotype switching to IgG2a, while anti-inflammatory cytokines support switching to IgG1 (Mosmann & Coffman 1989, Shyur et al. 1992, Pulendran et al. 1999, Dujovny et al. 2002).

The importance of the humoral response to the severity of human periodontal disease has been investigated in many studies (Gmür et al. 1986, Lopatin & Blackburn 1992, Lamster et al. 1998). Some studies showed that elevated serum levels of anti-*P. gingivalis* IgG have been directly linked to disease severity. However, others found that the levels of antibodies in the gingival crevicular fluid have been shown to be inversely related to the number of organisms at the site of sampling (Kinane et al. 1993, Ebersole et al. 1995a). In a clinical study, anti-*P. gingivalis* IgG levels at periodontitis sites were found to be lower than those at gingivitis sites in the same subjects, suggesting that a failure of a local antibody production may contribute to the transition from gingivitis to periodontitis (Mooney & Kinane 1997). IgG subclasses and their avidity have also been implicated as a factor in periodontal disease progression. Mooney et al. (1993) showed that patients who had not experienced attachment loss during a 3-month monitoring period had anti-*P. gingivalis* antibodies with a higher avidity than those patients who experienced attachment loss. In periodontal disease subjects, it was found that a humoral response involving IgG2 may be ineffective in clearing *P. gingivalis* infections (Lamster et al. 1998).

Although the current assumption suggests that a host-dependent exaggerated pro-inflammatory response is the main reason for periodontal destruction, it is possible that different bacterial strains may induce different levels of response. In order to test this possibility, investigators used different strains of *P. gingivalis* in various animal models, such as the mouse skin abscess model (Grenier & Mayrand 1987, Ebersole et al. 1995b, Griffen et al. 1999), the subcutaneous chamber model (Genco et al. 1991, Genco & Arko 1994) and the oral infection model (Evans et al. 1992, Baker et al. 2000). In these studies, many strains of *P. gingivalis* were classified as either virulent or avirulent; however, the correlation between alveolar bone loss (ABL) and the host immune response following infection with different *P. gingivalis* strains needs further clarification. We hypothesized that differences in the virulence of different *P. gingivalis* strains are derived from the type of host immune response induced by specific bacteria.

Using a mouse model of periodontitis, the purpose of this study was to determine whether different strains of *P. gingivalis* can modulate the host immune response differently and, consequently, induce various severities of periodontal disease.

Materials and Methods

Animals

Five- to 6-week-old female BALB/c mice (Harlane, Jerusalem, Israel) were used. The study was carried out in the specific pathogen-free unit of the animal facility. The mice were maintained on a 12-h light/dark cycle and received distilled water and food ad libitum. The experimental protocols were approved by the Internal Review Board of the Hadassah–Hebrew University Medical Center.

Bacteria

P. gingivalis ATCC 33277, 381 and 53977 strains were grown on blood agar plates in an anaerobic chamber with 85% N_2, 5% H_2 and 10% CO_2. After incubation at 37°C for 2–3 days, the bacterial cells were inoculated into Wilkins media (Oxoid, Basingstoke, UK) for a 2-day incubation under the same conditions. The bacteria were washed three times with sterile phosphate-buffered saline (PBS) before use (Houri-Haddad et al. 2000).

Oral infection

The mice were divided into four groups: *P. gingivalis* ATCC 33277-infected group ($n = 7$), *P. gingivalis* 381-infected group ($n = 7$), *P. gingivalis* 53977-infected group ($n = 7$) and control non-infected group ($n = 7$). Infection was carried out as described by Baker et al. (2000) and Lalla et al. (2003). In brief, all animals were treated by antibiotics for 10 days (sulphamethoxazole/trimethoprim 0.08% and 0.016%, respectively, in drinking water, ad libitum). Three days following the withdrawal of antibiotics (day 14), the four groups of animals were infected with the different *P. gingivalis* strains or vehicle only (10^{11} CFU/ml in 0.2 ml of PBS and 2% carboxymethylcellulose). The infection was carried out by gavage into the oesophagus, oral cavity and by local application of 20 μl of bacteria around the anus (Lalla et al. 2003) three times, once every other day. Forty-two days after the last infection, the mice were sacrificed and the hemi-maxillae were collected and prepared for bone loss measurements using the micro-computed tomography (μCT) technique (Wilensky et al. 2005). Serum samples were taken at baseline before the oral infection and at 42 days after the last infection. At the end of the experiments, mice were scarified using CO_2.

The subcutaneous chamber model

The mice were divided into four groups according to the specific injected bacteria and to three time points ($n = 7$) in each group. One group served as the non-infected control (saline injected), while the other three groups were infected by an intra-chamber injection with *P. gingivalis* ATCC 33277, *P. gingivalis* 381 or *P. gingivalis* 53977. One week following chamber implantation, all three test groups were infected by an intra-chamber injection of the various live *P. gingivalis* strains (5×10^8 CFU in 1 ml of PBS) while saline was injected into the control non-infected group. Exudates from the two chambers on each mouse were collected at baseline (immediately before the intra-chamber infection), 2, 48 and 96 h post-infection. Each chamber was sampled once and the individual mouse was chosen as the unit of analysis. The exudates were centrifuged for 10 min. at 290 *g*. The supernatants were removed and stored at −70°C until analysed. At the end of the experiments mice were sacrified using CO_2.

Quantification of alveolar bone loss

For quantitative three-dimensional (3D) analysis of the ABL, the hemi-maxillae

were examined by a desktop μCT system (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland). The sagittal plan of the specimens was set parallel to the X-ray beam axis. The specimens were scanned at a resolution of 12 μm in all three spatial dimensions. The scans were Gaussian filtered and segmented using a multi-level global thresholding procedure for the segmentation of enamel, dentin and bone. Residual supportive bone volume (RSBV) was determined separately for either root (bucco-mesial and bucco-distal) using a direct 3D approach (Hildebrand et al. 1999). The measured mesio-distal length of the alveolar bone was 204 μm and 120 μm for the mesio-buccal and the disto-buccal roots, respectively. The apical basis of the measured volume was set mesio-distally parallel to the cemento-enamel junction (CEJ) and bucco-palatinally parallel to the occlusal plane. The results represented the residual bone above the reference plane in mm^3 (Wilensky et al. 2005).

Quantification of anti-*P. gingivalis* IgG antibodies

Serum levels of anti-*P. gingivalis* IgG subclass were determined by a enzyme-linked immunosorbent assay (ELISA), as described previously (Houri-Haddad et al. 2001, 2002). In brief, 96-well ELISA plates were coated with the three different *P. gingivalis* strains (10 μg protein/ml), followed by overnight incubation at 4°C. The wells were blocked for 30 min. (RT) with 1% bovine serum albumin in PBS. Serum samples were incubated for 2 h at 37°C and myeloma proteins of each subclass were used as standards (100–1.56 μg/ml, Sakai et al. 2001). Alkaline phosphatase-conjugated, goat anti-mouse IgG1 or IgG2a antibodies (Southern Biotech, Birmingham, AL, USA) were added and incubated for 1 h at 37°C. *p*-nitrophenyl phosphate was used as a substrate. The reaction was stopped by the addition of 1 N sodium hydroxide, and the optical density was read using a V max microplate reader (Molecular Devices, Palo Alto, CA, USA) at 405 nm.

Analysis of cytokines

The presence of IL-1β, TNF-α and IL-4 in the chamber exudates were determined by ELISA, as described previously (Frolov et al. 1998). The assays were based on antibody pairs matched for ELISA obtained from Pharmingen (San Diego, CA, USA).

Data analysis

Data analysis was performed using a statistical software package (SigmaStat, SPSS Science, Chicago, IL, USA). The differences between IgG subclasses were evaluated using a paired *t*-test. One-way analysis of variance was used to test the significance of the differences in RSBV and cytokines between the treated groups. When significance was established, the inter-group differences were tested for significance by a *t*-test with the Student–Newman–Keuls method correction for multiple testing. The level of significance was determined at $p < 0.05$. All the results are presented as mean values ± the standard error of the mean.

Results

Alveolar bone loss

Forty-two days after the oral infection, significant differences were found in RSBV between the three infected groups and the control group. The *P. gingivalis* 53977-infected group showed the lowest RSBV $(4.38 \pm 0.19) \times 10^{-3}\,mm^3$, while the *P. gingivalis* 381-infected group showed the highest RSBV $(5.70 \pm 0.17) \times 10^{-3}\,mm^3$. All the infected groups showed significant differences in RSBV among themselves (Fig. 1).

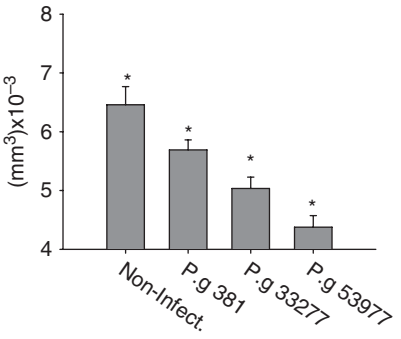

Fig. 1. Residual supportive bone volume 42 days following oral infection with *Porphyromonas gingivalis* 53977, *P. gingivalis* 381 and *P. gingivalis* 33277, compared with the non-infected (control) group. Bars represent mean values ± standard error of the mean. *Significantly different from all other groups. One-way analysis of variance, $p < 0.05$.

Antibody responses

Forty-two days after infection, significantly higher levels of serum IgG1 and IgG2a against *P. gingivalis* were found in the *P. gingivalis* 33277- and 381-infected groups, compared with *P. gingivalis* 53977, the non-infected group and baseline levels (Fig. 2). Antibody levels of the *P. gingivalis* 53977-infected group were very low, although a significant difference was observed between this group and the non-infected group in IgG2a levels only. Examination of the IgG2a/IgG1 ratio in the groups with a marked IgG response (381 and 33277) revealed that the IgG2a/IgG1 ratio in the *P. gingivalis* 33277-infected group was 1.60, suggesting a pro-inflammatory response, while the IgG2a/IgG1 ratio for the *P. gingivalis* 381-infected group was 0.51, suggesting an anti-inflammatory response.

Intra-chamber cytokine levels

IL-1β levels peaked 2 h post-infection in the three infected groups compared with the non-infected group. At 48 h, IL-1β levels continued to remain significantly higher in the three infected groups, but without significant differences between the groups. At 96 h IL-1β levels remained significantly higher only in the *P. gingivalis* 53977-infected group (Fig. 3a).

IL-4 levels also peaked 2 h post-infection in the three infected groups compared with the control non-infected group, without significant differences between the groups at 2 and 48 h post-infection. Ninty-six hours post-infection, significantly higher levels of IL-4 were found only in the *P. gingivalis* 381-infected group (Fig. 3b). The results show prolonged IL-1β secretion in the *P. gingivalis* 53977-infected group, in contrast to prolonged IL-4 secretion in the *P. gingivalis* 381-infected group.

No significant differences were found between the different *P. gingivalis* strains with regard to TNF-α levels (data not shown).

Discussion

In the present study, we analysed the relationship between the host's local inflammatory and immune response and ABL following infection with different *P. gingivalis* strains. The results from the oral infection model showed that although all three *P. gingivalis*

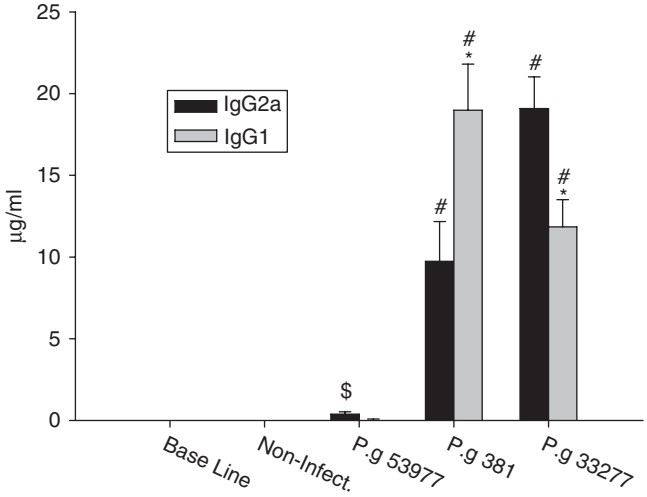

Fig. 2. Levels of serum anti-*Porphyromonas gingivalis* IgG1 and IgG2a antibodies 42 days following oral infection. *Significantly different from IgG2a group. #Significantly different from baseline, non-infected and *P. gingivalis* 53977 groups. $Significantly different from baseline and non-infected groups. Paired *t*-test, $p < 0.05$.

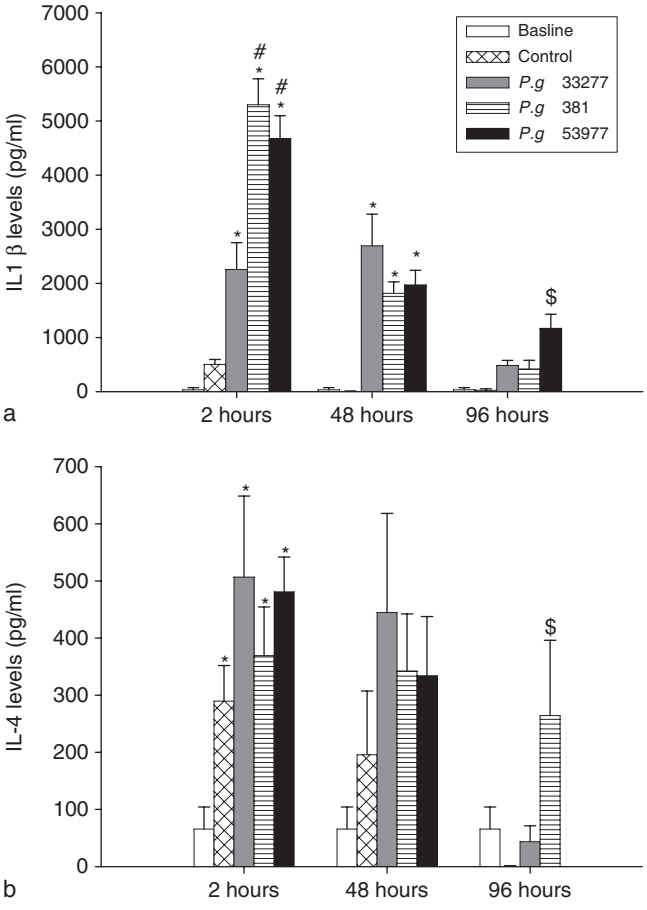

Fig. 3. Quantification of interleukin (IL)-1β (a) and IL-4 (b) levels in the subcutaneous chamber fluids, at different time intervals following intra-chamber infection. BALB/c mice were infected with *Porphyromonas gingivalis* 33277, 381, 53977 or saline. Cytokine levels in the chamber exudates were determined by enzyme-linked immunosorbent assay. Bars represent mean values ± standard error of the mean. *Results that are significantly different from baseline. #Results that are significantly different from *P. gingivalis* 33277. $Results that are significantly different from all other groups. One-way analysis of variance, $p < 0.05$.

strains induced significant ABL, there was a marked difference in their pathogenic potential.

The specific *P. gingivalis* strains were chosen due to their heterogenic virulence properties. For example, *P. gingivalis* 381 has only a moderate proteolytic activity (Birkedal-Hansen et al. 1988, Neiders et al. 1989) and does not produce disseminated disease when injected subcutaneously into mice (Van Steenbergen et al. 1982, Chen et al. 1987), while *P. gingivalis* 53977 is more proteolytic (Birkedal-Hansen et al. 1988, Neiders et al. 1989) and may produce widespread infection, cachexia and death (Chen et al. 1987, Neiders et al. 1989). Two of these strains also show different patterns with respect to the induction of gingival collagenase and gellatinase activity; *P. gingivalis* 53977 induced high host-collagenase and gellatinase activity 42 days after infection, while only moderate activity was induced by *P. gingivalis* 381 (Evans et al. 1992).

In addition, these *P. gingivalis* strains are different in their *fimA* genotype. *P. gingivalis* 53977, which has the type II *fimA* gene, caused severe clinical symptoms and increased serum inflammation markers, after being injected subcutaneously, compared with *P. gingivalis* 381 and *P. gingivalis* 33277 which have the type I *fimA* gene (Nakano et al. 2004).

In the present study, *P. gingivalis* 381, which was originally isolated from an adult with periodontitis (Tanner et al. 1979), induced the lowest ABL among the three strains, while *P. gingivalis* 53977 induced the highest ABL levels. *P. gingivalis* 53977 was originally isolated from a periodontitis patient with type II diabetes mellitus (Zambon et al. 1988), and its virulence has already been confirmed by previous findings (Baker et al. 2000). Using a similar model, Baker et al. (2000) had previously shown that *P. gingivalis* 53977- and 381-infected mice had similar levels of the bacteria in their oral cavities, 42 days following oral infection. This fact supports our results, that the modified immune response, rather than the levels of infection, was responsible for the different clinical outcome between the infected groups. However, it is important to mention that the presence of *P. gingivalis* in the mouths of mice was not evaluated in the present experiments and we cannot exclude the possibility that the variations in the host

response were due to different levels of colonization by each of the inoculated strains.

Another explanation for the differences in virulence may be related to the variance in activation of the host immune response. We demonstrated that oral infection with *P. gingivalis* 381 induced a significant elevation of serum IgG1 and IgG2a levels, while *P. gingivalis* 53977 induced only a minor elevation, and this was in IgG2a levels only. The inverse correlation between IgG subclasses levels and ABL confirms the importance of the humoral immune response in the protection against periodontal disease.

Our results showed that *P. gingivalis* 53977 induced high levels of IL-1β secretion, while it was not recognized by the humoral immune system. As stated above, the enhanced proteolytic activity of this strain may lead to a more stable chronic infection, which was previously described to attenuate T-cell activity (Bronstein-Sitton et al. 2003). T-cell activity is essential to B-cell development, and any changes in their function may lead to a defective humoral response.

A different possibility for the differences in the induction of periodontal destruction between the different strains is their ability to induce different pathways of T-cell response. IgG2a/IgG1 is used as a marker for T-helper (Th) cell response. High levels of this ratio are correlated with a Th1, pro-inflammatory response, while low levels (under 1) are correlated with a Th2, anti-inflammatory response (Mosmann & Coffman 1989, Shyur et al. 1992, Pulendran et al. 1999, Dujovny et al. 2002). In our study, the IgG2a/IgG1 ratio was high in the serum of *P. gingivalis* 33277, which induced significantly higher bone loss compared with *P. gingivalis* 381, while *P. gingivalis* 381 induced a reverse (low) IgG2a/IgG1 ratio. These results may also demonstrate the importance of T-cell response in *P. gingivalis*-induced experimental periodontitis.

It was suggested (Offenbacher 1996) that if the antibody response does not result in clearance of the pathogen, the outcome of the lymphocyte challenge is the secretion of catabolic cytokines and inflammatory mediators such as IL-1, IL-6, TNF-α and prostaglandin E_2 (PGE_2), which may induce the loss of periodontal-supporting tissues. The inability of specific antibodies to eliminate the causative microorganisms of periodontal disease could be due to a number of factors, including poor antigenicity (Slots 1999) and the production of anti-*P. gingivalis* antibodies with different avidities (Mooney & Kinane 1994). The ability of bacteria to induce a humoral response and a local inflammatory response may be at least partly due to their ability to induce periodontal destruction. In the present study, we found that different *P. gingivalis* strains cause different levels of cytokine secretion. IL-4 levels were found to be significantly higher in the *P. gingivalis* 381-infected group compared with the other two infected groups. IL-4 is considered to be an anti-inflammatory cytokine that suppresses cell-mediated responses and enhances the resistance associated with humoral immunity (Modlin & Nutman 1993). IL-4 encourages B cell activation and antibody production. Antibodies can protect the host by inactivation of bacterial toxins, prevention of bacterial adherence and by acting as opsonins for phagocytosis by neutrophils (Gemmell & Seymour 2004). In addition, IL-4 inhibits several macrophage functions and exerts a strong and consistent inhibitory effect on the activity of pro-inflammatory cells. The higher IL-4 secretion induced by infection with *P. gingivalis* 381 may explain the lower bone loss (higher RSBV) found following the oral infection. On the other hand, IL-1β was found to be significantly higher in the *P. gingivalis* 53977-infected group. In contrast to IL-4, IL-1β is a pro-inflammatory cytokine, that enables recruitment of inflammatory cells into sites of infection, promotes bone resorption, stimulates PGE_2 release in addition to stimulating the release of matrix metalloproteinases that degrade proteins of the extracellular matrix (Tatakis 1993). These findings can explain the significantly higher bone loss induced by *P. gingivalis* 53977 infection.

In summary, our study shows that infection of mice with different *P. gingivalis* strains can induce different levels and patterns of humoral and cytokine responses.

These results are in accordance with ABL found in each strain, and may explain the variances in virulence that were found between the tested strains. These findings may imply that today's bacterial tests looking for the presence of *P. gingivalis* may provide us with only partial information regarding the risk. Future microbiological tests should consider that the virulence of a periodontal pathogen may vary between strains, and is correlated to its ability to evade the humoral immune response as well as activate inflammation.

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Clinical Relevance

Scientific rationale for the study: Oral infection with *P. gingivalis* induced experimental periodontitis in mice. However, the correlation between the virulence of different strains and the mouse immune response needs further investigation.

Principal findings: *P. gingivalis* 53977-infected mice showed the highest ABL, which was significantly different from the other infected groups. ABL was correlated with an elevated pro-inflammatory response and inversely correlated with the humoral response.

Practical implications: Future microbiological tests should consider that the virulence of a periodontal pathogen may vary between strains, and is correlated with its ability to evade the humoral immune response as well as activate inflammation.