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Journal	Clinical and vaccine immunology : CVI, 18(5): 888-891
URL	http://hdl.handle.net/10130/2707
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HGP44 induces protection against *Porphyromonas gingivalis*-induced alveolar bone loss in mice

Running title: HGP44 protects against *P. gingivalis*-induced bone loss

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Abstract

The protective effect of DNA vaccines expressing the arg-gingipainA domain against bone loss induced by *Porphyromonas gingivalis* infection was investigated in a murine model. phgp44, which expresses the 44-kDa-adhesion/hemagglutinin domain of arg-gingipain A, prevented *P. gingivalis*-induced alveolar bone loss. The results indicate that phgp44 could be a candidate antigen for a vaccine against *P. gingivalis* infection.

Periodontitis, a highly prevalent chronic inflammatory disease which causes irreversible destruction of the supporting tissue of the teeth, affects more than 30% of the adult population (19). Periodontitis has also been reported to be involved in the development of systemic diseases such as bacterial endocarditis, atherosclerosis and diabetes (13).

Porphyromonas gingivalis is a major pathogen of chronic periodontitis (5, 21). *P. gingivalis* expresses several virulence factors, including proteases, fimbriae and endotoxin (1, 9, 18). Arg-gingipainA (RgpA) is a major virulence factor of *P. gingivalis* (16). RgpA is involved in activation of complement and bradykinin, and degradation of C3b, IL-8, IgG and MCP-1 (9). These activities may play an important role in the virulence of *P. gingivalis.* RgpA consists of a preproprotein, a catalytic domain and an adhesin/hemagglutinin (HA) domain which consists of HGP44, HGP15, HGP17, and HGP27 (Fig. 1). This HA domain has similarity to hemagglutinin A (HagA) genes and the HA domains of the lysine-specific gingipain (Kgp) (16). Antibody against gingipain was reported to have a protective effect against infection by *P. gingivalis* (7, 15, 23). In the present study, we investigated the protective effect of *rgpA*-domain DNA vaccines against *P. gingivalis*-induced alveolar bone loss, with the aim of clarifying their potential as candidate antigens for a novel vaccine.

We investigated the protective effect of immunization with *rgpA*-domain vaccines (Fig. 1) containing the *rgpA* catalytic domain (pcat), the HGP44 domain coding region (phgp44), the HGP15-27 domains coding region (phgp15-27), or the N-terminal (phgp44H) or C-terminal (phgp44T) halves of the HGP44 domain coding region against infection by *P. gingivalis*. All vaccines were constructed by

self-ligation of amplified fragments from the *rgpA* DNA vaccine (23) with the primers described in Table 1 using LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan). The plasmid used for construction of these vaccines, pVAX1, (Invitrogen, Carlsbad, CA) was used as a control.

Immunization with *rgpA*-domain DNA vaccines was carried out as described previously (23). Briefly, 6-week-old female BALB/c mice were separated into 8 groups of 4 mice each: a non-immunized group, and groups immunized with 2.5 μg *rgpA* DNA vaccine, pcat, phgp44, phgp15-27, phgp44H, phgp44T, or pVAX1 alone via the skin of the abdomen by a Gene Gun (Bio-Rad Laboratories, Hercules, CA), weekly for 5 weeks. Additional immunization with phgp44H and phgp44T was performed at 6 weeks as the antibody titers of the mice immunized with the DNA vaccines had not reached a plateau at 5 weeks. Levels and reactivity of antibodies against RgpA at days 0, 28, 35 and 42 after immunization were determined by ELISA and immunoblotting. Approval to conduct these studies was obtained from the Animal Use Committee of Tokyo Dental College (Chiba, Japan).

The protective effect of the vaccinations against *P. gingivalis* infection was investigated according to the method of Baker et al (2). Briefly, the mice were challenged with *P. gingivalis* at 6 weeks after the first immunization. Initially, the mice were given 5 mg each of kanamycin and ampicillin by gavage, once a day for 4 days. After a 3-day antibiotic-free period, all except the non-immunized control mice were orally infected with 1×10^9 CFU *P. gingivalis* ATCC 33277 in 2% carboxymethylcellulose. Challenge was carried out 3 times at 2-day intervals. Forty-two days after the last challenge, the mice were sacrificed and alveolar bone loss was assessed by at the defined landmark sites on the maxillary molars as described

previously (8). We performed measurements (14 sites) on each skull from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) with a stereomicroscope. Measurements were made under a dissecting microscope fitted with a video image-maker ,measurement system, MS-803 (MORITEX Co., Tokyo, Japan), standardized to yield measurements in millimeters. The 4 non-infected and non-immunized mice were used to determine the baseline value from the CEJ to the ABC in normal mice. The experiments were repeated to confirm reproducibility, and a one-way ANOVA and the Turkey post hoc tests were used to make multiple comparisons between groups in terms of antibody titers and protective effects against bone loss using the pooled data from the experiments.

Antibody titers elicited by the DNA vaccine plasmids pcat, phgp44, or phgp15-27 are shown in Fig. 2A. Significant elevation of specific IgGs against *P. gingivalis* was observed to similar levels in mice immunized with the *rgpA* DNA vaccine and in those immunized with phgp44. Only a small increase was seen in antibody levels with phgp15-27, and that with pcat was almost the same level as that in the controls. As shown in Fig. 2B, the specificities of IgG in mice immunized with pHGP44 or *rgpA* DNA vaccine were evaluated by immunoblots. In serum from mice immunized with the *rgpA* DNA vaccine, 52.6-, 43.8-, 40.8-, 33.5-, and 14.5-kDa bands were observed. In serum from mice immunized with phgp44, 43.8-, 33.5- and 14.5-kDa bands were observed. These multiple protein bands may have been degraded fragments of RgpA, Kgp and HagA, which share antigenicity with HGP44. In both groups, the predominant band was the 43.8-kDa band, suggesting high immunogenicity for HGP44. This agrees with the results of earlier reports (10, 12). The epitope for

the protective antibody was reported to be located within HGP44 (12). Twenty-one of 25 amino acid residues of the epitope were contained in phgp44H. The antibody titer of mice immunized with phgp44H was significantly high at week 5 (2.6 ± 1. 43, Fig. 4A) and the reciprocal titer reached 3.9 ± 1.64 at week 6. These results suggest that the N-terminal half of HGP44 is potentially a potent epitope in the induction of protective antibody by RgpA, although further study is required to confirm this.

The effects of immunization with *rgpA*-domain vaccines on *P. gingivalis*-induced alveolar bone loss are shown Fig. 3. The infected mice showed significantly greater alveolar bone loss in the maxillary molar area than did the uninfected control mice. Alveolar bone loss was reduced significantly in both the *rgpA* DNA vaccine-immunized group and the phgp44-immunized group, whereas the pcat- and phgp15-27-immunized groups showed no protection against alveolar bone loss. These results suggest that the HGP44 domain coding region plays a predominant role in the *rgpA* DNA vaccine. The pattern of protection observed in the present study is in accordance with earlier results showing that passive immunization with monoclonal antibody against *P. gingivalis* protected against recolonization by *P. gingivalis* (3). Specific IgGs against *P. gingivalis* RgpA protected against colonization and alveolar bone loss in a murine model (3, 6, 7, 15, 23). The HGP44 domain was involved in adherence to epithelial cells (4) and *Treponema denticola* (11). Moreover, antibody elicited by a *rgpA* DNA vaccine inhibited binding of *P. gingivalis* to collagen sponges and hemagglutination of *P. gingivalis* (23). Antibody induced by a repeated sequence in the HGP44 domain inhibited binding of the RgpA-Kgp complex to fibrinogen, fibronectin and collagen type IV (17). Antibody against anti-HGP44 also

enhanced opsonization and killing of both invasive and noninvasive strains of *P. gingivalis* (22). These results agree with the protective effects we deomonstated with phgp44.

In this study, phgp44H was observed to exert a protective effect in comparison with the non-immunized mice, but not in comparison with pVAX-immunized mice (Fig. 4B). The antibody titer of phgp44H immunized mice was lower than that of phgp44 immunized mice (Fig 4A). Further study is required to determine the dose and rate of immunization required to induce protection against infection by *P. gingivalis*. Taken together with the results of an earlier study, the present results suggest that antibody against the N-terminal half of the HGP44 domain has a major protective effect.

The protective effect of phgp44 in the present experiments was somewhat lower than that of the *rgpA* DNA vaccine, although the difference was not significant. Kuboniwa et al. (14) reported that a DNA vaccine encoding the catalytic subunits of Rgp and Kgp elicited antibody production. The antibody attenuated protease activity and showed a protective effect against lethal challenge by *P. gingivalis*. Genco et al. (6) reported that an N-terminal peptide of the RgpA catalytic domain showed a protective effect against colonization by *P. gingivalis*. One study reported that a T cell epitope in the catalytic domain of Kgp induced Th2 responses (20). It is possible that an additional effect in response to the catalytic subunit is necessary to elicit the same protective effect as that obtained with the *rgpA* DNA vaccine. Hgp44 was reported to be involved in the adherence by this microorganism (4, 11). Colonization by *P. gingivalis* was also reported in the mice model (2). It is possible that the protection against colonization by *P. gingivalis* plays a major role in the inhibition of bone loss in the present study. Based upon our results, we cannot exclude the

possibility that the proteolytic activity of RgpA plays an important role in bone loss since protection mediated by the adhesive and proteolytic domains may not be additive. Therefore, further analysis is required to investigate the effects of antibody against the catalytic subunit on bone loss.

In the present study, the HGP44 domain coding DNA vaccine could account for the protective effect of the *rgpA* DNA vaccine. The results of the present study indicate that phgp44 has the ability to induce protective immunity against *P. gingivalis*-induced alveolar bone loss and that the Hgp44 coding region is a candidate antigen for a DNA vaccine.

The authors would like to thank Professor Howard K. Kuramitsu for a critical review of this manuscript and Associate Professor Jeremy Williams for his assistance with the development of the manuscript. This study was supported by Grant HRC7 from the Oral Health Science Center of Tokyo Dental College, and a High-Tech Research Center Project for Private Universities Matching Fund Subsidy from MEXT, 2006-2010.

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Table 1 Primers used in this study

Primer name	Sequence
Metup	5’-CATGGTCGCTAGCTAGCCAGCTTGGGT-3'
Taildown	5’-TAATTCTGTCTTGGACTCGGAGCTCGAGTCTAG-3'
44down	5’-AGCGGTCAGGCCGAGATTGTTCTTGAA-3'
Catup	5’-GCGAAGAAGTTCGGGGGCATCGCTGACTGACA-3'
15down	5’-CGCAGACTTCACGGAAACGTTCGAGTCTTCTAC-3'
44up	5’-CGCTTGCCGTTGGCCTTGATCTCAACCTCATCA-3'
44Tdown	5’- CAGAACCTGACCGGTAGTGCAGTCGGCCAGA-3’
44Hup	5’- TACAGGAGCAAATTCATTGGATCCTTCTACC-3’

Underlining indicate start codon and termination codon in Metup and Taildown, respectively.

Figure legends

Fig. 1 Map of cloned *rgpA* in the *rgpA* DNA vaccine and primers used to construct the *rgpA* domain DNA vaccines. Small arrows below the map indicate the location of primers used in this study. Large arrows indicate fragments expressed by the DNA vaccines.

Fig. 2

A. Induction of *P. gingivalis*-specific IgGs in mice immunized with *rgpA*-domain DNA vaccines.

Serum IgG titers of mice against sonicates of *P. gingivalis* were determined on day 42 after primary immunization, and endpoint titers were evaluated by measuring serially diluted serum. Results represent mean ± standard deviation of $\log_2$ ELISA antibody titers. *$p < 0.05$ by one-way ANOVA compared with mice immunized with pVAX1.

B. Immunoblot analysis with serum from mice immunized with *rgpA* DNA vaccine or phgp44.

Sonicates of *P. gingivalis* were separated by SDS-PAGE and transferred to PVDF membranes. Blotted membranes were immunostained using serum from mice immunized with the *rgp*A DNA vaccine or phgp44. Lanes 1, 3: molecular size markers; Lane 2: serum from mice immunized with *rgpA* DNA vaccine; and lane 4: serum from mice immunized with phgp44. Molecular mass markers are shown in kilodaltons.

Fig. 3

Levels of alveolar bone loss elicited following *P. gingivalis* oral challenge after

immunization with the *rgpA*-domain DNA vaccine.

BALB/c mice immunized with *rgpA* DNA or *rgpA*-domain DNA vaccines. Control groups consisted of age-matched, non-vaccinated mice and pVAX1 immunized mice. After immunization, mice were orally challenged with *P. gingivalis* ATCC33277. At 42 days after oral challenge, all mice were sacrificed. *$p < 0.05$ by one-way ANOVA compared with mice infected by *P. gingivalis* without immunization. §$p < 0.05$ by one-way ANOVA compared with mice infected by *P. gingivalis* and immunized with pVAX1.

Fig. 4

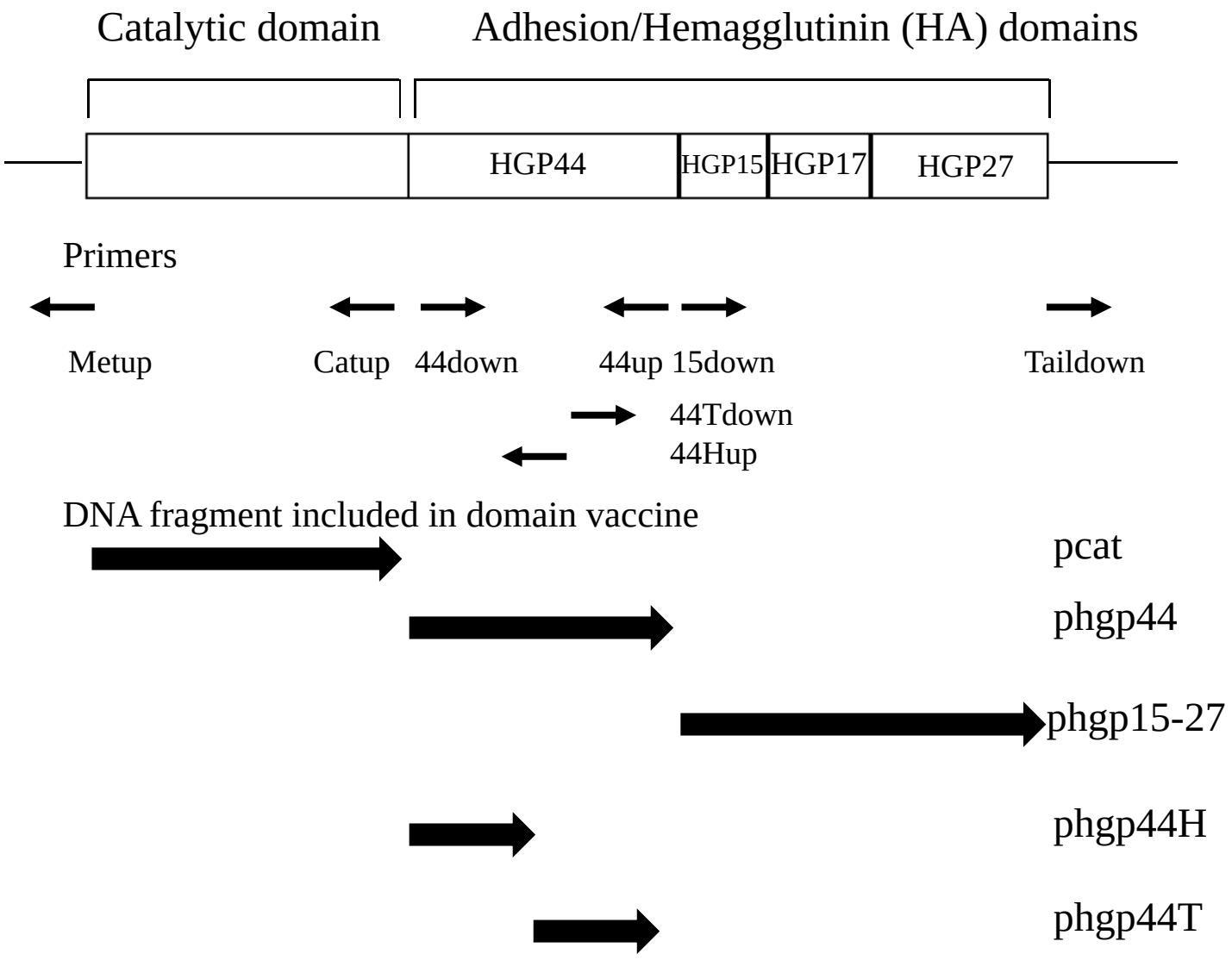
A. Induction of *P. gingivalis*-specific IgGs in mice immunized with *rgpA*-domain DNA vaccines.

Serum IgG titers of mice against sonicates of *P. gingivalis* were determined on day 43 after primary immunization and endpoint titers were evaluated by measuring serially diluted serum. Results represent mean ± standard deviation of $\log_2$ ELISA antibody titers. *$p < 0.05$ by one-way ANOVA compared with mice immunized with pVAX1.

B. Levels of alveolar bone loss elicited following *P. gingivalis* oral challenge after immunization with the phgp44 derivative.

BALB/c mice immunized with *rgpA* DNA or *rgpA*-domain DNA vaccines. Control groups consisted of age-matched, non-vaccinated mice and pVAX1 immunized mice. After immunization, mice were orally challenged with *P. gingivalis* ATCC33277. At 42 days after oral challenge, all mice were sacrificed. *$p < 0.05$ by one-way ANOVA compared with mice infected by *P. gingivalis* without immunization. §$p < 0.05$ by one-way ANOVA compared with mice infected by *P. gingivalis* and immunized with

pVAX1.


Catalytic domain
Adhesion/Hemagglutinin (HA) domains
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HGP15
HGP17
HGP27
Primers
Metup
Catup
44down
44up
15down
Taildown
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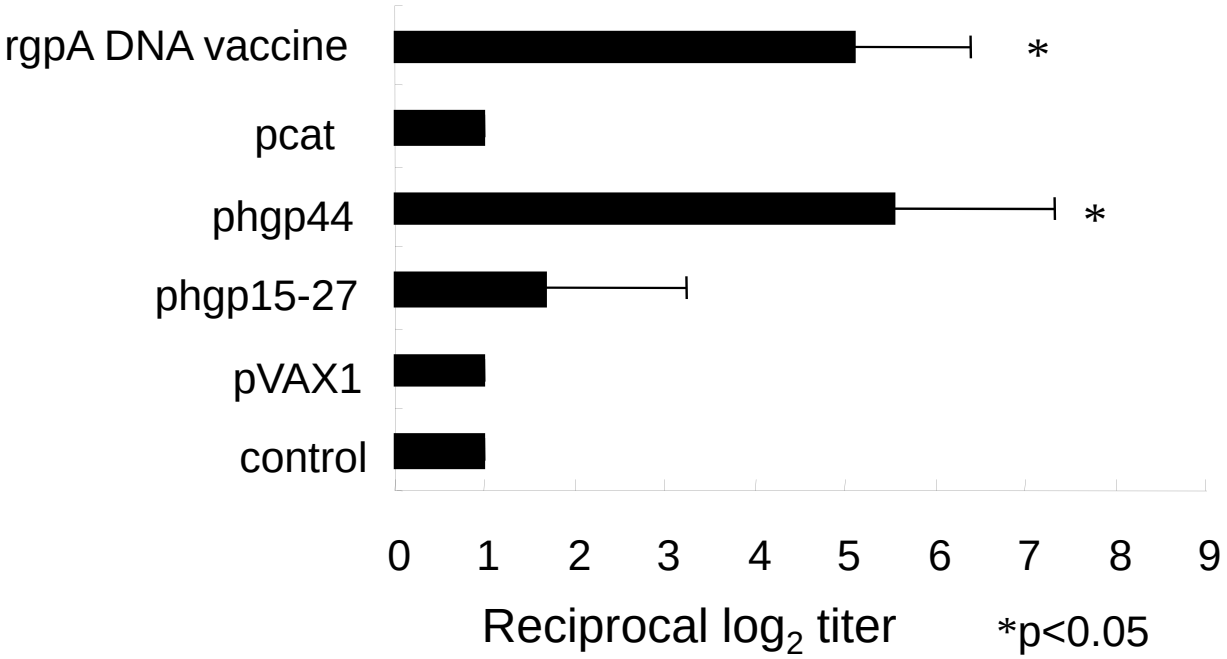
44Hup
DNA fragment included in domain vaccine
pcat
phgp44
phgp15-27
phgp44H
phgp44T

Fig. 1

A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pcat
phgp44
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pVAX1
control
*
*
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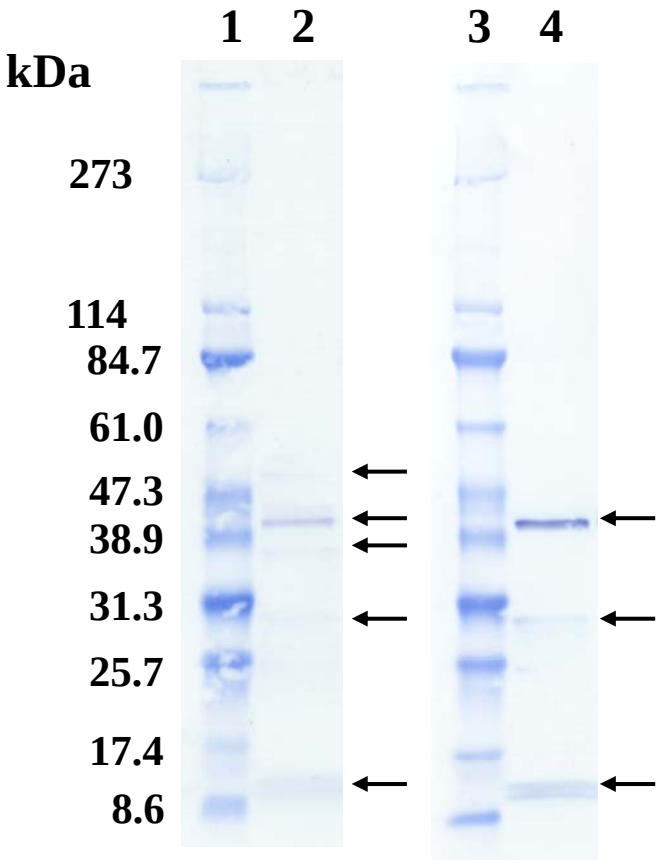
Reciprocal log2 titer
*p<0.05

B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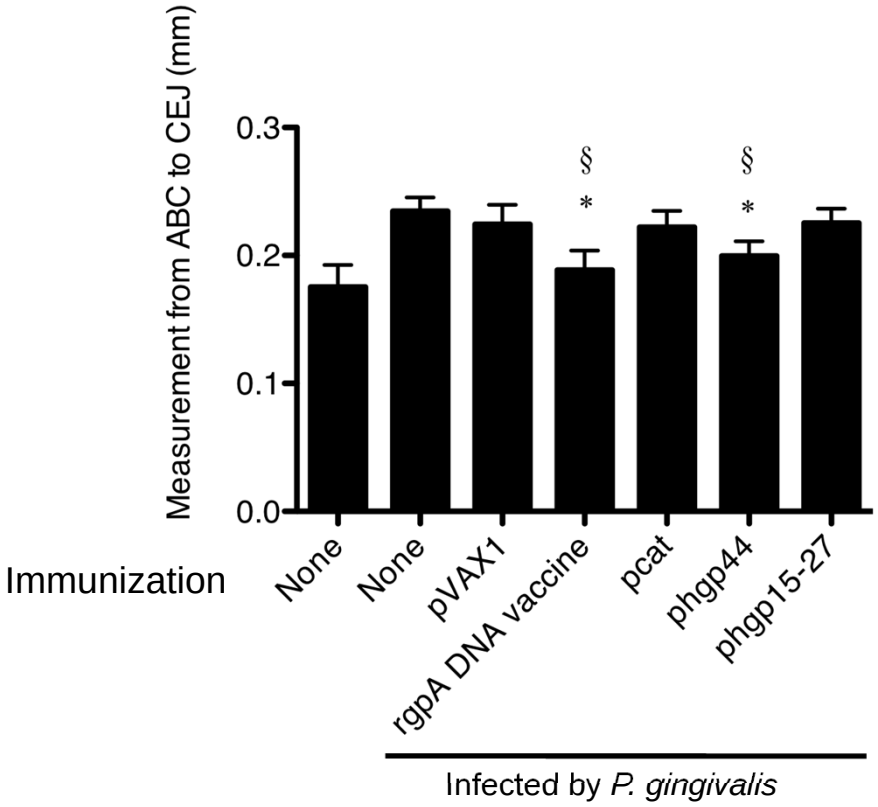
8.6

Fig. 2


Measurement from ABC to CEJ (mm)
0.3
0.2
0.1
0.0
§
*
§
*
Immunization
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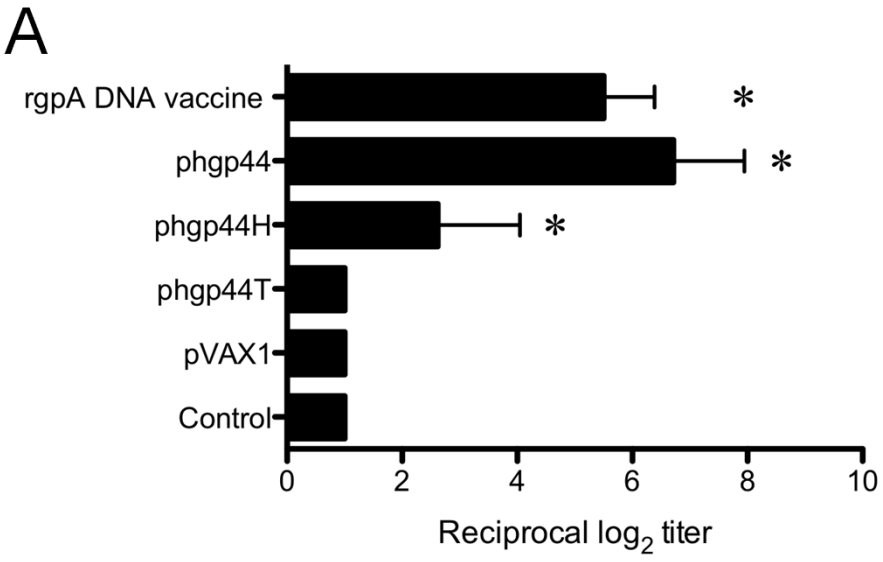
None
pVAX1
rgpA DNA vaccine
pcat
phgp44
phgp15-27
Infected by P. gingivalis

Fig. 3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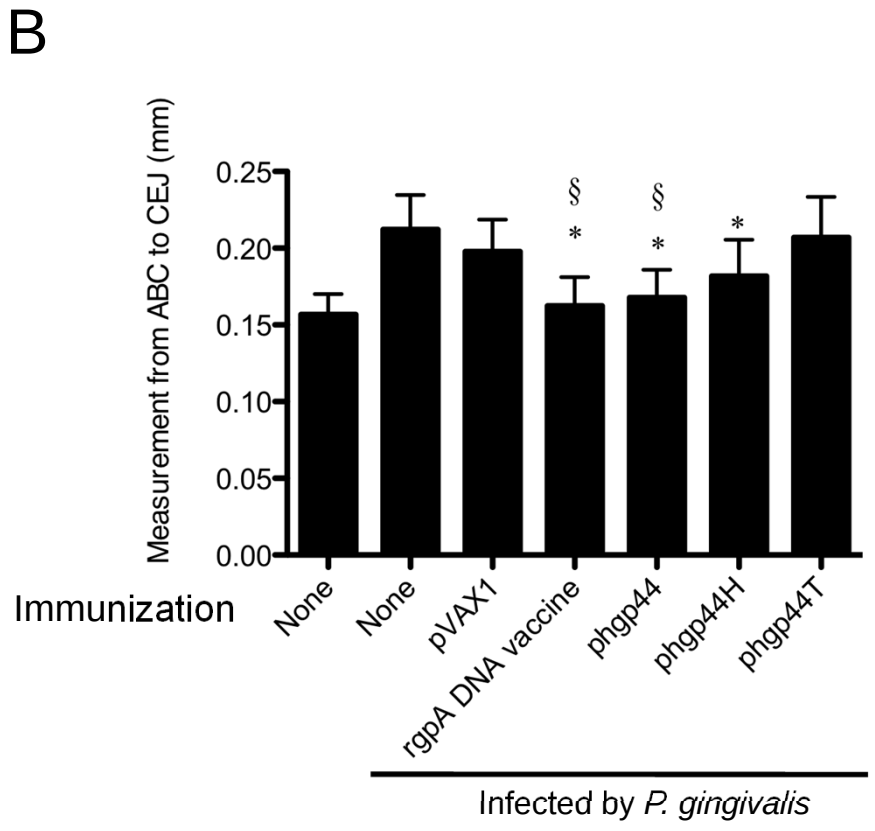
B
Measurement from ABC to CEJ (mm)
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0.10
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*
Immunization
None
None
pVAX1
rgpA DNA vaccine
phgp44
phgp44H
phgp44T
Infected by P. gingivalis

Fig. 4