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Immunization by Arg-gingipain A DNA vaccine protects mice against an invasive *Porphyromonas gingivalis* infection through regulation of interferon-γ production

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We previously demonstrated that a *Porphyromonas gingivalis rgpA* DNA vaccine induced protective immune responses against *P. gingivalis* infection in mice (Yonezawa et al. Infect Immun 2001: 69: 2858–2864). In the present study, reduction in lethality against infection by lethal doses of *P. gingivalis* was observed in the *rgpA* DNA vaccine-immunized mice. Cytokine levels in the mouse model with nonlethal doses of infection by *P. gingivalis* were evaluated to analyze the mechanism of protection by immunization with the *rgpA* DNA vaccine. After nonlethal challenge with invasive *P. gingivalis* W50, production of interleukin (IL)-2, IL-4, IL-5 and IL-12 was elevated; however, interferon (IFN)-γ was lower in the serum of the DNA vaccine-immunized mice than in the serum of nonimmunized mice. The regulation of IFN-γ production elicited by immunization with the *rgpA* DNA vaccine may play a significant role in protection against *P. gingivalis* infection in mice.

Key words: DNA vaccine; interferon-γ; *rgpA*

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Periodontitis is an infectious disease which causes chronic inflammation of the supporting tissue of teeth, accounting for a significant proportion of tooth loss in humans (2, 15, 23). One of the major pathogens of this disease is *Porphyromonas gingivalis.* Cysteine proteinases of *P. gingivalis*, gingipains, cleave synthetic and natural substrates after arginine (21) or lysine residues (20). These enzymes are thought to participate in pathogenicity by activation of host proenzymes such as plasminogen, by exposing host cell cryptotopes for colonization, altering blood clotting (5, 29) and dysregulating host immune responses via degradation of cytokines and their receptors (1, 3, 9, 35, 37).

Several research groups have established that inhibition of the enzyme activity of gingipains severely limits the colonization and infection capability of the microorganism (4, 28). A synthetic peptide based upon the structure of the gingipains stimulates humoral immunity and induces protection against *P. gingivalis* challenge in a murine model (11, 18). However, several reports showed cellular immune responses are intimately associated with *P. gingivalis* infection (6). In a previous study, we have constructed an *rgpA* DNA vaccine (34). Sera from the immunized mice inhibited hemagglutination and adherence to type I collagen by *P. gingivalis*, while vaccination induced an attenuation of abscess formation by *P. gingivalis* in a mouse model.

In the present study, we evaluated the protective potential of this DNA vaccine against a lethal challenge of *P. gingivalis* and also analyzed the induction of cellular immune responses by the DNA vaccine.

Material and methods

Immunization of experimental animals

For the mouse lethality model and evaluation of *in vivo* and *in vitro* cytokine production, female 4–6-week-old BALB/c mice were purchased from Sankyo Laboratories Service Co. Inc. (Tokyo, Japan) and immunized using the same protocol. All experiments described in this study were performed under the guidelines of the Animal Use Committee at Tokyo Dental College (Chiba, Japan). The regions encoding the catalytic and hemagglutinin domains of Arg-gingipain A (RgpA) were amplified by polymerase chain reaction (PCR) with *P. gingivalis* ATCC 33277 genomic DNA as the template. The 5133-bp amplified *rgpA* gene was digested with *Nhe*I and *Xho*I and ligated with vector pVAX1 (Invitrogen, Carlsbad, CA). pVAX1 was also used as the control plasmid. BALB/c mice were immunized with *rgpA* DNA vaccine using a Gene Gun as described previously (34). Briefly, mice received 2.5 μg of DNA administered with a Helios Gene Gun. Mice were immunized at weeks 0, 1, 2, 3, and 4. As controls, nonimmunized mice and those immunized with control plasmid pVAX1 (2.5 μg/mice) by Gene Gun were used. The kinetics of antibody responses were shown in our previous study (34). In that study, we confirmed that the antibody response reached a plateau 5–6 weeks after the first immunization. Increase in antibody was evaluated at day 42 by ELISA (enzyme-linked immunoabsorbant assay) as described previously (34).

Mouse infection model

The protective effects of immunization with the *rgpA* DNA vaccine were evaluated using a mouse model with a lethal challenge of invasive *P. gingivalis* W50. The lethal dose was determined before the experiments as the level that killed the mice after infection with *P. gingivalis* W50. Mice were immunized at weeks 0, 1, 2, 3, and 4. As controls, nonimmunized mice and those immunized with control plasmid pVAX1 (2.5 μg/mice) by Gene Gun were used. Overnight cultures of *P. gingivalis* W50 were harvested, washed three times with phosphate-buffered saline (PBS) and resuspended at a concentration of 3×10^{10} CFU in 200 μl of PBS. At the 49th day following the first immunization, 12 vaccinated mice were challenged with 3×10^{10} colony forming units (CFU) of *P. gingivalis* by intraabdominal injection. Eleven nonimmunized mice and 13 control plasmid injected mice were also challenged with the same dose of *P. gingivalis* as controls. Mice were then monitored for health status following challenge with *P. gingivalis* for 72 h.

Evaluation of *in vivo* cytokine profiles following nonlethal dose infection of *P. gingivalis*

To study the effects of cytokine production *in vivo*, a mouse model involving challenges with nonlethal doses of *P. gingivalis* was used. Before the experiments, we determined the highest infective dose that was not lethal in the mice. This dose was approximately 5×10^9 CFU. Six immunized mice, six naïve control mice, and four control plasmid immunized mice were used for this experiment. Mice were immunized at weeks 0, 1, 2, 3, and 4. As controls, nonimmunized mice and those immunized with control plasmid pVAX1 (2.5 μg/mice) by Gene Gun were used. On the 49th day following the first immunization, the DNA vaccine immunized, control plasmid injected and nonimmunized mice were intraabdominally infected with nonlethal doses (5×10^9 CFU in 200 μl) of *P. gingivalis* W50. After infection, sera were collected from mice 30 min, 2 h, 7 h, 12 h, and 24 h after challenge. The levels of cytokines in the mouse serum were evaluated as described below.

Serum collection. Blood samples were obtained from each mouse on day 0 (before the first immunization) and on day 42. On day 49 following the first immunization, blood samples were also obtained from mice infected with nonlethal doses of *P. gingivalis* 30 min, 2 h, 7 h, 12 h, and 24 h after challenge. The sera were collected by centrifugation following clotting at 4°C. Anti-RgpA immunoglobulins in all serum samples were determined by ELISA as described previously (34).

Evaluation of *in vitro* cytokine production

To evaluate the effects of *rgpA* DNA vaccination on cytokine production *in vitro*, the cytokine-producing activity of mouse spleen cells was measured. Mice were immunized at weeks 0, 1, 2, 3, and 4. As controls, nonimmunized mice and those immunized with control plasmid

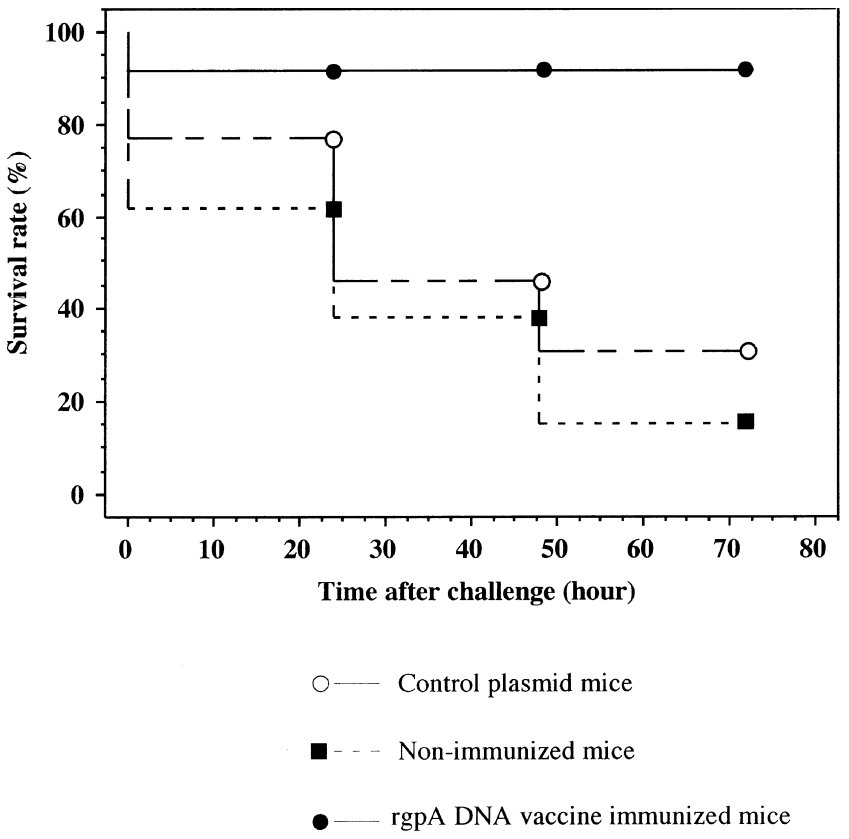

Fig. 1. Effects of plasmid DNA immunization against lethal challenge with *P. gingivalis* in female BALB/c mice. Mice immunized with the *rgpA* DNA vaccine were intraperitoneally challenged with viable *P. gingivalis* W50 (3×10^{10} CFU). Control plasmid pVAX1 or nonimmunized mice were used as controls. Survival was monitored over 72 h.

pVAX1 (2.5 μg/mice) by Gene Gun were used. Spleen cells from mice immunized with *rgpA* DNA vaccine or control mice were isolated 42 days after initial immunization and cell suspensions were prepared following lysis of erythrocytes. Cells were washed three times with PBS and resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Spleen cells (5×10^6/ml) were incubated in 24-well microtiter plates at 37°C in air containing 5% CO_2. After culturing for 36 h, 2 μl of sonic extracts of *P. gingivalis* were applied to the spleen cell cultures and incubated for 16 h. Cell cultures were collected and centrifuged at 15,000 × *g* for 15 min. The supernatant fluids were also collected and processed for cytokine assays. Bovine serum albumin (BSA) was used to monitor responses against nonspecific antigens. After collecting the culture supernatants, IL-2, IL-12, and IFN-γ were determined by the ELISA system described below.

Evaluation of cytokine profile

The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α and IFN-γ from sera as well as IL-2, IL-12, and IFN-γ from culture supernatants of spleen cells were then measured using an ELISA system according to the manufacture's recommended procedures (ENDOGEN Mouse ELISA, Endogen, Woburn, MA).

Results

Antibody titer after *rgpA* DNA immunization

Antibody titer was evaluated by ELISA 6 weeks after the first immunization in all mice immunized with *rgpA* DNA vaccine. The mean reciprocal $\log_2$ titer of antibody against *P. gingivalis* immunized mice was 4.7 ± 0.68 and the levels are almost the same as for serum obtained in our previous study (34). The reciprocal titer of *P. gingivalis*-specific antibodies was approximately zero in the naïve mice and mice immunized with control plasmid.

Protective effect of *P. gingivalis* immunization against mouse lethal challenge

Up to 72 h, the challenge killed 9 of 11 mice which were not immunized and 9 of 13 mice which had been injected with intact pVAX1 (survival rates were 18.2% and 30.8%, respectively). On the other hand, 11 of 12 mice vaccinated with the *rgpA* DNA vaccine survived up to 72 h (survival rate was 91.7%) (Fig. 1). The survival rate of the *rgpA* DNA vaccine immunized mice at 72 h was significantly higher than that of naïve control and control plasmid injected mice ($P < 0.0005$ and $P < 0.005$, respectively, by χ^2 test).

Levels of inflammatory cytokines

The TNF-α levels were slightly elevated up to 7 h after infection and IL-1β levels were low 7–12 h after infection. These changes, however, were not statistically different (Fig. 2).

In vivo Th1 cytokine profile

The Th1 cytokine levels, such as those for IFN-γ, IL-2, IL-12, in mice immunized with the DNA vaccine were evaluated after *P. gingivalis* infection *in vivo*. In mice immunized with the DNA vaccine, the levels of IFN-γ were statistically lower than levels in the sera of control mice from 7 to 12 h (Fig. 3a). A peak of IFN-γ levels in control sera was observed around 12 h after *P. gingivalis* challenge but the IFN-γ levels of the vaccine immunized mice showed no marked increase. This result was still unchanged up to 48 h after *P. gingivalis* challenge (data not shown). In contrast, the levels of IL-2 production following infection of *P. gingivalis* were significantly higher in sera from vaccinated mice up to 7 h (Fig. 3b). The IL-2 levels of nonimmunized mice were slightly increased 30 min after *P. gingivalis* challenge. Interestingly, IL-2 levels increased in the sera from mice injected with control plasmid, pVAX1, compared with sera from nonimmunized mice. The IL-2 titers of sera from vaccinated mice or control

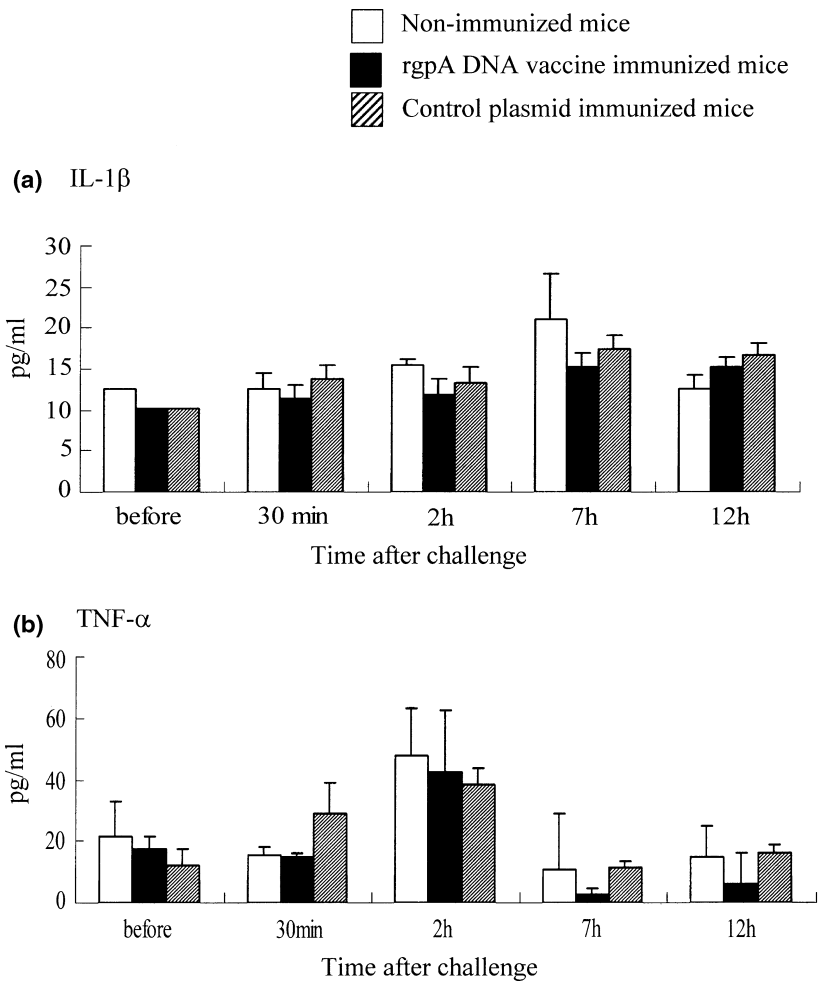

Fig. 2. Response and time course analysis of inflammatory cytokines in mouse sera following challenge with *P. gingivalis* W50 (5×10^9 CFU/200 μl). Serum samples were collected from mice 0 min, 30 min, 2 h, 7 h, and 12 h after challenge. The production of IL-1β (a) and TNF-α (b) in sera from mice was measured using ELISA. Data are expressed as mean ± standard deviation.

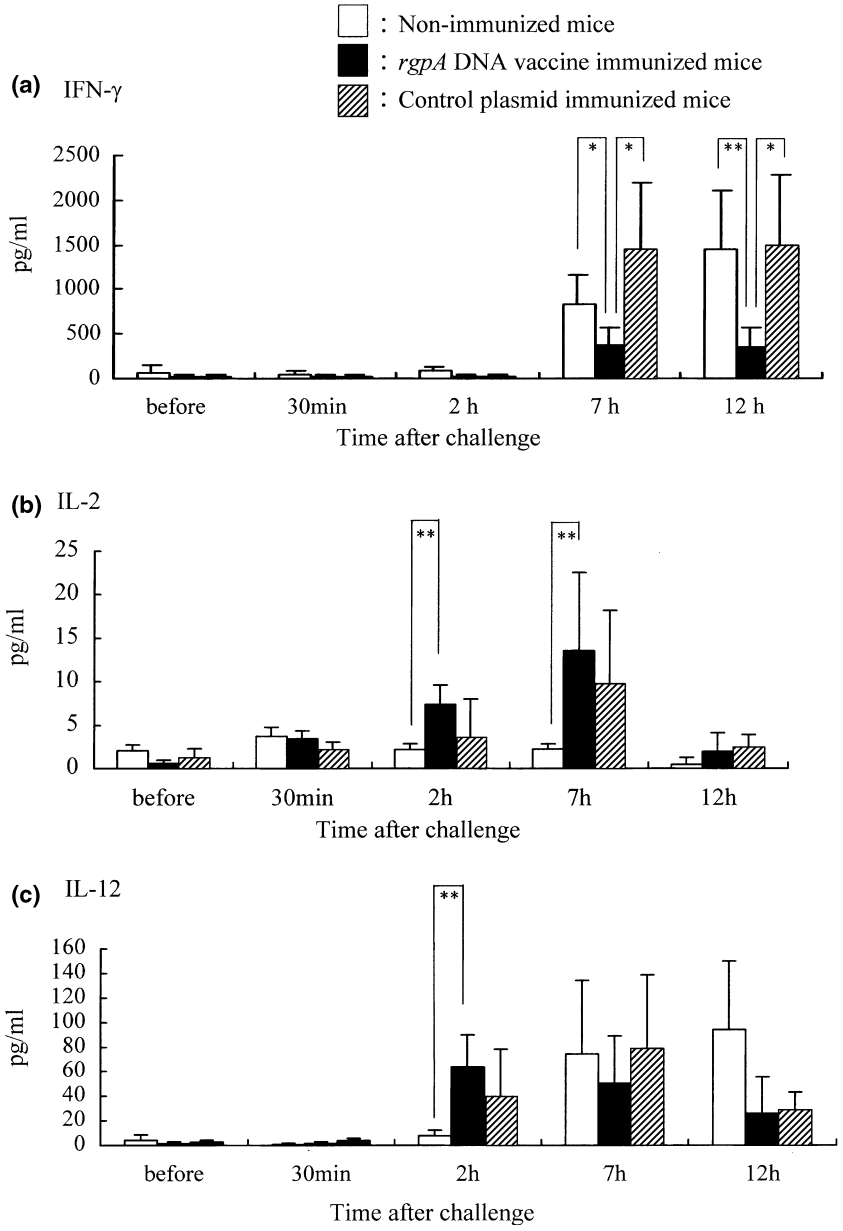

Fig. 3. Dose–response and time course analysis of Th1 type cytokines in mouse sera following challenge with *P. gingivalis* W50 (5×10^9 CFU/200 µl). Serum samples were collected from mice 0 min, 30 min, 2 h, 7 h, and 12 h after challenge. The production of IFN-γ (a), IL-2 (b), or IL-12 (c) in sera from mice was measured using ELISA. Data are expressed as mean ± standard deviation. *Significantly different by Mann–Whitney *U*-test ($P < 0.05$). **Significantly different by Mann–Whitney *U*-test ($P < 0.005$).

plasmid injected mice were near preinfection levels 12 h after challenge. IL-12 production in DNA vaccine immunized mice was increased relative to nonimmunized mice at 2 h after challenge but did not increase up to 7 h (Fig. 3c).

In vitro Th1 cytokine profile

In vitro cytokine release was not detected in spleen cells from vaccinated mice and control mice after stimulation by BSA (data not shown). Following stimulation with sonic extracts of *P. gingivalis,* spleen cells from the DNA vaccine immunized mice produced about 40-fold more IFN-γ than did spleen cells from the nonimmunized mice spleen cells (Fig. 4a). The release of IL-2 in spleen cells from immunized mice was also significantly higher than that of controls (Fig. 4b). However, IL-12 production of immunized spleen cells was not different from that of the control groups (Fig. 4c). In control plasmid immunized mice, levels of IFN-γ and IL-2 were increased.

In vivo Th2 cytokine profile

We also investigated the induction of the levels of Th2 cytokines such as IL-4, IL-5, IL-6, and IL-10 in sera following *P. gingivalis* infection *in vivo*. Two distinct cytokine profiles were noted based on the expression of selected Th2 cytokines. IL-10 levels increased in all groups 7 h after challenge (Fig. 5b). The sera from animals immunized with the DNA vaccine exhibited elevated levels of IL-10 at 12 h compared with sera from nonimmunized mice ($P < 0.05$) (Fig. 5b). IL-5 production in the control groups was not significantly altered after challenge (Fig. 5a). At 12 h, the sera from animals immunized with the DNA vaccine contained significantly higher levels of IL-5 compared with controls ($P < 0.05$). For IL-4 and IL-6, all of the groups following challenge with *P. gingivalis* demonstrated transient increases in IL-4 and IL-6 levels at 2 h (Fig. 5c, d). The vaccinated mice sera showed significantly higher levels of IL-4 expression at 2 h compared with control mice sera ($P < 0.005$). However, production of IL-4 quickly decreased 7 h after challenge in sera from the DNA vaccine immunized mice.

Discussion

Trials for vaccination against periodontitis using components of *P. gingivalis* have been performed. Gibson et al. (12) reported that immunization with RgpA stimulates the production of hemagglutinin domain-specific antibodies, which contributes to the prevention of *P. gingivalis*-mediated periodontal disease. Rajapakse et al. (25) demonstrated that immunization with RgpA-kgp restricted colonization by *P. gingivalis* and periodontal bone loss in the rat. We and other groups showed the protective effect of *rgpA* or *kgp* DNA vaccines (13, 34). This indicated that an *rgpA* DNA vaccine could be a candidate for development of a human vaccine for periodontitis. The increase of the survival rate in the mouse lethality challenge model demonstrated the protective immune response generated by the DNA vaccine against *P. gingivalis* infection and agreed with the results obtained recently in a murine abscess model (34). It is possible that the protective effect was primarily induced by specific antibody against RgpA. Genco et al. (11) also reported the protective effect of induction of anti-RgpA

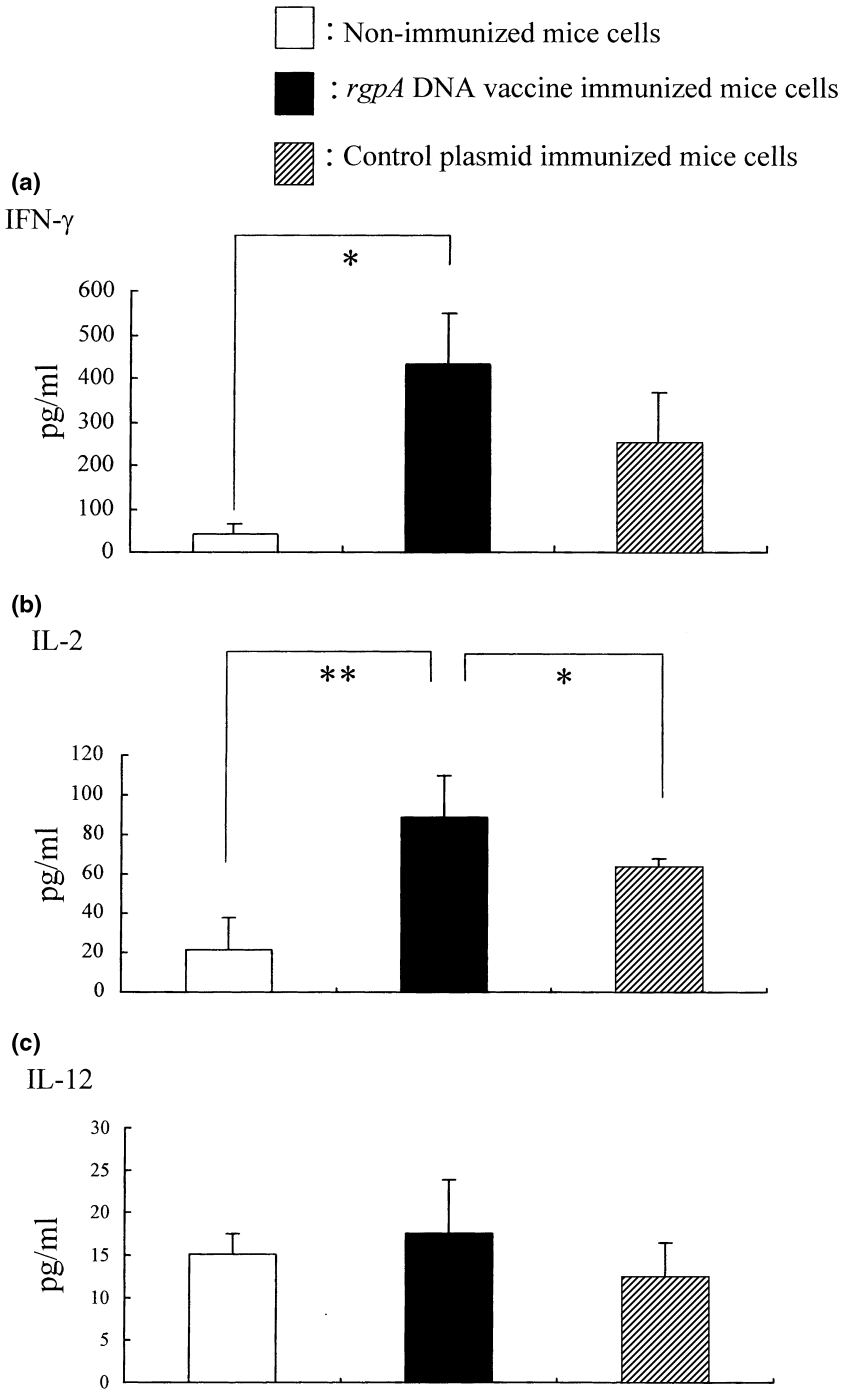

Fig. 4. Th1 type cytokine levels in supernatants of stimulated murine spleen cell cultures. Mice were immunized with *rgpA*-DNA vaccine or control plasmid at weeks 0, 1, 2, 3, and 4. Sonic extracts of *P. gingivalis* (2 μg) were added to spleen cell cultures obtained from the DNA vaccine immunized mice, control plasmid immunized mice, and naïve mice, and were incubated for 16 h. The cell culture supernatants were collected and evaluated for Th1 type cytokines. The release of IFN-γ (a) or IL-2 (b) from spleen cell cultures was measured using ELISA. Data are expressed as mean ± standard deviation. *Significantly different by Mann–Whitney *U*-test ($P < 0.05$). **Significantly different by Mann–Whitney *U*-test ($P < 0.005$).

antibody following immunization with an RgpA peptide.

We have explored the mechanism of protection following immunization by evaluating cytokine profiles in the animal model. DNA vaccines are known to induce both humoral and cellular immunity, so it is possible that immunization with DNA vaccines affects the balance between Th1 and Th2 type reactions. To evaluate this balance we infected *P. gingivalis* at non-lethal doses and evaluated cytokine levels *in vivo*. Our results indicated that serum IL-12 levels increased 2 h after the challenge and serum IL-2 levels increased 2–7 h after the challenge in immunized mice. However, serum IFN-γ levels in immunized mice were significant lower than in naïve control and control plasmid immunized mice. IL-12, an important factor for the differentiation of Th1 cells (30), has been reported to increase Th1 cell maturation and production of IFN-γ (32). Production of IFN-γ in response to infection is a hallmark of innate and adaptive immunity. Although IFN-γ plays a pivotal role in host defense mechanisms, its excessive release has been associated with the pathogenesis of chronic inflammatory and autoimmune diseases (7, 8, 17, 27). Oberholtzer et al. (19) reported that the expression of IL-10 in dendritic cells using adenovirus vectors increased the survival rate in sepsis. In the present study, of the Th1 type cytokines tested, serum IFN-γ was down-regulated, suggesting that low levels of IFN-γ expression subsequent to challenge with the organism play an important role in the decrease of lethality following *P. gingivalis* infection. It is possible that the down-regulation of serum IFN-γ may reflect the down-regulation of Th1 type responses. Further analysis is required to clarify the relationship between the protective effect of the vaccination and a shift to Th2 immunoresponse reactions.

To analyze the production of Th1 type cytokines, *in vitro* cytokine profiles were evaluated using spleen cells from *rgpA* DNA vaccine-immunized mice, control plasmid-immunized mice, and naïve mice. Production of IFN-γ and IL-2 from spleen cells was induced significantly by sonic extracts of *P. gingivalis* but not by BSA in immunized mice. Therefore, the *rgpA* DNA vaccine strongly induced Th1 type immune responses. We also observed the delayed hypersensitivity reaction using footpad swelling assays in *rgpA* DNA vaccine immunized mice (data not shown). IL-12 and IFN-γ were previously reported to be hydrolyzed by *P. gingivalis* (35). However, Yum et al. (36) also reported that gingipains associated with lipopolysaccharide in the outer membranes or in outer membrane vesicles released from the organism could have stimulatory effects on Th1 responses. In immunized mice, although there is an increase in the *in vitro* IFN-γ production evaluated from spleen cells, the levels in serum are low compared with naïve mice and control plasmid immunized mice. Hydrolysis of IFN-γ by

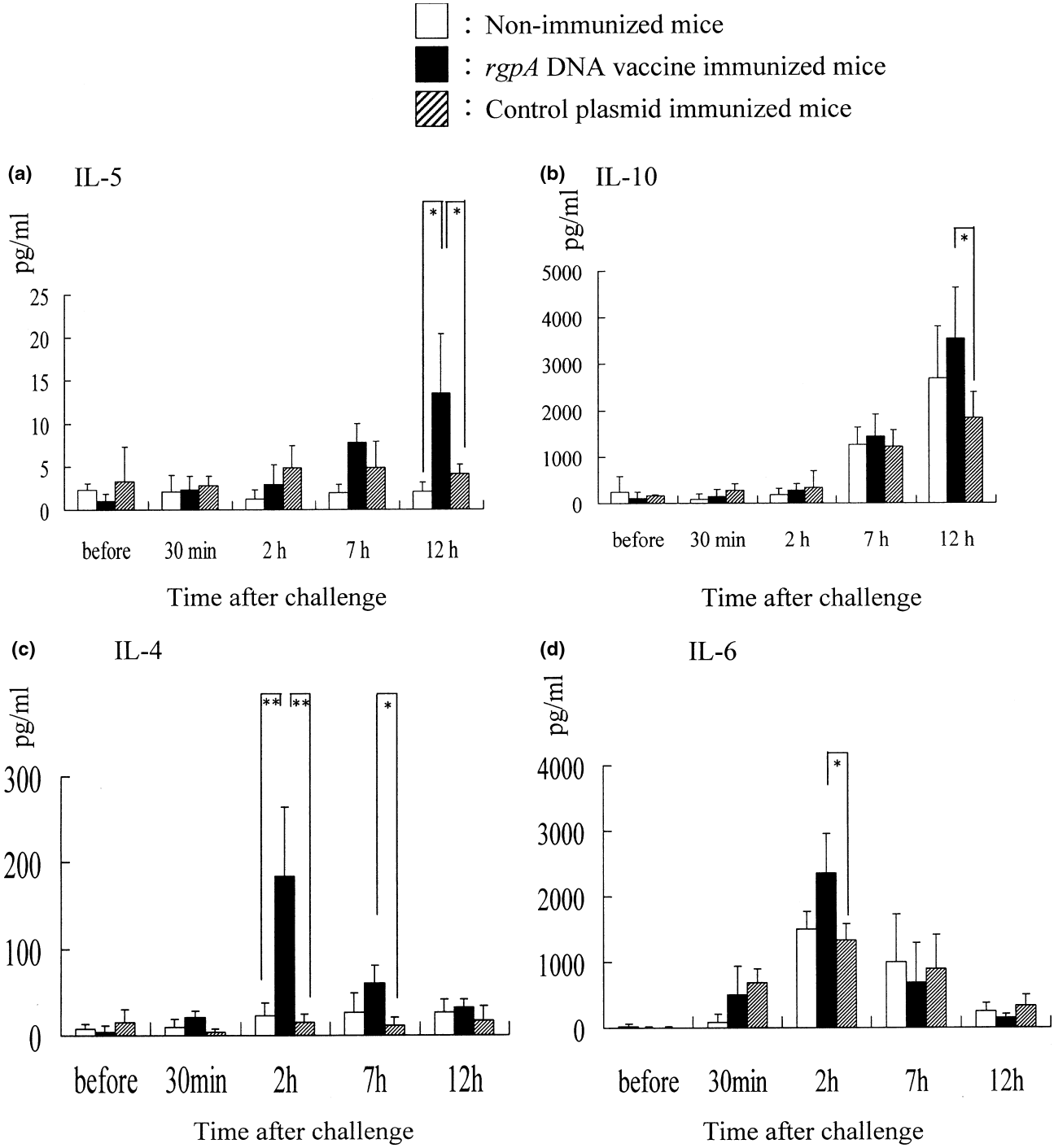

Fig. 5. Dose–response and time course analysis of Th2 type cytokine levels in mouse sera following challenge with *P. gingivalis* W50 (5×10^9 CFU/200 µl). The production of IL-5 (a), IL-10 (b), IL-4 (c), or IL-6 (d) from mice was measured using ELISA. Data are expressed as mean ± standard deviation. *Significantly different by Mann–Whitney *U*-test ($P < 0.05$). **Significantly different by Mann–Whitney *U*-test ($P < 0.005$).

gingipains from *P. gingivalis* could not be implicated in this reaction because the reduction was not observed in naïve mice, suggesting that down-regulation of IFN-γ was induced.

It is possible that the reduction of serum IFN-γ was induced by Th2 type cytokines. Therefore, the levels of Th2 type cytokines were evaluated in the nonlethal dose mouse model. Th2 type cytokines such as IL-4, IL-5, IL-6, and IL-10 were increased in immunized mice *in vivo*. These types of cytokines have been shown to be involved in many aspects of the humoral mouse response. For example, IL-4 and IL-5 stimulate growth and differentiation of B cells. The down-regulation of Th1 inflammatory responses may involve the anti-inflammatory cytokines such as IL-10 and IL-4 (16, 22, 24, 31). It is possible that increased IL-4 and IL-10 production and specific antibodies against RgpA in sera following immunization with the *rgpA* DNA vaccine may contribute to

restraining excessive IFN-γ expression induced by *P. gingivalis* infection and increase the survival rate.

Dysregulation of the immune response by *P. gingivalis* and its components, such as lipopolysaccharide, can induce the host to express a variety of cytokines. Cytokines play a major role in the regulation of immune responsiveness. A balanced regulation of cytokines induced by the injected *rgpA* DNA vaccine may be important in maintaining homeostasis of the immune system after *P. gingivalis* infection. The reduction in IL-4 production at diseased sites could be a common feature of periodontitis (26, 33). In contrast, in the present study *rgpA* DNA vaccine-immunized mice demonstrated an increased IL-4 expression compared to control mice. These results suggested that the humoral immune response to *P. gingivalis* is dominant in the DNA vaccine immunized mice. Several reports suggested that infection by *P. gingivalis* induces Th1 type immunoresponses (10, 14). Based upon these results, it would appear that immunization with the *rgpA* DNA vaccine may induce both humoral and cellular immune responses for protection against *P. gingivalis* challenge. Further analysis involving alterations in the immunization dosages or evaluation of cytokine profiles after infection can now be undertaken. Taken together, the present results suggest that attenuated excessive IFN-γ production in animals immunized with an *rgpA* DNA vaccine may play a key role in protection against *P. gingivalis* infection.

Acknowledgments

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