molecular oral microbiology

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Oral immunization with *Porphyromonas gingivalis* outer membrane protein and CpG oligodeoxynucleotides elicits T helper 1 and 2 cytokines for enhanced protective immunity

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SUMMARY

The aim of this study was to evaluate the efficacy of an oral vaccine containing the 40-kDa outer membrane protein of *Porphyromonas gingivalis* (40K-OMP) and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG ODN) to control oral infection by *P. gingivalis.* Oral immunization with 40K-OMP plus CpG ODN induced significant 40K-OMP-specific serum immunoglobulin G (IgG), IgA, and saliva IgA antibody responses. The 40K-OMP-specific $CD4^+$ T cells induced by oral 40K-OMP plus CpG ODN produced both T helper type 1 (Th1; interferon-γ) and Th2 (interleukin-4) cytokines. Furthermore, increased frequencies of $CD11c^+$ $B220^+$ dendritic cells (DCs) and $CD11c^+$ $CD11b^+$ DCs with upregulated expression of CD80, CD86, CD40, and major histocompatibility complex class II molecules were noted in spleen, Peyer's patches, and cervical lymph nodes. Immunized mice were then infected orally with *P. gingivalis* to determine whether the immune responses induced by oral 40K-OMP plus CpG ODN were capable of suppressing the bone resorption caused by *P. gingivalis* infection. Mice given 40K-OMP plus CpG ODN showed significantly reduced bone loss associated with oral infection by *P. gingivalis*. Oral administration of 40K-OMP together with CpG ODN induces Th1-type and Th2-type cells, which provide help for protective immunity against *P. gingivalis* infection. This may be an important tool for the prevention of chronic periodontitis.

INTRODUCTION

Oral health is threatened by chronic periodontitis destroying periodontal tissues and thereby causing tooth loss (Cutler *et al.*, 1995). Moreover, periodontal diseases have been linked to a number of systemic diseases, such as cardiovascular diseases and diabetes, as well as to osteoporosis (DeStefano *et al.*, 1993; Beck *et al.*, 1996; Grossi & Genco, 1998; Kinane, 1998; Meyer & Fives-Taylor, 1998; Mealey, 1999; Reddy, 2002). The prevention of periodontitis might then be relevant for both oral and systemic health.

Porphyromonas gingivalis has been shown to be one of the major pathogens of chronic periodontitis (Cutler *et al.*, 1995). An outer membrane protein with

molecular mass of 40 kDa produced by *P. gingivalis* is important for the coaggregation activity of *P. gingivalis* (Hiratsuka *et al.*, 1992; Saito *et al.*, 1997; Hamajima *et al.*, 2007). Furthermore, this outer membrane protein (designated 40K-OMP) has been shown to be a hemin-binding protein (Shibata *et al.*, 2003). The 40K-OMP is found in various strains of *P. gingivalis*, and resides both on the cell surface and in extracellular vesicles (Hiratsuka *et al.*, 1992; Saito *et al.*, 1997; Shibata *et al.*, 2003; Hamajima *et al.*, 2007). Previous studies have shown that monoclonal antibodies to recombinant 40K-OMP inhibited coaggregation of several strains of *P. gingivalis* with *Actinomyces oris* (*viscosus*) and possessed complement-mediated bactericidal activity against *P. gingivalis* (Hiratsuka *et al.*, 1992; Saito *et al.*, 1996, 1997; Katoh *et al.*, 2000). These studies suggest that induction of 40K-OMP-specific antibodies in the oral cavity might be a logical approach for the prevention of *P. gingivalis* infection. Indeed, previous studies have demonstrated that nasal administration of 40K-OMP plus non-toxic chimeric enterotoxin adjuvant elicited 40K-OMP-specific secretory immunoglobulin A (IgA) antibodies in saliva, and serum immunoglobulin G (IgG) antibodies, that reduced alveolar bone loss caused by oral infection with *P. gingivalis* (Momoi *et al.*, 2008). Furthermore, when apolipoprotein E-deficient spontaneously hyperlipidemic mice were nasally immunized with 40K-OMP plus cholera toxin (CT) as adjuvant before *P. gingivalis* infection, atherosclerotic plaque accumulation in the aortic sinus was significantly reduced when compared with that in non-immunized mice (Koizumi *et al.*, 2008). These studies indicate that 40K-OMP may be an effective vaccine antigen for the prevention of *P. gingivalis* infection.

It is well established that mucosal immunization can elicit antigen-specific immune responses in both mucosal and systemic compartments. In particular, oral immunization offers several advantages over other antigen delivery systems. First, oral vaccines are easier to administer and are expected to have much greater acceptability than injected vaccines. Second, oral vaccine administration could help to simplify vaccine manufacture, thereby increasing the potential for local vaccine production in developing countries. Third, oral immunizations can be administered by volunteers with limited training, allowing larger numbers of people to be immunized. However, mucosal vaccines, including oral vaccines, generally require the use of adjuvants to enhance specific immunity (Holmgren *et al.*, 2003). Bacterial toxins, such as CT, are commonly used as mucosal adjuvants in animal models; however, toxicity prevents their use in humans (Spangler, 1992). Genetically detoxified CT mutants have been developed by site-directed mutagenesis and they appear to be non-toxic in animal models but retain their adjuvanticity (Yamamoto *et al.*, 2001). Despite this progress, there remains a need for novel safe and effective mucosal adjuvants.

An alternative adjuvant class includes synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides (CpG motifs). The CpG ODN interact with Toll-like receptor 9 expressed by B cells and dendritic cells (DCs), and induce T helper type 1 (Th1) and proinflammatory cytokine responses (Krieg *et al.*, 1995; Klinman *et al.*, 1996). A number of studies have reported that parenteral immunization of animals with various antigens together with CpG ODN as adjuvant induces Th1-type responses, as indicated by high levels of IgG2a antibodies and Th1 cytokines, such as interleukin-12 (IL-12) and interferon-γ (IFN-γ) (Chu *et al.*, 1997; Lipford *et al.*, 1997; Roman *et al.*, 1997; Weiner *et al.*, 1997; Davis *et al.*, 1998). Furthermore, it has been shown that CpG ODN is a potent adjuvant when given nasally (McCluskie & Davis, 1998) or orally (McCluskie *et al.*, 2000).

In this study, we evaluated the efficacy of an oral vaccine to prevent oral infection by *P. gingivalis*. The results suggest that oral 40K-OMP plus CpG ODN is an effective and practical candidate for the induction of antigen-specific antibody responses in both oral mucosal and systemic compartments.

METHODS

Animals

All experiments were performed using female BALB/c mice that were purchased from Sankyo Lab Services (Tokyo, Japan) and were maintained in the experimental facility under pathogen-free conditions. Mice received sterile food and water, and were 8–12 weeks old when used for experiments. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals (Nihon University School of Dentistry at Matsudo).

Antigen and adjuvant

Plasmid pMD125 expressing 40K-OMP was kindly provided by Dr Yoshimitsu Abiko (Nihon University). The 40K-OMP was purified to homogeneity from a cell suspension of *Escherichia coli* K-12 harboring pMD125, as described previously (Kawamoto *et al.*, 1991). The purity of 40K-OMP was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and no contaminating protein bands were noted. Possible residual endotoxin in the preparation was assessed with an LAL pyrochrome kit (Associates of Cape Cod Inc., Woods Hole, MA). The 40K-OMP contained as little as 0.3 pg of endotoxin per mg protein. CpG ODN (5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Coley Pharmaceutical Group, Inc. (Wellesley, MA) and the CT was obtained from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

Immunization groups were primed on day 0 and boosted on days 7 and 14. Before immunization, each mouse was deprived of food for 2 h and then given an isotonic solution (250 μl per mouse). After 30 min, mice were orally immunized with 200 μl phosphate-buffered saline (PBS) containing 200 μg 40K-OMP alone or combined with 10 μg CT or 500 μg CpG ODN. Serum and saliva were collected from each group to examine antigen-specific antibody responses. To evaluate the effects of oral vaccine on alveolar bone loss by *P. gingivalis* infection, mice were orally immunized with 40K-OMP plus CT, 40K-OMP plus CpG ODN or PBS 7 days before oral infection with *P. gingivalis*.

Detection of antigen-specific antibody responses

Antibody titers were determined by enzyme-linked immunosorbent assay (VanCott *et al.*, 1996). Briefly, plates were coated with 40K-OMP (5 μg/ml) and blocked with PBS containing 1% bovine serum albumin. After blocking, serial dilutions of serum or saliva samples were added in duplicate. The starting dilution of serum was $1 : 2^6$, and that of saliva was $1 : 2^1$. Four hours after incubation at room temperature, plates were washed and goat horseradish peroxidase-conjugated anti-mouse γ, γ1, γ2a, γ2b or α heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) with H_2O_2 (Moss, Inc., Pasadena, MD) was added for color development. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 415 nm of 0.1 greater than non-immunized control samples after 15 min of incubation.

ELISPOT employed for assessment of antibody-forming cells

Single-cell suspensions were obtained from the spleen and submandibular gland (SG), as described previously (Mega *et al.*, 1992; van Ginkel *et al.*, 1997). Briefly, SG were carefully excised, teased apart, and dissociated using 0.3 mg/ml collagenase (Nitta Gelatine Co. Ltd., Osaka, Japan) in RPMI-1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan). Mononuclear cells from SG were obtained at the interface of the 50 and 75% layers of a discontinuous Percoll gradient (GE Healthcare UK, Ltd., Amersham Place, Little Chalfont, UK). To assess numbers of antigen-specific antibody-forming cells (AFCs), an ELISPOT assay was performed as previously described (Yamamoto *et al.*, 1998). Briefly, 96-well nitrocellulose plates (BD Biosciences, Franklin Lakes, NJ) were coated with 40K-OMP (5 μg/ml), incubated for 20 h at 4°C, and were then washed extensively and blocked with RPMI-1640 containing 10% fetal calf serum. After 30-min, the blocking solution was discarded and lymphoid cell suspensions at various dilutions were added to wells and incubated for 4 h at 37°C in 5% CO_2 in moist air. Detection antibodies included goat horseradish peroxidase-conjugated anti-mouse γ or α heavy chain-specific antibodies (Southern Biotechnology Associates). Following overnight incubation, plates were washed with PBS and developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 m sodium acetate buffer containing H_2O_2 (Moss, Inc.) to each well. Plates were incubated at room temperature for 25 min and were washed with water. AFCs were then counted with the aid of a stereomicroscope (Olympus, Tokyo, Japan).

Analysis of cytokine responses

Levels of cytokines in $CD4^+$ T-cell culture supernatants from spleen or cervical lymph nodes (CLNs)

were determined by cytokine-specific enzyme-linked immunosorbent assay. $CD4^+$ T cells from spleens and CLNs were isolated with the Imag system (BD Biosciences), as described elsewhere (Hashizume *et al.*, 2008). Briefly, mononuclear cells were mixed with anti-CD4 antibodies and incubated at 4°C for 30 min and then $CD4^+$ T cells were separated using a magnet. $CD4^+$ T cells (2.0×10^6 cells/ml) were cultured with 5 μg 40K-OMP per ml in the presence of T-cell-depleted, mitomycin-treated splenic feeder cells (2.5×10^6 cells) in RPMI-1640 supplemented with 10% fetal bovine serum, 50 μm 2-mercaptoethanol, 15 mm HEPES, 100 U/ml penicillin, and 10 U/ml recombinant IL-2. Cultures were incubated for 5 days at 37°C under 5% CO_2 in air. Levels of IL-4, IL-5, IL-6, and IFN-γ in culture supernatants were determined using commercially available assay kits (Pierce Biotechnology, Inc., Rockford, IL) in accordance with the manufacturer's instructions.

Flow cytometry

DC-enriched cell populations from spleen, CLNs and Peyer's patches (PPs) were prepared as described previously (Jang *et al.*, 2006). Briefly, lymphoid tissues were digested with collagenase D (Roche Diagnostics GmbH, Mannheim, Germany) and DNase I (Roche Diagnostics) in RPMI-1640 supplemented with 10% fetal bovine serum albumin, with continuous stirring at 37°C for 45–90 min. Ethylene diaminetetraacetic acid was added (10 mm final concentration), and the cell suspension was incubated for an additional 5 min at 37°C. Cells were spun through a 15.5% Accudenz (Accurate Chemical & Scientific Corp., Westbury, NY) solution to enrich for DCs. The purity of $CD11c^+$ cells was routinely > 50%. DC-enriched cell populations were analysed for the expression of various cell surface molecules with fluorescence-labeled antibodies. Aliquots of mononuclear cells (0.2×10^6–1.0×10^6 cells) isolated from various tissues were stained with fluorescein isothiocyanate-conjugated anti-mouse CD80, CD86, CD40 or $I\text{-}A^d$ monoclonal antibodies; phycoerythrin-labeled anti-mouse CD11c monoclonal antibodies; Alexa-labeled anti-mouse B220, CD11b, CD8α monoclonal antibodies (BD Biosciences). Samples were then subjected to fluorescence-activated cell sorting analysis (BD Biosciences).

Oral infection

Mice were orally infected with *P. gingivalis* as described previously (Baker *et al.*, 1994) with minor modifications. Briefly, mice were given sulfamethoxazole-trimethoprim (Sulfatrim; Goldline Laboratories, Ft. Lauderdale, FL) at 10 ml per 600 ml in deionized water *ad libitum* for 10 days. This was followed by a 3-day antibiotic-free period. Mice were then administered 10^9 colony-forming units of *P. gingivalis* suspended in 100 μl PBS with 2% carboxymethylcellulose via oral topical application over 3 weeks for a total of 15 inoculations. Control groups included sham-infected mice, which received antibiotic pretreatment and carboxymethylcellulose without *P. gingivalis*. Forty-seven days after the first gavage, mice were sacrificed using CO_2.

Measurement of alveolar bone loss

Horizontal bone loss around the maxillary molars was assessed by the morphometric method as described previously (Klausen *et al.*, 1989). Briefly, skulls were defleshed after 10 min of treatment in boiling water under 15 psi pressure, immersed overnight in 3% hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cemento–enamel junction to the alveolar bone crest was measured at a total of 14 buccal sites per mouse. Bone measurements were performed a total of three times by two evaluators using a random and blinded protocol.

Statistical analysis

Results were expressed as means ± standard error (SE). Statistical significance ($P < 0.05$) was determined by unpaired Student' *t*-test.

RESULTS

Induction of 40K-OMP-specific serum antibody responses

To evaluate the ability of oral immunization with 40K-OMP to induce serum antibody responses, a group of mice was orally immunized with 40K-OMP alone. 40K-OMP-specific IgG antibody responses were detected; however, the responses were low.

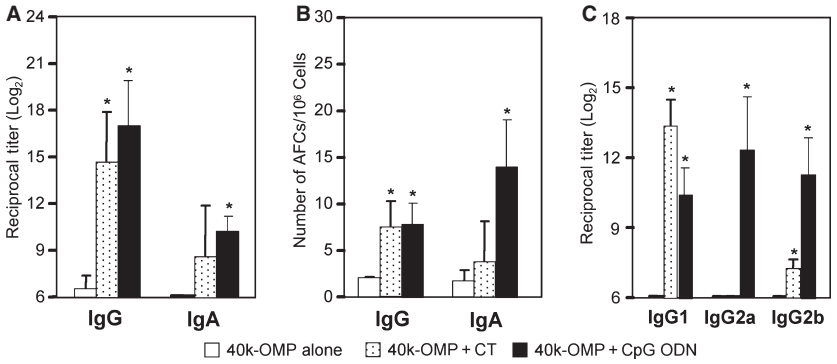

Figure 1 Detection of 40-kDa outer membrane protein (40K-OMP)-specific immunoglobulin G (IgG) and IgA responses in serum (A), numbers of IgG and IgA antibody-forming cells in spleen (B) and 40K-OMP-specific IgG subclass responses (C). Mice were orally immunized with 200 μg 40K-OMP either alone or together with 10 μg cholera toxin (CT) or 500 μg CpG oligodeoxynucleotide (ODN) on days 0, 7 and 14. Serum and saliva samples were collected at 7 days after the last immunization and were assessed for 40K-OMP-specific antibody titers. Results are expressed as means ± SE obtained for five mice per group. $^*P < 0.05$ vs. mice given 40K-OMP alone.

Furthermore, no IgA responses were induced (Fig. 1 A). In contrast, mice orally immunized with 40K-OMP plus CpG ODN as adjuvant showed significant increases in 40K-OMP-specific serum IgG and IgA antibodies, which were comparable to or greater than those induced by oral 40K-OMP plus CT as adjuvant (Fig. 1A). As expected, administration of CpG ODN or CT alone did not induce 40K-OMP-specific antibody responses that were above the dilution cutoff (log_2 of 6). The results of the serum antibody titers were confirmed by AFC responses, which indicated significant numbers of 40K-OMP-specific IgG and IgA AFCs in the spleens of mice given 40K-OMP plus CpG ODN as adjuvant (Fig. 1B). Analysis of IgG subclass responses in mice given 40K-OMP plus CpG ODN revealed that the major subclasses were IgG1, IgG2a and IgG2b, whereas CT predominantly induced IgG1 antibody responses (Fig. 1C).

Oral administration of 40K-OMP plus CpG ODN induced high levels of 40K-OMP-specific IgA antibody responses in saliva after immunization (Fig. 2A). Furthermore, the IgA antibodies induced by oral immunization with 40K-OMP plus CpG ODN were significantly higher than those induced by 40K-OMP plus CT (Fig. 2A). In contrast, essentially no IgA was detected in the saliva of mice orally immunized with 40K-OMP alone (Fig. 2A). As expected, oral delivery of CpG ODN or CT alone failed to elicit 40K-OMP-specific antibody titers greater than log_2 of 1. The AFC analysis confirmed the above results by revealing high numbers of IgA AFCs in the SG following oral administration of 40K-OMP plus CpG ODN and by showing low AFC responses in SG of mice given 40K-OMP alone (Fig. 2B).

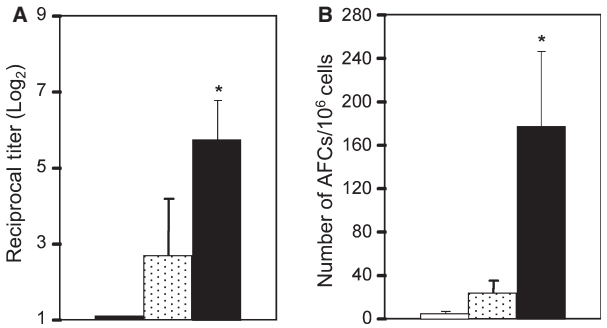

Figure 2 Detection of 40-kDa outer membrane protein (40K-OMP)-specific immunoglobulin A (IgA) antibody responses in saliva (A) and numbers of IgA antibody-forming cells (AFCs) in salivary glands (B). Mice were orally immunized with 40K-OMP alone (open bars) or together with cholera toxin (dotted bars) or CpG oligodeoxynucleotide (solid bars) as described in the legend for Fig. 1. Saliva samples were collected at 7 days after the last immunization and were assessed for 40K-OMP-specific IgA titers. Mononuclear cells from salivary glands were assessed for IgA AFCs. Results are expressed as means ± SE obtained for five mice per group. $^*P < 0.05$ vs. mice given 40K-OMP plus cholera toxin.

Analysis of cytokine responses

As oral immunization with 40K-OMP plus CpG ODN induced 40K-OMP-specific antibody responses in both mucosal and systemic compartments, it was important to establish the nature of the $CD4^+$ T cells

supporting 40K-OMP antibody responses. We therefore assessed Th1-type and Th2-type cytokine profiles induced by oral 40K-OMP plus CpG ODN. The 40K-OMP-stimulated $CD4^+$ T cells from spleen, as well as CLNs, which are draining lymph nodes of the maxillofacial mucosal compartments, of mice given oral 40K-OMP plus CpG ODN induced high levels of IFN-γ and IL-6 production. Furthermore, restimulation of 40K-OMP-specific $CD4^+$ T cells from spleen or CLNs also produced IL-4 and IL-5 (Fig. 3). These results clearly show that oral administration of 40K-OMP plus CpG ODN induces both Th1-type and Th2-type cytokine responses to support 40K-OMP-specific mucosal IgA, as well as serum IgG1, IgG2a, and IgG2b antibody responses.

Oral administration of CpG ODN expands DCs in both mucosal and systemic tissues

We next investigated the frequencies of $CD11c^+$ DCs in various lymphoid tissues. The results showed major increases in the percentage of $CD11c^+$ $B220^+$ plasmacytoid DCs (pDCs) and $CD11c^+$ $CD11b^+$ myeloid DCs (mDCs) in the spleen, PPs, and CLNs of mice given oral 40K-OMP plus CpG ODN when compared with mice given 40K-OMP alone. In contrast, frequencies of $CD11c^+$ $CD8\alpha^+$ DCs were not altered (Fig. 4). Furthermore, these expanded DCs expressed higher frequencies of costimulatory molecules (CD40, CD80, and CD86) and major histocompatibility complex (MHC) class II when compared with DCs from mice given 40K-OMP alone (Table 1). These results indicate that oral administration of 40K-OMP plus CpG ODN preferentially expands mature DCs and induces their activation in both mucosal and systemic compartments.

Oral 40K-OMP plus CpG ODN reduces alveolar bone loss caused by oral infection with *P. gingivalis*

As oral 40K-OMP plus CpG ODN elicited antigen-specific antibody responses in serum and saliva, we sought to determine whether these antibodies were capable of suppressing bone resorption caused by oral infection with *P. gingivalis*. Hence, mice given 40K-OMP plus CpG ODN or 40K-OMP plus CT were infected orally with *P. gingivalis* 7 days after immunization. Oral immunization of mice with 40K-OMP plus CpG ODN resulted in reduced bone loss associated with *P. gingivalis* infection. In contrast, mice immunized with 40K-OMP plus CT exhibited less reduced bone loss (Fig. 5). As expected, mice immunized with 40K-OMP, CT, or CpG ODN alone did not exhibit reduced bone loss associated with *P. gingivalis* infection (data not shown). These findings indicated that antibody responses elicited by oral 40K-OMP plus CpG ODN were protective against oral infection by *P. gingivalis*.

DISCUSSION

The observation that 40K-OMP can elicit protective immune responses when given nasally with CT or non-toxic mutant derivative (Momoi *et al.*, 2008) has caused 40K-OMP to be considered as a candidate

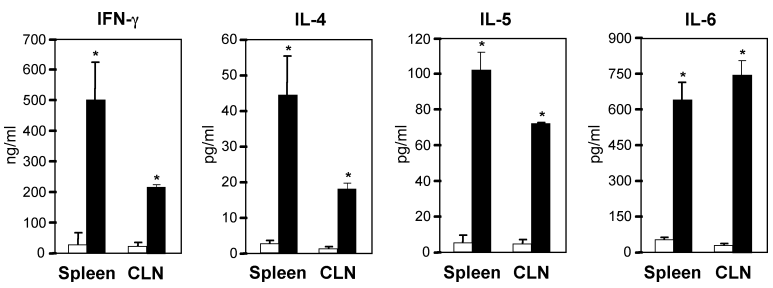

Figure 3 T helper type 1 (Th1) and Th2 cytokine responses in splenic (SP) or cervical (CLN) lymph node $CD4^+$ T cells. Mice were orally immunized with 40-kDa outer membrane protein (40K-OMP) plus CpG oligodeoxynucleotide as described in the legend for Fig. 1. $CD4^+$ T cells were isolated from SP or CLN of immunized mice and were cultured with (solid bars) or without (open bars) 40K-OMP in the presence of splenic feeder cells. After incubation, culture supernatants were harvested, and the levels of secreted cytokines were assessed by cytokine-specific enzyme-linked immunosorbent assay. The results are expressed as means ± SE obtained for five mice per group. $^*P < 0.05$ vs. cells cultured without 40K-OMP.

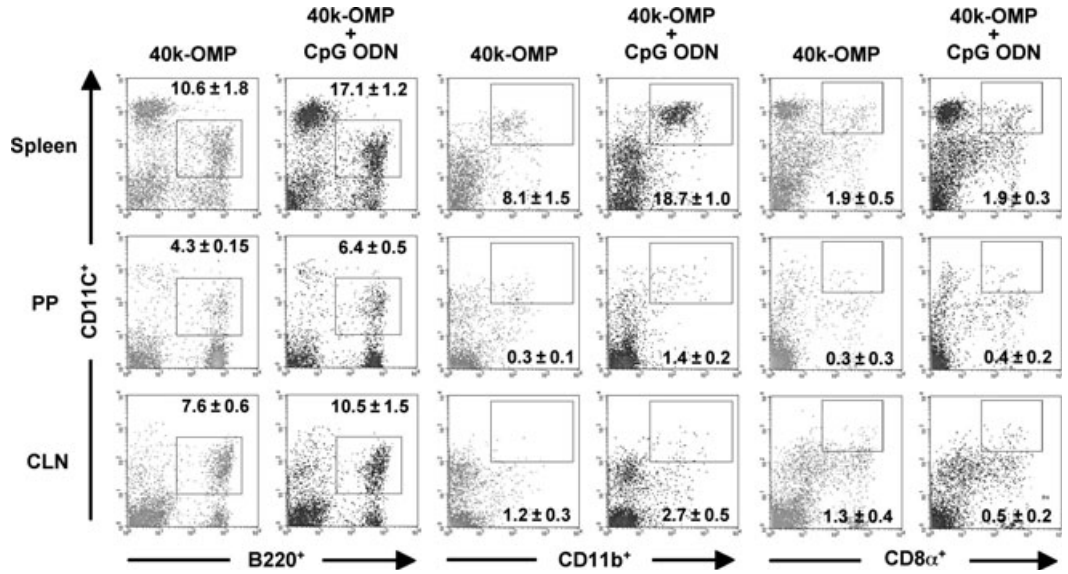

Figure 4 Comparison of the frequencies of $CD11c^+$ dendritic cells (DCs) in various lymphoid tissues. Mice were orally immunized with 40-kDa outer membrane protein (40K-OMP) alone or together with CpG oligodeoxynucleotide (ODN) as described in the legend for Fig. 1. DC-enriched cell populations isolated from spleen (SP), Peyer's patches (PPs), and cervical lymph nodes (CLNs) were stained with fluorescence-conjugate monoclonal antibodies and subjected to flow cytometry. Results are representative of three separate experiments. The numbers shown in the graph give the percentage of cells contained by the square region.

Table 1 Comparison of costimulatory molecule expression by $CD11c^+$ $B220^+$ and $CD11c^+$ $CD11b^+$ dendritic cells in several lymphoid tissues of mice given oral 40-kDa outer membrane protein (40K-OMP) plus CpG oligodeoxynucleotide (ODN)[1]

		Mean fluorescence intensity							
		$CD11c^+$ $B220^+$				$CD11c^+$ $CD11b^+$			
	Vaccine	CD80	CD86	CD40	MHC II	CD80	CD86	CD40	MHC II
SP	40-OMP40-OMP+CpG ODN	2.9 ± 0.3	1.5 ± 0.2	6.8 ± 0.7	10.9 ± 1.1	3.9 ± 0.4	2.8 ± 0.3	4.8 ± 0.5	34.7 ± 3.5
		5.0 ± 0.4*	3.7 ± 0.4*	9.4 ± 0.9*	24.9 ± 2.5*	12.6 ± 1.3*	6.5 ± 0.7*	14.8 ± 1.5*	77.9 ± 7.8*
PP	40-OMP40-OMP+CpG ODN	7.4 ± 0.6	2.1 ± 0.2	5.2 ± 0.5	14.4 ± 1.4	4.9 ± 0.5	3.4 ± 0.3	10.6 ± 1.1	83.5 ± 8.4
		9.8 ± 0.8	4.2 ± 0.4*	16.9 ± 1.7*	72.5 ± 7.3*	24.9 ± 2.5*	8.2 ± 0.8*	17.8 ± 1.8*	112.1 ± 12.1*
CLN	40-OMP40-OMP+CpG ODN	1.0 ± 0.1	1.3 ± 0.1	2.7 ± 0.3	4.3 ± 0.4	5.8 ± 0.6	5.7 ± 0.6	13.3 ± 1.2	81.0 ± 9.5
		2.6 ± 0.3*	2.6 ± 0.3*	5.6 ± 0.4*	12.1 ± 1.1*	9.1 ± 0.9*	11.0 ± 1.2*	30.3 ± 3.1*	146.3 ± 13.7*

[1]Dendritic cell-enriched cell populations from spleen (SP), Peyer's pathches (PP), and cervical lymph nodes (CLN) of mice immunized with 40K-OMP alone or 40K-OMP plus CpG ODN were stained with fluoroscein isothiocyanate-conjugated anti-CD80, anti-CD86, anti-CD40 or $I\text{-}A^d$ and phycoerythrin-labeled anti-CD11c antibodies.

*$P < 0.05$, compared with mice given 40K-OMP alone.

MHC, majpr histocompatibility complex.

antigen for the development of a human vaccine. Nasal administration of vaccine antigens has been widely used for mucosal immunization because it delivers antigen directly to IgA-inductive sites, termed nasal-associated lymphoid tissues, without the influence of enzymes and acids in the gastrointestinal tract. However, nasally administered CT and adenovirus vectors accumulate in the olfactory nerves and epithelial regions via GM-1 ganglioside (van Ginkel *et al.*, 2000; Lemiale *et al.*, 2003). A clinical study suggested a strong association between nasal influenza vaccine and Bell's palsy (Mutsch *et al.*, 2004). These findings raised concerns about nasal administration and the potential threat posed

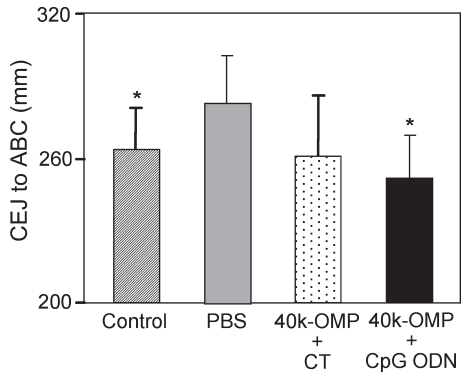

Figure 5 Oral vaccine containing 40-kDa outer membrane protein (40K-OMP) plus CpG oligodeoxynucleotide (ODN) reduces alveolar bone loss caused by *Porphyromonas gingivalis* infection. Mice were immunized orally with 40K-OMP plus CpG ODN, 40K-OMP plus cholera toxin (CT) or phosphate-buffered saline (PBS), as described in the legend for Fig. 1. Seven days after immunization, mice given PBS, 40K-OMP plus CT, or 40K-OMP plus CpG ODN were inoculated orally with 10^9 colony-forming units of *P. gingivalis* in 2% carboxylethylcellose. Control mice were sham-infected (inoculated with 2% carboxymethylcellose only). Results are expressed as means ± SE for six mice per group. $^*P < 0.05$ vs. mice given PBS.

by vaccines trafficking to neural tissues, including the central nervous system. A significant milestone would be the development of a practical oral vaccine.

In the present study, we assessed the potential of a combined oral vaccine, 40K-OMP with CpG ODN, to induce an immune response after host challenge. We have demonstrated that oral administration of 40K-OMP plus CpG ODN as adjuvant induced significant 40K-OMP-specific IgG and IgA antibody responses in serum and IgA antibody in saliva. Induction of antibody responses was associated with elevated numbers of activated pDCs and mDCs in PPs, CLNs, and spleen. Hence, increased frequencies of pDCs, as well as of mDCs in PPs, CLNs, and spleen with upregulated expression of MHC II, CD40, CD80, and CD86 molecules were noted in mice given 40K-OMP plus CpG ODN when compared with DCs from mice given oral 40K-OMP alone. Furthermore, CpG ODN as oral adjuvant induced $CD4^+$ T cells producing Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines. Importantly, antibody responses induced by oral immunization provided significant protection against oral infection with *P. gingivalis*. These findings suggest that oral immunization with 40K-OMP plus CpG ODN is a practical and effective route of immunization for the induction of specific immunity against oral infection with *P. gingivalis*.

Parenteral immunization with CpG ODN as adjuvant induces Th1-dominant immune responses primarily consisting of IFN-γ production (Chu *et al.*, 1997; Lipford *et al.*, 1997; Roman *et al.*, 1997; Weiner *et al.*, 1997; Davis *et al.*, 1998). Furthermore, our previous study also showed that nasal administration of CpG as adjuvant elicits Th1-type responses (Fukuiwa *et al.*, 2008), suggesting that CpG acts as an adjuvant that induces Th1-type immunity. Interestingly, however, the results presented in this study clearly show that oral immunization with 40K-OMP plus CpG induced IL-4 and IL-5 production in addition to IFN-γ and IL-6. The IgG subclass responses confirmed the cytokine profile showing that CpG as adjuvant elicited both IgG1 and IgG2a anti-40K-OMP antibody responses. The induction of both Th1-type and Th2-type cytokine responses may be explained by the route of immunization. Hence, oral immunization may favor the induction of Th2-type responses. In this regard, earlier studies have shown that PP or spleen T cells immunized orally with sheep red blood cells, and then restimulated by the antigen *in vitro*, give rise to 1.5-fold to 2.0-fold more IL-5-producing cells than IFN-γ-producing cells (Xu-Amano *et al.*, 1992). Furthermore, while parenteral immunization with CT induces both Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokine responses in the spleen, oral immunization elicits solely Th2-type responses in PP and spleen (Xu-Amano *et al.*, 1994). However, it has been shown that freshly isolated PP T cells contained an approximately equal number of T cells that spontaneously produce IFN-γ and IL-5 and that the ratio of Th1 to Th2 responsiveness was similar to that found in freshly isolated spleen T cells (Taguchi *et al.*, 1990). Furthermore, stimulation of PP T cells via gastrointestinal infection with microorganisms such as *Toxoplasma gondii* (Liesenfeld *et al.*, 1997) and *Salmonella typhimurium* (George, 1996; Hess *et al.*, 1996; VanCott *et al.*, 1996; Everest *et al.*, 1997), results in strong Th1 responses. These studies suggest that both Th1-type and Th2-type responses readily occur in PPs. It may be that oral administration of protein antigen favors the induction of Th2-type responses. Indeed, oral immunization with protein antigen (e.g. hepatitis B surface antigen and tetanus toxoid) elicited both Th1-type and Th2-type responses based on the IgG subclass profile and CTL responses (McCluskie *et al.*, 2000).

Alternatively, the effects of CpG ODN on mDCs may particularly induce Th2-type responses. It is well known that DCs play a crucial role in directing the differentiation of $CD4^+$ T cells into either Th1 or Th2 cells (Boonstra *et al.*, 2003). In this regard, CpG has been shown to stimulate pDCs that in turn lead to Th1 responses (Boonstra *et al.*, 2003). Indeed, our results also showed that CpG ODN as adjuvant stimulated pDCs in both mucosal and systemic lymphoid tissues, and enhanced the expression of costimulatory molecules and MHC II. Thus, CpG ODN as adjuvant stimulates pDCs, which enhance Th1 cell development. Interestingly, however, oral administration of 40K-OMP plus CpG ODN stimulated mDCs in addition to pDCs. In this regard, it has been shown that Toll-like receptor 9, a specific receptor for CpG, is expressed by both pDCs and mDCs in mice, whereas expression of this receptor in humans is limited to pDCs (Boonstra *et al.*, 2003). Furthermore, a recent study has shown that mDCs and pDCs have different capacities to produce IL-10 and IL-12 in response to CpG, and that CpG stimulation of mDCs resulted in IL-10 production with low IL-12p70 whereas pDCs do not produce IL-10 and show vigorous IL-12p70 production (Boonstra *et al.*, 2006). Interestingly, endogenous IL-10 suppressed IL-12p70 production. These studies, together with our results, suggest that oral administration of CpG ODN as adjuvant stimulates mDCs in addition to pDCs, and activated mDCs induce Th2-type responses resulting from inhibition of IL-12 production, whereas pDCs stimulated with CpG ODN induce Th1-type responses. However, as described above, nasal immunization of mice with CpG ODN as adjuvant does not enhance mDCs with subsequent Th2-type responses (Fukuiwa *et al.*, 2008). The characteristics of mDCs in gut-associated lymphoid tissues and nasal-associated lymphoid tissues may therefore differ, and this would explain why oral immunization favors the induction of Th2-type responses. Studies are underway to elucidate these additional steps.

It is known that immune responses in the oral cavity are derived from both the mucosal and systemic immune systems. The salivary glands, part of the mucosal immune system, are known to produce secretory IgA antibodies in saliva. On the other hand, serum-derived IgG antibodies are present in crevicular fluid, which continuously flows from the gingival capillaries and is part of the systemic immune system (Challacombe & Shirlaw, 1999). Because *P. gingivalis* colonizes subgingival and supragingival biofilm (Gibbons & Nygaard, 1970; Theilade *et al.*, 1982), generation of serum-derived IgG antibodies in crevicular fluid may be more effective in preventing *P. gingivalis* infection when compared with the IgA antibody response in saliva. Interestingly, however, the present results indicate that oral administration of 40K-OMP with CpG ODN, but not with CT, provides significant protection and reduces the bone loss caused by *P. gingivalis* infection, although both immunization regimens induce identical serum IgG anti-40K-OMP antibody responses. It is important to note that oral 40K-OMP plus CpG ODN induced significant 40K-OMP-specific IgA antibodies in saliva while 40K-OMP plus CT elicited the IgA response only slightly. These findings suggest that IgA responses in saliva play more crucial roles in the prevention of *P. gingivalis* infection than serum-derived IgG.

It should be noted that oral immunization with 40K-OMP plus CT induced only low levels of 40K-OMP-specific IgA antibodies in saliva. The basis for the low IgA response by 40K-OMP plus CT is unknown but could be explained by the nature of 40K-OMP, as antigen nature greatly influences the immune response. As a consequence, CT may not be an effective adjuvant for 40K-OMP when inducing mucosal IgA responses. However, our previous studies have demonstrated that CT acts as an adjuvant and significantly enhances mucosal IgA, as well as serum IgG and IgA, when co-administered with 40K-OMP nasally or transcutaneously (Namikoshi *et al.*, 2003; Maeba *et al.*, 2005). Oral immunization with 40K-OMP plus CT therefore may not be particularly effective for induction of mucosal IgA responses. Alternatively, CpG ODN may be simply a more effective mucosal adjuvant for the induction of salivary IgA antibody responses when compared with CT. In this regard, it is known that CT elicits strong Th2 cytokine responses when given orally (Xu-Amano *et al.*, 1993; Marinaro *et al.*, 1995; Yamamoto *et al.*, 1996). On the other hand, our results showed that oral administration of CpG ODN induced both Th1 and Th2 responses. The difference in cytokine responses between CT and CpG ODN may influence the induction of the mucosal IgA response to 40K-OMP.

In summary, the combination of 40K-OMP plus CpG ODN provides a very effective means of eliciting

both IFN-γ-producing Th1-type and IL-4-producing Th2-type $CD4^+$ T cells for the induction of serum IgG, IgA and mucosal IgA antibody responses. The mechanisms responsible for the effects of CpG ODN are mediated by increased pDCs and mDCs. Finally, 40K-OMP-specific immune responses induced by 40K-OMP plus CpG ODN provide protective immunity against alveolar bone loss caused by *P. gingivalis* infection. These findings suggest that oral administration of 40K-OMP with CpG ODN effectively elicits protective levels of antibodies against 40K-OMP, and should therefore be considered as a candidate vaccine to immunize humans against *P. gingivalis* infection.

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