Published in final edited form as:

Mol Oral Microbiol. 2010 May 4; 25(3): 178–189. doi:10.1111/j.2041-1014.2009.00560.x.

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

Chenlu Liu^{1,2}, Tomomi Hashizume¹, Tomoko Kurita-Ochiai¹, Kohtaro Fujihashi³, and Masafumi Yamamoto¹

¹ Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

² Tianjin Stomatology Hospital, Tianjin 300041, China

³ The Immunobiology Vaccine Center, Departments of Pediatric Dentistry and Microbiology, The University of Alabama at Birmingham, AL35294-0007, USA

Abstract

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*. [run on]

40K-OMP40K-OMP40K-OMP Oral immunization with 40K-OMP plus CpG ODN induced significant 40K-OMP-specific serum IgG, IgA and saliva IgA antibody responses. The 40K-OMP-specific CD4⁺ T cells induced by oral 40K-OMP plus CpG ODN produced both Th1 (IFN- γ) and Th2 (IL-4) cytokines. Furthermore, increased frequencies of CD11c⁺B220⁺ DCs and CD11c⁺CD11b⁺ DCs with up-regulated expression of CD80, CD86, CD40 and MHC 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 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*.

Thus, oral administration of 40K-OMP together with CpG ODN induces Th1- and Th2-type cells, which provide help for protective immunity against *P. gingivalis* infection. This may be an important tool for prevention of chronic periodontitis.

INTRODUCTION

Oral health is threatened by chronic periodontitis destroying periodontal tissues and thereby causing tooth loss (7). Moreover, periodontal diseases have been linked to a number of systemic diseases, such as cardiovascular diseases and diabetes, as well as osteoporosis (2,9,14,23,35,37,41). The prevention of periodontitis might then be relevant for both oral and systemic health.

Masafumi Yamamoto, Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan, Tel: +81-47-360-9336; Fax: +81-47-361-2712; yamamoto.masafumi@nihon-u.ac.jp.

Porphyromonas gingivalis has been shown to be one of the major pathogens of chronic periodontitis (7). An outer membrane protein with molecular mass of ~40 kDa produced by *P. gingivalis* is important for the coaggregation activity of *P. gingivalis* (15,18,44). Furthermore, this outer membrane protein (designated 40K-OMP) has been shown to be a hemin-binding protein (45). The 40K-OMP is found in various strains of *P. gingivalis*, and resides both on the cell surface and in extracellular vesicles (15,18,44,45). Previous studies have shown that monoclonal antibodies (Abs) 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* (18,21,43,44). These studies suggest that induction of 40K-OMP specific antibodies in the oral cavity might be a logical approach for prevention of *P. gingivalis* infection. Indeed, previous studies have demonstrated that nasal administration of 40K-OMP plus nontoxic chimeric enterotoxin adjuvant elicited 40K-OMP specific secretory immunoglobulin A (S-IgA) antibodies in saliva, and serum immunoglobulin G (IgG) antibodies, that reduced alveolar bone loss caused by oral infection with *P. gingivalis* (38). 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 non-immunized mice (26). These studies indicate that 40K-OMP may be an effective vaccine antigen (Ag) for the prevention of *P. gingivalis* infection.

It is well established that mucosal immunization can elicit Ag-specific immune responses in both mucosal and systemic compartments. In particular, oral immunization offers several advantages over other Ag 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 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 (19). Bacterial toxins, such as CT, are commonly used as mucosal adjuvants in animal models; however, toxicity prevents their use in humans (46). Genetically detoxified CT mutants have been developed by site-directed mutagenesis, which appear to be non-toxic in animal models but retain adjuvanticity (57). 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). CpG ODN interact with TLR9 expressed by B cells and dendritic cells, and induce Th1 and proinflammatory cytokine responses (25,27). 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 IL-12 and IFN- γ (6,8,30,42,52). Furthermore, it has been shown that CpG ODN is a potent adjuvant when given nasally (33) or orally (34).

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 induction of Ag-specific Ab 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 to 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-OMP40K-OMP was kindly provided by Dr. Yoshimitsu Abiko (Nihon University). The 40K-OMP40K-OMP was purified to homogeneity from a cell suspension of *Escherichia coli* K-12 harboring pMD125, as described previously (22). The purity of 40K-OMP40K-OMP was determined by SDS-PAGE, 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-OMP40K-OMP contained as little as 0.3 pg of endotoxin per mg protein. CpG ODN (5'-TCCATGACGTTTCCTGACGTT-3') was purchased from Coley Pharmaceutical Group, Inc. (Wellesley, MA). 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 food for 2 h and then given an isotonic solution (250 μ l per mouse). After 30 min, mice were orally immunized with 200 μ l of phosphate-buffered saline (PBS) containing 200 μ g of 40K-OMP40K-OMP alone or combined with 10 μ g of CT or 500 μ g of CpG ODN. Serum and saliva were collected from each group in order to examine Ag-specific antibody responses. To evaluate the effects of oral vaccine on alveolar bone loss by *P. gingivalis* infection, mice were orally immunized with 40K-OMP40K-OMP plus CT, 40K-OMP 40K-OMP plus CpG ODN or PBS 7 days before oral infection with *P. gingivalis*.

Detection of Ag-specific antibody responses

Antibody titers were determined by ELISA (51). Briefly, plates were coated with 40K-OMP 40K-OMP (5 μ g ml⁻¹) and blocked with PBS containing 1% bovine serum albumin. After blocking, serial dilutions of serum or saliva samples were added in duplicate. Starting dilution of serum was 1:2¹⁰, and that of saliva was 1:2². Four hours after incubation at room temperature, plates were washed and goat horseradish peroxidase-conjugated anti-mouse γ , γ 1, γ 2a, γ 2b or α heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) with H₂O₂ (Moss, Inc., Pasadena, MD) was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 415 nm of 0.1 greater than nonimmunized 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 (36,50). Briefly, SG were carefully excised, teased apart, and dissociated using 0.3 mg ml⁻¹ 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, England). To assess numbers of Ag-specific antibody-forming cells (AFCs), an ELISPOT assay was performed as previously described (56). Briefly, 96-well nitrocellulose plates (BD Biosciences, Franklin Lakes, NJ) were coated with 40K-OMP (5 $\mu\text{g ml}^{-1}$), incubated for 20 hs at 4°C, and were then washed extensively and blocked with RPMI 1640 containing 10% fetal calf serum. After 30 min, 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% CO₂ in moist air. Detection antibodies included goat horseradish peroxidase-conjugated anti-mouse γ or α heavy chain-specific Abs (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 H₂O₂ (Moss) to each well. Plates were incubated at room temperature for 25 min and were washed with water, and 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 ELISA. CD4⁺ T cells from spleens and CLNs were isolated with the Imag system (BD Biosciences), as described elsewhere (16). Briefly, mononuclear cells were mixed with anti-CD4 Abs and incubated at 4°C for 30 min and then CD4⁺ T cells were separated using a magnet. CD4⁺ T cells (2.0 $\times 10^6$ cells ml⁻¹) were cultured with 5 μg of 40K-OMP in the presence of T cell-depleted, mitomycin-treated splenic feeder cells (2.5 $\times 10^6$ cells) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 15 mM HEPES, 100 U of penicillin ml⁻¹ and 10 U of recombinant IL-2 ml⁻¹. Cultures were incubated for 5 days at 37°C under 5% CO₂ in air. Levels of IL-4, IL-5, IL-6 and IFN- γ in culture supernatants were determined by commercially available assay kits (Pierce Biotechnology, Inc., Rockford, IL) in accordance with the manufacturers' instructions.

Flow cytometry

DC-enriched cell populations from Spleen, CLNs and Peyer's patches (PPs) were prepared as described previously (20). Briefly, lymphoid tissues were digested with collagenase D (Roche Diagnostics GmbH, Mannheim, Germany) and DNase I (Roche) in RPMI 1640 supplemented with 10% fetal bovine serum albumin, with continuous stirring at 37°C for 45–90 min. EDTA 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 analyzed for the expression of various cell surface molecules with fluorescence-labeled antibodies. Aliquots of mononuclear cells (0.2 – 1.0 $\times 10^6$ cells) isolated from various tissues were stained with FITC-conjugated anti-mouse CD80, CD86, CD40 or I-A^d monoclonal antibodies; PE-labeled anti-mouse CD11c monoclonal Abs; Alexa-labeled anti-mouse B220, CD11b, CD8 α monoclonal Abs (BD Biosciences). Samples were then subjected to FACS analysis (BD Biosciences).

Oral infection

Mice were orally infected with *P. gingivalis*, as described previously (1) 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 10⁹ CFU of *P. gingivalis* suspended in 100 μl of PBS with 2% carboxymethylcellulose via oral topical application over three weeks for a total 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 euthanized using CO₂.

Measurement of alveolar bone loss

Horizontal bone loss around the maxillary molars was assessed by the morphometric method as described previously (24). Briefly, skulls were defleshed after 10 min of treatment in boiling water under 15-lbin² 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 (CEJ) to the alveolar bone crest (ABC) was measured at a total of 14 buccal sites per mouse. This measurement is referred to below as CEJ to ABC. 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 $\pm$ standard error (S.E.). Statistical significance ($p < 0.05$) was determined by unpaired Student's *t* test.

RESULTS

Induction of 40K-OMP-specific serum antibody responses

In order 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. Furthermore, no IgA responses were induced (Fig. 1A). 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, while 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. 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).

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 CD4⁺ T cells supporting 40K-OMP antibody responses. Thus, we assessed Th1- 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- and Th2-type cytokines responses to support 40K-OMP-specific mucosal IgA, as well as serum IgG1, IgG2a and IgG2b Ab 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 α ⁺ DCs were not altered (Fig. 4). Furthermore, these expanded DCs expressed higher frequencies of co-stimulatory molecules (CD40, CD80, and CD86) and 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 Ag-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*. Thus, mice given 40K-OMP plus CpG ODN or 40K-OMP plus CT were infected orally with *P. gingivalis* seven 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 nontoxic mutant derivative (38) has caused 40K-OMP to be considered as a candidate Ag for the development of a human vaccine. Nasal administration of vaccine antigens has been widely used for mucosal immunization because it delivers Ag 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 (28,49). A clinical study suggested a strong association between nasal influenza vaccine and Bell's palsy (39). These findings raised concerns about nasal administration and the potential threat posed by vaccines trafficking 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, in order 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. Thus, increased frequencies of pDCs, as well as mDCs in PPs, CLNs and spleen with up-regulated 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 IL5) 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 (6,8,30,42,52). Furthermore, our previous study also showed that nasal administration of CpG as adjuvant elicits Th1-type responses (11), suggesting that CpG acts as an adjuvant that induced 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. 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- and Th2-type cytokine responses may be explained by the route of immunization. Thus, 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- to 2.0-fold more IL-5-producing cells than IFN- γ -producing cells (53). 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 (54). 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 (47). Furthermore, stimulation of PP T cells via gastrointestinal infection with microorganisms such as *Toxoplasma gondii* (29) and *Salmonella typhimurium* (10,12,17,51), results in strong Th1 responses. These studies suggest that both Th1- and Th2-type responses readily occur in PPs. It may be that oral administration of protein Ag favors the induction of Th2-type responses. Indeed, oral immunization with protein Ag (e.g., hepatitis B surface antigen and tetanus toxoid) elicited both Th1- and Th2-type responses based on the IgG subclass profile and CTL responses (34).

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 (3). In this regard, CpG has been shown to stimulate pDCs that in turn lead to Th1 responses (3). Indeed, our results also showed that CpG ODN as adjuvant stimulated pDCs in both mucosal and systemic lymphoid tissues, and enhanced the expression of co-stimulatory 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 TLR9, a specific receptor for CpG, is expressed by both pDCs and mDCs in mice, while expression of this receptor in humans is limited to pDCs (3). Furthermore, a recent study has shown that mDC 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 while pDCs do not produce IL-10 and show vigorous IL-12p70 production (4). 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 (11). 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 Abs in saliva. On the other hand, serum-derived IgG Abs are present in crevicular fluid, which continuously flows from the gingival capillaries and is part of the systemic immune system (5). Because *P. gingivalis* colonizes subgingival and supragingival biofilm (13,48), 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, provide significant protection and reduce 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 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 Abs 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 immune response. Thus, 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 (31,40). Thus, oral immunization with 40K-OMP plus CT may not be particularly effective for induction of mucosal IgA responses. Alternatively, CpG ODN may be simply a more effective mucosal adjuvant for 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 (32,55,58). 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 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- and IL-4-producing Th2-type CD4⁺ T cells for the induction of serum IgG, IgA and mucosal IgA Ab 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 Abs against 40K-OMP, and should therefore be considered as a candidate vaccine to immunize humans against *P. gingivalis* infection.

Acknowledgments

This work was supported by grants-in-aid for scientific research (18592270 and 19791624) from the Japan Society for the Promotion of Science and an “Academic Frontier” Project for Private Universities matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, 2007-2011 as well as by NIH grants DE 12242 and AG 025873.

REFERENCES [alphabetical without numbering]

1. Baker PJ, Evans RT, Roopenian DC. Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol.* 1994; 39:1035–1040. [PubMed: 7717884]
2. Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. *J Periodontol.* 1996; 67:1123–1137. [PubMed: 8910831]
3. Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, O’Garra A. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med.* 2003; 197:101–109. [PubMed: 12515817]
4. Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP, Bates EE, Akira S, Vieira P, Liu YJ, Trinchieri G, O’Garra A. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol.* 2006; 177:7551–7558. [PubMed: 17114424]
5. Challacombe, SJ.; Shirlaw, PJ. Immunity of diseases of the oral cavity. In: Ogra, PL.; Mestecky, J.; Lamm, ME.; Strober, W.; Bienenstock, J.; McGhee, JR., editors. *Mucosal Immunology*. San Diego, CA: Academic Press; 1999. p. 1313-1337.
6. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med.* 1997; 186:1623–1631. [PubMed: 9362523]
7. Cutler CW, Kalmar JR, Genco CA. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. *Trends Microbiol.* 1995; 3:45–51. [PubMed: 7728384]
8. Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol.* 1998; 160:870–876. [PubMed: 9551923]
9. DeStefano F, Anda RF, Kahn HS, Williamson DF, Russell CM. Dental disease and risk of coronary heart disease and mortality. *BMJ.* 1993; 306:688–691. [PubMed: 8471920]
10. Everest P, Allen J, Papakonstantinou A, Mastroeni P, Roberts M, Dougan G. *Salmonella typhimurium* infections in mice deficient in interleukin-4 production: role of IL-4 in infection-associated pathology. *J Immunol.* 1997; 159:1820–1827. [PubMed: 9257845]
11. Fukuiwa T, Sekine S, Kobayashi R, Suzuki H, Kataoka K, Gilbert RS, Kurono Y, Boyaka PN, Krieg AM, McGhee JR, Fujihashi K. A combination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. *Vaccine.* 2008; 26:4849–4859. [PubMed: 18625280]
12. George A. Generation of gamma interferon responses in murine Peyer’s patches following oral immunization. *Infect Immun.* 1996; 64:4606–4611. [PubMed: 8890214]
13. Gibbons RJ, Nygaard M. Interbacterial aggregation of plaque bacteria. *Arch Oral Biol.* 1970; 15:1397–1400. [PubMed: 5280139]
14. Grossi SG, Genco RJ. Periodontal disease and diabetes mellitus: a two-way relationship. *Ann Periodontol.* 1998; 3:51–61. [PubMed: 9722690]
15. Hamajima S, Maruyama M, Hijiya T, Hatta H, Abiko Y. Egg yolk-derived immunoglobulin (IgY) against *Porphyromonas gingivalis* 40-kDa outer membrane protein inhibits coaggregation activity. *Arch Oral Biol.* 2007; 52:697–704. [PubMed: 17275778]
16. Hashizume T, Togawa A, Nochi T, Igarashi O, Kweon MN, Kiyono H, Yamamoto M. Peyer’s patches are required for intestinal immunoglobulin A responses to *Salmonella* spp. *Infect Immun.* 2008; 76:927–934. [PubMed: 18086815]

17. Hess J, Ladel C, Miko D, Kaufmann SH. *Salmonella typhimurium* aroA-infection in gene-targeted immunodeficient mice: major role of CD4⁺ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J Immunol.* 1996; 156:3321–3326. [PubMed: 8617956]
18. Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H. Role of *Porphyromonas gingivalis* 40-kDa outer membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. *Arch Oral Biol.* 1992; 37:717–724. [PubMed: 1329700]
19. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine.* 2003; 21 (Suppl 2):S89–95. [PubMed: 12763689]
20. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, Guo Z, Umemoto E, Ebisuno Y, Yang BG, Seoh JY, Lipp M, Kiyono H, Miyasaka M. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol.* 2006; 176:803–810. [PubMed: 16393963]
21. Katoh M, Saito S, Takiguchi H, Abiko Y. Bactericidal activity of a monoclonal antibody against a recombinant 40-kDa outer membrane protein of *Porphyromonas gingivalis*. *J Periodontol.* 2000; 71:368–375. [PubMed: 10776923]
22. Kawamoto Y, Hayakawa M, Abiko Y. Purification and immunochemical characterization of a recombinant outer membrane protein from *Bacteroides gingivalis*. *Int J Biochem.* 1991; 23:1053–1061. [PubMed: 1664808]
23. Kinane DF. Periodontal diseases' contributions to cardiovascular disease: an overview of potential mechanisms. *Ann Periodontol.* 1998; 3:142–150. [PubMed: 9722698]
24. Klausen B, Evans RT, Sfantescu C. Two complementary methods of assessing periodontal bone level in rats. *Scand J Dent Res.* 1989; 97:494–499. [PubMed: 2617150]
25. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA.* 1996; 93:2879–2883. [PubMed: 8610135]
26. Koizumi Y, Kurita-Ochiai T, Oguchi S, Yamamoto M. Nasal immunization with *Porphyromonas gingivalis* outer membrane protein decreases *P. gingivalis*-induced atherosclerosis and inflammation in spontaneously hyperlipidemic mice. *Infect Immun.* 2008; 76:2958–2965. [PubMed: 18426881]
27. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* 1995; 374:546–549. [PubMed: 7700380]
28. Lemiale F, Kong WP, Akyurek LM, Ling X, Huang Y, Chakrabarti BK, Eckhaus M, Nabel GJ. Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system. *J Virol.* 2003; 77:10078–10087. [PubMed: 12941918]
29. Liesenfeld O, Kosek JC, Suzuki Y. Gamma interferon induces Fas-dependent apoptosis of Peyer's patch T cells in mice following peroral infection with *Toxoplasma gondii*. *Infect Immun.* 1997; 65:4682–4689. [PubMed: 9353050]
30. Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur J Immunol.* 1997; 27:2340–2344. [PubMed: 9341778]
31. Maeba S, Otake S, Namikoshi J, Shibata Y, Hayakawa M, Abiko Y, Yamamoto M. Transcutaneous immunization with a 40-kDa outer membrane protein of *Porphyromonas gingivalis* induces specific antibodies which inhibit coaggregation by *P. gingivalis*. *Vaccine.* 2005; 23:2513–2521. [PubMed: 15752838]
32. Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, Okahashi N, Yamamoto M, Kiyono H, Bluethmann H, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J Immunol.* 1995; 155:4621–4629. [PubMed: 7594461]

33. McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J Immunol.* 1998; 161:4463–4466. [PubMed: 9794366]
34. McCluskie MJ, Weeratna RD, Krieg AM, Davis HL. CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine.* 2000; 19:950–957. [PubMed: 11115721]
35. Mealey BL. Influence of periodontal infections on systemic health. *Periodontol* 2000. 1999; 21:197–209. [PubMed: 10551183]
36. Mega J, McGhee JR, Kiyono H. Cytokine- and Ig-producing T cells in mucosal effector tissues: analysis of IL-5- and IFN-gamma-producing T cells, T cell receptor expression, and IgA plasma cells from mouse salivary gland-associated tissues. *J Immunol.* 1992; 148:2030–2039. [PubMed: 1545116]
37. Meyer DH, Fives-Taylor PM. Oral pathogens: from dental plaque to cardiac disease. *Curr Opin Microbiol.* 1998; 1:88–95. [PubMed: 10066462]
38. Momoi F, Hashizume T, Kurita-Ochiai T, Yuki Y, Kiyono H, Yamamoto M. Nasal vaccination with the 40-kilodalton outer membrane protein of *Porphyromonas gingivalis* and a nontoxic chimeric enterotoxin adjuvant induces long-term protective immunity with reduced levels of immunoglobulin E antibodies. *Infect Immun.* 2008; 76:2777–2784. [PubMed: 18411288]
39. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med.* 2004; 350:896–903. [PubMed: 14985487]
40. Namikoshi J, Otake S, Maeba S, Hayakawa M, Abiko Y, Yamamoto M. Specific antibodies induced by nasally administered 40-kDa outer membrane protein of *Porphyromonas gingivalis* inhibits coaggregation activity of *P. gingivalis*. *Vaccine.* 2003; 22:250–256. [PubMed: 14615153]
41. Reddy MS. Oral osteoporosis: is there an association between periodontitis and osteoporosis? *Comp Dent Educ.* 2002; 23:21–28. [PubMed: 12790013]
42. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, Kornbluth RS, Richman DD, Carson DA, Raz E. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med.* 1997; 3:849–854. [PubMed: 9256274]
43. Saito S, Hayakawa M, Hiratsuka K, Takiguchi H, Abiko Y. Complement-mediated killing of *Porphyromonas gingivalis* 381 by the immunoglobulin G induced by recombinant 40-kDa outer membrane protein. *Biochem Mol Med.* 1996; 58:184–191. [PubMed: 8812738]
44. Saito S, Hiratsuka K, Hayakawa M, Takiguchi H, Abiko Y. Inhibition of a *Porphyromonas gingivalis* colonizing factor between *Actinomyces viscosus* ATCC 19246 by monoclonal antibodies against recombinant 40-kDa outer-membrane protein. *Gen Pharmacol.* 1997; 28:675–680. [PubMed: 9184801]
45. Shibata Y, Hiratsuka K, Hayakawa M, Shiroza T, Takiguchi H, Nagatsuka Y, Abiko Y. A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. *Biochem Biophys Res Commun.* 2003; 300:351–356. [PubMed: 12504090]
46. Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev.* 1992; 56:622–647. [PubMed: 1480112]
47. Taguchi T, McGhee JR, Coffman RL, Beagley KW, Eldridge JH, Takatsu K, Kiyono H. Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of CD4⁺ and CD8⁺ T cells that secrete IFN-gamma and IL-5. *J Immunol.* 1990; 145:68–77. [PubMed: 1972722]
48. Theilade E, Theilade J, Mikkelsen L. Microbiological studies on early dento-gingival plaque on teeth and Mylar strips in humans. *J Periodontal Res.* 1982; 17:12–25. [PubMed: 6211535]
49. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J Immunol.* 2000; 165:4778–4782. [PubMed: 11045998]
50. van Ginkel FW, McGhee JR, Liu C, Simecka JW, Yamamoto M, Frizzell RA, Sorscher EJ, Kiyono H, Pascual DW. Adenoviral gene delivery elicits distinct pulmonary-associated T helper cell responses to the vector and to its transgene. *J Immunol.* 1997; 159:685–693. [PubMed: 9218583]
51. VanCott JL, Staats HF, Pascual DW, Roberts M, Chatfield SN, Yamamoto M, Coste M, Carter PB, Kiyono H, McGhee JR. Regulation of mucosal and systemic antibody responses by T helper cell

- subsets, macrophages, and derived cytokines following oral immunization with live recombinant *Salmonella*. *J Immunol*. 1996; 156:1504–1514. [PubMed: 8568254]
52. Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc Natl Acad Sci U S A*. 1997; 94:10833–10837. [PubMed: 9380720]
53. Xu-Amano J, Aicher WK, Taguchi T, Kiyono H, McGhee JR. Selective induction of Th2 cells in murine Peyer's patches by oral immunization. *Int Immunol*. 1992; 4:433–445. [PubMed: 1534253]
54. Xu-Amano J, Jackson RJ, Fujihashi K, Kiyono H, Staats HF, McGhee JR. Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. *Vaccine*. 1994; 12:903–911. [PubMed: 7975832]
55. Xu-Amano J, Kiyono H, Jackson RJ, Staats HF, Fujihashi K, Burrows PD, Elson CO, Pillai S, McGhee JR. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J Exp Med*. 1993; 178:1309–1320. [PubMed: 8376936]
56. Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J Immunol*. 1998; 161:4115–4121. [PubMed: 9780183]
57. Yamamoto M, McGhee JR, Hagiwara Y, Otake S, Kiyono H. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. *Scand J Immunol*. 2001; 53:211–217. [PubMed: 11251876]
58. Yamamoto M, Vancott JL, Okahashi N, Marinaro M, Kiyono H, Fujihashi K, Jackson RJ, Chatfield SN, Bluethmann H, McGhee JR. The role of Th1 and Th2 cells for mucosal IgA responses. *Ann NY Acad Sci*. 1996; 778:64–71. [PubMed: 8611017]

Fig. 1.

Detection of 40K-OMP-specific IgG and IgA responses in serum (A), numbers of IgG and IgA AFCs in spleen (B) and 40K-OMP-specific IgG subclass responses (C). Mice were orally immunized with 200 μ g of 40K-OMP alone or together with 10 μ g of CT or 500 μ g of CpG 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 $\pm$ S.E. obtained for five mice per group. * $p < 0.05$ vs. mice given 40K-OMP alone.

Fig. 2.

Detection of 40K-OMP-specific IgA antibody responses in saliva (A) and numbers of IgA AFCs in salivary glands (B). Mice were orally immunized with 40K-OMP alone (open bars) or together with CT (dotted bars) or CpG ODN (solid bars) as described in the legend for Figure 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 $\pm$ S.E. obtained for five mice per group. * $p < 0.05$ vs. mice given 40K-OMP plus CT.

Fig. 3.

Th1 and Th2 cytokine responses in splenic or CLN CD4⁺ T cells. Mice were orally immunized with 40K-OMP plus CpG ODN as described in the legend for Figure 1. CD4⁺ T cells were isolated from SP or CLN of immunized mice and were cultured with (solid bars) or without 40K-OMP (open bars) in the presence of splenic feeder cells. After incubation, culture supernatants were harvested, and the levels of secreted cytokines were assessed by cytokine-specific ELISA. The results are expressed as means $\pm$ S.E. obtained for five mice per group. * $p < 0.05$ vs. cells cultured without 40K-OMP.

Fig. 4.

Comparison of the frequencies of CD11c⁺ DCs in various lymphoid tissues. Mice were orally immunized with 40K-OMP alone or together with CpG ODN as described in the legend for Figure 1. DC-enriched cell populations isolated from spleen, PPs and CLNs were stained with fluorescence-conjugate monoclonal Abs 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.

Fig. 5.

Oral vaccine containing 40K-OMP plus CpG ODN reduces alveolar bone loss caused by *P. gingivalis* infection. Mice were immunized orally with 40K-OMP plus CpG ODN, 40K-OMP plus CT or PBS, as described in the legend for Figure 1. Seven days after immunization, mice given PBS, 40K-OMP plus CT, or 40K-OMP plus CpG ODN were inoculated orally with 10^9 CFU of *P. gingivalis* in 2% carboxylethylcellulose. Control mice were sham-infected mice inoculated with 2% carboxymethylcellulose only. Results are expressed as means $\pm$ S.E. for six mice per group. * $p < 0.05$ vs. mice given PBS.

Table 1

Comparison of co-stimulatory molecule expression by CD11c⁺B220⁺ DCs and CD11c⁺CD11b⁺ DCs in several lymphoid tissues of mice given oral 40K-OMPplus CpG ODN^a

		Mean fluorescence intensity							
		CD11c ⁺ B220 ⁺				CD11c ⁺ CD11b ⁺			
Vaccine		CD80	CD86	CD40	MHC II	CD80	CD86	CD40	MHC II
SP	40-OMP	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
	40-OMP+ CpG ODN	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-OMP	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
	40-OMP+ CpG ODN	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-OMP	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
	40-OMP+ CpG ODN	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 *

^aDC-enriched cell populations from spleen (SP), PP and CLN of mice immunized with 40K-OMP alone or 40K-OMP plus CpG ODN were stained with FITC-conjugated anti-CD80, -CD86, -CD40 or I-Ad and PE-labeled anti-CD11c Abs.

^{*} *p*<0.05, compared with mice given 40K-OMP alone.