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Nasal Immunization with a Fusion Protein Consisting of the Hemagglutinin A Antigenic Region and the Maltose-Binding Protein Elicits CD11c$^+$ CD8$^+$ Dendritic Cells for Induced Long-Term Protective Immunity$^\nabla$

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We assessed the efficacy of a fusion protein consisting of the 25-kDa antigenic region of *Porphyromonas gingivalis* hemagglutinin A and the *Escherichia coli* maltose-binding protein (25k-hagA-MBP) as a nasal vaccine for the prevention of oral infection with *P. gingivalis*. Nasal immunization with 25k-hagA-MBP induced high levels of 25k-hagA-specific serum IgG, serum IgA, and salivary IgA antibodies in a Toll-like receptor 4 (TLR4)-dependent manner. These antibody responses were maintained for at least 1 year after immunization. Analysis of cytokine responses showed that nasal administration of 25k-hagA-MBP induced antigen-specific CD4$^+$ T cells producing interleukin 4 (IL-4) and IL-5, but not gamma interferon (IFN-γ), in the spleen and cervical lymph nodes (CLNs). Furthermore, increased numbers of CD11c$^+$ CD8α$^+$, but not CD11c$^+$ CD11b$^+$ or CD11c$^+$ B220$^+$, dendritic cells with upregulated expression of CD80, CD86, CD40, and major histocompatibility complex class II (MHC II) molecules were noted in the spleen, CLNs, and nasopharynx-associated lymphoreticular tissues (NALT). Interestingly, when 25k-hagA-MBP or cholera toxin (CT) was given intranasally to enable examination of their presence in neuronal tissues, the amounts of 25k-hagA-MBP were significantly lower than those of CT. Importantly, mice given 25k-hagA-MBP nasally showed a significant reduction in alveolar bone loss caused by oral infection with *P. gingivalis*, even 1 year after the immunization. These results suggest that 25k-hagA-MBP administered nasally would be an effective and safe mucosal vaccine against *P. gingivalis* infection and may be an important tool for the prevention of chronic periodontitis in humans.

Chronic periodontitis is a common oral inflammatory disease that causes the breakdown of periodontal tissues, including resorption of alveolar bone and tooth loss (7). Furthermore, recent studies have demonstrated that periodontitis is much more than a localized oral infection: it may cause adverse changes in systemic physiology, such as cardiovascular disease, diabetes, and osteoporosis (2, 9, 15, 23, 28, 38, 39, 45). Hence, prevention of periodontitis is important for both oral and systemic health.

Subgingival Gram-negative bacteria are associated with the onset and progression of chronic periodontitis; populations of a few opportunistic pathogens, including *Porphyromonas gingivalis*, are increased during development from a healthy site to a diseased site (7, 22). Molecules such as fimbriae, aggregation factors, lipopolysaccharides, and numerous proteolytic enzymes responsible for colonization have been identified as virulence factors (22, 37). Hemagglutinin, which is known to be located on the cell surface and in vesicles of *P. gingivalis*, has been proposed to mediate bacterial attachment to, and penetration of, host cells and may also agglutinate and lyse erythrocytes in order to take up heme, an absolute requirement for the growth of the bacterium (6, 8, 41). Multiple hemagglutinin genes have been cloned from *P. gingivalis* by functional screening (35, 42, 44, 49). Among these, hemagglutinin A (HagA) has been thought to contain the functional domain of hemagglutinin and to be a potentially useful immunogen that elicits a protective immune response against subsequent colonization by *P. gingivalis* (30). The *hagA* gene is 7,887 bp long and encodes a protein of 2,628 amino acids, with a molecular mass of 283.3 kDa (17). This gene has four large, contiguous direct repeats, and the repeat unit is believed to contain the hemagglutinin domain (17). Previous studies have demonstrated the molecular cloning of a 200-kDa antigenic protein (200-k AP) from *P. gingivalis* and have shown that 200-k AP is identical to HagA (10, 19). Furthermore, the DNA sequence of a subclone encoding the 25-kDa antigenic region of 200-k AP (25k-hagA) is identical to that of the first repeat of *hagA* (10). These studies suggest that 25k-hagA may be a useful vaccine antigen (Ag) for the prevention of periodontitis caused by *P. gingivalis* infection.

Maltose-binding protein (MBP) is a high-affinity maltose/maltodextrin-binding protein responsible for the capture and transport of maltodextrins from the periplasmic space in Gram-negative bacteria (4). MBP is used as a fusion partner for recombinant protein expression to improve the yield and to

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facilitate the purification of fusion proteins (5, 47). Furthermore, the stability and solubility of a passenger protein can be improved by fusing it to MBP (12). Recently, MBP was used as a chaperone component in various vaccines and was shown to enhance antigen-specific immune responses (32, 46, 48, 51, 52). In this regard, a previous *in vitro* study has shown that MBP induces dendritic cell (DC) activation and increases Iκβ phosphorylation in treated cells. Furthermore, phosphorylation of Iκβ is largely abrogated by the addition of antibodies against toll-like receptor 4 (TLR4) (11). These findings suggest that MBP stimulates DCs via TLR4, and this may account for the adjuvanticity of MBP.

In the present study, we assessed the potential of a fusion protein consisting of the 25-kDa antigenic region of *P. gingivalis* HagA and MBP (25k-hagA-MBP) as a nasal vaccine for the prevention of oral infection with *P. gingivalis*. The results suggest that nasal 25k-hagA-MBP is a practical, effective, and safe vaccine candidate for the induction of protective immunity against alveolar bone loss caused by *P. gingivalis* infection.

MATERIALS AND METHODS

Mice. BALB/c mice and Toll-like receptor 4 gene-disrupted ($TLR4^{-/-}$) BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The mice were maintained under pathogen-free conditions at the experimental facility of the Nihon University School of Dentistry at Matsudo. All mice were randomly assigned to control or experimental groups of 4 to 6 mice each and were provided *ad libitum* access to sterile food and water. They were used at the age of 8 to 12 weeks in accordance with the Guidelines for the Care and Use of Laboratory Animals (Nihon University School of Dentistry at Matsudo).

Antigen. Plasmid pMD157, encoding the 25k-hagA-MBP fusion protein, was constructed as described previously (25). Briefly, plasmid pMD101, encoding 200-k AP (19), was partially digested with HaeIII, methylated, and ligated with EcoRI polylinkers. Plasmid vector pMAL-c2 (New England Biolabs, Ipswich, MA), which carries the MBP gene, was digested with EcoRI. Both digested DNAs were ligated with T4 DNA ligase and were then transformed into *Escherichia coli* K-12 cells. The constructed clones were screened by Western blot analysis with antibodies against recombinant 200-k AP and MBP. 25k-hagA-MBP and MBP-free 25k-hagA were purified to homogeneity utilizing amylase resin affinity chromatography (New England Biolabs). The purity of the proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and no contaminating protein bands were noted. Furthermore, the possible presence of residual endotoxin in the preparation was assessed with a Pyrochrome *Limulus* amoebocyte lysate (LAL) kit (Associates of Cape Cod Inc., Woods Hole, MA). 25k-hagA-MBP and 25k-hagA contained as little as 0.3 pg of endotoxin, which did not induce cell activation *in vitro* (data not shown). Cholera toxin (CT) was obtained from List Biological Laboratories (Campbell, CA).

Immunization and sample collection. Mice were immunized nasally on days 0, 7, and 14 with a 10-μl aliquot (5 μl per nostril) of phosphate-buffered saline (PBS) containing 20 μg of either 25k-hagA, 25k-hagA-MBP, or MBP. In some experiments, mice were given 20 μg of 25k-hagA plus 1 μg of CT (as a mucosal adjuvant) nasally. Serum and saliva samples were collected from each group, as described elsewhere (56), in order to examine 25k-hagA-specific antibody responses.

Detection of antigen-specific antibody responses. Antibody titers in serum and saliva were determined by enzyme-linked immunosorbent assays (ELISA). Briefly, plates were coated with 25k-hagA (5 μg/ml) or a sonicated extract of *P. gingivalis* (5×10^8 cells/ml) and were 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 $1:2^5$, while that of saliva was $1:2^1$. The plates were incubated for 4 h at room temperature, washed, and then incubated with horseradish peroxidase-conjugated goat anti-mouse heavy chain γ-, γ1-, γ2a-, γ2b-, γ3-, or α-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) at 4°C for 20 h. 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 giving an optical density at 415 nm of 0.1 greater than that of nonimmunized control samples after 15 min of incubation.

Assessment of antibody-forming cells. Single-cell suspensions were obtained from the salivary gland 7 days after the last immunization. Briefly, salivary glands were carefully excised, teased apart, and dissociated using 0.3 mg/ml collagenase (Nitta Gelatin Co. Ltd., Osaka, Japan) in RPMI 1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan). Mononuclear cells were obtained at the interface of the 50% and 75% layers of a discontinuous Percoll gradient (GE Healthcare UK, Ltd., Little Chalfont, United Kingdom) (36). To assess numbers of antigen-specific antibody-forming cells, an enzyme-linked immunospot (ELISPOT) assay was performed as described previously (57). Briefly, 96-well nitrocellulose plates (BD Biosciences, Franklin Lakes, NJ) were coated with 25k-hagA (5 μg/ml), incubated for 20 h at 4°C, and then washed extensively before being blocked with RPMI 1640 containing 10% fetal calf serum. After 30 min, the blocking solution was discarded, and cell suspensions at various dilutions were added to wells and incubated for 4 h at 37°C under 5% CO_2 in moist air. The cells were washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse heavy chain α-specific antibodies (Southern Biotechnology Associates) at 4°C for 20 h. Following incubation, the plates were washed with PBS and were developed by the addition to each well of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 (Moss). Plates were incubated at room temperature for 25 min and were washed with water, and antibody-forming cells were then counted with the aid of a stereomicroscope (Olympus, Tokyo, Japan).

25k-hagA-specific $CD4^+$ T cell responses. $CD4^+$ T cells from spleens and cervical lymph nodes (CLNs) were isolated 7 days after the last immunization by using the IMag system (BD Biosciences) as described elsewhere (18). Briefly, mononuclear cells were mixed with anti-CD4 antibodies and were incubated at 4°C for 30 min, after which $CD4^+$ T cells were separated using a magnet. The purity of $CD4^+$ T cells was determined by flow cytometry (FACSCalibur; BD Biosciences) and was routinely >90%. The $CD4^+$ T cells (2.0×10^6/ml) were then cultured with 5 μg/ml of 25k-hagA in the presence of T cell-depleted, mitomycin-treated splenic feeder cells (2.5×10^6 cells) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 15 mM HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 U/ml of recombinant interleukin 2 (IL-2). Cultures were incubated for 5 days at 37°C under 5% CO_2 in air. To measure 25k-hagA-specific cell proliferation, 1.0 μCi of [^{3}H]thymidine was added to the culture 18 h before harvesting, and incorporated radioactivity was measured by scintillation counting. Culture supernatants were harvested after 5 days of incubation. The levels of IL-4, IL-5, and gamma interferon (IFN-γ) in the culture supernatants were determined using commercially available assay kits (Pierce Biotechnology, Inc., Rockford, IL) in accordance with the manufacturer's instructions.

DC analysis. Dendritic cell (DC)-enriched cell populations were isolated from spleens, CLNs, and nasopharynx-associated lymphoreticular tissues (NALT) 7 days after the last immunization, as described previously (26). 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 to 90 min. EDTA was added (final concentration, 10 mM), 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. DC-enriched cell populations were analyzed for the expression of various cell surface molecules by using fluorescence-labeled antibodies. The purity of $CD11c^+$ cells was routinely >50%. Aliquots of mononuclear cells (0.2×10^6 to 1.0×10^6 cells) isolated from various tissues were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD80, CD86, CD40, or $I\text{-}A^d$ monoclonal antibodies; phycoerythrin (PE)-labeled anti-mouse CD11c monoclonal antibodies; and Alexa-labeled anti-mouse B220, CD11b, and CD8α monoclonal antibodies (BD Biosciences). Samples were then subjected to fluorescence-activated cell sorter (FACS) analysis (BD Biosciences).

Oral infection. Mice were orally infected with *P. gingivalis* as described previously (1, 14, 33), with minor modifications. Briefly, mice were given *ad libitum* access to deionized water containing sulfamethoxazole-trimethoprim (Sulfatrim; Goldline Laboratories, Fort Lauderdale, FL) at 10 ml per pint for 10 days. This was followed by a 3-day antibiotic-free period. Mice were then administered 10^9 CFU of *P. gingivalis* suspended in 100 μl of PBS with 2% carboxymethylcellulose via oral topical application. Mice were inoculated 5 times a week (from Monday to Friday) for 3 weeks, for a total of 15 inoculations. Control groups included sham-infected mice, which received antibiotic pretreatment and carboxymethylcellulose without *P. gingivalis*.

Measurement of alveolar bone loss. Forty-seven days after the first gavage, mice were euthanized using CO_2 for assessment of alveolar bone loss. Horizontal bone loss around the maxillary molars was assessed using a morphometric method as described previously (29). Briefly, skulls were defleshed after 10 min of treatment in boiling water under 15-lb/in^2 pressure, immersed overnight in 3%

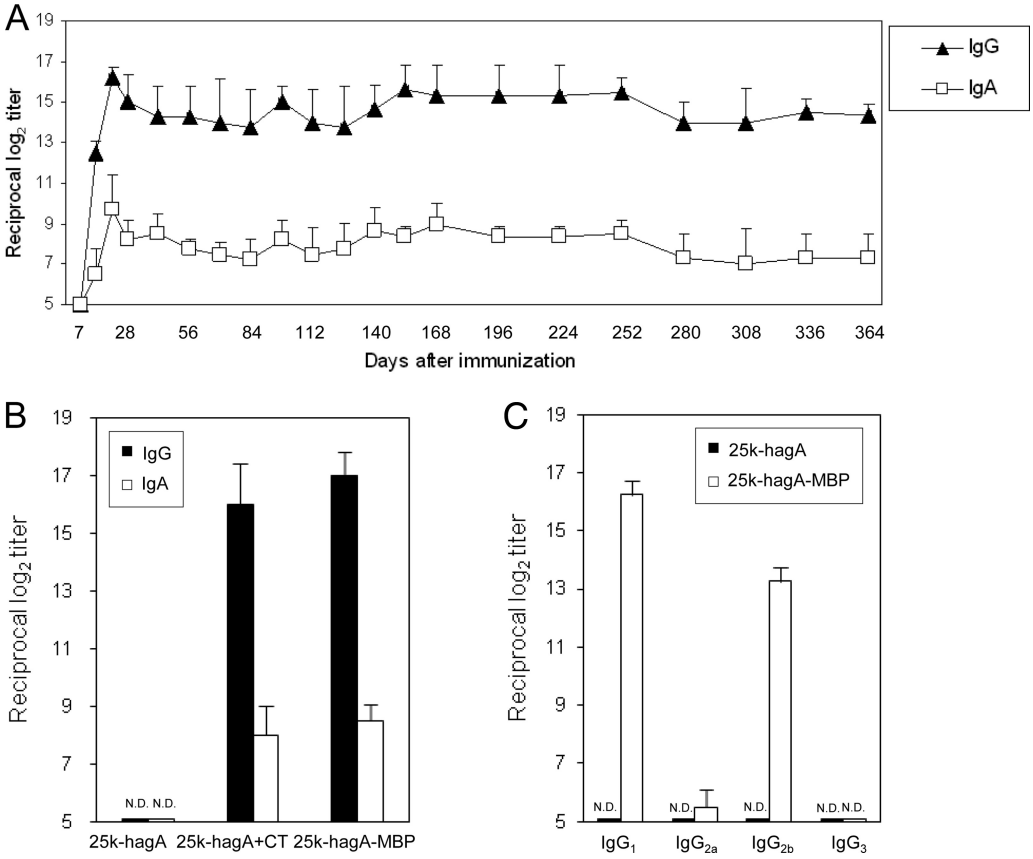

FIG. 1. 25k-hagA-specific IgG and IgA antibody responses in serum. Groups of mice were immunized nasally with either 20 μg of 25k-hagA, 20 μg of 25k-hagA-MBP, or 20 μg of 25k-hagA plus 1 μg of CT on days 0, 7, and 14. (A) Time course of 25k-hagA-specific IgG and IgA antibodies induced by nasal 25k-hagA-MBP. (B and C) One week after the final immunization, serum samples were collected in order to compare antibody titers between 25k-hagA-MBP and 25k-hagA plus CT, as well as IgG subclass responses. (B) Comparison of antibody responses in mice given 25k-hagA, 25k-hagA-MBP, or 25k-hagA plus CT. (C) IgG subclass responses in mice given 25k-hagA or 25k-hagA-MBP. Results are expressed as means ± SE for 4 to 6 mice per group in a total of three experiments. The *P* values for the comparison of antibody titers with 25k-hagA-MBP versus 25k-hagA were <0.05 at all times except day 7. ND, not detectable.

hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at a total of 14 buccal sites per mouse. Measurements were made under a dissecting microscope (magnification, ×50) fitted with a video image marker measurement system (VHX-100; Keyence, Osaka, Japan) standardized to give measurements in micrometers. Bone measurements were performed a total of three times by two evaluators using a random and blinded protocol.

Distribution of 25k-hagA-MBP. The 25k-hagA-MBP protein and CT were labeled with acridinium ester (Assay Designs, Inc., Ann Arbor, MI) in accordance with the manufacturer's instructions. Mice were given acridinium ester-labeled 25k-hagA-MBP (10 μg and 20 μg) or acridinium ester-labeled CT (1 μg and 5 μg) via the nasal route. All nasal applications were given in a final volume of 10 μl (5 μl per nostril) to naïve mice. At 24 h after administration, mice were sacrificed, and the olfactory nerves and epithelium (ON/E), olfactory bulbs (OB), and brains were removed as described previously (54). Each tissue was homogenized; the homogenates were centrifuged at 10,000 × *g* for 10 min; and the supernatants were tested for light activity by a luminometer (ARVO MX; Perkin-Elmer, Waltham, MA).

Statistics. Data are expressed as means ± standard errors (SE) and were compared using an unpaired Student *t* test.

RESULTS

Nasal 25k-hagA-MBP elicits long-term antibody responses in a TLR4-dependent manner. In an initial study, mice were immunized nasally with various amounts (10, 20, and 50 μg) of 25k-hagA-MBP or 25k-hagA in order to determine optimal concentrations for the induction of antibody responses. All of the dosages of 25k-hagA failed to induce 25k-specific serum IgG or IgA antibody responses that were above the dilution cutoff ($\log_2$ of 5) used in our experiments. In contrast, 20 μg of 25k-hagA-MBP induced high 25k-hagA-specific serum antibody titers that were comparable to those induced by 50 μg of the fusion protein (data not shown). Therefore, 20 μg of 25k-hagA-MBP was used throughout this study. The time course of anti-25k-hagA-specific serum antibody responses is shown in Fig. 1A. Mice immunized nasally with 25k-hagA-MBP showed significant levels of serum IgG and IgA antibodies. Furthermore, the serum IgG and IgA antibody responses induced by 25k-hagA-MBP persisted for at least 1 year. As expected, administration of MBP alone or PBS did not induce 25k-hagA-specific antibody responses (data not shown). To provide a direct comparison of antibody responses to 25k-hagA-MBP, mice were given 25k-hagA plus CT as the adjuvant nasally. Interestingly, serum anti-25k-hagA IgG and IgA antibody titers induced by 25k-hagA-MBP were comparable to those induced by 25k-hagA plus CT (Fig. 1B). Analysis of IgG sub-

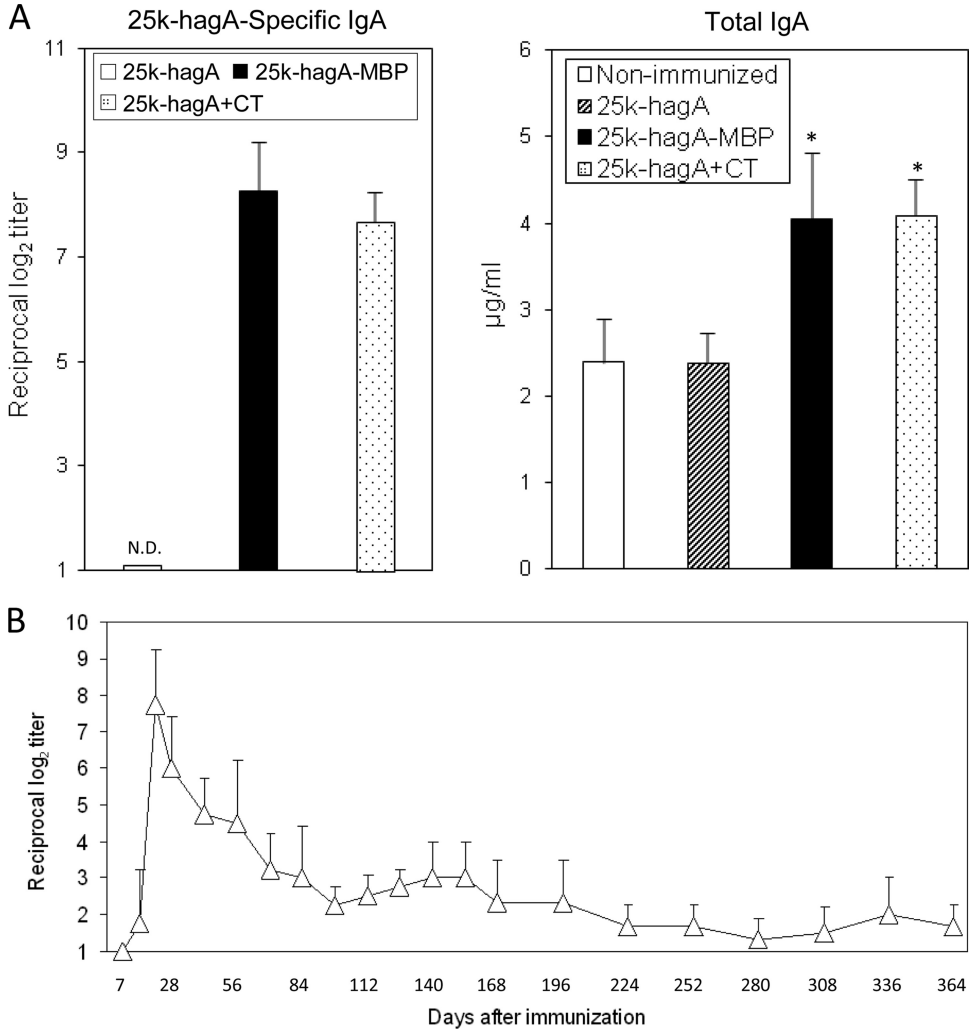

FIG. 2. 25k-hagA-specific IgA antibody response in saliva. Groups of mice were nasally immunized with either 25k-hagA, 25k-hagA-MBP, or 25k-hagA plus CT as described in the legend to Fig. 1. (A) Saliva samples were collected 7 days after the final immunization and were assessed for 25k-hagA-specific IgA (left) and total-IgA (right) antibody responses. (B) The time course of 25k-hagA-specific IgA antibodies induced by nasal 25k-hagA-MBP was also examined. Results are expressed as means ± SE from 4 to 6 mice per group in a total of three experiments. The *P* values for the comparison of IgA antibody titers with 25k-hagA-MBP versus 25k-hagA were <0.05 at all times except days 7 and 280. ND, not detectable.

classes in mice given 25k-hagA-MBP revealed that the major subclass was IgG1 and that IgG2b had the second-highest titer (Fig. 1C).

Nasal administration of 25k-hagA-MBP induced high levels of total and 25k-hagA-specific IgA antibody responses in saliva samples obtained a week after the third immunization, and these levels of antibodies were comparable to those induced by nasal 25k-hagA plus CT (Fig. 2A). In addition, IgA anti-25k-hagA antibodies in saliva were maintained for 1 year, although the responses decreased gradually from day 28 (Fig. 2B). As expected, nasal delivery of 25k-hagA, MBP alone, or PBS failed to elicit 25k-hagA-specific antibody titers in the starting dilution ($\log_2$ of 1) used in these experiments. Analysis of antibody-forming cells confirmed the results described above by revealing high numbers of IgA antibody-forming cells in salivary glands following nasal administration of 25k-hagA-MBP and by showing low numbers of antibody-forming cells in the salivary glands of mice given 25k-hagA alone [(170 ± 16) × 10^6 cells for 25k-hagA-MBP versus (34 ± 21) × 10^6 cells for 25k-hagA].

To test the possibility that MBP adjuvanticity is mediated via signaling through TLR4, we examined antibody responses in TLR4$^{-/-}$ mice. To this end, TLR4$^{-/-}$ mice were immunized nasally with 25k-hagA-MBP or 25k-hagA by using an immunization regimen identical to that described above. As expected, only low 25k-hagA-specific serum IgG antibody responses, and no detectable serum IgA antibody responses, were induced in TLR4$^{-/-}$ mice on day 21, while nasal administration to TLR4$^{+/+}$ mice resulted in the induction of high IgG and IgA antibody responses. Furthermore, nasal immunization of TLR4$^{-/-}$ mice with 25k-hagA-MBP failed to elicit anti-25k-hagA IgA antibodies in the saliva (Fig. 3). Although we continued to examine antibody levels until day 49, the responses in the TLR4$^{-/-}$ mouse group did not change (data not shown). As expected, nasal immunization of TLR4$^{-/-}$ mice with 25k-hagA failed to induce 25k-hagA-specific serum IgG, serum

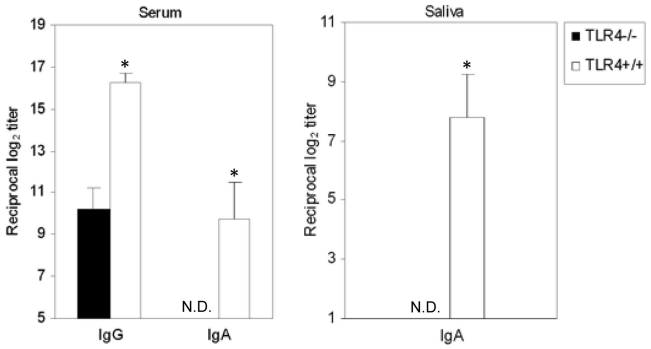

FIG. 3. 25k-hagA-specific serum IgG and IgA and salivary IgA antibody responses in TLR4$^{-/-}$ mice. Groups of TLR4$^{-/-}$ or TLR4$^{+/+}$ mice were immunized nasally with 25k-hagA-MBP as described in the legend to Fig. 1. Serum and saliva samples were assessed for 25k-hagA-specific serum IgG and IgA and salivary IgA antibody responses. Results are expressed as means ± SE from 5 mice per group. *, $P < 0.05$ for comparison with TLR4$^{-/-}$ mice. ND, not detectable.

IgA, or salivary IgA antibody responses. Taken together, these results indicate that 25k-hagA-MBP is a potential nasal vaccine for the induction of Ag-specific mucosal and systemic antibody responses and that signaling through TLR4 is involved in, and is necessary for, the mucosal adjuvanticity of MBP.

25k-hagA-specific CD4$^+$ T cell responses. Since nasal immunization with 25k-hagA-MBP elicited 25k-hagA-specific antibody responses in both mucosal and systemic compartments, it was important to establish the nature of the CD4$^+$ T cell help supporting the 25k-hagA-specific antibody responses. When CD4$^+$ T cells from the spleens or CLNs (which are draining lymph nodes of the maxillofacial mucosal compartments) of immunized mice were restimulated with 25k-hagA *in vitro*, significant levels of proliferative responses were induced (Fig. 4A).

Analysis of the cytokine responses revealed that 25k-hagA-specific CD4$^+$ T cells from the spleen and CLNs produced high levels of IL-4 and IL-5 (Fig. 4B). In contrast, CD4$^+$ T cells from immunized mice had an impaired ability to produce IFN-γ (data not shown). These results clearly show that nasal administration of 25k-hagA-MBP induces Th2-type cytokine responses to support antigen-specific mucosal IgA, as well as serum IgG and IgA, antibody responses.

Nasal administration of 25k-hagA-MBP expands CD11c$^+$ CD8α$^+$ DCs in mucosal and systemic lymphoid tissues. We next investigated the frequency of CD11c$^+$ DCs in various mucosal and systemic lymphoid tissues. Our results showed large increases in the percentages of CD11c$^+$ CD8α$^+$ DCs in the spleens, CLNs, and NALT of mice given nasal 25k-hagA-MBP relative to those in mice given 25k-hagA. In contrast, the proportions of CD11c$^+$ CD11b$^+$ DCs and CD11c$^+$ B220$^+$ DCs were not altered (Fig. 5). Furthermore, these expanded DCs expressed greater numbers of costimulatory molecules (CD40, CD80, CD86, and major histocompatibility complex class II [MHC II]) than DCs from mice given 25k-hagA (Table 1). The activation of CD11c$^+$ CD8α$^+$ DCs by 25k-hagA-MBP was due to the adjuvanticity of MBP, because nasal administration of MBP induced this DC subset (Fig. 5 and Table 1).

Since some activated T cells can also express CD11c, the proportions of CD11c$^+$ CD8α$^+$ DCs were reevaluated with MHC class II. The results showed that the percentages of MHC class II^{high} CD8α$^+$ DCs in CD11c$^+$ cells of the spleens, CLNs, and NALT of mice given nasal 25k-hagA-MBP or MBP alone were higher than those for mice given 25k-hagA (Table 2). Taken together, these results indicate that nasal administration of 25k-hagA-MBP preferentially expands CD11c$^+$ CD8α$^+$ DCs and also induces their activation in both mucosal inductive and systemic lymphoid tissues.

Nasal 25k-hagA-MBP reduces alveolar bone loss caused by oral infection with *P. gingivalis*. Since nasal 25k-hagA-MBP elicited long-term antigen-specific antibody responses in sera and saliva, we sought to determine whether these antibodies were protective. Thus, mice given 25k-hagA-MBP were infected orally with *P. gingivalis*. Mice immunized with 25k-hagA-MBP or 25k-hagA plus CT showed a significant reduction in alveolar bone loss caused by *P. gingivalis* infection 7 days

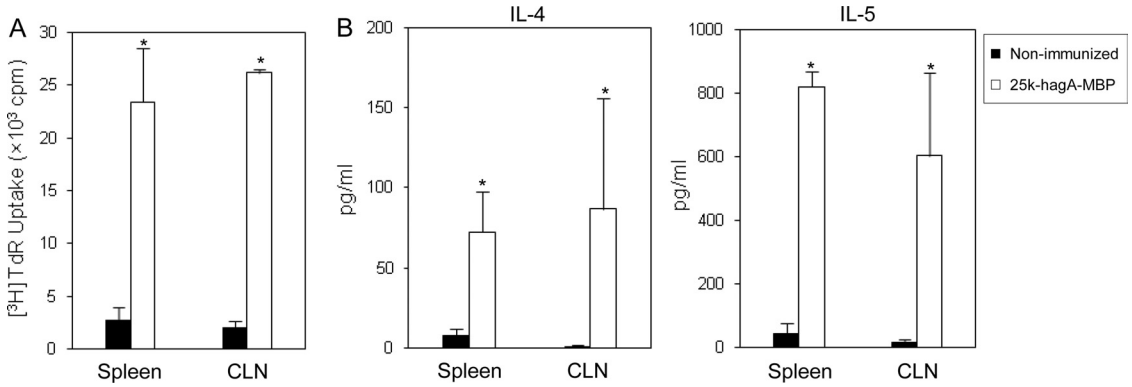

FIG. 4. 25k-hagA-specific CD4$^+$ T cell responses in the spleen and CLNs. Groups of mice were immunized nasally with 25k-hagA-MBP as described in the legend to Fig. 1. CD4$^+$ T cells were isolated from the spleens or CLNs of nonimmunized or immunized mice and were cultured with 25k-hagA in the presence of splenic feeder cells. (A) To measure cell proliferation, 1.0 μCi of [^{3}H]thymidine was added to the culture 18 h before harvesting, and incorporated radioactivity was measured by scintillation counting. (B) In order to analyze cytokine synthesis, culture supernatants were harvested, and the levels of secreted IL-4 and IL-5 were assessed by cytokine-specific ELISA. IFN-γ was not detectable. The results are representative of three separate experiments with 4 to 6 mice in each group/experiment. *, $P < 0.05$ for comparison with cells from nonimmunized mice.

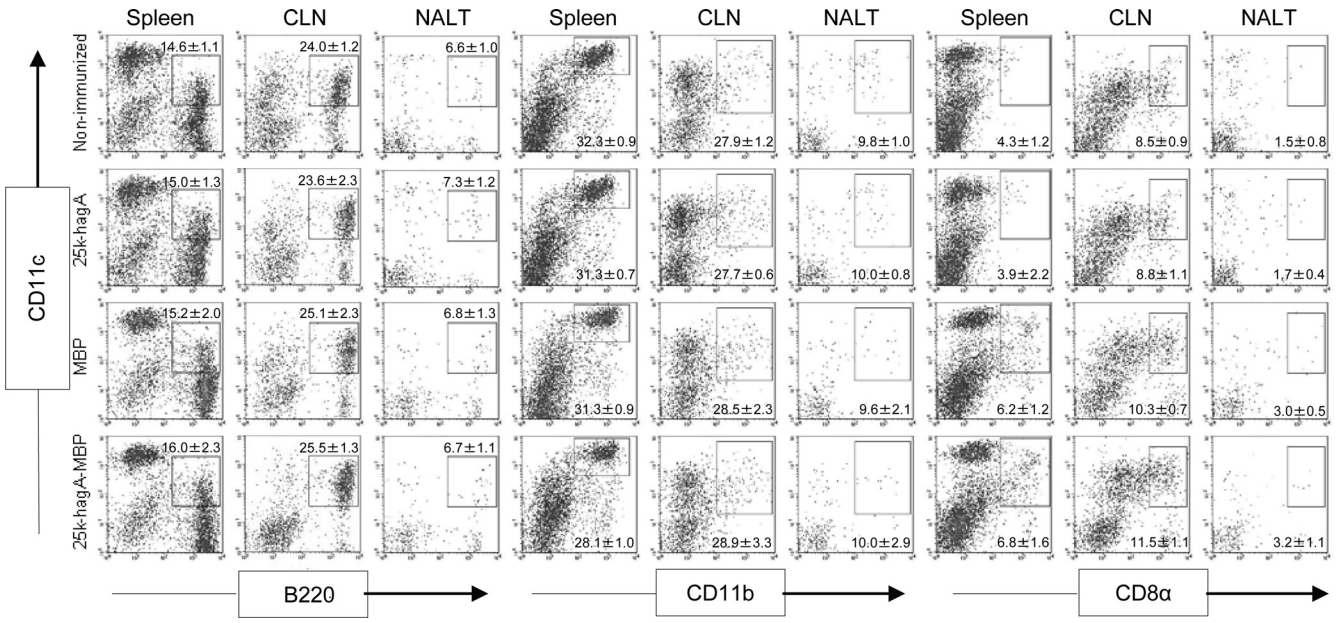

FIG. 5. Comparison of the proportions of CD11c$^+$ DCs in various lymphoid tissues. Mice were immunized nasally with 25k-hagA, MBP, or 25k-hagA-MBP as described in the legend to Fig. 1. DC-enriched cell populations isolated from the spleen, CLNs, and NALT were stained with fluorescent-dye-conjugated monoclonal antibodies and were then subjected to flow cytometry. The results are representative of three separate experiments. The percentage of cells contained in the boxed region is given in each panel.

after immunization. In contrast, mice given 25k-hagA alone or MBP alone failed to reduce alveolar bone loss (Fig. 6A). Furthermore, the antigen-specific antibody responses induced by nasal 25k-hagA-MBP provided significant protection and reduced bone loss caused by *P. gingivalis* infection, even at 1 year after immunization (Fig. 6B).

In order to elucidate whether specific antibodies induced by nasal 25k-hagA-MBP were protective, we examined anti-25k-hagA as well as anti-*P. gingivalis* antibodies in serum and saliva before and after *P. gingivalis* infection. The results indicated that the levels of serum IgG, serum IgA, and salivary IgA antibodies to both 25k-hagA and *P. gingivalis* were identical before and after infection (Fig. 6C and D). These results indicate that specific antibodies induced by nasal 25k-hagA-MBP provide protection against *P. gingivalis* infection. Taken together, these findings indicate that nasal immunization with 25k-hagA-MBP provides long-term protection against oral infection by *P. gingivalis*.

TABLE 1. Comparison of costimulatory-molecule expression by CD11c$^+$ CD8α$^+$ DCs in several lymphoid tissues of mice given nasal 25k-hagA-MBP[a]

Tissue and vaccine	Mean fluorescence intensity			
	CD80	CD86	CD40	MHC II
Spleen				
No immunization	26.8 ± 3.0	21.7 ± 2.0	177.3 ± 7.6	789.9 ± 41.8
25k-hagA	27.6 ± 4.9	20.9 ± 1.5	182.1 ± 8.2	804.4 ± 53.3
MBP	33.6 ± 3.8	25.9 ± 2.6	312.5 ± 16.8	1,080.0 ± 193.5*
25k-hagA-MBP	39.2 ± 10.2*	27.8 ± 3.7*	345.8 ± 35.4*	1,083.2 ± 215.3*
CLN				
No immunization	14.1 ± 2.8	25.9 ± 1.8	201.9 ± 42.8	803.2 ± 50.1
25k-hagA	13.8 ± 4.4	27.3 ± 2.9	222.5 ± 52.5	807.2 ± 43.6
MBP	22.9 ± 4.2*	40.2 ± 9.4*	306.1 ± 47.2*	992.6 ± 140.4*
25k-hagA-MBP	25.1 ± 7.9*	43.6 ± 12.7*	328.6 ± 49.8*	1,046.7 ± 188.5*
NALT				
No immunization	6.0 ± 1.2	10.8 ± 2.3	57.1 ± 15.2	228.1 ± 10.0
25k-hagA	6.7 ± 0.5	12.5 ± 3.0	56.8 ± 23.7	236.4 ± 5.7
MBP	12.9 ± 2.2*	18.8 ± 2.1*	206.1 ± 48.3*	766.2 ± 129.4*
25k-hagA-MBP	15.3 ± 4.9*	19.3 ± 2.9*	233 ± 84.9*	785.1 ± 142.1*

[a] DC-enriched cell populations from the spleens, CLNs, and NALT of mice immunized with 25k-hagA, MBP, or 25k-hagA-MBP were stained with FITC-conjugated anti-CD80, -CD86, -CD40, or -I-A^d antibodies, with PE-labeled anti-CD11c antibodies, and with Alexa-labeled anti-CD8α. *, $P < 0.05$ for comparison with mice given 25k-hagA.

TABLE 2. Comparison of frequency of MHC class II^{high} CD8α$^+$ DCs in CD11c$^+$ cells from several lymphoid tissues[a]

Vaccine	% MHC class II^{high} CD8α$^+$ DCs		
	Spleen	CLNs	NALT
None	8.6 ± 0.8	5.3 ± 0.4	8.1 ± 0.5
25k-hagA	8.2 ± 0.5	5.5 ± 0.9	7.8 ± 0.9
MBP	13.1 ± 1.0*	8.5 ± 0.6*	12.4 ± 0.7*
25k-hagA-MBP	13.6 ± 1.1*	8.9 ± 0.4*	13.5 ± 0.5*

[a] DC-enriched cell populations from the spleens, CLNs, and NALT of mice immunized with 25k-hagA, MBP, or 25k-hagA-MBP were stained with FITC-conjugated anti-I-A^d and PE-labeled anti-CD11c antibodies and with Alexa-labeled anti-CD8α antibodies. *, $P < 0.05$ for comparison with nonimmunized mice and with mice given 25k-hagA.

Nasal 25k-hagA-MBP does not target neuronal tissues. The distribution of 25k-hagA-MBP in various tissues was analyzed after nasal administration of acridinium ester-labeled 25k-hagA-MBP and was compared with that in groups inoculated with acridinium ester-labeled CT. Significantly lower levels of 25k-hagA-MBP accumulation were observed in the ON/E, OB, and brain samples isolated from the group given 25k-hagA-MBP nasally than from the group given CT (Fig. 7). These results indicate that nasal administration of 25k-hagA-MBP does not target neuronal tissues.

DISCUSSION

Previous studies have shown that *hagA* has four large, contiguous direct repeats, and the repeat unit is thought to contain the hemagglutinin domain (17, 43). Furthermore, the DNA sequence of 25k-hagA is identical to that of the first repeat of *hagA* (10). These findings have led to the consideration of 25k-hagA as a candidate Ag for the development of human vaccines. Our present study explored the potential of 25k-hagA-MBP as a nasal vaccine by using it as a model system with which to study the nature and kinetics of 25k-hagA-MBP-induced Ag-specific antibody responses and their protective ability against oral infection by *P. gingivalis*. In the initial study, 25k-hagA was administered nasally for the investigation of Ag-specific antibody responses. The results demonstrated that

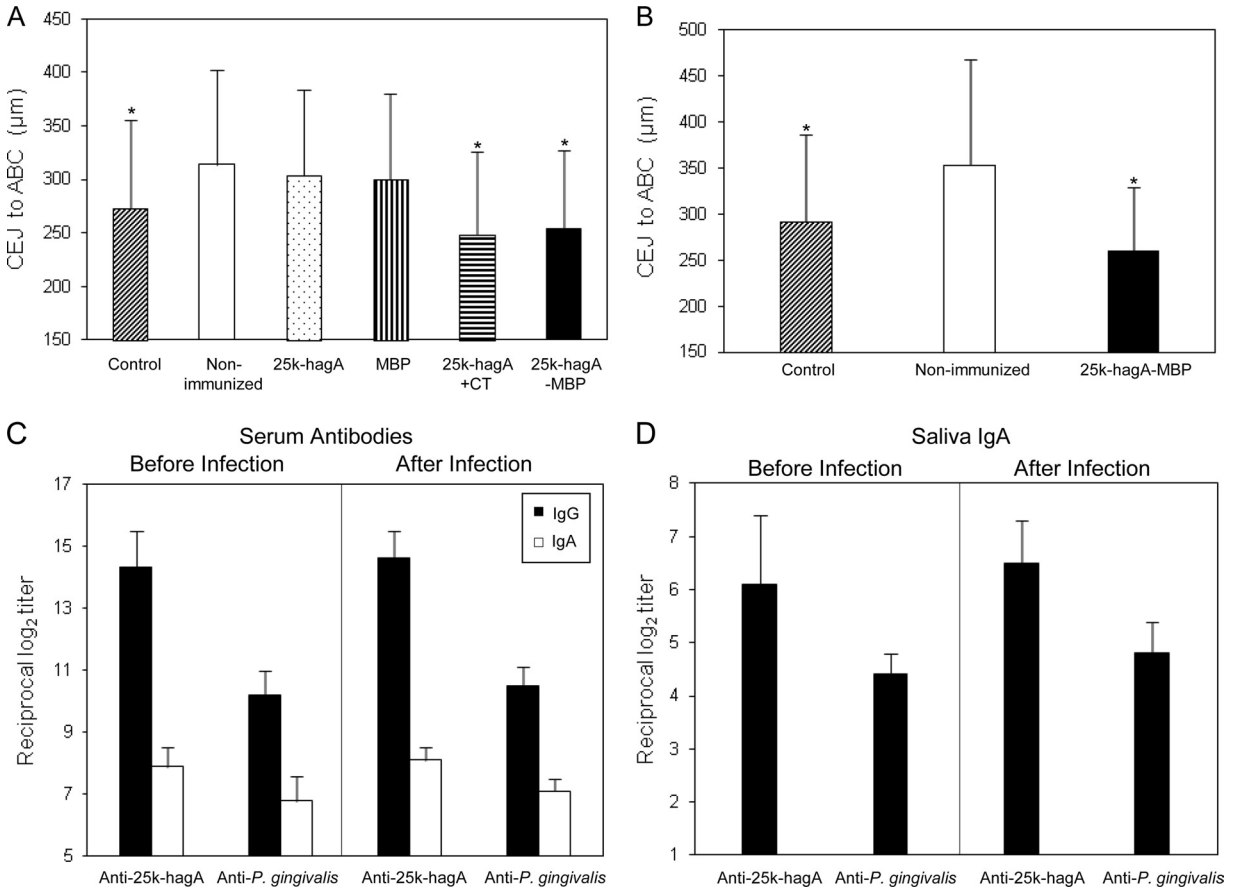

FIG. 6. Antibodies induced by nasal immunization with 25k-hagA-MBP reduced *P. gingivalis*-induced alveolar bone loss. Groups of mice were immunized nasally with either 25k-hagA, MBP, 25k-hagA plus CT, 25k-hagA-MBP, or PBS, as described in the legend to Fig. 1. (A and B) Seven days (A) or 1 year (B) after immunization, immunized mice were inoculated orally with 10^9 CFU of *P. gingivalis* in 2% carboxymethylcellulose, as described in Materials and Methods. Control mice were sham-infected mice inoculated with 2% carboxymethylcellulose only. The distance (in micrometers) from the CEJ to the ABC was measured at 14 predetermined sites in defleshed maxilla and was totaled for each mouse. *, $P < 0.05$ for comparison with nonimmunized mice. (C and D) Furthermore, 25k-hagA-specific or *P. gingivalis*-specific serum IgG and IgA (C) and salivary IgA (D) antibody responses before and after infection with *P. gingivalis* were examined. The results are expressed as means ± SE for 6 mice per group.

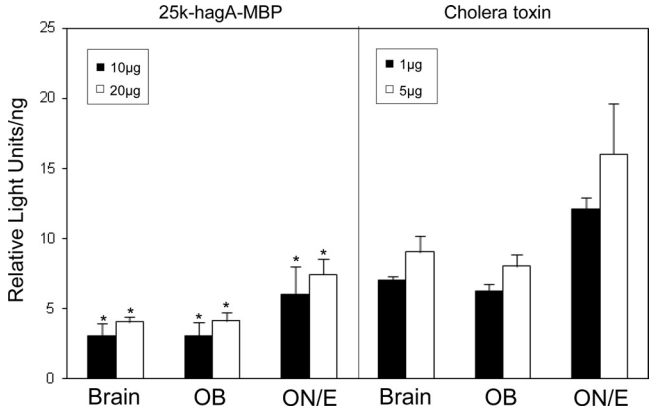

FIG. 7. Accumulation of 25k-hagA-MBP or CT in neuronal tissues after nasal challenge with acridinium ester-labeled 25k-hagA-MBP (10 μg or 20 μg) or acridinium ester-labeled CT (1 μg or 5 μg). The results are expressed as the mean relative light units for a particular tissue ± SE and are from two separate experiments with 5 mice per group. *, $P < 0.05$ for comparison with mice given 1 μg and 5 μg of CT.

nasal administration of 25k-hagA alone failed to induce anti-25k-hagA serum IgG, serum IgA, and salivary IgA antibody responses. These findings indicate that 25k-hagA is a weak immunogen when given via the nasal route. Indeed, numerous studies have demonstrated that nasal administration of proteins is only weakly immunogenic and requires appropriate Ag delivery systems for the induction of sufficient immune responses in both mucosal and systemic compartments (21, 31, 58).

MBP has been widely used as a chaperone component in vaccines and has been shown to enhance Ag-specific humoral and cellular immune responses (32, 46, 48, 51, 52). Thus, in the present study, we assessed the potential of a nasal vaccine, 25k-hagA fused to MBP, to induce an immune response after host challenge. We have demonstrated that nasal administration of 25k-hagA-MBP induces high levels of 25k-hagA-specific serum IgG, serum IgA, and salivary IgA antibody responses that are comparable to those induced by 25k-hagA plus an established mucosal adjuvant, CT. Furthermore, these antibodies persist for at least 1 year. Induction of antibody responses is associated with elevated numbers of activated $CD11c^+$ $CD8\alpha^+$ DCs in both mucosal and systemic lymphoid tissues. Thus, increased proportions of $CD11c^+$ $CD8\alpha^+$ DCs with upregulated expression of MHC II, CD40, CD80, and CD86 molecules in NALT, CLNs, and the spleen were noted in mice given 25k-hagA-MBP. Furthermore, 25k-hagA-MBP induced $CD4^+$ T cells producing predominantly Th2 cytokines (IL-4 and IL-5), as well as IgG1 and IgG2b responses. Importantly, mice given 25k-hagA-MBP were significantly protected against alveolar bone loss caused by oral infection with *P. gingivalis*, even 1 year after immunization. Interestingly, however, our separate study has shown that when 25k-hagA mixed with MBP was given nasally to mice, no detectable antibody titers were induced (Y. Du et al., unpublished observations). Thus, unlike other adjuvants, MBP should be fused to the target antigen. These studies demonstrate that MBP is an effective adjuvant for nasal immunization and that when used as a fusion partner for 25k-hagA, it facilitates the development of a long-term protective antibody response.

A previous study confirmed by Western immunoblotting that oral immunization with recombinant avirulent *Salmonella enterica* serovar Typhimurium expressing HagA induces HagA-specific serum antibody responses (30). In addition, a hen egg yolk antibody (IgY) against a truncated HagA protein has been developed (53). However, none of these studies have quantitatively evaluated the immunogenicity as well as the protective efficacy of HagA vaccines. Thus, our results are the first to show that a nasal vaccine combining MBP and 25k-hagA induces a long-term protective antibody response against oral infection with *P. gingivalis*.

It is well known that $CD4^+$ T cells and their derived Th cytokines are essential for the induction of Ag-specific antibody responses. We found that $CD4^+$ T cells from the spleens and CLNs of mice immunized nasally with 25k-hagA-MBP secreted IL-4 and IL-5 in response to stimulation with 25k-hagA. On the other hand, no detectable IFN-γ production was found. IgG subclass responses confirmed the cytokine profile, showing that 25k-hagA-MBP elicited anti-25k-hagA IgG1 and IgG2b antibodies. These results suggest that the adjuvant activity of nasally administered MBP is mediated by IL-4-producing Th2-type $CD4^+$ T cells. However, a previous study has shown that intraperitoneal immunization with a *Rickettsia tsutsugamushi* antigen fused with MBP induces IFN-γ-producing Th1-type responses (48). The basis for the different T helper cytokine patterns induced by MBP is not known. One possible explanation is the route of immunization. In this regard, it has been shown that DCs freshly isolated from Peyer's patches, but not from the spleen, induce the differentiation of Th2 cells (24). Furthermore, single-cell reverse transcription-PCR (RT-PCR) analysis revealed high numbers of Th2 cytokine-specific mRNA molecules expressed by $CD4^+$ T cells in nasal passages, while $CD4^+$ T cells with a Th0 profile were present in NALT (20). Moreover, although CT is known to induce Th2 cells that secrete high levels of IL-4 when it is given via mucosal routes (59), parenteral administration of CT elicited both Th1- and Th2-type cytokine responses (55). Thus, immunization via mucosal routes, such as the nasal or oral route, may favor the induction of Th2-type responses. However, in a previous study, nasal immunization with *P. gingivalis* fimbrial protein plus CT induced both Th1- and Th2-type cytokine responses in $CD4^+$ T cells of the nasal passage and submandibular glands (60). It may be that the combination of nasal immunization and MBP is a particularly effective vaccine regimen for the induction of Th2-type cytokine responses.

Alternatively, the effects of MBP on $CD11c^+$ $CD8\alpha^+$ DCs may particularly induce Th2-type responses. $CD11c^+$ $CD8\alpha^+$ DCs are nonmigrating resident DCs derived from a precursor, distinct from monocytes, that continuously seed the lymphoid organs from the bone marrow. This DC subset has in common with other DCs the ability to take up exogenous antigens and to process them for presentation on MHC class II molecules (50). It is known that DCs play a critical role in directing the differentiation of $CD4^+$ T cells into either Th1 or Th2 cells (3). In this regard, previous studies have demonstrated that nasal administration of a DNA plasmid encoding the Flt3 ligand as an adjuvant stimulates $CD11c^+$ $CD8\alpha^+$ DCs, which, in turn, lead to Th2 responses (13, 27). In support of this, our separate

in vitro study has shown that MBP-treated DCs induced IL-4 production in $CD4^+$ T cells (Du et al., unpublished). These findings suggest that MBP as a mucosal adjuvant stimulates $CD11c^+$ $CD8\alpha^+$ DCs, which enhance Th2 cytokine responses. However, as described above, intraperitoneal immunization with a *Rickettsia tsutsugamushi* antigen fused with MBP induces IFN-γ-producing Th1-type responses (48). It may be that the phenotype of DCs induced by nasal administration of MBP is distinct from that of DCs induced by intraperitoneal administration of MBP. This would explain why nasal but not parenteral immunization induces Th2 responses. This interesting possibility is currently under investigation in our laboratory.

It should be noted that nasal immunization of TLR4-deficient mice with 25k-hagA-MBP induced only low levels of 25k-hagA-specific serum IgG antibody responses. Neither serum IgA nor salivary IgA antibodies against 25k-hagA were detected. In this regard, a previous study has shown that MBP innately activates NF-κB-mediated cytokine signaling pathways via TLR4 (11). To further support this, one of our separate studies has demonstrated that $CD11c^+$ $CD8\alpha^+$ DCs were not activated in TLR4-deficient mice after nasal immunization with 25k-hagA-MBP (Du et al., unpublished). These findings suggest that MBP as an adjuvant stimulates $CD11c^+$ $CD8\alpha^+$ DCs in a TLR4-dependent manner for the induction of 25k-hagA-specific Th2-type cytokine responses in both mucosal and systemic compartments with subsequent serum IgG and IgA and mucosal IgA antibody responses.

Nasal administration of vaccines has been widely used for mucosal immunization because it delivers the antigen directly to nasopharynx-associated lymphoid tissues without the influence of enzymes and acids in the gastrointestinal tract. However, previous studies have shown that nasally administered CT or adenovirus vectors accumulate in the ON/E regions via the GM1 ganglioside (34, 54). Furthermore, CT as an adjuvant redirects coadministered protein Ag into these neuronal tissues (54). A clinical study suggested a strong association between nasal influenza vaccine and Bell's palsy (40). These findings raise concerns about a potential role of GM1-binding molecules that target neural tissues, including the central nervous system, in nasal immunization. However, another study has provided evidence that deposition of CT via the olfactory tissues does not lead to obvious pathological changes in brain tissues after nasal administration (16). Although the exact biological and pathological significance of vaccine deposition in the central nervous system is uncertain, we also investigated the distribution of 25k-hagA-MBP in ON/E and OB after nasal immunization. Interestingly, 25k-hagA-MBP accumulations in the ON/E, OB, and brain were significantly lower than those of CT. The biological effects of residual 25k-hagA-MBP on the neural tissues needs to be elucidated in the next study. Taken together, these results suggest that nasal administration of MBP as a chaperone component in vaccines is safer than that of GM1-binding molecules in terms of the potential threat posed by vaccines trafficking in neural tissues, including the central nervous system.

In summary, the fusion protein containing 25k-hagA and MBP provides a very effective means of eliciting IL-4- and IL-5-producing Th2-type $CD4^+$ T cells for the induction of serum IgG, serum IgA, and mucosal IgA antibody responses. The mechanisms responsible for the effects of MBP are mediated by increased levels of $CD11c^+$ $CD8\alpha^+$ DCs in a TLR4-dependent manner. Finally, 25k-hagA-specific immune responses induced by 25k-hagA-MBP provide protective immunity against alveolar bone loss caused by *P. gingivalis* infection. These findings suggest that nasal administration of 25k-hagA-MBP effectively elicits protective levels of antibodies against 25k-hagA and may be an effective and safe nasal vaccine for the immunization of humans against *P. gingivalis* infection.

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