Atheroprotective nasal immunization with a heat shock protein 60 peptide from *Porphyromonas gingivalis*

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**ABSTRACT**

**Purpose:** Immunization with *Porphyromonas gingivalis* heat shock protein 60 (PgHSP60) may have an immunoregulatory effect on atherogenesis. The aim of this study was to determine whether nasal immunization with a PgHSP60 peptide could reduce atherosclerotic plaque formation in apolipoprotein E knockout (ApoE KO) mice.

**Methods:** Seven-week-old male ApoE KO mice were assigned to receive a normal diet, a Western diet, a Western diet and challenge with PgHSP60-derived peptide 14 (Pep14) or peptide 19 (Pep19), or a Western diet and immunization with Pep14 or Pep19 before challenge with Pep14 or Pep19.

**Results:** Atherosclerotic plaques were significantly smaller in mice that received a Western diet with Pep14 nasal immunization than in mice that received a Western diet and no Pep14 immunization with or without Pep14 challenge. An immunoblot profile failed to detect serum reactivity to Pep14 in any of the study groups. Stimulation by either Pep14 or Pep19 strongly promoted the induction of CD4+CD25+forkhead box P3 (FoxP3)+ human regulatory T cells (Tregs) in vitro. However, the expression of mouse splenic CD4+CD25+FoxP3+ Tregs was lower in the Pep14-immunized mice than in the Pep14-challenged or Pep19-immunized mice. Levels of serum interferon gamma (IFN-γ) and transforming growth factor beta were higher and levels of interleukin (IL) 10 were lower in the Pep14-immunized mice than in the other groups. Induction of CD25+IL-17+ T helper 17 (Th17) cells was attenuated in the Pep14-immunized mice.

**Conclusions:** Nasal immunization with Pep14 may be a mechanism for attenuating atherogenesis by promoting the secretion of IFN-γ and/or suppressing Th17-mediated immunity.

**Keywords:** Atherosclerosis; Cardiovascular diseases; Heat-shock proteins; Periodontitis; *Porphyromonas gingivalis*; Vaccines

**INTRODUCTION**

Periodontitis is a chronic infectious disease and acts as an independent risk factor for cardiovascular disease [1] by triggering early-stage atherogenesis and inflammation of blood vessels [2]. The mechanism by which an infection is initiated and promotes the progression...
of atherosclerosis relates to the innate and adaptive immune responses of the host to bacterial heat shock proteins (HSPs) [3]. One such protein, HSP60, has a well-conserved amino acid sequence and has been suggested to be a regulator that either promotes or suppresses autoimmunity. HSP60 in Porphyromonas gingivalis, a major periodontal pathogen, is thought to be a key link between periodontosis as an infectious disease and atherosclerosis as an autoimmune-mediated disease [4]. HSP-derived peptides have been found to promote the production of anti-inflammatory cytokines, indicating that they have immunoregulatory potential [3]. Furthermore, a pathogen-driven antigenic peptide derived from P. gingivalis HSP60 (PgHSP60) has been observed to trigger and aggravate autoimmune atherosclerosis [5]. Various epitope peptides of bacterial HSPs may function as effector or regulatory molecules in the autoimmune response to infection-triggered atherosclerosis. Jeong et al. [6] proposed that 2 PgHSP60 peptides, peptide 14 (Pep14) and peptide 19 (Pep19), may play distinct antiatherogenic and proatherogenic roles in an apolipoprotein E knockout (ApoE KO) mouse model.

Given the evidence suggesting that an autoimmune response to PgHSP60 may contribute to the progression of atherosclerosis, immunization with HSP60 could induce an atheroprotective response. Hagiwara et al. [7] assessed the atheroprotective ability of a vaccine that targeted a recombinant HSP60 from P. gingivalis (GroEL) and found that it induced a protective mucosal immune response. Based on this observation, they suggested that sublingual vaccination with GroEL attenuates atherosclerosis [7]. However, a whole protein-based vaccine strategy may trigger an unwanted off-target immune reaction, whereas a subunit vaccine may afford a safer margin of tissue protection. In an epitope mapping study of PgHSP60, researchers identified several immunodominant peptides that are involved in the periodontitis-associated immunoregulation seen in autoimmune atherosclerosis [8]. These findings prompted our research interest in the potential anti-atherogenic and pro-atherogenic properties of Pep14 and Pep19 [6], and we hope to develop a peptide vaccine that protects against atherosclerosis using a better-defined peptide as a substitute for whole PgHSP60.

Nasal antigen delivery appears to be an effective route of immunization for the induction of specific protective immunity against P. gingivalis infection even in aged mice [9]; hence, it is an attractive approach for immunization and has several advantages, including both mucosal and systemic tolerizing immune responses and ease of application [10,11].

The aims of this study were (1) to determine whether nasal immunization using specific peptides derived from PgHSP60 could reduce atherosclerotic plaque formation in an ApoE KO mouse model and (2) to assess the feasibility of nasal immunization as a vaccine strategy for immune tolerance.

MATERIALS AND METHODS

Synthetic peptides
Pep14 (TVEVEGMQRDRGYISPYFV) and Pep19 (TLVNRGSLKCAKVAPG) were synthesized from the 37 overlapping peptides spanning the entire PgHSP60 protein sequence using 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Peptron Inc., Daejeon, Korea).

Mice
Homozygous male ApoE KO mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on clean temperature-controlled racks with a 12-hour light-dark
cycle. All animal protocols were approved by the Animal Care and Use Committee of Pusan National University (PNU 2017-1778) and conducted in accordance with the Animal Research: Reporting In Vivo Experiments guidelines. At 7 weeks of age, the mice were divided into 6 groups (n=5 per group). Nasal immunization was performed twice weekly for 3 weeks, after which intravenous (IV) inoculation was performed 3 times weekly for 2 weeks.

The mice were divided into 6 groups as follows. Mice in the first and second groups received nasal immunization with 50 μL of phosphate-buffered saline (PBS) and were then challenged with 100 μL of IV-administered PBS. Mice in the third and fourth groups received nasal immunization with 50 μL of PBS and were then challenged with 100 μL of IV Pep14 or Pep19 (100 μg/mL). Mice in the remaining 2 groups received nasal immunization with 50 μL of Pep14 or Pep19 (100 μg/mL) and were then challenged with 100 μL of IV Pep14 or Pep19 (100 μg/mL). After the final injection, mice in group 1 were fed a chow diet, while those in groups 2–6 were fed a Western diet (0.2% cholesterol, 21.2% fat, 13.7% saturated fatty acids, 7.3% total unsaturated fatty acids; D12079B, Research Diets, New Brunswick, NJ, USA), for 13 weeks for all groups. All mice were euthanized at 13 weeks after the final injection (Figure 1).

**Histometric analysis of aortic lesions**
Frozen aortic roots were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin and Oil Red O stains to visualize atherosclerotic plaque and lipid droplets in the atherosclerotic lesions. The percentage of lesion plaque area was calculated as the plaque area relative to the total aortic area as analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Total plasma cholesterol levels were determined using a total cholesterol assay kit (Cell Biolabs Inc., San Diego, CA, USA).
Dot immunoblot analysis

Pep14 and Pep19 were spotted onto a nitrocellulose membrane to assess the dot blot intensity as a measure of serum reactivity to each peptide. The membranes were blocked separately and incubated with individual serum samples from each group of mice. The membranes were then washed, and horseradish peroxidase-conjugated goat anti-mouse IgG was added. The membranes were then washed again, after which tetramethylbenzidine was added for color development. An identical procedure was repeated for PgHSP60 and oxidized low-density lipoprotein (oxLDL).

In vitro stimulation of naïve human CD4⁺CD25⁺CD45RA⁺ regulatory T cells (Tregs) with Pep14 or Pep19

Isolation of naïve human CD4⁺CD25⁺CD45RA⁺ Tregs
Naïve Tregs were isolated as described previously [12]. Human CD4⁺ T cells were stained with cyanine 5–anti-CD4, fluorescein isothiocyanate (FITC)–anti-CD25, and phycoerythrin (PE)–anti-CD45RA. The stained Tregs were sorted on the basis of the CD4⁺CD25⁺CD45RA⁺ T-cell phenotypic markers using a fluorescence-activated cell sorting (FACS) machine (FACSria III high-speed cell sorter, BD Biosciences, San Jose, CA, USA) and were incubated in OpTmizer CTS T-cell Expansion SFM (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing 2% heat-inactivated human serum (Sigma-Aldrich, St. Louis, MO, USA).

Establishment of human Pep14-specific or Pep19-specific CD4⁺CD25⁺ Tregs
Isolated CD4⁺CD25⁺CD45RA⁺ Tregs were co-cultured with human monocyte-derived dendritic cells pulsed with Pep14 or Pep19 to establish antigen-specific human CD4⁺CD25⁺ Tregs. Once established, the Tregs were sorted using the FACS machine and incubated in the OpTmizer CTS T-cell Expansion SFM.

In vitro stimulation of mouse splenic CD4⁺CD25⁺CD127lo Tregs with Pep14 or Pep19

Generation and culture of tolerogenic bone marrow-derived dendritic cells
Bone marrow was extracted from the tibias and femurs of 7- to 10-week-old male C57BL/6 mice. The red blood cells were eliminated using ACK lysing buffer (Invitrogen, Waltham, MA, USA). The cells were plated in 12-well culture plates (1×10⁵ cells/mL) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), recombinant murine granulocyte-macrophage colony-stimulating factor (50 ng/mL), recombinant murine interleukin (IL) 4 (10 ng/mL), and retinoic acid (1 nM) at 37°C in 5% CO₂. On days 3 and 6, the floating cells were removed from the cultures, and fresh medium was added. On day 7, the cells were positively separated using Pan DC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and treated with mitomycin C (Sigma-Aldrich) for 20 minutes at 37°C.

Isolation of mouse splenic T cells
First, 2×10⁶ splenic CD4⁺ T cells harvested from mice in groups 1–6 were negatively separated using MACS beads (>90%) (Miltenyi Biotec, Auburn, CA, USA). The CD4⁺ T cells were stained with cyanine 5–anti-CD4, FITC–anti-CD25, and PE–anti-CD127lo antibodies. The stained Tregs were sorted on the basis of the CD4⁺CD25⁺CD127lo T-cell phenotypic markers by the FACS instrument and were then incubated in RPMI 1640 containing 10% heat-inactivated fetal bovine serum.
Establishment of Pep14-specific or Pep19-specific mouse CD4$^+$CD25$^+$CD127$^{lo}$ Tregs
The tolerogenic bone marrow-derived dendritic cells were then pulsed with Pep14 or Pep19 (20 mg/mL) for 5 hours at 37°C. To establish Pep14-specific or Pep19-specific mouse CD4$^+$CD25$^+$CD127$^{lo}$ Tregs, Pep14-pulsed or Pep19-pulsed dendritic cells were co-cultured with sorted CD4$^+$CD25$^+$CD127$^{lo}$ Tregs. Tregs isolated from negative control mice were stimulated with PBS-pulsed dendritic cells. Tregs isolated from positive control mice were stimulated with oxLDL-pulsed dendritic cells.

FACS staining
The Tregs were counted and analyzed to investigate the expression profiles of various markers, including CD4, CD25, CD45RA, and forkhead box P3 (FoxP3), using the FACS instrument after staining with the monoclonal antibody cocktails described above [6]. FoxP3 was stained with PE-conjugated antibodies (eBioscience, Inc., San Diego, CA, USA). A Cytofix/Cytoperm kit (BD Biosciences) was used for intracellular staining.

Measurement of cytokine levels
The levels of interferon gamma (IFN-γ), IL-10, and transforming growth factor beta (TGF-β) in mouse serum from the 6 study groups were determined using enzyme-linked immunosorbent assay kits (eBioscience, Inc.).

Expression of T helper 17 (Th17) cells
Splenic CD4$^+$ T cells were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences) and then stained using the cyanine 5-CD4 and FITC-CD25 antibodies. For intracellular cytokine staining, cells were stimulated with GolgiStop (BD Biosciences) and stained with PE–IL-17 monoclonal antibodies after fixation and permeabilization.

Statistical analysis
Statistical analysis was performed using SPSS version 24.0 (IBM Corp., Armonk, NY, USA). The data are presented as mean±standard deviation. The statistical significance of comparisons between groups was determined by 1-way analysis of variance followed by the Tukey post hoc test. A Fvalue <0.05 was considered to indicate statistical significance.

RESULTS
Histomorphometric analysis of aortic lesions
The amount of atherosclerotic plaque accumulation in ApoE KO mice inoculated with Pep14 or Pep19 was markedly higher than that in the negative control group. However, nasal immunization with Pep14 significantly attenuated the accumulation of atherosclerotic plaque in the Pep14-challenged group and the positive control group ($P$<0.01). Nasal immunization with Pep19 also attenuated the accumulation of atherosclerotic plaque in the Pep19-challenged group and positive control animals, although this effect was not statistically significant (Figure 2A and B). Total cholesterol levels were lower in the Pep14-immunized group than in the peptide-challenged groups and the positive control group; however, the difference lacked statistical significance (Figure 2C).

Dot immunoblot analysis
The intensity of the dot blot as a measure of serum reactivity to each peptide is shown in Figure 3. Serum reactivity to Pep14 was not detectable in any of the groups, including the
Pep14-immunized group. In contrast, serum reactivity to Pep19 was detectable in all groups, with the highest level detected in the Pep19-immunized group. Serum reactivity to PgHSP60, Pep19 from human HSP 60 (Hu19), and oxLDL was identified in all 6 groups. Serum reactivity to Pep9 from human HSP 60 (Hu9) was detected in the Pep19-challenged and Pep19-immunized groups (Figure 3).

**In vitro stimulation of Tregs with peptide-pulsed dendritic cells**

Stimulation by either Pep14 or Pep19 strongly promoted the induction of CD4⁺CD25⁺FoxP3⁺ human Tregs *in vitro* (Figure 4A). However, the expression of mouse splenic CD4⁺CD25⁺FoxP3⁺ Tregs was lower in the Pep14-immunized group than in the Pep14-challenged and Pep19-immunized groups (Figure 4B).
Effects of nasal immunization on cytokine production and Th17 cell expression in cultured spleen cells

In the Pep14-immunized and Pep19-immunized groups, the amount of IFN-γ produced by spleen cells was significantly higher than that produced by spleen cells in the control and Pep19-challenged groups. However, the level of IFN-γ in the Pep14-immunized group was not significantly different from that in the Pep14-challenged group (Figure 5A). The amount of TGF-β produced in the Pep14-immunized group was significantly higher than that produced in the control, Pep14-challenged, and Pep19-challenged groups. In contrast, although the Pep19-immunized and positive control groups demonstrated increased TGF-β production relative to the other groups, this difference was not statistically significant (Figure 5B). The IL-10 level was significantly lower in the Pep14-immunized group than in the Pep14-challenged group (Figure 5C). Induction of CD25⁻IL-17⁺ Th17 cells was significantly lower in the Pep14-immunized and Pep19-immunized groups than in the Pep14-challenged and Pep19-challenged groups (Figure 5D).

DISCUSSION

Chronic infections may trigger, sustain, or exacerbate autoimmune disease through epitope spreading via the antigen presentation of epitopes released within injured tissue [13]. Identification of the target antigenic peptide of an infecting pathogen responsible for epitope spreading may facilitate the development of a vaccination strategy for the suppression of autoimmune disease [8]. Hagiwara et al. [7] recently suggested that sublingual immunization with GroEL significantly suppresses atherosclerosis induced by naturally-occurring bacteria, inflammatory cytokines, and oxLDL levels accelerated by P. gingivalis infection. In the present study, we found that nasal immunization with Pep14, a peptide derived from PgHSP60, could attenuate Pep14-induced atherosclerotic plaque formation in an ApoE KO mouse model and confer atheroprotection equivalent to that achieved via whole HSP60 immunization [7]. We propose that nasal immunization with Pep14 would be preferable to using a whole
HSP60 molecule because it may circumvent unwanted global, non-specific, and off-target immunosuppression. Furthermore, peptide vaccines can be designed to be safe, economical, and effective with a more targeted approach than conventional whole peptide vaccines [14]. However, even though Pep14 immunization may attenuate atherosclerosis, it is still unclear whether this Pep14 immunization suppresses atherosclerosis accelerated by Pep14 challenge or that caused by consumption of a high-fat diet.

Based on the dot immunoblot profiles, we assume that Pep14 preferentially stimulates T cells, whereas Pep19 stimulates both T cells and B cells, and this is consistent with our previous observations [8,15]. In agreement with a report by Jeong et al. [6], the immunoblot profile in the present study demonstrated that serum reactivity to Pep14 was not detectable in any of the study groups, including the Pep14-immunized group; this is attributed to its poor ability to stimulate B cells, reflecting the inherent immunoregulatory function
of HSP60 in atherogenesis [16]. This reasoning is strengthened further by the previous observation that the activation of Tregs contribute to the regression of atherosclerotic lesions in mice that receive subcutaneous Pep14-immunization [6]. The cross-reactivity of immune sera with human self-antigens and the neoantigen oxLDL is consistent with the epitope spreading observed in patients with autoimmune disease and ongoing periodontal disease [17]. Epitope spreading by Pep19 to Hu19, Hu9, and oxLDL has been proposed as an
important phenomenon observed in autoimmune diseases induced by chronic infection [15]. A noteworthy finding in our study was that serum reactivity to oxLDL without infection was detected not only in mice that received a Western diet, but also in those that received a normal diet, whereas seroreactivity to oxLDL, a neoantigen in the ApoE KO mouse, was greater in the Pep19-challenged group than in the other study groups. The formation of oxLDL is known to be a critical event in the development of atherosclerosis, for which Pep19 has a potent inductive capability [18]. Seropositivity to oxLDL has recently been observed even in healthy teenagers, presumably suggestive of early P. gingivalis colonization of the subgingival niche [15,19]. It is tempting to speculate on the hitherto less appreciated role of Pep19 in triggering oxLDL-based pro-atherogenicity [6], which is suggestive of cross-reactivity to the cognate self-antigen and a neo-antigen via the initiation and exacerbation of atherogenesis.

Nasal immunization with HSP60 may attenuate the formation of atherosclerotic plaque by inducing CD4+CD25+ Tregs that maintain immune homeostasis by secreting IL-10, TGF-β, and a minimal amount of IFN-γ [20]. Even though Pep14 and Pep19 have been shown to substantially stimulate human CD4+CD25+FoxP3+ T cells in vitro, the results of nasal immunization with Pep14 in mice in the present study were not consistent with the in vitro results, as evidenced by low levels of splenic CD4+CD25+FoxP3+ T cells. Hence, whether nasal Pep14 immunization exerts its atheroprotective effect by stimulating Tregs remains uncertain. Tregs have been shown to be present within atherosclerotic plaques [21]. In the present study, the level of mouse splenic CD4+CD25+FoxP3+ T cells was highest in the positive control group.

It was recently suggested that an imbalance between Th17 cells and Tregs plays a role in plaque destabilization [22]. IFN-γ has been reported to suppress the induction of Th17 cells, which are key players in autoimmune disease [23]. TGF-β signaling suppresses the activity of Th17 cells [24]. Given that nasal immunization with Pep14 was shown to stimulate secretion of IFN-γ and TGF-β, it is possible that this peptide exerts its atheroprotective effect by suppressing the induction of IL-17–secreting Th17 cells [25].

Previous studies have demonstrated that the mucosal administration of self-antigens can induce peripheral antigen-specific immune tolerance and downregulate antigen-specific T cells, eventually leading to the inhibition of excessive immune responses and the maintenance of self-tolerance that prevents the initiation of autoimmune disease [26,27]. In subtle contrast with these concepts, we have previously shown that Pep14 immunization enhances the expression of transcription factors specific for Tregs when adoptively transferred into ApoE KO mice via the IV route [6]. Furthermore, recent information regarding the stability and plasticity of Tregs categorizes effector Tregs into 2 groups: Th1-like effector Tregs and Th1-suppressive Tregs. Th1-suppressive Tregs are considered more desirable in terms of stability and suppressive ability because proinflammatory cytokines, such as IFN-γ, are secreted primarily by proatherogenic type 1 effector T cells during atherogenesis [28,29]. Therefore, the role of IFN-γ in atheroprotection awaits further clarification. Tregs may use different suppressive mechanisms depending on the mode of immune cell-based vaccination. Nevertheless, tolerizing effector T-cell immunity via oral or nasal administration of an antigen-specific peptide may induce FoxP3 expression in Tregs in response to low doses of antigen [30] or TGF-β–secreting Th3 cells [31], which is problematic. On balance, oral or nasal immunization may not guarantee the immune tolerance regulated by the most desirable Tregs, and the preferred mode of immune regulation achieved by CD4+CD25+FoxP3+ Tregs in the context of atheroprotection has yet to be identified.
In conclusion, we propose that nasal PepI4 immunization may attenuate atherosclerosis by promoting the secretion of IFN-γ and suppressing Th17-mediated immunity. In a future study, we will evaluate the efficacy of the markers in terms of clinical roles or diagnostic tools.

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