Pep19 drives epitope spreading in periodontitis and periodontitis-associated autoimmune diseases


Background and Objective: Epitope spreading is one of valid mechanisms operating in immunopathological processes of infection-induced autoimmune diseases. We hypothesized that the peptide 19 from Porphyromonas gingivalis heat shock protein (HSP) 60 (Pep19) may be the dominant epitope from which epitope-specific immune response to subdominant epitopes may diversify sequentially into autoimmune responses directed at human neoepitopes in P. gingivalis-induced periodontitis and autoimmune diseases. However, the exact feature and mechanism on how Pep19 may drive epitope spreading into human autoantigens in chronic periodontitis or P. gingivalis-induced experimental periodontitis has not been clarified. The present study was performed with the following specific aims: (i) to delineate retrospectively the features of epitope spreading by human cross-sectional analysis; (ii) to demonstrate prospectively the epitope spreading into new antigenic determinants in an ordered, predictable and sequential manner in experimental periodontitis; and (iii) to clarify the mechanism on how immunization with Pep19 may mobilize helper T cells or elicit B-cell responses to human autoantigens and neoantigen.

Material and Methods: The study was devised for two independent investigations – a cross-sectional analysis on clinical subjects and a prospective analysis on experimental periodontitis – each being subdivided further into two additional independent observations. Cross-sectional dot immunoblot pattern against a panel of peptides of P. gingivalis HSP60 and human HSP60 was performed among age-dependent healthy subjects and between healthy subjects, patients with chronic periodontitis and patients with autoimmune disease, to identify epitope spreading. A peptide-specific T-cell line was established for phenotype analysis and for proliferation assay to an array of identical peptides. An identical prospective analysis was performed in P. gingivalis-induced experimental periodontitis or in Pep19-immunized mice. Cross-reactivity of anti-Pep19 monoclonal antibody was also investigated.

Results: A dominant immune response exclusively to Pep19 prevailed in healthy human subjects (before the age of 40) and mice that persisted in chronic periodontitis and autoimmune diseases without being replaced further by subsequent subdominant epitopes. A sequential epitope spreading provoked by Pep19 to subdominant autoantigen peptide 19 from human HSP60 (Hu19) in most healthy human subjects and mice, and to autoantigen peptide 9 from human...
HSP60 (Hu9) and neoantigen oxidized low-density lipoprotein (ox-LDL) in *P. gingivalis*-induced chronic periodontitis and autoimmune diseases could be demonstrated in a reproducible and predictable manner. T-cell proliferative activity to multiple autoantigens Hu19, Hu9 and ox-LDL, and cross-reactivity of anti-Pep19 monoclonal antibody to these epitopes may be proposed as cellular and molecular mechanisms responsible for the phenomenon. Moreover, the predictive value of Pep19 for Hu9 increased remarkably in the disease group when compared with that of the healthy group.

**Conclusion:** Taken together, epitope spreading to Hu19, Hu9 and ox-LDL provoked by Pep19 could be proposed as a solid phenomenon observed in *P. gingivalis*-induced chronic periodontitis and infection-induced autoimmune diseases in a reproducible and predictable manner. T-cell proliferative activity to these peptides and cross-reactivity of anti-Pep19 antibodies to multiple human autoantigens could be proposed as cellular and molecular mechanisms responsible for this phenomenon.

Infection-triggered autoimmunity can be induced by multiple mechanisms, including molecular mimicry, epitope spreading and bystander activation (1). Epitope spreading was initially defined as the diversification of epitope specificity from the initial dominant epitope-specific immune response to subdominant epitopes (2). The switch begins with molecular mimicry to the dominant epitope resulting in an autoimmune response directed at a neoepitope (3).

Epitope spreading entails the progression of an autoimmune response from initial activation to a chronic state involving increased targeting of autoantigens by T cells and antibodies that may occur intra- or intermolecular (4). Intra- or intermolecular epitope spreading is one of the four mechanisms on how antigens of infectious organisms may propagate into various human autoantigens to trigger adaptive autoimmune responses. Substantial evidence supports the hypothesis that tissue damage can lead to epitope spreading, which can then contribute to ongoing disease (5). Autoreactive cells are found in normal individuals, but normally do not cause clinical pathology, perhaps due to low precursor frequency and/or overlying regulatory controls. However, in genetically susceptible individuals, the regulatory controls on autoreactive lymphocytes primed during tissue damage may be defective (6). The pathogen may lead to disease via epitope spreading. In this model, the immune response to a persisting pathogen or direct lysis by the persisting pathogen of host cells can cause damage to self-tissues (7).

The immune response consists of both the initial magnification phase, which can be either harmful or beneficial, to restore the immune system to homeostasis. Knowledge of the pattern of epitope spreading in human autoimmune diseases or during transplant rejection could be used to design antigen-specific therapies that block ongoing tissue destruction or organ rejection, respectively (5).

Heat shock proteins (HSPs) from infectious organisms have been reported to either trigger or suppress an immune response depending on their stimulatory properties on effector or regulatory T cells (Tregs) in autoimmunity (8). HSPs can hence prevent or arrest inflammatory damage, and in initial clinical trials in patients with chronic inflammatory disease, HSP-derived peptides have been shown to promote the production of anti-inflammatory cytokines, indicating that HSPs have an immunoregulatory potential (9,10). Regulation of autoimmune arthritis by regulatory properties of self-HSPs might be also useful for therapeutic purposes (11). In addition, 60 kDa HSP could be potentially used for therapeutic vaccination against type I diabetes (12).

HSPs, particularly the HSP60 family of proteins, are thought to play important roles linking microbial infections to autoimmunity due to the conserved amino acid sequences and their strong immunogenicity. HSP60 can be a target for molecular mimicry, and identifying candidate T-cell epitopes capable of inducing immunological tolerance might offer appealing opportunities for antigen-tailored therapy against infectious diseases or infection-triggered autoimmune disease such as atherosclerosis (9). Serum antibody to the periodontopathic bacteria-derived HSP60 was also frequently detected in both patients with periodontitis and autoimmune diseases. It is hypothesized that because of the upregulation of HSP60, T cells specific for self-hsp60 might be activated as part of the normal inflammatory process (13).

Among HSP60 peptides from *Porphyromonas gingivalis*, *Mycobacterium tuberculosis* and *Chlamydia pneumoniae*, peptide 19 (Pep19: TLVVNRLRGLKICAVKAPG) from *P. gingivalis* HSP60 (PgHSP60) was most predominantly and consistently recognized by the serum samples from patients of at least four types of autoimmune diseases, including periodontitis, atherosclerosis, diabetes mellitus and rheumatoid arthritis (14). It is of particular interest to note that Pep19 was found to be an immunodominant T- and
cross-reactive B-cell epitope in the periodontitis-atherosclerosis axis (14,15), which might serve as an autoimmune target (16). Moreover, anti-Pep19 monoclonal antibody (mAb) was found to be polyclonal to Pep19 of HSP60 from multiple periodontopathic bacteria and human HSP60 (15). Recently, Pep19 is one of immunodominant epitopes of P. gingivalis HSP60 that was also shown to demonstrate proatherogenic property by elaborating type I helper T-cell responses (17).

Taken together, we hypothesize that the Pep19 may be the dominant epitope from which the epitope-specific immune response to subdominant epitopes may originate, resulting in the subsequent development of autoimmune responses directed at human neoepitopes in P. gingivalis-induced periodontitis and autoimmune diseases. However, the exact feature and mechanism of how Pep19 may drive epitope spreading into human autoantigens in chronic periodontitis or P. gingivalis-induced experimental periodontitis has yet to be clarified.

The present investigation was performed with the following specific aims: (i) to delineate retrospectively the features of epitope spreading by human cross-sectional analysis; (ii) to demonstrate prospectively the epitope spreading to new antigenic determinants in an ordered, predictable and sequential manner in experimental periodontitis; and (iii) to clarify the cellular immunological and molecular mechanisms of Pep19-induced epitope spreading to human autoantigens and neoantigen.

**Material and methods**

**Cross-sectional study in human subjects**

**Study subjects**— Five clinically healthy subjects consisting of the 10–19 and 20–29 years age groups, who were devoid of chronic periodontitis or systemic diseases, were recruited for age-matched comparison between healthy subjects for immunoblot patterns against an array of target antigens. Eight clinically healthy subjects consisting of age groups 10–19, 20–29 and 30–39 years were also recruited for age-matched comparison of immunoblot patterns among healthy subjects against another panel of target antigens. Four patients with chronic periodontitis aged 20–30 years were selected for aged-matched comparison (14). For additional comparison in patients with autoimmune diseases, five patients with diabetes, two with rheumatoid arthritis and three with atherosclerosis were selected according to guidelines described in our previous paper (14). Peripheral blood was drawn from all study subjects by venipuncture for collection of serum and peripheral blood mononuclear cells.

The study was approved by the Institutional Review Board of Pusan National University Hospital. Written, informed consent for blood collection was obtained from the study subjects. All experiments were performed under the principles of the declaration of Helsinki. The Animal Care and Use Committee of Pusan National University approved all animal experiment protocols.

**Measurement of anti-P. gingivalis IgG levels in teenagers**— Serum IgG antibody levels of teenagers to P. gingivalis were determined by an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, P. gingivalis ATCC 33277 (5 μg/mL) was coated on to 96-well plates (Maxisorb Immunoplates, Nunc, Roskilde, Denmark). Serially diluted serum samples were added, followed by horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (γ-chain specific; Jackson ImmunoResearch Laboratories) was added, and the membranes were incubated for 1 h. The membranes were washed with PBS-Tween 20, and tetramethyl benzidine was added for color development. For mouse serum samples, HRP-conjugated goat antimouse IgG (γ-chain specific; Jackson ImmunoResearch Laboratories) was used.

**Establishment of antigen-specific T-cell lines in human subjects and in mouse**— CD4+ T cells (2 × 10^5) from human peripheral blood mononuclear cells were negatively separated by MACS beads (90%; Miltenyi Biotec, Auburn, CA, USA). T cells were then cocultured with 2 × 10^5 mitomycin C-treated antigen-presenting cells and each antigen (10 μg/mL). For mouse T-cell lines, a mouse CD4+ T-cell isolation kit was used (Miltenyi Biotec).

**Flow cytometry in human and mouse T-cell lines**— Purified CD4+ T cells were stained using the following antibodies: phycoerythrin-cyanine 5-conjugated...
anti-CD4 and the respective isotype antibodies. For intracellular staining of interleukin (IL)-17A, cells were fixed and permeabilized with cytofix/cytopermeabilization buffer (eBioscience, San Diego, CA, USA) and then stained with fluorescein iso-thiocyanate-conjugated anti-human IL-17A mAbs. For intracellular cytokine staining, cells were stimulated with GolgiStop (BD Biosciences, Franklin Lakes, NJ, USA) and then stained with fluorescein isothiocyanate-conjugated anti-IL-17A mAbs after permeabilization and fixation. All antibodies were purchased from eBioscience. An identical procedure was applied in mouse T-cell lines except that blocking with antimouse CD16/CD32 antibodies has been added before purification of CD4+ T cells.

T-cell proliferation assay in human and mouse determined by carboxyfluorescein diacetate succinimidyl ester analysis—

Splenic CD4+ T cells from each group sorted by the MACS system were stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Waltham, MA, USA) in dimethyl sulfoxide for 10 min at 37°C. Cells were incubated in RPMI 1640 media with 10% fetal bovine serum for 15 min at 4°C, centrifuged and washed twice with PBS. CFSE-labeled 2 × 10^5 CD4+ T cells were plated in 96-well plates (SPL, Daejeon, Korea) and treated with each peptide (10 μg/mL) or protein (4 μg/mL). After 4 d of antibody incubation, T cells were stained with phycoerythrin-cyanine 5 (PE-Cy5)-conjugated anti-CD4 mAb for flow cytometric assay. An identical procedure was applied in mouse except that T cells were blocked with anti-human CD16/CD32 antibodies before staining.

Immunohistochemical localization of anti-Pep19 monoclonal antibody—

Gingival lesions from the patients with periodontitis and atheromatous plaque from the patients with atherosclerosis were harvested during the process of surgical intervention. The tissue specimens were placed in OCT compound, quenched and stored in liquid nitrogen. After cryosections, immunostaining was performed using the streptavidin–biotin complex method. In brief, the sections were incubated for 20 min in a solution of PBS containing 0.3% H2O2 to eliminate endogenous peroxidase activity. After washing in PBS, the sections were incubated with 3% bovine serum albumin (Sigma). The excess solution was shaken off and the sections were incubated for 16–18 h at 4°C with anti-Pep19 or anti-Pep29 mAb (as control), respectively. Following incubation with the mAb, the sections were washed three times with PBS for 5 min and incubated for 1 h at room temperature with goat antimouse biotinylated antibody (Jackson ImmunoResearch Laboratories). They were then rinsed in PBS and incubated for 60 min at room temperature with a streptavidin–biotin complex reagent (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. The sections were developed in 0.05% 3,3'-diaminobenzidine and 0.003% H2O2 medium under microscopic control at room temperature to visualize peroxidase activity. Light microscopy slides were observed and photographed using an Olympus BX50 microscope. All photomicrographs were taken with an Olympus C-3030 digital camera (Olympus Optical Co., Osaka, Japan).

Longitudinal prospective observations in experimental periodontitis in the mouse model

Growth and maintenance of the bacterial strains—
P. gingivalis ATCC 33277 (American Type Culture Collection, Manassas, VA, USA) was grown anaerobically in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with hemin and menadione. The number of bacterial colony-forming units was standardized by measuring the optical density at 600 nm.

Oral infection with P. gingivalis and immunization of Pep19 by splenic dendritic cells— Six mice and five mice grown in germ-free or specific pathogen-free facilities in Pohang Technology University served as the negative control group. Six-week-old female C57/BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were fed with conventional diet and grown in conventional cages throughout the experimental period in Pusan National University Animal Care Facility. Four mice were challenged orally with PBS in 2% carboxymethyl cellulose and four mice were challenged orally with P. gingivalis (~5 × 10^8 colony-forming units/mouse) in 2% carboxymethyl cellulose three times per week for a period of consecutive 3 wks. Another four mice were immunized by adoptive transfer of Pep19-pulsed splenic dendritic cells (5 × 10^6 cells/mouse, 100 μg of Pep19/mouse) three times via tail vein done on a weekly basis. Mice were sacrificed 4 wk after final bacterial challenge or peptide immunization for collection of serum samples and splenic T cells.

In a separate experiment, four mice were subjected to P. gingivalis infection plus Pep19 subcutaneous immunization three times to induce experimental periodontitis and evaluate T-cell polarization skewed to T-helper (Th)17 phenotype in the course of epitope spreading to subdominant epitopes. Mice were sacrificed 2 or 10 wk after final bacterial challenge plus peptide immunization for collection of serum samples, splenic T cells and measurement of alveolar bone destruction by micro-computed tomography (CT).

Measurement of alveolar bone level by micro-computed tomography—

After death, the maxillary bone was subject to micro-CT to measure the distance from the cementoenamel junction to the alveolar bone crest (five sites at buccal surface) of the lower right and left first and second molar as an indicator of chronic destructive periodontitis. The significance of differences between the PBS-sham-infected/immunized group and P. gingivalis-infected plus Pep19-immunized group was obtained by Student t-test.

Evaluation of cross-reactivity of anti-Pep19 monoclonal antibody—

Cross-reactivity of anti-Pep19 mAb to the
selected synthetic peptides or to ox-LDL was evaluated by dot immunoblot in an identical manner described in the method section.

Statistical analysis on causality or coincidence of epitope spreading—To delineate the issue of causality or coincidence of epitope spreading, we have estimated the positive predictive value of Pep19 to provoke epitope spreading into each subdominant epitope in healthy or diseased groups, respectively, based on the immunoblot analysis. The positive predictive value refers to the probability of epitope spreading in a subject with positive reactivity to Pep19 reactivity.

The significance of differences in alveolar bone level was determined using the Student’s t-test. p < 0.05 was considered significant.

Results

Cross-sectional retrospective study in human subjects

The outline of clinical features of healthy subjects aged 10–19, 20–29 and 30–39 years are summarized in Table 1.

Measurement of anti-\textit{P. gingivalis} IgG levels in teenagers

Serum IgG antibody levels of teenagers to \textit{P. gingivalis} were 2.1–4.3 µg/mL as determined by an ELISA technique (Table 1).

Comparison of dot immunoblot patterns between healthy teenagers and healthy subjects in their 20s

Most serum samples of five healthy teenagers and five healthy subjects in their 20s reacted strongest with PgHSP60 and Pep19 than the analogous targets from \textit{C. pneumonia} and \textit{M. tuberculosis} (Fig. 1A). Simultaneous reactivity to human autoantigen Hu19 could be observed in some healthy young people in their 20s (Fig. 1B, arrows).

Comparison of dot immunoblot patterns among healthy teenagers, healthy subjects in their 20s and 30s

Most serum samples from eight healthy teenagers, eight healthy people in their 20s and eight healthy individuals in their 30s demonstrated strong reactivity to Pep19 and to human autoantigen (Fig. 2) and ox-LDL (Fig. 2A–C, arrows) suggestive of intramolecular epitope spreading to Hu19 and intermolecular epitope spreading to human neoantigen ox-LDL, respectively. The relative intensity of antibody reactivity to Hu19 and ox-LDL became stronger with increasing subject age. Putative intramolecular epitope spreading into human autoantigen Hu9 could not be observed in any healthy subjects (Fig. 2).

Comparison of dot immunoblot patterns among healthy subjects, patients with periodontitis and patients with autoimmune disease

In addition to serum reactivity towards Pep19, human autoantigen Hu19, PgHSP60 and human neoantigen ox-LDL observed in most healthy subjects (Fig. 3A), sera from patients with chronic periodontitis demonstrated an expanded reactivity to new human autoantigenic epitope Pep9 from human HSP60 (Hu9) (Fig. 3B, arrows) though it was weak in three of four patients. A more pronounced reactivity was observed with serum samples from patients with autoimmune disease (atherosclerosis, diabetes, rheumatoid arthritis) (Fig. 3C, arrows).

T-cell proliferative responses to peptide 19 and human autoantigens Hu19 and Hu9 in patients with age-matched chronic periodontitis

T-cell proliferative responses to Pep19 and human autoantigens Hu19 and Hu9 could be distinctively observed in most instances of four patients with age-matched chronic periodontitis as analyzed by the CFSE assay (Fig. 3D).

Prospective study in infection-induced experimental periodontitis in the mouse model

Dot immunoblot pattern of sera from germ-free and specific pathogen-free mice—Dot immunoblot with mouse sera from germ-free and specific pathogen-free (SPF) mice did not show any reactivity to PgHSP60, Pep19, Hu19 or ox-LDL (Fig. 4).

Comparative dot immunoblot patterns between healthy and \textit{P. gingivalis}-infected mice—Sera from healthy mice reacted weakly with Pep19 and PgHSP60 (Fig. 5A), whereas serum from \textit{P. gingivalis}-infected mice showed a robust reactivity to Pep19 and weak reactivity HSP60 from

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**Table 1.** Clinical outlines of healthy subjects aged 10–19, 20–29, and 30–39 years and serum anti-\textit{P. gingivalis} IgG levels in teenagers aged 10–19 years

<table>
<thead>
<tr>
<th>Age group</th>
<th>Gingival index (mean ± SD)</th>
<th>% BOP (mean ± SD)</th>
<th>Probing depth (mean ± SD)</th>
</tr>
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<tr>
<td>10–19</td>
<td>0.03 ± 0.01</td>
<td>1.4 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>20–29</td>
<td>0.05 ± 0.01</td>
<td>1.7 ± 0.2</td>
<td>2.5 ± 0.4</td>
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<tr>
<td>30–39</td>
<td>0.06 ± 0.02</td>
<td>1.9 ± 0.1</td>
<td>2.6 ± 0.3</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Anti-\textit{P. gingivalis} IgG levels (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>2.1</td>
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<tr>
<td>2</td>
<td>13</td>
<td>3.2</td>
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<tr>
<td>4</td>
<td>18</td>
<td>2.7</td>
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<td>5</td>
<td>19</td>
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Gingival Index by Loe and Silness.

% BOP: % of sites showing bleeding on probing.
P. gingivalis, C. pneumonia and M. tuberculosis (Fig. 5A). Another set of comparative dot immunoblot patterns (Fig. 5B) demonstrated that serum from healthy mice barely reacted to any target antigens while those from P. gingivalis-infected SPF mice showed an enhanced reactivity predominantly to Pep19, human autoantigens Hu19 and Hu9 as well as to human neoantigen ox-LDL in most instances (Fig. 5B, arrows).

**Dot immunoblot patterns of mice immunized with peptide 19-pulsed dendritic cells—** Immunoblot pattern of serum samples obtained from four SPF mice at 1, 2 and 4 wk, after adoptive transfer of Pep19-pulsed splenic dendritic cells showed a distinct reactivity of immune sera to PgHSP60, Pep19, human autoantigens Hu19 and Hu9 as well as human neoantigen ox-LDL (Fig. 6A, arrows).

**T-cell proliferative responses to peptide 19 and human autoantigens Hu19 and Hu9 in mice immunized with peptide 19-pulsed dendritic cells—** T-cell proliferative responses to Pep19, Hu19 and, Hu9, as analyzed by CFSE dilution assay, became more pronounced in most instances with an increased observation period (1, 2 4 wk) following dendritic cell-based Pep19 immunization (Fig. 6B).

**Dot immunoblot pattern of an anti-peptide 19 monoclonal antibody to selected human autoantigens and neoantigen—** Anti-Pep19 mAb was found to be cross-reactive with human autoantigens Hu19, Hu9 and neoantigen ox-LDL as identified by dot immunoblot analysis (Fig. 7A).

**Immunohistochemical localization of anti-peptide 19 monoclonal antibody—** Preferential and robust localization of anti-Pep19 mAb was identifiable at the site of inflammation, gingival connective tissue lesions in periodontitis and atheromatous plaques in patients with atherosclerosis, when compared with that of anti-Pep29 mAb as evidenced by immunohistochemistry (Fig. 7B–I).

**Measurement of alveolar bone level by micro-computed tomography imaging analysis—** The distance from the cementoenamel junction to alveolar bone crest analyzed by micro-CT was 2.06 ± 0.10 mm in sham-infected/immunized mice, 2.73 ± 0.10 mm at 2 wk and 2.69 ± 0.10 mm at 10 wk following the final oral challenge with P. gingivalis plus Pep19 immunization (Fig. 8A–C). The difference in the distances between two groups was statistically significant both at 2 wk (p < 0.05) and at 10 wk (p < 0.01; Fig. 8D).
Polarization of peptide-specific T-helper 17 cells associated with spreading into subdominant epitope(s)— Serum samples obtained from four mice at 2 and 10 wk, after subcutaneous injection of PBS or Pep19 demonstrated a distinct reactivity of immune sera to PgHSP60, Pep19, human autoantigens Hu19 and Hu9, and to human neoantigen ox-LDL, though faintly (Fig. 9A). Skewing of peptide-specific helper T cells to Th17 phenotype was evident in cases for Pep19, Hu19 and Hu9 is demonstrated in a representative of four age-matched patients with chronic periodontitis as analyzed by carboxyfluorescein diacetate succinimidyl ester assay (D). FITC, fluorescein isothiocyanate; HSP, heat shock protein; Hu9, Hu14 or Hu19, peptide 9, peptide 14 or peptide 19 from human HSP60; ox-LDL, oxidized low-density lipoprotein; Pep9 or Pep19, peptide 9 or peptide 19 from PgHSP60; Pg, P. gingivalis.

Statistical analysis on causality or coincidence of epitope spreading

The positive predictive values of Pep19 for Hu19, Hu and ox-LDL were 95.7, 34.0 and 95.7 in the healthy group, while these values were 58.8, 100 and 87.5 in the disease group. A substantial decline in the positive predictive values of Pep19 for Hu19 (from 95.7 in the healthy group to 58.8 in the disease group), a
Fig. 5. Comparative dot immunoblot patterns between uninfected healthy animals and Pg-infected animals. Serum from healthy mice reacts faintly but exclusively with Pep19 and PgHSP60 (A), whereas serum from Pg-infected mice shows a robust reactivity to Pep19 from three tested bacteria and HSP60 from Pg, Cp and Mt (A). Another set of comparative dot immunoblot patterns (B) demonstrates that serum from healthy mice barely reacts to any target antigens while those from Pg-infected mice shows an enhanced reactivity predominantly to Pep19, human autoantigens Hu19 and Hu9 as well as to human neoantigen ox-LDL in most instances (B, arrows). Cp, *C. pneumoniae*; HSP, heat shock protein; Hu9, Hu14 or Hu19, peptide 9, peptide 14 or peptide 19 from human HSP60; Mt, *M. tuberculosis*; ox-LDL, oxidized low-density lipoprotein; Pep9 or Pep19, peptide 9 or peptide 19 from PgHSP60; Pg, *P. gingivalis*.

Fig. 6. Immunoblot pattern of serum samples obtained from four mice at 1, 2 and 4 wk, after adoptive transfer of Pep19-pulsed SpDCs (A). The image shows a distinct reactivity of immune sera to PgHSP60, Pep19 and human autoantigens Hu19 and Hu9 as well as human neoantigen ox-LDL (A, arrows). T-cell proliferative responses to Pep19, Hu19 and Hu9 as analyzed by carboxyfluorescein diacetate succinimidyl ester assay become more pronounced in most instances with an increased observation period (1, 2 and 4 wk) following Pep19 immunization (B). FITC, fluorescein isothiocyanate; HSP, heat shock protein; Hu9, Hu14 or Hu19, peptide 9, peptide 14 or peptide 19 from human HSP60; ox-LDL, oxidized low-density lipoprotein; Pep9 or Pep19, peptide 9 or peptide 19 from PgHSP60; Pg, *P. gingivalis*; SpDC, splenic dendritic cells.
remarkable increase in the predictive values of Pep19 for Hu9 (from 34.0 in the healthy group to 100 in the disease group), and a slight decrease in the predictive values of Pep19 for ox-LDL (from 95.7 in the healthy group to 87.5 in the disease group) could be observed.

Discussion

Since the advent of the first description of epitope spreading in an autoimmune disease (2), several lines of evidence are available suggesting that epitope spreading is one of valid mechanisms operating in immunopathological processes of infection-induced autoimmune diseases (1,5,6,18).

Based on our series observations (14,15,17,19), we hypothesized that the Pep19 may be a dominant epitope from which the epitope-specific immune response to subdominant epitopes may diversify sequentially into autoimmune responses directed at human neoepitopes in P. gingivalis-induced periodontitis and autoimmune diseases. To clarify this phenomenon and to identify the mechanism to support the hypothesis, a study has been devised for two independent investigations; a cross-sectional analysis on clinical subjects and a prospective analysis on experimental periodontitis, each being subdivided further into two additional independent observations.

It was our primary goal to demonstrate the qualitative phenomenon of epitope spreading by dot blot image analysis. If we had attempted the quantitative approach to demonstrate epitope spreading, the IgG antibody titer by ELISA would have been measured. However, it was our original idea that the phenomenon of epitope spreading could be better demonstrated by dot blot image analysis than antibody titer expressed in numerical values. In other words, we wanted to see how far the epitope spread (extent), rather than how strong it is (strength).

Although P. gingivalis has widely been recognized as a major infectious microorganism in chronic periodontitis (20), several researchers have reported that this organism is frequently isolated in young healthy individuals demonstrating a salient immunoreactivity to the organism (21–23). Moreover, Pep19 from P. gingivalis HSP60 was previously considered as a predominant epitope in patients with chronic periodontitis and autoimmune diseases (14,15,19). Based on two independent age-specific cross-sectional observations in human subjects, it is tempting to presume that Pep19 from P. gingivalis may be the prevailing immunoreactive epitope in most healthy subjects before the age of 40. Serum samples from healthy subjects aged 10–39 years demonstrated apparent serum reactivity exclusively to Pep19 and into Hu19 in most instances suggestive of intramolecular epitope spreading as well as to ox-LDL suggestive of intermolecular epitope spreading. The tendency became more pronounced with increasing age. However, putative intramolecular epitope spreading into another autoantigen Hu9 could not be observed in healthy individuals. Thus, intramolecular epitope spreading in healthy subjects seemed limited primarily to human proteins of high sequence homology.

We have expanded our investigation in patients with periodontitis and patients with autoimmune disease. While serum samples from healthy subjects demonstrated apparent serum reactivity exclusively to Pep19, Hu19, PgHSP60 and ox-LDL in most instances, those from patients with chronic periodontitis demonstrated a distinct reactivity to new human autoantigenic epitope Hu9, though

Fig. 7. Dot immunoblot pattern (A) demonstrating the cross-reactivity of anti-Pep19 monoclonal antibody (clones JC5–JC9) with PgHSP60, Pep19, human autoantigens Hu19 and Hu9, and human neoantigen ox-LDL, but not with Pep9. Anti-Pep19 monoclonal antibody demonstrated robust reactivity in both gingival lesions (B) and atheromatous plaque (D) when compared with the control specimen where anti-Pep29 monoclonal antibody has been applied: gingival lesion (C) and atheromatous plaque (E) (magnification ×100, bar length: 10 μm). An identical feature could be identifiable in gingival connective tissue (F) and at atheromatous plaque (H) when compared with the control specimen at higher magnification (magnification ×400, bar length: 2.5 μm) where anti-Pep29 monoclonal antibody has been applied: gingival connective tissue (G) and atheromatous plaque (I). HSP, heat shock protein; Hu9 or Hu19, peptide 9 or peptide 19 from human HSP60; ox-LDL, oxidized low-density lipoprotein; Pep9 or Pep19, peptide 9 or peptide 19 from PgHSP60; Pg, P. gingivalis.
faintly. The features became more pronounced in serum samples from patients with autoimmune disease (atherosclerosis, diabetes, rheumatoid arthritis). T-cell proliferative responses to Pep19 and human autoantigens Hu19 and Hu9 could be distinctively observed in most instances of four age-matched patients with chronic periodontitis. The phenomenon is strongly suggestive of epitope spreading to Hu19 and sequentially to Hu9 with onset of chronic periodontitis and infection-induced autoimmune diseases.

Despite multiphasic animal experiments to reinforce our novel concept on epitope spreading provoked by Pep19, consistent and simultaneous recognition of Pep19 and ox-LDL by sera from healthy humans and mice seemingly leaves the problem of coincidence to be clarified further. However, putative epitope spreading into neoepitope ox-LDL may be corroborated in part by the observation that Pep19 was capable of oxidizing native LDL (J.-Y. Joo, G.S. Cha, J.-Y. Lee, S.-J. Kim, J. Choi, Unpublished data). Furthermore, Pep19 has been shown to mobilize exclusively Th1 cells (17). These two findings may constitute an alternative but not mutually exclusive explanation for a proatherogenic role of Pep19 in experimental atherosclerosis (17) presumably though provoking epitope spreading to ox-LDL (4).

One may argue that the putative epitope spreading based on a cross-section human study may be coincidental rather than a sequential phenomenon. Certainly, Vanderlugt and Miller (5) stated that a pathological role for epitope spreading is difficult to verify in human disease because the initiating antigenic specificity is usually impossible to define. As an alternative, animal models have been proposed because the peptide specificity of the initial immune response can be manipulated, genetically identical animals can be used and the immune response can be serially assessed in different peripheral lymphoid organs, as well as in the target tissue of the disease (5). To circumvent the shortcomings of human study and to enrich the presumptive hypothesis, we have devised two inde-

Fig. 8. Micro-computed tomography image depicting the distance from cementoenamel junction to alveolar bone crest as a measure of alveolar bone resorption in a healthy control mouse (A), a mouse with chronic periodontitis at 2 wk (B), and at 10 wk (C) after oral challenge of *P. gingivalis* plus Pep19 immunization. Bar graph (D) shows the difference in bone level that is statistically significant both at 2 wk and at 10 wk (*p < 0.05, **p < 0.01, respectively).
pendent investigations to induce experimental periodontitis in a mouse model (P. gingivalis infection-induced experimental periodontitis and dendritic cell-based immunization of Pep19).

Sera from mice bred in germ-free and SPF facilities that had no reactivity to Pep19 did not show reactivity to PgHSP60, Pep19 or to ox-LDL either indicating that epitope spreading into human autoantigens or neoantigen could not be demonstrated. Sera from normal mice bred in conventional cages reacted faintly but exclusively with Pep19 and PgHSP60, while those from P. gingivalis-infected animals demonstrated enhanced serum reactivity predominantly to Pep19 to Hu9 and Hu9 (though faintly) indicating an intramolecular epitope spreading in some instances as well as to ox-LDL suggesting intermolecular epitope spreading.

All the sera from mice at 1–4 wk after dendritic cell-based immunization of Pep19 showed robust reactivity to Pep19, Hu19, Hu9, PgHSP60 and neoantigen ox-LDL. T-cell proliferative responses to Pep19, Hu19 and Hu9 were evident at 4 wk indicating that an epitope spreading of Pep19 into human autoantigens Hu19, Hu9 and neoantigen ox-LDL is seen in mice immunized by Pep19.

T-cell cross-reactivity to multiple autoantigens and neoantigen in accordance to the prevalence or onset of periodontal disease may be a plausible mechanism that could validate the phenomenon of epitope spreading into those epitopes. Anti-Pep19 mAb was found to be polyreactive in nature (15).

In the present study, the cross-reactivity of this molecule was confirmed also with Hu19, Hu9 and ox-LDL. On the other hand but interestingly, B-1 cell-derived clones producing anti-ox-LDL mAbs virtually reacted with an array of neoantigens, exogenous antigens and endogenous antigens, including mammalian HSPs (J.-Y. Joo, G.S. Cha, J.-Y. Lee, S.-J. Kim, J. Choi, unpublished observations). Furthermore, anti-Pep19 mAb demonstrated a preferential and robust localization at the site of inflammation (i.e. gingival connective tissue lesions and in atheromatous plaques). This finding strongly suggests that expression of autoantigen Hu19, a human cross-reactive cognate to Pep19, which is immunologically dominant both in periodontitis and relevant autoimmune diseases, can be upregulated strongly at the site of inflammation, and can be a potential target for HSP epitope-specific immunoregulatory therapy for infection-induced autoimmune diseases (24–27).

Taken together, these observations reinforce the concept that B-cell cross-reactivity may play a pivotal role on determinant spreading.
provoked by Pep19. Therefore, T-cell and B-cell cross-reactivity of Pep19 with subdominant epitopes would be proposed as cellular and molecular mechanisms pertinent to intramolecular and intermolecular epitope spreading observed in this study. The concept of T-cell cross-reactivity has been implicated in molecular mimicry (1,28), particularly in terms of its elusive role of molecular mimicry in autoimmune diseases (28). Vanderlugt and Miller (5) stated that, although immune responses are complex involving both humoral and cellular immune components, some autoimmune diseases are predominately CD4+ T-cell mediated, whereas others seem primarily antibody mediated.

Because either a Th17 or a Th1 effector response can drive autoimmunity, we hypothesized that epitope spreading would give rise to polarization of autoantigen-specific helper T cell skewed to Th17 as indicative of development of experimental periodontitis as an autoimmune disease entity (29,30). Indeed, 2 wk after *P. gingivalis* infection plus Pep19 immunization, there was a marked increase in proportions of Th17 cells specific for subdominant epitopes, including Hu19, Hu9 and ox-LDL in the early phase of chronic periodontitis, while it diminished during the late phase. It is yet unclear whether autoantigens such as Hu19 alone or in concert with Hu9 or ox-LDL would be responsible for triggering autoimmune cascades. Nonetheless, initiation and progression of periodontitis is corroborated with the putative epitope spreading triggered by Pep19. Considering that Pep19 play a distinct proatherogenic role in apolipoprotein E knockout mice by consistently and preferentially stimulating antigen-specific Th1 cells, polarization of Pep19-specific Th1 cells skewed to bystander antigen-specific Th17 cells should imply that the substantial shift in helper T-cell phenotypes may be associated with the development of autoimmune responses in *P. gingivalis*-infected ginvival lesion. However, Ulivieri and Baldari (31) cautioned that the differentiation of helper T-cell subsets is dynamically regulated by the local microenvironment. They proposed that Th17 responses are implicated both in host defense and in the development of autoimmunity and a better understanding of their pathogenic or protective role in each autoimmune disease is required to develop safe and effective therapeutics. Furthermore, Th17 cells and Treg share some molecular markers as well as factors that control their differentiation (31).

Substantial evidence supports the hypothesis that tissue damage can lead to epitope spreading, which can then contribute to ongoing disease (32–34). Mounting evidence for a pathological role for epitope spreading in autoimmune disease makes the development of epitope-specific therapies problematic (32). However, there is some evidence that epitope spreading is a critical component of protective immune responses to enhance the efficiency of the immune response, as seen in infection and cancer, and as a mechanism to downregulate immune responses, such as those occurring in autoimmunity (5,35,36). Knowledge of the sequential pattern of epitope spreading in human autoimmune disease or transplant rejection could be used to design peptide-specific immunotherapies that suppress ongoing tissue destruction (37). Comprehensive understanding of the cellular and molecular basis of the epitope spreading is essential for tailoring antigen-specific treatments for human autoimmune diseases (5).

Defining antigen specificities in epitope spreading is critical in both infectious diseases and infection-induced autoimmune diseases. From a diagnostic perspective, human autoimmune diseases are characterized by a secondary non-specific inflammatory process, a bystander activation that leads to enhanced processing and presentation of autoantigens inducing the immune response spread out toward different autoantigens (25). This can be either deleterious or beneficial. From an immunotherapeutic perspective of autoimmune disease, a bystander antigen should be immunodominant, recognized by the *in vivo* expanded peptide-specific Tregs restricted to the site of inflammation, specifically upregulated at the site of inflammation.

HSPs fulfill both these criteria for bystander antigens, which make them highly attractive targets for antigen-specific immunotherapy through the induction of Tregs for bystander suppression (37–41). Pep19 as the bystander antigen was specifically upregulated at the site of inflammation and immunologically dominant, hence, can be an attractive candidate for antigen-specific immunotherapy leading to bystander suppression (5,40). The capacity of Tregs to mediate bystander suppression makes them attractive targets for the development of novel therapeutic strategies in patients with autoimmune disease via mucosal tolerization (40,41).

Thus, Treg-inducing bystander epitope-specific immunotherapy has recently been introduced reporting the successful treatment of human autoimmune disease such as experimental arthritis, rheumatoid arthritis and type 1 diabetes (40–44). Beneficial tolerizing effects of oral antigen administration paved the way to making a therapeutic application in humans feasible as long as an immunogenic antigen is used that is presented at the same location as the self-antigens driving the immune response (39,40). From this perspective, mucosal tolerization with microbial antigen Pep19 would enhance specifically this population of cells as microbial HSP epitopes are promising antigens for Treg induction, thus harnessing their immunoregulatory potential holds promise for the treatment of autoimmune disease. Taken together, Pep19 meets all these above-mentioned criteria and thus could be a novel candidate for epitope-specific immunotherapeutic strategy through mucosal tolerization that would mobilize peptide-specific Tregs for bystander suppression targeting multiple bystander autoantigens.

One may argue with the issue of causality or coincidence of epitope spreading driven by Pep19. As evidenced by immunoblot analysis and statistical analysis, the predictive values declined substantially for Hu19, increased remarkably for Hu9 and declined slightly for ox-LDL in the
disease group. This could imply a shift in determinant spreading from Hu19 to Hu9 strongly driven by Pep19 as periodontitis or the periodontitis-associated autoimmune disease develops. The predictive values for ox-LDL in the healthy and disease groups remained quite similar.

Pep19 identified in our study is similar in its sequence to epitope peptide p1 reported by another group who have screened pan-DR-binding HSP60 self-epitopes using a computer algorithm (42). They reported induction of Tregs by peptide p1 for tolerogenic response in the management of juvenile idiopathic arthritis (41). However, Pep19 consistently and preferentially induced Th1 cells leading to its proatherogenic role in apolipoprotein E knockout mice (17). The reason for the dissimilar observations remains to be clarified. We are currently devising an immunotherapeutic strategy to induce peptide-specific tolerance or bystander suppression through self-peptide administration by oral or nasal routes that has been shown to suppress rheumatoid arthritis (39–41).

Taken together, epitope spreading to Hu19, Hu9 and ox-LDL provoked by Pep19 could be proposed as a novel finding in P. gingivalis-induced chronic periodontitis and infection-induced autoimmune diseases in a reproducible and predictable manner. T-cell proliferative activity to these peptides and cross-reactivity of anti-Pep19 to multiple human autoantigens could be proposed as cellular and molecular mechanisms responsible for this phenomenon.

Conflict of interest
The authors declare they have no conflicts of interest.

References
44. Luo X, Herold KC, Miller SD. Immunotherapy of type 1 diabetes: where are we and where should we be going? Immunity 2010;32:488–499.