Oral Microbiology Immunology 2004: 19: 1–5
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

Identification of T-cell epitopes of *Porphyromonas gingivalis* heat-shock-protein 60 in periodontitis

J.-I. Choi[1], H.-S. Kang[2], Y.-M. Park[3], S.-J. Kim[1], U.-S. Kim[1]

[1]Department of Periodontology and Research Institute for Oral Biotechnology, School of Dentistry, [2]Department of Molecular Biology, College of Natural Sciences, [3]Department of Microbiology, School of Medicine, Pusan National University, Pusan, Korea

Choi J-I, Kang H-S, Park Y-M, Kim S-J, Kim U-S. Identification of T-cell epitopes of Porphyromonas gingivalis *heat-shock-protein 60 in periodontitis.*
Oral Microbiol Immunol 2004: 19: 1–5. © Blackwell Munksgaard, 2004.

The heat shock proteins (hsp) of bacterial species are considered to be involved in regulating the autoimmune mechanism in human diseases due to the considerable homology of their sequences with human hsp. To elucidate how stress proteins contribute to the immunopathogenesis of periodontitis, mononuclear cells from gingival connective tissue of 10 periodontitis patients were simulated with *Porphyromonas gingivalis* hsp60. T-cell lines reactive to *P. gingivalis* hsp60 were established from each patient to define T-cell epitope specificities. Anti-*P. gingivalis* IgG antibody titres were elevated in all patients. We could establish *P. gingivalis* hsp-reactive T-cell lines from gingival mononuclear cells that were mixtures of $CD4^+$ and $CD8^+$ cells. Of 108 overlapping synthetic peptides spanning the whole *P. gingivalis* hsp60 molecule, 10 peptides with epitope specificities for T-cells were identified, and were identical to those reported be B-cell epitopes in periodontitis.

Key words: epitope; heat shock protein; periodontitis, *Porphyromonas gingivalis*; T-cell

Jeom-Il Choi, Department of Periodontology, School of Dentistry, Pusan National University 1-10, Ami-Dong, Seo-Ku, Pusan 602-739, Republic of Korea
Tel.: +82 240 7460;
fax: +82 255 9149;
e-mail: jrapa@pusan.ac.kr
Accepted for publication April 24, 2003

Due to the considerably high degree of sequence homology between bacterial and human heat shock proteins (hsp), it has been suggested that these proteins might be involved in autoimmune disease mechanisms in humans (5). Several authors have postulated that hsp might be involved in the periodontal disease process (1, 13, 15). It can be hypothesized that *Porphyromonas gingivalis* hsp could stimulate expression of proinflammatory cytokines and recruit T lymphocytes, macrophages and monocytes in gingival connective tissue.

It is critical to identify the immunodominant epitopes of an infecting pathogen that are recognized by T cells and/or B cells modulating the autoimmune disease mechanisms in the pathogenesis of periodontal disease (3). Recently Maeda et al. (2000) have reported an immunodominant B-cell epitope of *P. gingivalis* hsp in periodontitis (10). In the present study, we have characterized *P. gingivalis* hsp-reactive T-cell lines and identified T-cell epitopes of *P. gingivalis* hsp in periodontitis patients.

Materials and methods

Selection of patients

Ten adult periodontitis patients demonstrating elevated anti-*P. gingivalis* hsp60 antibody titers were selected. They were had no systemic diseases that might modify the course of periodontal disease. Ten subjects without evidence of periodontal disease were also included as the age-/sex-matched control group. Informed consent forms were obtained from the patients according to the Guidelines of the Institutional Review Board of Pusan National University Hospital.

Measurement of anti-heat shock protein IgG antibody titers by ELISA

Recombinant *P. gingivalis* hsp60 was produced and purified from *P. gingivalis* GroEL gene (a gift from Professor Yoji Murayama, Okayama University Dental School, Japan). Triplicate microtiter plates were coated with *P. gingivalis* hsp60 diluted (10 μg/ml) in phosphate buffer (2). The plates were washed and an aliquot of serum samples serially diluted was added and incubated. The plates were washed, and peroxidase-conjugated mouse anti-human IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) was added. After 2 h of incubation, the plates were washed and an aliquot of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for incubation followed by

the addition of 0.18 M H_2SO_4 to stop the reaction. Optical densities read at 450 nm were plotted as a function of the serum dilution factor. The serum dilution factor corresponding to an optical density of 0.5 in one subject of the control group was assigned an ELISA unit of 100 and serum IgG titers of the other healthy control subjects and periodontitis patients were determined accordingly. Antibody titer was considered to be elevated if it was higher than the mean antibody titer + 3× the standard deviation of healthy control group.

Western immunoblot analysis

Recombinant *P. gingivalis* hsp60 (2) was subject to SDS-PAGE and electro-transferred to nitrocellulose membrane. After blocking the membrane with 5% skim milk, human sera diluted 1 : 25 in PBS buffer were added for 2 h. The membrane was washed and horseradish peroxidase-conjugated mouse anti-human IgG added for 1 h. After washing the membrane with PBS-Tween 20, tetramethylbenzidine was added for color development.

Establishment and characterization of *P. gingivalis* heat shock protein-reactive T-cell lines

Gingival connective tissues collected from 10 patients were digested with collagenase (10 μg/ml, Boehringer-Mannheim, Berlin, Germany) to obtain a mononuclear cell population. Using 12-well tissue culture plates (Costar, Corning, Corning, NY), mononuclear cells were stimulated with *P. gingivalis* hsp (5 μg/well in 12-well culture plate) with antigen-presenting cells (APC). Peripheral blood lymphocytes were drawn from the patients and mononuclear cells were isolated by a gradient T-cell separation technique using Ficoll-Paque medium (Pharmacia, Upsala, Sweden) for use as APC after treatment with mitomycin C. After 2 weeks of incubation, T cells were allowed to rest for 1 week. After the resting period, fresh mitomycin-treated APC and *P. gingivalis* hsp antigen were added again to induce T-cell proliferation. *P. gingivalis* hsp-reactive T-cell lines were established in the same manner by adding the heat shock protein in alternating cycles to stimulate T cells. For characterization of T-cell lines, cells were double-stained with Per-CP-conjugated mouse anti-human CD3, FITC-conjugated mouse anti-human CD4 or PE-conjugated mouse anti-human CD8 monoclonal antibodies (PharMingen, San Diego, CA). Phenotypic expression of each T-cell line was screened by flow cytometry using an Epics Elite ESP (Coulter, Hialeah, FL).

Synthetic peptide

A total of 108 decapeptides spanning the entire amino acid sequence of *P. gingivalis* GroEL (10) were synthesized using an Epitope-Scanning Kit (Chiron Mimotopes, Clayton, Victoria, Australia) according to the manufacturer's instructions based on Fmoc chemistry. Peptides were designed to overlap by five amino acid residues. The fidelity of the synthesis was monitored by simultaneous synthesis of a positive (PLAQ) and a negative (GLAQ) control peptide and by subsequently testing their binding to the supplied monoclonal antibody.

T-cell epitope mapping

For defining epitope specificity of the synthetic peptides, 1×10^5 T cells from *P. gingivalis* hsp-specific T-cell lines were stimulated with increasing doses of synthetic peptides (0.1 μg/μl) and 1×10^6 APC. The plates were incubated at 37°C in a 5% CO_2 humidified incubator. After 48 h of incubation, the cells were labeled with 1 μCi/well of ^{3}H-thymidine, incubated for an additional 6 h and counted in a liquid scintillation counter. Proliferation was presented as the stimulation index (SI); the ratio of the mean counts per minute (cpm) with antigen to the cpm without antigen. SI values of 2 or greater were considered to be positive.

Results

Patient profile

Clinical parameters consisting of age, gender, mean probing pocket depths and mean probing attachment levels of periodontitis patients are summarized in the Table 1. Periodontitis patients were suffering from either moderate or severe periodontitis with varying ranges of probing pocket depths and attachment levels, respectively.

Serum IgG antibody titers by ELISA

In periodontitis patients, anti-*P. gingivalis* hsp IgG antibody titers ranged from 187.2

Table 1. Clinical profiles and anti-*P. gingivalis* hsp IgG titers of periodontitis patients

No.	Age/gender	Dx	Pocket depth (in mm) (mean ± s.d.)	Attachment level (in mm) (mean ± s.d.)	Anti-*P. gingivalis* hsp60 IgG
1	52M	MP	4.84 ± 0.88	4.97 ± 0.81	216.9
2	41M	MP	4.66 ± 0.51	4.86 ± 0.46	187.2
3	38F	MP	4.74 ± 0.67	4.85 ± 0.33	279.4
4	44F	SP	5.98 ± 0.54	6.11 ± 0.75	282.3
5	46F	MP	5.04 ± 0.65	5.17 ± 0.52	420.2
6	51F	MP	4.33 ± 0.39	4.45 ± 0.83	294.1
7	49M	MP	4.48 ± 0.80	4.55 ± 0.69	195.4
8	40F	MP	5.23 ± 0.86	5.38 ± 0.77	241.9
9	47F	SP	6.22 ± 0.79	6.54 ± 1.18	311.4
10	53F	MP	5.21 ± 0.44	5.30 ± 0.61	340.3
Control group (age/gender matched, $n = 10$)			3.13 ± 0.22	3.16 ± 0.30	108.2 ± 18.3 (mean)

hsp: heat shock protein. MP: moderate periodontitis. SP: severe periodontitis.

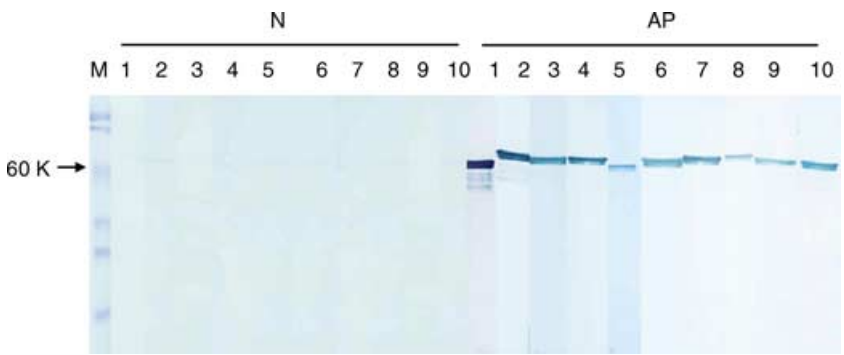

Fig. 1. Western immunoblot patterns against *P. gingivalis* hsp 60 by sera from 10 healthy control subjects (N1–N10) and 10 periodontitis patients (AP1–AP10) are demonstrated. Serum dilution factor was 1 : 25.

to 420.2, while mean antibody titer of healthy control subjects was 108.2 ± 18.3, respectively. Anti-*P. gingivalis* hsp antibody titers were elevated in all patients (Table 1).

Western immunoblot analysis

Sera from periodontitis patients (AP1–AP10) who had elevated IgG antibody to *P. gingivalis* hsp60 reacted strongly with *P. gingivalis* hsp60 as evidenced by Western immunoblot (Fig. 1). Sera from healthy control subjects (N1–N10) did not react with *P. gingivalis* hsp60.

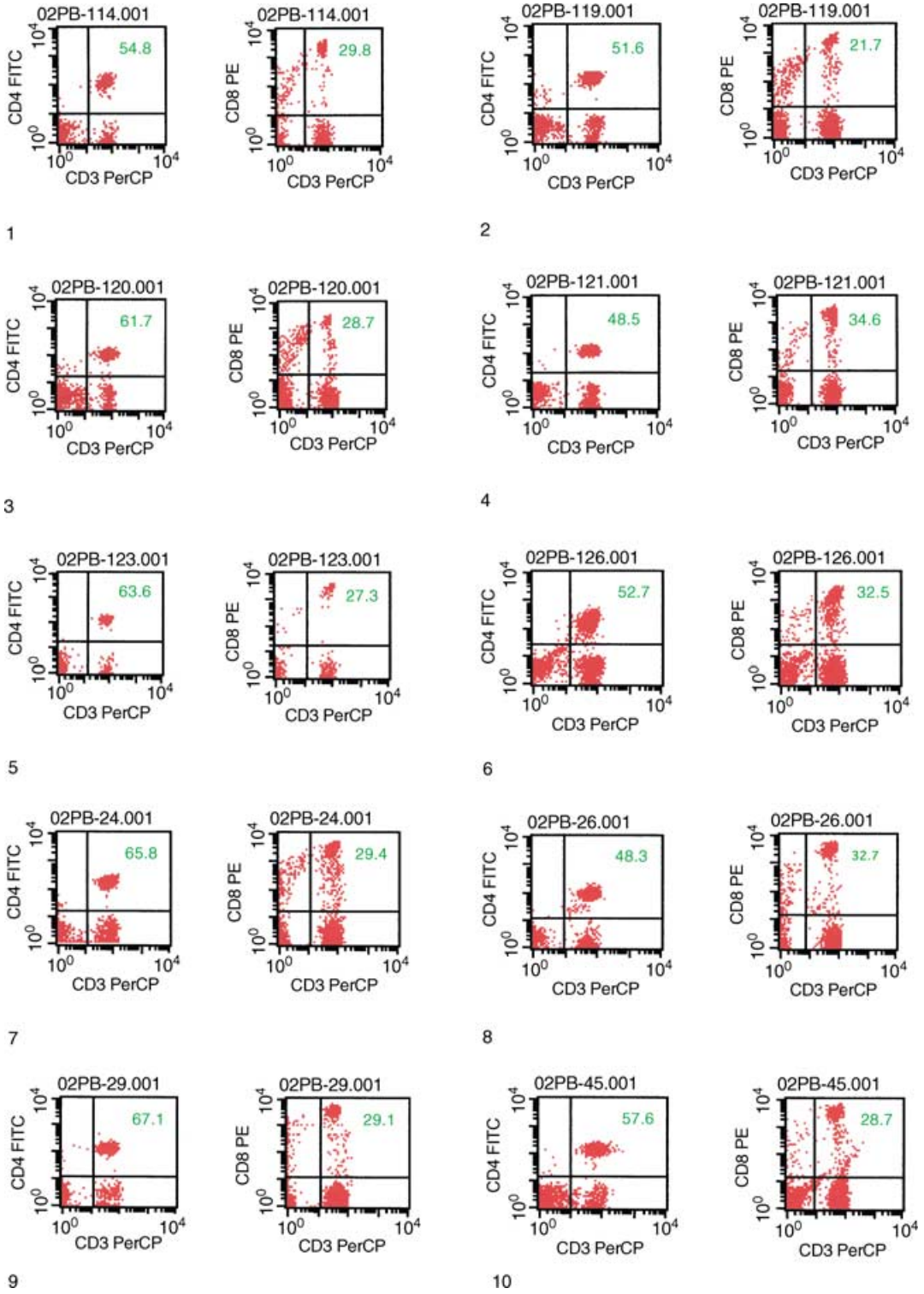

Fig. 2. Phenotype profiles of *P. gingivalis* hsp60-specific T-cell lines established from the 10 patients analyzed by flow cytometry. Proportions (%) of $CD3^+/CD4^+$ T cells (left) and $CD3^+/CD8^+$ T cells (right) of T-cell lines established from gingival connective tissue are demonstrated. The number indicates each patient.

Table 2. List of T-cell epitope peptide numbers and their amino acid sequences

Number	Position	One-letter amino acid sequence
P6	27–36	KVTLGPKVR
P15	73–82	VKEVASKTN
P24	117–126	RGIDKSVKSV
P29	142–151	QKIEHVAKIS
P33	162–171	IAEAMRKVKK
P39	193–202	MQFDRGYISP
P45	222–231	IYDKKISVLK
P53	262–271	LVVNRLRGSL
P56	279–288	PGFGDRRKAM
P74	366–375	QERLAKLAGG

Establishment and characterization of *P. gingivalis* heat shock protein-reactive T-cell lines

T cells isolated from gingival connective tissue in periodontitis patients demonstrated proliferation responses to *P. gingivalis* hsp. Proportions of $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T cells of *P. gingivalis* hsp-reactive T-cell lines were within varying ranges (48.3–67.1% for $CD4^+$ and 21.7–34.6% for $CD8^+$ T cells), respectively (Fig. 2).

T-cell epitope mapping

P. gingivalis hsp-reactive T cells from each patient showed multiple reactivity to 10 peptides from 108 overlapping peptides, spanning the whole sequence of *P. gingivalis* hsp60. Table 2 lists one-letter amino acid sequences of the 10 T-cell epitopes for which the SI values are 2 or greater in more than 8 out of 10 patients.

Discussion

The hsp60 was first described in *Escherichia coli* by Hendrix in 1979 and has been termed GroEL (5). Together with GroES, it facilitates protein translocation across membrane barriers and possibly secretion. A major task of the mammalian hsp60 is the protein folding and assembly in the mitochondrial matrix (9). Although it was originally thought that the localization of hsp60 was restricted to mitochondria, recent findings indicate their presence on the cell surface (14). It has also been demonstrated that hsp60 is present as a soluble form in circulation and correlated with severity of atherosclerosis (11). Due to considerably high degree of sequence homology between bacterial and human heat shock proteins (hsp), this protein might potentially be involved in autoimmune disease mechanisms in humans (5). T cells and antibodies with specificity for conserved sequences are potentially autoreactive and may contribute to pathogenesis of infectious diseases (8, 12).

To elucidate hsp-modulated autoimmune destructive mechanisms, it is critical to develop a strategy to identify the cross-reactive immunodominant epitope of hsp. Among hsp from several periodontopathogenic bacteria, we have selected *P. gingivalis* hsp as a candidate antigen for modulating T-cell mediated autoimmunity in periodontal disease because *P. gingivalis* is considered to be the most important key pathogen in destructive periodontal diseases (7).

Anti-*P. gingivalis* hsp60 IgG antibody titers in 10 periodontitis patients in the experimental group were elevated when compared with the control subjects. Sera from the patients reacted strongly with *P. gingivalis* hsp60 as evidenced by Western immunoblot. This finding is consistent with other reports implicating the possible involvement of the bacterial hsp's in the autoimmune pathogenic mechanisms of *P. gingivalis*-associated periodontitis (13, 15). Recently, several authors have claimed that bacterial stress proteins or human hsp play a critical role in recruiting immune cells which target antigens, leading to the development of periodontal lesions (1, 13, 15). The present study adds another line of evidence suggesting that *P. gingivalis* hsp-reactive T-cell immune response might be involved in immunopathogenesis of periodontal disease.

We could successfully establish *P. gingivalis* hsp-reactive T-cell lines from mononuclear cells isolated from gingival connective tissues in all the periodontitis patients; and these were a mixture of $CD4^+$ and $CD8^+$ cells. This finding suggests that T cells in the circulating peripheral blood may 'home' to periodontal lesions where *P. gingivalis* have infiltrated, potentially leading to T-cell response cross-reactive to mammalian hsp of gingival fibroblasts.

We have identified 10 epitopes of *P. gingivalis* hsp60 for T cells that concurrently belong to the 20 immunodominant B-cell epitopes reported by Maeda et al. (10). Therefore, the 10 T-cell epitopes identified in the present study might be the common T- and B-cell epitopes of *P. gingivalis* hsp60 in adult periodontitis. Among these, peptide no. 39 (MQFDRGYISP) is of potential interest, because this peptide is homologous to both other bacterial hsp and human hsp60 as clamed by Maeda et al. (10). Serologic cross-reactivity of hsp among periodontopathic bacteria has also been demonstrated (6). Based on our observation, peptide no. 15 (VKEVASKTND) of *P. gingivalis* hsp60 is a common T- and B-cell epitope in *P. gingivalis*-infected atherosclerosis patients and is also homologous to human hsp60 (unpublished observation). More interestingly, these two epitopes share a high sequence homology with other periodontopathic bacterial hsp60 (unpublished observation), and could be very important in modulating autoimmune disease mechanism in the pathogenesis of periodontal disease and possibly in atherosclerosis, though their defined role remains to be clarified.

Identifying cross-reactive antigenic epitopes of *P. gingivalis* hsp60 is critical in:

- understanding hsp-modulated autoimmune disease mechanisms operating in human periodontal diseases;
- identifying hsp epitopes cross-reactive with other hsp's from periodontopathic bacteria; and
- developing a peptide vaccine strategy for potential prevention of human periodontal disease.

Acknowledgments

We are thankful to Professor Yoji Murayama for providing *P. gingivalis* GroEL gene and S.W. Lee for his expertise in flow cytometry. This work was supported in part by a Grant #1999-2-20500-004-3 from KOSEF.

References

1. Ando T, Kato T, Ishihara K, Ogiuchi H, Okuda K. Heat shock proteins in human periodontal disease process. Microbiol Immunol 1995: **39**: 321–327.
2. Choi J-I, Chung S-W, Kang H-S, Rhim B-Y, Kim S-J, Kim S-J. Establishment of *Porphyromonas gingivalis* heat shock protein-specific T cell lines from atherosclerosis patients. J Dent Res 2002: **81**: 344–348.
3. Chung S-W, Kang H-S, Park H-R, et al. Immune responses to heat shock protein in *Porphyromonas gingivalis*-infected periodontitis and atherosclerosis patients. J Periodont Res 2003: **38**: in press.
4. Hansson GK. Immune mechanism in atherosclerosis. Arterioscler Thromb Vasc Biol 2001: **21**: 1876–1890.
5. Hendrix RW. Purification and properties of GroE, a host protein involved in bacteriophage assembly. J Mol Biol 1979: **129**: 375–392.
6. Hinode D, Nakamura R, Grenier D, Mayrand D. Cross-reactivity of specific antibodies directed to heat shock proteins from periodontopathogenic bacteria and of human origin. Oral Microbiol Immunol 1998: **13**: 55–58.
7. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000 1999: **20**: 168–238.
8. Lamb JR, Bal V, Mendez-Samperio P, et al. Stress proteins may provide a link between

the immune response to infection and autoimmunity. Int Immunol 1989: **1**: 191–196.
9. Langer T, Neupert W. Heat shock proteins hsp 60 and hsp 70: their roles in folding, assembly and membrane translocation of proteins. Curr Top Microbiol Immunol 1991: **167**: 3–30.
10. Maeda H, Miyamoto M, Kokeguchi S, et al. Epitope mapping of heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*. FEMS Immunol Med Microbiol 2000: **28**: 219–224.
11. Pockley A, Wu R, Lemne C, Kiessling R, de Faire U, Frostegard J. Circulating heat shock protein 60 is associated with early cardiovascular disease. Hypertension 2000: **36**: 303–307.
12. Schoenfeld Y, Isenberg DA. Mycobacteria and autoimmunity. Immunol Today 1988: **9**: 178–182.
13. Ueki K, Tabeta K, Yoshie H, Yamazaki K. Self-heat shock protein 60 induces tumour necrosis factor-alpha in monocyte-derived macrophage: possible role in chronic inflammatory periodontal disease. Clin Exp Immunol 2002: **127**: 72–77.
14. Xu Q, Schett G, Seitz CS, Hu Y, Gupta RS, Wick G. Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells. Circ Res 1994: **75**: 1078–1985.
15. Yamazaki K, Ohsawa Y, Tabeta K, et al. Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. Infect Immun 2002: **70**: 2492–2501.