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Expression of cutaneous lymphocyte-associated antigen (CLA) in tonsillar T-cells and its induction by in vitro stimulation with alpha-streptococci in patients with pustulosis palmaris et plantaris (PPP)

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Full title:

Expression of cutaneous lymphocyte associated antigen (CLA) in tonsillar T-cells and its induction by *in vitro* stimulation with alpha-*streptococci* in patients with pustulosis palmaris et plantaris (PPP)

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Running title: Expression of CLA in tonsils with PPP

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Abstracts

Pustulosis palmaris et plantaris (PPP) is known to be a one of the tonsil-related diseases because tonsillectomy is quite effective in curing this condition. However etiological association between tonsils and PPP have not fully clarified yet. Cutaneous lymphocyte associated antigen (CLA) is known to be a specific homing receptor that facilitates T-cell migration into skin. In this study, we investigated the expression of CLA on T-cells in tonsil, peripheral blood, and skin from patients with PPP. Two-color flow cytometric and two-color immunohistological analyses revealed that the numbers of CLA/CD3 double-positive cells in freshly isolated tonsillar mononuclear cells (TMC) and in tonsillar tissues were significantly higher in PPP patients than in non-PPP patients ($p<0.01$, each). *In vitro* stimulus with α-streptococcal antigens enhanced CLA expression of tonsillar T-cells and TGF-β production of TMC in PPP patients ($p<0.01$, each), but did not in non-PPP patients. In peripheral blood from PPP patients, the number of the CLA/CD3 double-positive cells significantly decreased at 6 months after tonsillectomy ($p<0.05$). The CLA/CD3 double-positive cells and the postcapillary venule that expressed with a ligand of CLA, E-selectin, were found more frequently in the plantar skin from PPP patients as compared to that from healthy volunteers ($p<0.01$, each). These data suggest that a novel immune response to α-streptococci may enhance CLA expression on tonsillar T-cells through TGF-β production in patients with PPP, resulting in homing of CLA-positive T-cells to skin and tissue damages. This may play a key role in pathogenesis of PPP.

Key words: cutaneous lymphocyte associated antigen (CLA), tonsil, tonsillitis, pustulosis palmaris et plantaris, α-streptococci, IL-6, TGF-β, E-selectin

INTRODUCTION

Pustulosis palmaris et plantaris (PPP) is characterized by symmetrical, erythematous, scaly plaques with numerous, sterile non-bacterial, pinpoint pustules that are restricted to the palms and soles [1, 2]. It has been reported that the incidence of PPP was about 0.05% [3]. PPP is seen more frequently in women and is most frequent between the ages of 30 and 60 years. The disease tends to have an unpredictable course with exacerbations that often occur during acute tonsillitis [1, 4]. PPP often disappears after tonsillectomy [4-6]. Therefore, PPP is considered one of the tonsil-related diseases. The etiology and pathogenesis of PPP remain unclear.

Previous studies have demonstrated increased serum levels of immune complexes and anti-keratin antibody in PPP; however, these return to normal after tonsillectomy with subsequent improvement in the skin lesions [6]. An animal model with PPP-like skin lesions has been established by reconstituting severe combined immunodeficiency (SCID) mice with tonsillar mononuclear cells (TMC) from patients with PPP [7]. Recently, Yamanaka et al. [8] grafted human PPP skin onto SCID mice and simultaneously injected them with TMC or peripheral blood lymphocytes (PBL) from subjects with PPP. A larger number of TMC infiltrated the area around the papillary dermis of the PPP skin than PBL, suggesting that TMC selectively home to the skin lesions of PPP. Furthermore, the mice exhibited increased level of human anti-keratin antibody in mice with the TMC [8]. These findings suggest a close association between tonsils and PPP.

Cutaneous lymphocyteassociated antigen (CLA), a glycoprotein recognized

by a monoclonal antibody HECA452, is known to be a specific homing receptor that facilitates T-cell migration into skin lesions [9]. The CLA expression on T-cells is reported to be up-regulated by TGF-β and IL-6 [10]. Previous reports demonstrated that the CLA expression on T-cells infiltrating skin lesions was increased in skin diseases such as cutaneous T-cell lymphoma [9], atopic dermatitis [9], and psoriasis [11-13]. E-selectin is a ligand of CLA and is predominantly expressed in postcapillary venules stimulated by inflammation [14]. E-selectin expression as well as expression of CLA is up-regulated in skin lesions of chronic leg ulcers [15]. These data suggest that CLA expressing T-cells, which infiltrate skin lesions through the E-selectin expressing postcapillary venules, play a key role in skin tissue damage in various skin diseases. There is no information regarding CLA expression in tonsil-related disease PPP.

Tonsils, which are lymphatic organs in Waldeyer's ring, appear to play an important role in the immune-defense mechanism of the upper respiratory airway against various foreign substances, especially bacteria colonized in the pharynx. In a certain condition, tonsils play a pathogenic role through a novel immune response to bacteria on several autoimmune diseases. In patients with PPP, α-*streptococci* has been detected at higher level in patients' tonsils [16]. Serum antibody levels to α-*streptococci* were also high in patients with PPP, especially in patients of whom skin lesions were markedly improved after tonsillectomy [16, 17]. We showed previously that TMC from PPP showed increased proliferation and proinflammatory cytokine productions including IFN-γ, TNF-α, and IL-6 in response to *in vitro* α-streptococcal stimulations [17]. These data suggest that hyper-immune response to indigenous bacterial specimens such as α-*streptococci* may be present in patients with tonsillar focal infections like PPP.

In this study, we performed tonsillectomy in patients with PPP for treatment of PPP and investigated (i) how CLA is expressed in tonsillar T-cells, (ii) how CLA expression of peripheral T-cells changes after tonsillectomy, (iii) how CLA and its ligand E-selectin is expressed in skin lesions, (iv) whether CLA expression of tonsillar T-cells is enhanced by *in vitro* stimulation with α-streptococcal antigens, and (v) whether TMC produce CLA-related cytokines TGF-β and IL-6 in response to *in vitro* stimulation with α-streptococcal antigens.

MATERIALS AND METHODS

Patients and samples

All patients were studied at Asahikawa Medical College. The study groups were composed of 2 groups, the PPP group and non-PPP group, undergoing tonsillectomy. PPP was diagnosed by dermatologists in our hospital on the basis of the findings characterized by symmetrical, erythematous, scaly plaques with numerous, sterile, pinpoint pustules restricted to the palms and soles. The non-PPP group was composed of patients with recurrent tonsillitis (RT) or obstructive sleep apnea syndrome (OSAS). Patients with RT required tonsillectomy because of recurrent episodes (more than 3 times per year) of acute tonsillitis. Patients with OSAS underwent uvulo-palate-pharyngoplasty together with tonsillectomy. Clinical findings which were collected from hospital chart of each PPP patient are listed in **table 1**. Seven patients in all PPP patients were treated with ointment of corticosteroid, however their skin lesions did not improve before tonsillectomy. Other treatments (e.g. psoralen plus ultraviolet A treatment) were not chosen in all cases. Eight patients (73%) in all PPP patients had

history of smoking. Exacerbation of skin lesion during upper air way infections such as tonsillitis were seen in 6 of all PPP patients. About Complication, sternocostoclavicular hyperosteosis (SCCH) were seen in only one patients of PPP, but other clinical symptoms and medical conditions potentially influencing the outcome of this study ware not found in all cases. The effects of tonsillectomy in 11 cases observed 6 months after tonsillectomy, and judged on 4 grade scale: 'Disappeared', 'Markedly effective', 'Effective', and 'Partially effective'. 'Disappeared' means the absence of an observed eruptive state. 'Markedly effective' means such improvement that the patients believes himself to be healed, though sometimes a few eruptions may persist. 'Partially effective' means persisting eruptions, though obviously improved. 'Effective' means the middle degree between 'Markedly effective' and 'Partially effective'. There were no cases with exacerbation or no improvement after tonsillectomy. The clinical pictures of skin condition of PPP before and after tonsillectomy were shown in Fig 1 (Case No 8, 'Markedly effective'). No Patients with RT or OSAS had skin diseases. All patients signed informed consent for therapy and tissue studies. The study was approved by the Institutional Review Board.

Cell preparation

Tonsillar and peripheral mononuclear cells were isolated from tonsils and peripheral blood by the gradient centrifugation method using Ficoll Paque Plus® (Amersham Pharmacia Biotech, Piscateway, NJ, USA), as described previously [18]. The cells were washed 3 times with sterile phosphate buffered saline (PBS), suspended in RPMI-1640 medium (GIBCO, Grand Island, NY, USA), and counted. The viability of the cell suspensions were over 95%.

Preparation of bacterial antigens

Frozen whole cell preparations from *Streptococcus* (*S.*) *sanguis* ATCC10556, *S. salivarius* ATCC7073 and *S. mitis* NCTC3265 were used in this study [17, 19, 20]. Bacteria were grown over night at 37°C in Todd-Hewitt Broth (DIFCO laboratories, Detroit, MI, USA) and incubated for 40 minutes at 60°C. The heat-inactivated cells were harvested by centrifugation at 9,000 *g* for 30 minutes at 4°C, resuspended in PBS, and centrifuged in the same conditions. The pellets were then lyophilized overnight in PBS and stored at -80°C until used.

Cell culture

The tonsillar mononuclear cells (TMC) were suspended at a concentration of 1×10^6/ml in 3 ml of RPMI-1640 culture medium (GIBCO) containing 10% fetal calf serum (FCS; GIBCO), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were then cultured without any mitogens or antigens, with 5 μg/ml phytohemagglutinin (PHA; Sigma-Aldorich, St. Louis, MO, USA), or with 100 μg/ml of lyophilized streptococci in 6-well culture plates in an atmosphere with 5% CO2 at 37°C. After 3 day in culture, the TMC and the culture supernatant fluids were collected and subjected to CLA expression and cytokine production determinations, respectively.

Two-color flow cytometry

Two-color immunofluorescence was performed as follows. One million cells were reacted with 50 μl of 40 μg/ml phycoerythrin (PE)-labeled anti-CLA monoclonal antibody (HECA-452; Pharmingen, San Diego, CA, USA) together with 50 μl of 50

μg/ml fluorescein isothiocyanate (FITC)-labeled anti-CD3 antibody (Pharmingen) for 20 min at 4°C. Isotype mouse IgG2a (Biodesign International, Saco, ME, USA) was used as a control. The cells were washed three times and then subjected to flow cytometric analysis using EPICS ELIETE flow cytometer (Coulter Electronics Inc., Hialeah, FL, USA). All dilutions and washings were done in ice-cold PBS containing 0.1% sodium azide and 2% FCS. The data were displayed as a contour cytogram.

Immunohistology

To examine CLA expression of T-cells in tonsils and skin, CLA/CD3 double immunohistological staining was employed. The formalin-fixed and paraffin-embedded specimens were obtained from tonsil and skin tissues. The specimens were cut into 5-μm sections. The slides were deparaffinized in xylene and ethanol. These sections were then incubated with 3% hydrogen peroxidase for 30 minutes. Slides were pressure cooked for 5 minutes in 1 mmol/EDTA buffer (PH 8.0) for antigen retrieval, and incubated with mouse anti-CD3 antibody (Pharmingen) for one hour at room temperature. They were rinsed twice in PBS and, then developed using peroxidase-labeled dextran polymer (Envision+; DAKO, Carpinteria, CA, USA) for 30 minutes at room temperature. They were visualized by immersing the slides in freshly prepared 0.02% diaminobenzidine (DAB) solution for 10 minutes. Slides were then washed for 15 minutes in PBS, and incubated with rat anti-CLA antibody (HECA-452; Pharmingen) for one hour at room temperature. They were rinsed twice in PBS, and then developed using alkaline phosphatase labeled dextran polymer (Envision AP+; DAKO) for 30 minutes at room temperature. New Fucsin Red substrate (DAKO) was applied for 15 minutes, and slides were counterstained with hematoxylin and mounted.

Sections with anti-mouse IgG1 monoclonal antibody was used as negative controls. In skin samples, we also analyzed expression of the ligand for CLA, E-selectin, by immunoperoxidase staining using anti-E-selectin monoclonal antibody (Santa Cruz Biotechnology Inc., CA, USA) and peroxidase-labeled dextran polymer (Envision+, DAKO).

The sections were examined microscopically at x400 magnification by three of the authors who had no knowledge of patients. In the interfollicular areas of tonsils, the number of CD3/CLA double-positive cells was counted in more than 1000 CD3-positive cells. The value was expressed as a percentage of CD3/CLA double-positive cells in CD3-positive cells. To examine skin, the CD3/CLA double-positive cells and the E-selectin positive vessels were counted in the whole length of the section and the average number per square millimeter was calculated in each case.

Enzyme-linked immunosorbent assay

Because the CLA expression of T-cells is reportedly enhanced by IL-6 and TGF-β [10], IL-6 and TGF-β production from TMC stimulated with or without α-streptococcal antigen were measured by commercialized enzyme-linked immunosorbent assay (ELISA) kits (Genzyme Diagnostics, Cambridge, MA, USA). Briefly, the 96-wells flat-bottomed plates coated with mouse anti-human cytokines were incubated with 200 µl of PBS containing 4% bovine serum albumin (BSA) for 2 hours at 37°C to cover the unreacted sites. The wells were washed with PBS containing 0.05% polysorbate 20 (Tween 20; PBS-T) and incubated overnight with 100 µl of supernatant culture fluids at adequate dilution at 4˚C. Each sample was assayed in duplicate. After

washing three times with PBS-T, the plates were then incubated sequentially with 100 μl of biotinylated anti-human cytokines at adequate dilution and with 100 μl of peroxidase-conjugated streptavidin diluted 1:1,000 in PBS-T with 1% BSA at 37°C for 60 minutes each. After each reaction the plates were washed three times with PBS-T. The wells were then reacted with 100 μl of substrate solution containing 0.1 mg/ml tetramethylbenzidine and 0.003% H_2O_2. After 10 minutes- of incubation at room temperature, the reaction was stopped by adding 100 μl of 5N sulfuric acid. The optical density of each well was measured by an automated spectrophotometer (SLT-Labinstruments, Grödig, Austria) at 450 nm. A serial diluted standard solution was run in each plate and the cytokine level in the sample was read from the standard curve. The lowest limit of detection in the assay was 8 pg/ml in IL-6 and 31.2 pg/ml in TGF-β.

Statistics

The data were expressed as median: 25th-75th percentiles. Two group comparisons were tested using nonparametric test procedures such as Mann-Whitney U test and Wilcoxon signed rank test. Statistical tests were based on a level of significance $p<0.05$.

RESULTS

CLA expression of tonsillar T-cells

Tonsillar tissues and mononuclear cells from 21 patients undergoing tonsillectomy were subjects to CLA expression. The two patient groups were composed of 11 patients with PPP and 10 patients with other tonsillar disease (8 recurrent

tonsillitis and 2 obstructive sleep apnea syndrome). The PPP group consisted of 3 males and 8 females, aged from 22 to 36 years with median age of 31 years, and the non-PPP group consisted of 7 males and 3 females, aged from 19 to 31 with median age of 25 years. There was no difference in age distribution between 2 groups.

The results of two-color flow cytometric analysis were summarized in Table 2. The percentage of CD3-positive cells was significantly higher in PPP patients (median: 25th-75th percentiles; 25%:20.5-28.0%) than in non-PPP patients (13.5%:11.3-19.3%; $p<0.001$). The percentage of CD3/CLA double-positive cells was significantly higher in PPP patients (10.2%:7.9-18.9%) than in non-PPP patients (6.3%:4.2-6.6%; $p<0.01$). The percentage of CLA-positive cells in CD3-cells was also significantly higher in PPP patients (24.6%:19.7-30.0%) than in non-PPP patients (13.1%:10.4-15.3%; $p<0.01$). However, the percentage of CLA-positive cells or CD3-negative cells were not different between PPP patients and non-PPP patients (12.6%:8.6-14.0% vs. 8.0%:5.0-12.6%; in the CD3-negative cells, 18.3%:16.7-18.7% vs. 13.1%:12.2-17.8%).

Representative immunohistological profiles of tonsils were shown in Fig. 2a. The CLA expression was mainly found in high endotherial venules (HEV) and CD3-positive cells in the T-cell nodules of interfollicular areas, but rarely found in lymphoid follicles. The percentage of CD3/CLA double-positive cells in CD3-positive cells was significantly higher in PPP patients (6.5%:4.6-8.3%) than in non-PPP patients (3.1%:1.9-5.0%; $p<0.01$; Fig. 2b).

Changes in CLA expression of tonsillar T-cells by *in vitro* α-streptococcal stimulus

Preliminary study under the culture conditions for 1-5 days of culture with 1,

10, and 100 μg/ml α-streptococcal antigens revealed that the highest levels of CLA expression on tonsillar T-cells were found at 3 days-culture with 100 μg/ml streptococcal antigens. Therefore, we analyzed CLA expression of tonsillar T-cells 3 days after stimulations with 100 μg/ml α-streptococcal antigens.

The percentages of CD3/CLA double-positive cells per CD3 cells stimulated with and without α-streptococcal antigens were shown in Fig. 3a. In both PPP and non-PPP patients, the PHA stimulation enhanced CLA expression on tonsillar T-cells ($p<0.01$). Under stimulus condition with PHA, there was no difference in percentage of CLA expression in CD3 cells between PPP patients (36.5%:31.0-41.7%) and non-PPP patients (28.9%:22.0-38.6). Stimulation with either *Streptococcus (S.) mitis* antigen or *S. salivarius* antigen enhanced CLA expression on tonsillar T-cells ($p<0.01$, each) in PPP patients, but did not in non-PPP patients. Under stimulus condition with *S. mitis*, *S. sanguis* and *S. salivarius*, the percentages of CLA expression on tonsillar T-cells were significantly higher in PPP patients (26%:25-33.7%, 21%:12.3-24%, 24:20-32.3%, respectively) than in non-PPP patients (11%:7.2-13%, 11%:5.2-16%, 13%:12-14%, respectively; $p<0.001$, each).

To clarify the effect of stimulus, the data were expressed as CLA stimulation rate (Fig. 3b). The stimulation rate was calculated with this formula: the percentage of CLA/CD3 double-positive cells in CD3 cells with stimulus / that without stimulus x 100. The CLA stimulation rate of PHA was not different between PPP patients (225:157-195) and non-PPP patients (210:182-335). However, the CLA stimulation rates of all three α̃streptococcal stimulus were statistically higher in PPP patients than in non-PPP patients (*S. mitis*; 174:161-199 vs. 88.9:81.1-116, $p<0.001$; *S. sanguis*; 126:109-145 vs. 74.7:61.0-100, $p<0.05$; *S. salivarius*; 135:120-189 vs. 91.4:67.6-106, $p<0.001$).

Cytokine production of TMC stimulated with α-streptococcal antigens

TGF-β levels produced by TMC are shown in Fig. 4a. There was no difference on TGF-β level under the no stimulus condition between PPP patients and non-PPP patients (545:484-593 pg/ml vs. 550:518-615 pg/ml). In both PPP and non-PPP patients, the PHA stimulation enhanced TGF-β production ($p<0.01$, each). Under stimulus condition with PHA, there was no difference on TGF-β production between PPP patients (706:655-721 pg/ml) and non-PPP patients (718:566-788 pg/ml). Stimulation with either *S. sanguis* antigen or *S. salivarius* antigen enhanced TGF-βproduction ($p<0.01$, each) in PPP patients, but did not in non-PPP patients. Under stimulus conditions with *S. sanguis* and *S. salivarius*, the TGF-β levels were significantly higher in PPP patients than in non-PPP patients (*S. sanguis*; 628:525-671 pg/ml vs. 527:452-588 pg/ml; $p<0.05$, *S. salivarius*; 615:585-656 pg/ml vs. 545:508-641 pg/ml; $p<0.05$). To clarify the effect of the stimulus, the data were expressed as cytokine stimulation rate (Fig 4b). The cytokine stimulation rate was calculated with this formula: the cytokine levels with stimulus / the cytokine levels without stimulus x 100. The stimulation rates of *S. sanguis* and *S. salivarius* stimulus were statistically higher in PPP patients than in non-PPP patients (*S. sanguis*; 110:102-113 vs. 96.3:89.3-103; $p<0.01$, *S. salivarius*; 115:106-121 vs. 100:92.8-112; $p<0.01$).

The results of IL-6 production by TMC were shown in Table. 3. There was no difference on IL-6 level in any culture condition between PPP patients and non-PPP patients. Although PHA stimulation enhanced IL-6 production ($p<0.01$, each), streptococcal stimulus did not in both PPP and non-PPP patients.

CLA expression of peripheral blood T-cells

Peripheral blood samples were obtained from 7 patients with PPP (3 males and 4 females, aged 28 to 36 years with median age of 32 years) and from 5 patients with RT (2 males and 3 females, aged from 26 to 31 years with median age of 30 years), 1 day before tonsillectomy, 3months, and 6 months after tonsillectomy.

There was no significant difference on CLA expression of peripheral blood CD3-cells before tonsillectomy between PPP patients and non-PPP patients (12.5:10.4-16.7% vs. 12.3:8.9-16.1%). Changes in CLA expression of peripheral blood T-cells in patients with and without PPP were shown in Fig. 5. The percentage of CD3/CLA double-positive cells in CD3 cells was not significantly different before tonsillectomy and after 3 months (12.2:10.5-16.1%). However, the CD3/CLA double-positive cells significantly decreased after 6 months of tonsillectomy (10.0%: 8.8-15.1%, $p<0.05$). On the other hand, in non-PPP group, this population was not significantly different among the three time points.

CLA and E-selectin expressions in the plantar skin

Skin samples were obtained from 7 PPP patients. Skin biopsies were taken from regional edges of the most active accessible plaque in plantar skin. Control plantar skin tissues were obtained from 5 healthy volunteers. The PPP group consisted of 1 male and 6 females, aged 25 to 36 years with median age of 33 years, and the healthy volunteer group consisted of 5 males, aged 30 to 42 with median age of 35 years. There was no difference on age distribution between 2 groups. PPP patients were also included in **table 1**

Representative immunohistological profiles were shown in Fig. 6. In PPP patients (Fig. 6A), numerous CD3/CLA double-positive cells were found around pustules in the epidermis and papillary dermis. However, the cells were rarely found in the plantar skin from healthy volunteers (Fig. 6B). The number of CD3/CLA double-positive cells infiltrating to the skin was significantly higher in PPP patients (135:48-147 /mm^2) than in healthy volunteers (5.0:2.3-6.3 /mm^2; $p<0.01$, Fig. 7A).

Numerous E-selectin-positive microvessels were found in the papillary and upper reticular dermis from PPP patients (Fig. 6C). However, the microvessels were rarely found in the plantar skin from healthy volunteers (Fig. 6D). As shown in Fig 7B, the number of E-selectin-positive microvessels in the plantar skin was significantly higher in PPP patients (38:21-44 /mm^2) than in healthy volunteers (8.0:6.7-10/mm^2; $p<0.01$).

DISCUSSION

Pustulosis palmaris et plantaris (PPP) is considered to be a one of the tonsil-related diseases based on marked clinical improvement of the skin lesions after tonsillectomy [1, 4-6]. Despite the accumulation of data showing the clinical efficacy of tonsillectomy for this skin lesion [4-6], fundamental etiological and pathophysiological issues have yet to be addressed. In the early stage of PPP, it has been reported that lymphocytes, predominantly T-cells, infiltrate the palmar and plantar skins [21, 22]. In this study, we found that numerous CLA-positive T-cells infiltrated plantar skin in PPP patients, as previously reported in psoriatic diseases and atopic dermatitis [9, 11-13]. In plantar skin lesions of PPP, we found numerous postcapillary venules expressed with a

ligand of CLA, E-selectin. Yokochi et al. [22] previously reported also increased expression of adhesion molecules ICAM-1 and E-selectin in the vessels surrounding the pustule of PPP. These findings suggest that CLA expressing T-cells may home to the palmar and plantar skins through the E-selectin expressing postcapillary venules and play a key role for formation of pustular lesions. Current interest has been focused on whether tonsillar mononuclear cells (TMC) home to the skin.

Recently, Yamanaka et al. [8] transferred human PPP skin grafts onto SCID mice and simultaneously injected the patients' TMC or peripheral blood lymphocytes (PBL). In these mice, a great number of human tonsillar T-cells from PPP patients infiltrated the area around the papillary dermis of the PPP skin, whereas only a few peripheral T-cells did, suggesting that tonsillar T-cells may selectively home to the skin lesions of PPP. Previous *in vitro* study using human samples demonstrated that CLA expressing tonsillar T-cells bind to E-selectin transfected cells [14]. In this study of human samples, we showed that CLA expression of tonsillar T-cells in patients with PPP was significantly higher than in those with non-PPP. The expression level of CLA in peripheral T-cells was not different between PPP and non-PPP before tonsillectomy. This is agree with previous report that there was no significant difference in the number of CLA expressing T-cells in peripheral blood between psoriatic disease and normal control [12]. However, we found that the CLA expression on peripheral T-cells in PPP patients significantly decreased at 6 months after tonsillectomy. The level of serum anti-keratin antibody, which was one of the candidates for autoantibodies associated with PPP, decreased also 6-8 months after tonsillectomy [6]. These results support the possibility that CLA expressing tonsillar T-cells may home to skin through peripheral blood circulation, resulting in skin damage in PPP patients. As an explanation of the

results about CLA expression on peripheral T-cells, we think that CLA positive T-cells which move from tonsils to peripheral circulation are similar numbers to those which moved from peripheral circulation to skins.

In the present study, we detected that stimulation of α-streptococcal antigens enhanced CLA expression on tonsillar T-cells and TGF-β production of TMC from patients with PPP, but did not in patients without PPP. As we already mentioned, the TMC from PPP patients showed increased cellular response and the proinflammatory cytokine productions to α-streptococcal antigens [16, 17]. Moreover, Cytokines IL-6 and TGF-β are reported to up-regulate CLA expression of peripheral T-cells [10]. Therefore, in patients with PPP, tonsillar T-cells are likely to be persistently stimulated with α-streptococci, resulting in overexpression of CLA on the T-cells through TGF-β̃ On the other hand, we failed to find IL-6 induction by any streptococcal stimulus in either PPP or non-PPP patients. This does not agree with our previous study that TMC from PPP patients showed a significant IL-6 induction in response to α-streptococcal antigens [17]. This discrepancy may be caused by the difference in culture periods. To determine cytokine profiles associated with α-streptococcal stimulation, we selected a 5 days-culture because highest cytokine levels were found in this culture period [17]. In the present study for determination of the cytokine associated with CLA expression, we selected a 3 days-culture because the highest level of CLA expression was found in a 3 days-culture rather than in a 5 days-culture. These data suggest that TGF-β rather than IL-6 may play an important role on enhancement of CLA expression in response to the α-streptococcal antigens.

We think that CLA positive tonsillar T-cells not strictly to be monoclonal if

main regulator of CLA expression is cytokine. Some of these cells may become sensitized to tonsillar epithelium or Heat-shock protein (HSP) in α-streptococcus which reportedly share an antigen with palmar and plantar skins [23-26]. After polyclonal CLA positive tonsillar T-cells form to palmar and plantar skins, sensitized T-cells may start to respond the common antigen in this target organ. These speculations are supported by reports that T-cell receptor repertoire in peripheral blood of patients with psoriatic disease, of which PPP is classified as a localized form, did not have specific pattern in spite of restricted usage in skin lesion [27, 28], and that selective clonal T-cells are not so accumulated in the early skin lesions of psoriasis vulgaris [28, 29]. Because E-selectin is also expressed in endothelial cells of lesions from psoriasis and atopic dermatitis, as well as PPP [30], movement of CLA positive T-cells from peripheral circulation to lesional skins through E-selectin is not only event for PPP. Therefore, we believed that the difference of symptoms among these diseases depend on kinds of sensitized antigen.

In summary, we demonstrated in this study of PPP patients that (i) the CLA expression of tonsillar T-cells increased, (ii) the CLA expression of peripheral T-cells decreased after tonsillectomy, (iii) the CLA-positive T-cells and the E-selectin-positive microvessels frequently distributed in the plantar skin, (iv) CLA expression of tonsillar T-cells was enhanced by *in vitro* stimulation with α-streptococcal antigens, (v) tonsillar T-cells produced CLA-related cytokine TGF-β in response to *in vitro* stimulation with α-streptococcal antigens. These results suggest that a novel immune response to α-streptococci may enhance CLA expression on tonsillar T-cells through TGF-β production, resulting in homing of CLA-positive T-cells to the plantar skin via E-selectin-positive microvessels and tissue damages. This may play a key role in pathogenesis of PPP, and we also think that these results suggest the possibility for new therapy by using CLA neutralization antibody to palatine tonsils.

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FIGURE LEGENDS

Figure 1. Skin lesion of pustulosis palmaris et plantaris (PPP) patients before (A) and 6 months after (B) tonsillectomy. The effect of tonsillectomy was judged as ‘Markedly improved’ in this case.

Figure 2. (a) Representative two-color immunohistological profiles of CD3 (brown) and CLA (red) expressions in tonsillar tissues from pustulosis palmaris et plantaris (PPP) patients (A: x100, B: x400) and non-PPP patients (C: x100, D: x400). The CLA expression is mainly found in high endothelial venules (HEV) and CD3+ cells in the T-cell nodules of interfollicular area, but hardly found in germinal center (gc) area. Numerous CLA/CD3 double-positive cells are seen in PPP patients (A, B), but only a few CLA/CD3 double positive cells are in non-PPP patients (C, D). (b) Results of two-color immunohistology for CLA expression on tonsillar T-cells in pustulosis palmaris et plantaris (PPP) patients (n=11) and non-PPP patients (n=10). Each value is expressed as a percentage of CD3/CLA double-positive cells in CD3-positive cells. The median values are displayed as short bar (-). Mann-Whitney U test was used to determine p-value. The percentage of CD3/CLA double-positive cells in CD3-positive cells was significantly higher in PPP patients than in non-PPP patients ($p<0.01$).

Figure 3. (a) Changes in CLA expression of tonsillar T-cells by *in vitro* α-streptococcal stimulus. Tonsillar mononuclear cells (TMC) from pustulosis palmaris et plantaris (PPP) patients (n=11) and non-PPP patients (n=10) were cultured for 3 days in the absence or the presence of phytohaemagglutinin (PHA) or α-streptococcal antigens. Each value is expressed as a percentage of CD3/CLA double-positive cells in CD3-positive cells, analyzed by two-color flow cytometry. The median values are displayed as short bar (-). Wilcoxon signed rank test was used to determine p-value. In both PPP and non-PPP patients, PHA stimulation enhanced CLA expression on tonsillar T-cells. Either stimulation with *S. mitis* antigen or *S. salivarius* antigen enhanced CLA expression on tonsillar T-cells in PPP patients, but did not in non-PPP patients. (b) The stimulation rate was calculated with this formula: (the percentage of CLA/CD3 double-positive cells in CD3-positive cells with stimulus / that without stimulus) x 100. The values were expressed as median: 25th-75th percentiles. Mann-Whitney U test was used to determine p-value. The CLA stimulation rate of PHA was not different between PPP patients and non-PPP patients. However, the CLA stimulation rates of all three α̃streptococcal stimulus were statistically higher in PPP patients than in non-PPP patients. *$p<0.05$. **$p<0.01$. ***$p<0.001$

Figure 4. (a) TGF-β production of tonsillar mononuclear cells (TMC) stimulated with α-streptococcal antigens. TMC from pustulosis palmaris et plantaris (PPP) patients (n=11) and non-PPP patients (n=10) were cultured for 3 days in the absence or the presence of phytohaemagglutinin (PHA) or α-streptococcal antigens.

TGF-β levels in the culture supernatant fluids were measured by enzyme-linked immunosorbent assay. The median values are displayed as short bar (-). Wilcoxon signed rank test was used to determine p-value. In both PPP and non-PPP patients, PHA stimulation enhanced TGF-β production. Either stimulation with *S. sanguis* antigen or *S. salivarius* antigen enhanced TGF-β production in PPP patients, but did not in non-PPP patients. (b) The stimulation rate was calculated with this formula: (the TGF-β levels with stimulus / the TGF-β levels without stimulus) x 100. The values were expressed as median: 25th-75th percentiles. Mann-Whitney U test was used to determine p-value. The stimulation rates of *S. sanguis* and *S. salivarius* stimulus were statistically higher in PPP patients than in non-PPP patients. *$p<0.01$.

Figure 5. Changes in CLA expression of peripheral blood T-cells in pustulosis palmaris et plantaris (PPP) patients (n=7) and non-PPP patients (n=5) before and after tonsillectomy. Each value is expressed as a percentage of CD3/CLA double-positive cells in CD3-positive cells, analyzed by two-color flow cytometry. The median values are displayed as short bar (-). Wilcoxon signed rank test was used to determine p-value. The percentage of CD3/CLA double-positive cells in CD3-positive cells significantly decreased at 6 months after tonsillectomy in PPP patients ($p<0.05$). On the other hand, in non-PPP group, this population was not significantly different among the three time points. ns: not significant.

Figure 6. Representative immunohistologic profiles of CLA/CD3 double staining (A, B; x200) and E-selectin staining (C, D; x200) in the plantar skin tissues from pustulosis palmaris et plantaris (PPP) patients (A, C) and healthy volunteers (B, D).

Numerous CD3/CLA double-positive cells (red) were found around pustules in the epidermis and papillary dermis from PPP patients (A), but the cells rarely found in the plantar skin from healthy volunteers (B). Numerous E-selectin-positive microvessels (brown) were found in the papillary and upper reticular dermis from PPP patients (C), but the microvessels were hardly found in the plantar skin from healthy volunteers (D).

Figure 7. Results of immunohistological analyses for CLA expression (a) and E-selectin expression (b) in the plantar skin tissues from pustulosis palmaris et plantaris (PPP) patients (n=7) and healthy volunteers (n=5). Each value is expressed as an average number of CD3/CLA double-positive cells or E-selectin positive microvessels per square millimeter. The median values are displayed as short bar (-). Mann-Whitney U test was used to determine p-value. The numbers of CD3/CLA double-positive cells and E-selectin positive microvessels in PPP patients were significantly higher than in healthy volunteers ($p<0.01$, each).

Fig. 1

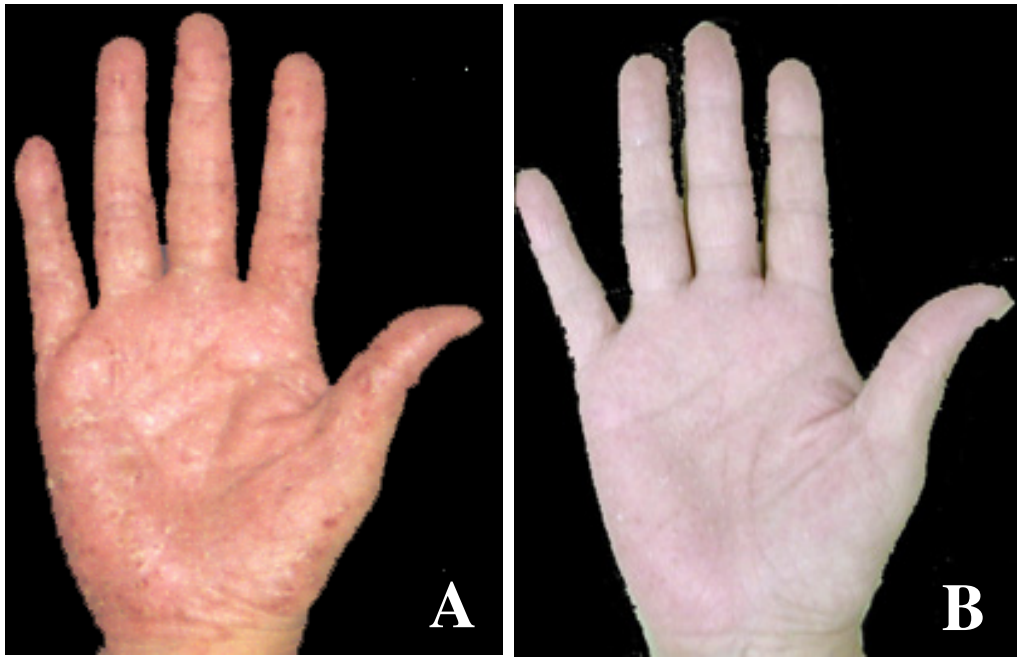

Fig. 2

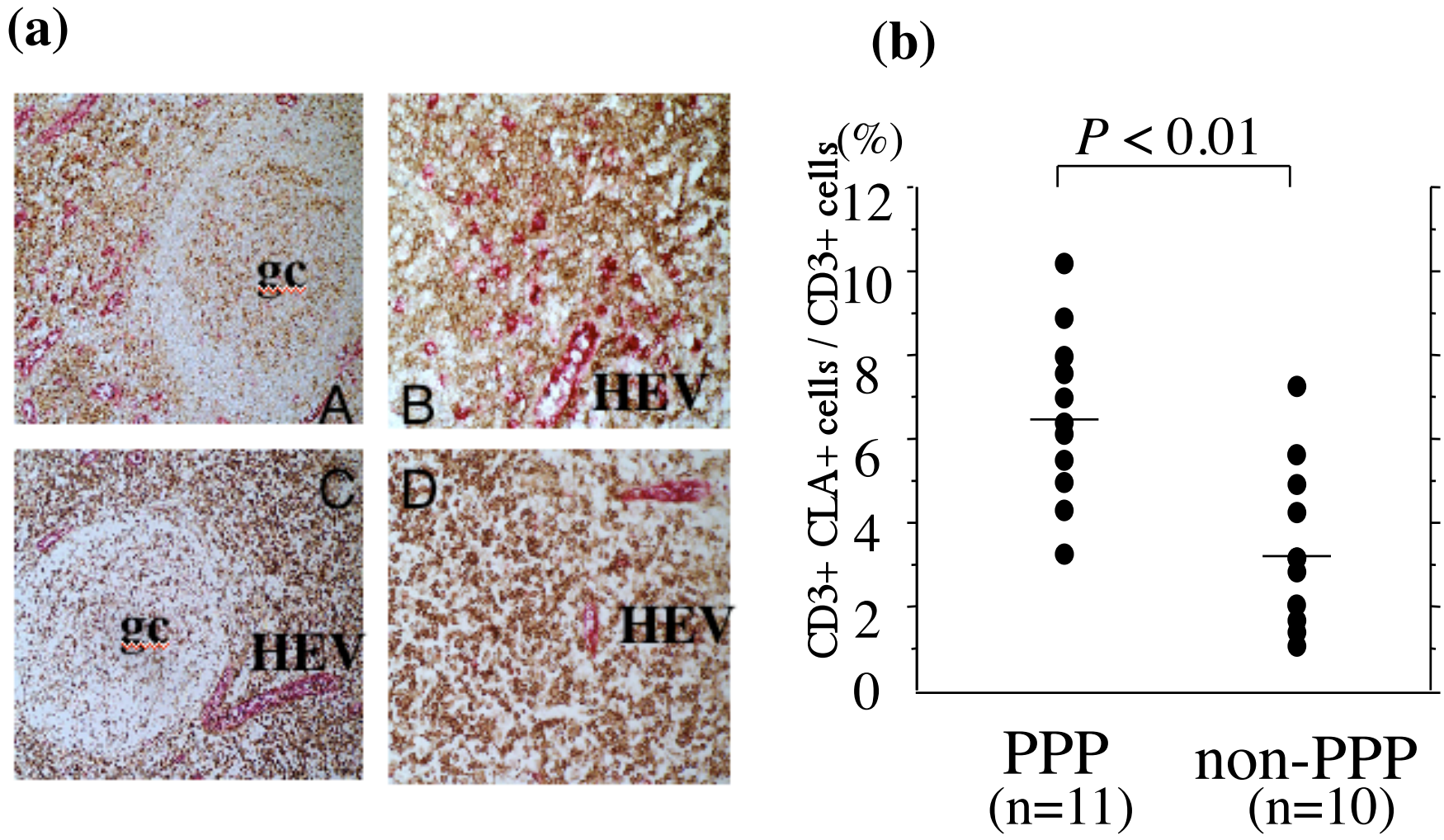
(a)
gc
A
B
HEV
C
gc
HEV
D
HEV
(b)
P < 0.01
CD3+ CLA+ cells / CD3+ cells (%)
0
2
4
6
8
10
12
PPP
(n=11)
non-PPP
(n=10)

Fig. 3

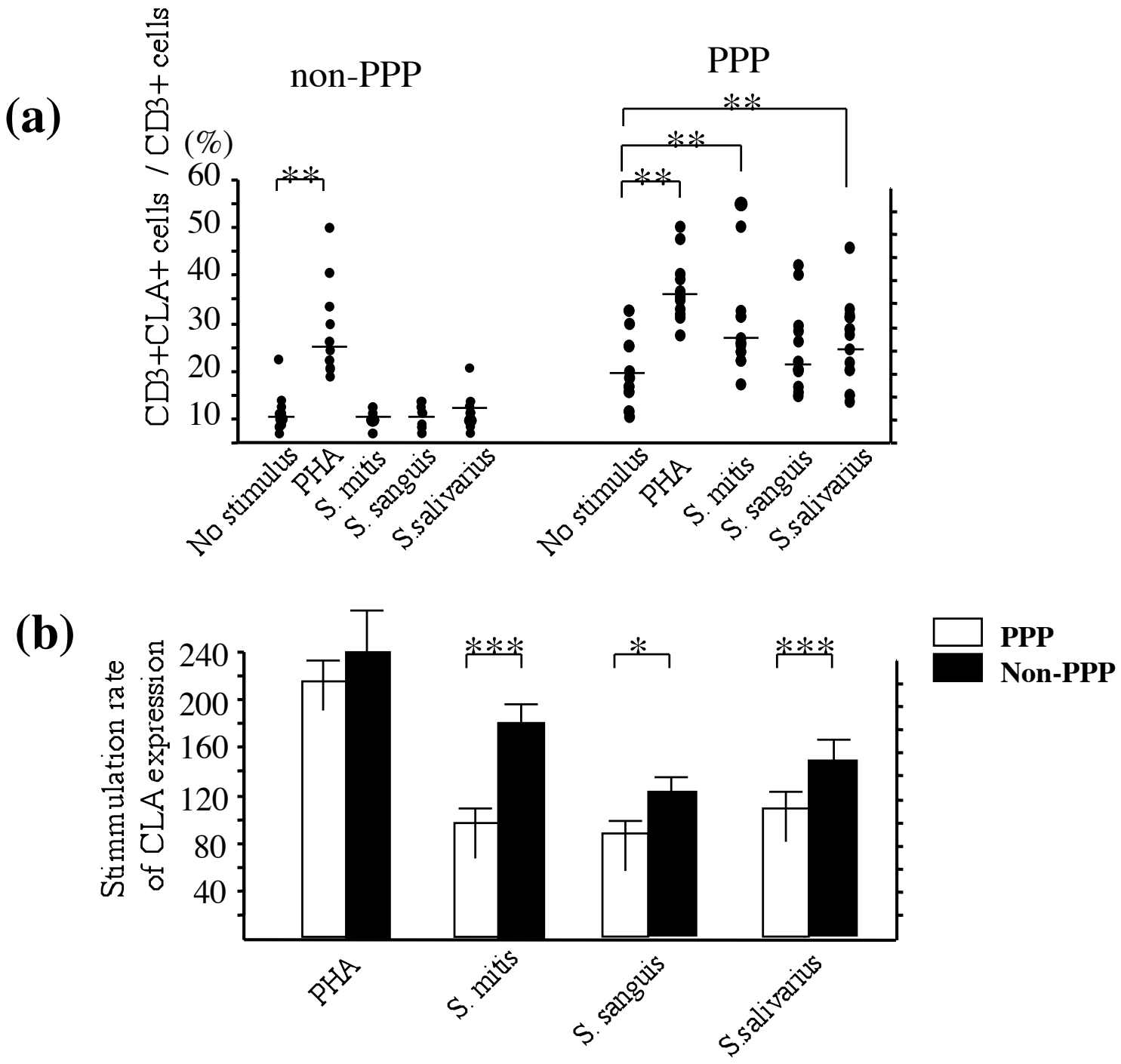
(a)
non-PPP
PPP
CD3+CLA+ cells / CD3+ cells
(%)
60
50
40
30
20
10
**
**
**
**
No stimulus
PHA
S. mitis
S. sanguis
S.salivarius
No stimulus
PHA
S. mitis
S. sanguis
S.salivarius
(b)
Stimmulation rate of CLA expression
240
200
160
120
80
40
***
*
***
PPP
Non-PPP
PHA
S. mitis
S. sanguis
S.salivarius

Fig. 4

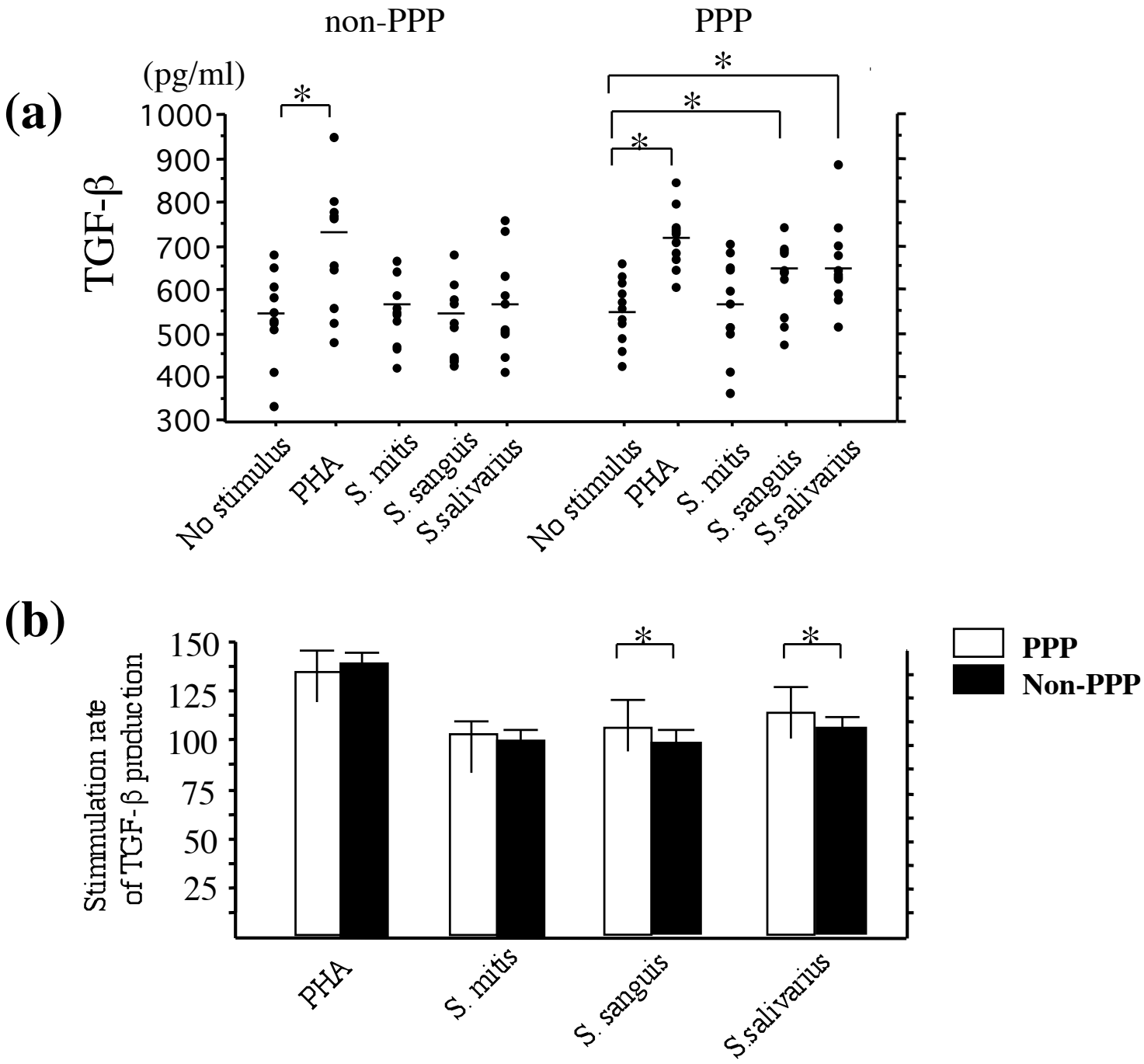
non-PPP
PPP
(a)
(pg/ml)
TGF-β
1000
900
800
700
600
500
400
300
*
*
*
*
No stimulus
PHA
S. mitis
S. sanguis
S.salivarius
No stimulus
PHA
S. mitis
S. sanguis
S.salivarius
(b)
Stimmulation rate of TGF-β production
150
125
100
75
50
25
*
*
PPP
Non-PPP
PHA
S. mitis
S. sanguis
S.salivarius

Fig.5

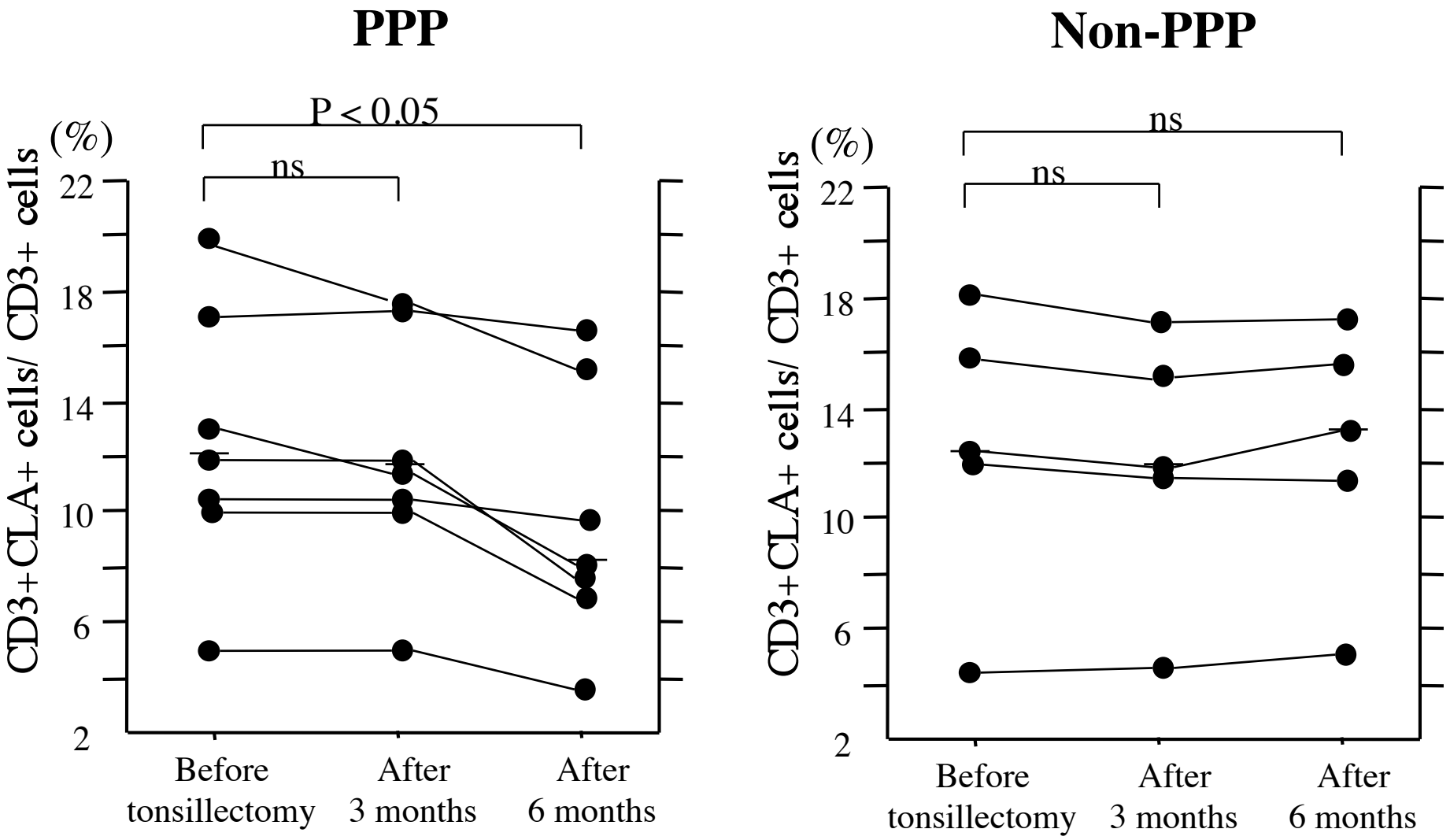
PPP
Non-PPP
P < 0.05
ns
ns
ns
(%)
(%)
CD3+CLA+ cells/ CD3+ cells
CD3+CLA+ cells/ CD3+ cells
22
18
14
10
6
2
Before tonsillectomy
After 3 months
After 6 months
Before tonsillectomy
After 3 months
After 6 months

Fig.6

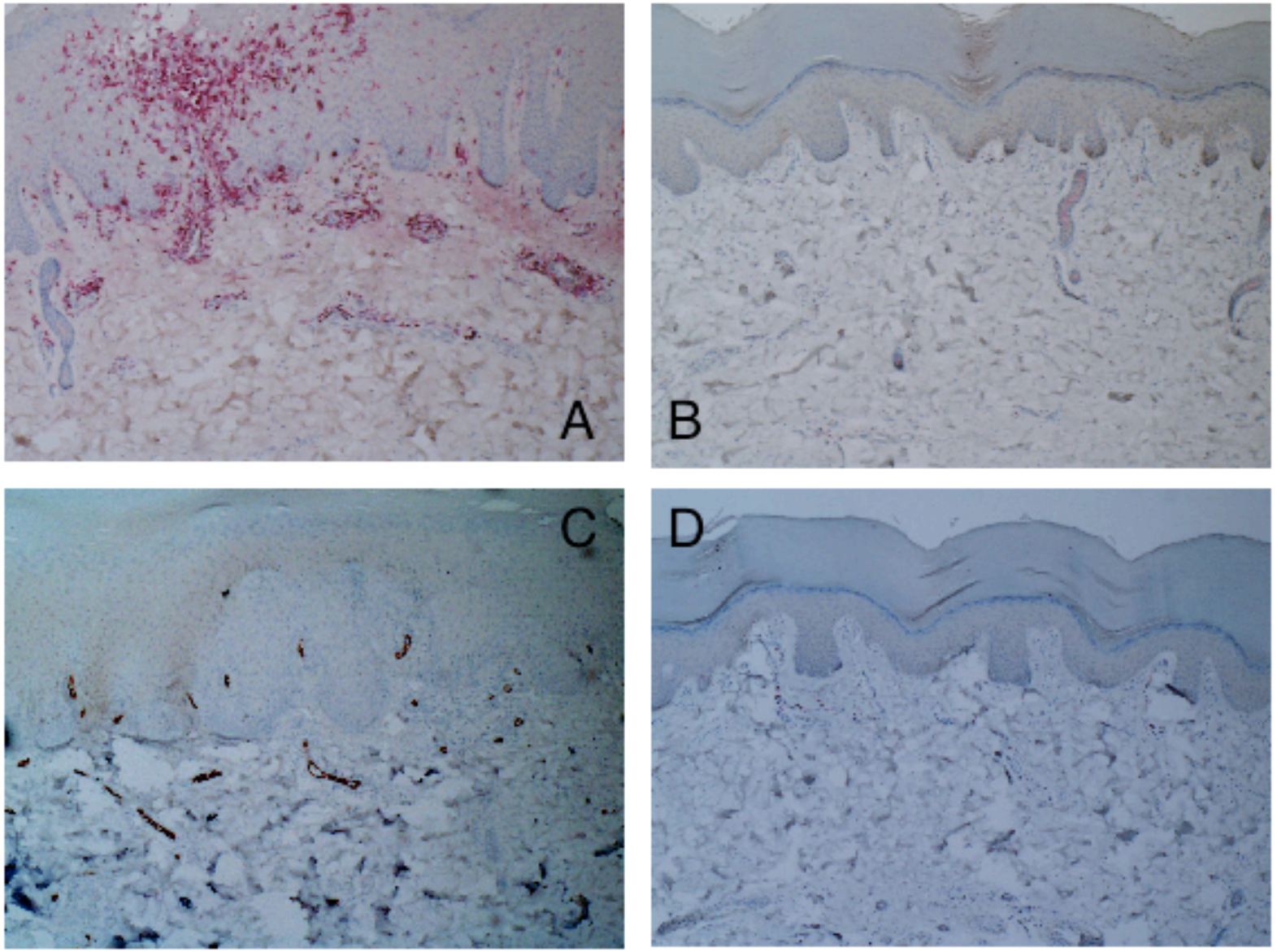

Fig. 7

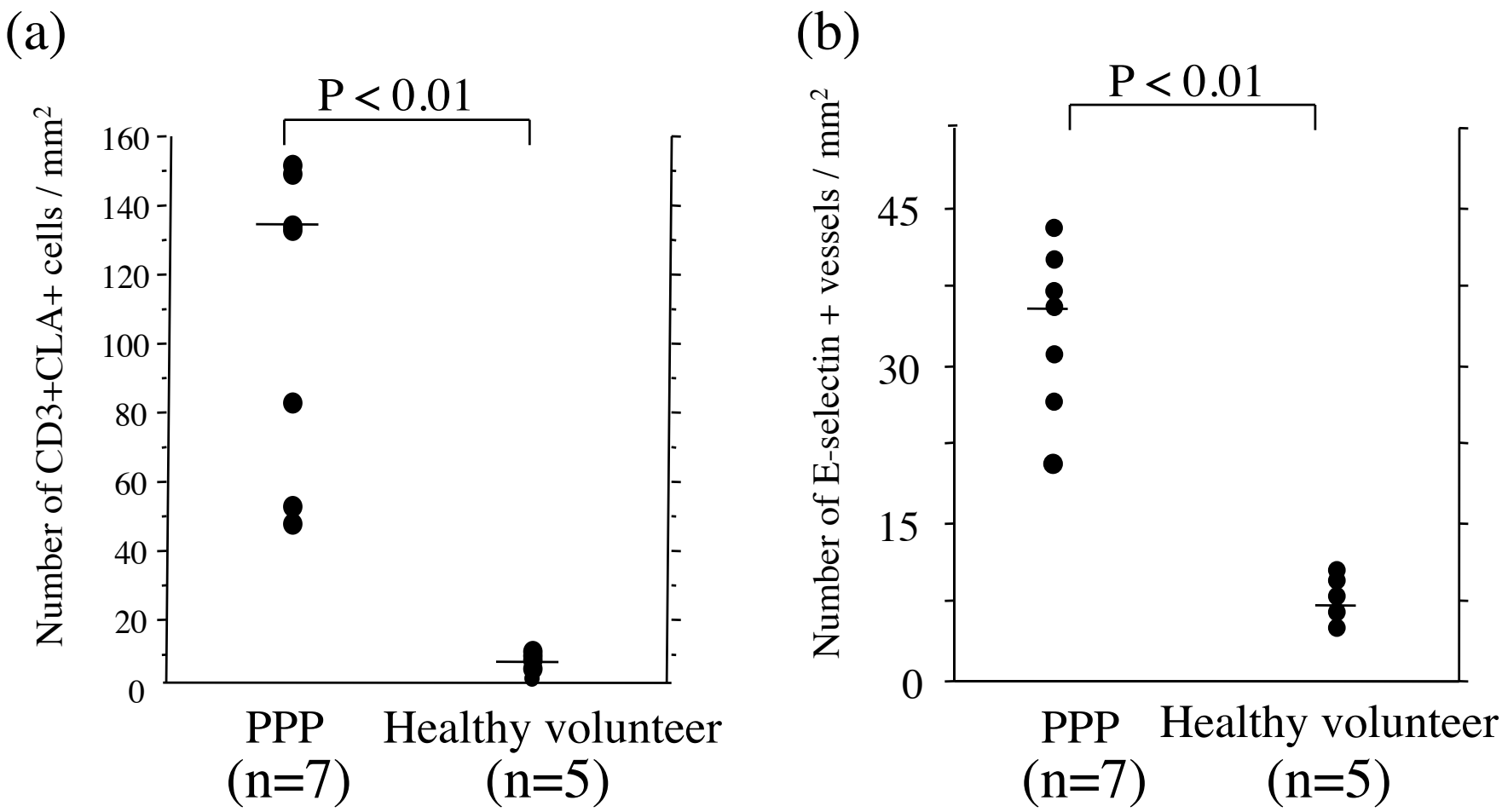
(a)
P < 0.01
Number of CD3+CLA+ cells / mm2
160
140
120
100
80
60
40
20
0
PPP
(n=7)
Healthy volunteer
(n=5)
(b)
P < 0.01
Number of E-selectin + vessels / mm2
45
30
15
0
PPP
(n=7)
Healthy volunteer
(n=5)

Table 1. Clinical findings of PPP patients in this study

Case No.	Age (y) on operation	Gender	Treatment before operation	History of smoking	Exacerbation of skin lesions during tonsillitis	Complication	Effect of tonsillectomy on skin lesion
1	22y	F	topical steroid	+	+	-	Disappeared
2	22y	F	none	-	-	-	Partially effective
3	26y	F	topical steroid	-	+	-	Markedly effective
4	29y	M	none	+	+	SCCH	Effective
5	30y	F	topical steroid	+	-	-	Partially effective
6	31y	F	topical steroid	+	-	-	Markedly effective
7	32y	M	none	+	+	-	Partially effective
8	34y	F	topical steroid	+	+	-	Markedly effective
9	35y	F	topical steroid	+	+	-	Disappeared
10	36y	F	topical steroid	-	-	-	Partially effective
11	36y	M	none	+	-	-	Effective

PPP: pustulosis palmaris et plantaris, F: female, M: male, SCCH: sternocostoclavicular hyperosteosis

Table 2. CLA expression on tonsillar mononuclear cells

Population	PPP (n=11)	non-PPP (n=10)	*p*-value
In whole TMC			
CD3+	25.0: 20.5-28.0	13.5: 11.3-19.3	< 0.01
CLA+	23.1: 18.6-26.4	12.4: 11.6-17.6	< 0.01
CD3+CLA+	10.2: 7.9-18.9	6.3: 4.2-6.6	< 0.01
CD3-CLA+	12.6: 8.6-14.0	8.0: 5.0-12.6	ns
In CD3+ cells			
CLA+	24.6: 19.7-30.0	13.1: 10.4-15.3	< 0.01
In CD3- cells			
CLA+	18.3: 16.7-18.7	13.1: 12.2-17.8	ns

Tonsillar mononuclear cells (TMC) were isolated from pustulosis palmaris et plantaris (PPP) patients (n=11) and non-PPP patients (n=10) and analyzed by two-color flow cytometry. Data are expressed as the median: 25th-75th percentiles of the percentage in whole TMC, CD3+ cells, or CD3- cells. Mann-Whitney U test was used to determine p-value. ns: not significant.

Table 3. IL-6 production by tonsillar mononuclear cells stimulated with or without α-streptcoccal antigens

Stimulus	PPP (n=11)	non-PPP (n=10)	*p*-value
Without stimulus	62.2: 40.5-86.7	51.5: 36.0-81.0	ns
PHA	110: 66.2-129	87.0: 65.0-110	ns
S.mitis	70.5: 51.2-112	51.5: 36.7-87.0	ns
S.sanguis	66.0: 46.1-97.5	62.5: 37.0-80.0	ns
S.salivalius	78.8: 44.9-88.7	51.5: 40.0-68.0	ns

Tonsillar mononuclear cells (TMC) from pustulosis palmaris et plantaris (PPP) patients (n=11) and non-PPP patients (n=10) were cultured for 3 days in the absence or the presence of phytohaemagglutinin (PHA) or α-streptococcal antigens. The IL-6 levels in the supernatant culture fluids were measured by enzyme-linked immunosorbent assay. The values were expressed as median: 25th-75th percentiles pg/ml. Mann-Whitney U test was used to determine p-value. ns: not significant.