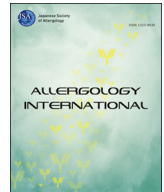




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Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Original Article

Role of whole saliva in the efficacy of sublingual immunotherapy in seasonal allergic rhinitis

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ARTICLE INFO

Article history:

Received 5 April 2018

Received in revised form

8 July 2018

Accepted 24 July 2018

Available online xxx

Keywords:

Allergic rhinitis

IL-10

Prediction

Sublingual immunotherapy

Whole saliva

Abbreviations:

AR, allergic rhinitis; DC, dendritic cell;
PMB, polymyxin B; SLIT, sublingual
immunotherapy; SMS, symptom-
medication scores; VAS, visual analog scale

ABSTRACT

Background: The development of methods to predict the clinical effectiveness of sublingual immunotherapy (SLIT) for allergic diseases is a crucial matter. We sought to determine whether whole saliva, which is the first body component that contacts allergen extracts during SLIT, is associated with the clinical effectiveness of SLIT in Japanese cedar pollinosis.

Methods: Blood monocytes or monocytic THP-1 cells were cultured in the presence or absence of either whole saliva or pure saliva with or without treatments including filtration and blockade of TLR2 and/or TLR4 signaling. IL-10 levels in the supernatants were then measured. Whole saliva-induced IL-10 production by THP-1 cells was compared between asymptomatic and disease-onset patients during peak pollen dispersal after SLIT.

Results: Both monocytes and THP-1 cells produced substantial amounts of IL-10 in response to whole saliva. IL-10 production was significantly reduced in response to pure saliva and 0.2 µm-filtered saliva. Simultaneous treatment with polymyxin B and TL2.1, a neutralizing antibody against TLR2, also reduced IL-10 production. IL-10 levels produced by THP-1 cells in response to whole saliva collected prior to SLIT were significantly higher in asymptomatic patients determined by symptom-medication scores than disease-onset patients following SLIT. Such differences were not seen in saliva collected 3 months after the initiation of SLIT or saliva collected during peak pollen dispersal.

Conclusions: Our results provide a basis for why the sublingual route is effective and preferable in allergen immunotherapy. Saliva-induced IL-10 levels produced by THP-1 cells may be a predictive marker for clinical remission after SLIT.

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Introduction

Sublingual immunotherapy (SLIT) is not only effective, but also induces long-term clinical remission in allergic rhinitis (AR).^{1,2} For example, a recent report showed that effects of timothy grass and ragweed SLIT tablets on nasal symptoms were nearly as great as that of mometasone furoate nasal spray and numerically greater than

those of montelukast and desloratadine.³ Unlike pharmacotherapies, SLIT can modify the natural history of AR such as prevention of new sensitization to other allergens and new onset of asthma.⁴ However, SLIT is a slow-acting therapy compared with the above pharmacotherapies and requires several years to achieve clinical remission and disease-modifying effects, leading to a low persistence in real-life settings.⁵ In addition, it is known that the efficacy of SLIT varies among patients.^{6–9} For example, a report showed that SLIT was considered clinically ineffective in 16.7% of patients, showing less than 30% reduction of visual analog scale (VAS) scores compared with baseline scores before starting SLIT.⁸ Thus, it is desired to develop methods to predict the clinical effectiveness of SLIT, especially prior to starting such treatment in patients.

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Peer review under responsibility of Japanese Society of Allergy.

<https://doi.org/10.1016/j.alit.2018.07.008>

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The main mechanism of allergen immunotherapy for allergic diseases is induction of immune tolerance to allergens.^{10–12} This includes the activation of regulatory T cells, which directly or indirectly suppress the activity of effector cells such as Th2 cells, Th17 cells, mast cells, eosinophils, basophils, and neutrophils.^{11–13} Regulatory B cells are also induced, leading to the production of IL-10, TGF- β , and granzyme B and antibodies such as IgG4, sialylated IgG, and IgA.^{12,14,15} In addition, immunotherapy regulates innate immune cells such as dendritic cells (DCs) and innate lymphoid cells with decreased type 2 immune reactions.^{9,12}

Based on these mechanisms, attempts have been made to establish biomarkers to predict the clinical effectiveness and/or immunological tolerance of SLIT.^{6–9,16} For example, a recent study showed that downregulation of DC2-associated markers concomitantly with upregulation of DCreg cell-associated markers in monocyte-derived DCs correlated with clinical benefits as early as 2 months after the start of SLIT.⁹ Conversely, changes in basophil activation or allergen-specific CD4⁺ T cell responses did not predict clinical efficacy of SLIT.^{7,16} Ideally, prediction of efficacy before the start of SLIT is desirable. Responders to SLIT showed high serum-specific IgE titers before SLIT.⁸ The serum-specific IgE/total IgE ratio before SLIT is a candidate marker for the prediction of treatment efficacy, however, contradictory results were seen.^{6,17} Patients with effective SLIT responses tended to have higher serum eosinophil cationic protein levels before SLIT than those with ineffective responses.¹⁸ More recently, a report showed that low body mass index is associated with the efficacy of SLIT for Japanese cedar pollinosis (JCP).¹⁹

The oral mucosa, the allergen application site of SLIT, has “immune privilege” where acute inflammatory and allergic reactions are rarely seen despite high bacterial colonization and frequent allergen contact.²⁰ Therefore, SLIT has site-specific advantages to induce immune tolerance via several mechanisms such as the absence of mucosa-associated lymphoid tissue, small numbers of effector cells including mast cells and eosinophils, stratified squamous epithelium which blocks allergen penetration into the sub-mucosal layer, and the presence of DCregs in the oral cavity.^{21–23} Oral Langerhans cells highly express Fc ϵ RI and TLR4 and produce IL-10 via signaling through these receptors.²² In mouse, oral CD103⁺CD11b⁺ classical DCs effectively induce Foxp3⁺ regulatory T cells as compared with other DCs in the spleen and small intestine.²³ Furthermore, the oral mucosa is covered with saliva (oral fluid). Although more than 99% of saliva is water, saliva contains ions, mucins, enzymes, antimicrobial proteins, immunoglobulins, and immune cells such as neutrophils, and some of the components such as sIgA have immunoregulatory functions.^{24,25} In addition, saliva contains microbes including viruses, fungi, and bacteria.²⁶ Among these, oral commensal bacteria play a role in the modulation of host responses.²⁷ For example, *Porphyromonas gingivalis* promoted IL-10 production in a TLR2-dependent manner in murine splenocytes.²⁸

In the present study, we determined the regulatory effect of human saliva on monocytes and monocytic THP-1 cells and investigated whether the clinical effectiveness of SLIT can be predicted using patients' saliva.

Methods

Subjects

Twelve Japanese volunteers (age range, 29–51 years; mean age, 39.0 years) and 105 patients with Japanese cedar pollinosis (JCP) (age range, 12–70 years; mean age, 33.8 years) were enrolled in the study. Written informed consent was obtained from each subject or their guardians. Patients had a history of

naso-ocular symptoms during the pollen season (February to March) over 2 seasons and showed sensitization to Japanese cedar pollen confirmed by the presence of serum-specific IgE Ab of >0.7 UA/ml as determined by ImmunoCAP (Phadia AB, Uppsala, Sweden). All patients began SLIT with Japanese cedar pollen extracts (Cedartolen™, Torii Pharmaceutical, Tokyo, Japan) in 2015. The clinical efficacy during the peak pollen season in 2016 was evaluated by a questionnaire to evaluate symptom scores and combined symptom-medication scores (SMS) and visual analog scale (VAS) scores reflecting naso-ocular symptoms as previously described.²⁹ The study was approved by the Human Research Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (approval number: 1507-039).

Antigen and reagents

We purchased the following materials for the study: polymyxin B sulfate salt (PMB, Sigma–Aldrich, St. Louis, MO), Pam3CSK4, Poly(I:C), LPS, CpG and mouse anti-human TLR2 mAb (TL2.1, Novus Biologicals, Littleton, CO), mouse IgG2a (R&D Systems, Minneapolis, MN); FITC-conjugated anti-human CD80 mAb (BD Biosciences, San Jose, CA), FITC-conjugated anti-human CD86 mAb (Agilent Technologies, Santa Clara, CA), PE-conjugated anti-human HLA-DR mAb (BD Biosciences), and Ficol–Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). FITC-conjugated anti-human B7-H1 mAb was gifted by Dr. Miyuki Azuma.³⁰ The culture medium (CM) used throughout the study was RPMI-1640 medium supplemented with 10% fetal calf serum and L-glutamine-penicillin-streptomycin solution (Sigma–Aldrich).

Collection of saliva

Oral fluids (whole saliva) were collected by the passive drool method using Saliva Collection Aid (Salimetrics, Carlsbad, CA) according to the manufacturer's instruction. In volunteers, pure parotid saliva was also collected by cannulation of Stensen's duct under stimulation with lemon juice (Pokka Sapporo Food & Beverage, Nagoya, Japan). Some oral fluids were passed through 30 or 0.2 μ m sterilized filters (Steradisc™, Kurabo Industries, Osaka, Japan) in order to eliminate particles including microbes. In addition, some fluids were passed through the Nab™ Protein L Spin Kit (Thermo Fisher Scientific, Waltham, MA) in order to eliminate immunoglobulins that have kappa light chains.³¹ In 105 patients with JCP, oral fluids were sampled three times: prior to the start of SLIT (Pre-SLIT saliva, n = 105), 3 months after initiation of SLIT (3M-SLIT saliva, n = 105), and peak pollen season (March 2016) (In-season saliva, n = 80). The fluids from all patients were stored at –20 °C until use.

Response to autologous saliva by peripheral blood monocytes

Monocytes were isolated from volunteers' peripheral blood mononuclear cells as described previously.³² Monocytes (0.5 $\times$ 10⁶/ml) in CM were incubated with various concentrations (0, 0.01, 0.1, 1, and 5%) of whole saliva at 37 °C in a 5% CO₂/air mixture for 24 h. In separate experiments, monocytes were cultured with 1% of filtered saliva or Protein L column-passed saliva. Alternatively, monocytes were cultured with 1% of saliva in the presence or absence of 100 μ g/ml of PMB and/or either 10 μ g/ml of anti-TLR2 Ab or control mouse IgG2a. Then, the supernatant was collected and stored at –80 °C until it was assayed for cytokine determination. The cell pellets were subsequently used for flow cytometry.

Response to patients' saliva by THP-1 cells

Human monocytic cell line THP-1 cells were gifted by Dr. Hideo Takahashi.³³ THP-1 cells ($0.8 \times 10^6/\text{ml}$) in CM were incubated with 1% of saliva from each patient at 37 °C in a 5% CO₂/air mixture for 24 h. Then, the supernatant was collected and stored at –80 °C until it was assayed for cytokine determination.

Cytokine determination

IL-10 levels in culture supernatants were measured by using Opt EIA sets (BD Biosciences) in accordance with the manufacturer's instructions. IL-12 and IFN- γ levels were measured by using a DuoSet ELISA development kit (R&D Systems). The detection limit of these assays was 4 pg/ml for IL-10, 2 pg/ml for IL-12 and 8 pg/ml for IFN- γ .

Flow cytometry

Cultured monocytes were collected and then stained with fluorescence-conjugated anti-human mAbs against CD80, CD86, HLA-DR, B7-H1, and controls as previously described.³¹ Mean fluorescence intensity was determined by flow cytometry. Alternatively, intracellular IL-10 expression was determined using the IL-10 secretion assay kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Statistical analysis

Values are given as medians. The nonparametric Mann–Whitney *U* test was used to compare data between groups, and the Wilcoxon signed-rank test was used for analysis within groups. The Friedman test followed by the Dunn test was used for multiple comparisons. Correlation analyses were performed using the Spearman rank correlations. Receiver operating characteristics

(ROC) analyses were performed to determine the cut-off values for IL-10 to predict clinical remission after SLIT. *P*-values of less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA).

Results

Activation of human monocytes by autologous whole saliva

Monocytes were stimulated with various concentrations of autologous whole saliva (oral fluids) for 24 h, then IL-10 and IL-12 levels in the culture supernatants were determined. A significant difference was observed in IL-10 ($P = 0.001$), IL-12 ($P = 0.009$) and IFN- γ ($P < 0.001$) production (Friedman test, $n = 12$). Furthermore, 0.1% ($P = 0.005$) and 1.0% ($P = 0.005$) autologous saliva significantly increased IL-10 production (Dunn test, Fig. 1A–C). Flowcytometric analysis revealed that proportion of IL-10-positive cells was increased in CD14⁺ cell in response to 1.0% saliva (data not shown). In addition, 1.0% saliva significantly upregulated the expression of CD80 ($P = 0.031$, Fig. 2A–C) and HLA-DR ($P = 0.031$, Fig. 2G–I) but not CD86 ($P > 0.999$, Fig. 2D–F) or B7-H1 ($P = 0.563$, Fig. 2J–L) ($n = 6$). According to these findings, we sought to characterize saliva-induced IL-10 production by monocytes.

Characterization of IL-10-inducing component in whole saliva

Because saliva contains abundant immunoglobulins such as IgA, and these immunoglobulins have a regulatory role in immune responses, we first sought to determine the role of salivary immunoglobulins in IL-10 production by monocytes. One percent Protein L column-passed saliva induced IL-10 production by autologous monocytes comparable to control saliva ($P = 0.240$, $n = 11$, Fig. 3A). Since whole saliva contains particles in the oral cavity such as host immune cells and microbes, we next sought to determine the role of salivary particles in IL-10 production. A significant decrease in IL-

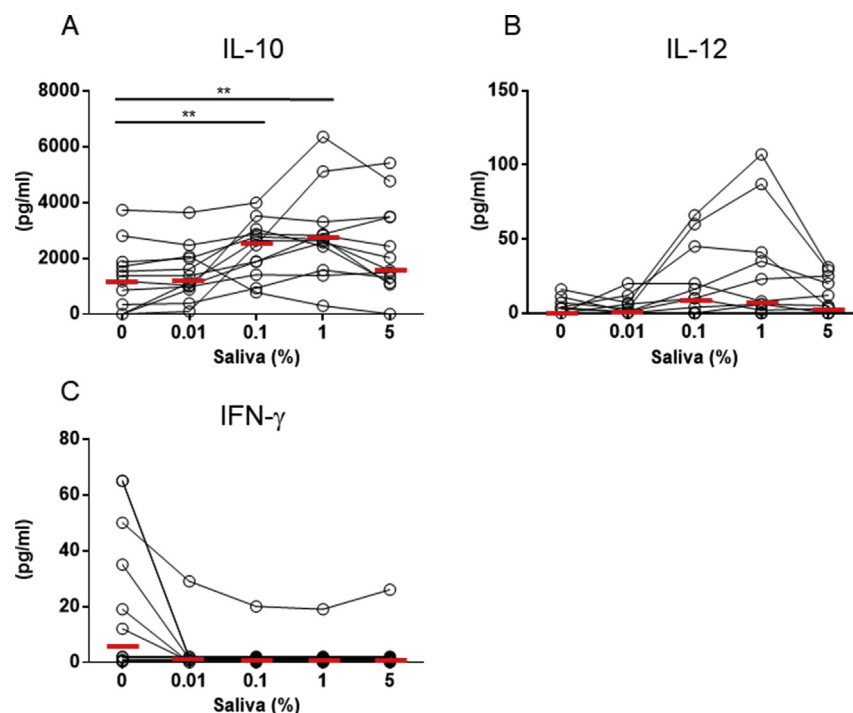


Fig. 1. Whole saliva-induced IL-10 (A), IL-12 (B) and IFN- γ (C) production by autologous monocytes. Bar represents the median. *P*-values were determined using the Dunn test (** $P < 0.01$).

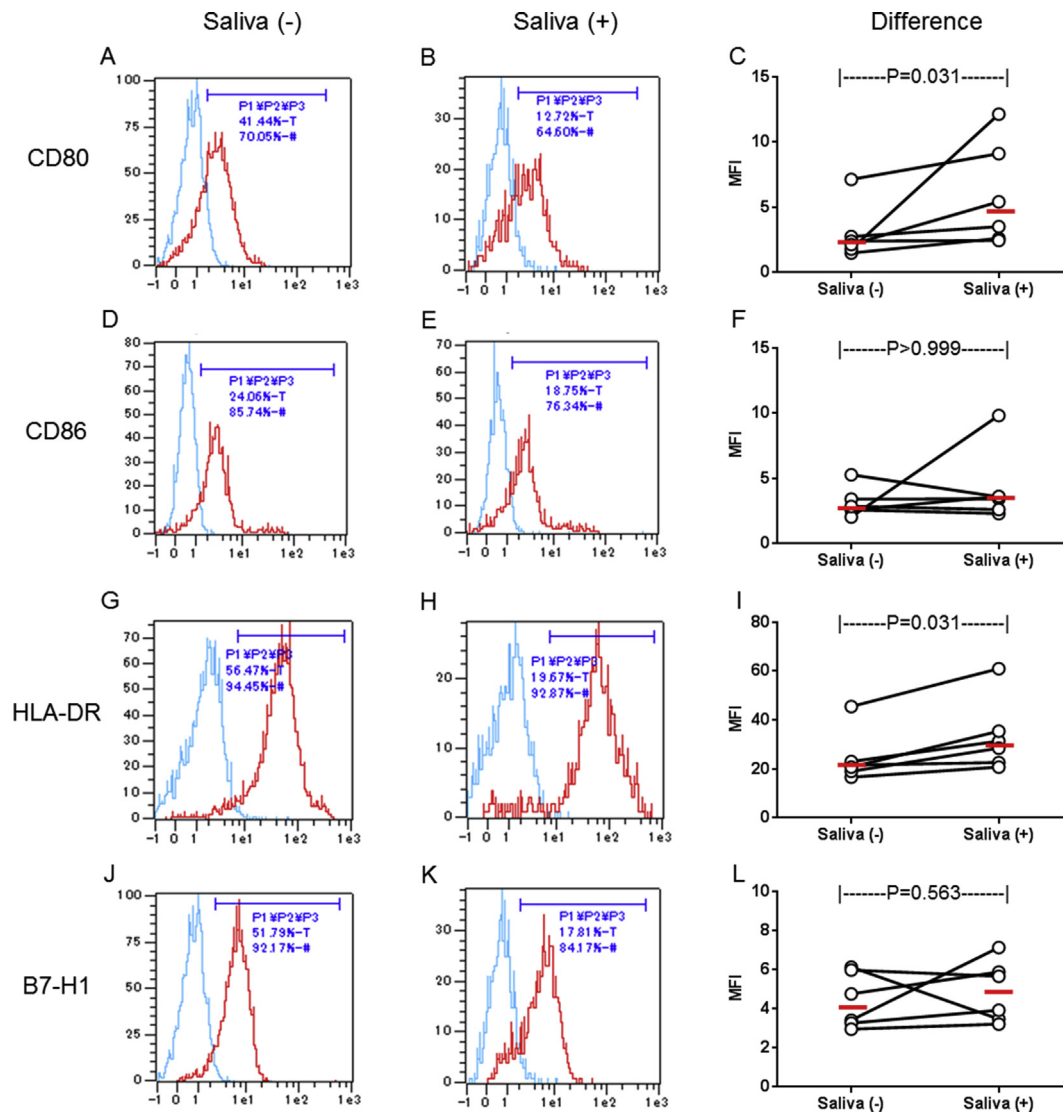


Fig. 2. Whole saliva-induced expression of surface molecules CD80 (A–C), CD86 (D–F), HLA-DR (G–I), and B7-H1 (J–L) in autologous monocytes. Monocytes were cultured in the presence (B, E, H, K) or absence (A, D, G, J) of 1% autologous saliva for 24 h. Differences in mean fluorescent intensity between active (red histogram) and control (cyan histogram) antibodies against CD80 (C), CD86 (F), HLA-DR (I), and B7-H1 (L) were compared between saliva-stimulated and unstimulated monocytes. Bar represents the median. *P*-values were determined using the Wilcoxon signed-rank test.

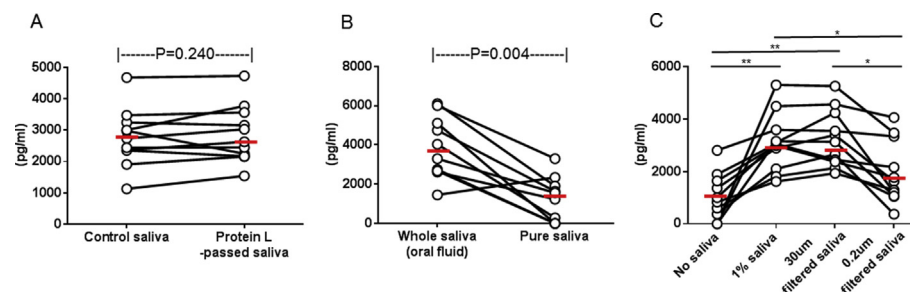


Fig. 3. Characterization of the IL-10-inducing component in whole saliva. (A) Comparison of IL-10-inducing effect between Protein L column treatment and control treatment. (B) Comparison of IL-10-inducing effect between whole saliva and pure saliva. (C) Effect of saliva filtration on IL-10-inducing effect. Bar represents the median. *P*-values were determined using the Wilcoxon signed-rank test (A, B) or the Dunn test (C; **P* < 0.05, ***P* < 0.01).

10 production was seen in response to 1% pure saliva directly collected from Stensen's duct as compared with whole saliva (*P* = 0.004, *n* = 10, Fig. 3B). In addition, filter treatment of saliva significantly lead to the alteration in IL-10 production (*P* < 0.001,

Friedman test, *n* = 11). Furthermore, 0.2-µm-filtered saliva showed a significant reduction in IL-10 production as compared with native (*P* = 0.039, Dunn test) and 30-µm-filtered (*P* = 0.023, Dunn test) saliva. In addition, IL-10 levels in response to 0.2-µm-filtered saliva

were comparable to that of negative control (no saliva stimulation) ($P = 0.592$, Fig. 3C).

Role of TLR2 and TLR4 signaling in whole saliva-induced IL-10 production by monocytes

Because both Gram-positive and Gram-negative bacteria exist in saliva, we sought to determine the role of TLR2 and/or TLR4 signaling in saliva-induced IL-10 production.²⁵ Monocytes were cultured in the presence or absence of Pam3CSK4 (TLR1/TLR2 agonist), Poly(I:C) (TLR3 agonist), LPS (TLR4 agonist), or CpG (TLR9 agonist). A significant difference in IL-10 production was seen among the stimulations ($P < 0.001$, Friedman test). Furthermore, stimulation with Pam3CSK4 ($P = 0.009$) and LPS ($P < 0.001$) but not Poly(I:C) ($P > 0.999$) or CpG ($P > 0.999$) significantly induced IL-10 production as compared with no stimulation (Dunn test, $n = 10$, Fig. 4A). Treatment with either TL2.1 ($P = 0.770$, Fig. 4B), a neutralizing Ab against TLR2, or PMB ($P = 0.275$, Fig. 4C), an antagonist of LPS, did not solely inhibit saliva-induced IL-10 production. Conversely, simultaneous treatment with PMB and TL2.1 marginally, albeit significantly, suppressed IL-10 production ($P = 0.049$, Fig. 4D).

Whole saliva-induced IL-10 production by THP-1 cells

With respect to the clinical application of saliva-induced IL-10 production by monocytes to predict the efficacy of SLIT, use of autologous monocytes has disadvantages in terms of invasiveness, pain, and cell viability. Therefore, we investigated whether saliva induced IL-10 production by THP-1 cells, an immortal human

monocytic cell line. Similar to autologous monocytes, $0.8 \times 10^6/\text{ml}$ of THP-1 cells produced a substantial amount of IL-10 in response to 1% whole saliva ($P = 0.004$, Wilcoxon signed-rank test, $n = 11$, Fig. 5A). Although no significant correlation in saliva-induced IL-10 production was seen between autologous monocytes and THP-1 cells ($r = -0.228$, $P = 0.489$, Fig. 5B), we used THP-1 cells instead of autologous monocytes for the following patient analysis.

Association between whole saliva-induced IL-10 production by THP-1 cells and symptom severity in SLIT-treated JCP patients

One percent of Pre-SLIT ($n = 105$), 3M-SLIT ($n = 105$), and In-season ($n = 80$) saliva were cultured with $0.8 \times 10^6/\text{ml}$ of THP-1 cells for 24 h, then IL-10 levels in the culture supernatants were determined. Significant, positive correlations in IL-10 production were seen among the three timepoints: Pre-SLIT saliva and 3M-SLIT saliva: $r = 0.579$, $P < 0.001$ by Spearman rank correlations ($n = 105$); Pre-SLIT saliva and In-season saliva: $r = 0.415$, $P < 0.001$ ($n = 80$); and 3M-SLIT saliva and In-season saliva: $r = 0.548$, $P < 0.001$ ($n = 80$). However, salivary IL-10 was only detected in 32 (30.5%), 29 (27.6%), and 32 (40.0%) of 105 Pre-SLIT, 105 3M-SLIT, and 80 In-season saliva samples, respectively. The median concentration of salivary IL-10 was under the detection limit (4 pg/ml) at all timepoints.

SMS and VAS scores during peak pollen season (early March) were monitored in all patients. Overall, no significant correlations between the levels of saliva-induced IL-10 production (Pre-SLIT, 3M-SLIT, or In-season) and SMS or VAS scores were observed ($P > 0.05$, Spearman rank correlations). However, levels of Pre-SLIT saliva-induced IL-10 production were significantly higher in asymptomatic

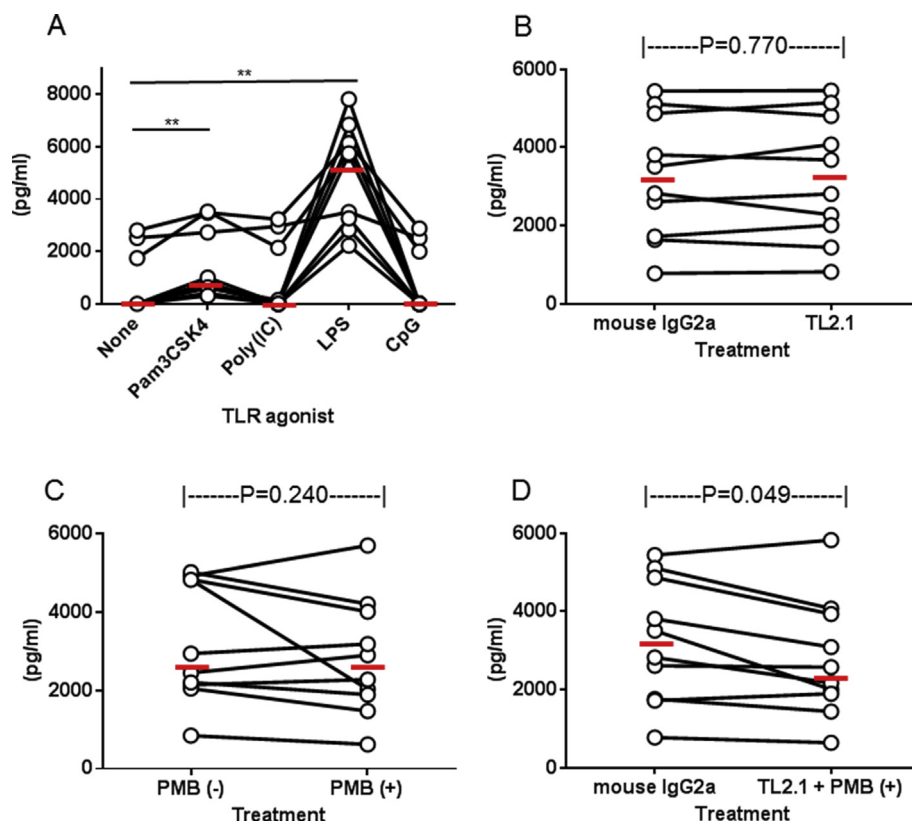


Fig. 4. Role of TLR2 and TLR4 signaling in whole saliva-induced IL-10 production by monocytes. (A) Effect of Pam3CSK4, Poly(I:C), LPS, and CpG on IL-10 production. (B–D) Effect of TL2.1 alone (B), PMB alone (C), and TL2.1 plus PMB (D) on IL-10 production. Bar represents the median. P -values were determined using the Dunn test (A; $**P < 0.01$) or the Wilcoxon signed-rank test (B–D).

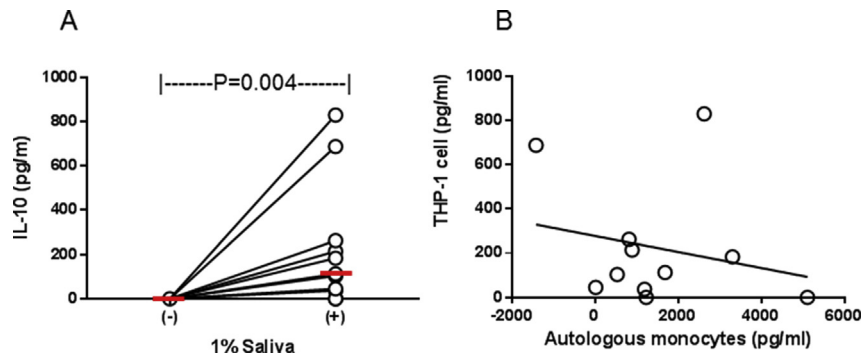


Fig. 5. Whole saliva-induced IL-10 production by THP-1 cells. **(A)** IL-10 production by THP-1 cells in the presence or absence of 1% whole saliva. **(B)** Correlation of saliva-stimulated IL-10 production between autologous monocytes and THP-1 cells. Bar represents the median. *P*-values were determined using the Wilcoxon signed-rank test.

patients ($n = 4$) showing zero SMS as compared with disease-onset patients ($n = 101$) showing positive SMS ($P = 0.018$, Fig. 6A). Similarly, IL-10 levels in response to Pre-SLIT saliva was higher in asymptomatic patients ($n = 8$) showing zero VAS scores as compared with disease-onset patients ($n = 97$) showing positive VAS scores ($P = 0.036$, Mann–Whitney *U* test, Fig. 6B). Such a

significant difference was lost if we set other scores such as one point or two point of SMS for cut-off level of symptom severity, suggesting that saliva-induced IL-10 production by THP-1 cells associated with onset of symptoms, in other words clinical remission, after SLIT. ROC analysis revealed that the IL-10 levels appeared to be a predictive marker for clinical remission (area under the curve

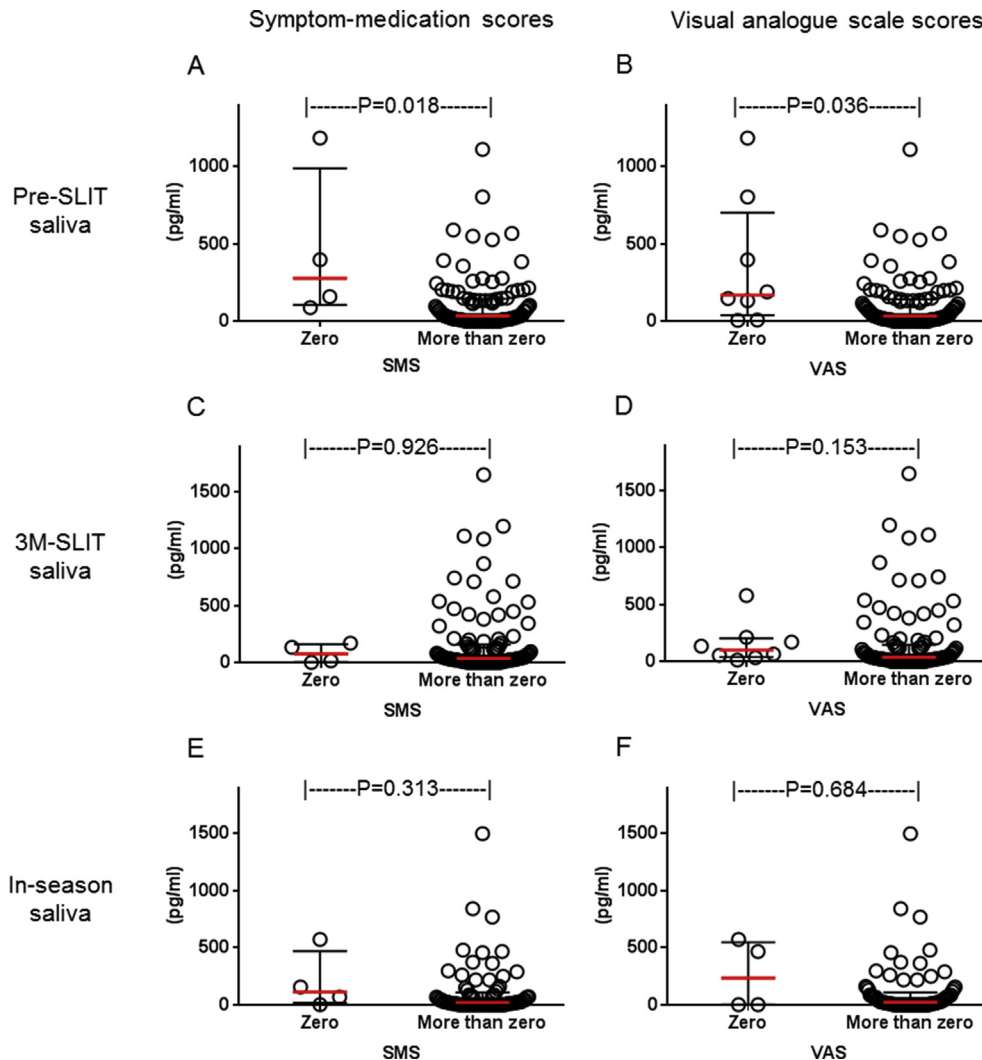


Fig. 6. Comparison of IL-10 production by THP-1 cells in response to Pre-SLIT saliva **(A, B)**, 3M-SLIT saliva **(C, D)**, and In-season saliva **(E, F)** between asymptomatic patients and disease-onset patients determined by SMS **(A, C, E)** and VAS scores **(B, D, F)**. Bar represents the median. *P*-values were determined using the Mann–Whitney *U* test.

(AUC): 0.720; 95% confidence interval (CI): 0.522–0.919, $P = 0.039$). This analysis showed that the best cut-off for IL-10 was 129 pg/ml, and the corresponding sensitivity and specificity for predicting clinical remission was 71.3% (95% CI: 61.1–79.9) and 75.0% (95% CI: 34.9–96.8), respectively. Conversely, such differences were not found in either 3M-SLIT saliva or In-season saliva (Fig. 6C–F).

Discussion

The present study shows that whole saliva has an IL-10-inducing effect on monocytes and monocytic THP-1 cells. In particular, we found that both pure saliva and 0.2- μ m-filtered saliva reduced this regulatory effect, and salivary components that simultaneously stimulate TLR2 and TLR4 play a role in this effect. Furthermore, Pre-SLIT saliva, but not those after initiation of SLIT from asymptomatic (no onset) patients following SLIT, induced higher IL-10 production by THP-1 cells than those from symptomatic patients.

Whole saliva concentrated from 0.1 to 1.0% in CM induced IL-10 production by autologous monocytes. Although the IL-10 level in saliva is associated with the pathophysiology of several diseases including Sjogren's syndrome and oral squamous cell carcinoma, to the best of our knowledge, this is the first report showing that human saliva induces monocytes to produce IL-10.^{34,35} On the other hand, our preliminary result found that the addition of Cry j 1, the major allergen of Japanese cedar pollen, did not alter saliva-induced IL-10 production by monocytes (data not shown). Our study showed that the majority of patients' saliva contained IL-10 levels below the detection limit; further, the median concentration of IL-10 was also below the detection limit. These findings suggest that IL-10 levels in culture supernatants may not reflect the level of salivary IL-10 added to the CM. These results suggest a regulatory activity of saliva on human monocytes.

Saliva contains immunoregulatory components including IgA, antimicrobial proteins, and microbes. First, we sought to determine whether salivary IgA exhibits an IL-10-inducing effect on human monocytes because IgA is a major immunoglobulin in saliva and it has immunoregulatory functions including the induction of IL-10.^{25,36,37} In addition, increased IgA production is seen following allergen-specific immunotherapy including SLIT.^{14,38} However, the present results showed that Protein L column-passed saliva still promoted IL-10 production by monocytes. In addition, pure saliva directly collected from Stensen's duct significantly reduced the ability to induce IL-10 production. These findings suggest that salivary IgA has a negligible effect on IL-10 production by monocytes.

Blockade of both TLR2 and TLR4 signaling significantly reduced whole saliva-induced IL-10 production by monocytes. Together with the finding that 0.2- μ m-filtered saliva showed a significant reduction in IL-10 production compared with native and 30- μ m-filtered saliva, these results suggest that microbes in whole saliva play an essential role in IL-10 production. The bacterial communities found in the oral cavity are highly complex with around 1000 species present and has been shown to be the second most complex in the body after the colon.²⁶ The oral bacterial community is dominated by the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria*, which account for 96% of species detected, suggesting that both Gram-positive and gram-negative bacteria profoundly exist in the oral cavity. TLR2 senses lipoproteins, peptidoglycans, and lipoteichoic acids from gram-positive bacteria whereas TLR4 senses LPS from gram-negative bacteria.³⁹ The present results suggest that symbiosis of gram-positive and gram-negative bacteria in saliva play, at least in part, a role in saliva-induced IL-10 production. However, the reduction of saliva-induced IL-10 production by TLR2 and TLR4 blockade was marginally significant ($P = 0.049$), suggesting that molecules other than TLR2 and TLR4 ligands in oral microbes may

participate in the induction of IL-10 production. Our preliminary result using microbiome analysis showed that storage at -20°C had little effect on bacterial community in phyla level.

THP-1 cells, a human monocytic cell line, also produced IL-10 in response to human saliva. THP-1 is an immortal cell line derived from a boy with acute monocytic leukemia.³³ This cell line has characteristics of human monocytes including TLR2 and TLR4 expression.⁴⁰ It grows easily in essential culture medium such as RPMI-1640 medium supplemented with fetal calf serum. Therefore, the use of THP-1 cells instead of autologous monocytes from peripheral blood has advantages for practical application in terms of noninvasiveness, easy handling, and stable supply. However, the levels of saliva-induced IL-10 production were not correlated between THP-1 cells and autologous monocytes. This is not surprising because THP-1 cells and peripheral monocytes are not identical; essentially, THP-1 cells are tumor cells whereas monocytes are primary cells. As compared with autologous monocytes, the degree of saliva-induced IL-10 production by THP-1 cells relatively reflects the saliva status because the degree of saliva-induced IL-10 production by autologous monocytes is influenced by the status of not only saliva but also monocytes themselves.

Levels of Pre-SLIT saliva-induced IL-10 production by THP-1 cells were significantly higher in asymptomatic patients compared with patients showing symptom onset during peak pollen dispersal. This suggests that the efficacy of SLIT, especially in achieving clinical remission after SLIT, is associated with the ability of IL-10 production by saliva collected prior to SLIT. Because SLIT is a time-consuming treatment and the efficacy of SLIT varies among patients from no symptoms to lack of a remarkable effect, attempts have been made to establish biomarkers to predict the clinical effectiveness and/or immunological tolerance of SLIT.^{6–9,16} To the best of our knowledge, this is the first report showing that saliva, which is the first body component that contacts allergen extracts during SLIT, plays a substantial role in the efficacy of SLIT.

Significant, positive correlations in IL-10 production by THP-1 cells were seen among Pre-SLIT, 3M-SLIT, and In-season saliva. This finding suggests that the ability of saliva to induce IL-10 production is comparable for at least 6 months in general. Conversely, a significant association with clinical remission was only seen in Pre-SLIT saliva. An early IL-10 response was seen after the initiation of allergen immunotherapy, which was associated with the suppression of late allergic response probably via induction of regulatory T cells and/or suppression of Th2 cells.^{11,41,42} Therefore, the present results suggest that the microenvironment in the oral cavity during the initiation phase, but not the maintenance phase, contributes to the efficacy of SLIT by induction of IL-10 production.

In the present study, we used monocyte due to easy sampling in order to examine the effect of saliva on antigen presenting cells instead of oral Langerhans cells which is known to play a regulatory role in oral cavity such as an induction of regulatory T cells.²² Future experiment using oral Langerhans cells will confirm whether IL-10 is over produced into saliva in real setting.

In conclusion, we showed evidence that saliva promotes IL-10 production in antigen-presenting cells including monocytes and monocytic THP-1 cells. This finding suggests that saliva, which is the first body component to contact therapeutic extracts during SLIT, has an adjuvant effect in this treatment. In fact, our preliminary result showed that the addition of 1% autologous saliva inhibited Cry j 1-induced IL-5 production by PBMCs from patients with JCP. In addition, this might provide a basis why the sublingual route is effective and preferable in allergen immunotherapy. In particular, IL-10 levels produced by THP-1 cells in response to saliva collected prior to the initiation of SLIT are associated with the efficacy of SLIT. Thus, the present observations might provide a basis for the use of saliva as a predictive marker of SLIT. It is interesting to

investigate whether results of the present study become the same about SLIT for other allergens except the Japanese cedar pollen, and the investigation targeting house dust mite allergy is currently underway.

Acknowledgement

The authors would like to thank Yuko Okano for her editorial assistance, Hiromi Miyashita for saliva collection, Miyuki Azuma for her gift of anti-human B7-H1 mAb, and Hideo Takahashi for his gift of THP-1 cells. This work was supported in part by grants from Ministry of Education, Culture, Sports, Science and Technology, Japan (15H04987 and 26670742).

Conflict of interest

AY received lecture fees from Torii Pharmaceutical. The rest of the authors have no conflict of interest.

Authors' contributions

TakeH, AY, and KN and MO designed the study and wrote the manuscript. YO contributed to patient collection. TF, TakeH, AO and PZ contributed to experiments regarding IL-10. KK and YH contributed to flowcytometry. SK performed the statistical analysis and interpretation of the results.

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