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Role of salivary microbiome in IL-10 production and efficacy of sublingual immunotherapy

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To the Editor

The development of methods to predict the clinical effectiveness of sublingual immunotherapy (SLIT) for allergic diseases as well as adjuvants to improve effectiveness are crucial matters. In this letter, we show that the salivary microbiome, especially *Prevotella*, is involved in the efficacy of SLIT and may be used as an adjuvant for SLIT.

We have recently shown that human monocytes and monocytic THP-1 cells produce substantial amounts of IL-10 in response to saliva, which is the first body component that contacts allergen extracts during SLIT (1). Because saliva contains immunoregulatory bacteria, and because we showed that signals through TLR2 and TLR4 are involved in saliva-induced IL-10 production in a previous report, we sought to determine the association between the composition ratio of the salivary microbiome and the efficacy of SLIT in addition to saliva-induced IL-10 production (1, 2).

Saliva was collected prior to the start of SLIT in 104 patients with Japanese cedar pollinosis. Salivary microbiomes were determined according to the 16S metagenomic sequencing library preparation protocol. Monocytic THP-1 cells were cultured in the presence or absence of 1%

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whole saliva, then IL-10 levels in the supernatants were measured. The clinical efficacy during the peak pollen season was evaluated by a visual analog scale reflecting naso-ocular symptoms (for details, see Appendix S1).

At the phylum level, organisms in the salivary microbiome included *Firmicutes* (median: 38.42%, interquartile range (IQR): 30.51-47.54%), *Bacteroidetes* (median: 16.89%, IQR: 8.80-27.18%), *Proteobacteria* (median: 14.37%, IQR: 5.69-22.53%), unclassified (median: 12.47%, IQR: 4.90-17.97%), *Fusobacteria* (median: 5.45%, IQR: 3.44-8.47%), *Actinobacteria* (median: 5.26%, IQR: 3.65-7.98%), and others (median: 0.07%, IQR: 0.03-0.20%) (Fig. S1). Thus, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are the three major phyla in salivary microbiomes of patients with Japanese cedar pollinosis. In addition, our preliminary result showed that the composition ratios of *Bacteroidetes* (median: 9.55%, IQR: 6.70-13.48%, $P < 0.001$) and *Firmicutes* (median: 31.83%, IQR: 23.66-39.87%, $P = 0.008$) were significantly less, and conversely that of *Proteobacteria* (median: 37.31, IQR: 25.82-44.57%, $P < 0.001$) was significantly higher, in nonallergic controls as compared with patients, although this result was limited in that the mean age was different between the groups ($P = 0.022$) (Fig. S2).

Saliva induces IL-10 production by THP-1 cells, and this production is regulated by signaling through TLRs (2). The composition ratio of *Bacteroidetes* in saliva was significantly and positively correlated with the level of IL-10 production ($r = 0.652$, $P < 0.001$, Fig. 1A). Conversely, the composition ratio of *Proteobacteria* was significantly and negatively correlated with the level of IL-10 production ($r = -0.517$, $P < 0.001$, Fig. 1B). In contrast, the composition ratio of *Firmicutes* showed no significant correlation ($r = -0.116$, $P = 0.240$, Fig. 1C), suggesting that *Bacteroidetes* and *Proteobacteria* in saliva affect IL-10 production. Treatment with 100 $\mu\text{g/ml}$ of polymyxin B, an antagonist of lipopolysaccharide, significantly reduced saliva-induced IL-10 production by THP-1 cells (Fig. S3).

Next, we sought to determine the genera and species of *Bacteroidetes* for which the composition ratio was associated with the level of IL-10 produced by THP-1 cells. At the class level, *Bacteroidia* ($r = 0.646$, $P < 0.001$, Fig. S4A) but not *Flavobacteria* ($r = 0.036$, $P = 0.715$, Fig. S4B) was correlated with the level of IL-10 production. *Bacteroidia* includes only one order: *Bacteroidales*. At the family level of *Bacteroidales*, *Prevotellaceae* ($r = 0.583$, $P < 0.001$, Fig. S4C) showed the strongest correlation compared with *Porphyromonadaceae* ($r = 0.229$, $P = 0.019$, Fig. S4D) and *Bacteroidaceae* ($r = 0.089$, $P = 0.367$, Fig. S4E). At the genus level of *Prevotellaceae*, *Prevotella* ($r = 0.597$, $P < 0.001$, Fig. S4F) but not *Alloprevotella* ($r = -0.222$, $P =$

0.024, Fig. S4G) showed a significant, positive correlation, suggesting that saliva-induced IL-10 production by THP-1 cells was mainly due to the presence of *Prevotella* in saliva.

In our microbiome analysis, 37 species of *Prevotella* could be identified. Among these, *P. melaninogenica* (median: 5.68%, IQR: 1.74-11.12%), *P. histicola* (median: 0.43%, IQR: 0.04-1.98%), *P. jejuni* (median: 0.41%, IQR: 0.08-1.24%), *P. pallens* (median: 0.30%, IQR: 0.11-1.19%), *P. nanceiensis* (median: 0.22%, IQR: 0.11-0.52%), *P. salivae* (median: 0.22%, IQR: 0.06-0.58%), and *P. shahii* (median: 0.11%, IQR: 0.02-0.29%) showed a composition ratio above 0.1% (Table S1). Among these species, the composition ratio of *P. melaninogenica* showed the strongest correlation with the level of IL-10 ($r = 0.562$, $P < 0.001$), suggesting that saliva-induced IL-10 production by THP-1 cells was mainly caused by *P. melaninogenica* in saliva (Table S1, Fig. 1D). The composition ratio of *P. pallens* ($r = 0.475$, $P < 0.001$; Fig. 1E), *P. jejuni* ($r = 0.413$, $P < 0.001$; Fig. 1F), *P. salivae* ($r = 0.313$, $P = 0.001$; Fig. 1G), and *P. histicola* ($r = 0.296$, $P = 0.002$; Fig. 1H) also showed significant, positive correlations with the level of IL-10 (Table S1). However, other *Prevotella* species showed median composition ratios of 0%.

IL-10 levels produced by THP-1 cells in response to saliva are significantly higher in asymptomatic patients with Japanese cedar pollinosis following SLIT with Japanese cedar pollen extract than disease-onset patients following SLIT (2). At the phylum level, the composition ratio of *Bacteroidetes* was significantly higher in asymptomatic patients showing a visual analogue scale (VAS) of 0 after SLIT compared with symptomatic patients showing a VAS above 0 after SLIT ($P < 0.001$, Fig. 2A). Conversely, the composition ratio of Firmicutes was significantly lower in asymptomatic patients compared with symptomatic patients ($P < 0.001$, Fig. 2B). No significant difference was seen between the composition ratio of *Proteobacteria* and the effectiveness of SLIT ($P = 0.334$, Fig. 2C).

Finally, we determined whether the composition ratios of various *Prevotella* species were associated with the effectiveness of SLIT. Similar to *Bacteroidetes*, the composition ratio of the genus *Prevotella* was significantly higher in asymptomatic patients compared with symptomatic patients ($P = 0.011$, Fig. 2D). In addition, the composition ratio of *P. pallens* was significantly higher in asymptomatic patients compared with symptomatic patients ($P = 0.001$, Fig. 2E). The composition ratio of *P. melaninogenica* was also higher in asymptomatic patients, but the difference did not reach a statistically significant level ($P = 0.050$, Fig. 2F). The composition ratio of *P. salivae* was significantly higher ($P = 0.033$) in asymptomatic patients (Fig. 2G). The ratios of *P. oulorum* ($P = 0.030$, Fig. 2H), *P. loescheii* ($P = 0.027$, Fig. 2I), and *P. scopos* ($P = 0.002$, Fig.

2J) were also significantly different between these patients. However, the composition ratios of other *Prevotella* species were not significantly different between asymptomatic and symptomatic patients after SLIT (Table S1). These results suggest that the oral environment containing these *Prevotella* species during initiation of SLIT is important for clinical remission after SLIT (see detailed discussion in Appendix S2).

In conclusion, the present observation provides a basis for why the sublingual route is effective and preferable for allergen immunotherapy as well as the use of *Prevotella* as an adjuvant for SLIT.

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Figure legends

Figure 1: Significance of the composition ratio of organisms in the salivary microbiome on the level of saliva-induced IL-10 production by THP-1 cells. A-C: Correlation between the IL-10 level and the composition ratio of *Bacteroidetes* (A), *Proteobacteria* (B), and *Firmicutes* (C) at the phylum level. D-H: Correlation between saliva-induced IL-10 production by THP-1 cells and the composition ratios of *Prevotella* species including *P. melaninogenica* (D), *P. pallens* (E), *P. jejuni* (F), *P. salivae* (G), and *P. histicola* (H).

Figure 2: Comparison of the composition ratios of organisms in the salivary microbiome between asymptomatic patients showing a VAS of 0 and symptomatic patients showing a VAS above 0 after SLIT. A-C: phyla *Bacteroidetes* (A), *Firmicutes* (B), and *Proteobacteria* (C). D: genus *Prevotella*. E-G: species *P. pallens* (E), *P. melaninogenica* (F), *P. salivae* (G), *P. oulorum* (H), *P. loescheii* (I) and *P. scopos* (J). P-values were determined with the Mann-Whitney U test.

Figure S1: Composition ratio of organisms of the salivary microbiome at the phylum level.

Figure S2: Comparison of salivary microbiomes at the phylum level between nonallergic controls and patients with Japanese cedar pollinosis(JCP). A: *Bacteroidetes*. B: *Firmicutes*. C: *Proteobacteria*.

Figure S3: Effect of treatment with polymyxin B (PMB) on saliva-induced IL-10 production by THP-1 cells. P-values were determined with the Wilcoxon signed-rank test.

Figure S4: Correlation between the level of saliva-induced IL-10 production by THP-1 cells and the composition ratios of class *Bacteroidia*(A), class *Flavobacteria*(B), family *Prevotellaceae*(C), family *Porphyromonadaceae*(D), family *Bacteroidaceae*(E), genus *Prevotella*(F), and genus *Alloprevotella*(G).

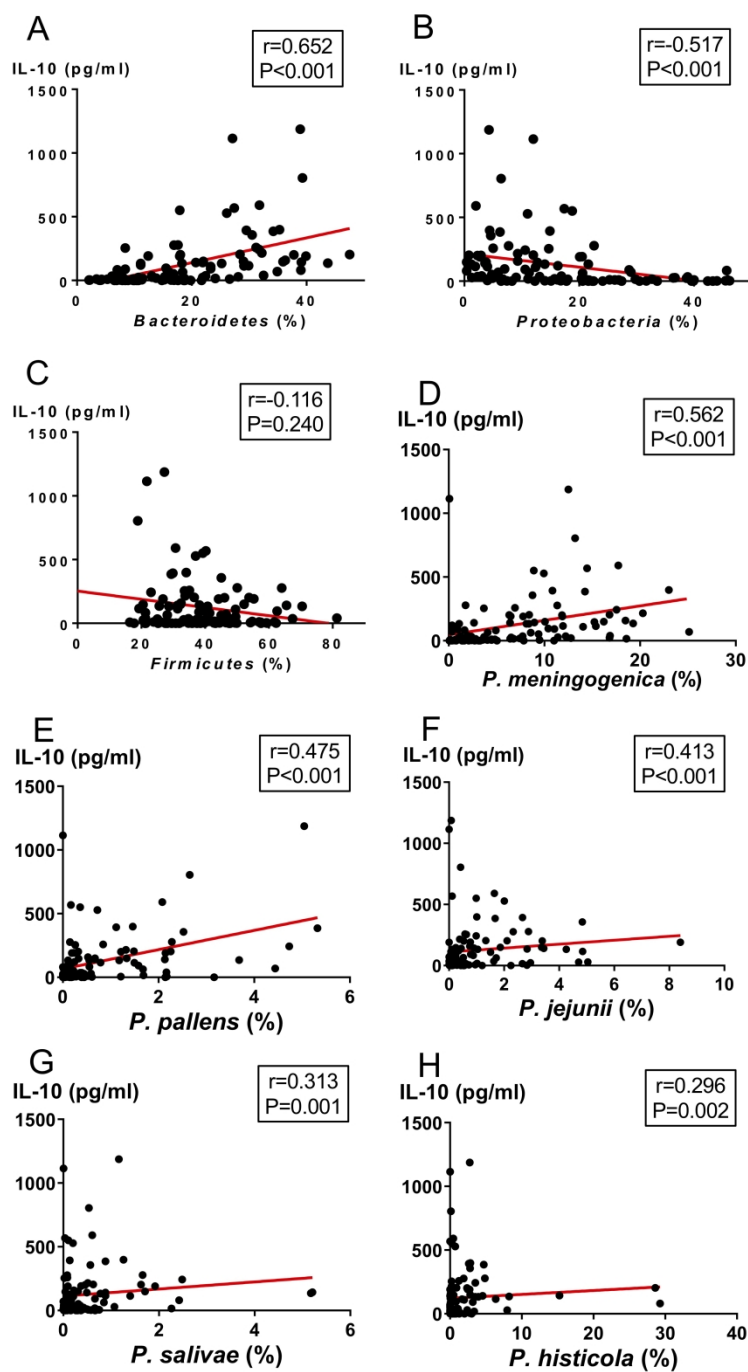
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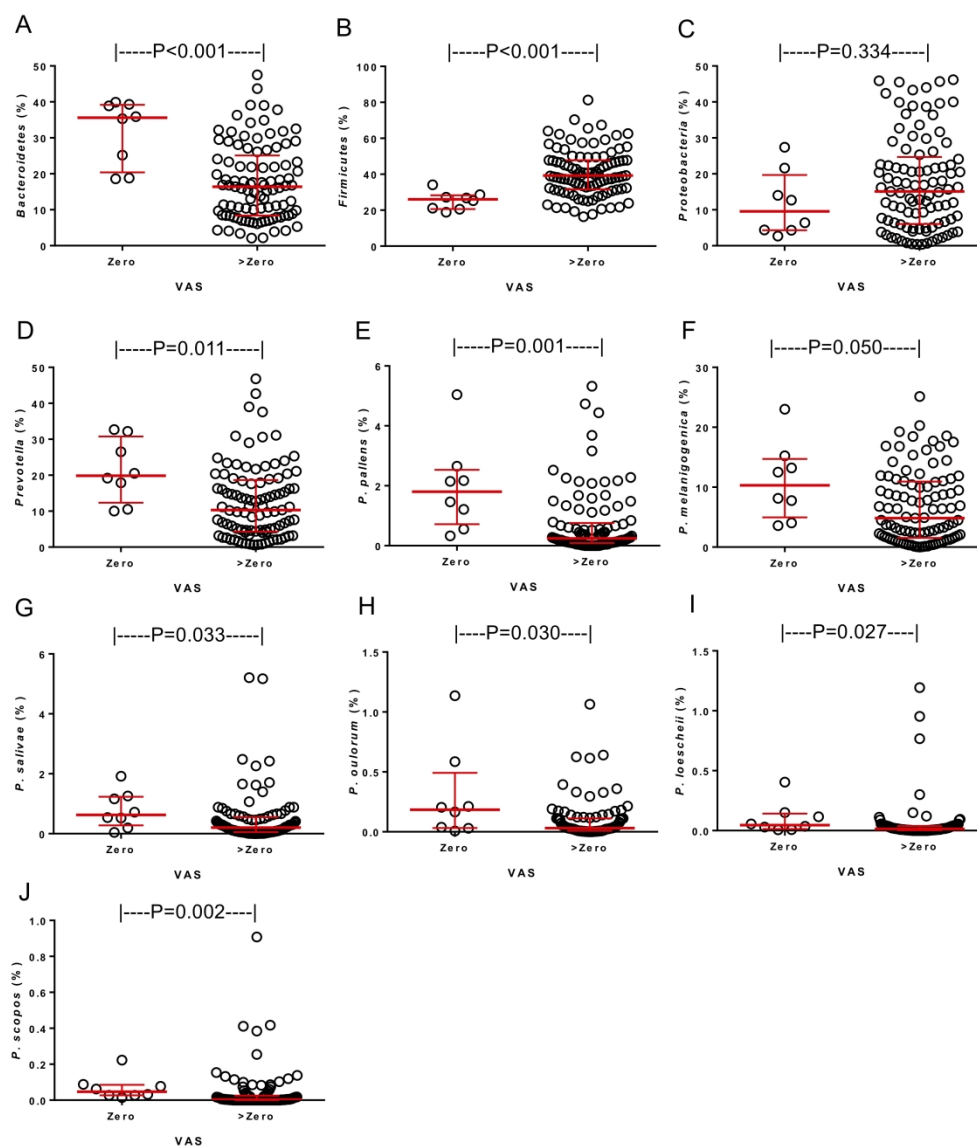
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Figure 1



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Figure 2



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Appendix S1

Methods

Subjects

We conducted a prospective study investigating the role of the saliva microbiome in the efficacy of SLIT in 104 Japanese patients with Japanese cedar pollinosis (age range, 12–70 years; mean age, 33.8 years) as previously described (1). In brief, patients had a history of naso-ocular symptoms during the pollen season (February to March) over two seasons and showed sensitization to Japanese cedar pollen as confirmed by the presence of serum-specific IgE Ab of >0.7 UA/ml as determined with ImmunoCAP (Phadia AB, Uppsala, Sweden). All patients began SLIT with Japanese cedar pollen extracts (Cedartolen™, Torii Pharmaceutical Co., Tokyo, Japan) in 2015. In brief, patients daily received extract drops from 40 to 2,000 JAU using a sublingual-swallow method during an escalation period of the first 2 weeks, and after that they continued to receive 2,000 JAU extract once daily. The clinical efficacy during the peak pollen season in 2016 was evaluated with a questionnaire with VAS reflecting naso-ocular symptoms as previously described (1). After the season in 2016, the VAS was quantified, and patients were stratified into asymptomatic (VAS of 0) and symptomatic (VAS above 0). The patients had insignificant symptoms at the initiation of SLIT from our chart records. For comparison of salivary microbiomes in the presence or absence of pollinosis, nonallergic controls (age range, 21–58 years; median age, 41.5 years) were also enrolled. Written informed consent was obtained from each subject or their guardians. The study was carried out in accordance with the Helsinki Declaration and was approved by the Human Research Committee of Okayama University Graduate School of Medicine, Dentistry

and Pharmaceutical Sciences (approval number: 1507-039).

Microbiome analysis

Prior to the start of SLIT, oral fluids were collected using the passive drool method with the Saliva Collection Aid (Salimetrics, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at -80°C until use (2). After thawing at 4°C , 100 to 800 μl saliva were centrifuged at $20,400 \times g$ for 15 min at 4°C , and then pellets were collected (3). DNA in the pellets was extracted using the DN-Sure Blood Mini Kit (APRO Science Inc., Naruto, Japan) according to the manufacturer's instructions. The concentration of DNA was determined by absorbance at 260 nm (A_{260}) using an optical density meter (DS-11+, DeNovix, Wilmington, DE, USA).

PCR amplification, amplicon cleaning, adding to index, and sequencing with the Illumina MiSeq were performed according to the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the following recommended primers:

Primer name	Sequence (5' to 3')
341F	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT</u> <u>ACGGGNGGCWGCAG</u>
806R	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA</u> <u>CTACHVGGGTATCTAATCC3</u>

The primers contain Illumina adapter overhang sequences as indicated by underlining. The initial PCR contained 2.5 μl DNA (5 ng/ μl), 5 μl each forward and reverse primers (1 mmol/L), and 12.5 μl KAPA HiFi HotStartReadyMix (Roche

Sequencing Solutions Inc., Pleasanton, CA, USA) in a total volume of 25 μ l. PCR was performed with the following program: 95°C for 3 min; followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina) according to the manufacturer's instructions. The second PCR contained 5 μ l initial amplified DNA, 5 μ l each Nextera XT Index 1 and 2 Primers (Illumina), 25 μ l KAPA HiFi HotStartReadyMix (Roche Sequencing Solutions Inc.), and distilled water to bring the total volume to 50 μ l. PCR was performed with the following program: 95°C for 3 min; followed by eight cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The quality of the purified DNA was determined using an Agilent 2100 Bioanalyzer or TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA). The final library was paired-end sequenced at 2×300 bp using a MiSeq Reagent Kit v3 (Illumina) on the Illumina MiSeq platform. The pair-end fastq datasets were merged and subjected to local Basic Local Alignment Search Tool (BLAST) (4) with the reference database (GenBank-release 221) using the Homology Analysis Tool (World Fusion, Tokyo, Japan). Then, the local BLAST data were assigned to operational taxonomic units (minimum identity $\geq$ 98% and cut-off E-value $\leq$ 1.0E-30) using Metagenome@KIN (World Fusion). We identified 10,567,121 sequences in total after demultiplexing and filtering for read quality, with a median of 51,218 sequences per experimental sample.

Induction of cytokine production by THP-1 cells using patients' saliva

Saliva-induced cytokine production by THP-1 cells including IL-10, TGF- β , TNF- α , IL-8, and IL-6 was measured as previously described (2). The human monocytic THP-1

cell line was gifted by Dr. Hideo Takahashi, Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan. This immortalized cell line was established from peripheral blood from a one-year-old boy with acute monocytic leukemia (5). There is no restriction regarding its use for basic research (6). Briefly, THP-1 cells ($0.8 \times 10^6/\text{ml}$) in conditioned media (RPMI-1640 medium supplemented with 10% fetal calf serum and L-glutamine-penicillin-streptomycin solution (Sigma-Aldrich)) were incubated with 1% 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 measurement. Levels of IL-10, TGF-β, TNF-α, IL-8, and IL-6 in culture supernatants were measured with Opt EIA sets (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's instructions. The detection limits of these assays for IL-10, TGF-β, TNF-α, IL-8, and IL-6 were 4 pg/ml, 31 pg/ml, 4 pg/ml, 4 pg/ml, and 4 pg/ml, respectively.

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 Spearman rank correlations. ROC analyses were performed to determine the cut-off values for IL-10 to predict the dominant flora. P-values of less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA).

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Appendix S2

Discussion

Our previous report showed that signals through TLR4 and TLR2 are involved in saliva-induced IL-10 production, which is associated with the clinical efficacy of SLIT (Haruna paper). Based on this information, we focused on the salivary microbiome to predict the clinical efficacy of SLIT. In the present study, we showed that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are the three major phyla of the salivary microbiome in Japanese subjects with Japanese cedar pollinosis. The composition ratios of *Bacteroidetes*, especially *P. melaninogenica* and *P. pallens* species, in saliva were significantly and positively correlated with the level of saliva-induced IL-10 production by THP-1 cells. Analysis of IL-10 production using autologous monocytes instead of monocytic THP-1 cells may be preferable. However, the use of autologous monocytes has practical disadvantages such as invasiveness, pain, low cell viability, difficulty in handling, and stable supply. In fact, it was hard to isolate and incubate autologous monocytes from the 104 patients enrolled in this study. In addition, the composition ratio of *P. pallens* was significantly higher in asymptomatic patients showing a VAS of 0 after SLIT compared with symptomatic patients showing a VAS above 0 after SLIT. These results suggest that *Bacteroidetes*, especially *P. pallens*, in saliva have the substantial regulatory effect of inducing IL-10 production and play a role in the efficacy of SLIT.

We chose saliva to sample the oral cavity because saliva is easy to collect from patients. Also, the microbiome in saliva consists of bacteria from all parts of the oral cavity (1, 2). In our study, *Firmicutes* (median: 38.4%), *Bacteroidetes* (16.9%), and

Proteobacteria (14.4%) were the three major phyla of the salivary microbiome in Japanese subjects. Although the composition of the salivary microbiome is slightly different depending on various factors such as geography, race/ethnicity, and socioeconomic status (3,4), our result is consistent with previous reports (1,5,6). For example, Keijser et al. reported that *Firmicutes* (41%), *Bacteroidetes* (28%), and *Proteobacteria* (20%) are the three major phyla of the salivary microbiome in healthy adults from the Netherlands(7). From a report by Takeshita et al., the approximate composition ratios of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* in orally healthy individuals in Japan were 42%, 20%, and 11%, respectively (6). These results are similar to our present results. In the present study, patients who recently had dental operations such as tooth extraction and those having wounds or inflammation in the oral cavity were excluded according to the SLIT guidelines in Japan and the package leaflet accompanying the SLIT droplets (CedartolenTM). However, other factors may affect the salivary microbiome composition, such as dental health.

The composition ratios of *Bacteroidetes* and *Proteobacteria* in saliva were positively and negatively correlated with the levels of saliva-induced IL-10 production by THP-1 cells, respectively. An imbalance in the microbiota in organs such as the gut, called dysbiosis, is associated with altered host responses and illness (8, 9). In the oral cavity, dysbiosis is associated with periodontitis (10). The finding that the IL-10 levels appeared to predict *Bacteroidetes* as the major phylum in saliva suggests that a balanced microbiome composition in saliva is important in immune regulation in the oral cavity. *Proteobacteria* was negatively correlated with the level of IL-10. Because the present study showed a relative abundance of each phylum in the saliva, this may be due to the dominant effect of *Bacteroidetes*, especially *Prevotella* species, on IL-10 production.

IL-10 is a crucial anti-inflammatory cytokine that prevents inflammation-mediated tissue damage (11, 12). The relationship between the microbiome and the IL-10-mediated immune response, especially in the gut, has been studied (13, 14). The gut commensal *Bacteroides fragilis* of the *Bacteroidetes* phylum or its capsular polysaccharide A induces Foxp3⁺ Tcells and promotes IL-10 secretion (15). Gulden et al. reported that IL-10 in the serum is not increased when four types of antibiotics are given to mice, suggesting that several types of gut bacteria play a role in IL-10 production (16). Some bacteria in the oral cavity, for example, *Streptococcus mitis* and *Lactobacillus brevis* of the *Firmicutes* phylum, induce IL-10 production and reduce inflammatory responses (17-19). The composition ratio of *Firmicutes* was not related to IL-10 production in our study. However, some specific bacteria of the *Firmicutes* may affect IL-10 production. IL-10 levels produced by THP-1 cells after saliva stimulation may be affected by different inflammatory environments of saliva samples caused by several factors such as smoking and periodontitis. Although we found no association between the IL-10 levels and the presence of smoking or periodontitis, dietary habits (e.g. a protein-rich diet) may influence the salivary microbiome.

In our study, among members of the *Bacteroidetes* phylum, the composition ratios of the *Prevotella* genus, especially *P. melaninogenica* and *P. pallens*, in saliva were substantially associated with the level of saliva-induced IL-10 production by THP-1 cells, suggesting that *Prevotella* in saliva has the potential for immune regulation and anti-inflammatory functions. *Prevotella* are anaerobic gram-negative commensal bacteria that are found not only in the oral cavity but also in the gut and lung. Our previous report showed that signals through TLR4 and TLR2 are involved in saliva-induced IL-10 production (20). Because *Prevotella* is a gram-negative bacterium, lipopolysaccharide in

Prevotella may act as a powerful adjuvant to enhance SLIT. Regulatory effects of *Prevotella* have been reported (21-25). For example, the *Prevotella* genus diminishes neutrophilic airway inflammation via TLR2 in mouse lung tissue *in vitro* (21). *Prevotella* induces IL-10 production by eosinophils (22). Serum levels of IL-10 are significantly elevated in mice treated orally with *Prevotellahisticola*(23). In patients with asthma, the abundance of *Prevotella* in the lung is reduced while the abundance of *Proteobacteria* is significantly increased (24). A recent study showed that the composition ratio of *Prevotella* in stool is lower in allergic children (25). We also measured the saliva-induced production of TGF- β , another regulatory cytokine, by THP-1 cells. Unlike IL-10, no significant correlation was seen between the level of TGF- β and the composition ratios of *Bacteroidetes* ($r=0.025$, $P=0.905$; Fig. S5A) and *Prevotella* ($r=0.014$, $P=0.948$; Fig. S5B). Also, no significant difference in TGF- β production was seen between asymptomatic and symptomatic patients after SLIT ($P=0.239$, Fig. S5C).

Little is known about whether these *Prevotella* species promote IL-10 production or regulate immune responses. One report showed that the serum level of IL-10 is not affected by oral administration of *P. melaninogenico* mice (26). Responses to *P. melaninogenica* may be different between humans and mice. In humans with atopic dermatitis, another report showed that the abundance of *P. melaninogenica* in perioral skin lesions decreases gradually as disease severity increases, and that the abundance increases after treatment (22). Another report showed that preincubation of monocytes with *Haemophilusparainfluenzae* in bronchoalveolar lavage samples from asthma patients inhibits corticosteroid responses, but this does not occur with *P. melaninogenica*(26). One report showed that the ratio of *P. pallens* in salivary microbiota is decreased in patients with IgA nephropathy, which is associated with chronic tonsillitis

(27). Another report showed that the ratio of *P. pallens* in the gastric microbiota is decreased in patients with gastric cancer (28). If *P. pallens* induces IL-10 production, a decreased ratio of *P. pallens* in the local microbiota may lead to an increased prevalence of IgA nephropathy and gastric cancer through the impaired anti-inflammatory effect of IL-10.

The composition ratios of *P. pallens* and *P. salivae* in saliva collected prior to the initiation of SLIT were significantly higher in asymptomatic patients after SLIT compared with symptomatic patients, suggesting that the oral environment containing these *Prevotella* species during the initiation of SLIT is important for clinical remission after SLIT. One limitation in the present study is that it is possible that the differences come from unequal sample sizes between asymptomatic and symptomatic groups. In fact, no significant difference in the microbiome composition was seen if we divide patients into different groups according to VAS during the pollen season. In the present study, we divided patients into asymptomatic (VAS of 0) and symptomatic (VAS above 0) after SLIT, because our final goal is to establish a treatment strategy to achieve clinical remission of allergic rhinitis. This may be the first report showing an association between the microbiome and the efficacy of allergen immunotherapy (AIT). However, some reports have investigated the therapeutic role of bacteria used as probiotics (29-33). For example, *Lactobacillus* GG and *Lactobacillus rhamnosus* function as probiotics for the acquisition of tolerance to food allergens in children (29, 30). In other reports, *Escherichia coli*, *Clostridium butyricum*, *Bifidobacterium lactis*, and *L. rhamnosus* improve the efficacy of AIT, including subcutaneous immunotherapy and SLIT, in patients with allergic rhinitis and asthma (31-33). Our study suggests that *P. melaninogenica* plays a role in IL-10 production, because the composition ratio of this

species was positively correlated with IL-10 production. However, *P. pallens* and *P. salivae* in saliva are the most important oral adjuvants for SLIT via induction of IL-10.

In addition, we examined saliva-induced production of pro-inflammatory cytokines by THP-1 cells including TNF- α , IL-8, and IL-6. The production of TNF- α , IL-8, and IL-6 was detected in 51, 104, and 4 out of 104 samples, respectively. The levels of TNF- α ($r=0.628$, $P<0.001$: Fig. S5D) and IL-8 ($r=0.643$, $P<0.001$: Fig. S5G) but not IL-6 ($r=0.143$, $P=0.150$: Fig. S5J) were significantly and positively correlated with the composition ratio of *Bacteroidetes*. Similar correlations were seen between the composition ratio of *Prevotella* and the levels of TNF- α ($r=0.565$, $P<0.001$: Fig. S5E) and IL-8 ($r=0.600$, $P<0.001$: Fig. S5H) but not IL-6 ($r=0.056$, $P=0.572$: Fig. S5K). In addition, the levels of TNF- α ($P=0.046$ by Mann-Whitney U test: Fig. S5F) and IL-8 ($P=0.015$) but not IL-6 ($P=0.153$: Fig. S5L) were significantly higher in asymptomatic (VAS=0) patients as compared with symptomatic (VAS>0) patients. These results suggest that not only IL-10 but also IL-8 and TNF- α play a role in the effectiveness of SLIT, and are consistent with a report by Hsieh et al. showing that the level of IL-8 in culture supernatants of peripheral blood mononuclear cells is significantly higher in good responders to injection immunotherapy with mite allergens than in poor responders (34).

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Table S1

List of *Prevotella* species: correlation coefficients between the composition ratios and saliva-induced IL-10 production by THP-1 cells and comparison of the composition ratios between asymptomatic patients and symptomatic patients as determined with VAS scores.

Species	Composition ratio (median: IQR) (%)	Correlation with saliva-induced IL-10 production by THP-1 cells		Comparison of composition ratio between symptomatic and asymptomatic patients after SLIT (P value)
		Correlation coefficient	P value	
<i>P. melaninogenica</i>	5.6843 (1.7378-11.1192)	0.5618	<u><0.0001</u>	0.0504
<i>P. histicola</i>	0.4291 (0.0427-1.9752)	0.2956	<u>0.0023</u>	0.0886
<i>P. jejuni</i>	0.4080 (0.0815-1.2352)	0.4125	<u><0.0001</u>	0.1466
<i>P. pallens</i>	0.3004 (0.1051-1.1857)	0.4751	<u><0.0001</u>	<u>0.0013</u>
<i>P. nanceiensis</i>	0.2238 (0.1057-0.5232)	0.1155	0.2430	0.1012
<i>P. salivae</i>	0.2233 (0.0563-0.5839)	0.3125	<u>0.0012</u>	<u>0.0334</u>
<i>P. shahii</i>	0.1135 (0.0164-0.2931)	0.0624	0.5293	0.3208
<i>P. oris</i>	0.0667 (0.0256-0.1762)	-0.3120	0.0013	0.2242

<i>P. veroralis</i>	0.0450 (0.0097-0.1951)	0.1909	0.0522	0.0932
<i>P. oulorum</i>	0.0344 (0.0088-0.1173)	0.2291	0.0193	<u>0.0302</u>
<i>P. loescheii</i>	0.0157 (0.0046-0.0398)	-0.1276	0.1967	<u>0.0265</u>
<i>P. denticola</i>	0.0113 (0.0009-0.0427)	-0.2934	0.0025	0.1183
<i>P. maculosa</i>	0.0111 (0.0035-0.0291)	-0.2442	0.0125	0.5459
<i>P. scopos</i>	0.0048 (0.0000-0.0356)	0.2332	0.0172	<u>0.0023</u>
<i>P. nigrescens</i>	0.0046 (0.0000-0.0189)	-0.1935	0.0491	0.3386
<i>P. saccharolytica</i>	0.0027 (0.0000-0.0089)	-0.1424	0.1494	0.3589
<i>P. micans</i>	0.0020 (0.0000-0.0184)	-0.0156	0.8753	0.8197
<i>P. aurantiaca</i>	0.0006 (0.0000-0.1581)	0.0403	0.6845	0.1230

Supplementary figure legends:

Figure S1: Composition ratio of organisms of the salivary microbiome at the phylum level.

Figure S2: Comparison of salivary microbiome at phylum level between non-allergic

controls and patients with Japanese cedar pollinosis(JCP). A: *Bacteroidetes*. B: *Firmicutes*. C: *Proteobacteria*.

Figure S3: Effect of treatment with polymyxin B (PMB) on saliva-induced IL-10 production by THP-1 cells. P-values were determined with the Wilcoxon signed-rank test.

Figure S4: Correlation between the level of saliva-induced IL-10 production by THP-1 cells and the composition ratio of class *Bacteroidia*(A), class *Flavobacteria* (B), family *Prevotellaceae*(C), family *Porphyomonadaceae*(D), family *Bacteroidaceae*(E), genus *Prevotella*(F), and genus *Alloprevotella*(G).

Figure S5: Correlation between the level of saliva-induced TGF- β (A, B), TNF- α (D, E), IL-8 (G, H) and IL-6 (J, K) production by THP-1 cells and the composition ratio of *Bacteroidetes* (A, D, G, J) and *Prevotella* (B, E, H, K). Comparison of levels of saliva-induced TGF- β (C), TNF- α (F), IL-8 (I) and IL-6 (L) production by THP-1 cells between asymptomatic patients showing a VAS of 0 and symptomatic patients showing a VAS above 0 after SLIT.

Figure S1

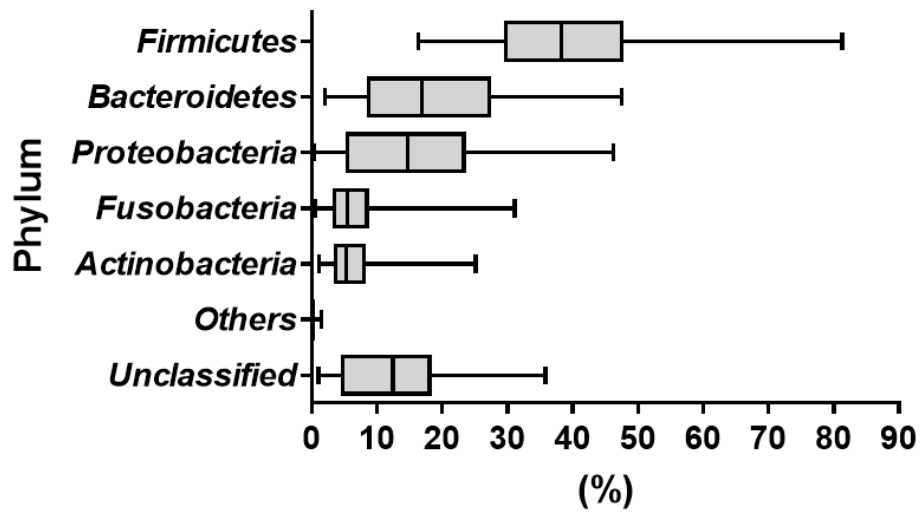


Figure S2

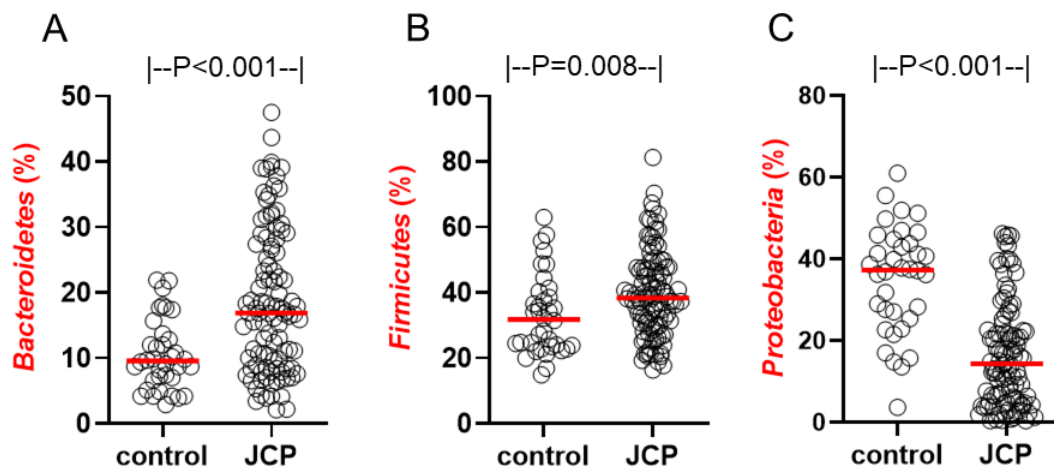


Figure S3

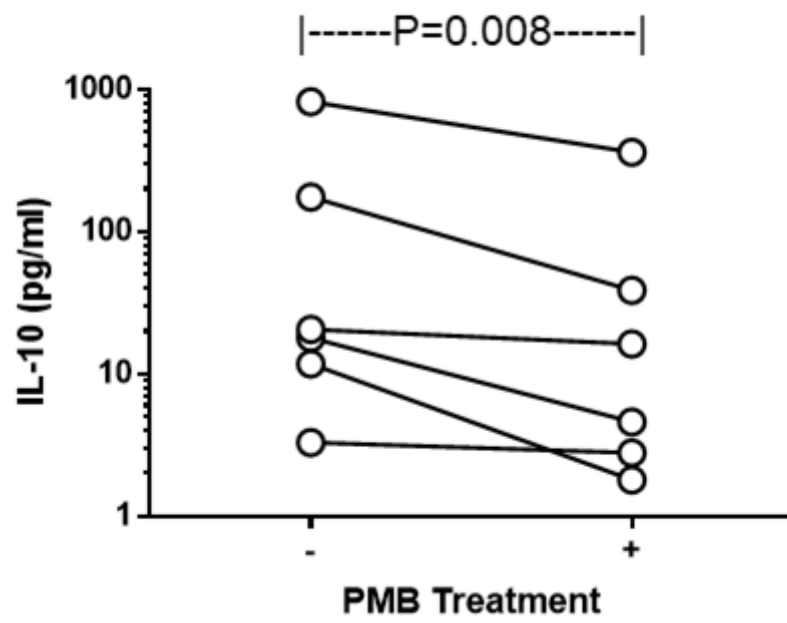


Figure S4

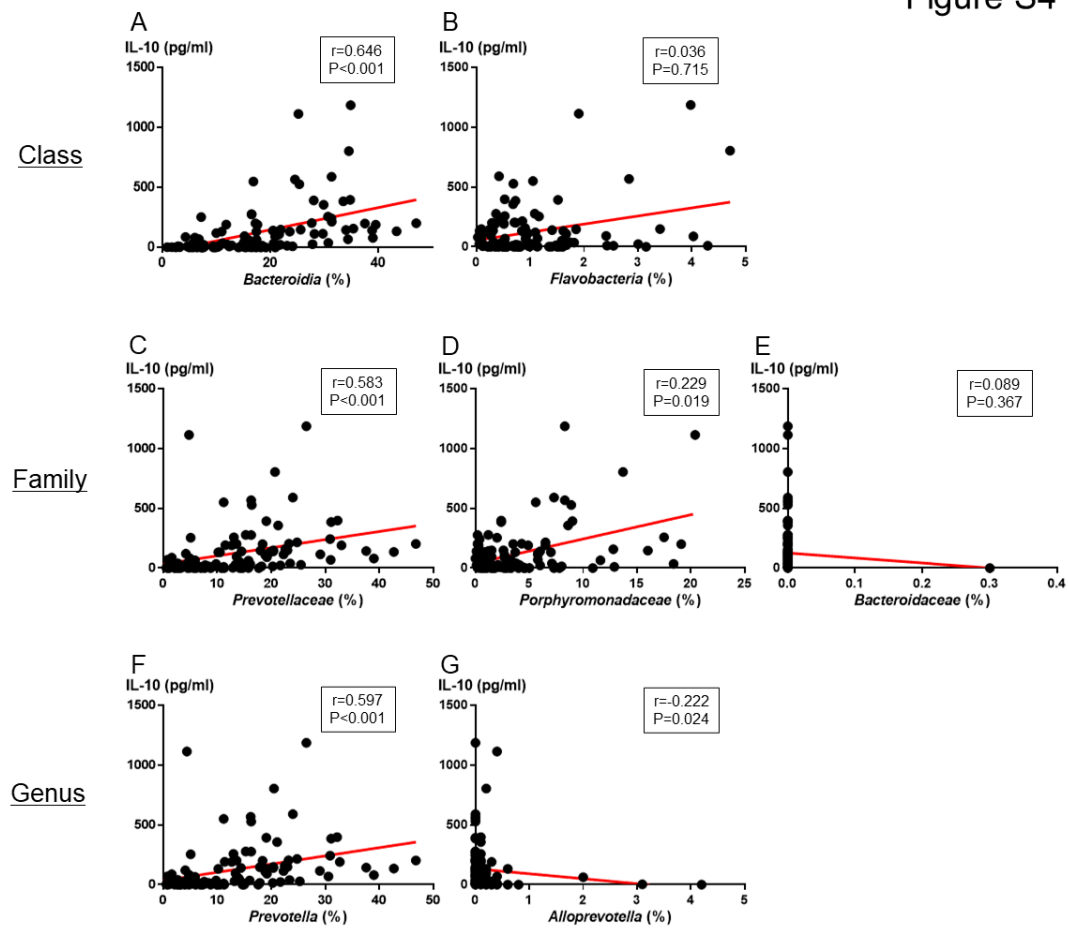


Figure S5

