

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Characterization of oral immune cells in birch pollen-allergic patients: impact of the oral allergy syndrome and sublingual allergen immunotherapy on antigen-presenting cells

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Abstract

Background: A detailed characterization of human oral immune cells is needed to better understand local mechanisms associated with allergen capture following oral exposure.

Methods: Oral immune cells were characterized by immunohistology and immunofluorescence in biopsies obtained from three healthy individuals and 23 birch pollen-allergic patients with/without oral allergy syndrome (OAS), at baseline and after 5 months of sublingual allergen immunotherapy (AIT).

Results: Similar cell subsets (i.e., dendritic cells, mast cells, and T lymphocytes) were detected in oral tissues from healthy and birch pollen-allergic individuals. CD207⁺ Langerhans cells (LCs) and CD11c⁺ myeloid dendritic cells (DCs) were found in both the epithelium and the papillary layer of the *Lamina propria* (LP), whereas CD68⁺ macrophages, CD117⁺ mast cells, and CD4⁺/CD8⁺ T cells were rather located in both the papillary and reticular layers of the LP. Patterns of oral immune cells were identical in patients with/without OAS, except lower numbers of CD207⁺ LCs found in oral tissues from patients with OAS, when compared to OAS⁻ patients ($P < 0.05$). A 5-month sublingual AIT had a limited impact on oral immune cells, with only a significant increase in IgE⁺ cells in patients from the active group. Colocalization experiments confirmed that such IgE-expressing cells mostly encompass CD68⁺ macrophages located in the LP, and to a lesser extent CD207⁺ LCs in the epithelium.

Conclusion: Two cell subsets contribute to antigen/allergen uptake in human oral tissues, including (i) CD207⁺ LCs possibly involved in the physiopathology of OAS and (ii) CD68⁺ macrophages likely critical in allergen capture via IgE-facilitated mechanisms during sublingual AIT.

Sublingual allergen immunotherapy (AIT) is a safe and efficient therapy for type I respiratory allergies in both adults and children (1–8). The impact of sublingual AIT on immune effector mechanisms in the blood and in target organs is

quite well understood, with a shift of the polarization of allergen-specific adaptive responses from a Th2 to a Th1/T regulatory (Treg) pattern (9, 10). However, local immune mechanisms associated with sublingual AIT remain to be

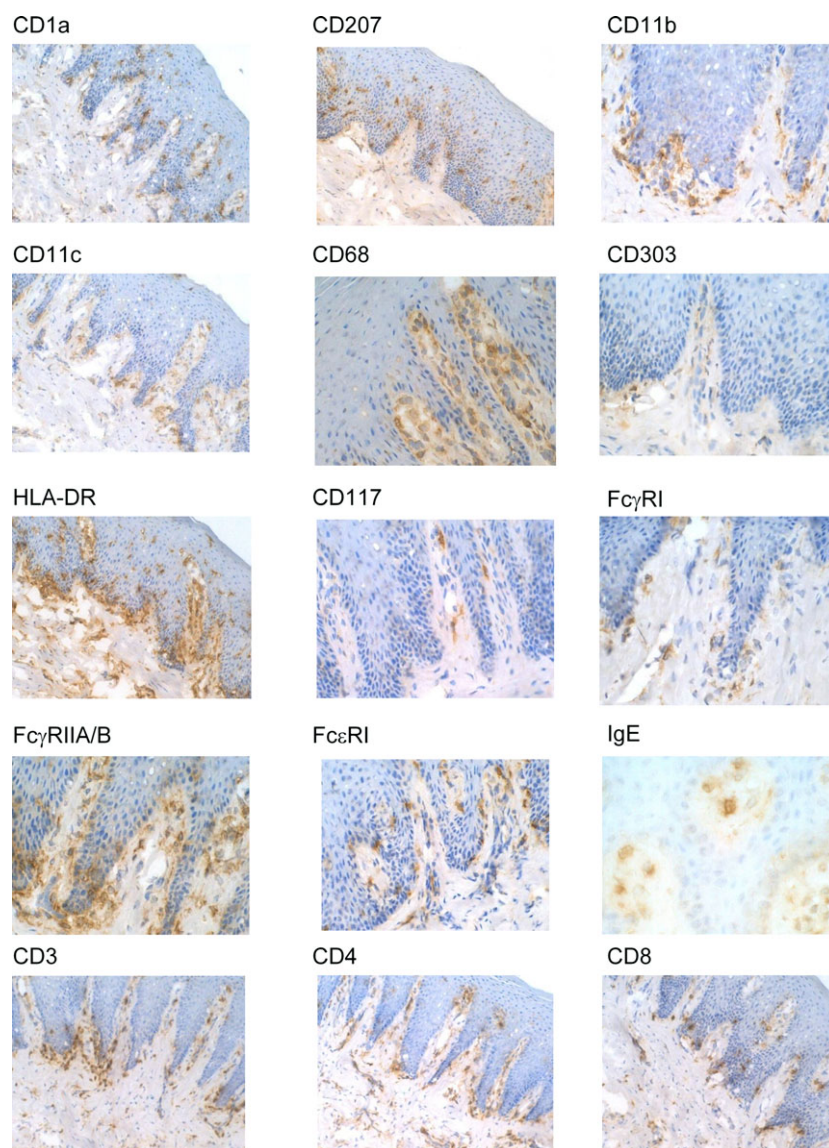


Figure 1 Mapping of immune cells in gingival tissues from healthy subjects. Gingival tissues from healthy subjects were processed for immunohistology analysis, as described in Methods. Slides were stained with the following specific antibodies: anti-CD1a, anti-CD207, anti-CD11b, anti-CD11c, anti-CD68, anti-CD303, anti-HLA-

DR, anti-CD117, anti-Fc γ RI, anti-Fc γ RIIA/B, anti-Fc ϵ RI, anti-IgE, anti-CD3, anti-CD4, and anti-CD8 (magnification $\times 100$). Photomicrographs of gingival sections are representative of data obtained in three healthy subjects.

further investigated, most particularly with respect to cells involved in allergen uptake at the administration site.

Mapping of the oral immune system in mice revealed the presence of various antigen-presenting cell (APC) subsets exhibiting distinct localization in the epithelium or the *Lamina propria* (LP), including Langerhans (LCs), myeloid, and plasmacytoid dendritic cells (DCs), as well as macrophages (11, 12). In mice, such oral macrophages are critical to capture and transport the allergen to proximal lymph nodes, where they elicit a strong Th1/Treg polarization of naïve T cells. In humans, *in vitro* experiments showed that oral LCs can rapidly capture the allergen(s) to induce specific Th1/Treg

responses (13–15). As of today, however, it is still unclear whether other APCs (e.g., myeloid DCs, macrophages) are involved during sublingual AIT.

Investigating the oral immune system is also relevant for a better understanding of specific inflammatory conditions of the mouth, such as the oral allergy syndrome (OAS). For example, birch pollen-allergic patients can experience severe local reactions when absorbing apple or hazelnut proteins, as a consequence of the IgE cross-reactivity between Bet v 1 (the major birch pollen allergen) and homologous Mal d 1 or Cor a 1 food allergens (16). A detailed analysis of local immune cells involved in the physiopathology of the OAS is

Table 1 Distribution of immune markers in the gingiva of healthy subjects

Markers	Epithelium	Lamina propria Papillary layer	Lamina propria Reticular layer
Antigen-presenting cells			
CD1a	++	+	–
CD207	++	+	–
CD11b	–	++	+
CD11c	+/-	+	+/-
CD68	+/-	+++	++
CD303	–	+/-	–
HLA-DR	+	+++	++
Lectin receptors			
CD169	–	++	+
CD205	–	+	+
CD209	–	+	+
Ig and Ig receptors			
IgE	+/-	++	++
FcγRI	–	+	+/-
FcγRIIA/B	+/-	+++	++
FcεRI	+	++	+
Mast cells and Lymphocytes			
CD117	–	+	+
CD3	+/-	+	+/-
CD4	+/-	+	+/-
CD8	+/-	+	+/-

The tissue distribution of immune cells was analyzed in the epithelium and *Lamina propria* (both the papillary and reticular layers) of human gingiva by immunohistology using a panel of specific antibodies, as described in Methods. Results are expressed in terms of presence (+/- very low, + low, ++ medium, +++ high) or absence (–) of a given marker in these different sites, combining data obtained in biopsies from three healthy subjects.

currently lacking, most particularly in relationship with the implication of oral antigen-presenting cells (APCs) and/or pro-inflammatory cells.

To better understand the physiopathology of OAS and the local mechanisms involved during sublingual AIT, we undertook a detailed characterization of human oral immune cells, including APCs as well as T lymphocytes and mast cells using biopsies from birch pollen-allergic patients. We document a detectable impact of both OAS and AIT on CD207⁺ LCs and IgE⁺-expressing CD68⁺ macrophages, respectively.

Methods

Biological samples from healthy and birch pollen-allergic individuals

After ethical committee approval and informed consent, gingival tissues were collected from healthy subjects undergoing preventive wisdom tooth extraction at the Department of Oral Surgery, University Hospital La Pitié-Salpêtrière, Paris, France. For ancillary studies conducted in three centers, oral biopsies were obtained after informed consent from a subset of 23 birch pollen-allergic volunteer patients during the VO68.10

trial (ClinicalTrials.gov: NCT01731249) (17). Briefly, this randomized, double-blind, placebo-controlled, Phase IIb study assessed the safety and efficacy of a birch pollen allergen extract (Staloral 300 IR solution; Stallergenes SA, Antony, France) administered sublingually over the 2011–2012 birch pollen seasons. Birch pollen-allergic patients were stratified according to their OAS status at baseline (Visit 1) prior to initiating sublingual AIT. Oral biopsies were performed following local anesthesia with Prilocain 1% (Xylonest® 1%). A lower lip pretreatment biopsy was obtained before the 2011 birch pollen season at Visit 1 on the right side of the mouth, whereas a posttreatment biopsy was collected at the end of the pollen season, that is, after 5 months of treatment (Visit 6) at a similar site on the left side of the mouth. Although no complications were observed in patients during the procedure, a minimum period of 2 weeks was observed between the biopsy and the first treatment administration, to allow complete healing. For both healthy and birch pollen-allergic individuals, biopsies were immediately snap-frozen without preservative in liquid nitrogen in a 2-ml Nunc tube, then stored in a freezer at –80°C until processing. All samples were coded and analyzed in a blind manner by the operators. Birch pollen-specific IgE titers were determined in sera obtained from blood collected at Visits 1 and 6 using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden).

Antibodies

The following monoclonal antibodies (mAbs) were used, either as purified antibodies for immunohistology or labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or Alexa 647 for immunofluorescence microscopy: anti-CD1a (HI149), anti-CD3 (UCH-T1), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD11b (ICRF-44), anti-CD11c (3.9), anti-CD117 (YB5.B8), anti-CD205 (MG38), anti-FcεRI (AER-37), anti-HLA-DR (LN3) (all from eBiosciences, San Diego, CA), anti-CD68 (KP-1), anti-CD169 (5D7), anti-CD209 (7D2), anti-FcγRI (2H7), anti-FcγRIIA/B (AT10), anti-IgE (4H10) (all from Abcam, Paris, France), anti-CD303 (Clone AC144; Miltenyi Biotec, Paris, France), and anti-CD207 (Clone DCGM4; Beckman Coulter, Villepinte, France). Anti-FcγRIIA (mouse IgG2b) and anti-FcγRIIB (human IgG1) antibodies were kindly provided by Dr. Pierre Bruhns (Institut Pasteur, Paris, France). In some labeling experiments, anti-rabbit IgG FITC (Lifespan Biosciences, Seattle, WA) and anti-mouse IgG PE (Sigma, Saint Louis, MA) antibodies were used as secondary antibodies. Corresponding isotype-matched mAbs were used as controls in all experiments.

Immunohistology and immunofluorescence microscopy

Biopsies of human gingiva from healthy subjects and lower lip from birch pollen-allergic patients were included in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Tissue sections (5 μm thick) were serially cut, mounted on glass slides (Tissue-Tek® Auto-Write® Starfrost® Adhesion microscope slides; Sakura Finetek

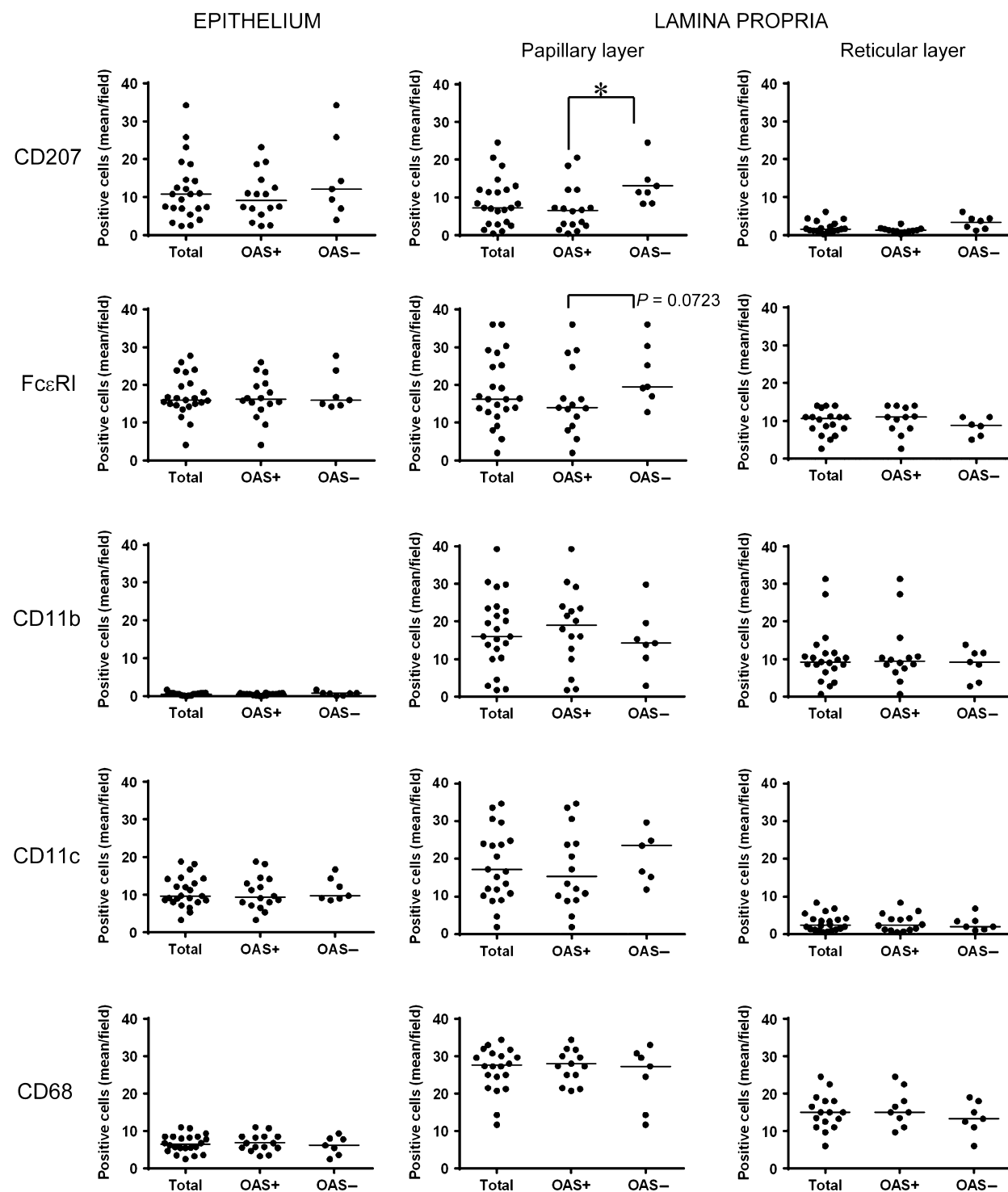


Figure 2 Quantification of antigen-presenting cells in lower lip tissues from birch pollen-allergic patients, with or without OAS. Lower lip biopsies from birch pollen-allergic patients ($n = 23$; 16 OAS+ and 7 OAS-) were processed for immunohistochemistry analysis, as described in Methods. A quantitative counting was performed on slides labeled with Abs specific for APC markers (anti-CD207, anti-FcεRI, anti-CD11b, anti-CD11c, and anti-CD68) to evaluate the

mean number of positive cells per field using a light microscope (magnification $\times 400$). The high power field surface corresponds to 0.246 mm^2 . All areas (epithelium, papillary, and reticular layers of the *Lamina propria*) were scored. Horizontal bars represent the median. Statistical significance was determined by the Mann-Whitney test ($*P < 0.05$).

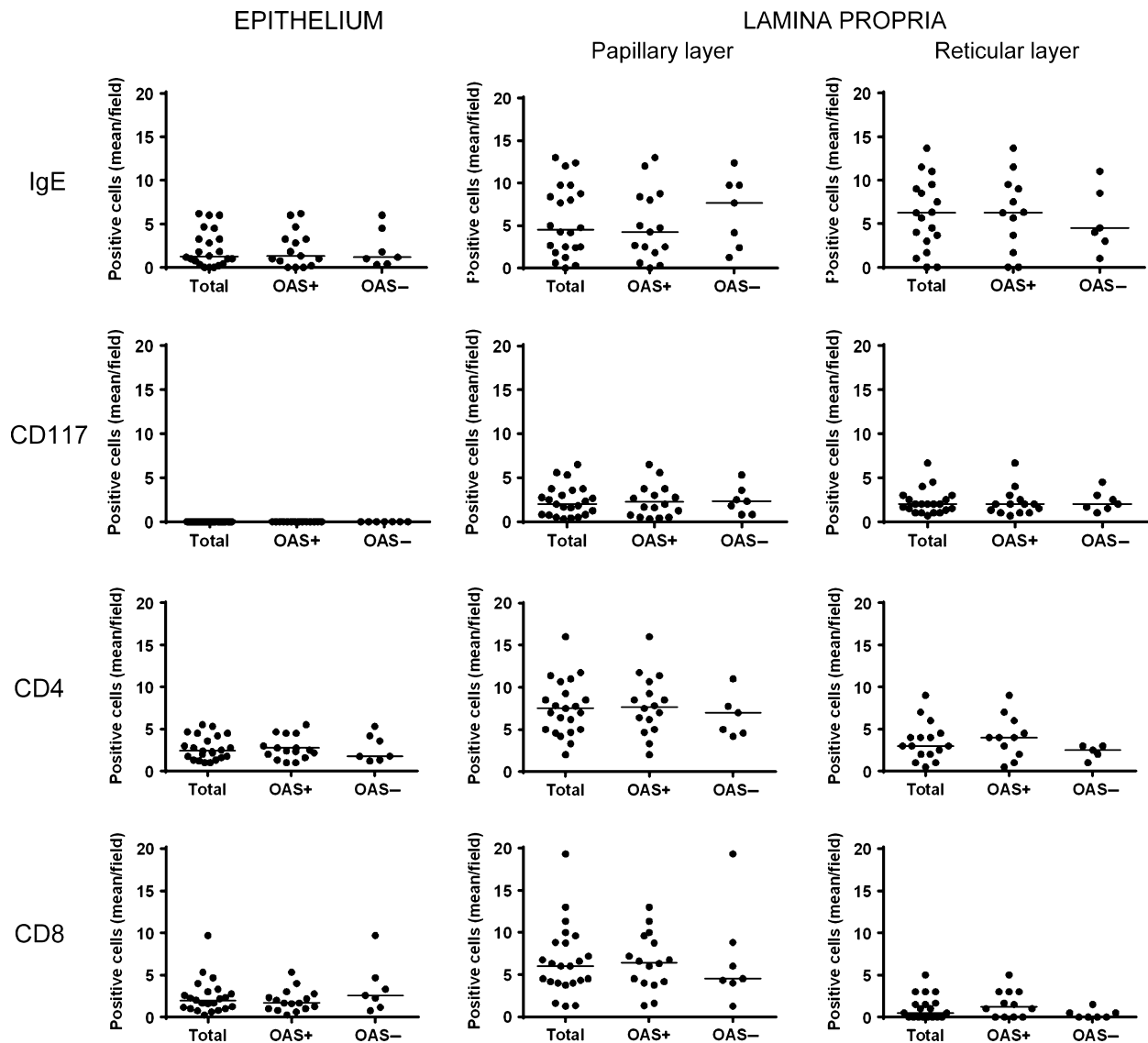


Figure 3 Quantification of IgE-expressing cells, CD117⁺ mast cells, and CD4⁺/CD8⁺ T lymphocytes in lower lip tissues from birch pollen-allergic patients, with or without OAS. Lower lip biopsies from birch pollen-allergic patients ($n = 23$; 16 OAS⁺ and 7 OAS⁻) were processed for immunohistology analysis, as described in Methods. A quantitative counting was performed on slides labeled

with mAbs specific for immune markers (anti-IgE, anti-CD117, anti-CD4 and anti-CD8) to evaluate the mean number of positive cells per field using a light microscope (magnification $\times 400$). The high power field surface corresponds to 0.246 mm^2 . All areas (epithelium, papillary and reticular layers of the *Lamina propria*) were scored. Horizontal bars represent the median.

Europe BV), fixed in cold acetone for 5–10 min, and then rehydrated. After blocking endogenous peroxidase activity (EnVision[®]+ System-HRP (DAB); Dako, les Ulis, France), slides were incubated with diluted specific mouse or rabbit primary antibodies for 1 h at room temperature. After washing, slides were incubated with appropriate anti-mouse or anti-rabbit Abs coupled to peroxidase (EnVision[®]+ System-HRP (DAB); Dako) for 30 min. Corresponding isotype-matched or secondary mAbs were utilized as negative controls. Slides were washed, and specific staining was visualized after a 5-min incubation with a diaminobenzidine substrate solution (EnVi-

sion[®]+ System-HRP (DAB); Dako). For cell quantification, samples were coded and scored blindly. Countings were performed on at least three continuous fields per area under a light microscope (Olympus BH2 and BX50; Olympus Optical Co., Tokyo, Japan) using a $40\times$ objective lens and $10\times$ eyepiece lenses. The high power field diameter was determined as 0.56 mm, which corresponds to a surface of 0.246 mm^2 . The mean number of positive cells/field for a given immune marker was calculated at the level of both the epithelium and *Lamina propria*, including the papillary and reticular layers, for each allergic patient. In selected experiments, slides were analyzed

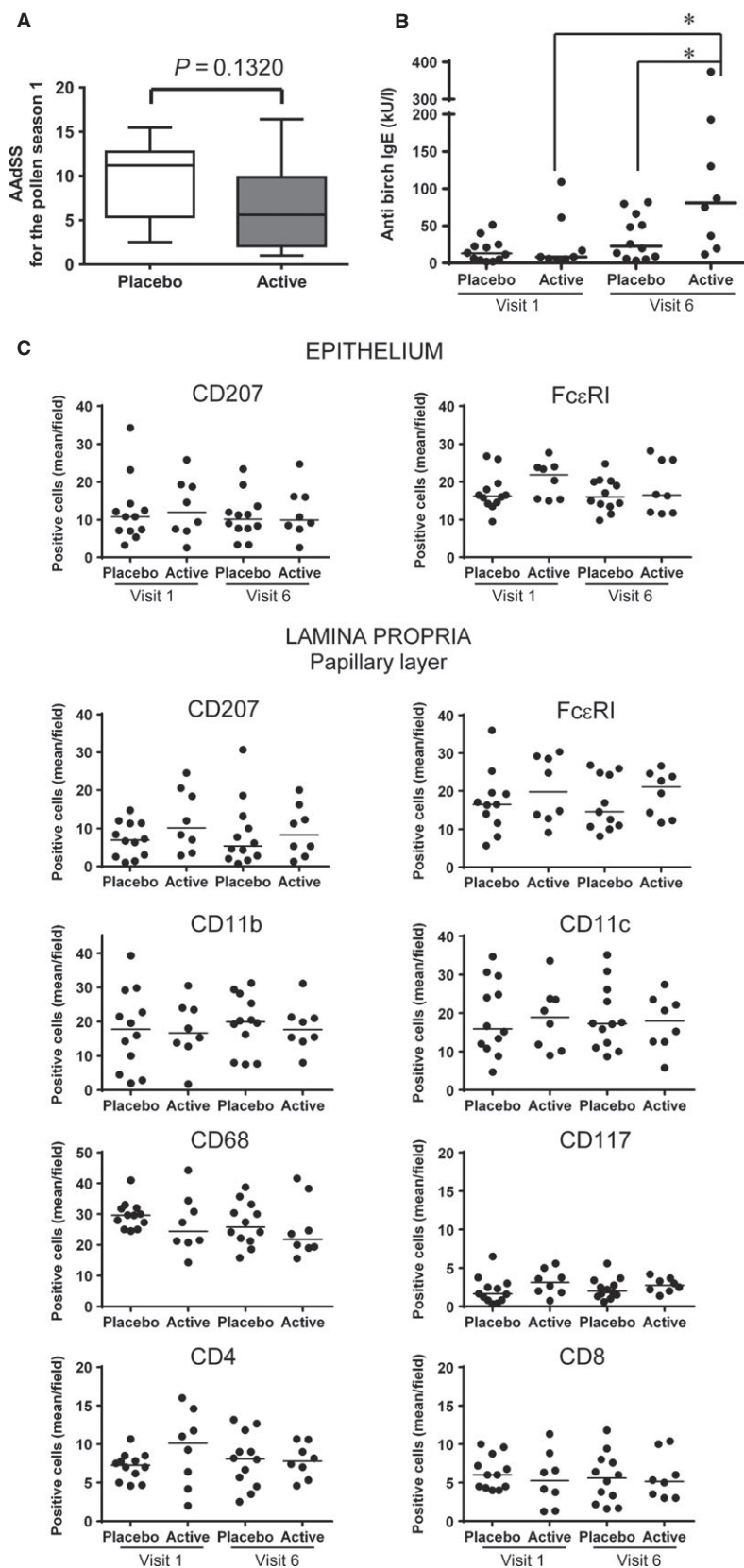


Figure 4 Evaluation of clinical scores, quantification of antigen-presenting cells, CD117⁺ mast cells and CD4⁺/CD8⁺ T lymphocytes in lower lip tissues and detection of birch pollen-specific IgE in serum from birch pollen-allergic patients, before and after sublingual AIT. (A) Average Adjusted Symptom Scores (AAdSS) are presented after one season of immunotherapy for 20 patients (12 receiving placebo and eight receiving the active treatment). Statistical analyses were performed using the Mann–Whitney test. (B) IgEs specific for birch pollen allergens were quantified by ImmunoCAP in sera from patients ($n = 12$ receiving placebo and $n = 8$ receiving the active treatment) before (Visit 1) and after 5-month AIT (Visit 6). Statistical significance was determined by the Wilcoxon test ($*P < 0.05$) for comparison between patients receiving the active treatment between Visits 1 and 6. Statistical significance was determined by the Mann–Whitney ($*P < 0.05$) test for comparison

between patients receiving either the placebo or the active treatment at Visit 6. (C) Lower lip biopsies from birch pollen-allergic patients ($n = 20$; 12 receiving placebo and eight receiving the active treatment) were processed for immunohistology analysis, before (Visit 1) and after 5 months of AIT (Visit 6), as described in Methods. A quantitative counting was performed on slides labeled with specific immune markers (anti-CD207, anti-FcεRI, anti-CD11b, anti-CD11c, anti-CD68, anti-CD117, anti-CD4, and anti-CD8) to evaluate the mean number of positive cells per field using a light microscope (magnification $\times 400$). The high power field surface corresponds to 0.246 mm^2 . All areas (epithelium, papillary, and reticular layers of the *Lamina propria*) were scored, with results only presented for the epithelium and the papillary layer of the *Lamina propria*, selected as representative for immune cell distribution in all these areas. Horizontal bars represent the median.

by immunofluorescence microscopy using an Eclipse Ti-S apparatus (Nikon, Champigny sur Marne, France).

Statistical analysis

Statistical differences between groups were assessed using the Wilcoxon and Mann–Whitney tests and the Prism6 software (GraphPad, La Jolla, CA). P -values < 0.05 were considered as significant.

Results

Mapping of oral immune cells in healthy subjects

In a first set of experiments, the phenotype and tissue distribution of immune cells were assessed by immunohistology at three different sites (i.e., epithelium, papillary, and reticular layers of the *Lamina propria* (LP)) from the gingival mucosa of healthy subjects, using a panel of specific antibodies, as described in Methods. Staining in the absence of primary antibodies was always negative in our experiments (data not shown). Qualitative and semi-quantitative results obtained in three healthy subjects are presented in Fig. 1 and Table 1, respectively. In those analyses, HLA-DR⁺ antigen-presenting cells (APCs) were detected throughout the mucosa, encompassing both CD1a⁺ CD207⁺ Langerhans cells (LCs) and CD11c⁺ myeloid dendritic cells (DC) in the epithelium and the papillary layer of the LP, as well as CD11b⁺ CD68⁺ macrophages in the papillary and reticular layers of the LP. CD303⁺ plasmacytoid dendritic cells were barely detectable in tissue sections. The presence of lectin receptors commonly associated with either DCs (such as CD205 and CD209) or macrophages (CD169) was detected in cells only in the papillary and reticular layers of the LP (Table 1). Similarly, cells expressing IgG and IgE receptors (including FcγRI, FcγRIIA/B, and FcεRI) as well as IgE-bearing cells were mostly detected in the papillary and reticular layers of the LP. Low numbers of CD117⁺ mast cells as well as oral resident CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were also predominantly located in the papillary and reticular layers of the LP (Fig. 1 and Table 1). We failed to identify B

(CD20⁺) or natural killer (CD336⁺/NKp44⁺) cells in human gingiva (data not shown).

Distribution of oral immune cells in birch pollen-allergic patients with or without an oral allergy syndrome (OAS)

To extend these observations and analyze the impact of the oral allergy syndrome (OAS) on oral immune cells, we took advantage of lower lip biopsies collected from a subset of 23 birch pollen-allergic patients enrolled in a clinical study (VO68.10) assessing the safety and efficacy of a birch pollen allergen extract administered sublingually (17). Patient demographics and baseline characteristics were similar in the two groups at study entry (Supplementary Table S1). Immunohistology analyses conducted on biopsies obtained before immunotherapy (i.e., at Visit 1) revealed patterns of expression of markers similar to the ones observed in gingival tissues from healthy subjects (Figs 2–3; total population, left columns). In particular, CD207⁺ LCs, FcεRI⁺, and CD11c⁺ cells were mainly detected in the epithelium and the papillary layer of the LP, whereas CD11b⁺ CD68⁺ macrophages, few CD117⁺ mast cells, and CD4⁺ and CD8⁺ T cells as well as IgE⁺, FcγRI⁺, and FcγRIIA/B⁺ cells were rather located in the papillary and reticular layers of the LP (Fig. S1; total population, left columns). Together, these results confirm that comparable patterns of immune cells are found in oral tissues obtained from healthy subjects and birch pollen-allergic patients.

Birch pollen-allergic patients were further stratified according to their OAS status, with 16 patients having an OAS and seven being OAS[−]. Overall, no dramatic differences were found between OAS⁺ and OAS[−] patients regarding their oral immune cells (Figs 2–3 and Fig. S1, center and right columns). In particular, OAS⁺ patients did not exhibit higher numbers of potential pro-inflammatory cells, such as CD117⁺ mast cells or effector CD4⁺ or CD8⁺ T cells. Nonetheless, as shown in Fig. 2, we detected a significantly lower number ($P < 0.05$) of CD207⁺ LCs in OAS⁺ in comparison with OAS[−] patients in the papillary layer of the LP, but not in the epithelium. In line with this observation, the numbers of FcεRI⁺ cells were also lower in the papillary

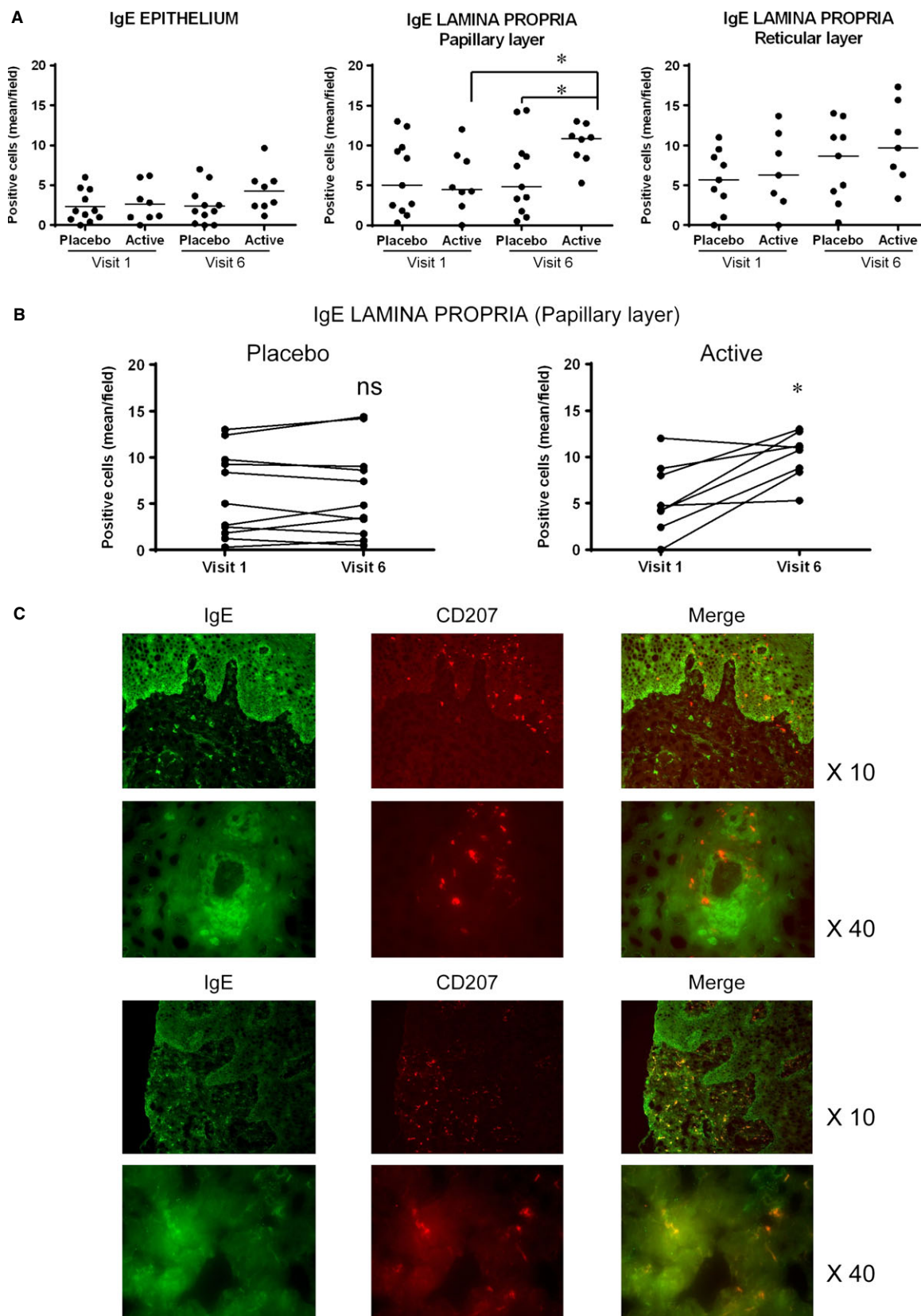


Figure 5 Quantification and identification of IgE-expressing cells in lower lip tissues from birch pollen-allergic patients, before and after sublingual AIT. (A) Lower lip biopsies from birch pollen-allergic patients ($n = 20$; 12 receiving placebo and eight receiving the active treatment) were processed for immunohistology analysis, before (Visit 1) and after 5 months of AIT (Visit 6), as described in Methods. A quantitative counting was performed on slides labeled with antibodies against IgE to evaluate the mean number of positive cells per field using a light microscope (magnification $\times 400$). The high power field surface corresponds to 0.246 mm^2 . All areas (epithelium, papillary and reticular layers of the *Lamina propria*) were scored. Horizontal bars represent the median. Statistical significance was determined by the Wilcoxon test ($*P < 0.05$) for com-

parison between Visit 1 and Visit 6 data among patients receiving the active treatment. Statistical significance was determined by the Mann–Whitney ($*P < 0.05$) test for comparison between patients receiving either the active treatment or a placebo at Visit 6. (B) Quantitative counting of IgE-positive cells in the papillary layer of the *LP* in lower lip tissues from birch pollen-allergic patients ($n = 20$) obtained at Visit 1 and Visit 6. $*P < 0.05$ was considered significant (Wilcoxon's test). (C) Colocalization of CD207⁺ LCs and CD68⁺ macrophages with IgE expression using specific monoclonal antibodies followed by fluorescence microscopy analysis. *Left panels*, IgE⁺ cells (green); *center panels*, CD207⁺ or CD68⁺ cells (red); *right panels*, overlay of green and red fluorescences.

layer of the *LP* from OAS⁺ vs OAS⁻ patients, although the difference was not statistically significant ($P = 0.0723$). As FcεRI and CD207 are at least partially colocalized (Fig. S2, upper panels), this low number of FcεRI⁺ cells detected in the papillary layer of the *LP* from OAS⁺ patients likely reflects a downregulation of CD207⁺ LCs.

IgE expression is increased on the surface of oral CD68⁺ macrophages from birch pollen-allergic patients following sublingual allergen immunotherapy (AIT)

We subsequently monitored the effect of sublingual AIT on oral immune cells, in a subset of 20 birch pollen-allergic patients, for which biopsies were obtained at both Visit 1 (prior to AIT) and Visit 6 (after 5 months of daily AIT). Among them, eight patients received the active sublingual treatment and 12 patients received a placebo. Both groups were matched for age, gender, IgE titers, and skin prick test reactivity to birch pollen allergens as well as polysensitization profiles (Table S2). Although not statistically significant due to the small size of the cohort subset, the Average Adjusted Symptom Score (AAdSS), which adjusts symptom scores for the use of rescue medication, was decreased in the active group when compared to placebo (Fig. 4A, $P = 0.1320$). As expected, immunotherapy resulted in higher titers of birch pollen-specific IgE in the blood of patients receiving the active treatment, vs placebo (Fig. 4B and Fig. S3). Overall, sublingual immunotherapy had little impact on the composition and numbers of oral immune cells, including APCs, mast cells, or T cells from the whole cohort of 20 birch pollen-allergic individuals (Fig. 4C and Fig. S4), including 16 OAS⁺ patients (data not shown). One noticeable exception was an increase in oral IgE⁺ cells in the papillary layer of the *LP* from patients receiving immunotherapy, but not placebo (Fig. 5A). Particularly, this increase concomitant with the upregulation of seric IgEs was obvious in 6 of 8 patients in the active group (Fig. 5B, right panel), whereas no specific trend was observed in the placebo group (Fig. 5B, left panel). To better characterize such IgE⁺ cells, we performed costaining experiments using mAbs labeled with distinct fluorescent dyes. As shown in Fig. 5C, most IgE⁺ cells were found to be CD68⁺ macrophages, even if some IgE⁺ CD207⁺ LCs were also detected. Noteworthy, the numbers

of oral CD11b⁺ CD68⁺ cells were unaffected by immunotherapy, suggesting that only changes in the expression of IgEs at the surface of CD68⁺ macrophages occurred during sublingual AIT, likely as a consequence of a boost of specific IgE responses (Fig. 4B). Consistent with this hypothesis, FcεRI expression was also detected in oral CD68⁺ macrophages (Fig. S2).

Discussion

Improving our understanding of the physiology of the oral immune system in humans is of interest in multiple regards. First of all, some poorly understood inflammatory conditions involving the oral cavity have been reported in allergic patients, such as the oral allergy syndrome (OAS) (16). It remains as of today unclear whether pro-inflammatory cells, such as mast cells or effector T cells, contribute to those plant-food cross-reactive allergic symptoms. In addition, in the context of an increasing interest for sublingual allergen immunotherapy (AIT) (1–9), dedicated investigations are needed to better understand local immune mechanisms occurring at the administration site, most particularly in relationship with allergen uptake.

In this context, we undertook a detailed mapping of immune cells in oral tissues from healthy and birch pollen-allergic individuals. Although this analysis was performed on a limited number of individuals, we confirm the presence of CD207⁺ Langerhans cells (LCs) as a major APC subset mainly located in the epithelium of the human oral mucosa, in agreement with previous reports (13–15). In line with our previous studies in mice (11, 12), we also document in human tissues the presence of CD11c⁺ myeloid dendritic cells detected within the epithelium and the papillary layer of the *Lamina propria* (*LP*), as well as numerous CD11b⁺ CD68⁺ macrophages rather located in the papillary and reticular layers of the *LP*. Only low numbers of mast cells were found, also in the papillary and reticular layers of the *LP*, that is at some distance from the administration site, as previously documented in mice (18). The latter is also in agreement with previous evidence that the mouth contains limited amounts of mast cells compared, for example, to the skin, thereby explaining the good safety profile of sublingual AIT (19, 20). Resident CD4⁺ and CD8⁺ T cells were

mostly found in the papillary and reticular layers of the *LP*, that is, in the proximity of oral APCs, thus allowing a rapid onset of adaptive immune responses to antigens/allergens. Characterization studies of oral T cells in mice and humans suggest that they encompass both regulatory as well as Th1, Th2, and Th17 cells (18, 21). In contrast, we could not detect B lymphocytes nor NK cells in oral tissues, even if such cells are known to be present in tonsils (22, 23). Collectively, we found little differences in the distribution of oral immune cells when comparing healthy and birch pollen-allergic individuals. Our results also show comparable patterns in tissues originating from gingiva or the lower lip, although some local differences in oral immune cell subpopulations may exist depending upon the site considered (for example, with higher numbers of LCs reported in the vestibulum and lower numbers of mast cells found in the palatum and lingual areas) (20).

Our study allowed to compare the local distribution of oral immune cells in 16 OAS⁺ and seven OAS⁻ birch pollen-allergic patients. Quantitative immunohistology analyses failed to detect any substantial difference between oral tissues from those patients with respect to potential pro-inflammatory cells (e.g., mast cells and effector T cells). Unfortunately, we failed to implement successfully tetramer-based *in situ* detection of Bet v 1-specific CD4⁺ T cells (24) in tissue sections and thus, could not investigate whether OAS⁺ patients have more Th2 and/or Th17 resident T cells. Nonetheless, we observed significantly lower numbers ($P < 0.05$) of CD207⁺ LCs in the papillary layer of the *LP* from birch pollen-allergic OAS⁺ patients when compared with OAS⁻ patients. Similarly, cells expressing FcεRI, a marker also expressed by LCs (13), were in lower numbers ($P = 0.0723$) in OAS⁺ patients. One possible explanation for this observation is that when OAS⁺ patients are exposed to food allergens cross-reactive with Bet v 1 (25), a sustained migration of oral LCs occurs toward draining lymphoid organs, thus decreasing their numbers in the mucosa. Alternatively, high numbers of tolerogenic LCs in oral tissues could contribute to the induction of protective immune responses in OAS⁻ patients (13–15, 26). The hypothesis that oral CD207⁺ LCs may contribute to the physiopathology of OAS is also consistent with the observation that these cells represent a prominent subset of oral APCs involved in allergen capture (13–15, 26).

Another goal of our study was to investigate the immunological impact of sublingual AIT at the administration site. We took advantage of the randomized, placebo-controlled, VO68.10 study assessing the safety and efficacy of sublingual AIT with a birch pollen allergen extract in 572 birch-allergic patients (17). This study clearly demonstrated a statistically significant and sustained clinical efficacy with a decrease in the Average Adjusted Symptom Scores (AAdSS) in the active group compared to placebo, leading to a relative least squares (LS) mean difference of -19.0% and -30.6% observed during treatment over the first and second pollen seasons, respectively (17). In the context of this clinical study, we could investigate by immunohistology the impact of sublingual AIT on oral immune cells in only 20 patients, includ-

ing eight patients receiving the active treatment and 12 patients receiving placebo. Although not statistically significant due to the small size of this patient subset, the efficacy data were consistent with the overall conclusion from the clinical study that patients receiving AIT exhibit clinical benefit when compared with patients receiving placebo (Fig. 4A). For safety reasons, we elected to biopsy the lower lips of patients as opposed to the tongue which is highly vascularized. Noteworthy, sublingual AIT allows allergens to diffuse to other oral tissues beyond the tongue, including the lower lip, and consequently is often associated with lip edema, confirming allergen exposure in this area. We did not find any major differences, before and after treatment, regarding the numbers of APCs or pro-inflammatory cells (i.e., CD117⁺ mast cells, CD4⁺ or CD8⁺ T cells) detected in oral tissues. These results are in agreement with previous studies conducted in grass pollen-allergic patients concluding that numbers of oral APCs and T cells are not significantly impacted by sublingual AIT, with the exception of an increase in Foxp3⁺/IL-10⁺ cells (27, 28). Noteworthy, although local reactions associated with sublingual AIT are known to be due to oral mast cell activation following IgE engagement (9, 29), no changes in numbers or distribution of such cells were observed in the present study in the course of sublingual AIT. Obviously, our results obtained after 5 months of AIT do not preclude that mast cells may have been recruited and/or expanded in oral tissues at the beginning of AIT, that is, when most local reactions are observed (29). Interestingly, the numbers of IgE⁺ cells were significantly increased in the papillary layer of the *LP* in birch pollen-allergic patients receiving active therapy, when compared to the placebo group. Colocalization experiments revealed that such IgE⁺ cells include mostly CD68⁺ macrophages and possibly, to some extent, CD207⁺ LCs. Interestingly, none of those cell subsets were expanded in oral tissues following sublingual AIT, thus implying that the main impact of AIT is to arm APCs with IgEs at their cell surface as a consequence of a boost in birch pollen-specific IgE levels. These findings strongly suggest that in humans, oral CD68⁺ macrophages are involved *in vivo* in the uptake of allergens *via* IgE-facilitated mechanisms and thus likely contribute to sublingual AIT efficacy. This hypothesis is in agreement with results from our previous studies performed in asthmatic mice, demonstrating the enhanced therapeutic benefit on airway inflammation of vector systems targeting the allergen to such oral macrophages, thereby resulting in powerful allergen-specific Th1/Treg responses (11, 12, 30). Another implication is that sublingual AIT should be performed with allergens in a wild-type conformation, as opposed to hypoallergens, to take advantage of IgE addressing to FcεRI-expressing oral APCs (31).

Collectively, our findings establish that in humans, two major subsets of oral APCs, that is, CD207⁺ LCs and CD68⁺ macrophages, contribute to allergen uptake *in vivo*. These observations pave the ground for specific vectorization strategies to better target the allergen onto those APCs in allergic patients, in order to improve tolerance induction during sublingual AIT.

Conflict of interest

This work was funded by Stallergenes. LM, MM, SS, RZ, VB, and PM are employed by Stallergenes. The rest of the authors declare no competing financial interest.

Author contributions

LM designed and supervised the study and wrote the manuscript. SR and MW supervised the clinical study. RZ and MM contributed to clinical study design and analysis. SS contributed to statistical analyses. GL and FL supervised and performed oral biopsies on healthy subjects. FJ and SP performed immunofluorescence analyses. LC performed immunohistology analyses. BH and CB supervised and performed oral biopsies on birch-allergic patients. VB contributed to writing of the manuscript. PM supervised the study and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographics and baseline characteristics of OAS[−] and OAS⁺ patients.

Table S2. Demographics and baseline characteristics of patients receiving sublingual immunotherapy or placebo.

Figure S1. Quantitative counting of FcγRI, FcγRIIA and FcγRIIB bearing cells in lower lip tissues from birch pollen-allergic patients, with or without OAS.

Figure S2. Co-localization of FcεRI with CD207⁺ LCs and CD68⁺ macrophages.

Figure S3. IgE responses to birch pollen allergens during sublingual AIT.

Figure S4. Quantitative counting of FcγRI, FcγRIIA and FcγRIIB bearing cells in lower lips from birch pollen-allergic patients, before and after sublingual AIT.

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Supplementary data Table S1:

Demographics and baseline characteristics of OAS⁻ and OAS⁺ patients

	OAS ⁻ N = 7	OAS ⁺ N = 16
Age; mean years (SD)	34.7 (10.6)	43.6 (8.9)
Gender; male (%) : female (%)	5 (71%) : 2 (29%)	9 (56%) : 7 (44%)
Birch pollen-specific IgE; mean kU/L (SD)	10.1 (13.4)	23.6 (26.1)
Skin prick test to birch natural extract; mean mm (SD)	6.6 (1.5)	7.1 (2.1)
Poly-sensitized; n (%)	7 (100%)	10 (62.5%)
Presence of asthma; n (%)	3 (43%)	5 (31%)

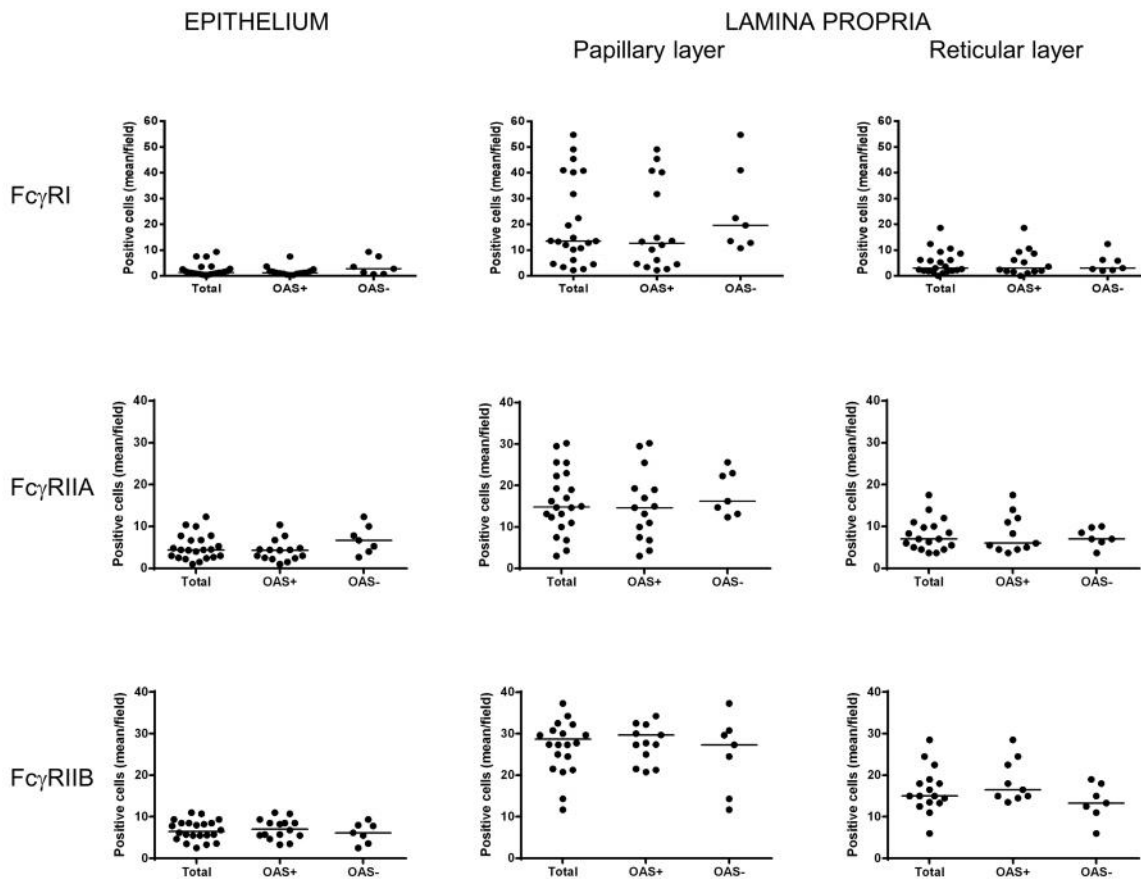
Results describing continuous variables are expressed as means (SDs). Results describing categorical variables are expressed as numbers of patients and percentages relative to numbers of OAS⁻ or OAS⁺ patients.

Supplementary data Table S2:

Demographics and baseline characteristics of patient receiving sublingual immunotherapy or placebo

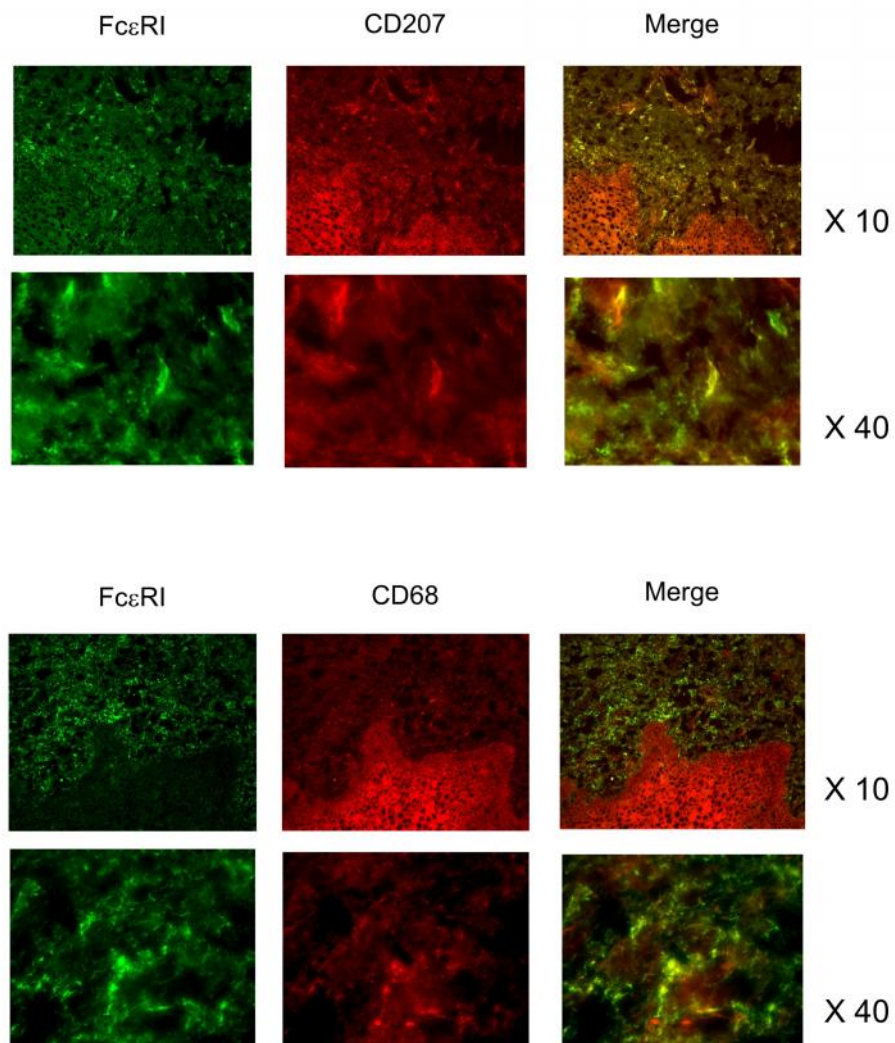
	Immunotherapy N = 8	Placebo N = 12
Age; mean years (SD)	42.3 (11.9)	39.7 (10.0)
Gender; male (%) : female (%)	5 (63%) : 3 (37%)	6 (50%) : 6 (50%)
Birch pollen-specific IgE; mean kU/L (SD)	26.1 (35.4)	16.9 (15.9)
Skin prick test to birch natural extract; mean mm (SD)	7.1 (1.9)	6.9 (2.1)
Poly-sensitized; n (%)	6 (75%)	9 (75%)
Presence of asthma; n (%)	4 (50%)	4 (33%)

Results describing continuous variables are expressed as means (SDs). Results describing categorical variables are expressed as numbers of patients and percentages relative to numbers of immunotherapy or placebo-treated patients.



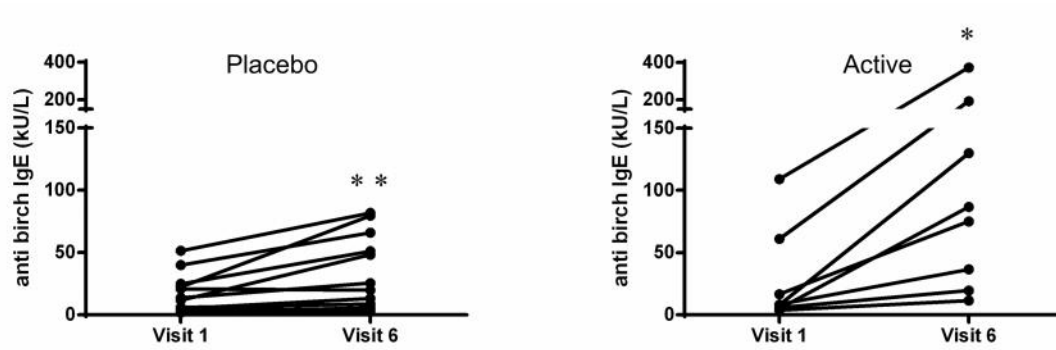
Supplementary data Figure 1: Quantitative counting of Fc γ RI, Fc γ RIIA and Fc γ RIIB bearing cells in lower lip tissues from birch pollen-allergic patients, with or without OAS.

Lower lip biopsies from birch pollen-allergic patients (n=23; 16 OAS⁺ and 7 OAS⁻) were processed for immunohistology analysis, as described in Methods. A quantitative counting was performed on slides labeled with antibodies against Fc γ RI, Fc γ RIIA or Fc γ RIIB to evaluate the mean number of positive cells per field using a light microscope (magnification x400). The high power field surface corresponds to 0.246 mm². All areas (epithelium, papillary and reticular layers of the *Lamina propria*) were scored. Horizontal bars represent the median.



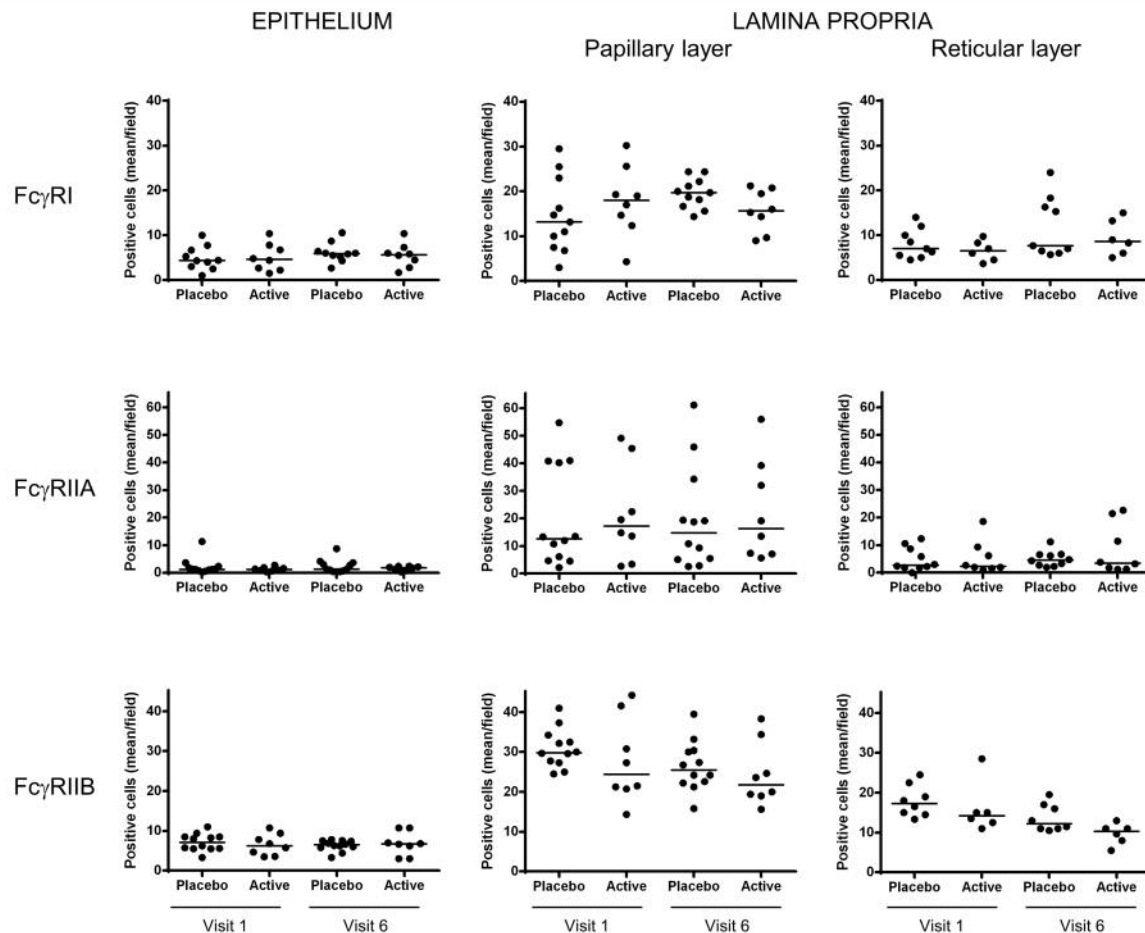
Supplementary data Figure 2: Co-localization of FcεRI with CD207⁺LCs and CD68⁺ macrophages.

Co-localization of FcεRI with CD207⁺LCs and CD68⁺ macrophages was performed using specific monoclonal antibodies followed by fluorescence microscopy analysis. *Left panels*, anti-FcεRI-FITC (*green*); *center panels*, anti-CD207-PE or anti-CD68-PE (*red*); *right panels*, overlay of green and red fluorescences.



Supplementary data Fig. 3: IgE responses to birch pollen allergens during sublingual AIT.

IgEs specific for birch pollen allergens were quantified by ImmunoCAP in sera from patients (n=12 receiving placebo and n=8 receiving the active treatment) before (Visit 1) and after 5-month AIT (Visit 6). * $p < 0.05$, ** $p < 0.01$, were considered significant (Wilcoxon test).



Supplementary data Figure 4: Quantitative counting of Fc γ RI, Fc γ RIIA and Fc γ RIIB bearing cells in lower lips from birch pollen-allergic patients, before and after sublingual AIT.

Lower lip biopsies from birch pollen-allergic patients (n=20; 12 receiving placebo and 8 receiving the active treatment) were processed for immunohistology analysis, before (Visit 1) and after 5 months of AIT (Visit 6), as described in Methods. A quantitative counting was performed on slides labeled with antibodies against Fc γ RI, Fc γ RIIA or Fc γ RIIB to evaluate the mean number of positive cells per field using a light microscope (magnification x400). The high power field surface corresponds to 0.246 mm². All areas (epithelium, papillary and reticular layers of the *Lamina propria*) were scored. Horizontal bars represent the median.