

Phl p 5 resorption in human oral mucosa leads to dose-dependent and time-dependent allergen binding by oral mucosal Langerhans cells, attenuates their maturation, and enhances their migratory and TGF- β 1 and IL-10-producing properties

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Background: Sublingual immunotherapy (SLIT) is safe and effective as treatment of allergic rhinitis and mild asthma. Oral mucosal Langerhans cells (oLCs) play a central role. However, little is known about allergen binding by oLCs during mucosal allergen resorption and its impact on oLC functions.

Objective: Binding of Phl p 5 to oLCs was studied in a standardized *ex vivo* model to investigate mechanisms important for SLIT.

Methods: Human oral mucosal biopsies were incubated with the grass pollen allergen Phl p 5. Migration, binding of Phl p 5, phenotype and cytokine production, and T-cell priming of Phl p 5-binding oLCs were analyzed.

Results: Significant uptake required more than 5 minutes, and dose-dependent binding of Phl p 5 to oLCs was saturated at 100 $\mu\text{g/mL}$ Phl p 5. Furthermore, Phl p 5 significantly increased the migratory capacity of oLCs but attenuated their maturation

and strongly promoted the release of TGF- β 1 and IL-10 by oLCs themselves as well as by cocultured T cells.

Conclusion: Oral mucosal Langerhans cells bind Phlp5 in a dose-dependent and time-dependent manner, leading to an increased production of tolerogenic cytokines and an enhanced migratory capacity but decelerated maturation of oLCS. (J Allergy Clin Immunol 2010;###:###-###.)

Key words: Sublingual immunotherapy, oral mucosa, Langerhans cells, Phl p 5, tolerance

Sublingual immunotherapy (SLIT) has been accepted as a safe and efficient alternative to subcutaneous immunotherapy (SCIT) and treatment for allergic rhinitis and mild asthma.¹⁻⁴ In human beings, it has been shown that orally applied allergen is detected within oral mucosal tissue for at least 20 hours after application.^{5,6} Antigen-presenting cells (APCs) such as dendritic cells (DCs) play a pivotal role in SLIT and SCIT because they are known to bind and process antigens and to induce antigen-specific T-cell responses.⁷ In this regard, it has been shown in mice that local CD11b⁺/CD11c⁻ and CD11b⁺/CD11c⁺ DCs process sublingually applied antigens.⁸ Because in human beings, Langerhans cells (LCs) account for the major DC population within the oral mucosa and these oral mucosal LCs (oLCs) have been demonstrated to express the high-affinity receptor for IgE (FcεRI), which is mainly occupied by IgE molecules, it is very likely that oLCs also bind and process allergens during SLIT.⁹ However, in human beings, little is known about the binding of allergen to oLCs, the duration of allergen contact necessary, and allergen concentrations sufficient to achieve the desired effects. A shift from a T_H2 to a T_H1 immune response along with the induction of allergen-specific regulatory T cells has been postulated to be a key mechanism in SLIT, similar to the mechanisms known for SCIT.^{10,11} To expand our knowledge about optimal allergen concentrations, application time, and functional changes of oLCs after exposure to allergens currently used during SLIT in the clinical practice, an *ex vivo* model was established by using fresh human oral mucosal tissue to study binding of the major grass pollen allergen Phl p 5 to human oLCs with standardized protocols.

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Abbreviations used

APC: Antigen-presenting cell
 DC: Dendritic cell
 FITC: Fluorescein isothiocyanate
 FoxP3: Forkhead box protein 3
 GPA: Grass pollen allergy
 LC: Langerhans cell
 oLC: Oral mucosal Langerhans cell
 SCIT: Subcutaneous immunotherapy
 SLIT: Sublingual immunotherapy

METHODS**Oral mucosa specimen**

Specimens of oral mucosal tissue from the vestibular region were obtained from patients undergoing intraoral surgery at the Department of Oral and Maxillofacial Surgery, University of Bonn (n = 90; age, 27.1 ± 12.8 years; females, n = 46; age, 25.7 ± 9.0 years; males, n = 44; age, 27.8 ± 15.5 years). Patients with tumors were excluded, and only clinically noninflamed tissue was collected. Specimens from individuals with a history of grass pollen sensitization confirmed by skin prick test and/or serum specific IgE with patients with allergic rhinitis alone or in combination with allergic asthma and/or atopic dermatitis were designated as having grass pollen allergy (GPA; n = 40; age, 28.1 ± 11.9 ; females, n = 21; age, 28.0 ± 11.1 ; males, n = 19; age, 28.2 ± 13.0 years), whereas specimens from individuals without any history of allergic disease were indicated as nonatopic (n = 49; age, 26.2 ± 13.5 ; females, n = 24; age, 24.5 ± 6.3 ; males, n = 25; age, 27.6 ± 17.4 years). All specimens were obtained with the approval of the local ethics committee and the medical board ethics committee and after informed consent from patients had been obtained.

Standardized *ex vivo* model for allergen resorption

Collected human oral mucosal tissue was placed mucosal side up on a sterile Petri dish and incubated at 37°C with PBS containing fluorescein isothiocyanate (FITC)-coupled Phl p 5 or dextran at concentrations ranging from 10 to 1000 µg/mL. For repetitive allergen challenge, 10 µg/mL Phl p 5 was administered on oral mucosal tissue every 6 minutes by removal of previous and application of new allergen solution without washing between. As control conditions, oral mucosal tissue was incubated with PBS alone or cooled to 4°C before incubation with FITC-coupled Phl p 5 or dextran to determine unspecific allergen binding. After incubation, oral mucosal tissue was washed with PBS and placed in RPMI 1640 medium (Invitrogen, Carlsbad, Calif) containing 10% heat-inactivated FCS (Sigma, Deisenhofen, Germany), 1% antibiotics/antimycotics (Invitrogen), and 500 IU/mL GM-CSF (Berlex Laboratories, Richmond, Calif) at 37°C. In a time kinetic, 24 and 36 hours after allergen exposure, cells that migrated out of the tissue were collected and further processed for flow-cytometric analysis. Medium was exchanged, and incubation of the tissue was continued. After 48 hours, migrated cells were finally collected, and resting tissue was further processed and digested to prepare a single cell suspension by trypsin treatment in 0.5% trypsin buffer without Ca^{2+} for 1 hour at 37°C as described elsewhere.¹² Binding of Phl p 5 to oLCs was calculated by setting gates of the major CD1a⁺ population in FITC fluorescence, which migrated from tissue at 4°C. Specific binding of Phl p 5 was calculated by subtracting the percentage of FITC-positive CD1a cells at 4°C from the percentage of FITC-positive CD1a cells at 37°C. The major grass pollen allergen Phl p 5, which was coupled to FITC or used pure, was provided by ALK-Abelló (Horsholm, Denmark), and FITC-dextran was purchased from Molecular Probes (Invitrogen Europe, Paisley, United Kingdom). Circular dichroism spectrum analysis of Phl p 5-FITC could demonstrate same conformational state compared to native Phl p 5.

Calculation of migrated oLCs

Oral mucosal Langerhans cells were gated by their CD1a expression and location in the forward scatter/sideward scatter 24, 36, and 48 hours after

allergen/dextran exposure. The remaining tissue was treated with trypsin, and resting oLCs were identified by their CD1a expression. Gated cells were counted at each migration time point as well as in the remaining tissue. The percentage of migrated oLCs at each time point was calculated by adding the number of oLCs at the particular time point and previous time points to divide the sum by the total number of oLCs detected.

Reagents

See the subsection on reagents in this article's Online Repository at www.jacionline.org.

Immunolabeling of cell suspensions

A direct extracellular or intracellular staining for a number of 0.5 to 2×10^5 unfixed or fixed cells was performed as described elsewhere.¹² The production of IL-10 and TGF-β1 by oLCs and T cells was detected by intracellular staining using the BD Cytotfix/Cytoperm kit (BD Biosciences, Heidelberg, Germany), whereas FoxP3 expression of cocultured T cells was detected by using the forkhead box protein 3 (FoxP3) staining kit (eBiosciences, San Diego, Calif) as described in the instruction manuals. Finally, the cells were measured on a FACS-Canto (BD Biosciences) as described in detail before and analyzed by FACSDiva (BD Biosciences) and FlowJo (TreeStar Inc, Ashland, Ore) software. For quantitative evaluation, dead cells were excluded by 7-amino-actinomycin-D staining. The CD1a population was gated out manually and expressed in the percentage of positive cells.

T-cell cocultures

See the subsection on T-cell cocultures in this article's Online Repository at www.jacionline.org.

Statistical analysis

For statistical evaluation of significance, the Mann-Whitney *U* test or Wilcoxon test was performed. These tests were realized by using the SPSS 17.0 for Windows software (SPSS Software, Munich, Germany). Results are shown as arithmetic mean $\pm$ SEM (# or **P* < .05; ***P* < .01; no indication, not significant unless otherwise indicated).

RESULTS**Binding of Phl p 5 to oLCs is time-dependent and displays saturation characteristics**

It has been reported in mice that DCs bind ovalbumin during resorption.⁸ However, little is known about the application time sufficient for allergen binding to human DCs, in particular human oLCs. For this reason, *ex vivo* human oral mucosal tissue was incubated with FITC-coupled Phl p 5 (1000 µg/mL) at 4°C and 37°C for 20 seconds, 60 seconds, 5 minute, 10 minutes, and 60 minutes and placed in cell culture medium afterward to allow oLCs, which had taken up allergen, to migrate out of the tissue. Thereby we observed time-dependent binding of Phl p 5 to oLCs. In contrast with 20-second and 60-second incubation times, 5 minutes of application of Phl p 5 resulted in significant allergen binding with a strong scattering of the individual binding capacity between oLCs of different donors ($32.6\% \pm \text{SEM}$, 10.5; n = 10). Interestingly, extending incubation time reduced the relatively wide range of the individual binding capacities and led to comparable percentages of oLCs binding Phl p 5 of the different donors, suggesting a saturation of oLC-allergen binding (Fig 1, A). However, because Phl p 5 binding to CD1a-negative cells such as keratinocytes could also be detected (data not shown), oral mucosal tissue was preincubated with pure Phl p 5

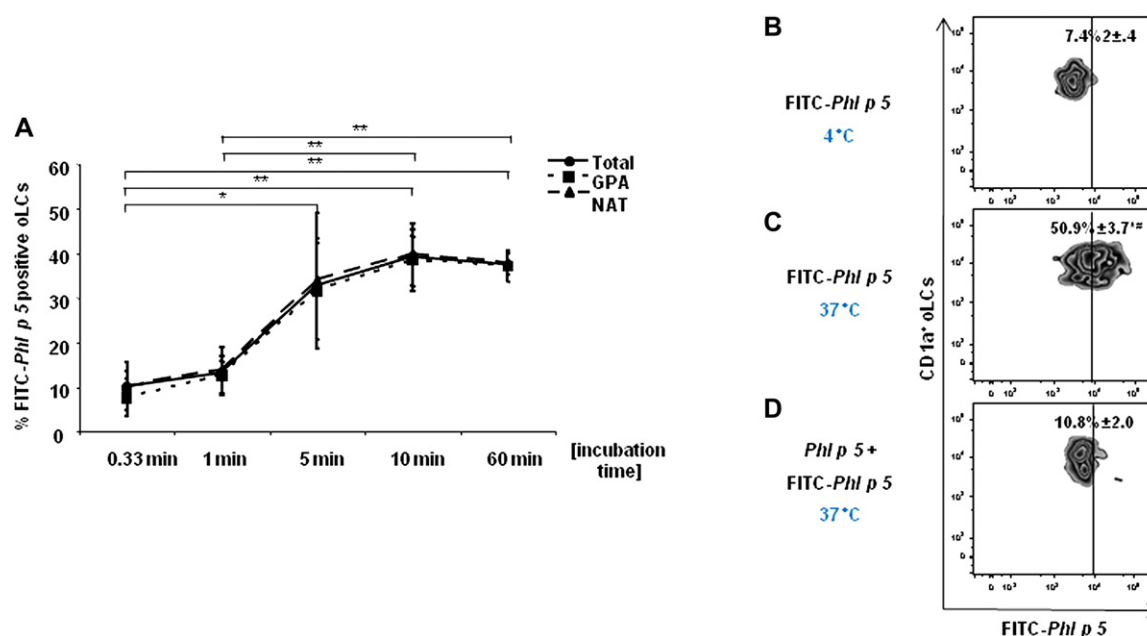


FIG 1. Allergen exposure for 5 minutes is sufficient to induce Phl p 5 binding to oLCs, and saturation of Phl p 5 binding to oLCs is reached at a dose of 1 mg/mL Phl p 5. **A**, Oral mucosal tissue was incubated with FITC-coupled Phl p 5 (1 mg/mL) for 20 seconds, 60 seconds, 5 minutes, 10 minutes, and 60 minutes ($n = 10$) at 37°C and 4°C. Significant binding of Phl p 5 to oLCs could be detected after 5 minutes of allergen exposure (solid black line, circles). oLCs from samples of individuals with GPA (dotted line, squares; $n = 5$) and non-atopic individuals (NAT; dashed line, triangles; $n = 5$) displayed comparable dose-dependent binding capacity. * $P < .05$; ** $P < .01$. Oral mucosal tissue was incubated for 10 minutes with FITC-coupled Phl p 5 (1 mg/mL) at 4°C (**B**) or FITC-coupled Phl p 5 (1 mg/mL) at 37°C (**C**) or with pure Phl p 5 (1 mg/mL, 10 minutes) at 37°C before addition of FITC-coupled Phl p 5 (1 mg/mL, 10 minutes) at 37°C (**D**), and oLCs migrating out of the tissue were analyzed 36 hours after exposure. Values are depicted as means of percent-positive CD1a cells $\pm$ SEMs. Preincubation of oral mucosal tissue with pure Phl p 5 at a concentration of 1 mg/mL inhibited binding of FITC-coupled Phl p 5 (1 mg/mL); * $P < .05$ compared with oLCs + FITC-Phl p 5 at 4°C; [#] $P < .05$ compared with oLCs + Phl p 5 + FITC-Phl p 5 at 37°C. Figures show representative plots.

(1 mg/mL; 10 minutes) before FITC-Phl p 5 challenge (1 mg/mL; 10 minutes), which abrogated FITC-Phl p 5 binding to oLCs, indicating that saturation of oLC-allergen binding was achieved (Fig 1, B-D).

From this set of experiments, we conclude that allergen binding to oLCs is time-dependent, with saturation of all oLCs capable reached after 5 minutes of allergen exposure.

Dose-dependent Phl p 5 binding to CD1a⁺ oLCs

Because randomized controlled trials could demonstrate dose-dependent efficacy of sublingually applied grass allergen tablets, incubation of *ex vivo* isolated human oral mucosal tissue with Phl p 5 at different concentrations was investigated.^{3,4} Oral mucosa was challenged with FITC-coupled Phl p 5 for 60 minutes before tissue culture because previous experiments revealed stable binding of Phl p 5 to DCs. After migration of oLCs out of the tissue 36 hours later, oLCs were analyzed for Phl p 5 binding. Thereby, a dose-dependent binding of Phl p 5 to oLCs could be demonstrated. Although incubation of human *ex vivo* isolated oral mucosal tissue with 10 μ g/mL Phl p 5 did not lead to a significant binding by migrating oLCs, 100 μ g/mL Phl p 5 resulted in significant allergen binding of migrating oLCs, and a further increase of Phl p 5 concentration to 1000 μ g/mL did not lead to a stronger binding of allergen to oLCs (Fig 2, A and B). Repetitive stimulation with Phl p 5 at 10 μ g/mL (every 6 minutes) up to a cumulative total dose of 100 μ g/mL did not result in any significantly

increased allergen binding of oLCs, suggesting that allergen concentration during single contact but not the number of allergen contacts is critical (data not shown). Interestingly, binding of Phl p 5 to oLCs was comparable in samples obtained from individuals with GPA and nonatopic individuals (Table I). From this set of experiments, we conclude that besides exposure time, allergen concentrations applied during single exposure are critical for efficient binding to local oLCs.

Stable kinetics of Phl p 5 binding to oLCs is associated with increased oLC migration

Next, the kinetics of Phl p 5 binding to oLCs and their migration were investigated. To address this issue, freshly *ex vivo* isolated oral mucosal tissue was incubated with Phl p 5 at 100 μ g/mL for 1 hour and placed in cell culture medium to let the oLCs that had taken up allergen migrate out of the tissue. Migrated oLCs were analyzed 24, 36, and 48 hours later for allergen binding. It has been shown that LCs display high dextran uptake.¹³ Thus, oral mucosal tissue incubated with FITC-dextran was used as a reference for oLCs binding to Phl p 5. In this context, the total percentage of oLCs binding Phl p 5 was lower than the percentage of oLCs binding dextran 24 hours and 36 hours after exposure (Fig 3). Interestingly, the percentage of oLCs binding Phl p 5 was comparable 24 hours (40.7% $\pm$ SEM, 6.7), 36 hours (37.1% $\pm$ SEM, 3.8), and 48 hours (36.3% $\pm$ SEM, 3.4) after exposure, whereas the percentage of oLCs binding dextran was

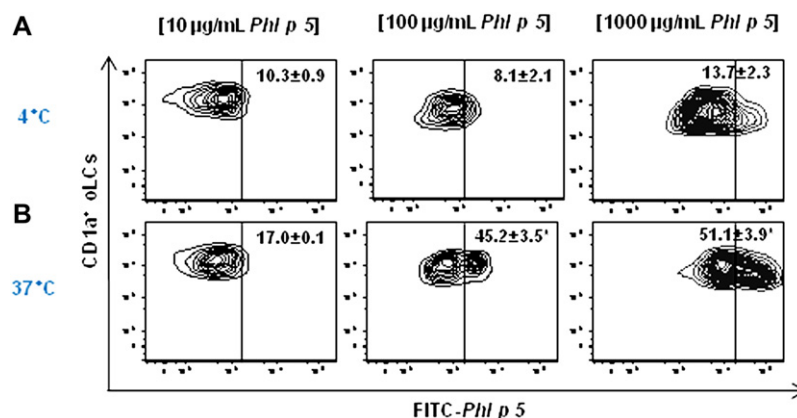


FIG 2. Amount of Phl p 5 binding to CD1a⁺ oLCs is dose-dependent. Oral mucosal tissue was incubated with FITC-coupled Phl p 5 at 4°C (**A**) and at 37°C (**B**) for 60 minutes by using 10 µg/mL (first column, $n = 10$), 100 µg/mL (second column, $n = 16$), and 1000 µg/mL (third column, $n = 10$) Phl p 5. Thirty-six hours after allergen exposition, migrated cells were harvested. OLCs were detected by CD1a expression (y-axis) and analyzed for Phl p 5 binding (x-axis). Values are depicted as means of percent-positive CD1a cells $\pm$ SEMs (upper right corner of the counter plots). A dose-dependent binding of Phl p 5 to CD1a⁺ oLCs could be detected. Compared with 10 µg/mL, significant Phl p 5 binding to CD1a⁺ oLCs could be demonstrated for 100 µg/mL (second column) and 1000 µg/mL (third column) Phl p 5. * $P < .05$.

highest 24 hours after exposure ($56.3\% \pm \text{SEM}, 5.5$) and decreased continuously 36 hours ($44.2\% \pm \text{SEM}, 7.1$) and 48 hours after exposure ($35.6\% \pm \text{SEM}, 5.8$). Binding of Phl p 5 to oLCs in this time kinetic was comparable in samples from individuals with GPA and nonatopic individuals (data not shown).

Furthermore, migratory capacity of oLCs was investigated 24 hours, 36 hours, and 48 hours after allergen exposure in GPA and nonatopic samples ($n = 14$). Thereby, a significant increase of oLC migration was detected 36 hours after exposure to Phl p 5 at 37°C (Fig 4, A-D). Forty-eight hours after exposure, most of oLCs migrated out, whereas in control conditions with PBS and Phl p 5 at 4°C, significant numbers of oLCs rested within the tissue (Fig 4, A and B). However, in GPA, exposure to Phl p 5 at 37°C (Fig 4, A and C, red graph) led to a significant increase of oLCs compared with dextran (Fig 4, C, gray and black graphs) already after 24 hours, whereas in nonatopic samples (right column) 24 hours after exposure to Phl p 5 at 37°C (Fig 4, B and D, green graph) a significantly higher migration could be detected compared with Phl p 5 at 4°C (Fig 4, B and D, black graph) and PBS (Fig 4, B, gray graph).

Furthermore, enhanced migration of oLCs was seen only in tissue samples incubated with Phl p 5 at 37°C, not in tissue samples incubated with dextran at 4°C or 37°C (Fig 4, A and B). These data indicate that Phl p 5 binding to oLCs enhances their migratory capacity in a specific way.

Phl p 5 prevents full maturation of migrating oLCs

The increased migratory capacity of oLCs from oral mucosal tissue on Phl p 5 challenge implies accelerated activation and maturation of oLCs. Thus, oLC maturation was investigated by analyzing the expression of CD207, CCR6, CCR7, and CD83 of Phl p 5-negative versus Phl p 5-positive oLCs. Furthermore, the expression of the coinhibitory molecules B7-H1 and B7-H3 was analyzed because it has been reported that oLCs upregulate these molecules on activation.¹⁴ In this regard, comparable expression of B7-H1 and B7-H3 by Phl p 5-negative and Phl p 5-positive oLCs migrating from oral tissue challenged with Phl p 5

TABLE I. Similar binding of Phl p 5 to CD1a⁺ oLCs of atopsics and nonatopsics

Phl p 5	GPA	NAT
10 µg/mL	7.1% $\pm$ 2.5	6.0% $\pm$ 2.2
100 µg/mL	33.6% $\pm$ 6.9	40.2% $\pm$ 2.6
1000 µg/mL	37.1% $\pm$ 3.5	37.7% $\pm$ 2.6

Comparable binding of Phl p 5 to oLCs migrated from tissue of GPA and nonatopic (NAT) individuals ($n = 10$ for each concentration). Values represent mean percentages of FITC-Phl p 5⁺ CD1a cells from tissue incubated at 37°C versus 4°C $\pm$ SEM.

(100 µg/mL) at 37°C could be detected (data not shown). However, a significantly lower expression of CD83 and CCR7 was detected on Phl p 5-positive oLCs in contrast with Phl p 5-negative oLCs (Fig 5, A-E). Moreover, C-type lectin Langerin (CD207) and CCR6, which is restricted to immature oLCs, could be detected on resting as well as migrated oLCs. Interestingly, comparable expression could be detected in GPA and nonatopic samples (data not shown). This set of experiments demonstrates that binding of Phl p 5 to oLCs during resorption retains their immature phenotype and decelerates oLC maturation.

Phl p 5 binding to oLCs upregulates their TGF-β1 and IL-10 production and induces TGF-β1 and IL-10-producing T cells

Because tolerance induction is thought to be a key immunologic mechanism in allergen-specific immunotherapy,^{15,16} the production of the tolerogenic cytokines TGF-β1 and IL-10 was investigated in oLCs migrating out of oral mucosal tissue after incubation with Phl p 5 at either 4°C (negative control) or 37°C. In this regard, an upregulated TGF-β1 and IL-10 production could be detected in oLCs migrated from tissue samples incubated with Phl p 5 at 37°C (Fig 6). The same expression pattern could be detected in GPA and nonatopic samples (data not shown). Moreover, coculture of these oLCs with allogeneic T cells upregulated the TGF-β1 and IL-10 production of cocultured T cells

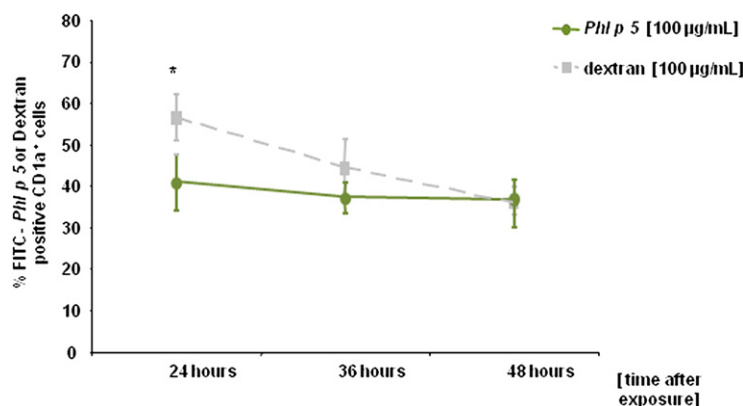


FIG 3. Percentage of oLCs binding Phl p 5 and emigrating from the tissue remains stable whereas the percentage of migrating oLCs that bound dextran decreases continuously after exposure. Oral mucosal tissue was incubated with FITC-coupled Phl p 5 (100 µg/mL; dark green line, circles) or FITC-coupled dextran (100 µg/mL; gray line, dashed) at 4°C and at 37°C for 60 minutes. Twenty-four, 36, and 48 hours after exposure, migrated cells were harvested while tissue remained in culture. OLCs were detected by CD1a expression and analyzed for Phl p 5 or dextran binding. Specific binding of Phl p 5 was calculated as described in the Methods section. Values are depicted as means of percent-positive CD1a cells $\pm$ SEMs. Binding of Phl p 5 to CD1a⁺ oLCs was stable at all investigated time points, whereas binding of dextran decreased. The strongest binding of dextran could be detected after 24 hours, which was significantly higher than Phl p 5 binding at this time point.

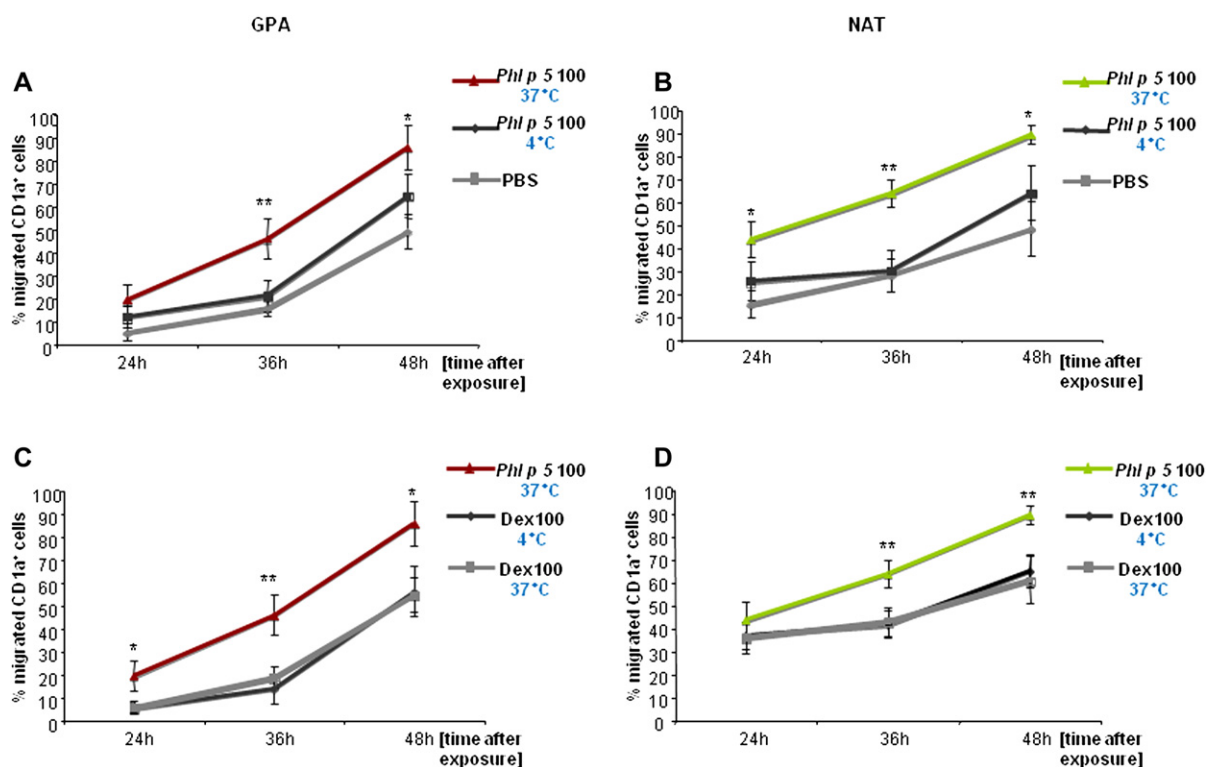


FIG 4. Phl p 5 stimulation enhances migratory capacity of oLCs. Oral mucosal tissue (n = 14) was incubated with FITC-coupled Phl p 5 (100 µg/mL; 60 minutes) at 4°C (black lines, diamond) and at 37°C (red line, triangles) in GPA (A and C) and nonatopic (NAT) samples (B and D), with PBS (60 minutes; gray line, squares; A and B) or with FITC-coupled dextran (100 µg/mL; 60 minutes) at 4°C (60 minutes; black line, diamonds) and at 37°C (gray line, squares; C and D). Twenty-four, 36, and 48 hours after exposure, migrated cells were harvested and tissue was trypsinized and analyzed for oLCs resting in the tissue. OLCs were detected by CD1a expression. A significantly higher percentage of CD1a⁺ cells migrated out of the oral mucosal tissue 36 and 48 hours after exposure to Phl p 5 at 37°C compared with oLCs incubated with Phl p 5 at 4°C, PBS, or with dextran at 4°C and 37°C. *P < .05; **P < .01.

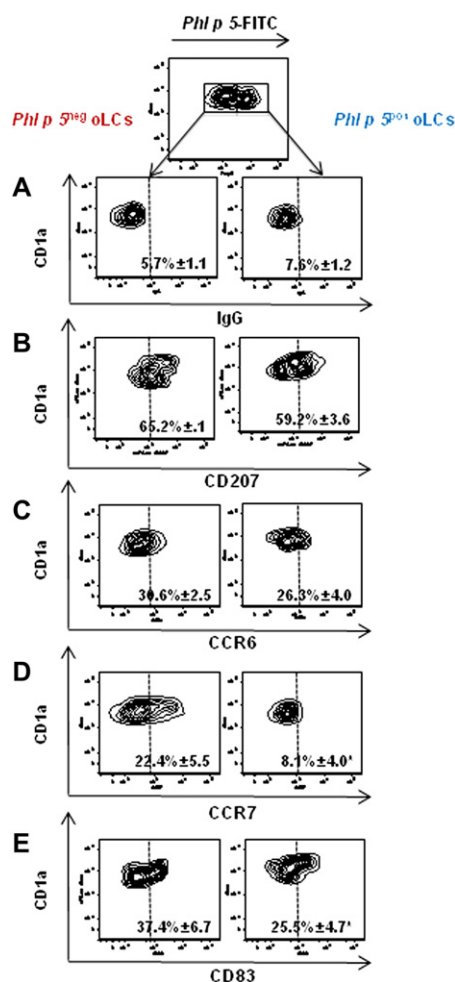


FIG 5. CD1a⁺ oLCs that have taken up Phl p 5 and migrate out of the tissue are retained in an immature state. Oral mucosal tissue was incubated with FITC-coupled Phl p 5 (100 µg/mL; 60 minutes) at 37°C. Thirty-six hours after allergen exposure, migrated cells were harvested. CD1a⁺ cells were gated manually depending on their Phl p 5 binding (counter plots, upper row). Gated CD1a⁺ cells were discriminated in CD1a⁺ without Phl p 5 binding (first column) and with Phl p 5 binding (second column) compared with isotype control (A); expression of CD207, n = 11 (B); CCR6, n = 6 (C); CCR7, n = 6 (D); and CD83, n = 15 (E) was analyzed. Thereby we could detect a comparable expression of CD207 and CCR6 on both populations, clearly identifying CD1a⁺ as oLCs. Moreover CCR7 and CD83 expression was decreased on Phl p 5-positive oLCs compared with Phl p 5-negative oLCs. **P* < .05.

in all samples (Fig 7) as well as in GPA and nonatopic samples (data not shown). However, a significant induction of the transcription factor FoxP3, which is specific for regulatory T cells, could not be detected (data not shown). From these data we conclude that allergen binding of oLCs within oral mucosal tissue enforces the tolerogenic character of both oLCs and primed T cells.

DISCUSSION

Although the clinical efficacy of SLIT is well documented and widely accepted, the underlying immunologic mechanisms remain uncertain. Most likely, APCs such as DCs are key players because they take up allergen to induce an adequate allergen-specific immune response. However, neither the critical APC population nor allergen concentration or application time sufficient for

allergen uptake has been investigated in human beings on a cellular level. In this report, we demonstrated for the first time that oLCs bind allergen in a time-dependent and dose-dependent manner. Furthermore, we showed that allergen binding enforces the production of TGF-β1 as well as IL-10 by oLCs and cocultured T cells. Moreover, allergen binding by oLCs enhances their migratory capacity but attenuates their maturation.

The data reported here demonstrate dose-dependent binding of Phl p 5 to oLCs with a saturation of binding, which is in line with clinical SLIT studies demonstrating a dose-dependent clinical efficacy.^{17,18} The observed time-dependent saturation of Phl p 5 binding to oLCs supports data obtained from a rhinitis mouse *in vivo* model, in which a time-dependent efficacy was observed.¹⁹ However, in this mouse model, sufficient allergen contact time to oral mucosal tissue was less than 1 minute, which might be because an *ex vivo* model was used in our study. A study in human beings reported a higher efficacy of repetitive allergen administration in SLIT patients, demonstrating that dosing frequency plays an important role *in vivo*.²⁰ The missing benefit of repetitive allergen administration observed here might result from the fact that only short-time repetitive application (every 6 minutes) with low Phl p 5 doses (10 µg/mL) was investigated in our *ex vivo* model and that single as well as cumulative allergen concentrations in this *ex vivo* system might differ from allergen concentrations required *in vivo* to achieve any additional effect. Furthermore, the presented data are in accordance with an earlier study demonstrating a dose-dependent and time-dependent uptake of Phl p 1 and the major birch pollen allergen (Bet v 1) by *in vitro*-generated LC-like DCs.²¹ In the same study, the authors showed that Phl p 1 and Bet v 1 were internalized by LC-like DCs mainly through macropinocytosis, whereas blocking of receptor-mediated internalization by inhibitors did not alter uptake of Phl p 1 and Bet v 1 significantly. However, the saturation character of Phl p 5 binding to oLCs observed in our study by time and dose depending binding as well as blocking effect of FITC-Phl p 5 binding by preincubation of unlabeled Phl p 5 leads to the assumption that receptor-mediated processes rather than macropinocytosis seem to be in the foreground, because macropinocytosis represents a constitutive feature of DCs.²² In this context, Fc receptors such as FcεRI, which is expressed by oLCs of atopic and nonatopic individuals, might contribute in part to allergen uptake *in vivo*. It has been shown that in particular FcεRI expression on oLCs of atopic individuals is high and that this receptor is mostly occupied by IgE molecules, allowing allergens to bind and crosslink this structure.²³ Because FcεRI expression was profoundly downregulated from the surface of migrating oLCs that had taken up Phl p 5, it is tempting to speculate that oLCs bind Phl p 5 via FcεRI (data not shown). Nevertheless, we could demonstrate similar binding of Phl p 5 to oLCs of individuals with GPA and nonatopic individuals. Furthermore, we could not detect a different binding pattern in individuals with GPA with elevated specific Phl p serum IgE and nonatopic individuals (data not shown). However, it is difficult to draw a final conclusion about the involvement of FcεRI on oLCs in Phl p 5 binding because local mucosal IgE appears more critical than specific serum IgE. Also, it remains a matter of further investigations whether oLCs of grass pollen-sensitized individuals bind Phl p 5 via specific IgE bound to FcεRI in general or whether only oLCs in a subgroup of patients such as monosensitized individuals bind Phl p 5 via FcεRI. In view of the data obtained in this study, it is most likely that other mechanisms at least in part predominate. In this regard, a previous study demonstrated that C-type lectins are

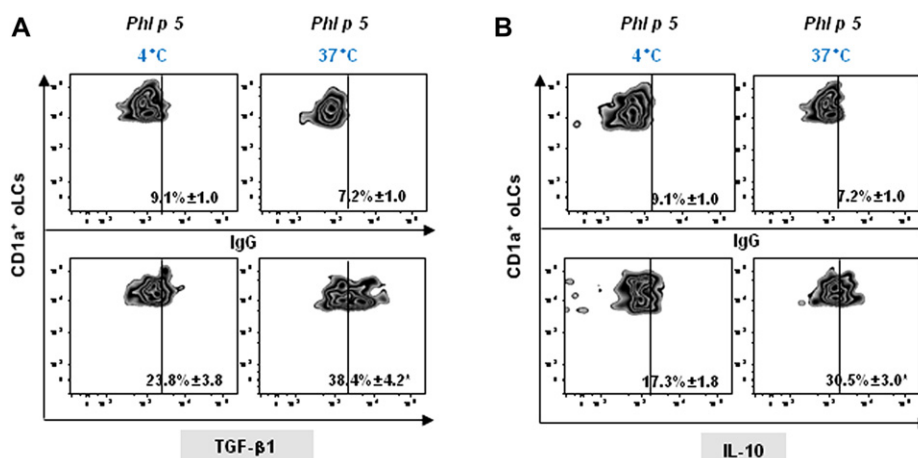


FIG 6. Phl p 5 binding to oLCs increases their TGF-β1 and IL-10 production. Oral mucosal tissue was incubated with Phl p 5 (100 μg/mL; 60 minutes) at 4°C and 37°C (n = 17). After migration of oLCs for 3 hours, intracellular staining of CD1a⁺ cells for TGF-β1 (A) and IL-10 (B) with respective isotype controls (upper row) was performed. A significantly higher production of TGF-β1 and IL-10 could be detected in oLCs, which migrated from tissue incubated with Phl p 5 at 37°C. *P < .05.

involved in the uptake of dextran by DCs.¹³ Because we showed that oLCs are capable of binding dextran and Phl p 5 at comparable levels, C-type lectins might also be involved in the binding of Phl p 5. In this context, besides CD207, CD209 might be involved, which plays a pivotal role in dextran uptake²⁴ and which could be detected in significantly higher amounts on migrating Phl p 5-positive oLCs in this study (data not shown). Further studies need to reveal which particular receptors are involved in allergen binding to oLCs. Moreover, future studies will show whether adjuvants¹⁴ or mucoadhesive formulations²⁵ applied during SLIT might enhance allergen uptake by local oLCs. Nonetheless, because an *ex vivo* model was used in our study, it has to be considered that mechanisms other than oLC migration play a role *in vivo* and that the efficiency of allergen uptake by oLCs may exceed the sensitivity of our *ex vivo* assay. However, because Phl p 5 binding to oLCs led to an enhanced migration out of mucosal tissue compared with the control condition *in vitro*, it appears that specific migration of oLCs after Phl p 5 binding plays a role.

In relation to immunomechanisms underlying SLIT, allergen-specific tolerance induction has been postulated as a key mechanism. In this report, we demonstrated for the first time that Phl p 5 binding to oLCs leads to an enhanced production of TGF-β1 and IL-10 by oLCs. It is well accepted that both cytokines are important for the induction of tolerance.²⁶ The induction of TGF-β1 and IL-10-producing T cells by oLCs after uptake of Phl p 5 further supports this suggestion. Because we could not detect profound induction of FoxP3 in cocultured T cells, it is most likely that type 1 regulatory T cells as well as T_{H3} cells, which produce IL-10 and TGF-β1 in a cell contact-dependent way, rather than naturally occurring CD4⁺CD25⁺FoxP3⁺ T regulatory cells are induced in this system.²⁶ The decelerated maturation of oLCs on Phl p 5 binding observed here underlines in addition the tolerogenic properties of oLCs, because it has been shown that immature and semimature DCs preferentially induce tolerance.^{27,28} Nevertheless, the accelerated migration in combination with the immature phenotype of oLCs after Phl p 5 binding remains in contrast with other investigations, which demonstrated that migrating DCs from skin are fully matured.²⁹⁻³¹ Of course,

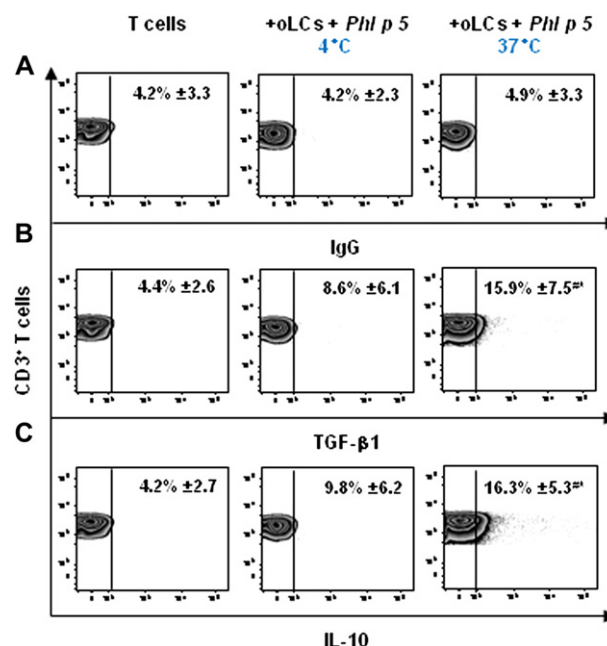


FIG 7. Phl p 5 binding to oLCs promotes TGF-β1 and IL-10 production of cocultured T cells. T cells were cultured alone (first column), together with oLCs migrated from tissue, which was incubated with Phl p 5 (100 μg/mL) at 4°C (second column) or oLCs migrated from tissue that was incubated with Phl p 5 (100 μg/mL) at 37°C (third column; n = 9). After 5 days of coculture, T cells were detected by CD3 expression (y-axis, zebra plots) and analyzed for expression of TGF-β1 (B) and IL-10 (C) compared with isotype controls (A). T cells cocultured with oLCs from tissue that was previously incubated with Phl p 5 at 37°C produced increased amounts of TGF-β1 and IL-10; *P < .05 compared with T cells + oLCs + Phl p 5 (100 μg/mL) at 4°C; #P < .05 compared with T cells alone.

this phenomenon might result from the culture condition used in our system. However, we demonstrated that LCs that migrated from epidermal sheets after allergen stimulation under the same culture conditions as in our mucosal model displayed high expression of the DC maturation marker CD83 and the DC homing factor CCR7 (data not shown). In view of these data, a picture

emerges that migration of immature oLCs might be a hallmark of mucosal DCs. In this regard, the oral mucosal microenvironment might play a pivotal role because it represents a preferential tolerogenic site, which might preserve the immature phenotype of oLCs by soluble factors such as TGF- β 1.^{32,33} However, the CD83 and CCR7 are important for DC maturation and migration to regional lymph nodes, where they present allergen to naive T cells.^{30,34} This might emphasize that oLCs display prolonged homing to regional lymph nodes or local lymphatic tissue. In addition, it is possible that oLCs contact T cells to elicit an immune response apart from the regional lymph nodes locally in the tissue. In this context, it has been reported that oLCs and T cells colocalize directly within the rete ridges and lamina propria in the oral mucosa.^{35,36}

Nonetheless, further investigations need to focus on the specificity of the Phl p 5-associated mechanisms. Although we could observe TGF- β 1 and IL-10 production in oLCs on Der p 2 binding (data not shown), more studies are needed to confirm whether tolerogenic mechanisms prevail in the oral mucosa in response to other proteins or protein aggregates.

In conclusion, this report provides evidence that oLCs bind Phl p 5 during resorption in a time-dependent and dose-dependent manner. The reported induction of tolerogenic cytokines supports the concept of tolerance induction in SLIT mediated by local oLCs. The presented data might serve as a basis for further studies developing new strategies to optimize SLIT with the help of adjuvants or mucoadhesive formulations.

Clinical implications: These results point out the role of oLCs in allergen binding during SLIT and shed light on underlying tolerogenic mechanisms mediated by oLCs.

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METHODS

Reagents

CD1a was detected by mouse antibody (mAb) phycoerythrin-labeled T6RD1 (Beckman-Coulter, Krefeld, Germany) or phycoerythrin-carbocyanin 5-labeled CD1a (clone HI149; BD Biosciences, Heidelberg, Germany). Phycoerythrin-labeled TGF- β 1 (clone TB21) was from IQ Products (Groningen, The Netherlands). Unconjugated mAb clone 130002 detecting B7-H1, mAb clone 185504 detecting B7-H3, mAb clone 53103.111 against CCR6, and mAb clone 150503 detecting CCR7 was purchased from R&D Systems (Minneapolis, Minn). mAb clone hFoxy against FoxP3 was from eBioscience (Nattutec, Frankfurt, Germany). Phycoerythrin-conjugated antibody (clone JES3-19F1) was used to detect IL-10, mAb (clone HB15e) to detect CD83, mAb clone DCN46 to detect DC-Sign/CD209, and allophycocyanin-carbocyanin 7-labeled clone SK7 to detect CD3 (BD Biosciences). mAb against CD207 (clone DCGM4) was from Beckman Coulter (Fullerton, Calif). MOPC-141 (IgG_{2b}), MOPC-21 (IgG₁), UPC10 (IgG₂; Sigma, Deisenhofen, Germany), and IgG₁ (RD1; Beckman Coulter) were used as appropriate isotype controls. APC-conjugated goat antimouse (GaM/FITC) antibody was from Jackson Laboratories (West Grove, Pa). Normal mouse serum for blocking

purposes was obtained from Dianova (Hamburg, Germany), and 7-amino-actinomycin-D was from Sigma. All other reagents were from Sigma unless otherwise stated.

T-cell cocultures

Migrated CD1a⁺ cells were purified by magnetic microbead-labeled CD1a antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) and the AUTOMACs technique (Miltenyi Biotec) as described in the manufacturer's instructions. Proliferation assays were performed with allogeneic T cells as responder cells. Allogeneic CD4⁺ T cells were isolated from PBMCs from healthy donors by magnetic microbead-labeled antibodies (Miltenyi Biotec) and the AUTOMACs technique (Miltenyi Biotec) as described in the manufacturer's manual. Triplicates of CD1a⁺ cells containing 200 viable CD1a⁺ cells/well were incubated with 100,000 viable allogeneic T cells at 37°C for 3 days before activation with ionomycin and phorbol 12-myristate 13-acetate. After 6 hours, brefeldin A was added for another 3 hours before staining. IL-10 and TGF- β 1 cytokine production was detected in CD3⁺ T cells by intracellular staining using the BD Cytotfix/Cytoperm kit (BD Biosciences) following the instruction manual.