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Dendritic Cells in Distinct Oral Mucosal Tissues Engage Different Mechanisms To Prime CD8+ T Cells

Itay Nudel,* Mazal Elnekave,* Karina Furmanov,* Moran Arison,* Björn E. Clausen,† Asaf Wilensky,‡ and Avi-Hai Hovav*†

Although oral dendritic cells (DCs) were shown to induce cell-mediated immunity, the identity and function of the various oral DC subsets involved in this process is unclear. In this study, we examined the mechanisms used by DCs of the buccal mucosa and of the lining mucosa to elicit immune responses. After plasmid DNA immunization, buccally immunized mice generated robust local and systemic CD8+ T cell responses, whereas lower responses were seen by lining immunization. A delayed Ag presentation was monitored in vivo in both groups; yet, a more efficient presentation was mediated by buccal-derived DCs. Restricting transgene expression to CD11c+ cells resulted in diminished CD8+ T cell responses in both oral tissues, suggesting that immune induction is mediated mainly by cross-presentation. We then identified, in addition to the previously characterized Langerhans cells (LCs) and interstitial dendritic cells (iDCs), a third DC subset expressing the CD103+ molecule, which represents an uncharacterized subset of oral DCs expressing the langerin receptor (Ln+iDCs). Using Langerin-DTR mice, we demonstrated that whereas LCs and Ln+iDCs were dispensable for T cell induction in lining-immunized mice, LCs were essential for optimal CD8+ T cell priming in the buccal mucosa. Buccal LCs, however, failed to directly present Ag to CD8+ T cells, an activity that was mediated by buccal iDCs and Ln+iDCs. Taken together, our findings suggest that the mechanisms engaged by oral DCs to prime T cells vary between oral mucosal tissues, thus emphasizing the complexity of the oral immune network. Furthermore, we found a novel regulatory role for buccal LCs in potentiating CD8+ T cell responses. The Journal of Immunology, 2011, 186: 891–900.

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Although oral dendritic cells (DCs) were shown to induce cell-mediated immunity, the identity and function of the various oral DC subsets involved in this process is unclear. In this study, we examined the mechanisms used by DCs of the buccal mucosa and of the lining mucosa to elicit immune responses. After plasmid DNA immunization, buccally immunized mice generated robust local and systemic CD8+ T cell responses, whereas lower responses were seen by lining immunization. A delayed Ag presentation was monitored in vivo in both groups; yet, a more efficient presentation was mediated by buccal-derived DCs. Restricting transgene expression to CD11c+ cells resulted in diminished CD8+ T cell responses in both oral tissues, suggesting that immune induction is mediated mainly by cross-presentation. We then identified, in addition to the previously characterized Langerhans cells (LCs) and interstitial dendritic cells (iDCs), a third DC subset expressing the CD103+ molecule, which represents an uncharacterized subset of oral DCs expressing the langerin receptor (Ln+iDCs). Using Langerin-DTR mice, we demonstrated that whereas LCs and Ln+iDCs were dispensable for T cell induction in lining-immunized mice, LCs were essential for optimal CD8+ T cell priming in the buccal mucosa. Buccal LCs, however, failed to directly present Ag to CD8+ T cells, an activity that was mediated by buccal iDCs and Ln+iDCs. Taken together, our findings suggest that the mechanisms engaged by oral DCs to prime T cells vary between oral mucosal tissues, thus emphasizing the complexity of the oral immune network. Furthermore, we found a novel regulatory role for buccal LCs in potentiating CD8+ T cell responses.
unique for LCs (18–20). LN-dCcs present different origin and functional qualities than that of LCs and were found to mediate T cell priming in some experimental models (21–23). Nevertheless, whereas data are rapidly accumulating emphasizing the complexity of DC function in the body, DCs of the oral mucosa have been generally overlooked, and most of our knowledge of these cells is based on research of other tissues. Several works studying sublingual immunotherapy have analyzed oral DCs with regard to their tolerogenic function (24, 25). In contrast, oral DCs were found to prime T cells in other systems (14, 26–28), suggesting their involvement in immune induction. Yet, our understanding of the precise mechanism used by oral tissues to elicit T cell responses and the contribution of each oral DC subset to this process is very limited. In this work, we focused our analysis on two representative oral areas: the buccal mucosa, covering the inside of the cheek, and the lining mucosa anterior to the murine incisors, including the gingival papilla and the mucosa toward the midline of the lower lip (hereinafter termed lining mucosa). These anatomically distinct tissues contain DC subsets that were found to be involved in various oral diseases such as lichen planus and periodontitis (29, 30). We believe that by studying these oral DCs, we can increase our understanding of their role during disease and harness their capacity for eliciting protective oral immunity.

Materials and Methods

Abs and reagents

The Abs used in this study were directly coupled to FITC, PE, allophycocyanin, Pacific blue, or PerCP-Cy5.5. The following monoclonal Abs were used: anti-CD8α (53-6.7; BD Biosciences), anti-CD103 (2E7; BioLegend), anti-CD11c (HL3; BD Biosciences), and anti–Ep-CAM (G8.8; BioLegend). The SⅠNFEKⅣLH-2Kc tetramer was purchased from Beckman Coulter. CFSE was purchased from Molecular Probes (Invitrogen).

Mice

Six- to twelve-week-old knock-in mice expressing the human diphtheria toxin receptor (DTR) under transcriptional control of the endogenous Langerin/CD207 promoter (Lang-DTR) were bred in our facility and maintained under specific pathogen-free conditions (31). The identity of the mice used for experiments was confirmed by genotyping with the following PCR primers: forward, 5′-GCCACCATGAACTGCTGCGC-3′; reverse, 5′-ATAAGTTATCCCGGTCCTTACTTTGAC-3′. OT-I mice were purchased from The Jackson Laboratory and used at the age of 6–8 wk. C57BL/6 (B6) and BALB/c mice were purchased from Harlan Laboratories (Jerusalem, Israel). Research on mice was approved by the Hebrew University (Jerusalem, Israel) Institutional Animal Care and Ethics Committee.

Vectors and immunizations

Mice were anesthetized with a ketamine/xylazine mixture prior to injection into the buccal mucosa, lining mucosa, or ear pinna using a 31-gauge needle. For buccal or ear pinna immunizations, 50 μg DNA was diluted in 80 μl PBS, and 40 μl was delivered into each side. For lining immunization, a single injection of 50 μg DNA was administered anterior to mouse incisors. Intramuscular injection was performed using insulin syringes, and the mice received 50 μg DNA in 100 μl total injection volume (50 μl was delivered into each quadriceps muscle). The pACB-OWA plasmid was a gift from Dr. Maripat Corr (University of California, San Diego, La Jolla, CA). The CD11c-OVA and K14-OVA plasmids were kindly provided by Dr. Boris Jacob (Emory Vaccine Center, Atlanta, GA). Plasmid encoding the firefly luciferase (VRC-LUC) was generously provided by Dr. Norman Letvin (Division of Viral Pathogenesis, Harvard University, Boston, MA). For the protection assay, the highly immunogenic (in BALB/c genetic background) catatlytic fragment of arginine-specific gingipain A (RgpA) of Porphyromonas gingivalis (aa 228–688) was cloned (32). The relevant segment of rgpA was amplified by PCR from genomic DNA of P. gingivalis strain 53977 using the following primers: forward, 5′-TAAGCTTATGGAGGAAACAAATTGTC-3′; reverse, 5′-TAGCTTATGGTCTGGCCGTG-3′. The gene segment was then inserted into pcDNA3.1(+)(Invitrogen) to generate the DNA-RgpAc Clone. All plasmids used in this study were prepared using the Endo-Free plasmid Giga kit (Qiagen), and the levels of endotoxin in the DNA preparations were always lower than 0.1 endotoxin unit/μg DNA.

Ablation of langerin-expressing cells in vivo

Mice were treated i.p. with 1 μg diphertheria toxin (DT) (Sigma-Aldrich) in 100 μl PBS every 10 d starting 3 d before immunization. To deplete selectively only LCs, the mice were treated once with DT 7 d before immunization. Similar administration of DT into wild-type B6 mice did not influence the magnitude and kinetics of the examined immune responses.

Tetramer analysis

Blood was collected from individual mice in RPMI 1640 medium containing 40 U heparin per ml, and PBMCs were isolated using Lympholyte-M (Cedarlane). Cells were washed with PBS containing 2% FCS and stained for 15 min at room temperature (RT) with H-2Kb/SⅠNFEKⅣL tetramers. The cells were then stained with magnetic anti-CD8a Ab for an additional 15 min, RT, and washed with PBS containing 2% FCS. Samples were collected on an LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Bioimaging of luciferase protein expression

Bioimaging of in vivo firefly luciferase gene expression was performed using the Roper Chemiluminescence Imaging System (model LN/CCD-1300EB; Roper Scientific, Princeton Instrument, Trenton, NJ). Mice were anesthetized with ketamine/xylazine mix and injected i.p. with 500 μl of an isotonic salt solution containing 30 mg/ml D-Luciferin (Xenogen). Ten minutes after luciferin injection, photon emissions were measured, and raw data were analyzed using the MetaView software to assess photon flux in regions of interest in each mouse.

FITC painting

FITC (Sigma) was dissolved as a 20% (w/v) solution in DMSO (Sigma-Aldrich) and then diluted to 2% (v/v) FITC solution prepared in acetate and dibutyl phthalate (DBP, 1:1). Mice were painted on both sides of the buccal mucosa or on the lining mucosa anterior to the murine incisors 3 d before harvesting the draining lymph nodes (LNs).

In vivo T cell proliferation assay

Splenocytes were obtained from OT-I mice, washed with PBS, and incubated with equal volume of 10 μM CFSE in HBSS for 10 min at 37°C to achieve a final concentration of 5 μM. Labelling was quenched by adding an excess of ice-cold RPMI 1640 complete medium, and the cells were washed twice with PBS. CFSE-labeled splenocytes (2 × 10⁶) in 200 μl PBS were transferred into plasmid DNA-immunized B6 mice by i.v. tail injection. Three days after the cell transfer, the mice were sacrificed, and the draining LNs were harvested. The level of CFSE dilution was determined by flow cytometry using anti-CD8α Ab.

Ag-presentation assays

The draining LNs were collected from immunized mice and treated with collagenase type II (1 mg/ml; Worthington Biochemicals) and DNase I (1 mg/ml; Roche) solution in PBS plus 2% FCS for 20 min at 37°C in a shaking bath. Then, 20 μl 0.5 M EDTA per 2-ml sample was added to the digested LNs and incubated for an additional 10 min. The cells were then washed and filtered. CD11c+ cells were obtained from the digested LNs by positive isolation using MACS Microbeads according to the manufacturer’s instructions (Miltenyi Biotec). The enriched CD11c+ cells were stained with Abs against CD8α, CD103, CD11c, and Ep-CAM and then subjected to sorting by flow cytometry (FACS Aria; BD Biosciences). OT-I CD8+ T cells were purified by negative selection with the EasySep mouse CD8+ T cell enrichment kit according to the manufacturer’s instructions (Stem Cell Technologies). The purified T cells were cocultured (5 × 10⁵/well) with the different DC populations (3 × 10⁶/well) in 96-well U-bottom plates (Nunc). The cultures were then incubated for 60 h, and the supernatants were collected and stored at −80°C immediately for measurement of IFN-γ secretion.

Isolation of lymphocytes and DCs from oral tissues

The buccal and lining tissues were excised and pooled from 4 or 16 mice to achieve a final concentration of 5 μM. Labelling was quenched by adding an excess of ice-cold RPMI 1640 complete medium, and the cells were washed twice with PBS. CFSE-labeled splenocytes (2 × 10⁶) in 200 μl PBS were transferred into plasmid DNA-immunized B6 mice by i.v. tail injection. Three days after the cell transfer, the mice were sacrificed, and the draining LNs were harvested. The level of CFSE dilution was determined by flow cytometry using anti-CD8α Ab.
anti-CD8α Ab to identify Ag-specific CD8+ T cells. For DC isolation, the cells were washed, filtered, and CD11c+ cells were enriched using MACS Microbeads as described earlier followed by staining with Abs against CD103, CD11c, and Ep-CAM. The stained lymphocyte and DC samples were further analyzed by flow cytometry.

**IFN-γ secretion**

The level of IFN-γ in the supernatants was measured using an ELISA MAX mouse IFN-γ kit (BioLegend) according to the manufacturer’s instructions. Cytokine levels were determined using standard curves of recombinant cytokines and are expressed as picograms per milliliter.

**Alveolar bone loss after oral infection with P. gingivalis**

The DNA-RgpA/Acet plasmid was injected into the lining mucosa or quadriceps muscle of BALB/c mice as described earlier, and the injections were repeated two more times at 4-wk intervals. The control group was injected with empty plasmid in the lining mucosa. Four weeks after the last immunization, the mice were treated with 0.4% trimethoprim and sulfamethoxazole solution (Resprim; Teva) in the drinking water for 10 d followed by 3 d without antibiotics. The mice were then infected via oral gavage, three times at 2-d intervals, with 4 × 10^7 CFU P. gingivalis strain 33277 in a 2% carboxymethylcellulose solution (Sigma). Uninfected mice were treated with vehicle alone. Six weeks later, the mice were sacrificed, and the hemimaxillae were harvested and scanned using micro computerized tomography (μCT; Scanco Medical). Three-dimensional alveolar bone loss was quantified as previously reported (33). Approximately 200 12-μm-wide slices were scanned for each sample, covering the entire buccopalatal aspect.

**Serum analysis**

Three weeks after immunization, blood was drawn from the mice, and the sera were stored at −80°C. Ninety-six-well plates (Nunc) were coated overnight at 4°C with 1 µg OVA protein/well in 0.1 M bicarbonate buffer (pH 9). The plates were washed twice with PBS-0.02% Tween 20 and blocked with PBS 10% FCS (2 h at RT). Subsequently, mouse serum samples diluted serially in PBS were added to the wells for 3 h incubation at RT. This was followed by four washes in PBS-0.02% Tween 20 and the addition of anti-mouse peroxidase-conjugated IgG Ab (Jackson Immunoresearch Laboratories). After incubation for 2 h at RT, the plates were washed five times, and 100 µl/well TMB solution (Southern Biotech) was added for 5 min followed by the addition of 100 µl TMB stop solution (Southern Biotech). Absorption was read at 450 nm using an iMARK microplate reader (Bio-Rad).

**Statistical analysis**

Data were expressed as mean ± SEM. Statistical tests were performed using one-way ANOVA and Student t test. A p value <0.05 was considered significant.

**Results**

**Kinetics of Ag expression and immune induction by oral mucosal tissues after plasmid DNA immunization**

We were interested in exploring the capacity of distinct oral tissues, the buccal mucosa and the lining mucosa, to generate cell-media
ted immunity after exposure to an Ag. Because expression of a transgene was shown to influence the development of T cell responses (34), we characterized the kinetics of Ag expression in each oral tissue. B6 mice were immunized in the buccal or lining mucosa (anterior to the murine incisors) with plasmid DNA en
coding the luciferase gene (VRC-LUC), and expression of lucif
erase was monitored in vivo. After both injections, expression of luciferase was localized at the sites of injection; however, the subsequent kinetics of transgene expression varied with the site of plasmid DNA administration (Fig. 1A, 1B). After buccal immu
nization, expression of the transgene was maximal 1 d after in
jection and then decreased gradually over the ensuing days. Administration of plasmid DNA into the lining mucosa resulted in a higher peak level of transgene expression, and this expression reached its maximum level at a slower pace, with peak luciferase protein expression observed around 1 wk postimmunization. Nevertheless, in both sites the level of Ag expression decreased eventually and was comparable 2 wk after immunization.

To evaluate the generation of Ag-specific CD8+ T cells by these oral tissues, we immunized mice with a plasmid DNA encoding the OVA gene (pACB-OVA). The immunized mice were bled 2 wk after immunization to assess the magnitude of OVA-specific CD8+ T cells using H-2Kb tetramers directed against the OVA MHC class I epitope SIINFEKL. This time point was selected because it represents the time of peak CD8+ T cell responses in the immunized mice (data not shown). A higher percentage of SIINFEKL-
specific CD8+ T cells was found in PBMCs of mice immunized buccally than in PBMCs of mice immunized in the lining mucosa (p < 0.05; Fig. 1C). These SIINFEKL-specific CD8+ T cell re
sponses in buccal- and lining-immunized mice were comparable with those obtained by intradermal (i.d.; p = 0.21) or i.m. (p = 0.31) immunization with plasmid DNA, respectively. We further examined whether SIINFEKL-specific CD8+ T cells could be found in the site of injection (i.e., the buccal mucosa and lining mucosa). As shown in Supplemental Fig. 1, although low numbers of CD8+ T cells were isolated, we were able to identify tetramer-positive CD8+ T cells in these oral tissues, suggesting the de
velopment of local immunity in addition to systemic immune responses. We next measured the elicitation of Ag-specific IgG Abs in sera drawn from the immunized mice 3 wk after immu
nization. Substantial titers of OVA-specific IgG were produced by each group; nevertheless, unlike the CD8+ T cells responses, no significant differences were seen across the various immunized groups (Fig. 1D). Of note, no OVA-specific IgA Abs were found in the saliva of buccally or lining-immunized mice (data not shown). Taken together, these findings demonstrate that the buccal mucosa and lining mucosa vary in their capacity to express an Ag and elicit CD8+ T cell immunity after immunization with plasmid DNA. In addition, targeting of these oral tissues as immune inductive sites can generate robust systemic as well as local mucosal immune responses.

**Characterization of oral tissue-induced immunity**

To study further the mechanisms of T cell induction by buccal and lining immunization, we restricted Ag expression to DCs by immu
nizing mice with plasmid DNA in which expression of the OVA Ag was driven by the CD11c promoter (CD11c-OVA). This CD11c-OVA plasmid was previously shown to express high levels of the OVA transgene after i.d. immunization (35). After administra
tion of CD11c-OVA, we detected a significant decrease in the CD8+ T cell responses in both oral tissues compared with that of mice immunized with the pACB-OVA plasmid, a construct using the ubiquitous CMV promoter (Fig. 2A). This suggests that direct presentation is not the major mechanism used by oral DCs to prime CD8+ T cells. In agreement, limiting OVA expression to keratinocytes, by using the keratinocyte-specific K14 promoter (K14-OVA), was able to restore, in part, CD8+ T cell responses in the lining mucosa, which consists of keratinized cell layers (Supplemental Fig. 2) (36). Such effect, however, was limited in the nonkeratinized buccal mucosa, probably due to the rarity of K14-expressor cells in this tissue (37). Next, our earlier data demon
strated that buccal and lining immunizations seem to mimic i.d. and i.m. immunization, respectively. We previously reported that i.
d. boosting with plasmid DNA failed to generate enhanced sec
dondary T cell responses, whereas i.m. boosting was able to do so (38). Therefore, we tested the capability of the oral tissues to elicit secondary CD8+ T cells after homologous prime-boost immunization with plasmid DNA. As demonstrated in Fig. 2B, similar to i.
d. immunization, 2 wk after immunization buccally boosted mice developed a secondary CD8+ T cell response that was comparable.
with that obtained after priming ($p = 0.319$). In contrast, lining immunization generated a robust secondary response, reaching $\sim 8$ times more CD8$^+$ T cells than that measured after priming ($p = 0.028$). Finally, we examined the protective efficacy of oral immunization by exposing susceptible BALB/c mice to the oral pathogen $P. gingivalis$, which induces resorption of alveolar bone after infection. We focused our analysis on lining immunization for two important reasons: First, $P. gingivalis$ establishes infection in the gingival pocket, thus making it preferable to immunize mice in the lining mucosa that constitutes the gingiva. Second, as we showed earlier in this study, lining immunization, but not buccal immunization, induces enhanced immune responses after boosting, a feature important for generating protective immunity. As demonstrated in Fig. 2C, repeated immunizations with DNA-RgpAcat in the lining mucosa were able to protect the mice against $P. gingivalis$-induced alveolar bone loss as determined by $\mu$CT ($p < 0.0001$ compared with immunization with sham plasmid). The protection level achieved was comparable with that obtained by i.m. immunization. Collectively, these findings demonstrate that immune induction by buccal mucosa and lining mucosa depends on Ag expression in non-CD11c cells. In addition, this orally induced immunity can confer protection against a periodontal pathogen.

In vivo Ag presentation after administration of plasmid DNA to the buccal mucosa or lining mucosa

To study further the mechanisms used by the buccal and lining mucosal sites to prime CD8$^+$ T cell, we examined the kinetics of Ag presentation in vivo. Groups of B6 mice were immunized in the buccal or lining tissues with pACB-OVA (50 μg), and the induction of SIINFEKL-specific CD8$^+$ T cells was examined in the peripheral blood of each group using H-2K$^b$/SIINFEKL tetramers. Data are shown as the percentage of CD8$^+$ tetramer-positive T cells and represent the mean of four to five mice per group ± SEM. *$p < 0.05$ compared with CMV-OVA–immunized mice.

C, BALB/c mice were immunized three times at 4-wk intervals, with DNA-RgpAcat encoding the catalytic area of the $P. gingivalis$ RgpA protease. One month later, antibiotic-pretreated mice were infected via oral gavage, three times at 2-d intervals, with $4 \times 10^8$ CFU $P. gingivalis$ strain 53977. After 6 wk, maxillas of the mice were harvested and examined for alveolar bone loss using $\mu$CT. Data are presented as the volume of alveolar bone in the buccal plate and represent the mean of six to eight mice per group ± SEM. *$p < 0.003$ compared with the other groups.
transgenic mice whose CD8+ T lymphocytes express a TCR that recognizes the SIINFEKL epitope. Three days after transfer, the draining LNs were collected, and the CFSE dilution was monitored by flow cytometry to calculate the intensity of T cell proliferation resulting from Ag presentation (Fig. 3). Administration of pACB-OVA to the buccal mucosa resulted in a moderate level of Ag-presenting activity (∼29–38% of the transferred OT-I cells) until day 6 postimmunization. Ag presentation increased gradually thereafter reaching a level of ∼50% between day 6 and 9 and ∼82% on days 9–12. After immunization in the lining mucosa, low levels of Ag-presenting activity were detected until day 6 (∼13%), and the presentation increased moderately during the second week of immunization (∼38–56%). These findings suggest that the buccal mucosa and lining mucosa induced a delayed and prolonged Ag-presentation kinetics after immunization with naked plasmid DNA. Still, a more potent Ag-presenting activity was monitored in buccally immunized mice, as opposed to lining-immunized mice, which could explain the higher magnitude of Ag-specific CD8+ T cells found in this group.

Migration of oral DCs under inflammatory conditions

Because DCs mediate T cell responses after plasmid DNA immunization (39), we sought to characterize the type of DCs migrating from the buccal mucosa or lining mucosa to the draining cervical LNs under local inflammatory conditions. For this, we carefully applied on the buccal or lining mucosal tissues 4 μl FITC/DBP solution, and the painted area was kept isolated for a few minutes for localized absorption. Three days later, the cervical LNs were collected, processed, and CD11c+ cells were enriched by magnetic bead-based positive selection. The cells were then stained with anti-CD11c, CD103, and Ep-CAM Abs to identify the various oral DC subsets based on their phenotype in the skin as previously described (23, 40): the CD11c+FITC- (iDCs) and Ep-CAM- population was separated according to the expression of CD103 (Ln+iDCs), and the CD103+ cells were further segregated into Ep-CAM+ (iDCs) and Ep-CAM+ (LCs). As demonstrated in Fig. 4A, the cell populations were easily identified in the cervical LNs by FITC painting, whereas no FITC- cells were found in unpainted mice. The figure presents FITC-positive cells migrating from the lining mucosa as an example. In addition, the three DC subsets LCs, iDCs, and Ln+iDCs were clearly identified in both buccal and lining migratory DC populations (CD11c+FITC+) with similar percentages (Fig. 4B). To assess further the presence of the three DC subsets directly in the buccal and lining tissues, the noted tissues were collected, processed, enriched for CD11c+ cells, and stained as described earlier. Supplemental Fig. 3 clearly indicates that all three DC subsets found to migrate after DBP treatment can be identified also in each oral tissue. Our data suggest that in addition to iDCs and LCs that were previously identified in the buccal and lining tissues, the recently identified Ln+iDCs also reside in these oral sites. Furthermore, under inflammatory conditions, the three DC subsets migrate to the draining cervical LNs from each oral tissue.

Different function for buccal and lining langerin+ DCs in T cell induction

To evaluate the role of each oral DC subset in T cell induction, we used the Lang-DTR mouse model that allows conditional ablation of langerin-expressing cells in vivo by the administration of DT. Mice were i.p. administered DT (1 μg per mouse) 3 d before immunization and every 10 d thereafter to eliminate both LCs and Ln+iDCs as we previously reported for the skin model (40). This DT injection schedule led to a considerable decrease in the generation of SIINFEKL-specific CD8+ T cells in the blood of buccally immunized mice but not in lining-immunized mice (Fig. 5A). To better discriminate between the contribution of LCs and Ln+iDCs of the buccal mucosa, we used an approach that takes advantage of the differential repopulation kinetics of these DC subsets (41, 42). Lang-DTR mice were treated with a single dose of DT 7 d before immunization (Fig. 5B), a strategy previously reported to delete skin LCs but not Ln+iDCs at the time of immunization (40). To confirm this approach for the buccal mucosa, we painted the buccal mucosa of mice with FITC/DBP solution and treated them with either a single injection of DT or with repeated DT injections. The draining LNs were collected from the mice 2–3 d later, and the frequencies of migratory LCs and Ln+iDCs were analyzed. As shown in Fig. 6, a single injection of DT on day ∼7 considerably diminished the percentage of buccal-derived LCs in the LNs, whereas the Ln+iDC population was fully restored. In contrast, repeated DT injections resulted in a significant reduction of both DC subsets in the LNs. We then examined the influence of LC ablation on T cell induction in buccally immunized mice and found diminished magnitude of SIINFEKL-specific CD8+ T cells due to LC ablation (Fig. 5B). Notably, DT treatment resulted also in elevated OVA-specific IgG titers in both buccally and lining-immunized mice (Fig. 5C). These results suggest that the role of langerin-expressing DCs vary between the buccal and lining tissues. Whereas LCs and Ln+iDCs are dispensable for T cell induction in the lining mucosa, LCs of the buccal mucosa play a critical role in potentiating the elicitation of CD8+ T cells. Finally, as we reported earlier the
differential boosting capacity of each tissue after secondary immunization with plasmid DNA, we examined now the impact of DT treatment during priming on secondary immune responses. Our analyses indicated that whereas secondary CD8+ T cell responses were not affected by the treatment, ablation of langerin+ DCs in both tissues resulted in higher elicitation of Ag-specific Abs (Supplemental Fig. 4).

**Oral mucosal DCs present Ag to CD8+ T cells**

The ability of tissue-resident DCs to present Ag directly to T cells was challenged recently and seems to be vector-dependent. To address this issue in our system, we examined the contribution of each oral DC subset in Ag presentation. Based on our in vivo results, Ag-presenting activity can be hardly detected during the first week of immunization (Fig. 3), and examination of DC function before this time is not accurate. Thus, we collected LNs first week of immunization (Fig. 3), and examination of DCs was performed in situ using unique presentation of the Ag by DCs. In addition, the inability of DCs to induce IFN-γ secretion by OT-I cells, whereas their ablation in vivo reduced T cell expansion, suggests a regulatory role for these cells during T cell priming.

**Discussion**

The existence of distinct mucosal tissues in the oral cavity suggests that these areas may act differently during infection or immunization. In this study, we provided evidence that DCs of the buccal mucosa and lining mucosa used diverse mechanisms to prime CD8+ T cells after immunization with naked plasmid DNA. Our data demonstrated that LNs and Lndef DCs are dispensable for generating cytotoxic lymphocytes in the lining mucosa. In contrast, buccal-resident LCs were found to play a major role in potentiating plasmid DNA-induced CD8+ T cell responses. This role of LCs is probably not mediated by direct activation of CD8+ T cells, as buccal LCs failed to induce IFN-γ secretion by these cells. Therefore, it is more likely that buccal LCs act as positive regulators during plasmid DNA immunization via a mechanism that is not yet clear. Unfortunately, examining the sole contribution of buccal Lndef DCs in vivo is impossible in our system, as Lang-DTR mice allow elimination of LCs or both LCs and Lndef DCs but not Lndef DCs alone (43). Nevertheless, the ability of Lndef DCs to present Ag ex vivo and to induce IFN-γ secretion by Ag-specific CD8+ T cells suggests that these cells contribute to the overall T cell responses.

Previous studies have demonstrated that DCs of the buccal mucosa resemble skin DCs with regard to the various DC subsets populating the tissue and their migratory behavior (14, 15). Recent works studying another mucosal site, the vaginal mucosa, found large numbers of langerin+ DCs in the epithelium, whereas LCs represent only a small fraction of the DCs in this location (44, 45). Our study suggests that organization of DCs in the oral mucosa (at least in the buccal mucosa and lining mucosa) is similar to the skin rather than to the vaginal mucosa. Indeed, we were able to identify three major DC subsets migrating from the buccal mucosa under inflammatory conditions that were similar to those migrating from the skin (40). To our knowledge, this work is the first to show the presence of Lndef DCs in the buccal mucosa and lining mucosa. In the skin, this recently identified DC subset is located in the dermis in very low numbers as opposed to epidermal LCs, which made it hard to discover them before the development of Lang-DTR transgenic mice (18–20). It is very likely that these cells were overlooked in oral mucosa tissues because of the same reason. A recent work proposed the presence of two Lndef DC subsets in the skin, CD103+ Lndef DCs and CD103+ Lndef DCs (46). In our system, ablation of Lndef DCs by repeated DT injections resulted in the elimination of CD103+ DCs in the buccal mucosa (Fig. 6). This suggests that buccal-resident CD103+ DCs are similar to their counterparts in the skin, because in both tissues some of the CD103+ DCs express langerin. It is likely to assume that CD103+ Lndef DCs are also present in the buccal mucosa considering the resemblance between these tissues.
The similarities between the skin and buccal mucosa are also evident when examining kinetics of luciferase expression, Ag presentation, and CD8+ T cell responses after plasmid DNA immunization as previously reported (34). Nevertheless, our data propose that the buccal mucosa and skin diverge when it comes to the role of particular DC subsets in T cell priming. In contrast to the buccal mucosa, we recently reported that LCs of the ear skin are dispensable for CD8+ T cell priming, whereas Ln+dDCs play a critical role during this process (23). Moreover, we showed that expression of plasmid DNA-encoded Ag in skin CD11c+ cells is sufficient to prime CD8+ T cells, whereas in the current study restricting Ag expression to buccal-resident CD11c+ cells resulted in diminished CD8+ T cell responses (23). Thus, the widespread comparison of buccal DCs and skin DCs is not always correct, and care should be taken in the future to avoid such assumption in other experimental systems.

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FIGURE 5. CD8+ T cell responses after ablation of langerin-expressing DCs. A, Lang-DTR mice were injected i.p. with DT (1 μg per mouse) 3 d before immunization with pACB-OVA (50 μg) and every 10 d thereafter. *p < 0.02 compared with untreated mice. B, LCs were specifically depleted from Lang-DTR mice by a single administration of DT 7 d before immunization. In the following weeks, blood was collected from the different groups of mice to analyze the elicitation of SIINFEKL-specific CD8+ T cells. Results are shown as tetramer-positive CD8+ T cells and represent the means of five mice per group ± SEM. *p < 0.05 compared with the control group. C, Sera were collected from the Lang-DTR mice 3 wk after immunization, and the titers of OVA-specific IgG Abs were measured by standard ELISA. Data are presented as the OD values at 450 nm and represent the mean of five mice per group ± SEM. *p < 0.05 compared with DT-treated mice.

FIGURE 6. Percentages of buccal-derived LCs and Ln+dDCs in the LNs after DT treatment. Lang-DTR mice were injected i.p. with DT on days −7, −4, and −1 or alternatively received a single injection of DT on day −7. In each injection schedule, the buccal mucosa of the DT-treated mice was painted with FITC/DBP solution on day −3. The draining LNs were then collected, enriched for CD11c+ cells, stained with anti-CD11c, CD103, and Ep-CAM Abs, and analyzed by flow cytometry. Data presented are the percentages of LCs, Ln+ iDCs, and iDCs that have migrated from the skin to the LNs in DT-treated and untreated mice.
The function of LCs in vivo is still unclear. Whereas several studies have shown that LCs can present Ag to T cells and activate them, other works propose a role for LCs in immune regulation and tolerance (reviewed in Ref. 47). This knowledge, however, was mainly generated by studying epidermal LCs, whereas LCs of the oral mucosa were given little attention. The surprising discrepancy we found among buccal LCs and skin LCs after plasmid DNA immunization could be explained in several ways. First, it has been reported that LCs are more important when immunization is performed through the flank skin as opposed to ear skin, probably due to differences in the thickness of the dermis effecting Ag accessibility (48). With this regard, the buccal anatomy mimics the flank skin better than it does the ear skin (Supplemental Fig. 5). Another explanation could be the amount and location of the Ag expressed in the injection site that may facilitate LC function in the buccal mucosa but not in ear skin (47). Of note, it is unlikely to explain the unique role of buccal LCs simply by the differential repopulation kinetics of langerin+ cells in the buccal versus skin. We clearly demonstrated the ablation of buccal mucosa LCs, but not Ln+iDCs, in mice treated with DT 7 d prior to immunization; these kinetics are identical to that observed in the skin as we recently published (40). Overall, our findings suggest that LCs in various tissues may have a different role in T cell priming even when an identical immunogen is used. Notably, in contrast to the beneficial role of LCs described in this study, LCs of the genital mucosa were recently reported to dampen CD8+ T cell responses after immunization with a recombinant protein by a mechanism that may involve IL-17 production (45). Secretion of IL-17, however, was reported to be

FIGURE 7. The contribution of various buccal-derived DC subsets to Ag-presenting activity. A, B6 mice were immunized in the buccal mucosa with pACB-OVA, and 8 d later the LNs were collected from 12 mice, pooled, and enriched for CD11c+ cells. The cells were stained with Abs against CD11c, CD8α, CD103, and Ep-CAM for further separation by flow cytometry as demonstrated. B, The various DC subsets were incubated with purified OT-I CD8+ T cells, and supernatants were collected 60 h later to measure IFN-γ secretion by ELISA. *p < 0.001 compared with secretion by T cells only.

FIGURE 8. Ag presentation by lining-derived DCs. A, LNs were collected from 12 B6 mice 8 d after immunization with pACB-OVA in the lining mucosa. CD11c+ cells were enriched and stained with Abs against CD11c, CD8α, CD103, and Ep-CAM for further separation by flow cytometry as demonstrated. B, Supernatants collected 60 h after incubation of the DC subsets with purified OT-I CD8+ T cells were measured for IFN-γ secretion by ELISA. *p < 0.001 compared with secretion by T cells only. n.d., not determined.
downregulated after plasmid DNA immunization (49, 50), explaining, in some way, the differences between the buccal and genital mucosal tissues.

We found that the absence of LCs resulted in an enhanced production of OVA-specific Abs. In the case of buccal immunization, where ablation of LCs potentiates T cell responses, an increase CD4+ T cell responses may facilitate helper function for efficient activation of B cells (51). A similar phenomenon was also observed recently after gene gun immunization, reporting that LCs were required for maximal IgG1 responses (43). This, however, cannot explain the higher Ab titers in lining-immunized mice in which T cells were not affected by the ablation. Notably, the ability of LC to regulate Ab production but not T cell responses was also reported by us after intradermal plasmid DNA immunization (23). We thus suggest that LCs may have the ability to regulate Ab production in a T cell-independent manner.

It has been demonstrated that after infection or immunization, Ag presentation occurs rapidly in the LNs, reaching its maximal level between 24 and 48 h postimmunization (52–54). In our study, Ag presentation was delayed in both the buccal mucosa and lining mucosa, and a considerable activity was measured in vivo only during the second week of immunization. This unique pattern of Ag presentation seems not to be related to the oral tissue tested, as this may explain the relatively low concentrations of IFN-γ and IL-12p70 that we measured in our ex vivo assays, as T cell proliferation would have increased the number of IFN-γ-secreting CD8+ T cells in our cultures.

This work increases our understanding of the role of oral DCs in T cell priming, highlighting the unique mechanisms engaged by DCs of each oral tissue to activate CD8+ T cells. Besides demonstrating the presence of the Ln+dDC population in both buccal and lining tissues, we propose that these tissues can be promising sites for Ag presentation as they are involved in the pathogenesis.

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Disclosures
The authors have no financial conflicts of interest.

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