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Chapter 4

Comparative phenotypic and functional profiling of migratory dendritic cell subsets from human gingiva and skin

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4.1 ABSTRACT

Antigen exposure to oral mucosa is generally thought to lead to immune tolerance induction. However, very little is known about the subset composition and function of dendritic cells (DC) migrating from human oral mucosa. Here we show that migratory DC from healthy human gingival explants consist of the same phenotypic subsets in the same frequency distribution as DC migrating from human skin. Notably, the gingival CD1a+ Langerhans cell and dermal DC subsets lacked CXCR4 expression in contrast to their cutaneous counterparts, pointing to different migration mechanisms, consistent with previous observations in constructed skin and gingival equivalents. Remarkably, without any exogenous conditioning, gingival explants released higher levels of inflammatory cytokines than human skin explants, resulting in higher DC migration rates and a superior ability of migrated DC to prime allogeneic T cells and to induce type-1 effector T cell differentiation. From these observations we conclude that rather than inducing T cell tolerance, DC migrating from oral mucosa may induce effector T cell immunity and maintain a high state of alert against possible pathogenic intruders in the steady state. As such oral immunization may prove to be a viable therapeutic strategy for cancer and viral infections.

4.2 INTRODUCTION

Dendritic cells (DC) that are located in epithelia at the interface with the outside environment form a primary barrier of defense against pathogenic intruders. They are powerful antigen presenting cells (APC), linking innate to adaptive immunity. As such they perform a delicate balancing act, maintaining immune tolerance under steady-state conditions but also inducing T cell immunity when needed. During homeostasis, migrating immature DC from peripheral tissues take up antigen but do not acquire the capacity to promote functional T cell-mediated immune responses (Steinman, 2003;Steinman, 2007a). However, upon their recognition of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs respectively) through specialized receptors, they are activated, migrate to the draining Lymph Nodes (LNs), and mature into potent immune stimulators that can drive T cell induction, expansion and differentiation (Ouwehand *et al.*, 2008;Palucka *et al.*, 2010;Steinman, 2007b).

In human skin, at least five major DC subsets have been described, primarily distinguishable by their differential expression of CD1a and CD14, i.e. epidermal Langerhans cells, characterized by high levels of CD1a and Langerin expression, and four dermal DC (DDC) subsets, including CD1a$^+$ and CD14$^+$ DDC (Lindenberg *et al.*, 2013). Lindenberg et al., previously showed that the frequency distribution between these migrating subsets and thereby the eventual T cell activation outcome, depended on the activating versus regulatory cytokine balance in the skin microenvironment (Lindenberg et al., 2013). Under the influence of suppressive IL-10, migration of CD14$^+$ DDC prevails, resulting in abortive T cell priming and regulatory T cell (Treg) induction and expansion (Lindenberg et al., 2013). Under pro-inflammatory conditions (e.g. high levels of GM-CSF and/or IL-4) migration of CD1a$^+$ LC and DDC subsets is dominant, leading to Th1 and cytotoxic T cell (CTL) induction and expansion. Thus, the frequency distribution of migratory DC subsets from human skin determines subsequent T cell activation or tolerance induction. In the steady state the net outcome is maintenance of peripheral tolerance (Nestle et al., 2009;Steinman et al., 2003).

The oral cavity is daily exposed to a high burden of antigens emanating from food, bacteria, viruses, fungi, and their by-products. The oral mucosa thus forms a major interface with the outside world, and its integrity and appropriate response to antigens are crucial to maintain health (Ahlfors et al., 1996). Like gut mucosa, oral mucosa is generally assumed to be instrumental in maintaining immune tolerance against the daily onslaught of harmless food antigens and commensal bacteria. As such, the distribution of migratory DC subsets (and consequently their net T cell skewing capacity) might be expected to differ from that in skin, where in the steady-state CD1a$^+$ LC and DDC migration predominates with default priming of a type-1 T cell response in the allogeneic

mixed leukocyte response (Lindenberg et al., 2013). As yet, very little is known about DC subsets in human oral mucosa. No flow cytometric analyses of migrated DC from oral mucosa explants have been reported, due to a general scarcity of available tissue. So far LC have been mainly studied, showing their presence in oral mucosa (Barrett et al., 1996;Cruchley et al., 1989;Allam et al., 2008) and their superior ability to prime allogeneic T cells as compared to their skin counterparts (Hasséus et al., 2004). Of note, oral LC were further shown to differ from their skin counterparts by their expression of lipopolysaccharide receptor/CD14 and the high affinity receptor for IgE (FcεRI), possibly allowing for more efficient activation by gram-negative bacteria and allergen uptake, respectively (Allam et al., 2008). In addition to LC, DC-SIGN[+] DC were observed in the lamina propria of oral mucosa (Hovav, 2014).

To assess the distribution and maturation state of human oral mucosa associated migratory DC subsets, we performed flowcytometric analyses of DC migrated from gingival explants and we determined the inflammatory cytokine release profile from these explant cultures. Subsequently, the ability of the migrated DC to prime and skew a T cell response was assessed. Comparative analyses with their skin counterparts showed a similar subset distribution and activation state among gingiva-migrated DC, and even revealed their superior type-1 T cell skewing capacity. These data call for a reappraisal of the functionality of oral mucosa-associated DC subsets and shed new light on mechanisms underlying oral tolerance and immunisation.

4.3 MATERIALS AND METHODS

4.3.1 Tissue samples

Human adult skin was obtained from 15 healthy donors undergoing corrective breast or abdominal plastic surgery. Human adult gingival tissue was obtained from 15 healthy donors receiving dental implants or undergoing wisdom tooth extraction. Tissue specimens were collected after informed consent, and used in an anonymous fashion in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl) and following procedures approved by the institutional review board of the VU University medical center. The study was conducted according to the Declaration of Helsinki Principles. Skin and gingiva samples were not donor matched.

4.3.2 Immunohistochemistry

For immunohistochemical staining of gingiva and skin biopsies the samples were either snap-frozen in liquid nitrogen or embedded in paraffin. Vertical 5 µm cryostat frozen sections were cut from 10 different donors, and air-dried at room temperature on SuperFrost® gold slides (Menzel GmbH & Co KG, Braunschweig, Germany). The cryostat frozen sections were fixed in acetone (VWR, Amsterdam, the Netherlands) for 10 minutes and incubated for 60 minutes with primary monoclonal antibodies directed against the different surface markers, as listed in Table I. After washing, sections were incubated with goat anti-mouse conjugated to HRP (Envision, DakoCytomation). Subsequently slides were rinsed and incubated for 10 minutes with 3-amino-9-ethylcarbazole (Invitrogen, San Francisco, CA, USA). All sections were counter-stained with Mayer's haematoxylin (Sigma Chemical Co., St Louis, MO, USA). Negative controls were prepared by omitting the primary antibody and substituting an isotype control antibody. The sections were embedded in Aquatex® (Merck).

The 5 µm paraffin embedded sections were deparaffinised and rehydrated in preparation for immunohistochemical analysis, carried out as previously described (Ouwehand et al., 2011). In brief, antigen-retrieval was performed using citrate buffer and after cooling incubated O/N at RT with primary monoclonal antibodies directed against the different surface markers (see Table 1). After washing in PBS for 5 minutes, sections were incubated for another 30 minutes with human anti-mouse conjugated to HRP. After once again washing with PBS, the slides were incubated for 10 minutes with 3-amino-9-ethylcarbazole. All sections were counter-stained with haematoxylin. Negative controls were prepared by omitting the primary antibody and substituting an isotype control antibody. The sections were embedded in Aquatex®. The number of cells was quantified with the aid of Nis Elements AR version 3.2 Software (Nikon Instruments Europe B.V., Amstelveen, the Netherlands).

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Table I: Monoclonal antibodies used for immunohistochemical staining

Primary mAb	Species	Clone	Serial #	Manufacturer
Paraffin and cryo				
Langerin	mouse IgG2b	12D6	NCL-Langerin	Leica
HLA-DR	mouse IgG1	TAL.1B5		Dako
DC-Sign	mouse IgG2bκ	DCN46		BD Pharmingen
CD14	mouse IgG2aκ	TÜK4		Dako
CD83	mouse IgG1	1H4b		Monosan
CD86	mouse IgG1	2331		BD Pharmingen
CD68	mouse IgG1κ	EBM11	M0718	Dako
Paraffin only				
CD1a	mouse IgG1	MTB1	MONX10315	Monosan
Cryo only				
CD1a	mouse IgG1	JPM30	NCL-CD1a-220	Leica

4.3.3 Quantitation of cell populations

Assessment and quantitation of cell numbers after immunostaining were performed by two independent investigators. The number of positively stained cells in the epidermis, dermis, mucosal epithelium and subjacent lamina propria, were assessed for each sample per 100 μm^2 tissue at 200x magnification, with an ocular objective of 20×, an eyepiece of 10× and a tube factor 1. The average number of stained cells was then expressed per 100 μm^2 of tissue examined.

4.3.4 Skin and gingival explant preparation and culture

An exact punch biopsy of 6 mm diameter and 3 mm deep was taken from skin or gingiva, consisting of eptihelium and underlying dermis or lamina propria, respectively. Of note, the full-thickness skin and gingiva explants were of equivalent depths to ensure valid comparisons of numbers of emigrated DC and secreted cytokines. Explants were then placed in 1 ml culture medium (i.e. minimal essential media (MEM)-alpha (Gibco, Grand Island, NY) supplemented with 20% v/v heat inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin, 2mM L-glutamine (Invitrogen), 50μM 2-ME (Merck, Whitehouse Station, NY) allowing the cells to migrate from the biopsies for 48 h, after which they were harvested and analyzed by flowcytometry. The skin and gingiva explants were discarded. Conditioned medium and

migrated cells were harvested at this time and used for further analyses by flowcytometry, a mixed lymphocyte reaction (MLR) or Cytometric Bead Assay (CBA).

4.3.5 Inflammatory Cytometric Bead Assay (CBA)

Conditioned medium that was collected from the explant cultures after 48 h was analysed for secreted IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70 using the inflammatory CBA kit (BD, San Jose, CA) according to the manufacturer's instructions and using CBA analysis software (BD Biosciences).

4.3.6 Flow cytometry

Phenotypic analyses were performed by flow cytometry. Skin or gingiva emigrated cells were washed and resuspended in PBS supplemented with 0.1% BSA and 0.1% NaN$_3$ (PBA) and incubated for 30 min. at 4°C in the presence of appropriate dilutions in PBA of FITC, PE, PerCP or APC fluorochrome-conjugated specific mAbs to CD11c, CD14, CD1a, CCR6, CCR7, CXCR4, CD163, CD3, CD4, CD8, CD19 (BD, San Jose, CA), Langerin, CD86 or CD83 (Beckman Coulter Immunotech), or corresponding isotype-matched control mAbs (BD, San Jose, CA) as described previously (Lindenberg et al., 2013). The cells were subsequently analyzed, using a FACSCalibur and Cellquest-Pro FACS analysis software (BD, San Jose, CA).

4.3.7 Allogeneic T cell differentiation induction

Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Pharma AS, Oslo, Norway) gradient centrifugation from a buffy coat (Sanquin Blood supplies, Amsterdam, the Netherlands) and the monocytes were depleted after 2 h plastic adherence, leaving peripheral blood lymhocytes (PBL). From the explants, the migrated cells were harvested and the DC population was counted. Three thousand DC (pooled per condition) were co-seeded with 30,000 PBL in a 96-well round bottom plate, in duplicate for 6 days in IMDM supplemented with 10% Human Pooled Serum (Sanquin Blood Supply, Amsterdam, the Netherlands), 100IU/ml sodium penicillin (Yamanouchi Pharma), 100IU/ml streptomycin sulphate (Radiumfarma-Fisiopharma), 2 mM L-glutamine (Invitrogen), and 0.01 mM 2-ME (Merck). On day 6 the supernatants were collected for the simultaneous flowcytometric detection of IL-2, IL-4, IL-6, IL-10, TNFα, IL-17A and IFNγ, secreted by the T cells using a Th1/Th2/Th17 CBA kit (BD, San Jose, CA) following the manufacturer's instructions and using CBA analysis software (BD Biosciences).

4.3.8 Statistical Analysis

Differences between migrated DC subsets and cytokine release levels were analysed by the unpaired student t-test or Mann-Whitney U test, and considered significant when p<0.05.

4.4 RESULTS

4.4.1 DC density and localization in skin vs gingiva

Immunohistochemical analysis of full-thickness steady-state skin and gingiva tissue revealed higher density of CD1a^{+} LC per mm^2 epithelium in skin (**figure 1A-B**). However, as shown in figure 1A, the gingival stratified squamous epithelium was considerably thicker than the epidermis of the skin, which clearly accounted for this observation. Trans-epithelial assaults by e.g. pathogens, allergens or irritants, will effectively be countered by the DC encountered on the way. We therefore decided to quantify the number of LC and interstitial DC over the full-thickness (i.e. the epithelium and the underlying connective tissue layer) of skin or gingiva over a 100 µm cross-section (see Figure 1A). As shown in Figure 1B and Table II, this resulted in an opposite result with an effectively higher density of LC per 100 µm tissue cross-section in gingiva (identified by CD1a and Langerin staining in the epithelium). Similarly, DC-SIGN[+] cell numbers (located in the connective tissue underlying the epithelium) were higher in gingiva, as were HLA-DR^{+} LC and interstitial DC (Figure 1C and Table II). The CD14^{+} and CD68^{+} cell counts were comparable between the two tissue types, as were the CD83^{+} and CD86^{+} cells (all localized to the interstitial, connective tissue), although the latter two showed considerable inter-donor variation (**Figure 1C and Table II**).

4.4.2 DC subset frequency distribution upon migration from skin or gingiva

Full-thickness skin and gingiva explants of similar size and thickness were cultured for 2 days and crawl-out cells were subsequently harvested, counted and analyzed by flow cytometry. Of note, substantially higher numbers of migrated cells were observed for gingiva (mean 32,763 cells/explant, range 22,500-50,050, n=4) than for skin (mean 10,937 cells/explant, range 7,590-15,300, n=3) (*P*=0.039). Gating on CD11c^{hi} cells, we distinguished five emigrated DC subsets, based on CD1a and CD14 expression, as described previously[6], i.e. 1) CD1a^{hi} LC, 2) CD1a^{+}CD14^{-}, 3) CD1a^{+}CD14^{+} (double positive, DP), 4) CD1a^{-}CD14^{+} , and 5) CD1a^{-}CD14^{-} (double negative, DN) interstitial DC (see **Figure 2A**). Of note, the frequency distribution of these subsets within the migrated cell population was equivalent between skin and gingiva (**Figure 2B**), with CD1a^{+}CD14^{-} interstitial DC being the most frequent. The most striking (but not significant) difference was between the DP interstitial DC subsets, where skin-emigrated DC on average contained almost twice the amount of DP cells (skin: 15% vs. gingiva: 8%).

4.4.3 Phenotypic profiling of gingiva- vs skin-emigrated DC subsets

We next studied the phenotypes of the migrated DC subsets in more detail, focusing on maturation/differentiation state (CD83, Langerin, CD163) and migratory capacity (CCR6, CXCR4) (**Figure 3**). Of note, in general very similar expression levels of these
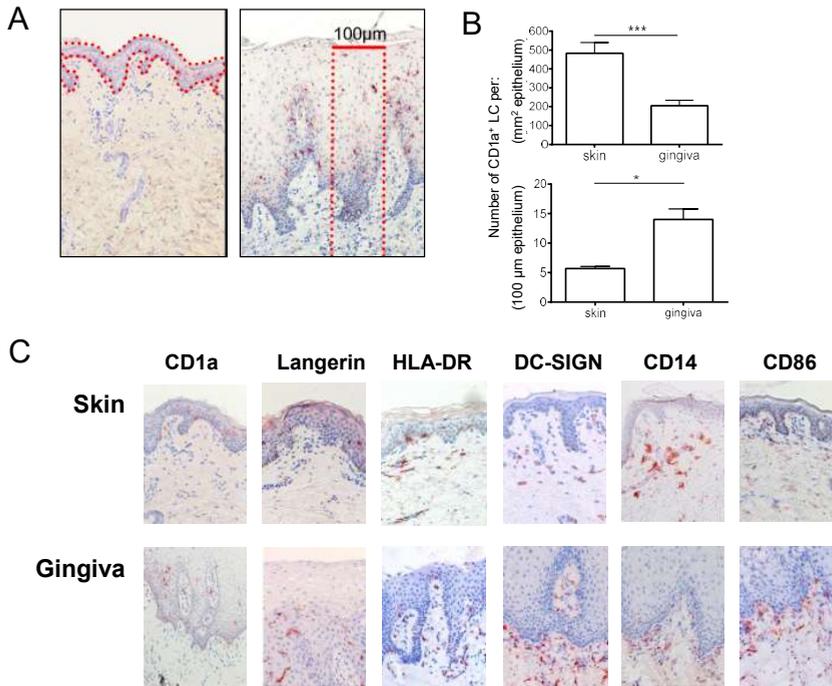
Figure 1. Dendritic cell (DC) marker expression, density and distribution over full-thickness human gingiva or skin.

A) CD1a staining of representative full-thickness skin (left panel) and gingiva (right panel) biopsies. Red dotted lines denote full-area epithelial surface (left panel, used for quantitation as shown in Figure 1B upper panel) and 100 μm wide full-thickness cross-section (right panel, used for quantitation as shown in Figure 1B lower panel, see also Materials and Methods). B) Quantitation of CD1a+ Langerhans cells (LC) according to total epithelium area (upper panel) or epithelium area in 100 μm wide full-thickness cross-section (lower panel), see areas denoted by red dotted lines in Figure 1A for the respective definitions (n=10). The number of positively stained cells in the epidermis, dermis, mucosal epithelium and subjacent lamina propria, were assessed for each sample per 100 μm^2 tissue. C) Representative staining of indicated additional DC maturation/differentiation markers shows distribution between epithelial and underlying connective tissue layers in skin and gingival biopsies (n=10). * P<0.05, ***P<0.001.

markers were observed for each particular subset, irrespective of tissue origins. Both in skin and gingiva, CD1a+ subsets were more mature as judged by CD83 expression levels, consistent with our previous observations in skin (Lindenberg et al., 2013). As expected, highest Langerin surface levels were observed in the LC subsets from both skin and gingiva. Lower but detectable Langerin expression levels were observed on the CD1a+ and DP subsets from gingiva, which were notably higher than on their counterparts from skin, although not significantly so. As previously found for skin, The M2 macrophage marker CD163 was highest on the CD14-expressing DC subsets. Expression of CCR6, generally associated with skin/epidermal homing, was very high on the CD1a+ and DN subsets, but, remarkably, for gingiva also on the CD14+ subsets.

**Table II: Comparison of cell density between full-thickness skin and gingiva per 100µm tissue cross-section**

Primary mAb	Skin*	Gingiva*
Paraffin and cryo		
CD1a	5.71 ± 1.5	14 ± 7.5
Langerin	4.83 ± 1.66	14.5 ± 9.3
DC-SIGN	6.42 ± 1.84	13.78 ± 4.7
HLA-DR	10.29 ± 1.38	18.67 ± 6.6
CD14	6.74 ± 2.32	6.63 ± 2.91
CD68	8.73 ± 2.37	8.76 ± 2.88
CD83	1.45 ± 2.16	2.24 ± 1.6
CD86	2.16 ± 2.23	0.87 ± 0.99

*Means and standard deviations, calculated over n=10

Finally, the only significant and most profound difference was found in CXCR4 surface expression levels between skin- and gingiva-emigrated LC and CD1a$^+$ interstitial DC, with high expression levels on the skin-emigrated subsets and virtually no expression on gingiva-derived subsets. This finding is highly suggestive of differential epithelium-to-connective-tissue migration mechanisms between these tissue types.

4.4.4 Gingiva explant cultures display a pro-inflammatory cytokine release profile and gingiva-emigrated DC a superior type-1 T cell induction ability compared to r skin-emigrated DC

We next tested the conditioned media from 48 h gingival and skin explant cultures for the release of inflammatory cytokines and observed strikingly higher levels of virtually all tested cytokines in the gingival cultures (**Figure 4**). IL-8, IL-6, IL-1β, IL-10 and TNFα were all significantly higher in the gingiva-conditioned cultures, whereas IL-12p70 levels were below the detection limit for both skin and gingiva. To assess and compare the ability of 48 h skin- and gingiva-emigrated DC to prime and skew T cell responses, crawl-out DC were co-cultured with a fixed number of allogeneic lymphocytes (3,000 DC: 30,000 lymphocytes) for seven days after which supernatants were harvested and the release of Th1-, Th2- and Th17-related cytokines were determined. As shown in figure 5, gingiva-derived DC turned out to be more powerful inducers of T cells. Taking IFNγ as an indicator of type-1 T cell priming, gingiva-emigrated DC on average induced 2.3-fold higher T cell reactivity. Whereas IL-6 and IL-10 may in part also derive from DC in the co-cultures, concerted release of significantly higher levels of IFNγ, IL-2
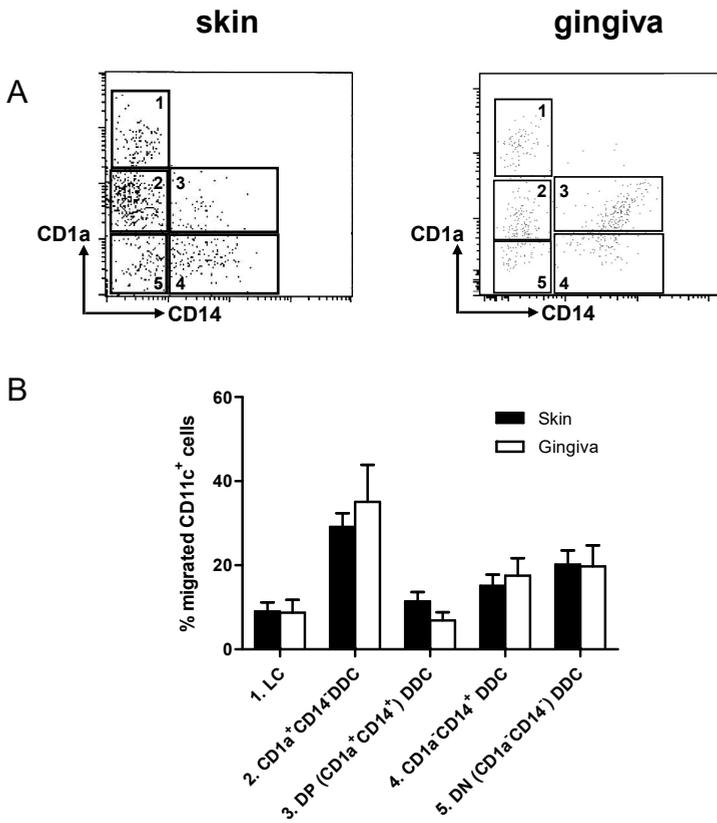
Figure 2. Dendritic cell (DC) subset definitions according to CD1aCD14 expression in DC migrated from human skin or gingiva.

A) Flow cytometry dot plots with gates denoting five migrated DC subsets (numbered 1 to 5) in skin and gingiva.

B) Frequency distribution of the five subsets among migrated DC (n=9).

and TNFα, together with a complete failure to release detectable levels of IL-4, point to a preferential Th1 skewing by gingiva-emigrated DC, and at superior levels to skin-derived DC.
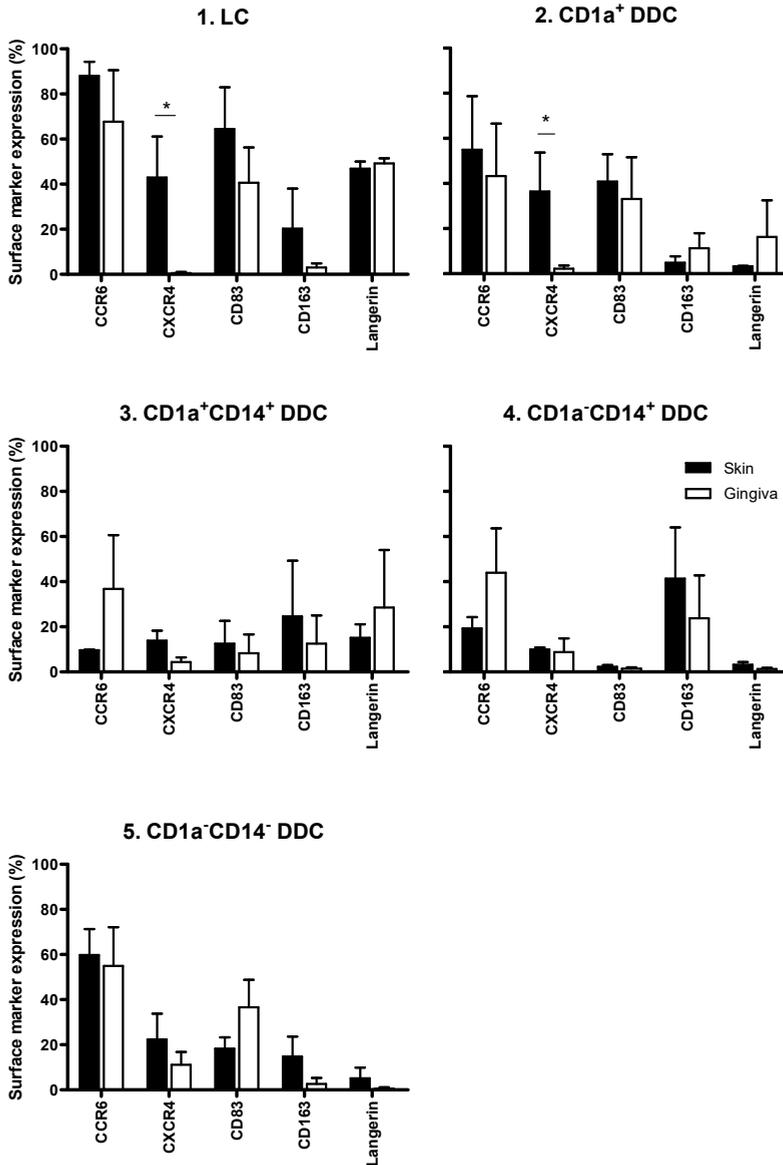
Figure 3. Phenotypic analysis of migratory dendritic cell (DC) subsets from skin and gingiva.

Chemotaxis, maturation and differentiation-associated marker expression on DC subsets 1-5 from skin vs. gingiva, shown per indicated subset (*P<0.05, n=4-9 for skin and gingiva).
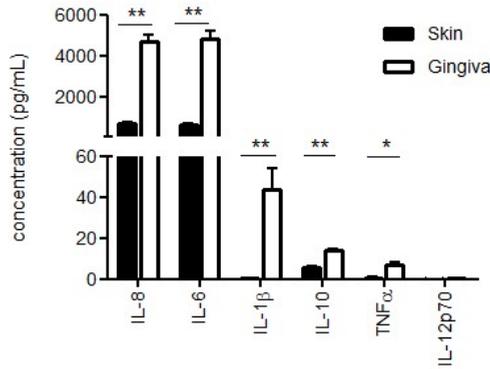
Figure 4. Inflammatory cytokine release profile of skin vs. gingival explants.

Shown in pg/ml and measured after 48 h of culture. IL-8, IL-6, IL-1β, IL-10 and TNFα were all significantly higher in the gingiva-conditioned cultures, whereas IL-12p70 levels were below the detection limit for both skin and gingiva (*P<0.05, **P<0.05; n=3 skin, n=3 gingiva).
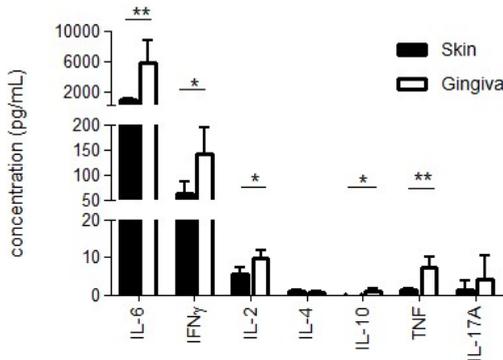
Figure 5. T cell cytokine release in allogeneic mixed leukocyte reactions with skin vs. gingiva emigrated dendritic cells (DC).

Shown in pg/ml and measured after 7 days of culture. Preferential Th1 skewing by gingiva-emigrated DC was demonstrated, and at superior levels to skin-derived DC (*P<0.05, **P<0.05; n=3 skin, n=3 gingiva).

4.5 DISCUSSION

Traditionally the oral route of antigen delivery is regarded as a sure way to induce immune tolerance. There is however a gap in our knowledge of differences in the phenotype and functionality between DC subsets of human skin and oral mucosa, which in large part will determine the outcome of T cell induction upon antigen exposure. The data provided in this manuscript are a first step towards a more detailed inventory and phenotypic and functional profiling of DC subsets in the oral mucosa, more specifically gingiva, in comparative analyses with human skin DC subsets. The skin is commonly regarded as an attractive gateway for the delivery of (tumor) vaccines whereas the oral mucosa is regarded as a gateway for the delivery of immune modulatory desensitisation therapies e.g. hyposensitisation sublingual immunotherapy (SLIT) (Allam et al., 2008;Allam et al., 2009a;Allam et al., 2009b;Allam and Novak, 2011;Novak and Allam, 2011;Novak et al., 2011). It is known that DC subsets are able to migrate to draining LN, even in the steady state, and so maintain peripheral tolerance. Remarkably we found equivalent LC and DC subsets migrating from skin and gingiva explants and in the same frequency distribution. As CD14+CD163+ subsets were previously identified as suppressive with the ability to expand Tregs (Chu et al., 2012;Lindenberg et al., 2013;Palucka and Banchereau, 2012), one might have expected a predominance of these subsets among gingiva-migrated DC. This however turned out not to be the case. Indeed, cytokine release profiling even pointed to a more pro-inflammatory microenvironment in the gingiva than in skin. Moreover, a superior ability of gingival-emigrated DC to prime T cells and skew them towards a type-1 functional state was observed.

It is well established that inflammatory responses and allergic reactions can occur in the oral cavity as well as the skin (Jotwani et al., 2001;Wray et al., 2000). Extensive literature on mucosal tolerization, generally refers to the gut, which has a clear immunosuppressive character compared to the inflammatory properties of the skin. When referring to "oral" tolerance, often "gut" tolerance is actually meant (Cassani et al., 2011;Wang and Toes, 2008;Weiner et al., 2011;Rescigno, 2011;Coombes and Powrie, 2008). Indeed, oral tolerance induction may be mediated by eventual antigen exposure to mucosa of the lower gastrointestinal tract rather than to oral mucosa. Our findings certainly support this notion.

Very little literature is available describing oral mucosal immunology. For oral mucosa, two alternative scenarios may be possible upon antigen exposure: i) tolerance may occur in response to the continuous exposure to antigens derived from commensal bacteria, and other non-pathogenic factors, which otherwise might lead to chronic inflammation (Hovav, 2014;Novak et al., 2008) or ii) an immune response may be induced to eliminate pathogenic or noxious factors (e.g. allergens, pathogenic microbes or toxins). Our results

strongly support the latter scenario as a default setting. This would suggest that rather than mediating immune tolerance, like the gut, the oral mucosa rather has immune stimulatory properties more akin to the skin. In line with our findings, Hasséus *et al.* demonstrated that LC in human oral buccal epithelium were more efficient primers of T cells than their counterparts in skin. Additionally, in their study CD83-positive cells were found in higher numbers in oral buccal epithelium than in skin epidermis, thus supporting the finding that oral LC are in a higher steady-state maturation state and have higher T cell stimulating capacity than skin LC (Hasséus et al., 2004). One could argue that like skin, the mouth is a gatekeeper and major barrier to the outside world where strong immune defenses should be up at all times, e.g. to keep harmful microbes from reaching the gastrointestinal tract. In contrast, in the gut it may be more important to prevent chronic inflammation, which could prove life threatening, and steady-state antigen exposure there may therefore rather lead to immune tolerance.

A striking difference between the more mature CD1a$^+$ DC subsets from gingiva and skin was the expression level of CXCR4: high in skin, absent in the oral mucosa. CXCR4 is a chemokine receptor which has a proven pivotal role in the migration of maturing LC from epidermis to dermis in response to dermal fibroblast-derived CXCL12: a first step en route to the draining lymph nodes (Ouwehand et al., 2008). Our findings indicate that gingival LC in contrast migrate to the lamina propria in a CXCR4/CXCL12 independent fashion. In keeping with this, researchers found that CXCL12 was not secreted by gingival fibroblasts, not even after their activation (Kosten et al., 2015a), and that in gingival equivalents LC migrated to the lamina propria in a CXCL12 independent manner (Kosten et al., 2015b). There are indications that LC in the oral mucosa may not even have to migrate to lymph nodes in order to direct a T cell immune response (Cutler and Jotwani, 2006). Indeed, it has been reported that oral LC present antigens to T cells in the lamina propria in so-called oral lymphoid foci, indicating that oral LC do not need to travel to nearby draining lymph nodes (Cutler and Jotwani, 2006) which would require sequential CXCL12 and CCL19/21 gradients (Lin et al., 1998;Ouwehand et al., 2008;Ouwehand et al., 2010;Saeki et al., 1999;Sallusto et al., 1998;Sozzani et al., 1998). DC migration to the lamina propria does not require maturation and could thus potentially contribute to immune tolerance induction, provided an immune tolerant milieu prevails in the lymphoid foci (Jotwani et al., 2001;Jotwani and Cutler, 2003). Observations reported by Allam and co-workers indeed support this scenario.(Allam et al., 2008) It is conceivable that the priming of type-1 T cell mediated immunity may require a higher maturation state of the migrating DC and subsequent T cell induction in draining lymph nodes.

Taken together, our data suggest the oral mucosa to be an attractive site for type-1 T cell immunity induction, possibly even more so than skin. Thus, one might even consider

oral immunization strategies to combat cancer or viral infection, as long as subsequent antigen exposure in the gut is prevented (e.g. by the use of aerosol-formulated antigens in the form of an oral spray). Clearly, more in-depth analysis is warranted to delineate the functional contribution of the different DC subsets to the induction of type-1 T cell responses. These studies may be seriously hampered by the scarcity of available healthy gingival tissue samples and may require the development of representative gingival equivalent models (Kosten et al., 2015a;Kosten et al., 2015b). Delineation of the functional abilities of the different DC subsets will eventually allow for the design of fine-tuned DC-targeted vaccine formulations combined with optimally effective oral adjuvants.

DISCLOSURE
S. Gibbs is co-founder of A-Skin BV which is a spin-off company (SME) of the VU university medical centre. The other authors have no conflicts of interest.

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Chapter 4