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Kosten, I.J.

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Chapter 5

MUTZ-3 Langerhans Cell maturation and CXCL12 independent migration in reconstructed human gingiva.

Ilona J. Kosten¹, Sander W. Spiekstra¹, Tanja D. de Gruijl², Susan Gibbs^{1,3*},

Departments of Dermatology¹ and Medical Oncology²,
VU University Medical Center, Amsterdam, the Netherlands;
Department of Oral Cell Biology, Academic Center for Dentistry (ACTA),
University of Amsterdam and VU University, Amsterdam, the Netherlands³

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Abbreviations: CA, cinnamaldehyde; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; DDC, dermal dendritic cell; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; GE, gingiva equivalent; iLC, immature (MUTZ-)LC; KC, keratinocyte; KGF, keratinocyte growth factor; LC, Langerhans cell; LP, lamina propria; mLC, matured (MUTZ-)LC; MUTZ-3 LC, MUTZ-3 derived Langerhans cell; SE, skin equivalent; TGF β , tumor growth factor beta; TNF α , tumor necrosis factor alpha
U, unexposed; V, vehicle exposed

5.1 ABSTRACT

Here we describe the first reconstructed full thickness human oral mucosa (gingiva) equivalent with integrated Langerhans Cells (GE-LC) and use it to compare LC activation and migration from oral versus skin epithelium. The physiologically representative models consist of a differentiated reconstructed epithelium (keratinocytes and Langerhans-like cells derived from the MUTZ-3 cell line) on a fibroblast-populated collagen hydrogel which serves as a lamina propria for gingiva and dermis for skin. Topical exposure of GE-LC and the skin equivalent (SE-LC) to sub-toxic concentrations of the allergen cinnamaldehyde resulted in LC migration out of the epithelia into the fibroblast populated collagen hydrogel. Neutralizing antibody to CXCL12 blocked allergen-induced LC migration in SE-LC but not in GE-LC. Also, gingival fibroblasts secreted very low amounts of CXCL12 compared to skin fibroblasts even when stimulated with rhTNF α or rhIL-1 α . Surprisingly, cinnamaldehyde exposure of GE-LC did not result in an increase in CD1a⁺ cells in the collagen hydrogel (as was observed for SE-LC), but did result in an increase in MUTZ-3 LC and CD83 mRNA. These results indicate that in gingiva, upon cinnamaldehyde exposure MUTZ-3 LC migrate in a CXCL12 independent manner from epithelium-to-lamina propria and in doing so mature and become CD1a⁺/CD83⁺ LC. These physiologically relevant in vitro models which not only are human but which also resemble specific tissues, may aid in the identification of factors regulating immune stimulation which in turn will aid the development of therapeutic interventions for allergy and inflammation, anti-cancer vaccines as well as improving diagnostics for skin and oral allergy.

5.2 INTRODUCTION

Skin and oral mucosa (e.g. gingiva, buccal mucosa, palatum) are both fully immunologically competent tissues participating in the induction and effector phase of the immune response. They both are constantly exposed to harmful environmental factors, such as pathogens and allergens, and therefore both play a key barrier role in maintaining homeostasis and regulating immune responses. However, evidence suggests that these tissues react differently to insults arising from the environment. For example, whereas nickel exposure via the skin can result in sensitization, oral exposure e.g. in the form of dental braces has been shown to induce specific tolerance (van Hoogstraten et al., 1992). This would suggest that skin is immune-stimulatory and oral mucosa tolerogenic and suggests differential roles for residential cells (e.g. keratinocytes (KC), fibroblasts and dendritic cells (DC)) residing in these tissues resulting in different cross-talk between cells within skin and oral tissue. Even so, allergies within the oral cavity have also been reported e.g. nickel and palladium allergy caused by dental restorative materials (Muris et al., 2009).

Although animals, in particular mice, are extensively used as skin research and testing models, misleading conclusions can be drawn due to very clear differences between human and animal immunology. This is illustrated by the fact that extensive differences have been reported between human and mouse DC subsets (Shortman and Liu, 2002;van de Ven et al., 2011). Importantly no suitable animal models exist to study oral immunity and in particular, no oral animal models exist to identify potentially hazardous substances which come into contact with the oral cavity (e.g. ingredients of toothpastes, mouth wash, dental restorative materials). Within Europe there is an increasing drive to refine, reduce and to replace (3Rs) test animals with *in vitro* alternatives wherever possible (EU Cosmetic Directive 7th Amendment, March 2009). With regards to skin, a number of *in vitro* models are available to investigate DC biology, in particular in the area of human safety and risk assessment of chemicals, and for testing novel drugs and therapeutic strategies (Roggen, 2014;dos Santos et al., 2009;Gibbs et al., 2013). Since large pieces of skin are regularly available from routine surgical procedures (e.g. abdominal dermolipectomy) fresh human skin explants provide a very relevant model to study Langerhans cell (LC) biology *in situ* (Ouwehand et al., 2008;Ouwehand et al., 2010;Jacobs et al., 2006;Lindenberg et al., 2013;Oosterhoff et al., 2013). Even so, the logistics around getting fresh tissue to the laboratory, the short viability of the tissue *ex vivo* (48 h) and the extremely limited size of oral mucosa (gingiva) biopsies in particular which are also often infected with microorganisms provide profound limitations for implementing these tissues directly as a research tool. As a first step towards overcoming these limitations, skin and gingiva equivalents (SE, GE respectively) have been developed. For example, reconstructed epidermis is now used as a validated method

for identifying corrosive and irritant chemicals (Fentem et al., 1998; Spielmann et al., 2007). Also a limited number of studies describe oral mucosa equivalents (Gibbs and Ponec, 2000; Macneil et al., 2011; Moharamzadeh et al., 2012). Significant applications of engineered oral mucosa include clinical transplantation (Vriens et al., 2008), *in vitro* investigations of the interaction of materials with oral mucosa (Moharamzadeh et al., 2008; Moharamzadeh et al., 2009), oral disease modelling (Andrian et al., 2007; Claveau et al., 2004; Rouabhia and Deslauriers, 2002; Yadev et al., 2011) and evaluation of drug delivery systems (Hearnden et al., 2012). However, none of these reconstructed models contain DC, and therefore their application is limited as an *in vitro* testing model. Only one study described integration of DC into a mucosa equivalent, and notably these were derived from primary CD34 positive cells isolated from fresh cord blood which creates a major logistical problem as well as donor variation (Sivard et al., 2003). Therefore, in this study, we developed a full thickness oral mucosa model with integrated human Langerhans-like cells derived from the human MUTZ-3 cell line.

The first phase in the activation of the immune system in both skin and oral mucosa after environmental assault involves triggering of the epithelium and connective tissue (known as dermis in skin and lamina propria in mucosa) to initiate LC migration out of the epithelium. The exact role of these migrated LC has yet to be established. With regards to skin sensitization, LC are recognized as an important immune stimulatory cell subset (Kimber et al., 2011). However, strong evidence now exists for LC also having an immune regulatory role related to induction and maintenance of tolerance (Shklovskaya et al., 2011). Whatever their function, clearly LC migration from the epithelium (epidermis) into the underlying dermis, as a first step en route to the draining lymph node, is pivotal for the orchestration of skin immune responses. With regards to oral mucosa, extremely little is known about mechanisms underlying LC migration due to the extreme scarcity of available healthy human tissue for functional analysis.

For skin, the key cytokines and chemokines involved in LC maturation and migration out of the epidermis and into the underlying dermis after topical exposure to harmful pathogens and contact allergens has been described in detail by us and others (Kosten et al., 2015b; Cumberbatch et al., 2003; Griffiths et al., 2005; Oosterhoff et al., 2013; Ouwehand et al., 2008; Kimber et al., 2000). Pro-inflammatory cytokines (e.g. TNF α , IL-1, IL-18) are rapidly released by epidermal cells and trigger the secretion of many chemokines from cells residing in the dermis thus initiating the inflammatory response (Kosten et al., 2015a). One of these chemokines is CXCL12 which is secreted by dermal fibroblasts (Ouwehand et al., 2008). CXCL12-secretion by fibroblasts coincides with up-regulation of its receptor, CXCR4, on the surface of maturing LC thus enabling the LC to migrate in a chemotactic manner from the epidermis into

the dermis (Ouwehand et al., 2008). It is currently unknown whether this CXCL12/CXCR4-axis is also pivotal for activated LC migration in oral mucosa.

We have previously described a human immune-competent skin equivalent which consists of a fully differentiated reconstructed epidermis, containing LC derived from the MUTZ-3 cell line, on a fibroblast populated collagen hydrogel (Ouwehand et al., 2011b;Kosten et al., 2015b). MUTZ-3 is an acute myeloid leukemic-derived human cell line with CD34⁺ proliferating progenitor cells which can be differentiated into LC (MUTZ-3 LC) in a cytokine dependent fashion. MUTZ-3 LC closely resemble their native counterparts, both phenotypically and functionally (Kosten et al., 2015b;Masterson et al., 2002;Santegoets et al., 2008). Indeed, in the past we have shown in skin equivalents with integrated Langerhans Cells (SE-LC) that upon allergen exposure MUTZ-3 LC mature and migrate in a CXCL12 dependent manner from the epidermis to the dermis whereas upon irritant exposure MUTZ-3 LC migrate in a CCL5 dependent manner and undergo an IL-10 dependent phenotypic switch into a macrophage like cell in the dermis closely mimicking our observations in excised skin (Kosten et al., 2015b;Ouwehand et al., 2008;Ouwehand et al., 2011b). Recently we have described a full thickness oral gingiva equivalent (GE) consisting of a reconstructed epithelium on a fibroblast populated collagen hydrogel (lamina propria), and shown that the GE secretes negligible amounts of key chemokines involved in LC migration in skin (Kosten et al., 2015a). In this study we have incorporated MUTZ-3 LC into the epithelium of this GE (GE-LC). We show that migration of maturing MUTZ-3 LC in GE-LC model after allergen exposure is CXCL12-independent in contrast to the CXCL12-dependent MUTZ-3 LC migration in SE-LC. Our results emphasize the need for physiologically relevant models which are not just of human origin but which also resemble the different tissues which are exposed to environmental hazards.

5.3 MATERIALS & METHODS

5.3.1 Cell culture

Human adult skin and gingiva were obtained after informed consent from patients undergoing abdominal dermolipectomy or wisdom tooth extraction respectively 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. Skin and gingiva samples were not donor matched.

Epithelial keratinocytes (KC): adult skin and gingiva KC were isolated and cultured under similar conditions. KC were isolated from 3 - 6 mm punch biopsies essentially as described earlier (Kroeze et al., 2012). KC were pooled and cultured in KC medium (Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland)/Ham's F-12 (Gibco, Grand Island, USA) (3:1) containing 1% UltrosorG (BioSeptra S.A. Cergy-Saint-Christophe, France), 1% penicillin-streptomycin (Gibco), 1 $\mu\text{mol/L}$ hydrocortisone, 1 $\mu\text{mol/L}$ isoproterenol, 0.1 $\mu\text{mol/L}$ insulin containing 2 ng/mL keratinocyte growth factor (KGF) for the skin or 2 ng/mL epithelial growth factor (EGF) for the gingival at 37°C, 7.5% CO₂. Cultures were passaged when 90% confluent, using 0.5 mM EDTA/0.05% trypsin (Gibco) and used for experiments at passage 2. Skin and gingiva KC were kept in culture for the same period of time (10-12 days) to eliminate confounding culture aging effects. Between 2-3 donors were pooled for each experiment. Skin and gingiva keratinocytes were not derived from the same donors.

Fibroblasts: adult skin and gingiva fibroblasts were isolated and cultured under identical conditions. In short, fibroblasts were enzymatically isolated from 3 - 6 mm punch biopsies and were cultured in DMEM containing 1% UltrosorG, 1% penicillin-streptomycin at 37°C, 5% CO₂. Cultures were passaged when 90% confluent, and used for experiments at passage 3. Skin and gingiva fibroblasts were cultured for the same period of time (28-35 days) to eliminate confounding culture aging effects. Between 2-3 donors were pooled for each experiment. Skin and gingiva fibroblasts were not derived from the same donors.

MUTZ-3 cell line: The MUTZ-3 progenitor cell line (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was maintained and differentiated into LC as previously described (Masterson et al., 2002; Ouwehand et al., 2008). Maturation was induced by a cytokine maturation cocktail, consisting of 100 ng/mL IL-6, 50 ng/mL TNF α , 25 ng/mL IL-1 β (Strathmann Biotec, Hamburg, Germany) and 1 $\mu\text{g/mL}$ PGE₂ (Sigma Chemical Co, St Louis, MO, USA) for at least 24 h. Cells were cultured at 37°C, 5% CO₂, 95% humidity. MUTZ-3 LC were labelled

with carboxyfluorescein succinimidyl ester (CFSE) as described previously (Kosten et al., 2015b).

SE-LC and GE-LC culture: Reconstruction of the human SE or GE containing MUTZ-3 LC was achieved by co-seeding CFSE labelled MUTZ-3 LC (1×10^6 cells) with KC (0.5×10^6 cells) onto fibroblast-populated collagen gels as previously described (Kosten et al., 2015b). Cells were submerged for 3 days in KC medium containing 1 ng/mL KGF for SE or 1 ng/mL EGF for GE. To induce epithelial differentiation, the constructs were lifted to the air-liquid interface and cultured for 7 days in KC medium containing 1×10^{-5} M L-carnitine, 1×10^{-2} M L-serine, and 50 μ g/mL ascorbic acid and 2 ng/mL KGF for SE or 2 ng/mL EGF for GE. Unless otherwise stated, all additives were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Chemical exposure of SE-LC and GE-LC: Finn Chamber filter paper discs of 11 mm diameter (Epitest, Oy, Finland) for SE-LC or gauze filters of 12 mm diameter for GE-LC (03-150/38, Sefar Nitex, Heiden, Switzerland) were impregnated with allergen cinnamaldehyde (CA) or the corresponding vehicle (H_2O with 0.1% DMSO v/v). The chemical or vehicle impregnated discs were applied topically to the cultures for 16 h at 37°C, 7.5% CO_2 in a non-toxic concentration as described previously (Ouwehand et al., 2010).

5.3.2 Ex vivo skin and gingival explants

Ex vivo skin and gingival explants: All fat was carefully removed from healthy human abdominal skin. Pieces of skin or gingiva of approximately 0.3 cm² were placed on 3.0 μ m polyester membrane transwell inserts, in a 12 wells plate (3462, Corning, Inc., New York, NY, USA) and topically exposed for 3 h to the vehicle as a control or CA (40 mM) in DMSO (applied at less than 0.1% v/v) at 37°C, 7.5% CO_2 , 95% humidity. After the 3 h incubation in MEM-alpha (Gibco, Grand Island, NY, USA) supplemented with 1% penicillin-streptomycin, 2 mM L-glutamine (Invitrogen) and 50 μ M 2-mercaptoethanol (Merck, Whitehouse Station, NJ, USA) at 37°C, 7.5% CO_2 , 95% humidity, explants were harvested and snap-frozen or embedded in paraffin for immunohistochemical analysis. At these chemical concentrations, no visible detrimental changes in tissue histology were observed by light microscopy.

5.3.3 Immunohistochemical staining

All procedures for paraffin embedded sections were performed as previously described (Ouwehand et al., 2008; Ouwehand et al., 2011b). In brief, antigen-retrieval was performed using citrate buffer followed by incubation overnight with primary monoclonal antibodies directed against CD1a (mouse IgG1 clone MTB1, MONX10315, Monosan), Langerin (mouse IgG2b clone 12D6, NCL-Langerin, Leica) or HLA-DR

(mouse IgG1 clone TAL.1B5, Dako). After washing and incubating with human anti-mouse conjugated to HRP the slides were incubated with 3-amino-9-ethylcarbazole as the chromogen. All sections were counter-stained with Mayer's haematoxylin. Negative controls were prepared by substituting the primary antibody with an isotype control antibody. For morphological analysis haematoxylin and eosin staining was used. The sections were embedded in Aquatex®.

5.3.4 Transwell chemotactic assay

This assay was performed essentially as described previously (Ouwehand et al., 2008). In brief, human fibroblasts, either from adult human skin or adult human gingiva (1×10^4 cell/well) were allowed to adhere overnight in the lower chamber of a 24 transwell culture plate (3421, Corning). Either immature MUTZ-3 LC (iLC) or cytokine matured MUTZ-3 LC (mLC) (5×10^4 cell/well) were placed in the transwell insert (5 μ m pore size, 3421, Corning). Lower chambers without fibroblasts were used as a control. Migrating MUTZ-3 LC entering the lower chamber were analysed by flow cytometry after 16 h incubation at 37°C, 5% CO₂, 95% humidity.

5.3.5 Quantitation of LC in epithelial sheets

The number of LC in epithelial sheets was quantified as described previously (Ouwehand et al., 2011b). In short, after removal of the epithelium from the collagen gel with fine forceps, the density of LC in the epithelia sheets was determined by incubating sheets for at least 1 h (max. overnight) with 50 μ l/mL PE-labelled anti-CD1a or anti-HLA-DR (BD Pharmingen, San Diego, CA, USA). Subsequently, the epidermal sheets were examined using a fluorescence microscope (Nikon Eclipse 80i), G-2a Ex510-560, DM575, BA590. The LC density was calculated by determining the measured fluorescence index with Image J software, and is expressed as CD1a fluorescence intensity.

5.3.6 Cytokine exposure and neutralization experiments

Exposure to rhTNF α or rhIL-1 α : Subconfluent fibroblast monolayer cultures, grown in 6 well plates, were exposed to serial dilutions of rhTNF α or rhIL-1 α (0, 100 or 200 International Units / mL) (Strathmann Biotech, Hamburg, Germany) for 4 h in 1.5 mL medium, after which time the cells were washed with PBS and new medium was added. After 24 h the culture supernatant was harvested and stored at -20°C for ELISA analysis.

CXCL12 neutralization assays: for blocking experiments 7 μ g/mL goat anti-human CXCL12 (AF-310-NA, R&D systems, Minneapolis, MN, USA) was added to the culture medium of SE-LC or GE-LC 30 min prior to chemical exposure (optimal blocking concentration was previously determined) (Ouwehand et al., 2008; Ouwehand

et al., 2010), according to manufacturer's data sheet. Isotype control to assess non-specific reactions was added at 7 µg/mL goat IgG (6-001-F, R&D systems).

5.3.7 ELISA

Duoset CXCL12 development systems (R&D systems), was used as described by the suppliers (R&D systems).

5.3.8 Flow cytometry

The number and phenotype of migrated MUTZ-3 LC was determined by flow cytometry in the SE-LC and GE-LC after disrupting the collagen gels with collagenase as described previously (Ouwehand et al., 2011b;Kosten et al., 2015b). Cell staining was performed using mouse anti-human CD1a-PE (IgG1, BD pharmingen, San Diego, CA, USA). Isotype control to assess non-specific binding was mouse IgG1-PE (BD pharmingen). Additionally, a staining with propidium iodine (Gibco) was performed to confirm the viability of the migrated LC. Cells were washed in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide, incubated with antibodies for 30 min and resuspended in the same buffer for FACS analysis on a FACScalibur flow cytometer (Beckton Dickinson, San Jose, CA, USA). The data were subsequently analyzed using CellQuestPro software.

Quantitation: an excess amount of Flow-Count fluorspheres (Beckman Coulter, Fullerton, CA) was added before analysis with a FACScan flow cytometer (Beckton Dickinson, San Jose, CA, USA) to the cells isolated from the collagen hydrogel as previously described (Ouwehand et al., 2008;Kosten et al., 2015b). The data were subsequently analysed using CellQuestPro software.

5.3.9 RT-qPCR

Epithelium was removed from the GE collagen hydrogel using fine forceps. Total RNA was isolated from the GE collagen hydrogel and RT-qPCR analysis was performed as described previously (Kosten et al., 2015b). cDNA was amplified by PCR using the following primer kits: RT² qPCR Primer Assay for Human CD83 and as housekeeping genes RT² qPCR Primer Assay for Human GAPDH and HPRT.

5.3.10 Statistical analysis

Differences between cytokine secretion of unexposed keratinocytes or fibroblasts and TNFα or IL-1α exposed keratinocytes or fibroblasts were determined using the Mann-Whitney test. Differences between the amount of LC in SE or GE were determined using the unpaired student's t-test. Differences between the amounts of migrated CFSE labelled cells in GE were determined using the paired student t-test. Differences were considered significant when P<0.05.

5.4 RESULTS

5.4.1 Allergen exposure initiates LC migration in ex vivo skin and gingiva

In order to confirm that LC migrate from gingiva epithelium to the underlying lamina propria (LP) upon allergen exposure in a similar manner to epidermis-to-dermis migration in skin, human gingiva and skin biopsies were exposed to the contact allergen, CA. Vehicle-exposed tissue was used as a control. For both gingiva and skin, CD1a⁺ and Langerin⁺ LC were already observed in the LP and dermis respectively after 3 h exposure to CA whereas no migration occurred after vehicle exposure (**Fig. 1**).

5.4.2 Mature MUTZ-3 LC migrate towards gingiva-derived fibroblasts

Previously we have shown that dermal fibroblasts play a pivotal role in mediating skin LC migration from the epidermis to the dermis (Ouweland et al., 2008). In order to determine whether gingival LP-derived fibroblasts also mediated LC migration, gingival fibroblasts were compared to skin fibroblasts and were placed in the lower chamber of a transwell system with iLC or mLC in the upper chamber (**Fig. 2**). Fibroblasts were omitted in the control condition to determine spontaneous migration. iLC did not migrate towards skin- nor towards gingiva-derived fibroblasts. However, mLC

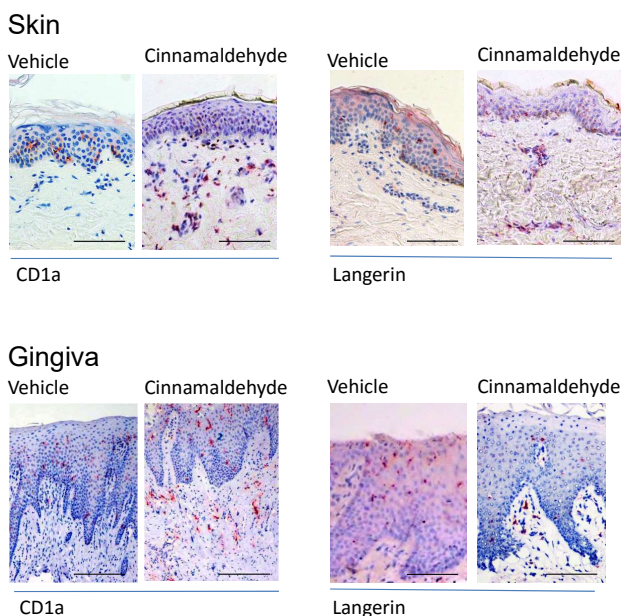


Fig. 1. Ex vivo LC migration upon exposure to allergen cinnamaldehyde.

Langerhans Cell migration in skin and gingival tissue sections is shown after topical exposure to vehicle (0.1% DMSO) or cinnamaldehyde (40 mM) for 3 h. Immunohistochemical staining with CD1a and Langerin is shown. Skin: 200x magnification; scale bar represents 50 μ m. Gingiva: 100x magnification; scale bar represents 100 μ m. Representative photographs from 3 independent experiments each with a different donor are shown.

were able to migrate towards both skin- (4-fold increase cf. spontaneous migration) and gingiva-derived fibroblasts (2.5-fold increase cf. spontaneous migration). These findings confirmed the ability of mature MUTZ-3 LC to migrate in response to gingiva fibroblast-derived soluble factors and justified their use in the construction of a gingiva equivalent model containing functional LC.

5.4.3 Allergen exposure induces LC migration in tissue-engineered skin and gingiva equivalents

The gingiva equivalent with integrated MUTZ-3 LC was compared with its previously described skin counterpart, (Ouweland et al., 2011b). Both models consisted of a reconstructed differentiated epithelium on a fibroblast populated collagen hydrogel, which functions as the dermis or LP respectively (**Fig. 3A**). Immunohistochemical staining of tissue sections with CD1a, Langerin and HLA-DR showed MUTZ-3 LC present in the epithelium of gingiva as well as in skin (**Fig. 3B**). Further immunostaining of isolated gingiva epithelial sheets showed CD1a positive and HLA-DR / CFSE double positive cells distributed evenly throughout the epithelium (**Fig. 3C**).

Next the SE-LC and GE-LC were topically exposed for 16 h to the highest non-toxic concentration of the allergen CA (skin: 1 mM; gingiva: 0.75 mM), a chemical known to induce migration and maturation of LC in both skin and oral mucosa (**Fig. 1**) (Wray et al., 2000). In both SE-LC and GE-LC, MUTZ-3 LC migrated out of the epithelium as observed by a decrease in the total fluorescence of CD1a⁺ MUTZ-3 LC in the epithelial sheets (**Fig. 4**). Exposure to the vehicle control did not result in a significant change the density of MUTZ-3 LC, as compared to unexposed SE-LC or GE-LC. Decreased MUTZ-3 LC densities in the epithelium were consistent with an allergen induced

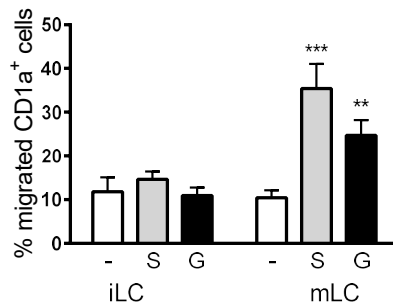


Fig. 2. MUTZ-3 LC migrate towards soluble mediators secreted by skin and gingival fibroblasts.

Skin (S; grey bars) or gingival fibroblasts (G; black bars) were seeded into the lower chamber of a transwell system and immature MUTZ-3 LC (iLC) or mature MUTZ-3 LC (mLC) were placed in the upper chamber for 16 h. Spontaneous migration in the absence of fibroblasts is represented by white bars. The percentage of CD1a⁺ LC migrating into the lower transwell chamber was determined by flow cytometry. Data represent the average of at least 6 individual experiments \pm SEM; ** $p < 0.01$ and *** $p < 0.001$ were calculated using the Mann-Whitney t-test

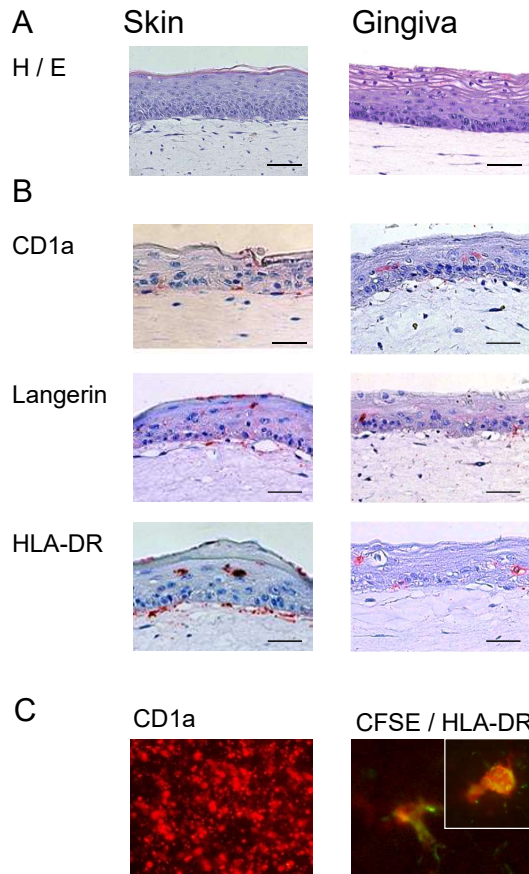


Fig. 3. Characterization of human reconstructed skin and gingiva containing MUTZ-3 LC in the epithelium.

A) Histology of skin and gingiva equivalents: haematoxylin and eosin (H/E) staining of tissue sections (200 x magnification; scale bar - 50 μ m). B) Visualization of MUTZ-3 LC: immunostaining of tissue sections with CD1a, Langerin and HLA-DR (200x magnification; scale bar - 50 μ m). C) CD1a immunostaining of MUTZ-3 LC in gingiva epithelial sheet (100x magnification) and high magnification detail of CFSE labelled MUTZ-3 LC stained with HLA-DR (400x magnification, including insert).

epithelium-to-dermis or LP migration of the maturing MUTZ-3 LC in the SE-LC and GE-LC (Fig. 1 and 4).

5.4.4 In contrast to skin, LC migration in gingiva is CXCL12 independent

In order to determine whether the LC migration in gingiva was CXCL12-dependent as it is in skin, a neutralizing antibody to CXCL12 was added to the culture medium of SE-LC and GE-LC constructs 30 min prior to and during exposure to CA (Fig. 4). Anti-CXCL12 was able to completely inhibit CXCL12-mediated migration of CD1a⁺ MUTZ-3 LC from the epidermis in SE-LC in line with our previous observations

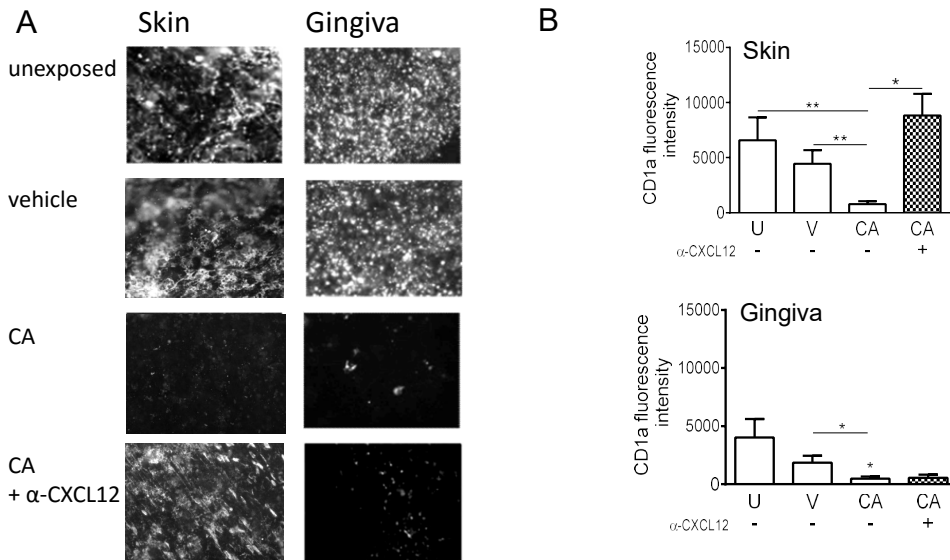


Fig. 4. CXCL12 independent migration of MUTZ-3 LC out of gingiva epithelium after cinnamaldehyde exposure.

A) Immunostaining of CD1a positive MUTZ-3 LC in epithelial sheets. SE-LC and GE-LC were unexposed or exposed to vehicle or cinnamaldehyde (CA) (skin: 1 mM; gingiva: 0.75 mM) for 16 h in the presence of a neutralizing antibody against CXCL12 or its isotype control. B) CD1a fluorescence intensity of MUTZ-3 LC in epithelial sheets. SE-LC and GE-LC were cultured in the presence of a neutralizing antibody against CXCL12 (+) or its isotype control (-). Unexposed (U); vehicle exposed (V); allergen cinnamaldehyde exposed (CA). Data represent the average of at least 4 individual experiments performed in duplicate \pm SEM; * $p < 0.05$ was calculated using the Mann-Whitney t-test

(Ouweland et al., 2011b; Ouweland et al., 2008). However, surprisingly, in GE-LC, anti-CXCL12 was not able to inhibit MUTZ-3 LC migration out of the epithelium. This indicates that LC migration in gingiva, in contrast to skin, is not dependent on the CXCR4 / CXCL12 axis.

In order to investigate this further, we next determined whether gingival fibroblasts were able to secrete the pivotal chemokine CXCL12. Gingival fibroblasts secreted only very low amounts of CXCL12 compared to skin derived fibroblasts (unexposed gingiva: 0.02 ng/mL; unexposed skin: 2 ng/mL) even when stimulated with rhTNF α or rhIL-1 α (Fig. 5). Taken together these results indicate that MUTZ-3 LC in GE-LC migrate in a CXCL12 independent manner after topical exposure to the contact sensitizer CA.

5.4.5 Migrated MUTZ-3 LC in GE-LC are CD1a⁻ /CD83⁺.

Next we determined by flow cytometric and PCR analysis, the phenotype of the migrated MUTZ-3 LC entering the collagen hydrogel after CA exposure (Fig. 6). In

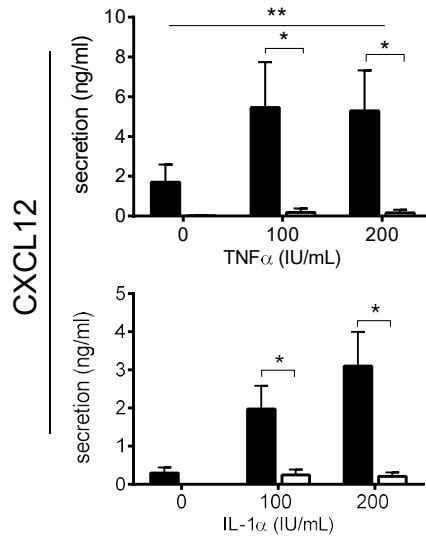


Fig. 5. Gingival fibroblasts secrete very low amounts of CXCL12 compared to skin fibroblasts.

Secretion of CXCL12 by fibroblasts upon exposure to serial dilutions of pro-inflammatory cytokines rhTNF α and rhIL-1 α . Skin fibroblasts: black bars; gingiva fibroblasts: white bars. Data represent the average of at least 3 individual experiments \pm SEM; * $p < 0.05$ was calculated using the Mann-Whitney t-test

line with previous results (Ouwehand et al., 2011b;Kosten et al., 2015b), CA exposure of SE-LC resulted in CD1a⁺ LC migrating into the dermis as indicated by an increase in the relative number of CD1a⁺ LC in the collagen gel. Furthermore, incubation with anti-CXCL12 totally abolished this increase in CD1a⁺ cells in the dermis, in line with blocking migration of CD1a⁺ cells out of the epidermis (c.f. Fig. 4). Surprisingly, CA exposure of GE-LC did not result in an increase in CD1a⁺ cells in the collagen hydrogel (Fig. 6A) but did result in an increase in CD83 transcripts (Fig. 6B) suggesting that the migrated MUTZ-3 LC have matured. Additionally, the migration of the CFSE labelled MUTZ-3 LC could not be blocked with anti-CXCL12 (Fig. 6C). These results indicate that in gingiva, upon CA exposure MUTZ-3 LC migrate in a CXCL12 independent manner into the LP and in doing so mature and become CD1a⁻ /CD83⁺.

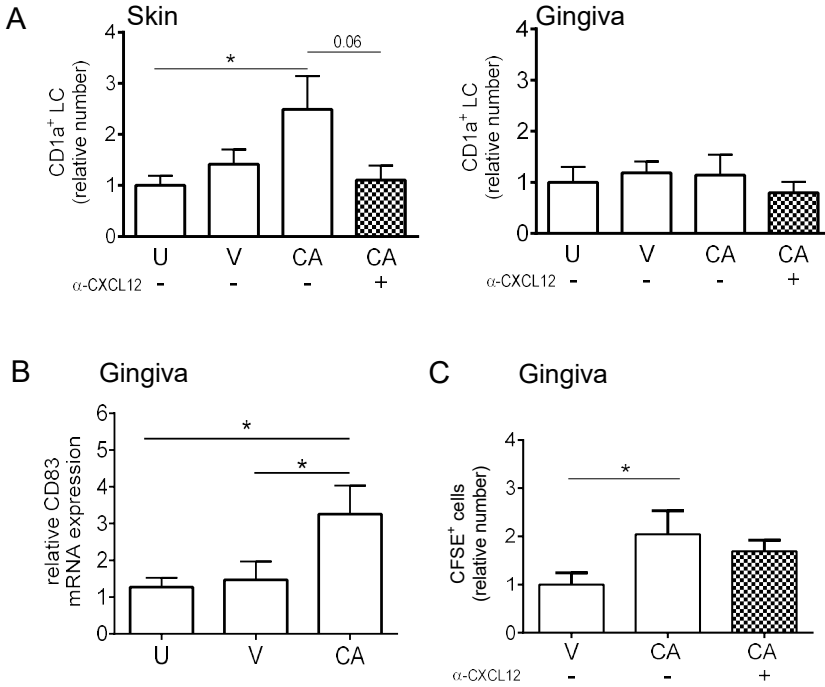


Fig. 6. MUTZ-3 LC become CD1a⁺/CD83⁺ and show CXCL12 independent migration into the collagen hydrogel after allergen exposure.

SE-LC and GE-LC were unexposed or exposed to vehicle or cinnamaldehyde (CA) (skin: 1 mM; gingiva: 0.75 mM) for 16 h in the presence of a neutralizing antibody against CXCL12 or its isotype control. The collagen hydrogel was then separated from the epithelium, and migrated MUTZ-3 LC assessed by flow cytometry or RT-qPCR. A) Relative number of CD1a⁺ MUTZ-3 LC compared to unexposed, isotype exposed SE-LC or GE-LC. B) RT-qPCR shows increased number of CD83 transcripts in the GE collagen hydrogel; data represent the average of at least 4 individual experiments. C) Relative number of migrated CFSE positive MUTZ-3 LC in the GE collagen hydrogel. Except for B), data represent the average of 6 individual experiments ± SEM; *p < 0.05 was calculated using the Mann-Whitney t-test.

5.5 DISCUSSION

In this study we describe the first full thickness oral mucosa (gingiva) equivalent with integrated Langerhans Cells derived from the MUTZ-3 cell line. It enables oral LC development, maturation and migration to be investigated in a human physiologically relevant model. Previously we have described the unique plasticity of the MUTZ-3 cell line. MUTZ-3 are able to differentiate in a cytokine dependent manner into DC-SIGN expressing dermal DC or Langerin expressing LC (Masterson et al., 2002; van de Ven et al., 2011b), which in turn are able to prime specific T cell responses (Santegoets et al., 2006; Santegoets et al., 2008). Furthermore, MUTZ-3 LC are functional when incorporated into full thickness skin equivalents. Upon topical irritant exposure, MUTZ-3 LC migrate in a CCL5 dependent manner into the dermis where they undergo an IL-10 dependent phenotypic change to a macrophage-like cell within the dermal compartment (Kosten et al., 2015b). In contrast, upon topical allergen exposure epidermal MUTZ-3 LC mature and migrate in a CXCL12 dependent manner (Ouweland et al., 2011b). We now show that in contrast to skin, in oral mucosa maturing CD83⁺ MUTZ-3 LC migrate in a CXCL12 independent manner and in doing so become CD1a⁺ in the LP upon allergen exposure.

In this present study we show that, similar to skin, mLC are able to migrate towards soluble factors secreted by gingiva fibroblasts. Since iLC did not migrate towards fibroblasts, we can conclude that LC migration is regulated by differential chemokine receptor expression on maturing LC compared to iLC which in turn enables the LC to migrate specifically towards chemokines secreted by gingiva fibroblasts as well as skin fibroblasts (Kosten et al., 2015b; Ouweland et al., 2008). However, even after stimulation with rhIL-1 α or rhTNF α , gingiva fibroblasts secreted much less CXCL12 which is a pivotal chemokine for skin epidermis-to-dermis migration. This, together with the finding that anti-CXCL12 was unable to block migration indicates that an as yet unknown chemokine / receptor pair is responsible for LC epithelium-to-LP migration in oral gingiva after allergen exposure. Indeed recently, we have shown that GE (without LC), topically exposed to an allergen or an irritant, secrete negligible amounts of key cytokines and chemokines (IL-18, CCL2, CCL20, CXCL12) involved in LC migration compared to skin, whereas the general inflammatory cytokine CXCL8 was secreted at similar levels by SE and GE (Kosten et al., 2015a). These results further support our current findings that the cytokines and chemokines triggering innate immunity and LC migration are very different in skin and gingiva. However, others have reported that gingival fibroblasts are indeed able to secrete (low amounts of) CXCL12 upon stimulation with rhTNF α , and also that biopsies obtained from periodontal diseased tissue show increased CXCL12 expression indicating that CXCL12 may yet be involved

in oral immunity e.g. in lymphocyte infiltration rather than LC migration (Hosokawa et al., 2005).

It was found that upon entering the LP of GE-LC, the migrating MUTZ-3 LC quickly lost their CD1a expression. This was in contrast to skin in which the migrating LC still clearly expressed CD1a after 16 hrs. Our finding is in line with others who reported that gingiva LC have a lower CD1a expression in comparison to the CD1a expression on skin LC (Novak et al., 2011). This strongly indicates a difference in antigen presentation in the oral mucosa as compared to skin (Cutler and Jotwani, 2006). Also, in contrast to skin where antigen presentation takes place in the draining lymph nodes, it has been reported that oral LC present antigens to T cells in the oral lymphoid foci within the LP, indicating that oral LC may not need to travel to nearby draining lymph nodes i.e. tonsils in order to induce an immune response (Cutler and Jotwani, 2006). This would make an up-regulation of CXCR4 or CCR7 and their respective chemokine ligands (CXCL12, CCL19 and CCL21) (Lin et al., 1998;Ouwehand et al., 2008;Ouwehand et al., 2010) possibly obsolete in gingiva (Allam et al., 2008). Whether or not a suspended state of iLC (no up-regulation of CXCR4, CCR7, CD86) in the oral cavity would actively induce tolerance still has to be determined. However, Jotwani et al. suggested that oral LCs which have taken up antigen mature only partially and migrate into the basal lamina (Jotwani et al., 2001;Jotwani and Cutler, 2003). However, we did find that MUTZ-3 LC migrating into the LP of GE-LC, in addition to becoming CD1a⁺, became mRNA CD83⁺. This is in line with our previous findings and those of others who reported HLA-DR⁺ DC subsets in human tonsils which lack CD1a expression (Summers et al., 2001;Lundberg et al., 2013) indicating that migration to the draining lymph nodes cannot be ruled out. Most likely the loss of CD1a upon migration is caused by differences in the LP in GE vs. dermis in the SE, consistent with our previous observations that the phenotype of migrating DC is highly plastic and dependent on the cytokine balance in the tissue microenvironment (de Gruijl et al., 2006;Ouwehand et al., 2011a).

Our study is an *in vitro* study using cultured GE-LC and therefore, even though this is a highly advanced technical model, we do have to recognize its limitations (e.g. lack of vasculature and diverse infiltrating immune cell subsets) when studying the complex mechanisms involved in regulating human immunity. However, the model is most suitable for studying DC biology and DC responses to substances which influence the innate immune system. Importantly, our study clearly emphasizes that physiologically relevant models which not only are human but which also resemble specific tissues are needed if we are to identify factors regulating immune activation and tolerization which in turn will aid the development of therapeutic interventions for allergy and inflammation, anti-cancer vaccines as well as improving diagnostics for skin and

oral allergy. Importantly, since many drugs are administered orally due to the greater efficiency of uptake in oral mucosa caused by its lower barrier competency compared to skin, our results hold great promise for implementation of relevant oral mucosa test models in this context.

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CONFLICT OF INTEREST

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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