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# Chapter 3

## MUTZ-3 derived Langerhans cells in human skin equivalents show differential migration and phenotypic plasticity after allergen or irritant exposure

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**Abbreviations:** ACD, allergic contact dermatitis; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ICD, irritant contact dermatitis; KC, keratinocytes; LC, Langerhans cell; MUTZ-LC, MUTZ-3 derived Langerhans Cell; NiSO<sub>4</sub>, nickel sulphate hexahydrate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SDS, sodium dodecyl sulphate; SE, skin equivalent; TGF-β, tumour growth factor beta; TNF-α, tumour necrosis factor alpha;

### 3.1 ABSTRACT

After allergen or irritant exposure, Langerhans cells (LC) undergo changes and exit the epidermis. Here we describe differences and similarities in the mechanisms regulating LC migration and phenotypic changes upon allergen or irritant exposure in a novel tissue-engineered full-thickness skin equivalent. The skin equivalent consisted of a reconstructed epidermis containing primary differentiated keratinocytes and CD1a<sup>+</sup> Langerhans-like cells derived from the human MUTZ-3 cell line (MUTZ-LC) on a primary fibroblast-populated dermis. Skin equivalents were exposed to a panel of allergens and irritants. Topical exposure to sub-toxic concentrations of allergens (nickel sulphate, resorcinol, cinnamaldehyde) and irritants (Triton X-100, SDS, Tween 80) resulted in LC migration out of the epidermis and into the dermis. Neutralizing antibody to CXCL12 blocked allergen-induced migration, whereas anti-CCL5 blocked irritant-induced migration. In contrast to allergen exposure, irritant exposure resulted in cells within the dermis becoming CD1a<sup>-</sup>/CD14<sup>+</sup>/CD68<sup>+</sup> which is characteristic of a phenotypic switch of MUTZ-LC to a macrophage-like cell in the dermis. This phenotypic switch was blocked with anti-IL-10. Mechanisms previously identified as being involved in LC activation and migration in native human skin could thus be reproduced in the *in vitro* constructed skin equivalent model containing functional LC. This model therefore provides an attractive and relevant research tool to study human LC biology *in situ* under controlled *in vitro* conditions, and will provide a powerful tool for hazard identification, testing novel therapeutics and identifying new drug targets.

### 3.2 INTRODUCTION

All skin diseases have an underlying immune component. In order to investigate human skin disease, physiologically relevant and immune competent human models mimicking the characteristic 3D tissue context of skin are therefore required. Although animal models, in particular mice, are used extensively, misleading conclusions can be drawn due to very clear differences between human and animal skin immunology. For example, murine TLR4 does not mediate a response to the most common human sensitizer nickel, whereas in man TLR4 plays a crucial role in elicitation of an allergic response to nickel (Schmidt et al., 2010). Indeed, extensive phenotypic and functional differences have been reported between human and mouse dendritic cell (DC) subsets (Shortman and Liu, 2002; van de Ven et al., 2011a). Furthermore, there is an increasing drive within Europe to refine, reduce and to replace test animals with *in vitro* alternatives wherever possible (Adler et al., 2011).

In order 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 a number of *in vitro* models have been proposed. Fresh human skin explants provide a very relevant model to study Langerhans cell (LC) biology *in situ* (Ouwehand et al., 2008; Ouwehand et al., 2010a; Jacobs et al., 2006; Lindenberg et al., 2013; Oosterhoff et al., 2013). However, the use of skin explants for research purposes and *in vitro* diagnostic assays requires a steady supply of large amounts of freshly excised skin, which creates a logistical bottleneck and can introduce high donor variation. To avoid the use of explant material, cultured, easily expanded cells have been used. DC (primary and immortalized cell lines, either human or animal-derived) have been extensively studied (dos Santos et al., 2009). A limitation in using these DC cultures is that they lack interactions with other cell types (e.g. keratinocytes and fibroblasts) which are essential for promoting *in vivo*-like DC maturation and migration. Furthermore, the skin barrier (stratum corneum) is absent. In order to overcome the absence of the skin barrier, the group of Schmidt incorporated cord blood-derived LC into human epidermis reconstructed from primary keratinocytes (Facy et al., 2005; Regnier et al., 1997). Widespread implementation of this model is limited however by its dependence on fresh cord blood which creates a major logistical problem as well as donor variation. Furthermore, the dermal component is absent which is essential for the regulation of LC migration from the epidermis (Ouwehand et al., 2008; Ouwehand et al., 2010a). Previously we have described a full thickness skin equivalent (SE) composed of a reconstructed epidermis on a fibroblast populated collagen gel (Spiekstra et al., 2005). Since it was not possible to distinguish allergens from irritants in this model by assessing increases in cytokine secretion, we next proceeded to incorporate LC into this *in vitro* SE model (Ouwehand et al., 2011b; Ouwehand et al., 2012).

The human Acute Myeloid Leukemia (AML) cell line MUTZ-3 were used for incorporation into SE. This cell line can be differentiated in a Transforming Growth Factor (TGF)- $\beta$ -dependent fashion into LC (MUTZ-LC), expressing Langerin and bearing LC-associated Birbeck granules (Masterson et al., 2002; Santegoets et al., 2006). In mono-culture, upon stimulation with maturation inducing factors (including allergens), MUTZ-LC expressed characteristic maturation markers, such as CD83, CXCR4 and CCR7, and acquired the ability to migrate towards CXCL12, thus closely resembling their *in vivo* counterparts (Larsson et al., 2006; Ouwehand et al., 2008; Ouwehand et al., 2010b). When MUTZ-LC were incorporated into full thickness SE they showed the ability to mature and migrate from the epidermis into the dermis after topical exposure of the stratum corneum to the allergens nickel sulphate and resorcinol (Ouwehand et al., 2011b).

Previously we have shown in fresh skin explant cultures that topical allergen and irritant exposure both result in LC migration, but mediated through different dermis-derived chemokines, i.e. CXCL12 (Ouwehand et al., 2008; Spiekstra et al., 2005) and CCL2/CCL5 (Ouwehand et al., 2010a), respectively. Whereas allergen-induced migration is accompanied by LC maturation and CXCR4 up-regulation, irritant-induced migration results in an intradermal LC-to-macrophage phenotypic switch by down-regulation of surface expression of Langerin and CD1a and up-regulation of CD14 surface expression and cytoplasmic CD68, in an IL-10 dependent fashion (de Gruijl et al., 2006; Ouwehand et al., 2011a). Even though the *ex vivo* skin explant model is clearly a valuable tool to study DC biology, it is greatly limited by logistics as mentioned above. But importantly since the skin is a complex organ containing many different cell types and precursors it is difficult to pin-point a phenotypic change within the tissue context exactly to a particular cell type. It cannot be entirely ruled out that the macrophage-like cells identified within the dermis after irritant exposure may also be derived from another cell type present in the skin biopsy.

In this study we further tested the validity and applicability of the SE-LC model by assessing and comparing the effects of allergen versus irritant exposure on LC migration and maturation. In particular, we show that MUTZ-LC are not only the first human DC cell line able to mature and migrate after allergen exposure but also that MUTZ-LC are capable of undergoing an IL-10 dependent phenotypic change into a macrophage-like cell upon migrating into the dermis after irritant exposure. This does not only confirm the irritant-induced phenotypic switch previously identified using skin explant cultures but, most importantly, illustrates the utility of the SE-LC to investigate DC biology *in vitro*.

### 3.3 MATERIALS & METHODS

#### 3.3.1 Cell culture

*Human skin explants:* explants were obtained after informed consent from patients undergoing corrective breast or abdominal plastic surgery 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](http://www.fmwv.nl)) and following procedures approved by the institutional review board of the VU University Medical Center.

*Epidermal keratinocytes and dermal fibroblasts:* cells were isolated and cultured as described previously (Kroeze et al., 2012; Ouwehand et al., 2011b).

*MUTZ-3 cell line:* The MUTZ-3 progenitor cell line (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was maintained as previously described by Masterson et al. (Masterson et al., 2002). MUTZ-3 progenitor cells were differentiated into LC by culturing at  $2 \times 10^5$  cell/mL in minimal essential media (MEM)-alpha (Gibco, Grand Island, NY, USA) supplemented with 20% v/v heat inactivated fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 1% penicillin-streptomycin, 2 mM L-glutamine (Gibco), 50  $\mu$ M 2- $\beta$  mercaptoethanol (Merck, Whitehouse Station, NY), 100 ng/mL recombinant human granulocyte macrophage-colony stimulating factor (Biosource, International Inc, Camarillo, CA, USA), 10 ng/mL TGF- $\beta$  (Biovision, Mountain View, CA, USA) and 2.5 ng/mL TNF- $\alpha$  (StrathmannBiotec, Hamburg, Germany) over a period of 7 days. Cells were cultured at 37°C, 5% CO<sub>2</sub>, 95% humidity.

*Construction of SE-LC:* Construction of the human SE containing MUTZ-LC (SE-LC) was achieved by co-seeding carboxyfluorescein succinimidyl ester (CFSE) labelled MUTZ-LC ( $1 \times 10^6$  cells) with KC ( $0.5 \times 10^6$  cells) onto fibroblast-populated collagen gels as previously described (Ouwehand et al., 2011b) followed by culturing for 4 days submerged and then 4 days at an air-liquid interface before chemical exposure. Unless otherwise stated, all additives were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For blocking experiments 7  $\mu$ g/mL goat anti-human CXCL12 (AF-310-NA, R&D systems, Minneapolis, MN, USA), 7  $\mu$ g/mL goat anti-human CCL5 (AF-278-NA, R&D systems) or 2  $\mu$ g/mL goat anti-human IL-10 (AF-217-NA, R&D systems) was added to the culture medium prior to chemical exposure (optimal blocking concentration was previously determined (Ouwehand et al., 2008; Ouwehand et al., 2010a; Ouwehand et al., 2011a). Isotype control to assess non-specific reactions was added at 7  $\mu$ g/mL polyclonal goat IgG (6-001-F, R&D systems).

### 3.3.2 Irritant or allergen exposure

Finn Chamber filter paper discs of 11 mm diameter (Epitest, Oy, Finland) were impregnated with vehicle (H<sub>2</sub>O), allergens (nickel sulphate hexahydrate (NiSO<sub>4</sub>·6H<sub>2</sub>O), cinnamaldehyde or resorcinol (Sigma Chemical Co.)), or irritants (Tween 80, Triton X-100 or SDS), dissolved in H<sub>2</sub>O as previously described (Gibbs et al., 2013). Chemical or vehicle impregnated discs were applied topically to the stratum corneum of SE-LC cultures for 16 h at 37°C, 7.5% CO<sub>2</sub>.

### 3.3.3 Immunohistochemical staining

From paraffin embedded full thickness human skin and *in vitro* cultured SE-LC, 5 µm tissue sections were cut. The sections were deparaffinised and rehydrated in preparation for morphological (haematoxylin-eosin staining) or immunohistochemical analysis which was performed as previously described (Ouwehand et al., 2008; Ouwehand et al., 2010a). In brief, antigen-retrieval was performed using citrate buffer and after cooling incubated overnight at room temperature with primary monoclonal antibody directed against HLA-DR (mouse IgG1 clone TAL.1B5, Dako, Denmark). Sections were incubated for another 30 minutes with human anti-mouse conjugated to horse radish peroxidase after washing with PBS. Subsequently, the slides were incubated for 10 minutes with 3-amino-9-ethylcarbazole as the chromogen. All sections were counterstained with haematoxylin. Negative controls were prepared by omitting the primary antibody and substituting an isotype control antibody. The sections were embedded in Aquatex® (Merck, Whitehouse Station, NJ, USA).

### 3.3.4 Quantitation of MUTZ-LC in epithelial sheets:

After chemical exposure, the epithelial sheets were harvested by separating the fibroblast populated collagen gel from the epithelial sheet with fine forceps. The density of LC in the epidermal sheets was determined by incubating the sheets for at least 1 h (max. overnight) with 50 µl/mL PE-labelled anti-CD1a (BD pharmingen, San Diego, CA) in FACS buffer. Subsequently, the epidermal sheets were washed three times in PBS and then examined using a fluorescence microscope (Nikon Eclipse 80i), G-2a Ex510-560, DM575, BA590). The density of CD1a<sup>+</sup> LC was calculated by determining the measured fluorescence index with Image J software and expressed as CD1a fluorescence intensity.

### 3.3.5 Flow cytometry

MUTZ-LC migration from the epidermis of SE was assessed by flow cytometry. Cell staining was performed using mouse anti-human CD1a-PE (IgG1, BD pharmingen, San Diego, CA, USA), mouse anti-human CD14-FITC/PerCP (IgG2a, Miltenyi Biotec, BergischGladbach, Germany), intracellular CD68-PE (kit 556078, IgG2b κ, BD pharmingen). Isotype controls to assess non-specific binding were mouse IgG1-PE,

IgG2b  $\kappa$  (BD pharmingen) and mouse IgG2a-FITC (MiltenyiBiotec). 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% BSA and 0.1% sodium azide, incubated with antibodies for 30 minutes 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.

### 3.3.6 Quantitation of MUTZ-LC in dermal compartments

In order to isolate cells (fibroblasts and MUTZ-LC) from within the collagen gel, gels were digested with 1% collagenase type II (Gibco) in Hanks buffered salt solution (Gibco) for 15 minutes at 37°C. An excess amount of Flow-Count fluorospheres (Beckman Coulter, Fullerton, CA, USA) was added to the harvested cells and these were then stained with mouse anti-human CD1a-PE, mouse anti-human CD14-FITC or intracellular CD68-PE (kit 556078, IgG2b  $\kappa$ , BD pharmingen) before analysis with a FACScalibur flow cytometer (Beckton Dickinson, San Jose, CA, USA). The data were subsequently analyzed using CellQuestPro software.

### 3.3.7 Real-time PCR

Epidermis was removed from the dermis using fine forceps. Total RNA was isolated from the dermis and RT-PCR analysis was performed as described previously (Ouweland et al., 2011b). cDNA was amplified by PCR using the following primer kits: RT<sup>2</sup> qPCR Primer Assay for Human CD68 and as housekeeping genes RT<sup>2</sup> qPCR Primer Assay for Human HPRT and RT<sup>2</sup> qPCR Primer Assay for Human GAPDH.

### 3.3.8 Statistical analysis

Statistical analysis of the number of LC under the different experimental conditions was performed using unpaired Student's t-test or Mann-Whitney tests by GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA). Differences were considered significant when  $P < 0.05$ .



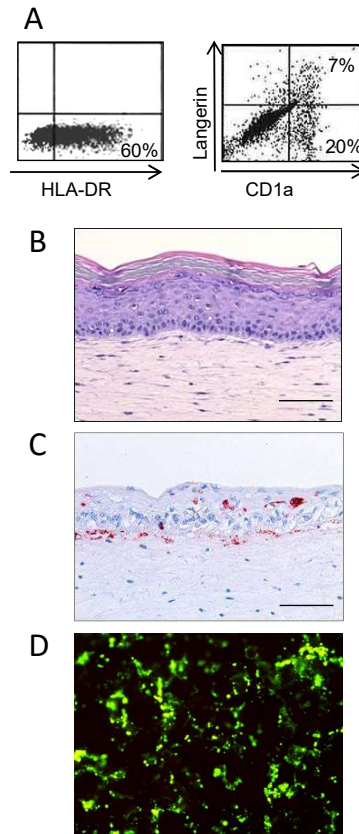
### 3.4 RESULTS

#### *Differential chemokine dependence of epidermis-to-dermis MUTZ-LC migration in SE after topical exposure to allergens versus irritants*

In order to investigate LC migration from the epidermis to the dermis, partially differentiated MUTZ-3 derived LC with low levels of CD1a, Langerin and HLA-DR (**Fig. 1A**) were co-seeded with human skin KC onto a fibroblast populated collagen gel. The SE containing MUTZ-LC closely resembled native human skin. It consisted of a fully differentiated epidermis reconstructed on a fibroblast populated collagen gel (**Fig. 1B**). The epidermis had a compact basal layer of keratinocytes, a spinous layer, granular layer and stratum corneum. MUTZ-LC were dispersed evenly throughout the 3D architecture of the epidermis and also at the epidermal-dermal junction (**Fig. 1C and D**). The model has been described in detail previously (Ouwehand et al., 2011b). In this study, we further tested the functionality of the SE-LC model by topically exposing SE-LC to a panel of allergens and irritants.

First, epidermis-to-dermis migration of MUTZ-LC in SE was investigated after exposure to chemical allergens. After topical exposure to NiSO<sub>4</sub> (10 mM), MUTZ-LC migrated out of the epidermis. This is demonstrated by the absence of CD1a<sup>+</sup> MUTZ-LC in epidermal sheets after NiSO<sub>4</sub> exposure compared to the presence of CD1a<sup>+</sup> MUTZ-LC in unexposed and vehicle exposed epidermal sheets (**Fig. 2A**). To assess CXCL12 dependence of allergen-induced MUTZ-LC migration from the epidermis the culture medium was supplemented with a neutralizing antibody against CXCL12 during chemical exposure. Indeed, confirming our previous explant data and illustrating the *in vivo* like properties of the SE-LC, incubation with anti-CXCL12 completely blocked NiSO<sub>4</sub> induced MUTZ-LC migration from the epidermis (**Fig. 2A and B**). The pivotal role of CXCL12 in mediating epidermis-to-dermis migration of MUTZ-LC upon allergen exposure was further confirmed for the allergens resorcinol and cinnamaldehyde (**Fig. 2B**).

After topical exposure of SE-LC to irritants (Triton X-100, Tween 80, SDS), CD1a<sup>+</sup> MUTZ-LC migrated out of the epidermal sheets similarly to allergen-exposed LC (**Fig. 2A and 2C**). Whereas incubation with the neutralizing antibody to CXCL12 failed to inhibit this irritant-induced migration from the epidermis, incubation with anti-CCL5 completely inhibited migration (**Fig. 2A, 2C and Fig. 3**). In contrast, MUTZ-LC migration after exposure to the allergen NiSO<sub>4</sub> was not inhibited by anti-CCL5 (**Fig. 3**).



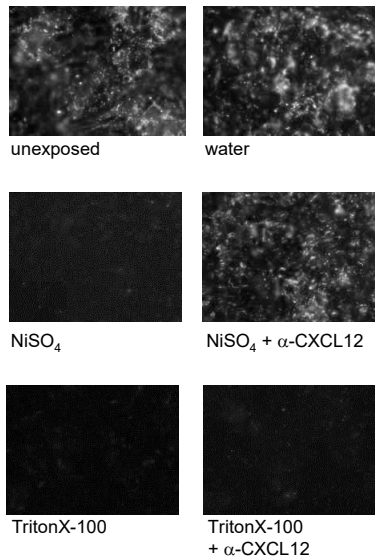
**Figure 1. Human full thickness skin equivalent with MUTZ-LC.**

A) Phenotypic characterization of MUTZ-LC after 7 days of differentiation. Flow cytometry dot plots show HLA-DR, CD1a, Langerin positive cells and double positive cells; percentage positive cells is written in the quadrants. B) Histology (haematoxylin-eosin staining) of SE-LC (200x magnification; scale bar represents 50  $\mu$ m). C) HLA-DR immunohistochemical staining of MUTZ-LC in SE-LC (200x magnification; scale bar represents 50  $\mu$ m). D) CFSE staining of the epidermal sheet isolated from SE-LC (100x magnification). Representative photographs from 5 independent experiments are shown.

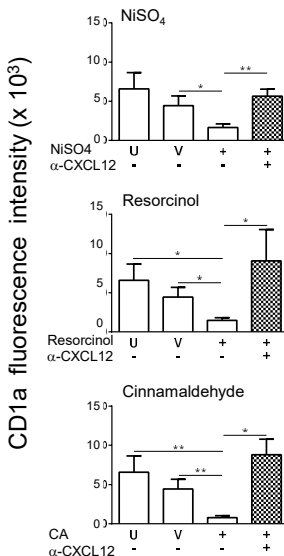
*Increased CD14 and CD68 expression and decreased CD1a surface levels on MUTZ-LC during irritant-induced epidermis-to-dermis migration.*

Next, the phenotype of the migrated MUTZ-LC was investigated. To this end, the isolated dermal compartments were digested and, upon live gating by forward and side scatter properties, migrated LC were analyzed by flow cytometry. Whereas an increase in CD1a<sup>+</sup> MUTZ-LC rates were observed in the collagen gel (dermis) after allergen exposure (control: 2%; vehicle: 4%; NiSO<sub>4</sub>: 65%), no corresponding increase in CD1a<sup>+</sup> MUTZ-LC were detected in the collagen gels after irritant Triton X-100 exposure (**Fig. 4**). In order to determine whether the post-migration MUTZ-LC were indeed

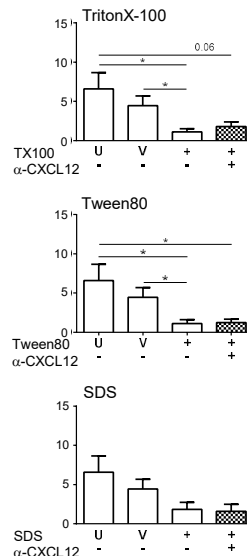
A Epidermis sheets



B allergens

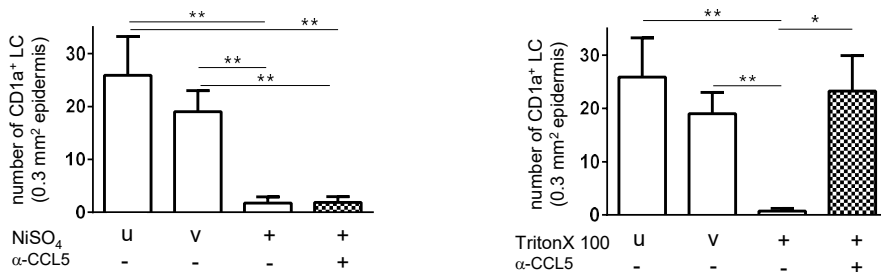


C irritants



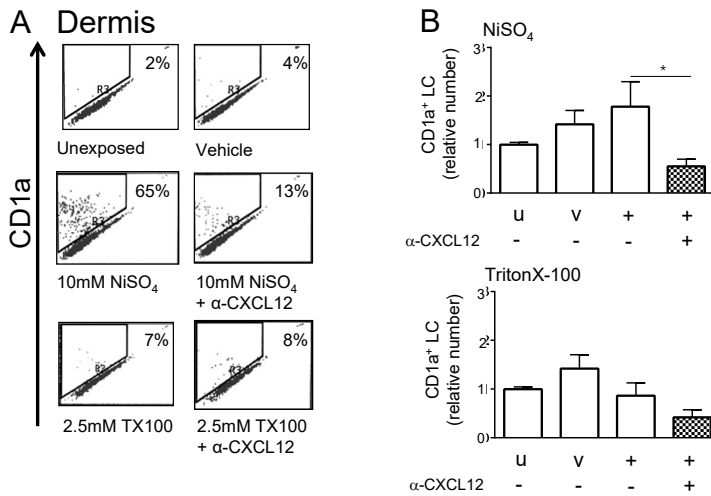
**Figure 2. MUTZ-LC migration out of the epidermis after allergen but not irritant exposure is CXCL12 dependent.**

A) Epidermal sheets isolated from SE-LC and stained with anti-CD1a-PE are shown. SE-LC were unexposed, water vehicle exposed or exposed to 10 mM NiSO<sub>4</sub> or 2.5 mM Triton X-100 for 16 h in the presence of the neutralizing antibody to CXCL12 or IgG1 isotype control as described in Materials and Methods. Fluorescence intensity (white) shows presence or absence of MUTZ-LC in epidermal sheets. B) SE-LC were unexposed (U), exposed to water vehicle (V), or allergens (10 mM NiSO<sub>4</sub>, 15 mM resorcinol, 1 mM cinnamaldehyde) or C) irritants (2.5 mM Triton X-100, 0.5 mM Tween 80, 5 mM SDS) for 16 h. Chemical exposure was performed in the presence of the neutralizing antibody to CXCL12 (+) or IgG1 isotype control (-). CD1a-PE fluorescence intensity of MUTZ-LC in epidermal sheets was quantified using Image J software. Data represent the average of 5 individual experiments performed in duplicate ± SEM. \*p < 0.05, \*\*p < 0.01 was calculated using the Mann-Whitney t-test



**Figure 3. MUTZ-LC migration out of the epidermis after irritant but not allergen exposure is CCL5 dependent.**

SE-LC were exposed to water vehicle (V), 10 mM NiSO<sub>4</sub> (N) or 2.5 mM Triton X-100 (T) for 16 h. Chemical exposure was performed in the presence of the neutralizing antibody to CCL5 (+) or IgG1 isotype control (-). Number of CD1a-PE positive MUTZ-LC in epidermal sheets was quantified using Image J software. Data represent the average of 4 individual experiments ± SEM. \*p < 0.05, \*\*p < 0.01 was calculated using the Mann-Whitney t-test



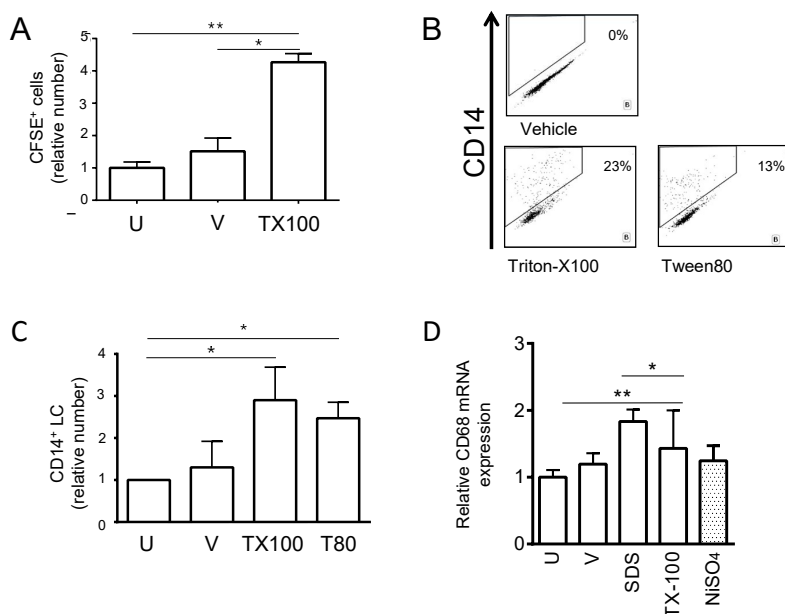
**Figure 4. MUTZ-LC migrating into the dermis are CD1a positive after allergen exposure but CD1a negative after irritant exposure.**

CD1a-PE flow cytometric staining of MUTZ-LC isolated from the dermal matrix (collagen gel) after allergen NiSO<sub>4</sub> or irritant Triton X-100 exposure in the presence of neutralizing antibodies to CXCL12 (+) or IgG1 isotype control (-). A) Flow cytometry dot plots of CD1a-PE positive MUTZ-LC; percentage positive cells are written in the upper right corner. B) Relative number of CD1a<sup>+</sup> MUTZ-LC in dermal matrix was quantified by flow cytometry using Flow-Count fluorospheres and CD1a-PE label. Data represent the average of 5 experiments performed in duplicate ± SEM. \*p < 0.05, \*\*p < 0.01 was calculated using the Mann-Whitney t-test.

still viable and detectable after irritant exposure, MUTZ-LC were labeled with CFSE before incorporating into SE. In contrast to unexposed or vehicle exposed SE-LC, after topical exposure to Triton X-100, CFSE labeled MUTZ-LC were clearly detected in the collagen gel proving that viable MUTZ-LC had indeed actively migrated from the epidermis-to-dermis upon irritant exposure (**Fig. 5A**). In order to determine whether MUTZ-LC were able to undergo a phenotypic switch to a macrophage-like state after irritant exposure, in line with their primary counterparts in native skin explants (de Gruijl et al., 2006; Ouwehand et al., 2011a), cells isolated from the collagen gels were stained with antibodies for CD14 or RNA was isolated for PCR with CD68 primers. After irritant exposure a clear increase in CD14<sup>+</sup> cells (**Fig. 5B and C**) and also an increase in CD68 transcripts (**Fig. 5D**) were detected in the collagen gels. This was not detected in the NiSO<sub>4</sub> exposed collagen gels (**Fig. 5D**). We therefore conclude that, after irritant exposure, MUTZ-LC in SE-LC are able to undergo a phenotypic switch to a macrophage-like state as shown by decreased CD1a and increased CD14 and CD68 expression during epidermis-to-dermis migration.

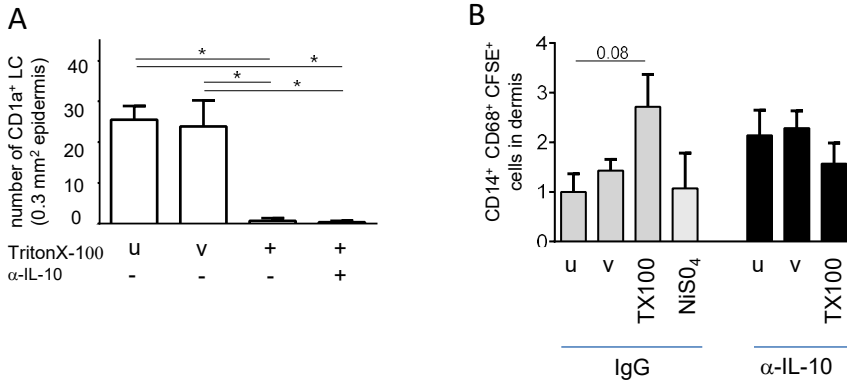
*Irritant-induced phenotypic switch from CD1a<sup>+</sup> LC to CD14<sup>+</sup>/CD68<sup>+</sup> macrophage-like cells is IL-10 dependent*

In the skin explant model we have previously shown that the irritant-induced LC-to-macrophage switch was dependent on IL-10 (Ouweland et al., 2011a). In order to confirm this observation in the SE-LC model, SE-LC were incubated with neutralizing antibodies to IL-10 during irritant exposure (Triton X-100). Whereas anti-IL-10 had no effect on irritant mediated MUTZ-LC migration from the epidermis to the dermis (**Fig. 6A**), the increase in CD1a<sup>+</sup> / CD14<sup>+</sup> / CD68<sup>+</sup> MUTZ cells accumulating in the dermis was totally inhibited by anti-IL-10 (**Fig. 6B**).



**Figure 5. MUTZ-LC migrating into the dermis after irritant exposure are CD14<sup>+</sup> / CD68<sup>+</sup>.**

A) Increased numbers of viable CFSE labeled MUTZ-LC are detected by flow cytometry in the dermal matrix after irritant Triton X-100 exposure compared to unexposed or water vehicle exposed SE-LC. B) Dot plots of flow cytometric staining show CD14 positive MUTZ-LC in the dermal matrix (collagen gel) after irritant (2.5 mM TritonX-100, 0.5 mM Tween 80) exposure. Percentage positive cells written in the upper right corner. C) The relative number of CD14 positive MUTZ-LC in dermal matrix compared to unexposed SE-LC was detected by flow cytometry and quantified using Flow-Count flurospheres. D) RT-PCR shows increased number of CD68 transcripts in dermis after irritant (2.5 mM TX-100, 5mM SDS) or allergen NiSO<sub>4</sub> exposure (10 mM). For C) and D) U = unexposed; V = vehicle exposed; TX-100 = Triton X-100; T80 = Tween 80. Data represent the average of at least 4 individual experiments ± SEM. \*p < 0.05, \*\*p < 0.01 was calculated using the Mann-Whitney t-test.



**Figure 6. MUTZ-LC phenotypic switch from CD1a<sup>+</sup> to CD1a<sup>-</sup> / CD14<sup>+</sup> / CD68<sup>+</sup> cells is IL-10 dependent.**

SE-LC were unexposed (U), exposed to water vehicle (V), or 2.5 mM Triton X-100 (T) for 16 h. Chemical exposure was performed in the presence of the neutralizing antibody to IL-10 (+) or IgG1 isotype control (-). A) CD1a-PE positive MUTZ-LC in epidermal sheets. B) The relative number of CD14<sup>+</sup> / CD68<sup>+</sup> CFSE labelled cells compared to IgG1 unexposed SE-LC were detected by flow cytometry in the dermal matrix. Unexposed (U), exposed to water vehicle (V), 10 mM NiSO<sub>4</sub> (N), 2.5 mM Triton X-100 (T). Data represent 5 individual experiments ± SEM. \*p < 0.05, \*\*p < 0.01 was calculated using the Mann-Whitney t-test.

### 3.5 DISCUSSION

In this study we show that the MUTZ-3 cell line is the most physiologically relevant LC-like cell line identified to date. In addition to being able to differentiate in a cytokine dependent manner into Langerin expressing LC or, indeed, DC-SIGN expressing dermal DC (Masterson et al., 2002; van de Ven et al., 2011b), which are both able to prime specific T cell responses (Santegoets et al., 2006; Santegoets et al., 2008) we now show that these MUTZ-LC remain functional when incorporated into full thickness skin equivalents. Upon topical allergen exposure epidermal MUTZ-LC mature and migrate in a CXCL12 dependent, and CCL5 independent, manner to the dermal compartment of SE, whereas after topical irritant exposure they migrate in a CCL5 dependent, and CXCL12 independent, manner and undergo an IL-10 dependent phenotypic change to a macrophage-like cell within the dermal compartment.

The SE-LC was designed to enable complex mechanisms concerning human DC biology to be investigated in an animal alternative, physiologically relevant test model. Whereas sensitization after allergen exposure (leading eventually to allergic contact dermatitis (ACD)) has been extensively described in the literature e.g. penetration of chemicals through the stratum corneum, activation of keratinocytes, maturation and migration of LC to the draining lymph nodes and stimulation of T cell responses (Roggen, 2014), extremely little is known about the mechanisms resulting in irritant contact dermatitis (ICD). Clearly, a localized acute inflammatory reaction is involved. Furthermore, it has been shown that the number of CD1a positive cells decreases in both the epidermis and dermis in ICD (Jacobs et al., 2006; Marks Jr. et al., 1987). Until now, the fate of these migrated LC was unknown. Previously we have shown using the explant skin model that irritant exposure resulted in a decrease in CD1a<sup>+</sup> cells in the epidermis and an increase in CD14<sup>+</sup> / CD68<sup>+</sup> cells in the dermis indicative of a phenotypic switch of LC to macrophage-like cells (de Gruijl et al., 2006; Ouwehand et al., 2011a). Since the SE-LC is a fully defined skin system, and contains no confounders such as unknown infiltrating (precursor) cells locally e.g. proliferative monocytes and since MUTZ-LC were labelled with CFSE before incorporating into the SE-LC, we can now unambiguously demonstrate that LC undergo a phenotypic trans-differentiation into macrophage-like cells upon irritant exposure. This alternative end-stage differentiation of LC to CD14<sup>+</sup> / CD68<sup>+</sup> macrophage-like cells could play an important role in rapidly removing damaged skin tissue after e.g. skin irritancy, UV radiation, and during wound healing, thus playing a key role in maintaining immunological ignorance and avoiding the generation of collateral autoimmunity. Our finding that anti-inflammatory cytokine IL-10 is responsible for the post-migrational switch of LC into a macrophage-like cell upon irritant exposure is fully in line with our previous *ex vivo* skin explant studies (de Gruijl et al., 2006; Ouwehand et al., 2011a).

A limited number of epidermal equivalent models (Facy et al., 2005; Regnier et al., 1997; Schaerli et al., 2005), dermal equivalent models (Guironnet et al., 2001) and full-thickness SE models (Bechetoille et al., 2007; Dezutter-Dambuyant et al., 2006; Uchino et al., 2009) have been described with integrated LC or DC. Moreover, all of these models are reliant on fresh blood-derived precursor cells for their source of LC or DC. This introduces extremely difficult logistics in fine-tuning the timely arrival of blood (LC or DC source) and skin tissue (primary keratinocyte and fibroblast source) to the laboratory, and fine-tuning the culture of the different cell types in order to have all cells ready to construct the skin model on the same day. A number of the epidermal equivalent models have been used to investigate LC responses to chemical sensitizers. However, as the fibroblast-populated dermis is absent, the LC do not migrate and only a limited number of LC maturation state changes have been reported. (e.g. increased CCR7, CD86 expression or different cytokine secretions) (Facy et al., 2005; Regnier et al., 1997; Schaerli et al., 2005). We have previously shown that topical exposure of our SE-LC to allergens results in MUTZ-LC starting to mature in the epidermis (increased IL-1 $\beta$ ) and migrating from the epidermis to the dermis where they further up-regulate CCR7 and CD83 (Ouweland et al., 2011b). Now we show that similarly to excised skin, this allergen and fibroblast-dependent MUTZ-LC migration can be completely blocked by a neutralizing antibody to CXCL12. The SE-LC is the only *in vitro* model to date that enables this degree of LC biology to be explored after allergen exposure of the skin and therefore it provides an alternative means to identify pathogenic sensitizers which are giving ambiguous results in other test models. Furthermore, since chemicals are applied topically to the stratum corneum the SE-LC can be used to test water insoluble chemicals.

The phenotypic change of LC to a macrophage-like cell is not only confined to irritancy. We and others have reported the existence of an inter-related population of cutaneous DC and macrophages in flux, trans-differentiating into each other in response to environmental stimuli (de Gruijl et al., 2006; Diao et al., 2012; Ouweland et al., 2011a). This finding has direct consequences for the type of immune responses that will follow, as different migratory DC sub-populations have now been directly linked to the induction of different types of immunity (Lindenberg et al., 2013). For example, CD1a<sup>+</sup> mature LC and dermal DC subsets have been linked to type-1 T cell mediated immunity, whereas CD14<sup>+</sup> immature dermal DC subsets have been linked to the induction of humoral immunity and expansion of regulatory T cells (Treg) (Banchereau et al., 2012; Chu et al., 2012). Further research will determine to what extent the full thickness SE-LC model will be able to replace animal tests for skin-based immunization studies, other pharmaceutical testing and even for incorporation into organ-on-a-chip models with future application in personalized medicine strategies (Huh et al., 2010).



In this study we have illustrated with the aid of allergens and irritants that MUTZ-LC integrated in the SE-LC are fully functional and that, depending on environmental danger or damage associated signals, MUTZ-LC will migrate and mature, or migrate and undergo a phenotypic change into a macrophage-like cell. From our findings we conclude that the SE-LC is most suitable for chemical hazard identification. Since the read-out consists of physiologically relevant functional changes in LC behaviour, it provides an alternative method in particular for the testing of chemical allergens and irritants which are difficult to distinguish from each other. This also makes the model suitable for a wide range of studies not just including hazard identification but also for investigating DC biology under normal steady-state conditions or in skin diseases and in particular cancer. It will enable vaccination research to occur in a standardized human model which in turn will lead to the identification of novel drug targets and therapeutic strategies.

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