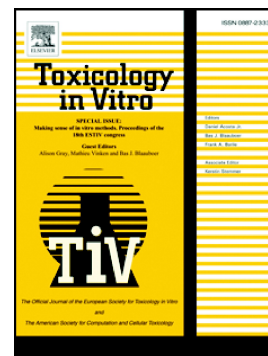


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Characterization of reconstructed human skin containing Langerhans cells to monitor molecular events in skin sensitization

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Abbreviations

ACD, allergic contact dermatitis; CMFDA, 5-chloromethylfluorescein diacetate; cSCC, cutaneous squamous cell carcinoma; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IF, immunofluorescence; IHC, immunohistochemistry; ITS, integrated testing strategy; LC, Langerhans cell; MoLC, monocyte-derived Langerhans cell; MUTZ-LC, MUTZ-3-derived Langerhans cell; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate buffered saline, RHE, reconstructed human epidermis; RHS, reconstructed human skin; RT-PCR, Reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulphate; TGF, transformung growth factor.

Abstract

Human cell-based approaches to assess defined key events in allergic contact dermatitis (ACD) are well-established, but lack cutaneous penetration and biotransformation as well as cellular cross-talk. Herein, we integrated *in vitro*-generated immature MUTZ-3-derived Langerhans-like cells (MUTZ-LCs) or monocyte-derived LC-like cells (MoLCs) into reconstructed human skin (RHS), consistent of a stratified epidermis formed by primary keratinocytes on a dermal compartment with collagen-embedded primary fibroblasts. LC-like cells were mainly localized in the epidermal compartment and distributed homogenously in accordance with native human skin. Topical application of the strong contact sensitizer 2,4-dinitrochlorobenzene (DNCB) induced IL-6 and IL-8 secretion in RHS with LC-like cells, whereas no change was observed in reference models. Increased gene expression of CD83, PD-L1, and CXCR4 in the dermal compartment indicated LC maturation. Importantly, exposure to DNCB enhanced mobility of the LC-like cells from epidermal to dermal compartments. In response to the moderate sensitizer isoeugenol and irritant sodium dodecyl sulphate, the obtained response was less pronounced. In summary, we integrated immature and functional MUTZ-LCs and MoLCs into RHS and provide a unique comparative experimental setting to monitor early events during skin sensitization.

Keywords:

Langerhans cells, personalized medicine, reconstructed human skin, skin sensitization

1. Introduction

Allergic contact dermatitis (ACD) is an inflammatory skin disorder with high prevalence initiated after cutaneous exposure to sensitizers (Martin, S.F. et al., 2011; Vocanson, M. et al., 2009). To ensure human safety, the hazard assessment of novel substances relies on relevant and reliable test methods. The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) focuses on an integrated testing strategy (ITS) using non-animal test batteries that combine *in silico*, *in chemico*, and *in vitro* methods to identify skin sensitizers (Jaworska, J. et al., 2011; Reisinger, K. et al., 2015; Rovida, C. et al., 2015; Urbisch, D. et al., 2015). The ITS includes defined key elements of the sensitization process and provides high accuracy for chemicals with adequate solubility. However, to further unravel the specific molecular mechanisms causing the key events in skin sensitization, cell based assays in monoculture lack complex skin architecture and signalling as neither cutaneous penetration nor complete biotransformation are addressed. Providing a physiological skin environment, human skin explants display the most relevant test system to assess skin sensitization (Jacobs, J.J. et al., 2006; Lehe, C.L. et al., 2006; Ouwehand, K. et al., 2008; Ouwehand, K. et al., 2010). Alternatively, three-dimensional multilayer skin constructs are considered as an organotypic system, but the current commercially available models including reconstructed human epidermis (RHE) and reconstructed human full-thickness skin (RHS), which is comprised of an additional dermis equivalent, largely lack immunocompetent cells.

Skin-resident DCs, including Langerhans cells (LCs) localized in the epidermis, are the relevant subset of immune cells during the sensitization phase of ACD (Kaplan, D.H. et al., 2012; Vocanson, M. et al., 2009). The incorporation of LCs into skin equivalents could overcome the above mentioned limitations and should allow the analysis of molecular key events in the pathogenesis of ACD (Facy, V. et al., 2005; Regnier, M. et al., 1997).

These events comprise the detection of chemicals covalently bound to skin peptides by LC through an intact stratum corneum, the subsequent intracellular processing and presentation of antigens on the cell surface, the extensive interaction with keratinocytes and fibroblasts of the environment and a chemokine-dependent migration to skin draining lymph nodes for the purpose of T cell interaction. *In vitro* generated MUTZ-3-derived (MUTZ-LCs) and monocyte-derived cells (MoLCs) express key markers associated with native LCs such as CD1a, CD207 and HLA-DR, display a LC-like morphology, and are functionally similar to human counterparts (Geissmann, F. et al., 1998; Rees, B. et al., 2011; Said, A. et al., 2015; Said, A. and Weindl, G., 2015). However, LC-like cells derived from different precursors revealed distinct functionality when exposed to microbial antigens and therefore might also differ in the response to sensitizers in an organotypic environment (Bock, S. et al., 2016a; Said, A. et al., 2015). Considering LCs as potential target in the prevention or treatment of ACD, possible discrepancies in their reactivity to sensitizers e.g. maturation profile or cytokine expression pattern, have to be investigated. To provide a reliable human model system, a comparative approach to identify the most relevant LC-like cell type for the assessment of skin sensitization is mandatory. MoLCs (Bechetoille, N. et al., 2007) and MUTZ-LCs (Ouwehand, K. et al., 2011) have been incorporated into RHS, however, no test protocol has been established for comparative integration of LCs derived from monocytes and the MUTZ-3 cell line. Furthermore, skin sensitizers have been investigated in MUTZ-LC-RHS (Kosten, I.J. et al., 2015), MoLC-RHE (Facy, V. et al., 2005) but not MoLC-RHS which allows the analysis of LC migration into the dermis equivalent.

In this study, we directly compared MUTZ-LC-RHS and MoLC-RHS with regard to their functional capability to respond to skin sensitizers in a human cell environment. This proof-of-concept study on the reactivity of both LC types within RHS and the comparative assessment of our data pave the way to advanced research on ACD pathophysiology.

2. Materials and methods

2.1. Human material

The use of human material was approved by the ethics committee of the Charité - Universitätsmedizin Berlin, Germany. All donor and patient samples were obtained after written informed consent and only anonymized samples were used for the experiments.

2.2. Maintenance of MUTZ-3 cell line and generation of MUTZ-LCs

The human acute myeloid leukaemia cell line MUTZ-3 (ACC 295; DSMZ, Braunschweig, Germany) was maintained in a 24-well tissue plate (BD Biosciences, Heidelberg, Germany) at a density of $0.5\text{--}1.0 \times 10^6$ cells/ml per well (Bock, S. et al., 2016b). Growth medium consisted of α -medium (w/o L-glutamine, with nucleosides; Biochrom, Berlin, Germany) supplemented with 20 % fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM L-glutamine and 10 % conditioned medium obtained from the human bladder carcinoma cell line 5637 (ACC 35; DSMZ, (Quentmeier, H. et al., 1997)). Medium was exchanged every 3 days. MUTZ-LCs were obtained after 10 days of differentiation (2.0×10^5 /ml) in a defined cytokine medium containing 10 ng/ml transforming growth factor (TGF)- β_1 , 100 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (all from MiltenyiBiotec, Bergisch-Gladbach, Germany), 2.5 ng/ml tumour necrosis factor (TNF) (eBioscience, Frankfurt am Main, Germany) and 50 μ M 2-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany), medium was exchanged completely at day 5. To ensure a consistent differentiation profile, solely MUTZ-3 progenitors at passage 15-25 were differentiated to CD1a⁺ CD207⁺ CCR6⁺ MUTZ-LCs (Bock, S. et al., 2016a) examined by a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). The identity of the MUTZ-3 cell line along experiments was confirmed by ATCC STR Cell Authentication Service (ATCC, LGC Standards, Wesel,

Germany) and the cell line was regularly tested negative for mycoplasma contamination (Venor[®] GeM Classic Mycoplasma PCR detection kit, Minerva Biolabs, Berlin, Germany).

2.3. Generation of MoLCs

MoLCs were differentiated from human monocytes as described previously (Geissmann, F. et al., 1998; Grohmann, L. et al., 2017; Said, A. et al., 2014; Said, A. et al., 2015). Briefly, buffy-coat samples were obtained from healthy donors (DRK-Blutspendedienst Nord-Ost, Berlin, Germany). Following density gradient centrifugation using NycoPrepTM 1.077 (Axis-Shield, Oslo, Norway), peripheral blood mononuclear cells resulted in the interphase. After washing with phosphate buffered saline (PBS, Sigma-Aldrich, Taufkirchen, Germany) for at least three times, monocyte adherence was used for purification. Differentiated MoLCs were obtained after 7 days of monocyte cultivation in complete medium, consisting of RPMI 1640 (Sigma-Aldrich, Taufkirchen, Germany) with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from PAA Laboratories, Cölbe, Germany) and 10 % heat inactivated FCS (Biochrom, Berlin, Germany), supplemented with GM-CSF (100 ng/ml), interleukin (IL)-4 (20 ng/ml) and TGF- β_1 (20 ng/ml; all from MiltenyiBiotec, Bergisch Gladbach, Germany). Cell separation via CD1a MicroBeads (clone HI149; MiltenyiBiotec, Bergisch Gladbach, Germany) was performed at day 5. Cytokine-dependant differentiation led to immature CD1a⁺ CD207⁺ CD324⁺ TROP-2⁺ Axl⁺ MoLCs (Said, A. et al., 2015) determined by flow cytometry analysis.

2.4. Incorporation of MUTZ-LCs or MoLCs into RHS

RHS (without MUTZ-LCs and MoLCs) were adapted from the normal skin models in the concurrent development of organotypic cutaneous squamous cell carcinoma (cSCC) models (Zoschke, C. et al., 2016). Primary normal human keratinocytes and fibroblasts (passage 3) were obtained from therapeutically indicated circumcisions. The dermal compartment was

constructed by pouring 4 mL collagen I (Biochrom, Berlin, Germany) into cell-culture inserts (0.4 μm pore size, BD Biosciences, Heidelberg, Germany) containing 0.6×10^6 normal human dermal fibroblasts. At day 7, the epidermal compartment was prepared by seeding 3.0×10^6 normal human keratinocytes and 1.0×10^6 MUTZ-LCs or MoLCs, respectively, onto the dermal compartment. Constructs were raised to the air-liquid interface the following day and the culture medium was exchanged 3 times a week. Two weeks after seeding keratinocytes and LC-like cells, the RHS, MUTZ-LC-RHS and MoLC-RHS were either cryo-fixed in liquid nitrogen, or directly processed to flow cytometry or quantitative RT-PCR analysis.

2.5. Quantification of LCs in human skin and LC-like cells in RHS

Human skin used for direct comparison with MUTZ-LC- and MoLC-RHS was obtained from abdominal or mamma reductions. Directly after the surgery subcutaneous fatty tissue was removed and human skin was either cryo-fixed in liquid nitrogen or dissected. The latter tissues were incubated with dispase (Sigma-Aldrich, Taufkirchen, Germany) over night at 4°C . The epidermis was removed from the dermal sheets and subsequently digested using trypsin/EDTA (Sigma-Aldrich, Taufkirchen, Germany) for 15 min at 37°C . Single cell suspensions were obtained using cell strainers with 70 μm pore size (BD Biosciences, Heidelberg, Germany). To assess the quantity of LC in human skin the $\text{CD1a}^+ \text{CD207}^+$ cell fraction was analysed by flow cytometry analysis.

RHS, MUTZ-LC-RHS, and MoLC-RHS were separated into epidermal and dermal compartments and dissected. Digestion for epidermal equivalents was performed by incubation with trypsin/EDTA for 10 min whereas dermal compartments were treated with collagenase (1 mg/ml; Sigma-Aldrich, Taufkirchen, Germany) for 20 min. The obtained cell suspensions were filtered through cell strainers with a pore size of 70 μm (BD Biosciences, Heidelberg, Germany). To assess quantity and migratory capacity of the integrated MUTZ-

LCs or MoLCs, the CD1a⁺ and/or CD207⁺ cell fractions were analysed by flow cytometry analysis.

2.6. CellTracker staining

Incorporated viable LC-like cells in RHS were visualized through staining with CellTrackerTM Green CMFDA (5-chloromethylfluorescein diacetate; Life Technologies, Darmstadt, Germany) for 30 min at 37 °C, directly before seeding onto the dermal compartment. Following the model cultivation period, CellTracker stained LC-like cells were observed within MUTZ-LC-RHS and MoLC-RHS using a BZ-8000 fluorescence microscope (Keyence, Neu-Isenburg, Germany).

2.7. Immunohistochemistry

Snap frozen RHS or human skin were cut into 10 µm cryosections, placed on lysine-coated slides, air dried and fixed with acetone (-20°C; Sigma-Aldrich, Taufkirchen, Germany) or 4 % paraformaldehyde (PFA, Sigma-Aldrich, Taufkirchen, Germany). After washing with PBS and washing buffer, consisting of PBS containing Aurion bovine serum albumin (BSA)-c (0.0025 %; Aurion, Wageningen, Netherlands) and Tween[®] 20 (0.025 %; Sigma-Aldrich, Taufkirchen, Germany), sections were incubated with PBS containing 20 % goat serum for 30 min at room temperature. Mouse anti-CD1a (1:250; clone O10; Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-langerin (1:250; clone DCGM4/122D5; Dendritics, Lyon, France), rabbit anti-collagen-IV (1:200; polyclonal; Abcam, Cambridge, UK), mouse anti-keratin-14 (1:50; clone LL002; Dianova, Hamburg, Germany) or mouse anti-filaggrin (1:50; clone FLG01; Dianova, Hamburg, Germany) were added for an overnight incubation at 4 °C. The following day, sections were either incubated for 45 min with the respective secondary antibody (1:200; AlexaFluor; Dianova, Hamburg, Germany) for immunofluorescence (IF) staining, or treated with 0.3 % hydrogen peroxide (H₂O₂, Sigma-Aldrich, Taufkirchen,

Germany) in PBS for immunohistochemistry (IHC) staining. Subsequent to H₂O₂ treatment, immunoenzymological staining was performed using a MaxPoly-Two™ Plus Polymer HRP Universal Detection Kit (Dianova, Hamburg, Germany) and a Signal Enhancer was applied prior to a Polymer HRP Secondary Antibody according to the manufacturer's instructions. Afterwards, samples were incubated with a substrate-chromogen solution, 0.05 % 3,3'-diaminobenzidine (DAB; Sigma-Aldrich, Taufkirchen, Germany) and 0.015 % H₂O₂ in PBS, until staining was detected. After IF or IHC staining, sections were mounted in ImmunoSelect Antifading Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Dianova, Hamburg, Germany). Samples analysis was performed using fluorescence or bright-field microscopy (BZ-8000 microscope).

2.8. Application of test substances

Two weeks after seeding keratinocytes and LC-like cells, the test substances 2,4-dinitrochlorobenzene (DNCB), isoeugenol, sodium dodecyl sulphate (SDS), the respective solvent controls, or α -medium were applied topically to the constructs (10 μ L/cm², finite dose approach) for 24 h. All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). DNCB was applied in a defined concentration of 0.07 % (w/v), since 0.05 % previously induced the release of IL-18, a cytokine related to skin sensitization, and concentrations up to 0.1 % DNCB caused only minor cytotoxicity in reconstructed human epidermis (RHE) (Gibbs, S. et al., 2013). In addition, 0.4 % (v/v) isoeugenol was selected based on previous studies in RHE demonstrating increased IL-8 levels (Frankart, A. et al., 2012) without reducing tissue viability (Saito, K. et al., 2013). The applied SDS concentration was set to 0.5 % (w/v). Genes related to sensitization remained unaltered in RHE following the application of 0.3 % SDS (Saito, K. et al., 2013), whereas higher SDS concentrations (> 0.5 %) caused cytotoxicity in RHE (Gibbs, S. et al., 2013).

2.9. Flow cytometry

Phenotypical characterization of integrated MUTZ-LCs and MoLCs, the evaluation of migratory capacity 24 h after the application of test compounds as well as analysis of human skin was assessed by flow cytometry. Cell suspensions were incubated with the following monoclonal antibodies: FITC-conjugated mouse anti-CD1a (clone HI149) and corresponding isotype control, PE-conjugated mouse anti-CD207 (clone 10E2) and corresponding isotype control (all from eBioscience, Frankfurt am Main, Germany). Dead cells and debris were excluded by scatter gates. A total of 25×10^3 events were counted and examined using a FACSCalibur (BD Biosciences, Heidelberg, Germany) flow cytometer.

2.10. ELISA

Culture medium was collected directly before (untreated) and 24 h after the test substances were applied to the RHS. IL-6-, IL-8- and IL-18-releases were quantified by ELISA using commercially available ELISA kits (ELISA-Ready Set Go; eBioscience, Frankfurt am Main, Germany and DuoSet; R&D Systems, Wiesbaden, Germany) and ratios (after/before application of test substances) were calculated.

2.11. RNA isolation and quantitative RT-PCR

To evaluate mRNA levels, RHS were separated into epidermal and dermal compartments and analysed separately. Total RNA isolation, cDNA synthesis and quantitative RT-PCR (qPCR) were performed as described previously (Weindl, G. et al., 2011). Primers (synthesized by TIB Molbiol, Berlin, Germany) with the following sequences were used: ATF3, 5'-CCTCGGAAGTGAGTGCTTCT-3' and 5'-ATGGCAAACCTCAGCTCTTC-3'; CD83, 5'-CCTCCAGCTTCTGCTCCTGA-3' and 5'-TCGGAGCAAGCCACCTTCAC-3'; CXCR4, 5'-GCCTTATCCTGCCTGGTATTGTC-3' and 5'-GCGAAGAAAGCCAGGATGAGGAT-

3'; IL-1 β (Hamidi, S. et al., 2014); IL-18 (Pfalzgraff, A. et al., 2016); PD-L1, 5'-CAAAGAATTTTGGTTGTGGA-3' and 5'-AGCTTCTCCTCTCTCTTGGA-3'; SDHA (Said, A. et al., 2015); YWHAZ (Weindl, G. et al., 2007). Fold differences in gene expression were normalized to the housekeeping gene SDHA or YWHAZ which showed the most constant level of expression.

2.12. Statistical analysis

Data are depicted as mean $\pm$ SEM from at least 3 biologically-independent experiments. Statistical significance of differences was determined by one-sample t test or one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis and considered significant at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad software Inc., La Jolla, CA, USA).

3. Results

3.1. LC-RHS show vital characteristics of epidermal differentiation and low basal cytokine levels

We investigated RHS after seeding $0.1-3 \times 10^6$ MUTZ-LCs and MoLCs, respectively, and found that the addition of 1×10^6 LC-like cells (ratio keratinocytes:LC-like cells 3:1) preferably simulated the *in vivo* situation as described below. To further improve the density of LC-like cells within the RHS, we systematically varied the medium composition as well as the co-culture period. However, altered medium composition, especially cholera toxin-free medium, did not improve the final outcome. The simultaneous seeding of keratinocytes with MUTZ-LCs or MoLCs at day 7 revealed best morphological results at the end of the culture period (day 22, Fig. 1a).

The integration of MUTZ-LCs or MoLCs into RHS did not change normal epidermal differentiation. The constructs showed normal morphology with keratinocytes attached to a collagen-IV-positive basal lamina (Fig. 1b). We found keratin-14 positive keratinocytes in all epidermal layers and filaggrin-positive corneocytes in the stratum corneum of both, MUTZ-LC-RHS and MoLC-RHS (Fig. 1b).

We did not detect any morphological hallmarks of skin inflammation such as hyperkeratosis, parakeratosis or spongiosis in the untreated models after two weeks of co-culture. With 1.5 to 3 ng/mL respectively, the secretion of IL-6 and IL-8 into the culture medium of RHS was remarkably low. Both, IL-6 and IL-8 levels were lower in untreated MUTZ-LC-RHS and MoLC-RHS when compared to RHS (Fig. 1c).

3.2. LCs incorporated into RHS resemble hallmarks of cutaneous LC morphology

To prove the incorporation of *in vitro* generated MUTZ-LCs and MoLCs into the RHS at a density comparable to epidermal LCs *in vivo*, LC-like cells were visualized through pre-

staining with fluorescent CellTracker Green CMFDA. Direct comparison displayed striking resemblance to longitudinal sections of human skin *ex vivo*, in which CD207⁺ LCs were visualized through immunofluorescence staining (Fig. 2a). Intriguingly, a clear extension of dendrites for MUTZ-LCs and MoLCs as a hallmarks of cutaneous LC morphology was observed (Fig. 2a, magnification), indicating a close conjunction with surrounding keratinocytes. Furthermore, in accordance to the LC distribution *in vivo*, cross sections of MUTZ-LC-RHS and MoLC-RHS revealed a localisation of incorporated LC-like cells mainly in suprabasal layers of the differentiated epidermal compartments. IHC staining confirmed CD1a and CD207 expression within the epidermal layer (Fig. 2b). RHS without LC-like cells was CD1a and CD207 negative (data not shown). Flow cytometry analyses of digested epidermal MUTZ-LC-RHS and MoLC-RHS compartments and human skin *ex vivo* resulted in approximately 3 % CD1a⁺ cells, further demonstrating the resemblance of MUTZ-LC-RHS and MoLC-RHS to human skin (Fig. 2c). However, *in vitro* generated MUTZ-LCs and MoLCs, respectively, expressed lower surface levels of CD1a and CD207 and therefore slightly differed in their expression pattern from human LCs *in vivo*.

3.3. Exposure to sensitizers augments cytokine release in LC-RHS

We chose three different test substances: the extreme sensitizer DNCB, moderate sensitizer and pre-hapten isoeugenol as well as the irritant SDS to address functional properties of MUTZ-LC-RHS and MoLC-RHS in comparison to RHS without integrated LC-like cells. The concentrations of test substances were adjusted to sufficiently induce functional responses in the context of sensitization assays on reconstructed equivalents of human skin, without reducing cell viability.

Regulation of skin homeostasis under normal and inflammatory conditions is controlled by NF- κ B activity mediating the expression of cytokines, including IL-6 and IL-8. The release of IL-8 by DCs is a commonly used cytokine for sensitization assessment and incorporating

immunocompetent MUTZ-LCs and MoLCs might further increase the sensitivity of RHS. In fact, DNCB induced IL-6 and IL-8 release into the culture medium of both MUTZ-LC-RHS and MoLC-RHS, with significantly increased cytokine levels in MoLC-RHS (Fig. 3). In contrast, isoeugenol caused a less pronounced cytokine induction. The application of the irritant SDS revealed no changes. The control (alpha medium) or solvents did not increase IL-6 or IL-8 levels in MUTZ-LC-RHS and MoLC-RHS. In RHS without LC-like cells, cytokine production remained largely unaffected by DNCB, isoeugenol or SDS application, indicating LC-dependent regulation of IL-6 and IL-8 release in the RHS. MoLC-RHS responded more sensitive compared to MUTZ-LC-RHS with statistically significant differences in cytokine releases between sensitizer and solvent exposure.

3.4. DNCB treatment distinctively alters gene regulation in LC-RHS

Gene expression analysis of established biomarkers for the detection of chemical sensitization in human skin (i.e. ATF3, IL-1 β , CD83, PD-L1, and CXCR4) was performed in epidermal and dermal compartments of the RHS. DNCB elevated ATF3 mRNA expression in epidermal compartments of MUTZ-LC-RHS, MoLC-RHS, and to a lesser extent in RHS, whereas no increase was obtained after the application of the solvent (Fig. 4). Compared to the solvent, the application of DNCB increased IL-1 β mRNA levels in MUTZ-LC-RHS and MoLC-RHS in both, epidermal and dermal compartments. However, augmented gene expression of IL-1 β was also found in RHS without immune cells (Fig. 4). Expression levels of the LC maturation markers CD83 and PD-L1 were similarly regulated in the epidermis of DNCB-treated models with or without LC-like cells. In the dermal compartments an upregulation of CD83 and PD-L1 mRNA was exclusively detected in MUTZ-LC-RHS and MoLC-RHS, which indicates a predominant localization of MUTZ-LCs and MoLCs in the dermal equivalent when treated with DNCB. Therefore we further analyzed mRNA levels of the chemokine-receptor CXCR4 which promotes LC mobility in the onset of migration. However, only MUTZ-LC-RHS

showed a slight increase of CXCR4 mRNA in the dermal compartment when compared to solvent whereas in MoLC-RHS and RHS without LC-like cells no differences in gene levels between solvent control and DNCB were noted. MoLC-RHS nevertheless revealed an increase in CXCR4 gene expression in the epidermal compartment.

In contrast to DNCB, isoeugenol regulated gene expression to a lesser extent. We detected increased PD-L1 gene levels only in MUTZ-LC-RHS, whereas ATF3 mRNA was upregulated in both, MUTZ-LC-RHS and MoLC-RHS. Isoeugenol and SDS caused increased CXCR4 gene transcripts in the epidermal compartment of MoLC-RHS. In general, only minor changes were obtained following SDS treatment (data not shown).

3.5. DNCB increases LC mobility in RHS

To confirm whether the incorporated LCs retain migratory ability, we assessed the mobility of incorporated LC-like cells 24 h after exposure to test substances. In response to DNCB, the number of CD1a⁺ and CD207⁺ MUTZ-LCs in the epidermal compartment was significantly reduced compared to the solvent control, indicating an increased mobility of the LC-like cells (Fig. 5). Additionally, the amount of MUTZ-LCs in the dermal compartment showed a significant increase, thus indicating increased mobility. We also observed a decrease of LC-like cells in the epidermal compartment of MoLC-RHS in response to DNCB and confirmed the presence of CD1a⁺ and CD207⁺ cells within the dermal compartment (Fig. 5), except for two experiments. This indicated an enhanced MoLC mobility after DNCB application which, however, appeared to be less pronounced and less reproducible compared to MUTZ-LCs. The number of immune cells in the epidermis and dermis remained unchanged following isoeugenol and SDS application.

4. Discussion

We comparatively characterized RHS with incorporated LC-like cells to evaluate the functional response towards sensitizers. Here, MUTZ-LC-RHS and MoLC-RHS appeared highly comparable to human skin *ex vivo* concerning quantity and localization of the LC-like cells and resemble non-inflamed cutaneous conditions. By using DNCB and isoeugenol as well as the irritant SDS, we evaluated the functionality of our test system according to the potency of the selected xenobiotics, which is crucial to allow future investigations of molecular key events of ACD in a human-based organotypic skin environment. Both LC containing RHS revealed a similar, but distinct regulation of cytokine release, mRNA expression, and LC mobility in response to sensitizers and thus are basically applicable for fundamental research. In accordance to *in vitro* studies, MUTZ-LCs show a less pronounced inflammatory phenotype, but closely mimic the migratory behaviour of native human LCs (Ouwehand, K. et al., 2010; Santegoets, S.J. et al., 2008). MoLC-RHS appear favourable for the evaluation of cytokine secretion which stands in line with MoLCs in monoculture (Said, A. et al., 2015). Thus, the eligibility of the integrated LC-like cells derived from different precursors largely depends on the endpoints of interest. The use of cell line-derived LC-like cells (e.g. MUTZ-LCs) additionally reduces donor specific biological variances whereas MoLC function is largely influenced by donor variability. At the same time, this latter approach reflects biological diversity thus fostering personalized approaches in ACD research.

Previous studies indicated that RHE with cord blood-derived LCs is suitable to analyse LC maturation markers and cytokine profiles in response to chemical sensitizers (Facy, V. et al., 2005). In agreement with MUTZ-LC-RHS (Ouwehand, K. et al., 2011), we confirmed increased mobility of MoLCs in skin models with a dermal equivalent in the presence of skin sensitizers. Thus, RHS with integrated LCs generated from the MUTZ-3 cell line but also

from primary monocytes is a suitable test system to evaluate early events during skin sensitization.

Cytokine release promotes local skin inflammation in the initiation of contact dermatitis (Lee, H.Y. et al., 2013; Vocanson, M. et al., 2009). An increased cytokine secretion after treatment with sensitizers and irritants was either observed in human skin explants or reconstructed skin with and without LCs (Coquette, A. et al., 2003; Lee, S. et al., 2014; Varani, J. et al., 2007). However, it remains unclear to which extent the incorporated LC-like cells contribute to IL-6 and IL-8 production, due to the low LC numbers in RHS. Nevertheless, our data clearly demonstrate higher IL-6 and IL-8 ratios for MUTZ-LC-RHS and MoLC-RHS when compared to reference RHS in response to DNCB and confirm a significant upregulation for both cytokines in MoLC-RHS. In MUTZ-LC-RHS, the ratio for IL-6 did not reach significance which is in line with an overall low cytokine secretion of MUTZ-LCs in monoculture (Bock, S. et al., 2016a). In contrast to DNCB, topical administration of isoeugenol in the selected concentration had little effect on cytokine production. Previously, exposure of isoeugenol at concentrations below 0.4 % significantly increased IL-8 levels in RHS co-cultured with MUTZ-LCs (Lee, S. et al., 2014). However, these results were obtained after a prolonged incubation period of 48 h. Considering isoeugenol as a pre-hapten, being activated through auto-oxidation, a time-dependent response might be apparent (Smith Pease, C.K., 2003).

Irritants are also known inducers of IL-6 and IL-8 secretion, however, SDS failed to modulate cytokine release in our models even in the presence of immunocompetent cells. Skin irritation by SDS is strictly concentration-dependent confirming a non-irritating effect at low concentrations (Ouweland, K. et al., 2008; Varani, J. et al., 2007). In previous studies, 0.05 % SDS significantly induced IL-8 gene expression levels in RHE after 18 h, whilst the release of IL-8 protein in RHE appeared to be concentration dependent and was confirmed in a range of 0.2 to 0.8 % SDS after 24 h (de Brugerolle de, F. et al., 1999; Niwa, M. et al., 2009).

However, improved RHE culture medium increased skin barrier function and significantly decreased IL-1 α cytokine release by keratinocytes upon exposure to SDS, leading to a concomitant decrease of IL-8 (Spiekstra, S.W. et al., 2009). In line with our data, 1 % SDS revealed no significant upregulation of IL-6 and IL-8 levels when added to RHS, whereas acquired skin barrier deficiency led to strikingly increased IL-6 and IL-8 release in response to SDS (Küchler, S. et al., 2011). Thus, considering the different outcomes of cytokine release in various test systems, the functional skin barrier appears important to assess SDS irritancy. In addition to cytokine secretion, gene expression profiling has been used to identify predictive markers involved in the initiation of ACD (Coleman, K.P. et al., 2015; Johansson, H. et al., 2013). In RHE, a dose dependent specificity for ATF3 in the identification of extreme sensitizers has been confirmed (Saito, K. et al., 2013). The ATF3 gene promoter is regulated by the Nrf2/Keap1/ARE signalling pathway and respond to direct cytotoxicity caused by accumulation of ROS due to cellular stress (Natsch, A. and Emter, R., 2008). Recent findings show that allergen-induced inflammatory responses are accompanied by elevated ROS levels (Byamba, D. et al., 2010) and additionally, the Nrf2/Keap1/ARE pathway is increased by extreme but not moderate sensitizers in reconstructed human skin models (McKim, J.M., Jr. et al., 2012). Our results extend these findings indicating enhanced ATF3 expression in the presence of immunocompetent cells, where the extreme sensitizer DNCB markedly induced ATF3 mRNA levels. Additionally, our data suggest that integrated LCs are dispensable for DNCB-induced IL-1 β expression and support the view that keratinocytes are the main source of IL-1 β (Facy, V. et al., 2005; Rambukkana, A. et al., 1996).

IL-18 plays a crucial role in the induction of skin sensitization and the release of IL-18 may be a promising endpoint for the identification and classification of skin sensitizing chemicals in RHE (Gibbs, S. et al., 2013). However, we failed to detect IL-18 in the supernatants and IL-18 gene levels remained unchanged in the models treated with the test substances (data not

shown). Additional studies are needed to clarify the role of IL-18 to serve as a marker for skin sensitizers using RHS.

The acquired mobility of integrated LCs after DNCB treatment was demonstrated by the significant increase of CD1a⁺ and CD207⁺ cells in the dermis and the concomitant significant decrease in the epidermis in MUTZ-LC-RHS. The observed effect is possibly triggered through a mobility promoting chemokine gradient provided by dermal fibroblasts (Ouweland, K. et al., 2008). The initiation of LC migration out of the epidermis is accompanied by an early upregulation of CXCR4 followed by CCR7 (Kabashima, K. et al., 2007). In our study, chemokine secretion might result from DNCB-induced inflammatory conditions within the RHS. However, MUTZ-LC-RHS only showed slightly altered CXCR4 mRNA levels, indicating that the observed increase in LC numbers in the dermal compartment is partly dependent on the chemokine gradient. Mobility of MUTZ-LCs appeared more reproducible compared to MoLCs. This confirms the observed CXCR4 expression pattern of both, MUTZ-LC-RHS and MoLC-RHS, with respect to the compartmentalization, since MoLC-RHS revealed an upregulation of CXCR4 mRNA only in the epidermis. Maturation of the migrated LC-like cells was also supported by increased CD83 and PD-L1 mRNA levels in the dermal compartment.

Conclusively, we found both *in vitro* test systems suitable to determine functional properties of LC-like cells in a physiologic cell environment. Although still in the early development stage, LC-containing RHS should closely resemble human skin *in vivo* in terms of number, morphology and phenotype of LCs. Importantly, strong skin sensitizers should induce phenotypic and functional changes in LCs, yet further screening tests are required to define quantitative criteria and applicability to study skin sensitization. MUTZ-LC-RHS emulate enhanced LC mobility in skin and thus are of importance in the evaluation of molecular mechanisms related with migratory behavior of human LC in response to sensitizers. MoLC-RHS outperformed MUTZ-LC-RHS with regard to cytokine release indicating a more

pronounced interaction with keratinocytes and fibroblasts. To what extent this distinct functionality affects T cell interaction, the last key event of the sensitization phase, needs to be elucidated. As it is known that allergic skin lesions are highly diverse in terms of cellular composition, including different subpopulations of CD4⁺/CD8⁺ T cells (Rovida, C. et al., 2013), mimicking the interaction of LC-like cells with skin resident T lymphocytes will be essential to further understand the pathophysiology of ACD in humans.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1 Construction and characterization of MUTZ-LC-RHS and MoLC-RHS. **a** The method to construct RHS containing LC-like cells as well as the subsequent analysis is schematically visualized in a procedure outline. **b** At the end of culture period (day 22), cross sections of MUTZ-LC-RHS and MoLC-RHS were stained for the differentiation markers Keratin-14, Filaggrin and Collagen-IV (green) using fluorescence microscopy. Cell nuclei were stained with DAPI (blue). Scale bar represents 100 μm . Pictures are representative of at least 3 independent experiments. **c** Basal IL-6 and IL-8 levels in untreated RHS, MUTZ-LC-RHS and MoLC-RHS. Cytokine levels were quantified by ELISA. Mean $\pm$ SEM, $n = 5-6$.

Fig. 2 MUTZ-LC and MoLC visualization and quantification in RHS compared to human skin. **a** MUTZ-LCs and MoLCs pre-stained with CellTracker Green CMFDA were visualized through high angle shots of separated epidermal sheets of the respective RHS. As direct comparison, longitudinal sections of human skin were stained for CD207 expression (red) using fluorescence microscopy. Cell nuclei were stained with DAPI (blue). Scale bar represents 50 μm . **b** Expression of CD1a and CD207 in MUTZ-LC-RHS, MoLC-RHS, and human skin was visualized by immunoperoxidase staining. Each dashed line denotes the epidermis–dermis boundary. Scale bar represents 50 μm . Pictures are representative of at least 3 independent experiments. **c** MUTZ-LCs, MoLCs, and primary LCs within single cells suspension of digested epidermal sheets were identified by the co-expression of the critical human LC markers CD1a and CD207, assessed by two-colour flow cytometry. Percentage of stained cells is indicated. Dot plots of $20-25 \times 10^3$ events of gated scatter plot are representative of 3 independent experiments.

Fig. 3 Cytokine release of MUTZ-LC-RHS and MoLC-RHS in response to DNCB and isoeugenol. Culture medium was collected over a period of 24 h directly before, and 24 h after

the test compound DNCB or isoeugenol was applied to constructs. IL-6 and IL-8 releases were quantified by ELISA and displayed as cytokine ratio. Alpha medium served as control. Mean $\pm$ SEM (n=3-5). * $p \leq 0.05$, ANOVA with post-hoc Bonferroni test.

Fig. 4 Gene expression in MUTZ-LC-RHS and MoLC-RHS in response to DNCB and isoeugenol. 24 h after application of 0.07 % DNCB or 0.4 % isoeugenol RHS were separated into epidermis and dermis and analysed separately. Gene expression values of ATF3, IL-1 β , CD83, PD-L1 and CXCR4 were normalized to YWHAZ and SDHA and displayed as fold change compared to control values (control assigned as 1.0). Mean $\pm$ SEM (n = 3-5).

Fig. 5 Mobility of LC-like cells in response to DNCB treatment. 24 h after application of 0.07 % DNCB, MUTZ-LC-RHS and MoLC-RHS were separated into epidermis and dermis sheets and single cell suspension of digested compartments was analysed. MUTZ-LCs and MoLCs were stained for expression of CD1a and CD207 and quantified by two-colour flow cytometry. Data represent mean values of 20-25 $\times 10^3$ events of gated scatter plot $\pm$ SEM (n = 3-5). * $p \leq 0.05$, ** $p \leq 0.01$, ANOVA with post-hoc Bonferroni test.

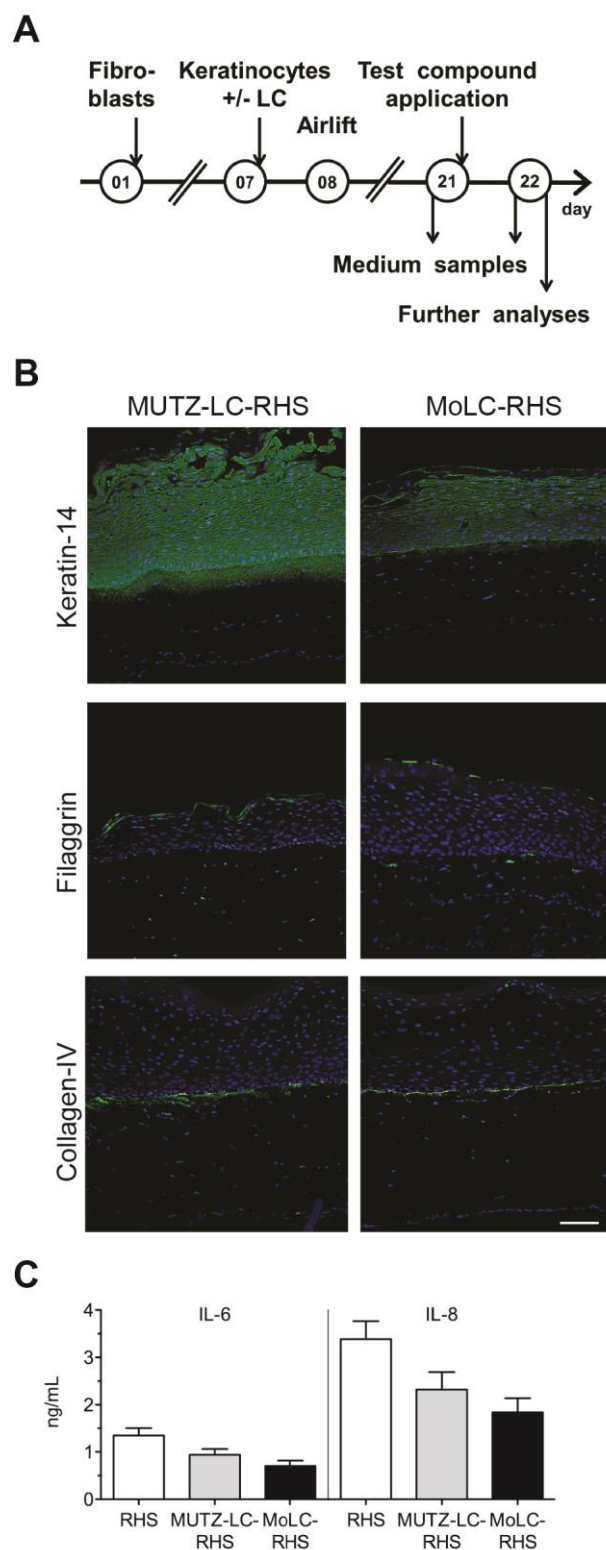


Figure 1

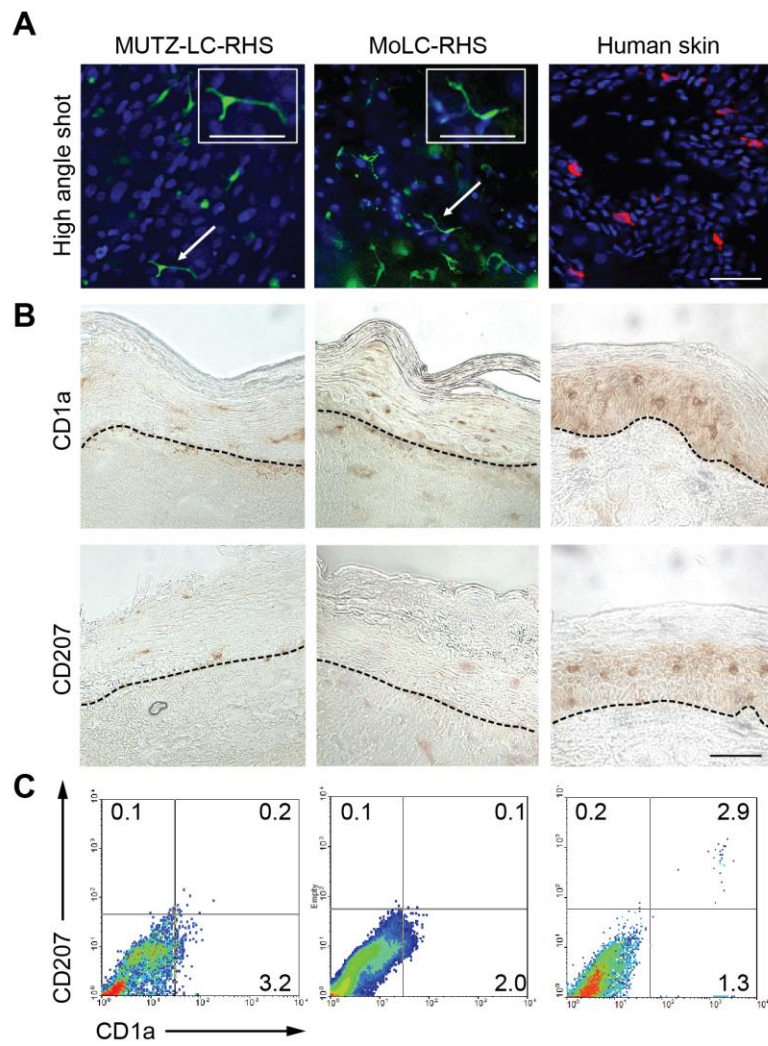


Figure 2

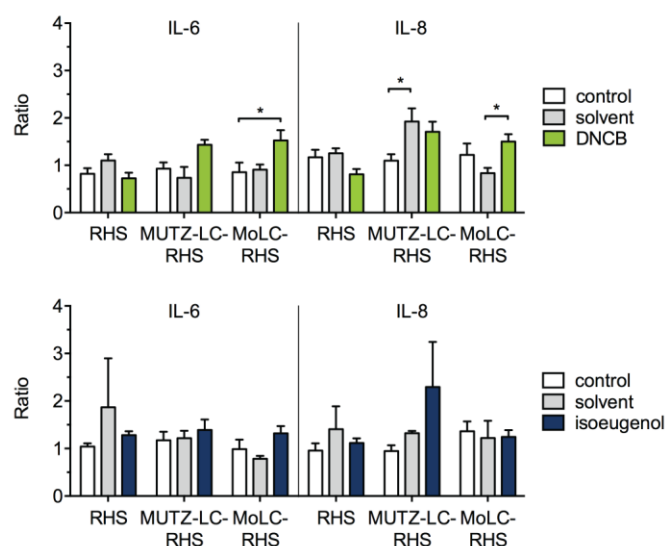


Figure 3

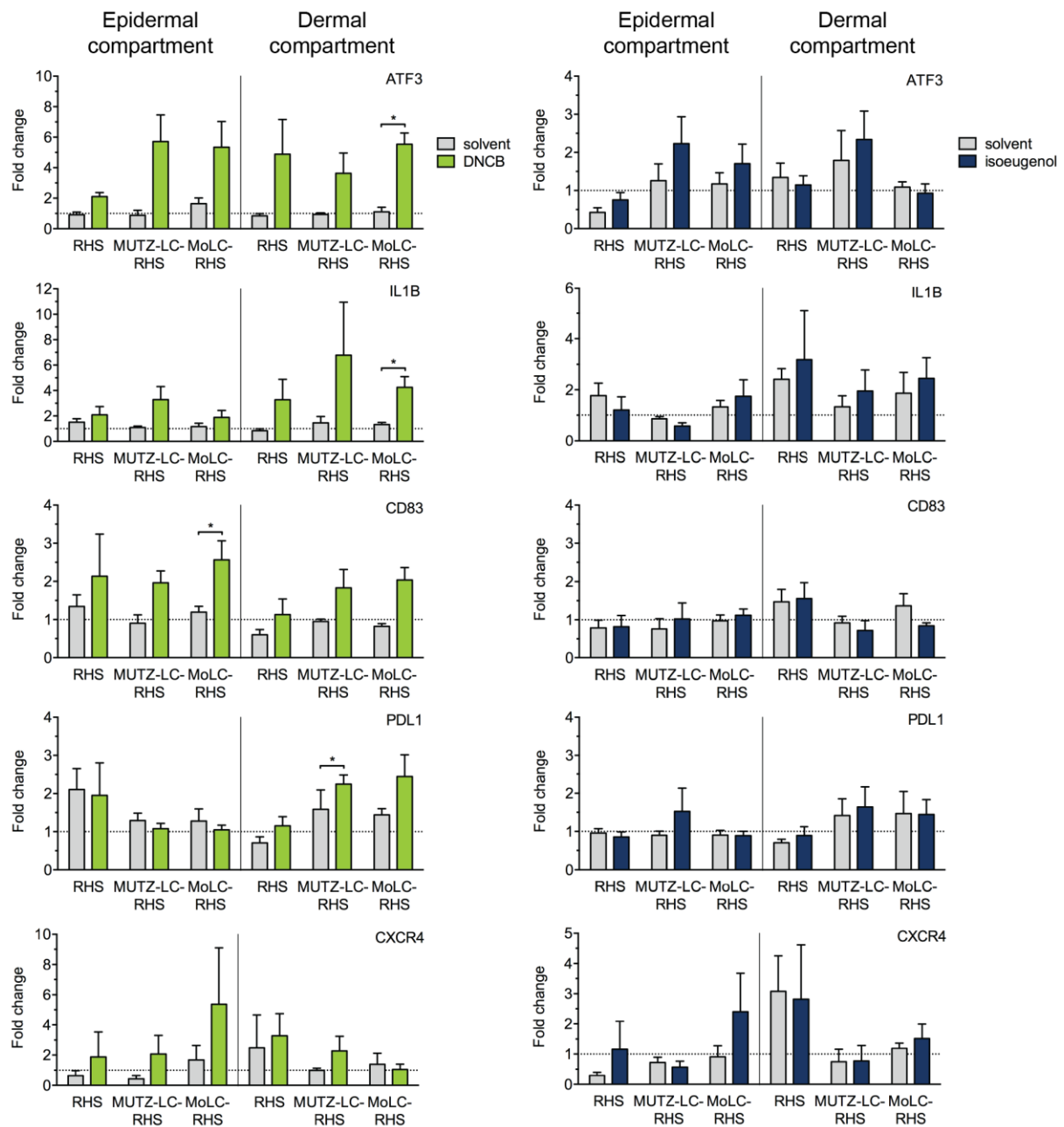


Figure 4

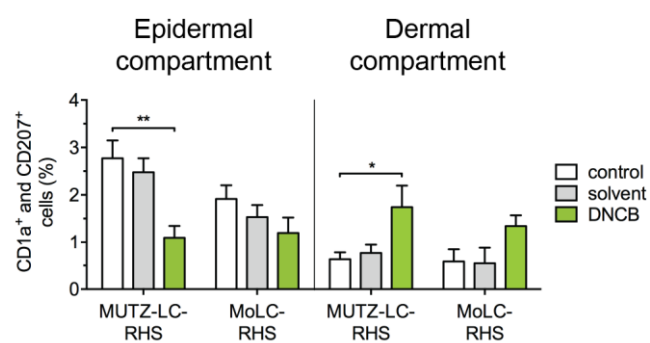


Figure 5

Highlights

- Reconstructed human skin (RHS) with LCs shows normal epidermal differentiation
- LCs incorporated into RHS resemble hallmarks of cutaneous LC morphology
- Exposure to sensitizers augments cytokine release in LC-RHS
- 2,4-Dinitrochlorobenzene increases LC mobility in RHS

ACCEPTED MANUSCRIPT