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

Stephanie Bock, André Said, Gerrit Müller, Monika Schäfer-Korting, Christian Zoschke, Günther Weindl*

Institute of Pharmacy (Pharmacology and Toxicology), Freie Universität Berlin, Berlin, Germany

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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.

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 signaling 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 drainig 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

* Corresponding author at: Institute of Pharmacy (Pharmacology and Toxicology), Freie Universität Berlin, Königin-Luise-Str. 2 + 4, D-14195 Berlin, Germany.
E-mail address: Guenther.Weindl@fu-berlin.de (G. Weindl).

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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 dictinct 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 0.5–1.0 × 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 μM 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 CD1a$^+$ 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

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