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Integration of MUTZ-Langerhans cells into a 3D full-thickness skin equivalent and influences of serum reduction and undefined medium supplements on differentiation

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ABSTRACT

The MUTZ-3 cell line is a surrogate for Langerhans cells (LCs) employed in New Approach Methodologies for assessing the skin sensitizing potential of chemicals. However, MUTZ-3 cells must first be differentiated to achieve the LC-typical phenotype. As all protocols use high fetal calf serum (FCS) concentrations, we aimed at reducing, or even replacing FCS, while maintaining MUTZ-LC characteristics. Additionally, we assessed the impact of the poorly defined 5637-conditioned medium (5637CM) on MUTZ-LC differentiation.

With reducing the FCS content by 75 %, the desired differentiation status was achieved after 7 instead of 14 days, identified by elevated CD207 and CD1a expression. Culture with Ultroser G, a synthetic surrogate for FCS, resulted in an insufficient number of MUTZ-LCs. 5 % FCS-differentiated MUTZ-LCs could be activated with DNCB, an extreme sensitizer, as demonstrated by increased CD83 expression. 5637CM did not affect MUTZ-LC differentiation and is therefore not needed as a supplement.

For their intended role in an immunocompetent skin model to assess the sensitizing potential of chemicals, MUTZ-LCs were successfully integrated into the Phenion® Full-Thickness skin model, as demonstrated by CD1a expression. These results are important steps towards medium standardization and the generation of an immunocompetent skin model according to the 3R principles.

1. Introduction

The EU Cosmetics Regulation EC 1223/2009 (2009) has put a complete marketing ban on cosmetic ingredients and formulations which have been tested on animals. Only non-animal tests, in particular *in vitro, in chemico* and *in silico* methods, are allowed for the toxicological risk assessment, including the skin sensitization potential, of chemicals used in cosmetic products.

In order to simulate the AOP for skin sensitization with non-animal-based methods, several *in vitro* and *in chemico* test methods, or New Approach Methodologies (NAMs), and Defined Approaches (DAs) which combine NAMs with *in silico* data, have been developed and validated (Hoffmann et al., 2018; Kleinstreuer et al., 2018). However, currently all NAMs accepted by the Organisation for Economic Co-operation and Development (OECD) address only one or two of the key events 1–3 of the AOP, with a reliable and validated method addressing T cell activation still missing. As a result, a combination of NAMs is mandatory to characterize the skin-sensitizing potential of chemicals within the concept of Next Generation Risk Assessment (NGRA; OECD TG 497, 2021; To et al., 2024).

To our knowledge, currently no single NAM for skin sensitization reflects the complexity of native human skin, comprising at least keratinocytes, fibroblasts and dendritic cells, the barrier function, and the skin-specific metabolism. Until today, three test methods for *in vitro* skin sensitization assessment, based on human reconstructed epidermal models, have been published so far. Whereas the EpiSensA method was recently adopted by the OECD (OECD TG 442D; 2024a), the Sens-IS (Cottrez et al., 2016) and IL-18 (Andres et al., 2020) tests are scientifically valid methods which can support the risk assessment of chemicals within the IATA framework. However, these test methods only reflect keratinocyte activation (KE 2), with the other key components, like immune cells, missing. Thus, it is our goal to develop an

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immunocompetent full-thickness (FT) skin model which has the prerequisite to reflect AOP key events 1–3 in a single system and in a more *in vivo*-like environment than in the OECD-approved NAMs. In addition, a skin model with a near-dry surface allows the application of nearly all kinds of test materials at the relevant skin sensitization dose metric, defined as dose of chemical allergen per unit area of skin (*e.g.* Kimber et al., 2008).

Langerhans cells (LCs), the dendritic cells (DCs) of the skin, play an important role in the induction of allergic contact dermatitis (ACD) after exposure of the skin with a sensitizing agent. The induction process can be divided into four key events (KE), which are detailed in the OECD Adverse Outcome Pathway (AOP; OECD, 2012). KE 1 comprises the haptenization of the sensitizing agent, KE 2 the keratinocyte inflammatory response, KE3 the dermal dendritic cell activation, and KE 4 the T cell activation in the local lymph node. Key events 2 and 3 are tightly connected to each other. After barrier penetration and haptenization, the epidermal keratinocytes are activated and start secreting different cytokines intended to attract and influence dendritic cells (KE 2). Once in place, the dendritic cells take up the hapten-protein complex, migrate to the next local lymph node, and pursues the processes subsumed under KE 3 (*e.g.* OECD AOP, 2012; Maxwell et al., 2014; Rodrigues Neves and Gibbs, 2018).

The LCs, a special type of DCs which reside in the lower strata of the human epidermis, are a central component in the induction and elicitation phases leading to allergic contact dermatitis (KE 3; OECD, 2012). Briefly, after haptenization, the antigen-protein complex is processed by epidermal LCs, which then start migrating out of the epidermis to the nearest local lymph node. In the lymph node, they present the hapten complex, together with major histocompatibility complexes, to the residing T-cells, thereby inducing their transformation into memory T cells. *In vitro* LC activation is currently addressed by the OECD-approved NAMs human cell line activation test (h-CLAT) and the U937 cell line activation test (U-SENS™, both part of TG 442E, 2024c). However, whereas they are not approved as stand-alone tests, they are integral components of the DAs published so far (OECD TG 497).

Due to limited supply, poor viability in culture, and putative donor variability of native LCs isolated from human skin biopsies (e. g. Régnier et al., 1998), the NAMs addressing KE 3 employ surrogate cell lines, derived from human leukemia cells (OECD TG 442E, 2024b). The human monocytic leukemia cell line THP-1 is used in the h-CLAT and the U-SENS test (OECD TG 442E). As read-out parameters for skin sensitization, the expression of two cell surface markers, CD54 and CD86, are determined by flow cytometry (only CD86 in the U-SENS assay). The human acute myeloid leukemia cell line MUTZ-3, which resembles native human $CD34^{+}$ dendritic cell precursors, is the basis for the GARD assay (Quentmeier et al., 1996; Larsson et al., 2006; Johansson et al., 2011, 2013). MUTZ-3 cells can be characterized by their CD14 expression, a ligand which binds bacterial lipopolysaccharides (Wright et al., 1990). This marker gets lost during differentiation (Masterson et al., 2002; Larsson et al., 2006). While in the GARD assay the MUTZ-3 cells are used in an undifferentiated status, they can be differentiated into a phenotype which reveals key characteristics of native LCs (Masterson et al., 2002; Santegoets et al., 2006; Larsson et al., 2006). These so-called MUTZ-LCs express CD207 (also called langerin), the main protein component of the Birbeck granules, characteristic intracellular structures of human epidermal LCs (Valladeau et al., 2000; Santegoets et al., 2006). Also, CD1a is expressed, which presents microbial lipid and glycolipid antigens to T cells (Melián et al., 1996). CD83 was reported as another maturation marker (Zhou and Tedder, 1995).

As a consequence of the differentiation process, the MUTZ-LCs elicit LC-typical reactions upon exposure to known sensitizers (e. g. Masterson et al., 2002; Santegoets et al., 2006; Nelissen et al., 2009; Groell et al., 2018). As a result of a comparative study including different cell lines, Santegoets et al. (2008) concluded that MUTZ-3 cells were the most suitable LC surrogates.

For the development of an immune-competent FT skin model enabling assessment of the skin sensitization potential and the potency of chemicals, differentiated MUTZ-LCs appear to be the preferred cell type compared to undifferentiated MUTZ-3 cells. The suitability of this concept was firstly demonstrated by integrating MUTZ-LCs in a FT skin model, comprising a fully differentiated epidermis and a dermis based on a collagen gel (Kosten et al., 2015). After culturing the models at the air-liquid interface, the MUTZ-LCs could be located immunohistochemically in the epidermis. Upon topical treatment of the tissues with sensitizers, the MUTZ-LCs could not be detected in the epidermis any longer, for the authors an indicator for dendritic cell migration. Based on these results, we decided to use this method as a template for integrating MUTZ-LCs in the Phenion® FT Skin Model (Mewes et al., 2007).

A key prerequisite for establishing an immunocompetent FT skin model is the generation of sufficient numbers of physiologically active MUTZ-LCs under optimum standardized conditions. This crucial demand was the starting point for a series of experiments and case studies intended to optimize and standardize the media used for MUTZ-3 culture and MUTZ-LC differentiation, which are the subject of this paper.

The cytokines GM-CSF, TGF-β and TNF-α are essential for successfully differentiating MUTZ-3 cells into MUTZ-LCs. GM-CSF is a cytokine known to induce dendritic cell development (Inaba et al., 1992). TGF-β, which is expressed and secreted by native LCs, also plays a role in DC development. This was shown by Borkowski et al. (1997), who reported that TGF-β1 deficient mice lacked LCs in their epidermis. Furthermore, Thomas et al. (2001) demonstrated, that TGF-β might act in an autocrine and paracrine way in LC differentiation in mice. TNF-α plays a role in maintaining the LCs in their immature/inactive state (Koch et al., 1990). This physiological state is required for the development of an *in vitro* method intended to identify sensitizing chemicals, as the MUTZ-LCs should only become activated after exposure with sensitizers. In addition to said cytokines, nearly all differentiation media published so far are supplemented with fetal calf serum (FCS), either at a concentration of 20 % (*e.g.* Santegoets et al., 2006; Nelissen et al., 2009; Ouwehand et al., 2010; Groell et al., 2018), or 10 % (Ruben et al., 2015). FCS can be considered an undefined cytokine cocktail, which is prone to lot-to-lot variabilities. In addition, FCS, an animal-derived component, is obtained under ethically questionable conditions which are the subject of ongoing debates (e. g. Kadam et al., 2020; Chelladurai et al., 2021; Cassotta et al., 2022; Duarte et al., 2023). Against this background, it is desirable to reduce or even replace FCS in the MUTZ-LC differentiation medium, while retaining all LC-typical expression markers and physiological pathways. This would improve the standardization of the differentiation protocol on the one hand, and contribute to the 3R principles, on the other hand (Russell and Burch, 1959).

The supplier of the MUTZ-3 cells used in our study, the German Collection of Microorganisms and Cell Cultures (DSMZ), recommends supplementing the culture medium with 20 % FCS and 5637-conditioned medium (5637CM). Cells of the human bladder carcinoma cell line 5637 are known to secrete cytokines into the culture medium, which support MUTZ-3 cell proliferation and viability in culture, namely GM-CSF, G-CSF, M-CSF, CSF and, to a lesser degree, IL-1A (Quentmeier et al., 1997). Although Quentmeier et al. present concentration ranges for the mentioned cytokines, these values cannot be generalized because they reflect results achieved under the specific culture conditions described in the paper. As culture conditions vary in each lab to a certain degree, the composition of the supernatant is also subject to fluctuations, and hence a source of uncertainty. Despite this apparent limitation, 5637CM has been demonstrated to be a key component for successful MUTZ-3 cell culture maintenance over a longer period of time, and no approach has been reported so far to replace the supernatant with high-purity cytokines at defined concentrations.

On the other hand, to our best knowledge, there is no information currently accessible about 5637CM use in the differentiation protocol. This is especially interesting because GM-CSF, a proven component of 5637CM, is pivotal for MUTZ-LC differentiation and must be added to the respective culture medium.

In order to eliminate possible sources of uncertainty, and to optimize standardization of the culture conditions, we investigated the influence of FCS at different concentrations on MUTZ-LC differentiation. The option to completely replace FCS was the subject of a case study with a commercially available, non-animal-derived surrogate, Ultroser G. In addition, due to lacking information, the impact of 5637CM on the MUTZ-LC differentiation process was analyzed. To determine the current physiological status of the MUTZ-3 and MUTZ-LCs, respectively, under the different experimental condition, the expression of key proteins like CD1a and CD207, were analyzed with flow cytometrically. The sensitization markers CD54 and C86, which are used in the human cell line activation test (h-CLAT, OEDC TG442E), as well as the maturation marker CD83, complemented the panel of proteins investigated in this study.

2. Materials and methods

2.1. Maintenance of MUTZ-3 cells

The acute myeloid leukemia cell line MUTZ-3 (ACC 295, DSMZ, Braunschweig Germany) was cultured in a 12-well tissue plate at a density of 0.8–1.2 × 10^6 cells per well. The cells were maintained in Minimal Essential Medium (MEM) alpha without nucleosides (Biochrom, Berlin, Germany) supplemented with 20 % FCS (Biochrom, Berlin, Germany), 10 % 5637-conditioned medium (5637CM), 1 % penicillin-streptomycin (Gibco, Carlsbad, CA, USA), 50 μM β-mercaptoethanol (Gibco). MUTZ-3 cells were passaged every three to four days. 5637CM was obtained with the following protocol: 5637 cells (ACC 35, DSMZ, Braunschweig Germany) were cultured in 175 cm^2 flasks (Greiner Bio-One International, Kremsmünster, Austria) and in RPMI-1640 (Invitrogen, Darmstadt, Germany) at a density of 3 × 10^6 cells and 30 ml medium per flask, supplemented with 10 % FCS, 1 % penicillin-streptomycin and 50 μM β-mercaptoethanol. The culture medium was changed every two to three days. For that, 5637 cells were washed with PBS and then detached after incubating the cells for seven minutes at 37 °C in 5 ml trypsin per flask. After two days of culture, when the 5637 cells were confluent, the medium was collected, filtered through 0.2 μm pore (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until further use.

2.2. Differentiation of MUTZ-3 cells into MUTZ-LCs and chemical exposure of MUTZ-LCs

The original differentiation protocol in our lab was as described as in the following: MUTZ-LCs were generated by culturing MUTZ-3 cells for 14 days in MEM alpha without nucleosides (Biochrom) supplemented with 20 % FCS, 1 % penicillin-streptomycin, 50 μM β-mercaptoethanol, 10 ng/ml TGF-β (Peprotech, Hamburg, Germany), 2.5 ng/ml TNF-α (Peprotech) and 100 ng/ml GM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured in a 12 well plate at a density of 5 × 10^5 cells per well (2 ml/well) and the medium was changed after seven days.

The differentiation protocol was optimized like this: MUTZ-3 cells were cultured for seven days without changing the medium in MEM alpha without nucleosides supplemented with 5 % FCS, 1 % penicillin-streptomycin, 50 μM β-mercaptoethanol, 10 ng/ml TGF-β, 2.5 ng/ml TNF-α and 100 ng/ml GM-CSF. The cells were cultured in a 12-well plate at a density of 5 × 10^5 cells per well (2 ml/well).

For induction of an allergic reaction MUTZ-LCs were treated for 24 h with DNCB (Sigma Aldrich, St. Louis, MO, USA). During the optimization process of the differentiation medium, 4 % Ultroser G (Pall BioSepra, Cergy-Saint-Christophe, France) was tested as an FCS substitute.

2.3. Flow cytometry analysis

The MUTZ-LCs were phenotypically characterized during differentiation. Cells were washed twice with flow cytometry buffer and incubated for 10 min at 4 °C in the dark with the following antibodies with the coupled fluorochrome in brackets: 1:200 anti-CD1a (PE), 1:200 anti-CD14, 1:200 anti-CD207 (PE-Vio770), 1:200 anti-CD54 (APC), 1:100 anti-CD83 (APC-Vio770) and 1:100 anti-CD86 (APC). To access non-specific binding, cells were stained with recombinant isotype controls, coupled with the fluorochromes, which were used for the marker-antibodies. After antibody incubation, the cells were washed twice with flow cytometry buffer and then measured with a MAQSQuant 10 flow cytometer. Dead cells and debris were excluded by propidium iodide (PI) staining and scatter plot. 10,000 PI-negative cells were analyzed, which was done with the MAQSQantify software. To determine the percentage of viable cells, a sufficient number of cells were analyzed to achieve a cell count of 10,000 PI-negative cells. The percentage of PI-negative cells was calculated as the ratio of PI-negative cells to the total number of cells (PI-negative and -positive cells). Only recombinant antibodies (REAfinity antibodies) were used to reduce the use of animal-derived products and to enhance standardization. All antibodies and products for flow cytometry were purchased from Miltenyi Biotec.

2.4. Integration of MUTZ-LCs into the Phenion® FT skin model

The MUTZ-LCs were integrated into the commercially available Phenion® FT skin model (Henkel, Düsseldorf, Germany). Parallel to the differentiation of the MUTZ-3 cells into MUTZ-LCs, the Phenion® FT dermis models as well as the keratinocytes were cultured, as described by Mewes et al. (2007). After the differentiation, 1.0 × 10^6 MUTZ-LCs were seeded with 0.25 × 10^6 keratinocytes on the Phenion® dermis model. Following a 1-2 h incubation, another layer of 0.25 × 10^6 keratinocytes was seeded to enclose the MUTZ-LCs between the keratinocytes.

2.5. Immunofluorescence staining of tissue sections

To prepare frozen tissue sections, one half of the skin model was embedded in tissue freezing medium in cryomolds. It was frozen at −20 °C and cut into 8 μm sections (Cryostat SM 1900, Leica Biosystems, Wetzlar, Germany) which were transferred onto slides. The immunofluorescence staining was performed by incubating the slides in pre-cooled acetone at −20 °C for at least 15 min to fix the tissue sections. Next, the frozen sections were incubated with normal goat serum 10 % (Thermo Fisher Scientific) to block non-specific binding of the antibodies (30 min, room temperature). The solution was replaced with the primary antibody CD1a (1:250; Santa Cruz Biotechnology, Dallas, TX, USA) in antibody diluent (Agilent Dako, Santa Clara, CA, USA). After at least 1 h incubation at 4 °C, the sections were washed three times with PBS, followed by an incubation for 1 h at room temperature with the secondary antibody goat anti-mouse IgG AlexaFluor 488 (FITC) (1:200; Invitrogen) in antibody diluent including DAPI (1:1000; Sigma-Aldrich). Lastly, the tissue sections were washed, covered with fluorescence mounting medium (Agilent Dako) and examined under a fluorescence microscope (BX51, Olympus, Germany) with a microscope-mounted digital camera DP71.

2.6. Statistical method

The number of independent experiments conducted are indicated in the figure legends (N = X). Each sample was measured once per

experiment (flow cytometry). Standard deviation of the independent experiments are depicted as error bars in the respective figures. The statistical analysis was done using Microsoft Excel.

3. Results

3.1. Impact of 5637CM on MUTZ-LC differentiation

We conducted a case study to determine whether 5637CM, an undefined and hence not standardized supplement, has also an impact on the MUTZ-LC differentiation. So far, 5637CM was seen as indispensable for MUTZ-3 culture, but its role MUTZ-LC differentiation has not yet been elucidated. We compared the influence of the 5637CM on MUTZ-LCs morphology, viability, and protein expression. Most of the non-adherent MUTZ-3 cells revealed a spherical morphology (Fig. 1A). Only a small fraction of cells acquired a more elongated shape. Moreover, the MUTZ-3 cells grew as single cells. The morphology of differentiated MUTZ-LCs differed from that of MUTZ-3 cells in some characteristic, but these differences were similar in media supplemented with and without 5637CM (Fig. 1B, C). The MUTZ-LCs were bigger in size, their granularity was enhanced, and they tended to aggregate with each other. Some cells even adhered to the bottom and exhibited small dendritic processes.

In addition, cell viability and the expression profile of proteins characteristic for differentiating DCs were analyzed during culture with flow cytometry (FC; Fig. 2). Before starting the differentiation culture, about 90 % of the MUTZ-3 cells were viable. Cell viability decreased during differentiation to 60 % on day 7 and to 50 % on day 14 under both culture conditions. Thus, the decrease over time was independent of the presence of 5637CM in the differentiation medium.

During differentiation, the cellular expression of LC-typical proteins in differentiation medium with or without 5637CM was similar. CD14 expression was lost during differentiation, whereas a *de novo* expression of CD1a and CD207 was detected. After 14 days of differentiation, nearly every cell expressed the LC-typical markers. The percentage of CD54-positive cells were continuously high. CD86 expression increased within the first 7 days in differentiation culture from 30 % to nearly 100 % and remained at this level until day 14.

3.2. FCS reduction accelerates MUTZ-LC differentiation

Another undefined medium supplement that is added to almost each published differentiation medium is FCS. Consequently, we performed another case study to test if the 20 % FCS can be reduced or even left out of the medium. For that, we tested four differentiation media containing 0 %, 5 %, 10 % and 20 % FCS and analyzed the MUTZ-LCs grown in these media with FC. The FC gating strategy to only analyze viable cells is shown in Fig. 3 A-C. Specifically, we included only signals derived from single cells (Fig. 3A), excluded cell debris (Fig. 3B), and included only living (PI-negative) cells (Fig. 3C). Thus, we ensured to analyze the relevant cell population, viable and single cells.

A completely FCS-free differentiation medium did not support MUTZ-LC survival. The count of viable cells in culture was too low for subsequent FC analysis (data not shown).

After 7 days of differentiation, FC analysis revealed a reduction in cell viability, a loss of CD14 expression, consistently high expression of CD54, increased CD86 expression and the *de novo* expression of CD1a and CD207 under all culture conditions (Fig. 3D). However, the numbers of CD1a- and CD207-positive cells were higher in differentiation media supplemented with 5 % and 10 % FCS than with 20 % FCS. Whereas CD54 and CD86 expression apparently did not depend on the FCS content, CD14 decrease was more pronounced in 5 % and 10 % FCS differentiation medium. After 14 days of differentiation, the numbers of CD1a- and CD207-positive cells in 20 % FCS differentiation media were similar to the numbers of cells cultured in 5 % and 10 % FCS differentiation medium at day 7. The numbers of CD54- and CD86-expressing cells remained high, but compared to day 7, there was no CD14 expression detected in any differentiation media.

Thus, the expression profiles of MUTZ-LCs cultured in 5 % and 10 % FCS-containing media showed only minor differences in the marker expression during differentiation. Moreover, the reduction of FCS accelerated the MUTZ-LC differentiation.

Cell viability massively dropped after transfer of the MUTZ-3 cells into differentiation culture. After 7 days of differentiation, only 40–45 % of the MUTZ-LCs were viable in 5 % and 10 % FCS medium, and about 55 % in 20 % FCS. After 14 days, cell viability even dropped to approximately 40 % of the initial population.

3.3. Impact of Ultroser G, an alternative to FCS, on MUTZ-LC differentiation

As the differentiation medium without FCS resulted in high cell death rates, we were interested to analyze whether an FCS substitute could replace the animal-derived FCS. Thus, we conducted a case study with Ultroser G, a commercially available FCS alternative. According to the protocol provided by the supplier, Ultroser G was used at a final concentration of 4 %, which is described to be equivalent to 20 % FCS. We then compared the MUTZ-LC differentiation in medium supplemented with either 4 % Ultroser G or 5 % FCS.

Compared to 5 % FCS (Fig. 4A), nearly no adherent cells with dendritic processes were observed in 4 % Ultroser G culture (Fig. 4B). In addition, the dendritic processes of the few adherent cells were not as long and branched as seen in 5 % FCS differentiation medium. Whereas the cell granularity was increased in both differentiation media, the cells cultured in Ultroser G-containing medium were smaller than in the FCS-supplemented media.

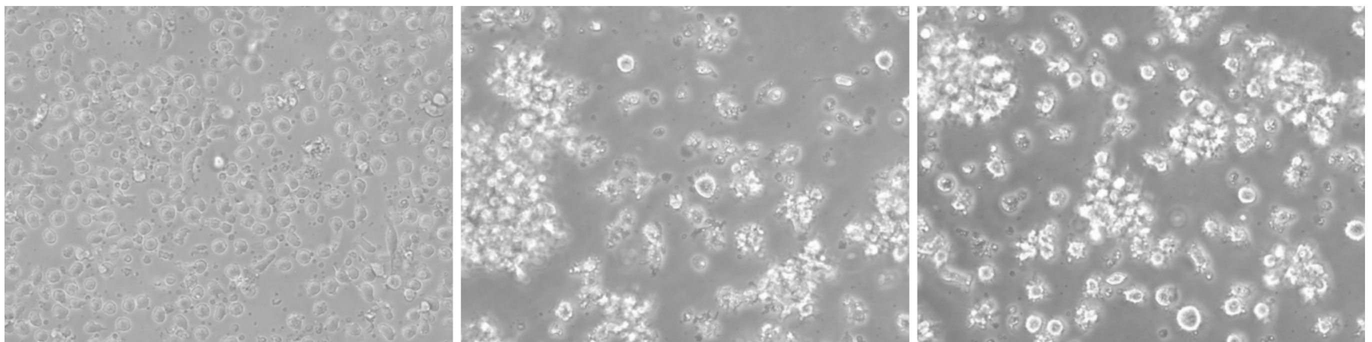

Fig. 1. Morphology of MUTZ cells before and after LC differentiation with and without 5637CM.
Representative phase contrast images of MUTZ-3 cells (A), MUTZ-LCs after 14 days differentiation in 20 % FCS with 10 % 5637CM (B) and without 5637CM (C). The images were taken at 20× objective magnification.

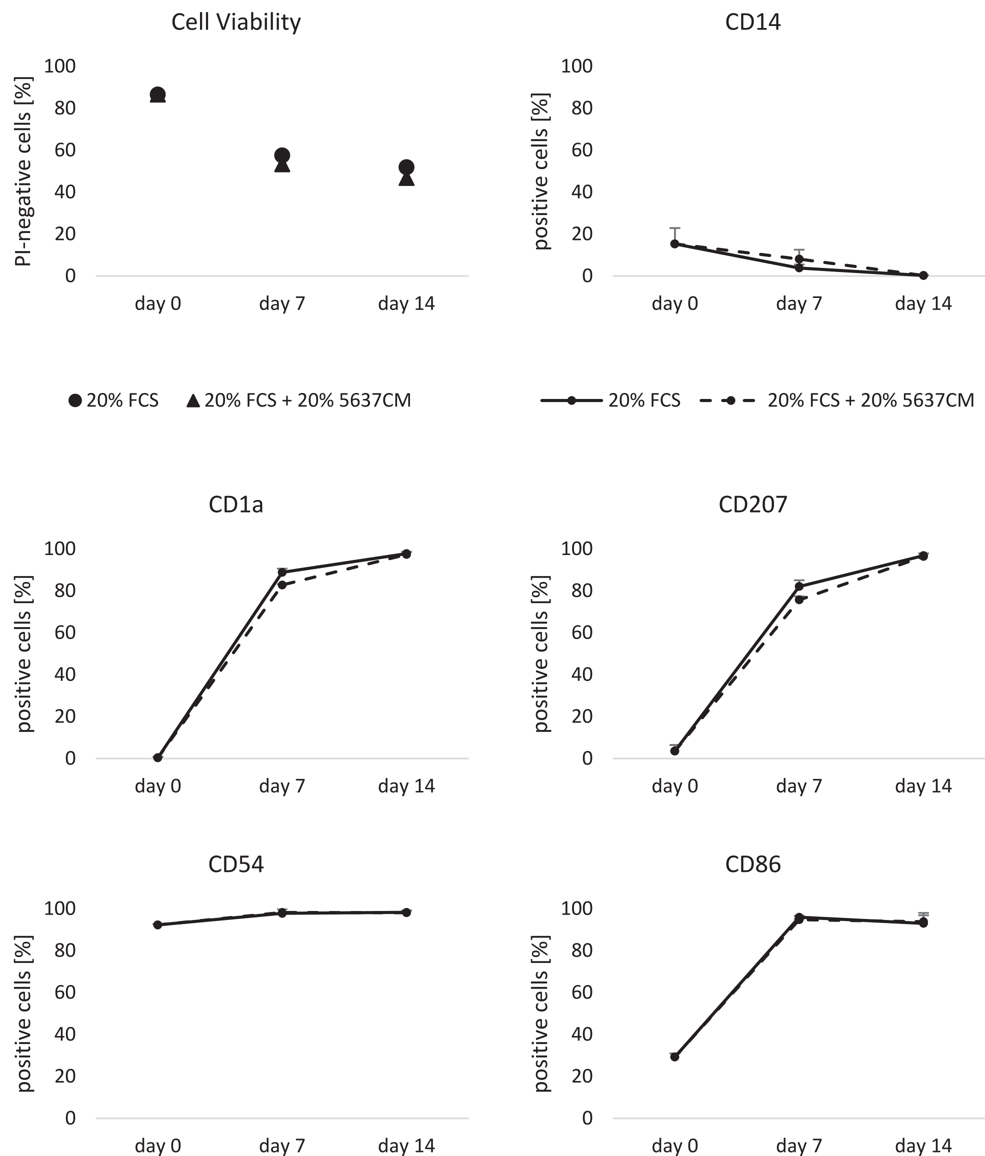

Fig. 2. Changes in cell viability and marker expression during MUTZ-LC differentiation with and without 5637CM over 14 days.
FC analysis of MUTZ-LCs cultured in 20 % FCS differentiation medium with (circle or dotted line) and without 5637CM (triangle or solid line). Day 0 represents the initial cell population, MUTZ-3 cells, before starting LC differentiation (day 7, 14). Cells were stained for following markers: CD14, CD1a, CD207, CD54 and CD86. A gate for PI-negative cells was drawn and a total of 10,000 PI-negative cells were analyzed. N = 3, error bars represent the standard deviation.

FC analysis at day 7 of differentiation revealed a higher number of viable cells in 4 % Ultroser G than in 5 % FCS differentiation medium (70 % *versus* 50 %; Fig. 5A). In both media CD1a and CD207 expression was induced (Fig. 5B), although at different levels. In Ultroser G-supplemented medium only approximately 60 % of the cells expressed CD1a and only about 25 % expressed CD207. In contrast, nearly 90 % of the cells, cultured in differentiation medium enriched with 5 % FCS, were positive for CD1a and CD207.

In addition, in Ultroser G-supplemented medium a fraction of around 55 % CD14-positive MUTZ-LCs remained, whereas hardly any CD14-positive cells were found in 5 % FCS differentiation medium. No differences occurred in respect of CD54 and CD86 expression, almost 100 % of the cells expressed these specific proteins, irrespective of the applied medium.

3.4. DNCB treatment of 5 % FCS-differentiated MUTZ-LCs

In order to test if the 5 % FCS-differentiated MUTZ-LCs (optimized protocol) can be activated after contact with a sensitizing chemical, the cells were treated with the extreme sensitizer DNCB. The DNCB concentration was determined with a dose finding assay according to the h-CLAT assay (OECD TG 442E, 2024b). After DNCB treatment, MUTZ-LC viability dropped to approximately 70 % (2.5 μg/ml DNCB) and 50 % (3.5 μg/ml DNCB), compared to the vehicle control (Fig. 6A). CD54 and CD86, two typical markers for dendritic cell stimulation after a sensitizing event, are already expressed in nearly every differentiated MUTZ-LC. Thus, after DNCB treatment no further increase in the number of positive cells was detected (Fig. 6A). The representative histograms for CD54 and CD86 expression show nearly identical signals of DNCB treated, 5 % FCS-differentiated MUTZ-LCs and the vehicle control (Fig. 6B). Because of that, we also analyzed the expression of CD83. FC analysis showed a three-fold increase of CD83-positive FCS-differentiated MUTZ-LCs after DNCB treatment. The similarity in CD54 and CD86 expression as well as the difference in CD83 expression after DNCB treatment is also shown in the representative FC histograms (Fig. 6B-D).

FC analysis of MUTZ-LCs, which were cultured in differentiation medium supplemented with 5 % FCS, after 24 h DNCB treatment. A gate for PI-negative cells was drawn and a total of 10,000 PI-negative cells

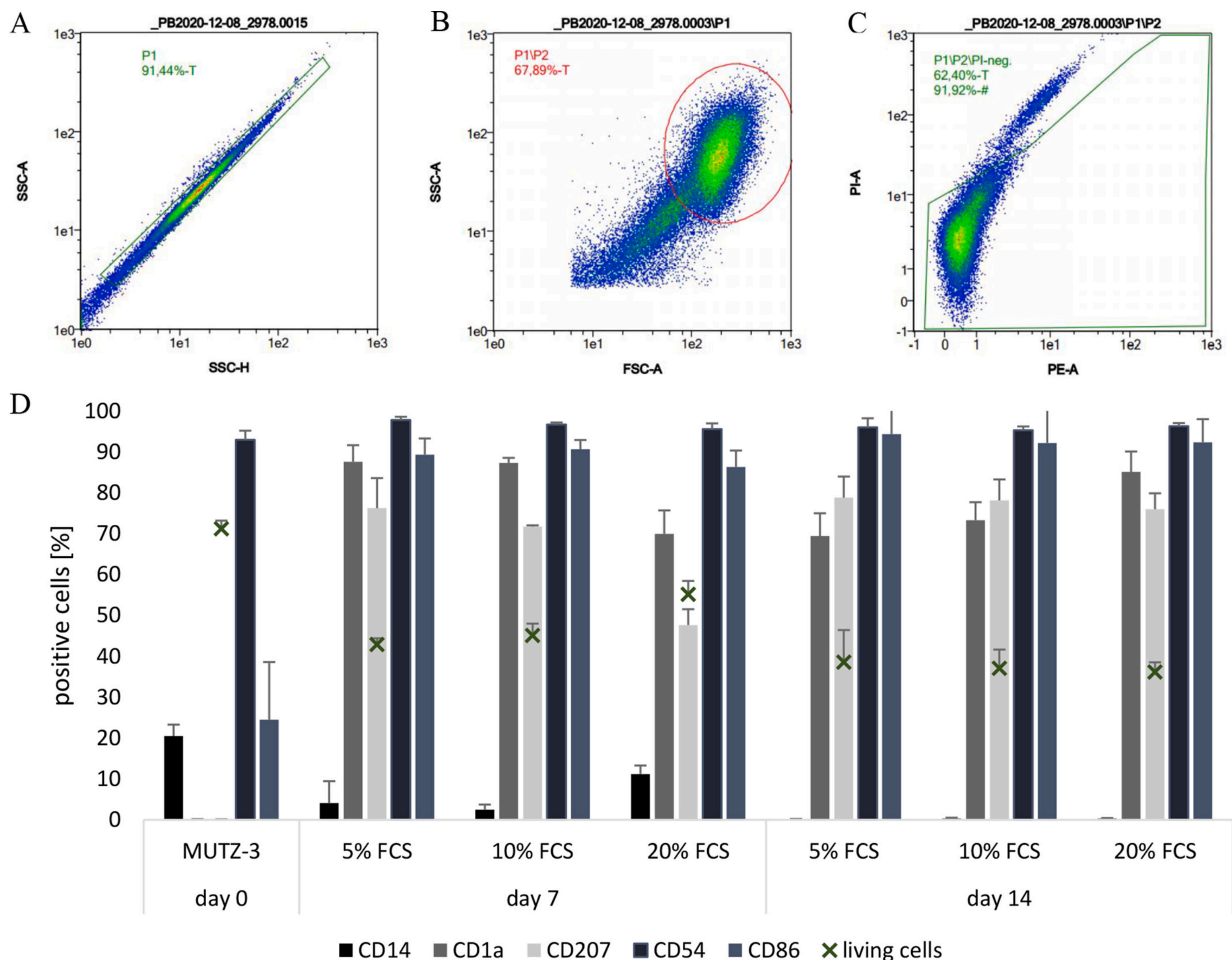

Fig. 3. FC analysis of MUTZ-LCs differentiated in medium supplemented with 5 %, 10 % and 20 % FCS over 14 days.
(A—C) Gating strategy applied for FC differentiation marker expression analysis. (A) Representative side scatter height *vs* side scatter area density plot for doublet exclusion showing gate P1 around single cells. (B) Representative side *vs* forward scatter plot of single cells P1 showing gate P2 around cell population to exclude cell debris. (C) Representative PI *vs* PE scatter plot of cells in P2 showing gate "PI-neg." around living (PI-negative) cells. (D) Expression of CD14, CD1a, CD207, CD54 and CD86 of MUTZ-LCs cultured in 5 %, 10 % and 20 % FCS differentiation medium on day 0 (MUTZ-3 cells before starting differentiation), 7 and 14. Crosses represent percentage of viable cells (PI-negative cells), reflecting the overall cell population. $N = 4$, error bars represent standard deviation. A total of 10,000 PI-negative cells were analyzed.

were analyzed. (A) Cells were stained for following markers: CD54, CD86, and CD83. PI-based cell viability detection compared to the vehicle control (crosses), reflecting the overall cell population. $N = 3$, error bars represent standard deviation. (B—D) Representative histograms of 5 % FCS differentiated MUTZ-LCs stained for CD54 (B), CD83 (C) and CD86 (D): vehicle control (green), 2.5 μg/ml (orange) and 3.5 μg/ml DNCB (red). Isotype controls are presented in black lines.

3.5. Successful integration of MUTZ-LCs into the Phenion© FT skin model

In subsequent studies, it was intended to investigate whether the optimized differentiation protocol had an impact on the integration of the MUTZ-LCs into the Phenion© FT skin model. For that, an immunofluorescence staining for the LC-typical marker CD1a on frozen tissue sections was performed. In the control skin models without integrated MUTZ-LCs, no CD1a staining was detected (Fig. 7A). In contrast, the skin models with integrated MUTZ-LCs exhibited CD1a-positive MUTZ-LCs, equally distributed in the epidermis, irrespective of the MUTZ-LC differentiation protocol (Fig. 7 B, C). These stained MUTZ-LCs exhibited the LC-typical long dendritic processes. Only a few positive signals were found in the dermis underneath the basement membrane. These signals were smaller in size compared to those observed in the epidermis. Fluorescence signals in the lower parts of the dermis were unequivocally identified as false positive signals caused by the auto-fluorescent collagen matrix.

4. Discussion

The standardization of culture conditions is a prerequisite to generate viable and physiologically competent immune cells for their subsequent integration into a 3D full-thickness skin model. With such an immunocompetent skin equivalent, the authors aim at developing a stand-alone test method to predict the skin-sensitizing potential and potency, respectively, of chemicals and formulations with higher physiological relevance compared to the already OECD-approved tests (e. g. OECD TG 442D, E). Isolation and subsequent culture of naïve Langerhans cells (LCs) from skin biopsies is generally possible but does not result in the high cell numbers needed to produce immunocompetent

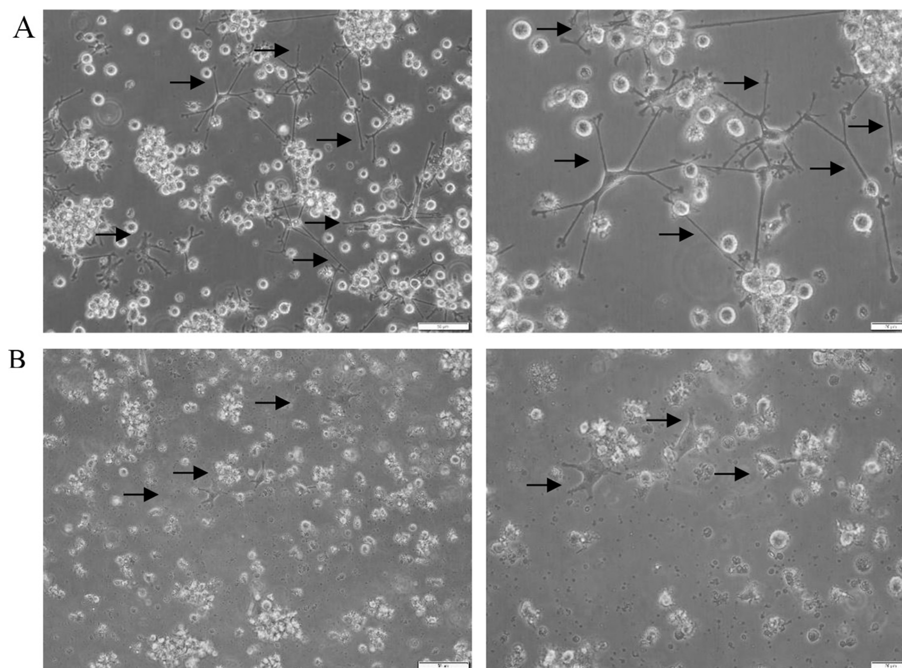

Fig. 4. Morphology of MUTZ-LCs after 7 days differentiation in medium supplemented with 5 % FCS or 4 % Ultroser G.
Representative phase contrast images of 5 % FCS-differentiated (A) and 4 % Ultroser G-differentiated MUTZ-LCs (B). Adhered MUTZ-LCs with dendritic processes are indicated with arrows. Scale bars on the left side represent 50 μm and on the right side 20 μm.

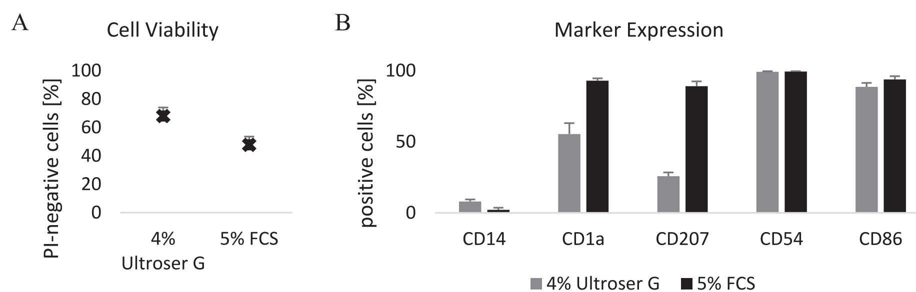

Fig. 5. Changes in cell viability and marker expression of 5 % FCS and 4 % Ultroser G differentiated MUTZ-LCs.
FC analysis of MUTZ-LCs after 7 days differentiation with 4 % Ultroser G or 5 % FCS. $N = 5$, error bars represent standard deviation. (A) PI-based cell viability detection. (B) Cells were stained for following markers: CD14, CD1a, CD207, CD54 and CD86. A gate for PI-negative cells was drawn and a total of 10,000 PI-negative cells were analyzed.

skin models at a larger scale (Larregina et al., 1996; Régnier et al., 1998; Richters et al., 2001; Tuschl and Kovac, 2001). Hence, for this innovative New Approach Methodology (NAM), we chose the MUTZ-3 cell line as a promising candidate out of a few other cell lines which had been identified as functional LC surrogates, too, e. g. THP-1 and U-937 (OECD TG 442E, 2024b). Hereby, the first step is to differentiate MUTZ-3 cells into MUTZ-LCs, a surrogate for the human epidermal LCs, in sufficient numbers. Before transferring the MUTZ-3 cells from the proliferation-stimulating maintenance medium into the differentiation medium, they were characterized both morphologically and by flow cytometry (FC). The expression profiles of proteins which are typical for undifferentiated dendritic cells (DCs) were nearly identical to those published by Azam et al. (2006).

An essential component of the maintenance medium used to culture the yet undifferentiated MUTZ-3 cells and to stimulate cell proliferation, is the 5637-conditioned medium (5637CM), an undefined cell culture supernatant. Quentmeier et al. (1997) identified and quantified the cytokines secreted by 5637 cells, a bladder carcinoma cell line, known to induce the proliferation of myeloid leukemia cell lines. Although indispensable for the maintenance medium, the 5637CM impact on MUTZ-LC differentiation has, according to the authors´ knowledge, not been investigated so far. We demonstrated that 5673CM does not affect MUTZ-LC differentiation, as the expression levels of the analyzed differentiation markers were nearly identical for cells cultured in differentiation medium with and without 5637CM.

FC analysis showed a loss of CD14 expression during MUTZ-LC differentiation, which was also described by Masterson et al. (2002) and Larsson et al. (2006). We have generated MUTZ-LCs with a high yield of at least 90 % CD1a-positive viable cells, compared to only 35 % to 80 % as demonstrated in some other studies (Larsson et al., 2006; Nelissen et al., 2009; Masterson et al., 2002). The number of CD207-positive MUTZ-LCs was nearly 3 times higher than in Masterson et al. (2002). In addition, we analyzed the cellular expression of the proteins CD54, CD86 and CD83, characteristic markers for LC maturation (OECD TG 442E, 2024b; Zhou and Tedder, 1995). After 7 days in differentiation culture nearly all MUTZ-LC's were CD54- and CD86- positive, which corroborates results obtained by Masterson et al. (2002). CD86 expression was even higher than documented by Nelissen et al. (2009).

Changes in MUTZ-3 cell morphology during differentiation were also observed. Phase microscopic images revealed enhanced cell granularity,

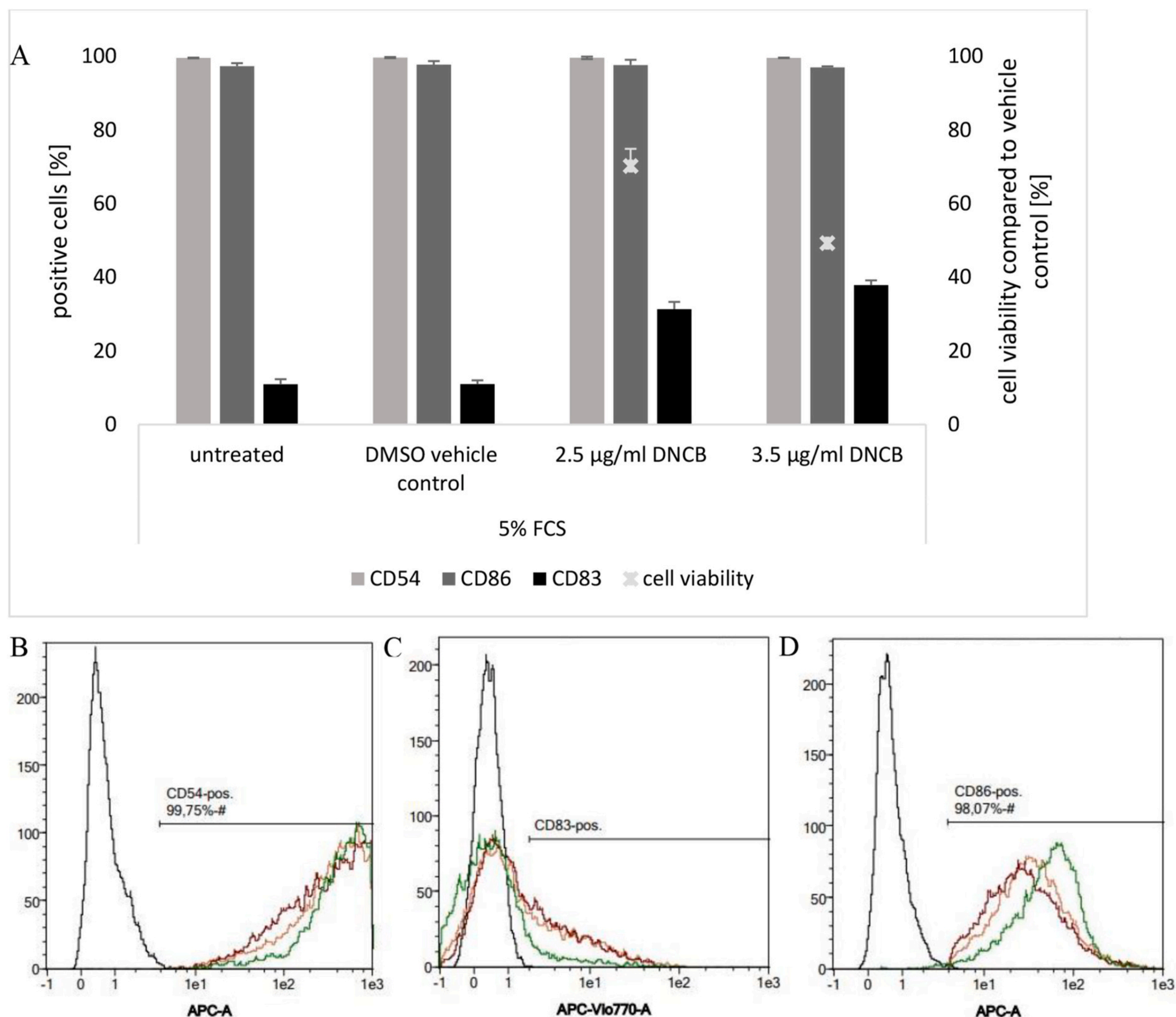

Fig. 6. 24 h DNCB exposure of 5 % FCS- MUTZ-LCs cultured with 5 % FCS.

which was confirmed in the forward/sideward scatter plots of flow cytometry analyses. The enhanced granularity might be the effect of the formation of LC-typical Birbeck granules, which Santegoets et al. (2008) and Laubach et al. (2011) detected in MUTZ-LCs by transmission electron microscopy. Although Birbeck granules cannot be identified light-microscopically, the presence of CD207, or langerin, can be seen as an indirect indicator for Birbeck granules. Furthermore, the cells increased in size, which might result from typical cytoplasmic protrusions, which were also described by Santegoets et al. (2008), enhancing the cell volume. All observed morphological changes were quite similar for cells differentiated with or without 5637CM supplement. Consequently, we continued MUTZ-LC differentiation without 5637CM, similar to protocols published by Kosten et al., 2015, and Bock et al., 2018.

The protocols for MUTZ-LC differentiation published so far stipulate the use of fetal calf serum (FCS), an expensive, animal-derived and hence not fully defined product (Santegoets et al., 2006; Nelissen et al., 2009; Ouwehand et al., 2010; Groell et al., 2018; DSMZ). Inherently, FCS is a major source of uncertainty in the cell culture process and hence defies standardization. Thus, for reasons of standardization and reproducibility as well as for ethical concerns (Baker, 2016), we have conducted a series of tests to assess if FCS can be reduced or even completely replaced in the differentiation medium while maintaining MUTZ-LC integrity and physiological properties.

Reduction of FCS from 20 % to 5 % resulted in MUTZ-LCs with nearly identical protein expression profiles compared with the original medium. This differentiation status was achieved in serum-reduced culture after only 7 days, while MUTZ-LCs cultured in medium supplemented with 20 % FCS reached this status only after 14 days. Shortening the culture time by 50 % just by serum reduction not only contributes to a better standardization but is also economically valuable in terms of cost savings. Moreover, with 5 %, the MUTZ-LC morphology was more LC-like, as a larger number of cells adhered to the bottom of the culture vessels and exhibited numerous long dendritic processes.

MUTZ-LC differentiation without FCS was not possible, as nearly all cells died within seven days of differentiation. An approach to overcome this apparent limitation despite complete serum deprivation might be the use of alternative, non-animal-derived serum replacement components. In the framework of a small case study, we assessed whether Ultroser G, a semi-defined animal-free supplement, was able to compensate for FCS elimination (suppliers data sheet, Pall BioSepra). MUTZ-LC's which were cultured in Ultroser G-containing medium expressed CD1a and CD207, although at a much lower percentage than with FCS. In addition, only few cells cultured with Ultroser G developed smaller and less numerous dendritic processes compared with FCS-differentiated cells. Cell size was reduced, too. Taken together, Ultroser G does not seem to be suited for MUTZ-LC differentiation when applied at the concentration recommended by the supplier. One reason for this observation might be that Ultroser G has been developed, and tested, especially for adherent cell types like fibroblasts, keratinocytes, amniotic and bone marrow cells, but not for DCs generally cultured

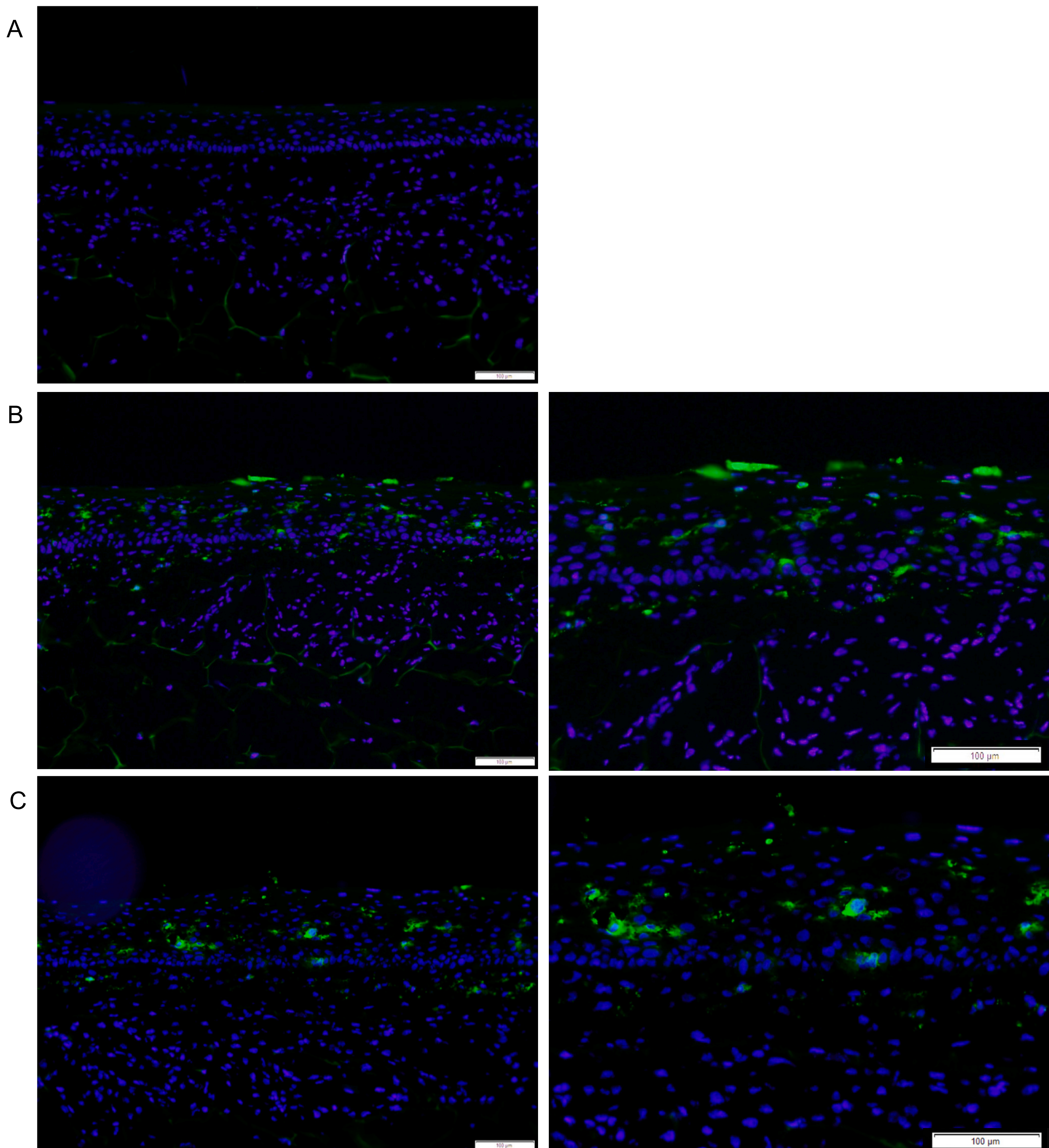

Fig. 7. CD1a expression in standard Phenion® Full-Thickness skin models with and without integrated 20 % FCS- or 5 %-differentiated MUTZ-LCs.
Representative microscopic images of frozen sections of Phenion® Full-Thickness skin models after ten days in air-liquid interface with an immunofluorescence staining for CD1a, which is expressed by MUTZ-LCs. Skin models without integrated MUTZ-LCs (A) and skin models with integrated MUTZ-LCs, which were differentiated for 14 days in 20 % FCS (B) or for 7 days in 5 % FCS medium (C). A magnification of the images is depicted on the right hand side to illustrate the morphological features. CD1a-stained MUTZ-LCs are depicted in green (FITC) and nuclei in blue (DAPI). Green, fluorescent signals in the lower part of dermis were false-positive ones, emitted by the matrix (signals are highlighted with asterisks, not exhaustive). Scale bars represent 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

under non-adherent conditions (Cytogen; www.cytogen.net/product/ultroser-g). However, we only tested one FCS alternative so far, thus this study is not representative for other FCS replacement products. In order to develop a completely FCS-free medium, additional alternative components should be tested in future.

Another key parameter for optimal cell culture conditions is the overall cell viability. To the authors ́best knowledge, cell viability during MUTZ-LC differentiation has not been critically discussed in any published protocol yet. However, the condition of the individual cells, and hence of the whole cell culture, is an important factor, as it can affect physiological reactions. Therefore, this parameter should be thoroughly monitored to ensure valid and reliable experimental results, especially in NAMs employed in the framework of New Generation Risk Assessment. For example, the OECD-validated h-CLAT method, based on THP-1 cells, prescribes a cell viability of at least 90 % for the negative control as an acceptance criterium for qualifying the test run (OECD TG 442E, 2024b). In addition, according to the prediction model, h-CLAT data must be excluded if cell viability after exposure with a test chemical drops below 50 % of the solvent control (OECD TG 442E, 2024b). At this low overall cell viability, it must be assumed that the cells do not react physiologically any longer, subsequently leading to unreliable test results.

During the MUTZ-LC differentiation process, we observed a decline in cell viability of nearly 50 %, a significant drawback when large numbers of viable cells are required. Consequently, the differentiation culture must be initiated with at least twice the number of MUTZ-3 cells to generate the number of MUTZ-LCs needed for an experiment. The documented decrease in cell viability might be the result, among others, of a proliferation arrest, which is characteristic for immature Langerhans cells (Masterson et al., 2002; Santegoets et al., 2008). If MUTZ-LCs, which do not divide any longer, are more susceptible for entering into apoptosis, or some other type of cell death, respectively, this might account for the massive decrease in overall cell viability. However, more analyzes are needed to dissect the physiological processes responsible for the observed cell loss,m prerequisite to develop culture conditions suited to maintain MUTZ-LC viability and to stimulate the desired differentiation processes.

After optimizing the standardization of the differentiation protocol and increasing the yield of CD1a- and CD207-positive cells, the capacity of the MUTZ-LCs to react in a DC-characteristic way upon exposure with sensitizing molecules had to be demonstrated. MUTZ-LCs were exposed to DNCB, known to be an extreme sensitizer and recommend as an h-CLAT proficiency chemical by the OECD (TG 442E, 2024b), and the expression profiles of characteristic DC markers were analyzed. Typical membrane-bound proteins, used as read-out parameters for the characterization of the sensitizing potential of chemicals, are CD54 and CD86, which are *e.g.* used in h-CLAT. The cell line used in the h-CLAT assay is THP-1, which is derived from the peripheral blood of an acute leukemia patient (product sheet, American Type Culture Collection), comparable to the MUTZ-3 cells derived from acute myeloid leukemia cells. However, both cell types reveal characteristic physiological differences. For example, Santegoets et al. (2008) compared the DC-phenotype of the 2 cell lines, among others, and listed several differences with the conclusion that MUTZ-LCs are the best DCs for being used in assessing the skin-sensitizing potential of chemicals. We observed that nearly all non-exposed MUTZ-LCs were already positive for CD54 and CD86, which stands in contrast to the situation described for THP-1 cells in the h-CLAT. Consequently, after treating the MUTZ-LCs with DNCB, no changes in CD54 and CD86 expression were observed, neither in the numbers of positive cells nor in the mean fluorescence intensity (MFI). Santegoets et al. (2008) demonstrated an MFI increase of CD54 and CD86 expression after maturation. However, the authors stimulated the MUTZ-LCs with a cytokine cocktail known to induce cellular reactions typical for allergic contact dermatitis, and not with a small organic molecule like DNCB, which might act differently on the cells. However, we demonstrated a 3-fold increase in CD83 expression on 5 % FCS-differentiated MUTZ-LCs upon DNCB exposure. CD83 is a known indicator of mature cells (Prechtel and Steinkasserer, 2007), and its induction after stimulation by sensitizers was also documented by Santegoets et al. (2008). Whether this physiological reaction can be generalized for all sensitizing agents, or at least for a subset of sensitizers, must be further evaluated with more sensitizing and non-sensitizing chemicals.

The MUTZ-LCs are foreseen to function as immunocompetent cells in a 3D skin model. Thus, as a next step, the MUTZ-LCs were integrated into the Phenion© Full-Thickness (FT) Skin Model which consists of primary human keratinocytes and fibroblasts isolated from human skin biopsies. The FT skin model comprises a fully differentiated epidermis with all layers seen in native human skin, including a functional skin barrier, and a dermal compartment characterized by a plethora of extracellular matrix proteins deposited by the fibroblasts embedded in the dermis (Mewes et al., 2007). To validate whether the serum reduction and accelerated differentiation has an impact on the MUTZ-LC integration into the skin model, we prepared FT skin models with MUTZ-LCs differentiated with 5 % FCS for 7 days and with MUTZ-LCs grown in 20 % FCS-containing medium for 14 days. According to immunofluorescent analyses of CD1a expression, the MUTZ-LCs were successfully integrated into the epidermal layers of the Phenion© FT skin model, irrespective of the MUTZ-LC differentiation protocol. This finding supports our hypothesis that the MUTZ-LCs differentiated under serum- and time-reduced conditions exhibit similar physiological traits as the MUTZ-LCs cultured under standard conditions. A few immune-positive signals were also detected in the dermis. However, the labelled structures were smaller and less defined as those observed in the epidermis, which might indicate towards cellular fragments. Only high-resolution microscopic analyses will help to elucidate the identity and origin of the dermally located fluorescent particles.

Based on the results presented in this paper the MUTZ-LCs, foreseen for their integration into an immunocompetent 3D skin model, will be differentiated only in FCS-reduced culture medium without 5637CM. With our optimized MUTZ-LC differentiation protocol we enable more standardized culture conditions, lower costs, and less animal-derived products. Moreover, the differentiation culture time could be halved from 14 to 7 days, similar to Nelissen et al. (2009) or Groell et al. (2018), and three days shorter than reported by Ouwehand et al. (2010) and Bock et al. (2018). Thus, with this work we contribute to the ambition to reduce, or even eliminate, animal-derived products from cell culture and NAMs.This approach is favorable both from an economic and an ethical point of view, and paves the way towards improved standardization of protocols.

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CRediT authorship contribution statement

Patricia Böttcher: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Steinmeyer:** Validation, Methodology, Investigation, Formal analysis. **Holger Stark:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Jörg Breitkreutz:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Karsten R. Mewes:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Karsten R. Mewes reports financial support was provided by German Federal Ministry of Food and Agriculture (BMEL), Project No. 281A308B18. Patricia Boettcher reports financial support was provided by German Federal Ministry of Food and Agriculture (BMEL), Project No. 281A308B18. Laura Steinmeyer reports financial support was provided by German Federal Ministry of Food and Agriculture (BMEL), Project No. 281A308B18. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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