Innovative *in vitro* 3D skin model for safety assessment: a case report for the skin sensitization

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Abstract

Prevalence of allergic contact dermatitis is increasing, justifying the need to assess skin sensitization potential for any chemical products. Current assessments of potential sensitization events of new compounds are performed according to three guidelines (OECD 442 C, D and E) and a battery of *in vitro* tests. Despite the advances of skin model equivalents (SME) validated by the governmental organizations, none of them consider the interplay between the epidermis/dermis cells and the resident immune cells. The aim of our study was to develop a SME that includes an endothelial barrier and some relevant resident immune cells involved in the skin sensitization cascade.

The model integrated adult Normal Human Epidermal Keratinocytes, Normal Human Dermal Fibroblasts, Human Umbilical Vein Endothelial cells, THP-1 (a human leukemia monocytic cell line), and THP-1 derived macrophages. Structure, cells and tissue-specific markers were revealed by immunocytochemistry techniques.

Our approach resulted in a multilayered, differentiated epidermis proliferating on top of the dermis as revealed by immunolabelling analyses. Presence of specific markers of human epidermis/de

rmis as well as presence/activation of immune resident cell phenotype was verified using immune labelling techniques.

This 3D skin model should allow in future to obtain more translatable indications regarding possible inflammatory events induced by drugs and medical devices.

Keywords

3D skin model; immunity; pseudo-vascularization; skin sensitization; cosmetic product safety

Introduction

Skin sensitization is a term used to refer to the regulatory hazard known as allergic contact dermatitis (ACD) in humans. This disease is a rather diffuse inflammatory disease originating from cell-mediated immune response to skin sensitizers, knowing that up to 20% of the population is affected at different degrees [1]. From a cosmetology/vigilance perspective, it is interesting to note that ACD is one of the most frequently observed adverse reactions related to the use of cosmetics. Skin sensitization is a complex immunological process, originating from the contact of sensitizing products with the first layers of the epidermis and resulting in a succession of physico-chemical and biological events that lead to the recruitment and activation of allergen-specific T lymphocytes. Different steps have been described as more specific in the development of this disease. Those steps have therefore been the target of the current tests, given that information on skin sensitization is required in the safety assessment of EU Cosmetics Regulation [2]. Following the revision of Annex VII of the Registration, Evaluation and Authorization of Chemicals (REACH) regulation [3], animal models, such as the Guinea Pig based assays (GPMT or the Buehler test) [4] are no longer allowed to meet the information requirements for substances exclusively intended for use in cosmetic products. It is nevertheless important to note that an *in vivo* sensitization study, preferably Local Lymph Node Assay, can only be performed if the in chemico/in vitro methods are not applicable.

New Approach Methods (NAMs), such as *in chemico* and *in vitro* methods, have been validated and incorporated since 2021 into the official test guidelines by the Organisation for Economic Co-operation and Development (OECD/ OECD TG 497: guidelines for chemical test), serving as viable replacements of animal modes [5]. These models focus on individual key events (KE) in the adverse outcome pathway (AOP) for skin sensitization for which the molecular initiating event (MIE) is covalent binding to proteins [6]. In short, the chemical sensitizer penetrates the stratum corneum, the uppermost layer of the skin, and subsequently binds covalently to proteins

(Key Event 1: KE1) to form hapten–protein conjugates, which can be immunogenic. Simultaneously, keratinocytes are stimulated both to release proinflammatory signals (e.g., pro-inflammatory cytokines or ATP) and activate antioxidative response genes (Key Event 2: KE2). Dendritic cells (DCs), which are antigen-presenting cells that playing a major role in the primary immune response and can be activated by allergenic molecules, then develop a mature phenotype involving the induction of various co-stimulatory molecules and production of pro-inflammatory cytokines and chemokines with formation of hapten–protein conjugates on major histocompatibility complex molecules (Key Event 3: KE3). The activated DCs are mobilized and then migrate from the skin to the draining lymph nodes to present the allergen to T cells (Key Event 4). Currently, three technical Test Guidelines (OECD TG 442 C, D and E) [7, 8, 9] describe a total of seven such methods. Those include: 1) the KE1 based Direct Peptide Reactivity Assay (DPRA) and the Amino acid Derivative Reactivity Assay (ADRA); 2) the KE2 based assays KeratinoSens and LuSens; 3) the KE3 based assays h-CLAT, U-SENS, and the IL-8 Luc assay (see Tab. 1). As previously reported [10], none of the NAMs methods, except the kDPRA, is considered a sufficient stand-alone replacements of animal data to draw conclusions about the skin sensitization potential of chemicals or to provide information for potency sub-categorization (sub-categories 1A and 1B). Each of the tests listed above have some known technical limitations, and data generated can incorrect safety assessment (e.g., false-negative). In the Table 1 are reported the test included in the OECD 497 [5]. Novel state-of-the-art scientific methods currently in the OECD Test Guideline Program, such as the SENS-IS, which is based on the toxicogenomic analysis on 3D reconstituted epidermidis model (Episkin®) to measure skin sensitization potency, are under evaluation by EURL-ECVAM. This test seems to address the three first key events of the Adverse Outcome Pathways: the KE1 through the expression of genes under the control of «sensor» proteins, the KE2 through the

expression of genes indicating keratinocytes activation and the KE3 through the expression of genes implicated in dendritic cells activation and maturation.

Despite the scientific literature has demonstrated the possibility of obtaining reconstructed skin models (skin model equivalents, SME), those models have not yet specifically aimed at recreating *in vitro* the complex cellular interplay between epidermis/dermis cells and the resident immune cells with the goal of providing a fully biomimetic skin equivalent suitable to assess sensitization potential of a given compound. The aim of our study was to tailor a new skin equivalent model that includes more key actors of the sensitization cascade. This new model included key immune resident cellular components as well as a functional endothelial barrier involved in the recruitment of immune cells; the aim is to investigate the possibility to have a model able to better predict the sensitization potency of single or multiple chemical compounds.

Table 1 List of Non-animal defined approaches (DAs) included in OECD 497

Key Events	**Mechanisms**	**OECD Tests**	**Latest Update**
Key Event 1	Protein / chemical compound binding	OECD 442 C [9] DPRA ADRA kDPRA	2021
Key Event 2	Keratinocyte activation	OECD 442D [10] LuSens Keratinosens ™	2018
Key Event 3	Dendritic Cell Activation	OECD 442E [11] OCDE 497 [7] Human Cell Line Activation test (h-CLAT) U937 cell line activation Test (U-SENSTM) Interleukin-8 Reporter Gene Assay (IL-8 Luc assay) Genomic Allergen Rapid Detection (GARD™) for assessment of skin sensitizers (GARDTMskin)	2018 2021

DPRA = Direct Peptide Reactivity Assay (DPRA); ADRA = Amino acid Derivative Reactivity Assay; kDPRA = kinetic Direct Peptide Reactivity Assay.

Materials and Methods

The model integrated Normal Human Epidermal Keratinocytes (NHEK), Normal Human Dermal Fibroblasts (NHDF), Human Umbilical Vein Endothelial cells (HUVECS), and THP-1 (a human leukemia monocytic cell line). Both keratinocytes and fibroblasts were obtained from Promocell, (PromoCell GmbH, Heidelberg) and the cells expanded until passage 3 according to the provider's guidelines. Adult pooled keratinocytes (forehead skin of women aged 34-64 years) were selected to reduce the bias introduced by a single donor. HUVECs were obtained from Lonza Bioscience (Lonza, Basel) while THP-1 (TIB-202) were obtained from ATCC (LGS, Molsheim) and expanded according to the ATCC guidelines. THP-1derived macrophages (MO) were obtained by treating THP-1 cells with 150 nM phorbol-12-myristate-13-acetate (PMA) for 24 hours. The skin model was constructed (Figure 1) using an 8um porous Brandt insert 2in1 for 12 MW (BrandTech Scientific, Essex). The dermal compartment consisted of an extra-cellular matrix, obtained by mixing rat tail collagen type I (Corning, Tewksbury), fibrinogen (Sigma Aldrich, Saint Louis) and 1 $UImg^{-1}$ of fibrinogen of thrombin (Sigma Aldrich, Saint Louis) together with fibroblasts and immune cells. 300 ul of hydrogel consisting of 9K THP-1 or M0 cells and 45K fibroblasts prepared in a final concentration of $4mg.ml^{-1}$ collagen and $3mg.ml^{-1}$ fibrinogen (Table 2) was added to each transwell and allowed to polymerize for 20 minutes at 37°C in a 5% CO_2 humidified incubator. HUVECs were seeded on the bottom of the transwells at a density of $49.1K.cm^{-2}$ and incubated for 1 hour in a 5% CO2 humidified incubator to permit the cells to adhere to the bottom.

Table 1 Cells concentration used to reconstruct the skin model equivalent

Cell Type	Cells Density
THP-1	$30K.ml^{-1}$
M0 derived THP-1	$30K.ml^{-1}$
Fibroblast	$400Kml^{-1}$

The transwells were then transferred to a 6 multi well (MW) with 3ml of EBM (Lonza, Basel). 300K Keratinoctyes suspended in 400 ul of Keratinocyte Medium 2 (Promocells, Heidelberg) were finally added on top of the dermal layer of each transwell (Figure 1). The tranwells were then transferred to the incubator. Two days post seeding, the keratinocytes were exposed to air and the medium inside the MW was lowered to 1.5 ml to avoid potential medium infiltration in the transwell due to hydrostatic pressure.

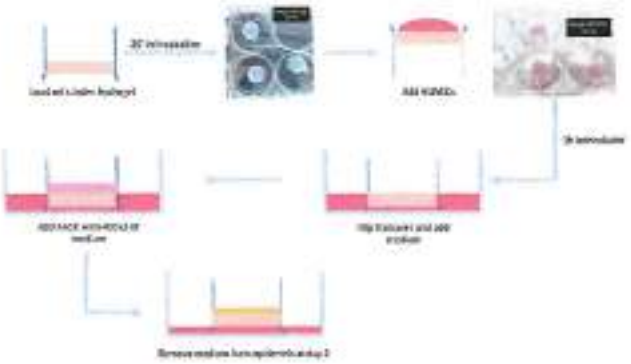

Figure 1. Graphical representation of the protocol steps used to obtain the reconstructed skin model equivalent

The full model was then cultured in the incubator and then perfused with the a Microphysiological System (MPS) to stimulate endothelial cell growth and differentiation with an adequate amount of shear stress (Figure 2). The MPS allows the control of gas concentration in the medium; we used 20% O_2, 4% CO_2 and 76% N_2 with the MPS, to compare results with

static incubator. MPS was used in a closed loop configuration, the medium was enriched for 10 minutes at 10sccm, and a flow of 150ul.min^{-1} was obtained with a peristaltic pump. For the whole duration of the perfusion, the medium flow rate was kept constant at 150 ul.min^{-1}. To keep the level of O_2 and CO_2 stable in the medium a continuous flow of 0.15 sccm was injected into the reservoir, and the temperature kept stable at 37 C by using a MPS heating plate (Figure 2).

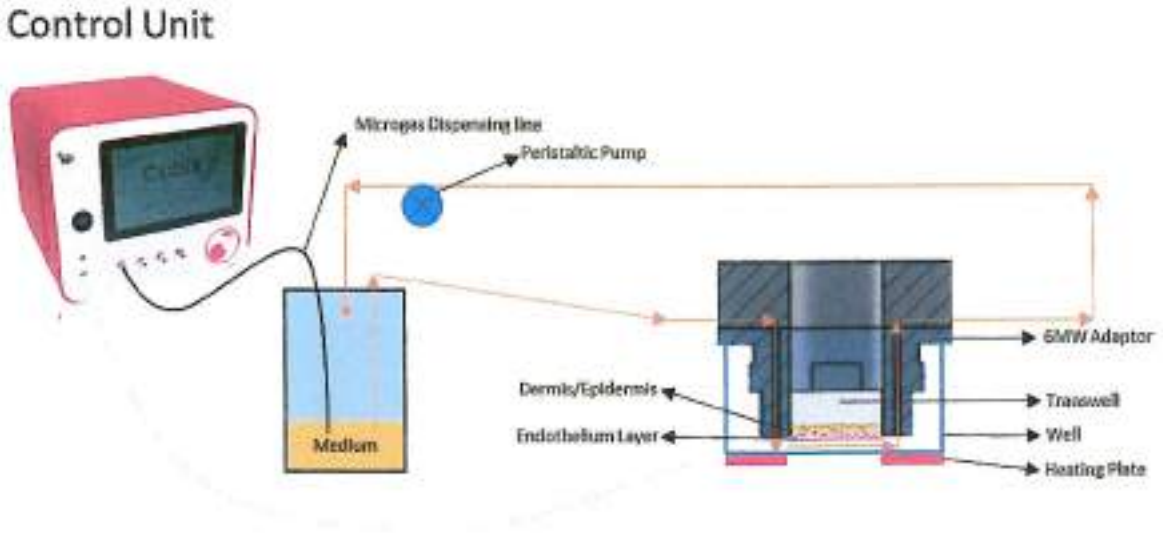

Figure 2. Schematic representation of the MPS configuration used to perfuse the model and condition the cell medium. Medium were recirculated using a peristaltic pump. The MPS was providing constant microvolumes of gas to keep the partial pressure of gasses constant among the duration of the experiment; at the same time the control unit guarantee a constant temperature inside the standard 6MW. The transwell is placed in a patented polycarbonate adaptor to seal the cells from environment and provide a laminar flow to stimulate the endothelial cells.

Viability of cells was assessed at the end of the maturation stage using a live dead assay (Merck, Burlington). To assess the expression of skin tissue-specific proteins (i.e., involucrin) and the presence and localization of the different cell populations sample were fixed, and paraffin

embedded or directly subjected to standard immune labelling and ICC and observed with a Leica Thunder microscope (Leica Microsystem, Wetzlar).

Immunofluorescence labelling was performed on slides and whole model. The slides were deparaffinised using a standard protocol. For whole mount labelling, the transwells were washed twice with phosphate buffer saline (PBS) and then fixed with cold 4% paraformaldehyde (PFA) in PBS at 4°C, overnight. Following 2 washes with PBS, both the slides/transwells were blocked with 5 % bovin serum albumin (BSA) in PBS for 1-2 hrs at room temperature. The primary antibodies to involucrin (Abcam #ab68, 1:200), CD31 (Thermofischer #MA5_18135, 1:100), CD54 (Abcam #ab214860, 1:100) and CD206 (Thermofischer #MA5_32498, 1:200) were diluted in 1% BSA/PBS containing 0.1% tween 20, and incubated with the slides/ transwells overnight at 4°C. After 3 washes with PBS, the slides/ transwells were incubated with the secondary antibodies to involucrin (Goat Anti-Mouse IgG H&L Alexa Fluor® 647, Abcam #ab150115, 1:500) and CD206 (Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488, Invitrogen# A32731, 1:500) diluted in 1% BSA/PBS containing 0.1% tween 20, for 2 hours at room temperature. Following 3 washes, the slides/transwells were labelled with Actin (ActinRed 555 Ready Probes reagent, Thermofisher # R37112) and DAPI (NucBlue Fixed Cell stain ready probes reagent, Thermofischer # R37606) according to the manufacturer's instructions, for 30 minutes at room temperature. The slides/ transwells were then mounted in PBS before imaging using a fluorescence microscope.

Results

The obtained reconstructed human skin model showed good viability of THP-1 whereas fibroblasts easily tent to proliferate too much when the endothelial cells monolayer is not present (Figure 3). Images showed no mortality of cells at the end of the experiment.

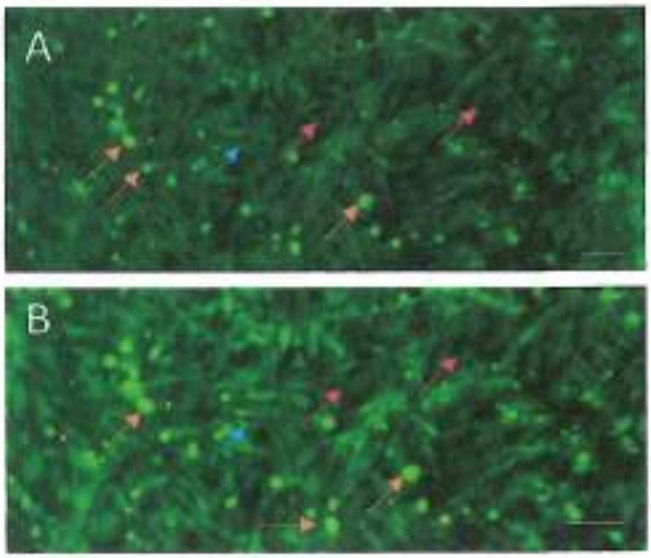

Figure 3. IF Live/dead micrograph of the dermis layers cultured in the incubator (A) and under MPS normoxic perfusion (B). In both the models' endothelial cells were not seeded on the bottom of the membrane; an excess of fibroblasts was observed. Green = calcein AM; orange = propidium iodine; orange arrows = THP-1; blue arrow = fibroblasts; red arrow = HUVECs. Scale bar = 50 um.

When endothelial cells were seeded on the bottom of the membrane, the presence of a continuous and cohesive monolayer of endothelial cells can be observed. Images obtained on the whole model using ActinRed as well as CD-31 immunolabelling on slices showed the presence of a well tight and aligned layer of cells on the bottom side of the transwell (Figure 4). A specific CD31 signal in some putative fibroblasts inside the dermis layer as well as the presence of the peculiar large monocyte nuclei were also observed (Figure 4).

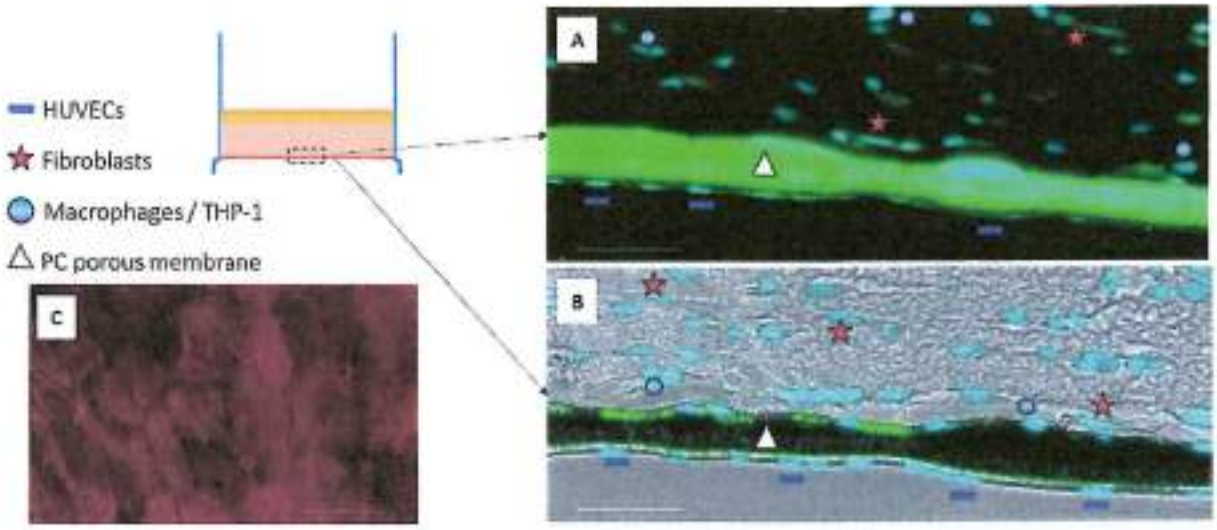

Figure 4. Micrograph showing the presence of a continuous and aligned monolayer of endothelial cells on the bottom side of the transwell. (A) = lateral view obtained from a thin slice labelled with anti-CD31 (green) and NucBlue (Nuclei, cyan). (B) = lateral view obtained from a thin slice labelled with anti-CD31 (green) and NucBlue (Nuclei, cyan) and phase contrast (greay). (C) = bottom-up view of aligned endothelial cells cytoskeleton revealed by ActinRed ICC after 5 days of perfusions using the MPS; pores of membrane are visible in the background. Bar = 50 microns.

Images of whole mount sections revealed the distribution and localization of cells included in the models as well as presence of involucrine, a standard marker of keratinocytes differentiation (Figures 5, 6).

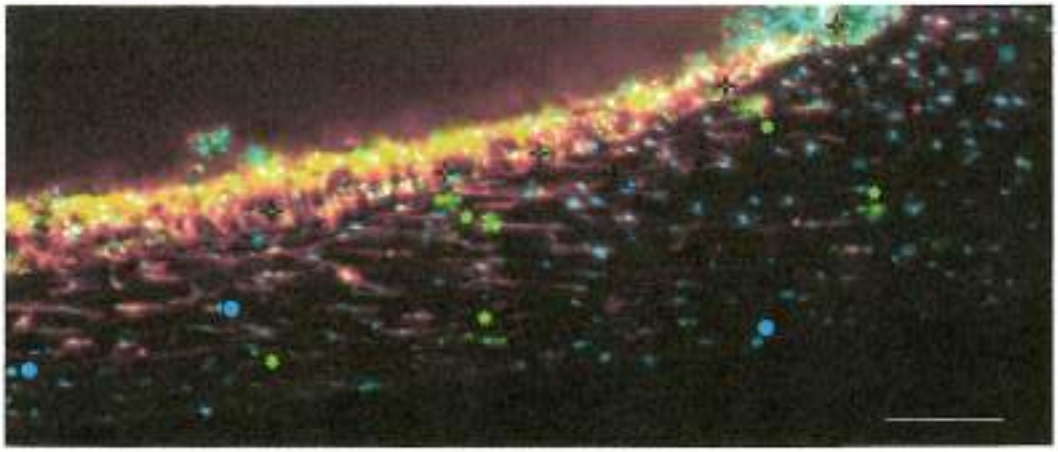

Figure 5. Cross section IF micrograph of a of a whole mount skin model. The disc-shaped dermis/epidermis layers were removed from the transwell, cut with a razor blade and mounted on a support to arrange the tissue orthogonal to the microscope objective. Red channel = ActinRed; cyan channel = NucBlue; green channel = CD206; yellow channel = involucrine; cyan circles = putative THP-1; green stars = putative macrophages; red stars = fibroblasts; yellow star = keratinocytes. Scale bar = 100 um.

Our data suggest a not homogeneous distribution of THP-1 and macrophages (Figure 5, 6). The presence of THP-1 was found more localized between the endothelial barrier and the first half of the dermis whereas the macrophages seemed more abundant in the dermis layer closer to the keratinocytes.

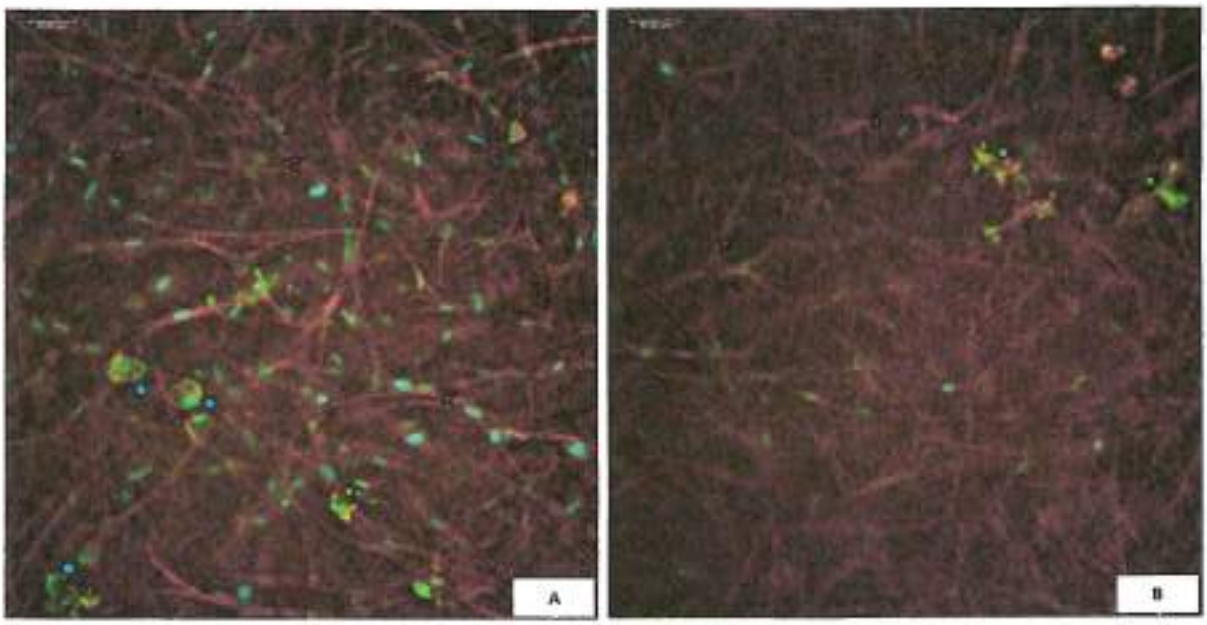

Figure 6. Bottom up IF optical sections obtained in the middle of the dermis (A) and close to the epidermis (B) of a whole mount skin model. Red channel = ActinRed; cyan channel = NucBlue; green channel = CD206; cyan circles = putative THP-1; green stars = putative macrophages; red stars = fibroblasts.

Discussion

European legislation has paved the way of more ethical test of chemicals envisaging cosmetic applications [11] banning test on animal models. Nevertheless, the chemical industry is constantly developing new and more performing/ecological chemicals. Each new chemicals need to undergo a rigorous risk assessment independently from the potential field of application. To date most of those assessment rely on the use of animal models that have the drawback of raising more and more ethical concerns as well as potential translatability of the results. To overcome those drawback new alternative methods are/have been developed. Those models to be accepted by the legislations must be reliable, reproducible, and relevant. With respect to skin and skin reactions to chemical compounds, SME based on human cell lines are currently the most promising technology to develop alternative guidelines aimed at replacing the animal

models. The increasing number of literatures on SME has demonstrated the superior capacity of those models to mimic native skin's cellular organization and function when compared with traditional 2D cells culture [12]. Different SME are currently commercialized and used as alternative methods to assess specific hazards of new compounds (e.g. EpiSkin®, SkinEthic® and T-Skin® (L'Oreal, France), Epi-Derm® (MatTek Corporation, USA), epiCS® (CellSystems, Germany), Phenion® (Henkel, Germany), NeoDerm® (Tego science, Korea), LabCyte® (J-TEC, Japan)). These models have the advantage of being reproducible and relatively simple as most consist of mono or bi-layered constructs with few cell types.

To further enlarge the availability of alternative animal model we decided to develop a model dedicated to skin sensitization assessment give that, to the best of our knowledge, none of the existing models have the needed cellular complexity to recapitulate the main sensitization-specific events. The presence of immune resident cells and of a functional vasculature are expected to increase the increase the relevancy of a new model dedicated to skin sensitization assessments.

SME all relies on some support (typically a transwell) to expose the epidermis to air and allow the maturation/differentiation of keratinocytes into corneocytes and formation of the stratus corneum. Whereas most of the models are cultured in static conditions inside incubators there have been more and more effort to integrate SME with microfluidic technologies. These combinations of technologies are referred as skin-on-a-chip models. Their main advantages are to enable physiologically relevant transport of nutrients and exogenous substances to the skin tissue, better control over physical and chemical factors in the cell microenvironment [13], and more reliable evaluation of drug candidates in terms of toxicity, efficacy, and delivery compared to static conditions. A vascularized SME could represent an invaluable tool for basic research (e.g. study vascular remodeling during wound healing, aging,) but has the potential to

become a new tool to assess topical and/or systemic skin reactions to specific chemical compounds and eventually test therapies to mitigate or treat such adverse reactions. Our data are the first encouraging step toward this direction. Our data demonstrated the capability to obtain a partially immunocompetent models that recapitulate the main structure and functional elements of human skin. The presence of a micro/milli fluidic circuit allowed us to obtain a continuous and aligned endothelial cells interface. The model is highly customizable in terms of cells population as well as vasculature to tissue ratio: this is expected to bring benefits when traying to recreate in vivo not only healthy and young skin models but also models with a pre-inflammatory condition or skin fragility.

Conclusion

Other groups created several SME models for the prediction of skin sensitization potential, but to our knowledge, none of them had integrated dermis, epidermis, THP-1 and HUVECS. For the first time, this study demonstrates the feasibility of such approach. The next steps will be to demonstrate the capability of our skin model to recapitulate a sensitization episode. This will be assessed by evaluating the shift in the macrophage/THP-1 population using: 1- reference chemicals representative of raw materials in cosmetics but also in medical devices; 2- chemicals representative of a balanced range of skin sensitizer potency (weak, moderate, and strong).

Acknowledgments

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Conflict of interest statement

Any conflict of interest

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