Lab on a Chip

PAPER

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In vitro micro-physiological immune-competent model of the human skin

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Skin allergy, in particular, allergic contact dermatitis and irritant contact dermatitis, are common occupational and environmental health problems affecting the quality of life of a significant proportion of the world population. Since all new ingredients to be incorporated into a product are potential skin allergens, it is essential that these ingredients be first tested for their allergenic potential. However, despite the considerable effort using animal models to understand the underlying mechanism of skin sensitization, to date, the molecular and cellular responses due to skin contact with sensitizers are still not fully understood. To replace animal testing and to improve the prediction of skin sensitization, significant attention has been directed to the use of reconstructed organotypic *in vitro* models of human skin. Here we describe a miniaturized immune competent *in vitro* model of human skin based on 3D co-culture of immortalized human keratinocytes (HaCaT) as a model of the epidermis barrier and human leukemic monocyte lymphoma cell line (U937) as a model of human dendritic cells. The biological model was fitted in a microfluidic-based cell culture system that provides a dynamic cellular environment that mimics the *in vivo* environment of skin. The dynamic perfusion of culture media significantly improved the tight junction formation as evidenced by measuring higher values of TEER compared to static culture. This setting also maintained the high viability of cells over extended periods of time up to 17 days. The perfusion-based culture also allows growth of the cells at the air–liquid interface by exposing the apical side of the cells to air while providing the cell nutrients through a basolateral fluidic compartment. The microsystem has been evaluated to investigate the effect of the chemical and physical (UV irradiation) stimulation on the skin barrier (*i.e.* the TJ integrity). Three-tiered culture differential stimulation allowed the investigation of the role of the keratinocyte layer as a protection barrier to chemical/biological hazards.

Introduction

Skin allergy is one of the major health problems affecting the quality of life of a significant proportion of the world population. In particular, allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) resulting from skin sensitization are common occupational and environmental health problems and have a major economic burden.[1] ACD involves an inflammatory process which is mediated through an immunologic reaction while ICD results from direct damage to the skin cells, which initiates an alternative inflammatory reaction, and it tends to be short lasting.[2,3]

Substances in products that come in contact with the skin, such as cosmetics, fragrances and clothing textiles play an important role as exogenous factors in the triggering of skin sensitization. Since all new ingredients incorporated into a product are potential sensitizers, it is necessary to conduct a thorough skin sensitization risk assessment prior to introduction of the product to the market to assure that the product/ingredient will not induce ACD or ICD. Traditionally, the identification of potentially sensitizing chemicals completely relies on animal testing such as the local lymph node assay in mice (LLNA)[4–6] and the Guinea pig maximization test (GPMT)[7] as no standardized, validated alternative exists. However, the chemical testing for allergenicity required by regulatory authorities is expected to utilize millions of animals annually. Several legislations call for significant reductions or even a complete ban on animal testing. It is estimated that the number of chemicals to be submitted for registration will increase from 68 000 to 101 000 chemicals between 2010 and 2022.[8] This suggests demand for millions of testing animals with an overwhelming cost which clearly challenges the feasibility of implementing this test without a major investment into alternative methodologies. Therefore,

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there is an urgent need for some reliable alternative animal-free assays that are able to predict the allergenic potential of chemicals.

Significant attention has been recently directed to the use of reconstructed organotypic *in vitro* models of human skin. Several reconstructed human *in vitro* skin equivalents are currently commercially available such as EpiDerm (MatTek, Ashland, MA, USA), Episkin (Episkin, Chaponost, France), Apligraf (Organogenesis Inc., MA, USA) and Skinethic (Skinethic, Nice, France) for *in vitro* toxicology studies. These models consist of differentiated human keratinocytes (KCs), seeded on matrices of either dermal components or non-biological origins.[9–11] The most commonly used parameter for skin sensitization is the measurement of cell viability. However, the European Centre for the Validation of Alternative Methods (ECVAM) agreed that measuring cytotoxicity alone does not always reveal the right prediction between irritants and non-irritants.[12] Therefore, additional biomarkers should be incorporated. In this respect, measuring the release of cytokines or other molecules might be a promising approach.

Cosmetics Europe (The European Cosmetics Industry Trade Association) member companies have developed three *in vitro* tests which include the Direct Peptide Reactivity Assay (DPRA),[13] the myeloid U937 skin sensitization test (MUSST),[14] and the human cell line activation test (h-CLAT).[15] The Procter & Gamble, Wella-Cosmital Laboratories has also developed an *in vitro* test that is based on human peripheral blood monocyte-derived dendritic cells (MoDCs).[16] Recently, the European consortium, SENS-IT-IV, has developed an *in vitro* test that uses a KC cell line (NCTC 2544) and it is based on the selective induction of IL-18 in the KCs by contact allergens. Cell-associated IL-18 was evaluated after 24 hours by ELISA.[17] For more details about the current technology of skin models, readers may refer to ref. 18.

Recent development in microfluidic-based cell culture technology has demonstrated the feasibility of using micro-scale *in vitro* physiological models "organs-on-a-chip" for drug screening. Microfluidic-based cell culture systems are capable of precisely tuning dynamic fluid flows and spatiotemporal gradients, as well as delivering nutrients, chemicals and stimuli to cells and removing soluble molecules in a controlled manner.[19,20] Additionally, microfluidic systems enhance cell–cell and cell–matrix interactions and allow controlled application of mechanical stresses by fluid flow and therefore, can potentially provide a better, faster and more efficient characterization of cell interaction with the surrounding environment. Various *in vitro* micro-physiological models have been conceptually developed over the last few years to mimic the physiological architecture and dynamics of human organs such as the human gut,[21,22] lung,[23] liver,[24] and blood–brain barrier.[25]

On-going research continues to provide new insights into the biological processes driving skin irritancy and sensitization. Chau *et al.*, in 2013, described the construction of a 3D human skin model comprised of dendritic cells co-cultured with keratinocytes and fibroblasts.[26] The authors reported that immune cells in the model are able to migrate and be responsive to stimulation with skin sensitizers (*e.g.* DNCB) even at low concentrations. Recently, Atac *et al.*, in 2013,[27] described the use of an EpiDerm *in vitro* model within a dynamically perfused chip-based bioreactor which is capable of applying variable mechanical shear stress and extending culture periods. The authors also reported the culture of single hair follicular units in the bioreactor.

Here, we describe a dynamic microfluidic-based *in vitro* immune-competent model of the human skin based on a co-culture of two cell lines simulating the epidermal and immune cells that are capable of recapitulating the interaction between KCs and dendritic cells (DCs) in extended culture periods. The Transepithelial Electrical Resistance (TEER) method is a very useful endpoint for quality control assessment to describe barrier function. The combination of perfusion based-microfluidic co-culture system, TEER probes and the capability of parallel differential stimulation experiments on the same chip provide a competitive and powerful tool to address the current industrial need.

Materials and methods

The biological model

After the stratum corneum, the epidermis epithelium is the outermost layer of the skin which mainly consists of KCs. KC acts as an effective barrier against a vast number of substances and plays crucial roles in the immune surveillance of the epidermis.[28] The human KC cell line (HaCaT) has been chosen for our study as in culture they form a fully differentiated epidermis in a 3D structure which very closely resembles real human epidermis. The underlying dermis contains many specialized immune cells, including dendritic cells (DCs), CD4+ T helper (Th) cells, natural killer T cells, macrophages and mast cells. Owing to the importance of the KCs as a major barrier of the skin and the DCs as the first line of the defense in the immunosystem, our biological model consists of a 3D structured of KCs/DCs co-culture in a double-layered microfluidic system as shown in Fig. 1a. This co-culture system would allow us to evaluate the importance of the skin barrier function in the prediction of the sensitizing potential of chemicals and to investigate the KCs/DCs cross-talk. When the chemical (allergen or non-allergen) to be tested is applied apically to the KC layer, the KCs respond to the chemical by releasing alarm signals in the form of inflammatory cytokines. These soluble mediators diffuse through the separating membrane (together with the metabolized chemical) to activate the DCs in the lower compartment. The DCs response is dependent on whether the chemical is an allergen or not.

Device design and fabrication

Fig. 1 shows schematically the structure of the microfluidic system that hosts the biological skin model and its perfusion setup. The human epidermis is approximated by a confluent layer of KCs which is cultured on top of a porous membrane.

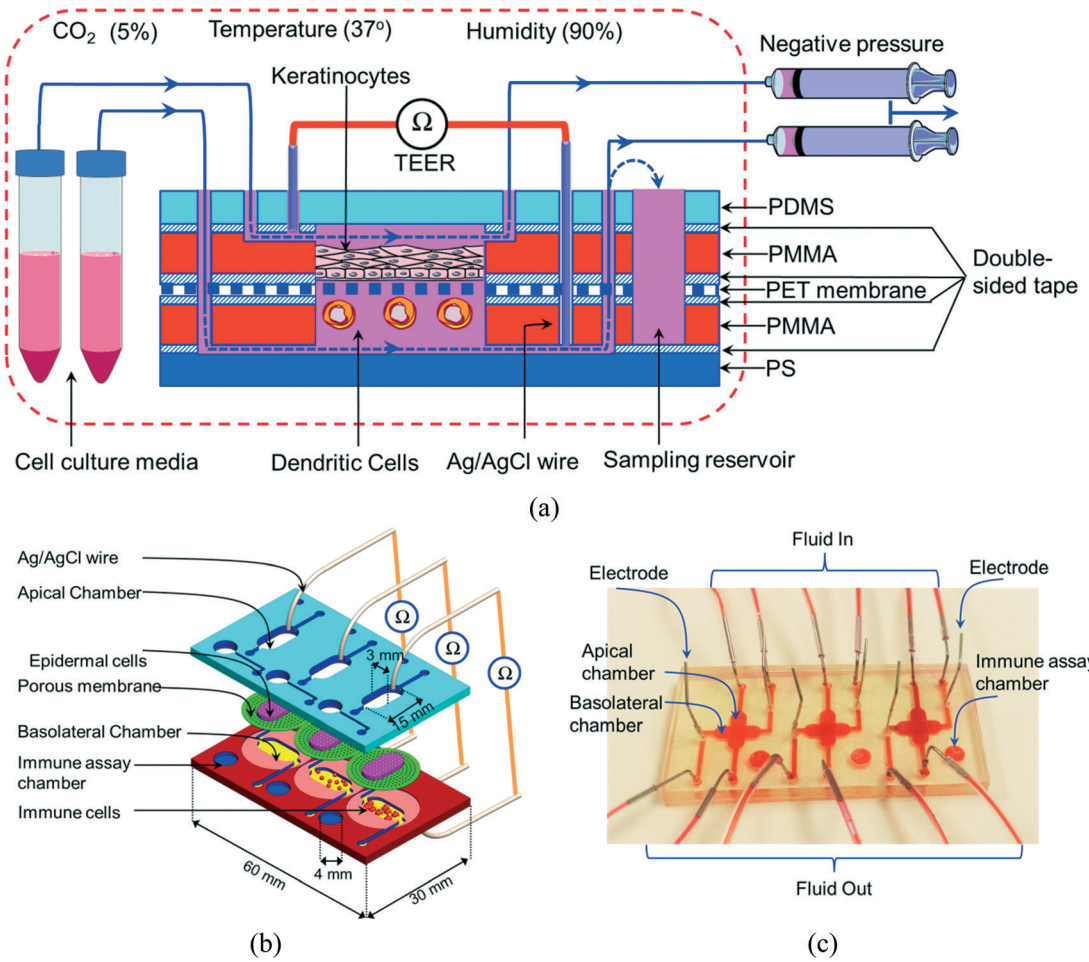

Fig. 1 (a) Schematic cross-sectional view of the cell culture device with the perfusion setup. (b) 3-Dimensional schematic view of the device with three parallel cell culture chambers and TEER measuring electrodes. (c) Image of the fabricated chip is shown in the inset.

A co-culture of immune cells (ICs) as a DC model is positioned beneath the KC layer, and the two cell cultures are separated by a porous membrane which allows KC–IC interaction as well as access of KCs from the basolateral side.

As a proof of concept, the skin-on-chip device was fabricated by assembling four polymer layers (two patterned poly(methyl methacrylate) (PMMA) sheets, a polystyrene (PS) sheet and a polydimethylsiloxane (PDMS) sheet) as shown in Fig. 1a. First, the microfluidic chambers and channels were cut into a one millimeter thick PMMA sheet that was previously laminated by using a double-sided tape (3M, USA). The polyethylene terephthalate (PET) membrane (Millipore, Singapore), with a pore size of 0.4 μm and pore density of 4×10^6 pores per cm^2,[29] was sandwiched between the two patterned PMMA sheets. To seal the fluidic device, the lower PMMA sheet was bonded to a PS sheet (0.5 mm) and the upper chamber was bonded to a PDMS sheet with a thickness of 1 mm. This PDMS lid allows gas exchange through the chip (Table 1). Each chip comprises three horizontally arranged fluidic chambers, with each chamber consisting of two vertically stacked compartments separated by a porous membrane (Fig. 1b).

Each culture chamber is connected to a sampling reservoir that is located next to the chamber outlet. Through our experiments, one chamber was used as a control and the other two chambers were used for differential stimulation experiments. The fluidic inlets of the culture chambers were connected to culture media reservoir through a set of PEEK tubing with an inner diameter of 0.5 mm while the fluidic outlets were connected to a set of syringe (5 mL). The fluidic setup was structured to allow storage of the culture media reservoirs

Table 1 Details of the chip dimensions

Component	Dimensions/volume
Chip (overall)	6 mm × 3 mm × 2.7 mm
Apical chamber	(15 mm × 3 mm × 0.5 mm)/17 μL
Basolateral chamber	(15 mm × 3 mm × 0.5 mm)/17 μL
PDMS cover	6 mm × 3 mm × 1 mm
Polystyrene sheet	6 mm × 3 mm × 0.5 mm
Double-sided tape	Thickness = 50 μm
Immune assay chamber	(Diameter = 4 mm, depth = 2.2 mm)/27.6 μL
Porous membrane	Thickness = 10 μm
Ag/AgCl electrode	Diameter = 0.5 mm

inside the cell culture incubator to maintain a steady culture environment (*i.e.* temperature and O_2 supply) during experiments. Negative pressures were applied onto the syringes using a set of syringe pumps (Harvard Apparatus, USA) to maintain continuous perfusion of cell culture media through the cell culture chambers. The perfusion was maintained through the course of the cell culture at a flow rate of 5–20 nL s^{-1}.

Ag/AgCl wires with a diameter of 0.5 mm (World Precision Instruments INC., USA) were inserted into the upper and lower compartments by punching holes in the PDMS and PMMA sheets. In each culture chamber, an Ag/AgCl wire was inserted above and beneath the PET membrane, respectively. These electrode wires were used to measure the TEER of the KC layer in order to monitor the cell layer integrity through the course of cell culture and to assess the cell layer confluence as well as detecting the impact of chemical/physical stimuli on the intercellular tight junction (TJ) integrity.

Cell culture

Immortalized human keratinocytes (HaCaT) (Addexbio Biotechnologies, San Diego, USA) were used as a model of human epidermis KCs. The KCs were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. In our experiments, cells from 5–7 passages were used. The human leukemic monocyte lymphoma cell line (U937) (Addexbio Biotechnologies, San Diego, USA) was used as a model of human DCs. The U937 cells were also maintained in the same media of the KCs. Before use in microchannels, the cells were maintained in the complete growth media and cultured in culture flasks according to the manufacturer's protocols. In general, KCs were cultured in the chip first and allowed to form a confluent layer before introducing the ICs. The cells were used in the microfluidic culture after 1–4 subcultures. The KCs were trypsinized and re-suspended in the culture medium before seeding into the chip. Before cell seeding, the chips were wiped with 70% ethanol, dried in the oven for 30 min at a temperature of 70 °C followed by UV irradiation for another 30 min. Then the chips were filled with culture media (no perfusion) and pre-incubated overnight. After filling the culture chambers with fresh media, the HaCaT cells were seeded at 10^5–10^6 cells per cm^2 into the upper chamber using a syringe pump, and the media flows were completely stopped to allow cell attachment onto the membrane. After 2–5 hours, steady flows of culture media were driven by the syringe pumps at a flow rate of 10–20 nL s^{-1}. The microfluidic culture was maintained at 37 °C in a humidified incubator with 5% CO_2. The U937 cells were seeded after one week of HaCaT seeding. A 3-tiered culture system was experimented on the same chip that includes: HaCaT monoculture, U937 monoculture, and HaCaT/U937 co-culture. To establish air–liquid interface (ALI) on the apical side of the HaCaT culture, the culture media were aspirated from the upper chamber while maintaining media flow through the lower chamber. Cell culture media were infused in the upper chamber at different time intervals to prevent a sharp drop in cell viability during the ALI experiments.

Cell viability

A mixture of calcein-AM (2 μM) and ethidium homodimer-1(4 μM) which was diluted in D-PBS was injected into the upper chamber and incubated in the cell culture incubator for 10 min. Then the cell culture was examined by fluorescence microscopy. Cell viability was quantified by the percentage of calcein AM-labeled cells. Images were taken from at least ten different observation locations, and the percentage value was averaged. Data were represented as mean ± SD.

TEER measurements

Ag/AgCl wires were sterilized by flushing with 70% ethanol and UV irradiation prior inserting into the chip through the PDMS lid. The wires were then connected to a Millicell® ERS-2 Volt-Ohm meter (Millipore, USA). TEER measurements were obtained at regular intervals (every day or every few hours at certain experiments), with each data point being a mean of three resistance measurements taken per culture chamber. The contribution of the membrane and culture medium is accounted for by subtracting the electrical resistance of the porous membranes without cells from the sample values and normalized with respect to the culture surface area by multiplying the net electrical resistance by the total surface area covered by the KC barrier to calculate TEER in Ω cm^2. One fluidic chamber was kept free of cells during the experiments as a control unit, and the TEER value was measured in parallel and subtracted from that of the TEER values of the chambers with cells.

Paracellular permeability

The apical-to-basolateral transport flux which is correlated to the paracellular transport route has been quantified by measuring the permeability of the KC barrier to fluorescein isothiocyanate (FITC)-4 kDa dextran (Life Technologies, Singapore) as a flux tracer. A 10 μg mL^{-1} solution of FITC-dextran was added at the apical side of the KC layer and the paracellular flux of the tracer molecule was quantified by serially sampling fluid from the basolateral compartment at different time intervals and the tracer concentration was determined using a fluorescent plate reader (Enspire, Perkin Elmer, USA). Simultaneous TEER measurements were also taken to monitor the integrity of the KC layer.

Immunofluorescence staining of intercellular tight junctions

The HaCaT Cells were grown to confluence in the upper compartment of the chip, and TJ staining was conducted one week post-confluence with well-formed TJs and a TEER value of more than 4000 Ω cm^2. The cells were rinsed with PBS and fixed in 3.7% formaldehyde at room temperature for 20 min. The cell layer was then rinsed in PBS and permeabilized in 0.2% Triton X-100 for 10 min at room temperature. The cells

were then rinsed in PBS followed by blocking with 1% bovine serum albumin (BSA) for 30 min at room temperature. The cells were incubated with 2 μg mL^{-1} anti-mouse occludin (Life Technologies, Singapore) at 4 °C overnight. After that, the cells were washed with PBS and incubated with anti-mouse IgG conjugated to FITC (Life Technologies, Singapore) at room temperature for one hour. After washing with PBS, the membrane, with the cell layer, was detached from the chip and mounted on a microscope glass slide and stored at 4 °C in the dark until analyzed. The fluorescence tight junction stain was visualized using an Olympus fluorescent microscope (Olympus, BX3-CBH, Japan).

Cell stimulation and secreted cytokine detection

To test the cellular system response to external stimuli, the cells were treated with LPS and the expression of two pro-inflammatory cytokines namely, IL-6 and IL-1β were measured using a magnetic bead-based sandwiched immune assay employing the Milliplex Map, Human Cytokine magnetic bead panel, Cat # HCYOMAG-60 k (Millipore, Singapore). Prior to conducting the cytokine detection on a chip, serial dilutions of IL-6 and IL-1β standards with known concentrations were prepared, and cytokine detection in a 96-well plate was conducted according to the manufacturer's instruction. However, the fluorescence signals were measured using a fluorescent plate reader instead of using the Luminex system (bead color was not detected). Cytokine detection was performed using chips with three different configurations: HaCaT monoculture, U937 monoculture, and HaCaT/U937 coculture. The U937 cells were exposed to a medium containing phorbol myristate acetate (PMA) (Sigma-Aldrich, Singapore) for 48 hours to initiate the differentiation to macrophages. The lower (basolateral) compartment of each chamber was connected to a downstream sampling reservoir (Fig. 1) where cell culture supernatant is collected for ~24 hours. Then, the magnetic bead-based immune assay was conducted in the reservoir in a similar manner to the 96-well protocol. Streptavidin-coated magnetic beads were incubated with biotinylated cytokine capture antibodies off-chip in PBS for 30 min. The antibody-coated beads were then separated from the supernatant by placing the tube on a magnet plate for 5 min and magnetically washed three times in PBS. The separated antibody-bead complexes were re-suspended to a concentration of 2×10^5 beads per mL and injected in the corresponding sampling reservoirs. The stimulus was injected into the upper compartment at a concentration of up to 1 μg mL^{-1} and incubated in the cell culture incubator overnight. The number of U937 cells which were used in the experiment is within the range of 20–300 cells per chamber. The supernatant from the basolateral compartments, where the immune cells are cultured, was allowed to infuse the sampling reservoir during the incubation time. Magnetic beads within the reservoirs were agitated and washed using an external magnet. For washing, a small NdFeB magnet was positioned under the reservoir, and the supernatant was aspirated. After the washing step, streptavidin-PE conjugated cytokine detection antibodies were injected into the reservoir containing the magnetic beads and incubated with the sample for 2 hours. Next, the magnetic bead/cytokine/streptavidin-PE complexes were washed with PBS and the fluorescence signal was measured using either the plate reader or fluorescence microscope. In the latter case, images were analyzed using the ImageJ software.

Results and discussions

Cell culture

Initial work focused on maintaining cell culture conditions that prolong the viable cellular system in dynamic flow environments. Experiments were conducted using 3-chambered chips (Fig. 1). The 2-layered structure of the chip allows feeding the KC culture from the apical and basolateral sides. In this configuration, the culture media is continuously replenished over the upper compartments and infused through the membrane where the upper and lower compartments intersect. The upper and lower compartments intersect through an area of 25 mm^2 that is separated by the porous membrane. The microsystem was first monitored for cell viability and the progress of confluent barrier layer formation in both in mono- and co-culture conditions. The formation of a confluent layer of KCs that covers the entire membrane is very important for evaluating the KC culture as a skin barrier model and to test the integrity and permeability characteristics of the layer. Fig. 2a shows optical images of the KC culture after 1, 5 and 20 days of seeding, respectively. The first two images show the upper culture chamber at a low magnification (2.5×) which display a void-less confluent layer (second image) that covers the entire surface of the chamber while the third image shows a higher magnification (20×) of the same culture.

It was found that the perfusion-based cell culture promotes a healthier barrier and prolongs the lifespan of the cellular system. This may be attributed to the continuous replenishing of cell nutrients and removal of cellular waste. Under the perfusion conditions, we observed that the KC culture survives up to 17 days with up to 85% cell viability (95% for 10 days) (Fig. 2b). To evaluate the ability of the system to mimic the skin–air interface conditions *in vivo*, two cell culture systems were monitored in parallel; these are the liquid–liquid interface (LLI) and air–liquid interface (ALI) systems. The LLI system maintains cell viability up to 95% and comparing to 88% and 67% for the ALI system, for 10 and 17 days, respectively. To enhance cell viability, the upper compartment of the ALI system was replenished with fresh culture media for 10 minutes every day, and the fluid was aspirated immediately to maintain ALI conditions. The HaCaT cells formed a monolayer when cultured *in vitro*, and the vast majority of cells are in direct contact with the porous membrane and are exposed to the flow streams of culture media in the lower chamber when cultured in ALI environments. Therefore, cell viability in the ALI conditions can be

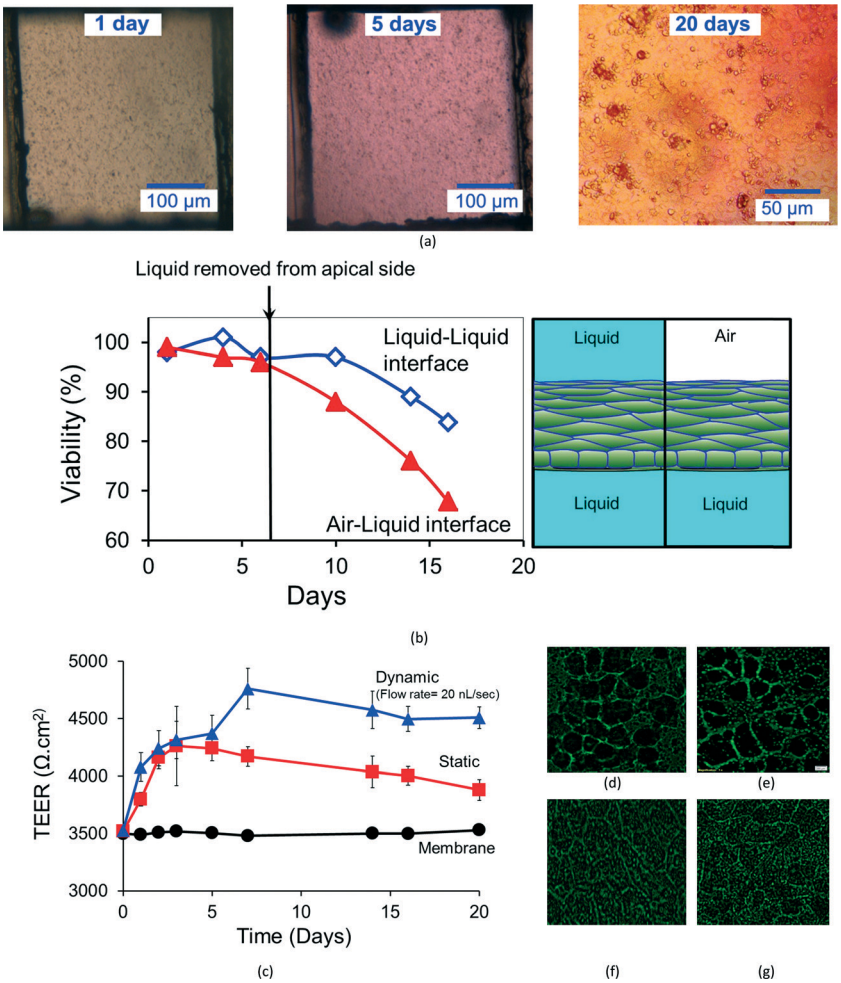

Fig. 2 (a) Optical images of KC culture after 1, 5 and 20 days of seeding. The first two images show the entire upper culture chamber at a low magnification (2.5×). The second image displays a void-less confluent KC layer that covers the entire surface of the chamber while the third image shows a higher magnification (20×) of the same culture. (b) The KC culture survives for up to 17 days with up to 85% cell viability (95% for 10 days). Cell viability was 88% and 67% for 10 and 17 days, respectively under ALI conditions. (c) Perfusion-based culture (dynamic culture) showed a higher TEER value after the first week of culture which suggests that perfusion accelerates the TJ formation and maintains healthier TJs over an extended period of culture compared to static culture. The TEER value is normalized by subtracting the TEER of the cell-free membrane from those with cells in both static and dynamic culture. The error bars represent the chamber to chamber variation. (d and e) No obvious difference was observed in the tight junction staining between the dynamic and static culture. (f and g) TJ staining appeared to be distorted after exposing the apical side to air in ALI culture.

enhanced by optimizing the porous membrane's design, mainly the pore's density, and size and membrane thickness. The residence time of culture media in each culture compartment at a perfusion flow rate of 10 nL s^{-1} is ~16.67 min.

TEER measurements show a rapid increase after the first day of culture until day 5 (Fig. 2c). *Fluctuation of the resistance followed this during the second and third weeks.* The perfusion-based culture showed a higher TEER value after the first week of culture. The constant supply of cell nutrients and waste removal may contribute to the stable growth of cells and improved differentiation and, in consequence, this would result in long-term cell survival. No obvious difference was observed in the tight junction staining between the dynamic and static culture (Fig. 2d and e). However, it was observed that the staining filaments of the intercellular TJs of the cells in the LLI system are clearer with less breakage compared to those of the ALI system (Fig. 2f and g). It should be noted that the voids in the cell layer in the images of the tight junction staining are due to some cell detachment from the membrane during the staining experiments. All experiments were conducted after assuring fully confluent layer of KCs.

KC monoculture stimulation

The KC layer in the monoculture was challenged with different concentrations of LPS within the range of 0.05–1 μg mL^{-1}. The TEER value was measured after 1 and 24 hours of stimulation/chemical treatment to investigate the effect of these chemicals on the integrity of the KC layer. In general, no significant decrease in TEER was observed after treatment of the KC layer with LPS (Fig. 3a). Also, TJ integrity/permeability was assessed by fluorescein labeling of occludin of the

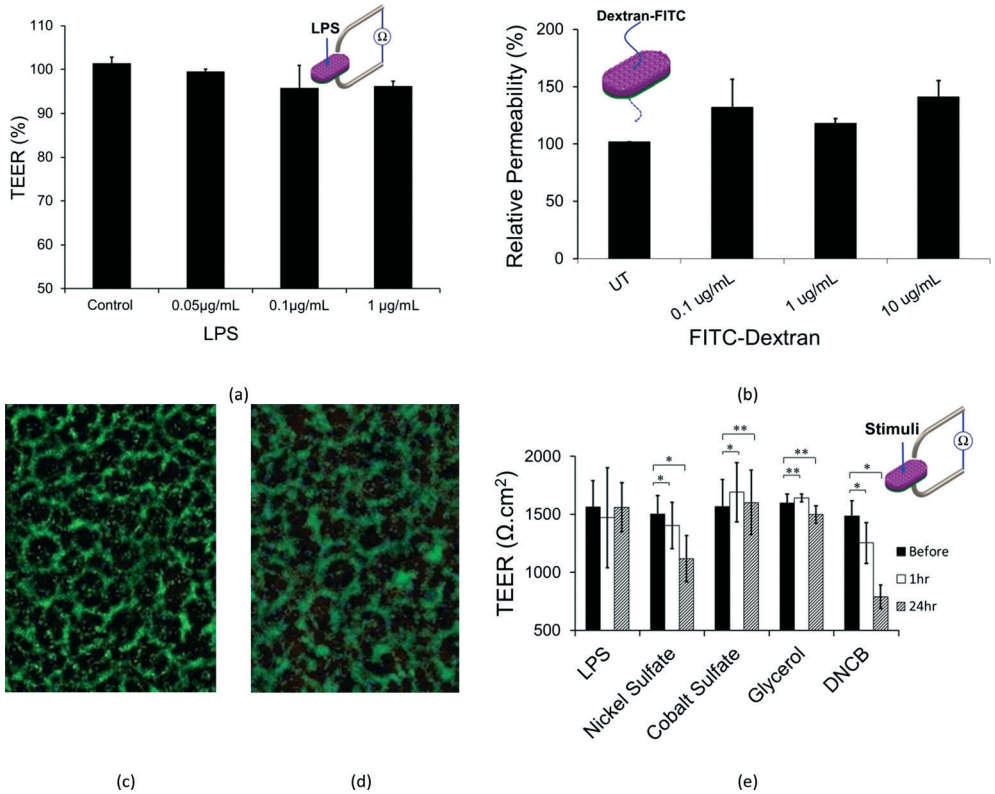

Fig. 3 (a) No significant change in TEER values was observed after treatment of the KC layer with a different concentration of LPS. (b) No significant change in the KC layer permeability after treatment with LPS. (c and d) The intercellular occludin staining signal intensity of the TJs of the KCs before and after LPS treatment appeared similar. (e) TEER significantly decreases after 24 hours of incubation with nickel sulfate while no obvious change after treating with LPS, cobalt sulfate, and glycerol (* $P < 0.05$, ** $P < 0.1$).

HaCaT layer. The permeability of the HaCaT layer was investigated by incubation of the cell culture with different concentrations of LPS. Then, the FITC-dextran flux through the layer was measured by collecting the supernatant from the basolateral chamber (sampling reservoir) and reading the fluorescence signals correlated to the dextran concentration. It was also found that LPS did not produce any disruption to the KC layer permeability (Fig. 3b). In the same manner, the intercellular occludin staining signal intensity of the TJs of the KCs before and after LPS treatment appeared similar (Fig. 3c and d).

To investigate the response of KCs to other stimuli, we treated the KC confluent layer with nickel sulfate and DNCB as skin allergens,[30] and cobalt sulfate and glycerol as neutral substances;[31] and the TEER was measured before treatment and after 1 and 24 hours of incubation. Fig. 3e shows that TEER significantly decreases after incubation with nickel sulfate and DNCB while no obvious change after treating with LPS, cobalt sulfate, and glycerol, which suggests that nickel sulfate and DNCB disturb the TJs of skin. We observed that, in some experiments, the TEER value decrease significantly after treating the cell culture with stimuli and tend to increase in a fluctuated manner after a few days. This suggests that the TJ structure is very dynamic. Therefore, it is very important to investigate further the structural parameters and signaling events that regulate the TJ protein stability in health and disease states. Understanding the TJ structure and function at the molecular level resolution would help to understand the critical role of TJ as a major component of the skin barrier, and paracellular transport in health and diseases, and may provide a tool for quantitative toxicology.

Effect of UV irradiation on TEER

Exposure to UVB irradiation is believed to induce biological changes in the skin including cutaneous barrier deterioration. UVB irradiation in human skin xenografts and human skin equivalent models was found to cause functional deterioration of TJs and down-regulation of Rac1 and kinase C in an *in vitro* KC culture system.[32] As a preliminary test, we exposed the HaCaT culture chip to a continuous UVB irradiation using a UV germicidal lamp (Osram Sylvania G30T, Osram, Singapore). The chip was positioned such that the apical side is facing the UV source, and the calculated radiant intensity was 75 mJ cm^{-2}. The TEER value was measured after 10 min and 30 min of continuous irradiation and compared to those of un-irradiated cell layer as a control. Fig. 4a shows that the TEER value decreased to 82% of its original value upon irradiation with UV for 30 min. The TEER value was measured after 24 hours of irradiation. It was also observed

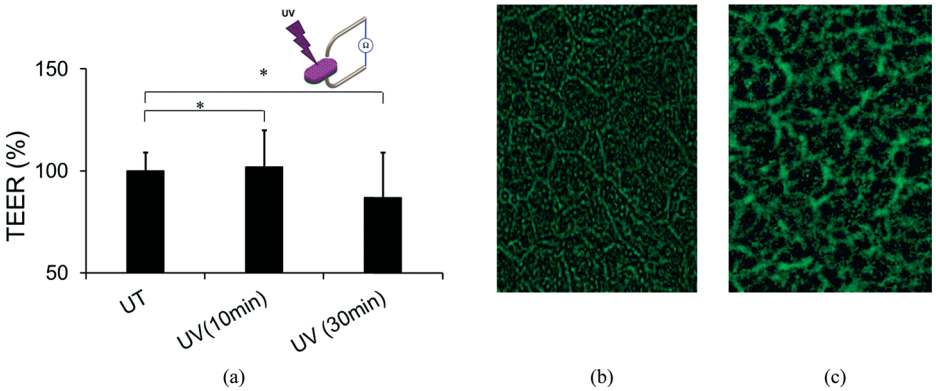

Fig. 4 (a) The TEER value decreased to 82% of its original value upon irradiation with UV for 30 min. TJ staining appeared as dotted filaments (b) compared to the more solid continuous filament in the case of those before UVB irradiation (c) (* $P < 0.05$).

that UV irradiation produced progressive disruption to TJs proteins. Fig. 4c shows that the TJ staining appeared as dotted filaments compared to the more solid continuous filament in the case of those before UVB irradiation (Fig. 4b). This suggests that UVB irradiation disturbs the TJ proteins of KCs.

LPS-induced expression of IL-6 and IL-1β

An effective skin barrier is an important factor in preventing exogenous invasion. Activation of U937 co-culture was used as an indicator of the barrier integrity and, in consequence, the potential allergenic properties of chemicals. U937 cells were inoculated into the basolateral compartment after ensuring the formation of a fully confluent layer of HaCaT cells, by observing the cells under a microscope and using TEER measurements. In general, U937 cells were introduced after 7 days of HaCaT culture. The maintenance of intact cell co-culture system for extended periods of time is crucial for valid *in vitro* models. The viability of the HaCaT cell after introducing U937 cells was measured using the live/dead assay and compared with that of the HaCaT monoculture. It was observed that the HaCaT cell viability dropped to 93% and 85% of its original value after 1 and 3 days of co-culture, respectively (Fig. 5a).

To assess the KC role as a protection barrier, a 3-tiered culture namely, HaCaT monoculture, U937 monoculture and HaCaT/U937 coculture was treated with LPS and the expression of the pro-inflammatory cytokines IL-6 and IL-1β was quantified in the basolateral compartment of each culture chamber using the magnetic bead-based sandwich immune assay. The LPS was applied equally to the upper compartment of the three systems and incubated for 24 hours. An additional untreated system was also monitored as a control. All stimulation experiments were conducted by applying the LLI conditions. A significant increase in IL-6 and IL-β secretion was observed in the basolateral media of U937 monoculture *i.e.* in the absence of KC barrier, compared to that of the HaCaT monoculture (Fig. 5b). However, a moderate increase in cytokine expression was observed upon treating the HaCaT/U937 co-culture with LPS, which suggests that the KC layer forms a robust barrier against LPS invasion. These results are in line with previously reported skin models that used conventional cell culture procedures.[26,33] It should be

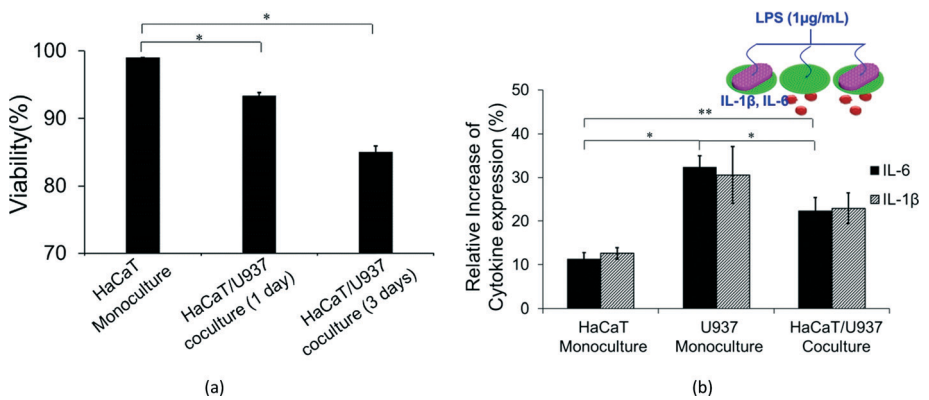

Fig. 5 (a) HaCaT cell viability was dropped to 93% and 85% from its original value after 1 and 3 day of co-culture, respectively. (b) Significant increase in IL-6 and IL-β secretion was detected in the basolateral media of U937 mono-culture *i.e.* in the absence of the KC barrier, compared to that of the HaCaT monoculture. On the other hand, a moderate increase in cytokine expression was observed upon treating the HaCaT/U937 co-culture with LPS, which suggest that the KC layer forms a robust barrier against LPS invasion (* $P < 0.01$, ** $P < 0.05$).

noted that the KC viability remains high (>90%) after 24 hours of LPS treatment. Therefore, the immune cell stimulation can be attributed to the paracellular transport of LPS.

The aim of this study was to demonstrate a miniaturized dynamic *in vitro* model of human skin based on 3D-co-culture of KCs and ICs. Our focus was on optimizing the cell culture environment that allows creating of an organotypic structure with close proximity to *in vivo* conditions which can be maintained viable for a sufficiently long time. The data presented using this model do not necessarily represent the biological process that cause skin allergy *in vivo*. But these preliminary results can be used as a precursor for further development in terms of the microsystem engineering as well as creating a biological model that mimics the structure and function of human skin in health and disease states. A major advantage of the *in vitro* organotypic technology is the employment of human cells to overcome the difference in metabolism between humans and animals. However, immortalized cell lines may behave differently in cell culture compared to primary cells. Therefore, they could provide less relevance. Due to ethical and regulation considerations, the primary cell source is not reliable for developing *in vitro* models. Therefore, adopting human pluripotent stem cells (hPSC) as a cell source could be a promising alternative. It should be noted that compared to the normal skin, skin *in vitro* models have a higher permeability that may result in an over-prediction of skin sensitization due to the higher penetration rate of applied substances. Therefore, experiments that compare *in vivo* (in human) and *in vitro* data are necessary. The screening of chemicals for skin allergy as required by the regulatory authorities is expected to utilize millions of animals annually while several legislations also call for significant reductions or even a complete ban on animal testing. This clearly indicates the necessities of investment in finding animal-alternative and high-throughput methodologies to perform the screening. However, the gap between the *in vivo* and *in vitro* conditions is still significant and many challenges are still ahead against closing this gap before adopting these tools as alternatives to the animal.

Conclusions and future outlook

The aim of this study was to demonstrate a miniaturized dynamic *in vitro* model of human skin based on a 3D-co-culture of KCs and ICs. The dynamic perfusion of culture media significantly improved the tight junction formation as evidenced by measuring higher values of TEER comparing to static culture. The perfusion-based culture also allows growth of the cells at ALI by providing the cell nutrients through the basolateral compartment. This configuration mimics the *in vivo* environment of skin. Also, perfusion allowed the prolongation of the cell culture period up to 17 days. The prolonged period is necessary to allow chemical screening over extended periods of time. In addition, the fully confluent layer of the KCs allowed the investigation of the effect of chemical and physical stimulation on the skin barrier *i.e.* the TJ integrity. Also, the 3-tiered culture differential stimulation allowed the investigation of the role of the KC layer as a protection barrier to chemical/biological hazards. This microsystem has the potential to be developed as a useful tool for skin sensitization and toxicity.

List of abbreviation

ACD	Allergic Contact Dermatitis
ICD	Irritant Contact Dermatitis
LLNA	Local Lymph Node Assay
GPMT	Guinea Pig Maximization Test
KCs	Keratinocytes
ECVAM	European Centre for the Validation of Alternative Methods
DPRA	Direct Peptide Reactivity Assay
h-CLAT	human Cell Line Activation Test
MoDCs	Monocytes Derived Dendritic Cells
DCs	Dendritic Cells
TEER	Transepithelial Electrical Resistance
ICs	Immune Cells
PMMA	Poly(methyl methacrylate)
PS	Polystyrene
PDMS	Polydimethylsiloxane
PET	Polyethylene terephthalate
ALI	Air-Liquid Interface
LLI	Liquid–Liquid Interface
FITC	Fluorescein isothiocyanate
PMA	Phorbol Myristate Acetate

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References

1 J. P. Thyssen, A. Linneberg, T. Menne and J. D. Johansen, *Contact Dermatitis*, 2007, **57**, 287–299.
2 P. J. Frosch, In: Cutneous irritation, ed. R. J. G. Rycroft, T. Menne and P. J. Frosch, *Textbook of Contact Dermatitis*, 2nd edn, Springer-Verlag, Berlin, 1995.
3 V. S. Beltrani, I. L. Bernstein, D. E. Cohen and L. Fonacier, Contact dermatitis: a practice parameter, *Ann. Allergy, Asthma, Immunol.*, 2006, **97**(3, supplement), S1–S38.
4 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Report (1999). The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemicals/compounds. NIH Publication No. 99–4494.
5 G. F. Gerberick, C. A. Ryan, I. Kimber, R. J. Dearman, L. J. Lea and D. A. Basketter, *Am. J. Contact Dermat.*, 2000, **11**, 3–18.
6 OECD Guidelines for Testing of Chemicals. (2002). Guideline No. 429. Skin sensitisation: Local lymph node assay.
7 B. Magnusson and A. M. Kligman, *J. Invest. Dermatol.*, 1969, **52**, 268–276.
8 T. Hartung and C. Rovida, *Nature*, 2009, **460**(27), 1080–1081.

9 T. Welss, D. A. Basketter and K. R. Schröder, *Toxicol. In Vitro*, 2004, **18**, 231–243.
10 M. A. Perkins, R. Osborne, F. R. Rana, A. Ghassemi and M. K. Robinson, *Toxicol. Sci.*, 1999, **48**, 218–229.
11 M. Ponec, *Int. J. Cosmet. Sci.*, 1992, **14**, 245–264.
12 J. H. Fentem, D. Briggs, C. Chesne, G. R. Elliott, J. W. Harbell, J. R. Heylings, P. Portes, R. Roguet, J. J. van de Sandt and P. A. Botham, *Toxicol. In Vitro*, 2001, **15**(1), 57–93.
13 G. F. Gerberick, J. D. Vassallo, R. E. Bailey, J. G. Chaney, S. W. Morrall and J. P. Lepoittevin, *Toxicol. Sci.*, 2004, **81**, 332–343.
14 F. Python, C. Goebel and P. Aeby, *Toxicol. Appl. Pharmacol.*, 2007, **220**(2), 113–124.
15 H. Sakaguchi, T. Ashikaga, N. Kosaka, S. Sono, N. Nishiyama and H. Itagaki, *Toxicol. Lett.*, 2007, **172**, S93.
16 P. Aeby, C. Wyss, H. Beck, P. Griem, H. Scheffler and C. Goebel, *J. Invest. Dermatol.*, 2004, **122**(5), 1154–1164.
17 E. Corsini, V. Galbiati, M. Mitjans, C. L. Galli and M. Marinovich, *Toxicol. In Vitro*, 2013, **27**, 1127–1134.
18 S. H. Mathes, H. Ruffner and U. Graf-Hausner, *Adv. Drug Delivery Rev.*, 2014, **69–70**, 81–102.
19 G. M. Whitesides, *Nature*, 2006, **442**, 368–373.
20 D. Huh, G. A. Hamilton and D. E. Ingber, *Trends Cell Biol.*, 2011, **21**(12), 745.
21 H. J. Kim, D. Huh, G. Hamilton and D. E. Ingber, *Lab Chip*, 2012, **12**, 2165–2174.
22 Q. Ramadan, H. Jafarpoorchekab, C. Huang, P. Silacci, S. Carrara, G. Koklu, J. Ghaye, J. Ramsden, C. Ruffert, G. Vergeres and M. A. M. Gijs, *Lab Chip*, 2013, **13**, 196–203.
23 D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin and D. E. Ingber, *Science*, 2010, **328**, 1662–1668.
24 D. A. Tatosian and M. L. Shuler, *Biotechnol. Bioeng.*, 2009, **103**(1), 187–198.
25 R. Booth and H. Kim, *Lab Chip*, 2012, **12**(10), 1784–1792.
26 D. Y. S. Chau, C. Johnson, S. MacNeil, J. W. Haycock and A. M. Ghaemmaghami, *Biofabrication*, 2013, **5**, 035011.
27 B. Ataç, I. Wagner, R. Horland, R. Lauster, U. Marx, A. G. Tonevitsky, R. P. Azarc and G. Lindner, *Lab Chip*, 2013, **13**, 3555–3561.
28 https://www.corning.com/media/worldwide/cls/documents/CLS-CC-027%20REV7%20DL%20%282%29.pdf.
29 T. Welss, D. A. Basketter and K. R. Schröder, In vitro skin irritation: facts and future. State of the art review of mechanisms and models, *Toxicol. In Vitro*, 2004, **18**, 231–243.
30 A. Lozsekova, H. W. Kaiser, T. Danilla, J. Buchvald and J. Simko, *Bratisl. Lek. Listy*, 2002, **103**(7–8), 254–259.
31 C. Rovida, S. F. Martin, M. Vivier, H. U. Weltzien and E. Roggen, *Advanced Tests for Skin and Respiratory Sensitization Assessment. Summary Report on the Sens-it-iv End Congress in Brussels*, 2011.
32 T. Yuki, A. Hachiya, A. Kusaka, P. Sriwiriyanont, M. O. Visscher, K. Morita, M. Muto, Y. Miyachi, Y. Sugiyama and S. Inoue, *J. Invest. Dermatol.*, 2011, **131**(3), 744–752.
33 I. H. Kuo, A. Carpenter-Mendini, T. Yoshida, L. Y. McGirt, A. I. Ivanov, K. C. Barnes, R. L. Gallo, A. W. Borkowski, K. Yamasaki, D. Y. Leung, S. N. Georas, A. De Benedetto and L. A. Beck, *J. Invest. Dermatol.*, 2013, **133**(4), 988–998.