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Immune-competent *in vitro* co-culture models as an approach for skin sensitisation assessment

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Abstract

There is a complex interplay between numerous cell types that act at different steps of the mechanism leading to allergic contact dermatitis. The validated *in vitro* methods for skin sensitisation assessment provide good statistical correspondence to local lymph node assay (LLNA) or to human data but, for the most part, poorly represent the actual *in vivo* situation as they generally involve single cell type culture in 2D. Significant progress has been made over the past decades to develop new technologies of data generation concurrently with novel approaches to improve the models especially by the use of co-culture. The importance of heterotypic cell-cell interactions in the *in vitro* assessment of skin sensitisation should not be overlooked. This review addresses the technical aspects to take into consideration when co-culturing depending on the desired objective and describes the different keratinocytes and dendritic cells co-cultures developed in 2D and 3D. To date, from a regulatory point of view, no alternative method to animal testing for skin sensitisation potential assessment using a keratinocytes and dendritic cells co-culture model is yet proposed. This review also presents several directions of further development.

Keywords: co-culture, skin sensitisation, predictive test

Introduction

Allergic contact dermatitis (ACD) is caused by repeated skin exposure to an allergen. ACD involves the activation of the innate and adaptive immune system and is divided in two phases: sensitisation and challenge. The sensitisation (or induction) phase starts at the first contact with an hapten and leads to the generation of hapten-specific T cells (Vocanson et al., 2009). Subsequent contact with the same hapten initiates the challenge phase (or elicitation) resulting in hapten-specific inflammation, the clinical expression of ACD.

ACD to at least one allergen affects nearly 20% of the general population and with the increasing prevalence of ACD, identification and evaluation of the sensitising potential of new compounds is important (Peiser et al., 2012). The prediction of skin sensitising potential and potency typically involves animal testing using method such as the murine local lymph node assay (LLNA). Given the questionable biological relevance of animal models to humans as well as ethical and regulatory pressure to limit or ban the use of animal models for safety testing, alternative methods are being developed.

The adverse outcome pathway (AOP) concept published by the Organisation for Economic Cooperation and Development (OECD) fostered the development of various alternative tests. The AOP describes the key events from the molecular initiating event through intermediate events to the adverse outcome, i.e. allergic contact dermatitis (OECD, 2012a, b). The four key events comprise the covalent binding of a chemical to proteins (key event 1), the inflammatory response by keratinocytes (key event 2), the dendritic cell activation (key event 3) and the T cell proliferation (key event 4).

Among these alternative assays, seven *in chemico* or *in vitro* tests are already validated by European Centre for the Validation of Alternative Methods (ECVAM) and test guidelines are edited by OECD, namely Direct Peptide Reactivity Assay (DPRA), Amino acid Derivative Reactivity Assay (ADRA), KeratinoSens™, LuSens, human Cell Line Activation Test (h-CLAT), U937 cell line activation test (U-SENS™) and the interleukine-8 reporter gene assay (IL-8 Luc assay). The DPRA and the ADRA, mimicking molecular initiating event (MIE) or key event 1 (KE1) within the skin sensitisation AOP, enables to have information on the hapten's reactivity

towards peptides containing cysteine or lysine using high performance liquid chromatography (Fujita et al., 2014; Gerberick et al., 2004) and are described in TG442C (OECD, 2019). The KeratinoSens™ and LuSens tests described in TG 442D (OECD, 2018a) model the KE2 by the evaluation of the activation of a relevant cyto-protective gene pathway (Keap 1/Nrf2) using a cell line derived from human keratinocyte transformed with a reporter gene of luciferase and luminescence detection (Emter et al., 2010; Ramirez et al., 2014). The TG 442E (OECD, 2018b) describes three different assays providing information on activation of dendritic cells, the KE3 of AOP: h-CLAT, U-SENS™ and IL-8 Luc assay. The h-CLAT and U-SENS™ are quantitative analysis of the up-regulation of cell surface markers, respectively CD86 and CD54 or CD86 alone, in cell lines, respectively a human monocytic leukemia cell line THP-1 or human histiocytic lymphoma cell line U937, used as surrogates of dendritic cells by flow cytometry (Alepee et al., 2015; Ashikaga et al., 2006; Piroird et al., 2015; Sakaguchi et al., 2006). The IL-8 Luc assay is a quantitative analysis of IL-8 expression associated with dendritic cell activation using a THP-1 derived reporter cell line (THP-G8) and luminescence detection (Kimura et al., 2015; Takahashi et al., 2011). Other assays in advanced step of development include the Electrophilic Allergen Screening Assay (EASA), the kinetic DPRA for KE1 (Chipinda et al., 2014; Wareing et al., 2017), the SENS-IS, the EpiSensA, the IL-18 assay targeting the KE2 (Cottrez et al., 2015; Gibbs et al., 2013; Saito et al., 2013) and the Genomic Allergen Rapid Detection skin assay (GARDskin™) for KE3 (Johansson et al., 2013).

Due to the complexity of the biological mechanisms underlying skin sensitisation, no single test but rather a combination of different methods is required to predict the skin sensitising potential, each providing information on one or more key step events of the mechanism (Ezendam et al., 2016). Based on these individual tests, different integrating approaches are derived, such as integrated testing strategies (ITS) or sequential testing strategies (STS) within an integrated approaches to testing and assessment (IATA) (OECD, 2016a, b). Some of these reported approaches show at least comparable performance to the LLNA (Kleinstreuer et al., 2018).

The co-culture systems provide different advantages over monocultures based assays such as the consideration of the intercellular crosstalk and the possible combination of several KE within a unique test. The applicability domain of the existing methods can be enlarged, using 3D co-culture, to mixtures and poorly water soluble substances. This review presents and discusses different approaches of co-culture models and sets them into the context of predictive *in vitro* toxicology for skin sensitisation potential taking into account the interplay of keratinocytes and dendritic cells.

Co-culture model

The relevance to combine keratinocytes and dendritic cells

As briefly described above, dendritic cells (DCs), T cells and keratinocytes (KCs) are key players in the ACD mechanism (Ainscough et al., 2013; Albanesi, 2010; Vocanson et al., 2009). KCs provides the environment favorable to mount the immune reaction, DCs process antigen for presentation to T cells, and T cells induce the clinical symptoms of ACD in elicitation phase. The interaction between these cells but also with microenvironment is important to maintain cutaneous homeostasis or to develop the appropriate immune response. The use of *ex vivo* skin to study the interplay is hampered by the fact that DCs spontaneously emigrate from the epidermis (Larsen et al., 1990; Lukas et al., 1996).

The approach of skin sensitisation assessment is based on integrating information from individual assays selected from available *in vitro* methods, using a single cell type model either KCs, DCs or T lymphocyte in 2D cell culture in combination with well-established endpoints. This artificial separation of skin sensitisation key events in monoculture-based tests poorly represents the temporal or spatial orchestration of cellular events involved *in vivo* and does not take into account neither the eventual crosstalk between cell types via heterotypic cellular communication nor the *in vivo* architecture organisation or extracellular conditions. Taken together, tests relying on co-culture are presumably more relevant to model the *in vivo*-like skin response than a combination of data from different isolated tests. Although 2D co-culture of DCs and T cells (Richter et al., 2013) or 3D co-culture of KCs and T cells (van den Bogaard et al., 2014) are developed, this review focus on the co-culture between KCs and DCs.

Technical considerations and challenges

The choice of the right co-culture framework depends on the context and the appropriate question to be addressed: understanding the cell and molecular mechanisms underlying skin sensitisation by replicating the *in vivo* modelling, studying the natural cells interplay, improving the culture of a cell type, or providing a practical perspective of novel assay development. The objectives being different, so will the specifications of the model. In the latter case, the model should also allow high throughput screening.

In the case of co-culture model, particular attention should be focused to different parameters. Table 1 summarizes the different interdependent parameters to consider while conducting experiments on skin sensitisation using co-culture system. Some of these parameters are common with regular monoculture, for example, the choice of human vs. animal cells, primary cells vs. cell line, 2D vs. 3D culture.

The experimental parameters concerning the model setting are of prime importance for the co-culture, as it will influence the stability, the microenvironment and the cellular communication (Abbott and Kaplan, 2015; Berg et al., 2014; Goers et al., 2014). The co-culture models require protocol optimisations and bring technical challenges such as the sourcing of different cell types, quality check prior to use, the involvement of scaffold and microfluidic systems, the retention of the desired state of cellular differentiation and the ratio among cell types over time. Long-term culture can alter signaling patterns due to over-confluency, decreased viability or dedifferentiation (Abbott and Kaplan, 2015). Moreover, technical considerations during testing must be carefully selected and controlled since it impacts the results. Thus, these models require extensive characterization and validation before use to be sure to discern the effect of the stimulation over the dynamic of the model (Abbott and Kaplan, 2015; Roth and Singer, 2014).

Mode of cellular crosstalk: direct *vs.* paracrine interaction

The cellular crosstalk can be studied via different degrees of contact between cells: with a direct cell contact or indirectly by paracrine interaction. Table 2 provides an overview of the different kind of co-cultures. The mixed co-culture enables study of the direct contact between cells involving tight or gap junctions as both cultures are set in the same medium without any kind of separation. The segregated co-culture, inhibiting direct cell contact, allows to identify and to evaluate the impact of secreted soluble factors on cell behavior in a unidirectional or bidirectional manner. Unidirectional segregated co-culture can be experienced by using conditioned media or using conditioned extracellular matrix which entrap the soluble factors obtained from culture of one cell type to culture the second cell type. The use of a physical separation via semipermeable divider, including membrane or hydrogel, enables to construct a bidirectional segregated co-culture.

Cell sources: primary cells *vs.* cell lines

Two opposite strategies are adopted concerning the selection of cells for *in vitro* tests: either the use of primary cells, culture of cells harvested directly from animal or human tissue or the use of cell lines which are characterised with an extended period of multiplication.

Many skin sensitisation tests are developed using human primary DCs or KCs in the form of submerged cell culture or commercialised KCs based-reconstructed human epidermis (RhE). Primary cell cultures are physiologically relevant to human biology, phenotypically similar to normal cells and retain the normal regulation of their growth pathways (Berg et al., 2014). Nevertheless, due to potential inter-donor variability, the procedure, selection of suitable donors, consideration of the number of donors required, pooling cells, characterization, can be cumbersome and expensive if pooled cells from multiple donors are not proposed by the cell supplier (Frombach et al., 2017). Besides decreasing the variability, pooling cells enables to overcome the relative limited cells numbers due to low availability or poor isolation and short life span set to avoid phenotypic drift (Abbott and Kaplan, 2015; Berg et al., 2014). Moreover, the use of primary cells for co-culture models requires fine-tuning the supply of tissue or blood and the culture in order to have the different cell types ready the day of co-culture. Specifically, the use of primary skin DCs is hampered by the low presence in the epidermis, the difficulty to extract and propagate *in vitro* with the limitation to a few passages. Therefore, peripheral blood, bone marrow or umbilical cord blood derived progenitor cells and monocytes are used as alternative sources of DCs. Nevertheless, variation in protocol for generating DCs led to variability of results (Ayehunie et al., 2009) and thus careful control and standardisation are mandatory for the development of a predictive *in vitro* method for skin sensitisation.

For the above reasons, many tests are designed using DC cell lines which are more readily available and easy to culture lowering the cost and allowing high reproducible results. Different DC-like cell lines with or without differentiation using a specific cytokine cocktail are used to predict the skin sensitisation potential and are reviewed elsewhere (dos Santos et al., 2009): CD34+ human acute myeloid leukemia cell line (MUTZ-3), human histiocytic lymphoma cell line (U-937) or human monocytic leukemia cell line (THP-1), human bone marrow-derived acute myelogenous leukemia cell line (KG-1) human acute pro-myeloid leukemia cell line (HL-60), and human erythroleukemia cell line (K562). Among the submerged cell culture of KCs cell lines, HaCaT,

NCTC2544 and N/TERT cell lines are the most used for skin sensitisation. Furthermore, RhE can also be engineered using cell lines (van den Broek et al., 2017). The use of KCs or DC surrogates cell lines enables the construction of reporter cell lines to monitor specific signaling pathway activation including cytokine/inflammation pathways or Keap1-Nrf2-ARE pathway (Natsch and Emter, 2015). However, cell lines are associated with difficulties such as genomic and phenotypic drift especially at high passages, metabolic deficiencies and dysregulated signaling mechanisms (dos Santos et al., 2009; Frombach et al., 2017).

A third strategy is to use human pluripotent stem cells (hPSC). The hPSCs retain the characteristics of stem cells with an unlimited growth potential and a wide variety of differentiated cell types can be produced. Due to ethical reasons, induced pluripotent stem cells (iPSC) originated from adult tissue is rather used than embryonic stem cells (Guenou et al., 2009). A few skin models are reconstructed using iPSC reverted mostly from fibroblasts because of the ease of extraction and culture. An iPSC derived-RhE shows similar differentiation and barrier properties to *in vivo* epidermis (Petrova et al., 2014). Another more elaborated iPSC derived-skin equivalent (SE) is reported comprising keratinocytes and fibroblasts embedded in collagen matrix (Gledhill et al., 2015; Itoh et al., 2013). Likewise, iPSC is used to derive DC mainly for clinical application and with the potential of large scale production (Li et al., 2014; Li and Ma, 2011). Nevertheless, none of the developed iPSC based-epidermal or full thickness skin models have incorporated immune cells to date.

Culture format: 2D *vs.* 3D model

2D models are supported by well-published culture, endpoint detection methods and equipment. Moreover, 2D models enable screening of molecules as high throughput studies or automation are possible. Despite the simplicity of use, cells have prevailing contact with plastic or medium and cell-to-cell interaction, by contact or by paracrine mediators, are limited (Hartung, 2014). In addition, the use of 2D KCs culture limits the testing to medium-soluble and stable substances that ensure a homogeneous treatment, otherwise, it is prone to a mistreatment as the exact concentration in contact to the KCs cannot be evaluated (Alepee et al., 2014).

3D models recapitulate the tissue-like structure complexity that is considered more physiologically relevant and thus hold the promise for being more predictive of *in vivo* response to compound treatment (Berg et al., 2014; Lelievre et al., 2017). 3D models favor homotypic and heterotypic interactions but also interaction with extracellular matrix (ECM) or with the microenvironment and result in tissue-specific signaling not found in 2D cultures (Abbott and Kaplan, 2015; Roth and Singer, 2014). While 3D culture is bridging the gap between traditional 2D culture and *in vivo* situation, the gain of complexity of these models should be supported by meaningful and reliable readouts that enable to study the heterogeneity especially when co-culturing. In the field of predictive toxicology, target effects due to compound treatment can be masked because of the use of assays methods that are not suitable to analyse the heterogeneity of responses throughout the 3D structure but rather assess the mean effect (Berg et al., 2014). Thus, new technology, devices, methods, miniaturisation and also data management enabling 3D models analysis with a higher throughput or single-cell resolution are increasingly being developed to circumvent these limitations.

The 3D epidermal models, also named reconstructed human epidermis (RhE), are generated by culturing human KCs at air-liquid interface (ALI) to induce the formation of a stratified epidermis. A more sophisticated full-thickness skin equivalent (SE) model, consists of a RhE onto a fibroblast-populated dermal compartment and other cell types can be incorporated such as melanocytes or immune cells. This improved model benefits from the crosstalk between epithelium and connective tissue, known to regulate epidermal morphogenesis and homeostasis. The scaffold choice, including collagen, chitosan gels, biochemically manufactured polymers or decellularised tissue among others, is another important aspect when reconstructing skin as this ECM analog guides the adhesion, proliferation and differentiation and thus provides the suitable artificial environment for cell development (Evangelatov and Pankov, 2013; Nicholas et al., 2016; Pridgeon et al., 2018).

The specificity of reconstructed 3D skin model is to replicate the *in vivo* architecture of the skin with the keratinocyte differentiation and to take into account the barrier function of the *stratum corneum*. The *stratum corneum* of reconstructed 3D skin models still has a weaker barrier function than human skin due to imperfect structural organisation of stratum corneum lipids (Dumont et al., 2015; Schafer-Korting et al., 2008). However, this barrier can be improved by decreasing the humidity during the culture (Sun et al., 2015) or using dynamic flow system within skin-on-chip platform (Sriram et al., 2018). Despite its poor reflect of the normal barrier function, the reconstructed 3D skin models have a strong impact on the bioavailability of the tested compound applied topically to reach viable skin compared to the dilution of the compound directly in the medium for

keratinocyte monolayer. To date, 3D skin models are devoid of desquamation leading to a short window for testing and could hamper the use of the models for repetitive applications because of the gradual thickening of the *stratum corneum* (Ponec et al., 2002).

Despite the presence of metabolic function in monolayer of KCs, the reconstructed 3D skin models better mirror the *in vivo*-like skin metabolism (Alepee et al., 2014; Dumont et al., 2015). The metabolic function of the reconstructed 3D skin models is of interest when assessing skin sensitisation and would avoid the use of rat S9 or peroxidase as in some developed tests (Gerberick et al., 2009; Natsch and Haupt, 2013).

The use of 3D skin model for skin sensitisation is thus a promising way to overcome limitations of tests designed with submerged cell culture systems. As the cell culture process to reconstruct human skin is longer and requires more manipulations, a well-defined experimental set-up and a substantial quality control are important to ensure the reproducibility of the results. The development and implementation of good cell culture and good *in vitro* methodology contributes to the maintenance of high standards as well as the meaningfulness and reproducibility of the obtained results (Coecke et al., 2005; Eskes et al., 2017).

Co-culture model for skin sensitisation

The different co-culture setting

Skin sensitisation potential prediction or mechanistic evaluation are performed thanks to keratinocytes and dendritic cell co-culture models in the form of segregated, mixed or multilayered co-culture (Tables 3 and 4).

The segregated co-culture is typically setup using a porous transwell membrane which enable the separation between the KCs and the DCs. The KCs, RhE or SE are seeded atop the compartment of the transwell and the DCs are cultivated submerged in the culture medium placed in the lower compartment.

Some multilayered co-culture (also named sandwich culture) models are developed. The construction of the co-culture is either done prior to or after keratinocyte differentiation and use hydrogel or synthetic membrane as semi-permeable divider for the cells. The cells can be fully embedded within ECM hydrogel with the main goal to approximate *in vitro* conditions to *in vivo*. However, the recovery of cells for downstream analysis is more complex than the segregated 3D co-culture.

The mixed co-culture system more closely replicates the *in vivo* skin by the presence of DC in the RhE or SE. This more sophisticated setting requires intensive study to develop a standardised model. Although extracellular release of cytokines in medium, immunohistochemistry or gene expression are performed, this co-culture suffers from limited technologies either for *in situ* evaluation or analysis without extensive technical expertise to study the direct cell-to-cell interactions. Likewise reliable cell isolation from the 3D mixed co-culture is required for downstream study of single cell type and this step is critical since it must not alter the cells (Schaerli et al., 2005) or have an unwanted effect. The labelling of cells using fluorescent dye (Schaerli et al., 2005) prior to co-culture can be an option to monitor the co-culture.

Some parameters are reported to be important while co-culturing KCs and DCs for skin sensitisation assessment. The co-culture setting, especially the choice between cell line or primary cells, depends on the endpoint of interest. A SE containing MUTZ-LCs seems to better mimic the epidermis-to-dermis motility whereas the use of monocyte-derived Langerhans cell (moLC) appears favorable for cytokine profile (Bock et al., 2018). Moreover, the presence of serum in the co-culture medium not only changes the basal cell surface markers on DCs but also leads to a higher sensitivity toward the sensitiser as reported by the increased CD86 upregulation (Frombach et al., 2017). The treatment procedure is important and defining the treatment duration, the post-incubation period and the modality of treatment, direct treatment or use of conditioned media, is necessary to avoid misleading results (Degeorge et al., 2014; Troese et al., 2015). Other reported parameters include the choice of scaffold and cell selection (Uchino et al., 2011).

Keratinocyte and dendritic cell crosstalk

In 2D models

An increased sensitivity of THP-1 cells is observed as lower concentration of product induce CD54 and CD86 expression on THP-1 cells in 2D segregated co-culture with keratinocyte compared to THP-1 monoculture and thus demonstrate the paracrine communication between both cell types (Cao et al., 2012). The paracrine

crosstalk is also reported with a model where monocyte-derived DCs (moDCs) are cultured with substance treated HaCaT keratinocytes conditioned media (Matjeka et al., 2012). The CD83 and CD86 expression levels on moDC are upregulated with conditioned media but to a lesser extent than the co-culture model in presence of HaCaT. Blockade of IL-1a using specific antibody in conditioned media before co-culture leads to a decreased CD83 and CD86 expression showing the importance of IL-1a released by KCs in DC activation. The presence of CD86 overexpression on moDC surface when monocultured is strongly enhanced (5 to 10 times) in sandwich tri-culture (Chau et al., 2013). Nevertheless, some protocol adjustments should be done as none of the previously studied cytokines (IL-1a, IL-6 and IL-8) are overexpressed after treatment with a sensitiser or irritant product.

Based on a previous work showing indirectly the importance of the cross-talk between HaCaT and moDC (Ohtani et al., 2009), another similar HaCaT and THP-1 2D co-culture is designed (Hennen et al., 2011) and an increased signal of CD86/CD54 compared to monoculture is observed (Hennen and Blomeke, 2016). An adaptation of this model is used to study the toxicity of sulfur mustard (Balszuweit et al., 2014). The team shows that the interaction between the two cell types is an active mechanism *via* indirect action of THP-1 cells meaning that THP-1 cells initiate a crosstalk with HaCaT, stimulating the latter to increase interleukin production.

In 3D models

The incorporation of DC precursors in RhE or SE model enables the study of their differentiation. A comparative study is done with co-cultures set either using cord blood derived CD34+ progenitors co-seeded with KCs or CD34+ derived-DC/LC incorporated in the same RhE, 2 days after the air-liquid exposure (Régnier et al., 1997). The resulting histological characteristics are the same highlighting the importance of the presence of KCs during the differentiation of hematopoietic progenitors into LC. In a second co-culture model, DC precursors from PBMC are added once epidermal stem cells reach confluency before the air-liquid exposure. This co-culture points out the importance of the continuous differentiation of KCs for DC development, especially the differentiation of DC precursors into LC-like cells with potent antigen presentation cell function (Schaerli et al., 2005). A SE containing moDCs recreates a microenvironment that favours a stable immature phenotype of DC and is adapted for DC differentiation (Bechetoille et al., 2007). Treatment of a murine macrophage cell line (RAW264.7) with lipopolysaccharides (LPS), which promotes the secretion of pro-inflammatory cytokines, in presence or absence of the SE outlines the inflammatory response modulatory property of the SE (Chung et al., 2014).

The origin of dermal DC is investigated using a co-culture of monocytes or moDCs inserted in a dermal equivalent (normal human fibroblasts in type-1 collagen lattice) in presence or absence of exogenous cytokines. Phenotypic and functional studies are performed and support an epidermal origin of dermal DC (Guironnet et al., 2001). Other 2D co-culture of fibroblast and DC highlights the potent immunoregulatory role of dermal fibroblasts in maturation and migration of DC (Saalbach et al., 2015; Saalbach et al., 2010; Saalbach et al., 2007). Two different SE including MUTZ-LCs evidence dendritic cell maturation and migration out of the epidermis toward the dermal compartment upon exposure to skin sensitisers (Ouwehand et al., 2011b). A more detailed study shows that exposure of both irritant or sensitiser result in LC epidermis-to-dermis migration but the migration is respectively dependent upon chemokine CCL5 or CXCL12 (Kosten et al., 2015) as found previously with *ex vivo* models (Ouwehand et al., 2008; Ouwehand et al., 2010). The group use another co-culture setting based on MUTZ-LC seeded underneath a SE (*ex vivo* epidermis onto a fibroblasts repopulated de-epidermised dermis) to study the LC migration toward the epidermis and evidences the role of keratinocyte-derived CCL5 and CCL20 as pivotal mediators in the chemo-attraction of MUTZ-LC (Ouwehand et al., 2012).

Predictive test development

The co-cultures models analysis for skin sensitisation mostly rely on the measure of various cell markers expression (CD54, CD83, CD86) at DCs surface or extracellular release of cytokine in medium after treatment on top of RhE. The main difference is the cells or RhE models used as well as the treatment conditions as shown in Table 5. The RhE provides a relevant barrier to the test chemical as higher concentrations are required to reach the same level of DC cytotoxicity compared to monoculture (Ouwehand et al., 2011a).

Beside the low number of reference chemicals used to assess the predictive performance of developed tests (Table 4), the preliminary results show at least comparable performance to monoculture tests (Cao et al., 2012; Thélu et al., 2019b). These developed models have to undergo extensive validation studies via the screening of a series of reference chemicals, reproducibility and repeatability assessment before they can be used as a

standardised method for the evaluation of skin sensitisation. Although a limited number of reference chemicals are tested using LCSA, the results are promising as the accuracy compared to the gold standard animal test, LLNA, is high and it is possible to distinguish sensitiser from non-sensitiser and even irritant (Wanner et al., 2010).

The commercialisation of SkinEthic™ RHE-LC, a reconstructed human epidermis containing LC or SkinEthic™ RHE-Cell Migration Model (CMM) a reconstructed human epidermis cultured on a double porosity insert providing the possibility for DC to migrate in the RhE, will enable the investigation of the potential of immune-competent RhE into the area of test method development for skin sensitisation. The progress in the pre-validation of the method is dependent on the complexity of the model and the RHE-LC or RHE-CMM could be a starting base to focus on establishment of a standardised method for regulatory acceptance.

The future directions include the assessment of the added value of the co-culture models for pro-haptens prediction and for potency determination. The pro-haptens require metabolic conversion prior to inducing sensitisation and preliminary results of pro-haptens prediction using co-culture is highlighted by the tests shown by Table 4. Moreover, there is already an improved correlation reported between the EC3 from LLNA and the concentration of chemicals to give a positive result, namely ECΔ10 (CD86) and ECΔ50 (CD54), from COCAT set-up compared to THP-1 monoculture suggesting a more fine-scale prediction of skin sensitisation potency (Hennen and Blomeke, 2016). Using another co-culture model comprising MUTZ-LC (Lee et al., 2018), skin sensitisation potency prediction is also experienced and shows that the co-culture of MUTZ-LC with KCs and fibroblasts outperforms the monoculture concerning the overall accuracy.

Skin-on-chip

A major improvement for skin culture is the control of nutrient supply and waste disposal that could be achieved using microfluidics. In spite of the constant nutrient renewal, the organ-on-chip technology offers the possibility to integrate different organotypic models to study the organ interplay and thus contributes to bring the *in vitro* models to a higher level of complexity improving the physiological relevance. With the dynamic microfluids technology also comes the idea to improve the long-term culture and thus development of non-destructive *in situ* assays are of prime interest to monitor the same culture over time (Abbott and Kaplan, 2015) or the time course of response to stimulations rather than endpoints (Hutson et al., 2016). A potential challenge to consider is the unexpected absorption onto plastic tubing which is part of the microfluidics hampering meaningful outcomes. The choice of the material used to elaborate the device should be carefully chosen depending on the type of test for which it is intended to prevent this unspecific binding (Roth and Singer, 2014).

Among the several skin-on-chip models developed and reviewed (van den Broek et al., 2017), only one model includes an immune component. Ramadan and Ting developed a skin-on-chip culture systems based on a segregated co-culture of a 3D reconstructed epidermis using HaCaT keratinocyte cell line and U937 cell line to model DCs separated by a porous membrane under dynamic perfusion conditions. This microfluidic system allows the *in situ* monitoring of both transepithelial electrical resistance (TEER) as a control of epidermal barrier function as well as IL-1β and IL-6 following LPS and UV stimulations (Ramadan and Ting, 2016). Despite demonstrating the promising potential of these chips, deeper investigations are needed concerning other endpoint readouts, comparison with *in vivo* skin, predictivity and validation, before the model can be fully implemented mainly for substance testing.

Conclusion and future outlooks

Co-culture systems represent promising tools for investigating cell cooperation in 2D and 3D networks and for practical perspectives of new assay development for skin sensitisation prediction. This review stresses the importance of experimental design to tailor the co-culture to achieve the desired goal. Test development requirements for predictive test or mechanistic study are not the same. While the availability of new technologies dedicated to co-culture system is important to study the heterogeneity of responses and to understand the contribution of each cell type to the observed effect; simple and inexpensive assays are requested for routine use as predictive tests. Some markers of interest to distinguish sensitiser from non-sensitiser on DCs or KCs have been reviewed (dos Santos et al., 2009; Koppes et al., 2017; Natsch and Emter, 2015) and should be deeper studied in the co-culture format. Likewise, genomic, proteomic and metabolomic can be deeply exploited to investigate and identify new biomarkers to be included.

The predictive tests based on co-culture are at different stages of development and the more advanced methods mainly rely on analysis of CD54 /CD86 expression of DC or extracellular release of cytokine. Most of the methods suffer from the use of a test panel of chemicals which is too limited to draw a conclusion. Moreover, the validation of the *in vitro* tests against LLNA due to the significant amount of historical data is questionable regarding its inconstancies in prediction of the human situation (Anderson et al., 2011). Although no current co-cultured based assays are ready for regulatory purpose of skin sensitisation potential assessment, these systems have great potential to provide insight and understanding of the KC and DC interplay.

There is a progression with more physiological relevant assay approaches. The cell-based assay tend to be more sophisticated using co-culture of multiple cell types, 3D models, presence of skin appendages, vasculature and models with microfluidic control. Moreover, 3D bioprinting, especially laser-assisted platform, can improve the defined spatial arrangement of cells and the respect of the actual ratios (Bauer et al., 2001; Proksch et al., 1996) of each cell types to reach highly standardised of 3D models (Augustine, 2018). The skin microbiota and the impact of host-microbe interactions should be extensively explored in the context of skin sensitisation (Byrd et al., 2018; Chen et al., 2018; Holland et al., 2009).

It is important to bear in mind that with complexity comes the idea to better mimic the *in vivo* situation but also comes increased difficulties for maintenance, reproducibility, cost, quality control, validation, readouts and interpretation of the results (Alepee et al., 2014; Mathes et al., 2014). Thus, test developers have to balance the expectations of the ideal model to tailor the model with the expected specifications. An assay using co-culture should primarily be able to distinguish sensitiser from non-sensitiser and correlate to human data. Other features can be expected such as the possibility to discriminate respiratory from skin sensitisers (Mizoguchi et al., 2017), to address the sensitising potency, sub-classification in extreme, strong, moderate, weak sensitisers, or to assess water-insoluble chemicals, mixtures, permeation, metabolism, repeated exposure to low doses and DC migration. Altogether, this review highlights that co-culture in the context of skin sensitisation is an area rich in opportunities for innovation.

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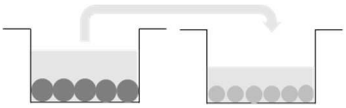
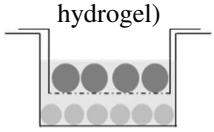
ACD: Allergic Contact Dermatitis
ALI: air-liquid interface
AOP: Adverse Outcome Pathway
CCL: Chemokine (C-C motif) Ligand
CXCL: Chemokine (C-X-C motif) Ligand
COCAT: Co-cultured activation test
DC: dendritic cell
DCrc: DC related cell
DPRA: Direct Peptide Reactivity Assay
EE: Epidermal Equivalent model
ELISA: enzyme-linked immunosorbent assay
ECM: Extracellular matrix
ECVAM: European Centre for the Validation of Alternative Methods
GM-CSF: Granulocyte-macrophage colony-stimulating factor
h-CLAT: human Cell Line Activation Test
hPSC: Human pluripotent stem cells
IATA: integrated approaches to testing and assessment
iPSC: induced Pluripotent Stem Cells
ITS: integrated testing strategies
KC: keratinocyte
KE: key event
LC: Langerhans cell
LCSA: Loose-fit Co-culture-based Sensitisation Assay
LLNA: Local Lymph Node Assay
LPS: lipopolysaccharides
MIE: molecular initiating event
moDC: monocyte-derived DC
moLC: monocyte-derived Langerhans cell
MUTZ-LC: MUTZ-3 acute myeloid leukemia cell line differentiated into LC
MTT: thiazolyl blue tetrazolium bromide
OECD: Organisation for Economic Cooperation and Development
PBMC: peripheral blood mononuclear cell
PI: propidium iodide
pDC: plasmacytoid dendritic cells
RhE: reconstructed human epidermis
RHS: reconstructed human full thickness skin
RT-qPCR: reverse transcription quantitative polymerase chain reaction
SE: skin equivalent
STS: sequential testing strategies
TEER: transepithelial electrical resistance

Table 1 Main experimental design parameters to consider for co-culture system addressing skin sensitisation

Goal of experiment	Model setting	Method development
Predictive / mechanistic	Cell source (primary, cell line, iPSC)	Presence of acclimation period between co-culture setting and treatment
Study of bioavailability	Number and ratios of cell types	Treatment before or after co-culture construction
Study of metabolism	Timeframe of culture	Basal or apical treatment when using semi-permeable divider
Low-dose evaluation	Requirement of *in vivo* tissue organisation, 2D / 3D format	Treatment duration
Mixture evaluation	Mode of cellular crosstalk and degree of physical separation	Ease of manipulation and readout
Potential / potency	Presence of flow	Endpoint selection
Regulatory validation	Presence of ECM or scaffold	Sophisticated device
Part of integrated approaches	Culture size	Throughput and automation
	Medium composition	Cost

ECM: Extracellular matrix; iPSC: induced pluripotent stem cell

Table 2 Co-culture models used for analysis of the cell-to-cell communication

	Mixed co-culture Co-culture with cell-cell contact: tight junction, gap junctions and adhesion proteins	**Segregated co-culture** Co-culture without cell-cell contact but with paracrine interaction	
		Conditioned media or extracellular matrix	Semi permeable divider (synthetic membrane or hydrogel)
			
Advantages		Individual response of each subpopulation of cells can be analyzed	
	Maximal cellular interaction via both direct contact and soluble factor signaling	Monitoring of the unidirectional effect of a cell type toward another one	Monitoring of the bidirectional effect between cell types
	Control of the relative levels of heterotypic and homotypic communication by altering the seeding densities of each cell type	Simplicity of detection of any soluble factors and their related effects	Identification of co-culture effects on individual populations
Drawbacks	Interpretation must take into account a possible dilution effect due to mixed culture as well as metabolic difference between cell types	Cumbersome multi-stage cell-seeding procedure	
	Awkward techniques are needed to separate cell types for downstream analysis	Difficulty to reproduce the optimal concentrations and temporal distribution of these secreted factors	Physical contact cannot be completely prevented in the long term
		Nutrient deficiency	Extensive cell grow can cover the pores of the inserts, limiting cellular interactions
	Contribution of each cell population to any observed effects is not readily discernible	Degradation and stability of the factors in the medium is questionable	Cell response and soluble factor transport depends on the properties of the divider

Based on (Bogdanowicz and Lu, 2013, 2014)

Table 3 Description of the models and methods used for 2D co-culture based skin sensitisation assessment

Model		Method	References
Segregated	Primary KC from foreskin ontop of a transwell + THP-1 cell line in the lower chamber	Co-culture is set 2h before treatment, 24h treatment of the upper and lower chamber CD54 and CD86 THP-1 cell expression by flow cytometry	(Cao et al., 2012)
Mixed	HaCaT KC cell line + THP-1 cell line	Co-cultured activation test (COCAT): 24h treatment CD54 and CD86 THP-1 cell expression and cell viability (PI) by flow cytometry	(Hennen et al., 2011); (Hennen and Blomeke, 2016)
		Co-culture is set 24h before treatment, 24h treatment of co-culture or 1h treatment of HaCaT cells and then co-culture with THP-1 cells for 24h Cell integrity, apoptosis and extracellular release of IL-6 and IL-8 by ELISA	(Balszuweit et al., 2014)
	HaCaT KC cell line + DC related cell (DCrc) (differentiation of PBMC with addition of cytokines cocktail after co-culture for 48h)	Loose-fit Co-culture-based Sensitization Assay (LCSA): 48h treatment CD86 DCrc expression and cell viability by flow cytometry	(Schreiner et al., 2007); (Schreiner et al., 2008); (Wanner and Schreiner, 2008); (Wanner et al., 2010); (Sonnenburg et al., 2012); (Sonnenburg et al., 2015); (Frohwein et al., 2016); (Frombach et al., 2017)
	Primary KC + PBMC with addition of cytokines cocktail after co-culture for 48h in serum free condition	LCSA-ly: 48h treatment CD44, CD119, CD124 and IL-23R lymphocyte cell expression and cell viability by flow cytometry, extracellular release of IL-4, IFN-ɣ and IL-17 by ELISA	(Frombach et al., 2018)
	NCTC2544 KC cell line + THP-1 cell line	24h treatment CD86, CD54, CCR7 and IL-8 co-culture expression by RT-qPCR	(Meloni et al., 2010)
	HaCaT KC cell line + moDC	2 treatment protocols: either 1h treatment of HaCaT cell line, removal of treatment and co-culture with DC for 20h or 1h treatment of HaCaT cell line, removal of treatment, post-incubation for 20h and conditioned supernatant in contact with DC for 20h CD83 and CD86 moDC cell expression by flow cytometry	(Matjeka et al., 2012)
	HaCaT KC cell line + primary dermal fibroblasts + MUTZ-LC	48h treatment Cell viability (Alamar Blue test), extracellular release of 27 cytokines using bio-plex assay and of IL-8 by ELISA	(Lee et al., 2018)

ELISA: enzyme-linked immunosorbent assay; moDC: monocytes-derived dendritic cell; MUTZ-LC: MUTZ-3 acute myeloid leukemia cell line differentiated into LC; PBMC: peripheral blood mononuclear cell; PI: propidium iodide

Table 4 Description of the models and methods used for 3D co-culture based skin sensitisation assessment

Model		Method	References
Segregated	MatTek EpiDerm™ RhE + of plasmacytoid dendritic cells (pDC) in the joint medium	2 treatment protocols: either 24h topical treatment onto EpiDerm™ of the co-culture or 4h topical treatment onto EpiDerm™ of the co-culture and 20h additional culture separately EpiDerm™ cell viability (MTT test) after 24h; CD86 pDC expression and pDC cell viability by flow cytometry after 24h; extracellular release of IL-18 by ELISA after 4 and 24h	(Degeorge et al., 2014); (Troese et al., 2015); (Troese et al., 2016)
	Episkin® RHE + MUTZ-3 cell line in the joint medium	48h topical treatment onto RhE RHE cell viability by MTT test, CD54 and CD86 MUTZ-3 cell expression and MUTZ-3 cell viability by flow cytometry	(Ouwehand et al., 2011a)
	Epidermal Equivalent model (EE) + MUTZ-LC in the joint medium	24h topical treatment onto EE and 24h post-incubation EE cell viability by MTT test; CD54 and CD86 MUTZ-LC cell expression and MUTZ-LC cell viability by flow cytometry	(Ouwehand et al., 2011a)
	EpiSkin™ RealSkin SE + MUTZ-LC in the joint medium	48h topical treatment onto RealSkin CD54 and CD86 MUTZ-LC expression by flow cytometry, extracellular release of 27 cytokines using bio-plex assay, migration ability of MUTZ-LC to migrate in presence of chemokine CCL19 by hemocytometer	(Lee et al., 2014)
	OS-Rep or SkinEthic™ RHE + THP-1 in the joint medium	24h topical treatment onto RhE CD86, CD54, CD40 and HLA-DR THP-1 cell expression and THP-1 cell viability (PI) by flow cytometry	(Schellenberger et al., 2019)
	VitroDerm RhE + THP-1 in the joint medium	39h topical treatment onto RhE RhE cell viability by MTT test, CD54 and CD86 THP-1 cell expression and THP-1 cell viability (PI) by flow cytometry, extracellular release of cytokines using secretome array and ELISA, gene microarray	(Thélu et al., 2019a)
Multilayered 3 layers sandwich model (from bottom to upper layer)	Primary fibroblasts on the lower side of transwell + DC precursors from PBMC on top of a transwell + EE using epidermal stem cells	CD14+ DC precursors tracking by fluorescence microscopy	(Schaerli et al., 2005)
	VD-KDF-skin: Normal Human Skin Fibroblasts (NHSF) 46 cell line in collagen vitrigel membrane + Normal Human Dendritic Cell (NHDC) in type-I collagen + primary Normal Human Epidermal Keratinocyte (NHEK)	1h topical treatment onto co-culture and 23h post-incubation Extracellular release of IL-1a, IL-4 and Il-8 by ELISA	(Uchino et al., 2007); (Uchino et al., 2008); (Uchino et al., 2009); (Uchino et al., 2011)
	2 days pre-cultured onto scaffold of primary fibroblasts + moDC from PBMC within an agarose–fibronectin gel + 2 days pre-cultured onto scaffold primary KC	24h topical treatment onto the co-culture CD11c, CD14, CD54, CD80, CD83, CD86, DEC205, CD206, CD209, HLA-DR, CD324, Annexin V moDC expression by flow cytometry, extracellular release of IL-1α, IL-6 and IL-8 by ELISA	(Chau et al., 2013)

Mixed	RhE containing LC derived from CD34+ cells or MUTZ-3 (named RHE-LC)	24h or 48h systemic or topical treatment onto RHE-LC CCR7, CD80 and CD83 gene expression by RT-PCR	(Régnier et al., 1997); (Facy et al., 2004); (Facy et al., 2005); (Ouwehand et al., 2011a)
	SE containing primary LC	Immunohistochemistry	(Fransson et al., 1998)
	SE containing CD14+ monocytes from PBMC (named Immune-reconstruced EE)	CD45 and CD1a staining in fluorescent microscopy Multiple cell markers expression by flow cytometry	(Schaerli et al., 2005)
	SE containing moDC and moLC from PBMC	Immunohistochemistry, cytokine and chemokine analysis of supernatant using array membrane, extracellular release of TNF-a, IL1b, IL-6, IL-8, and IL-10 using the BD Human Inflammation CBA assay kit, extracellular release of GM-CSF, TGF-b1, IL-4, IL-10, IL-13, and IL-15 by ELISA, transmission electron microscopy	(Bechetoille et al., 2007)
	SE containing MUTZ-LC	Immunohistochemistry	(Laubach et al., 2011)
	SE containing MUTZ-LC (named Langerhans cell containing skin equivalent (LC-SE))	16h topical treatment IL-1b, CCR7 gene expression by RT-PCR, CD83 immunostaining	(Ouwehand et al., 2011b); (Kosten et al., 2015)
	SE containing moLC or MUTZ-LC (named moLC-RHS & MUTZ-LC-RHS)	24h topical treatment LC-like cells tracking by fluorescence microscopy, extracellular release of IL-6, IL-8 and IL-18 by ELISA, ATF3, CD83, CXCR4, IL-1β, IL-18, PD-L1 gene expression by RT-PCR	(Bock et al., 2018)

ELISA: enzyme-linked immunosorbent assay; EE: epidermal equivalent; GM-CSF: Granulocyte-macrophage colony-stimulating factor; moDC: monocytes-derived dendritic cell; moLC: monocytes-derived Langerhans cell; MUTZ-LC: MUTZ-3 acute myeloid leukemia cell line differentiated into LC; MTT: thiazolyl blue tetrazolium bromide; PBMC: peripheral blood mononuclear cell; PI: propidium iodide; RhE: reconstructed human epidermis; RHS: reconstructed human full thickness skin; RT-qPCR: reverse transcription quantitative polymerase chain reaction; SE: skin equivalent

Table 5 Predictive performances of co-culture models for skin sensitisation

Model	Readouts	Chemicals	Predictive performance	References
2D segregated co-culture of KCs and THP-1 cells	CD54/CD86 cell markers	9 chemicals: 6 sensitisers (3 pro/pre-haptens)	100% accuracy 100% sensitivity	(Cao et al., 2012)
2D mixed co-culture of HaCaT and THP-1 cells (COCAT)	CD54/CD86 cell markers	24 chemicals: 14 sensitisers (8 pro/pre-haptens)	96% accuracy	(Hennen and Blomeke, 2016)
2D mixed co-culture of HaCaT, fibroblasts and MUTZ-LC	IL-8, MIP-1β and GM-CSF extracellular release	19 chemicals: 12 sensitisers (10 pre/pro-hapten)	Comparison to LLNA 91% accuracy	(Lee et al., 2018)
3D segregated co-culture of MatTek EpiDerm™ RhE and plasmacytoid dendritic cells (pDC)	CD86 cell marker and IL-18 extracellular release	6 chemicals: 4 sensitisers (1 pre/pro-hapten)	89% accuracy 100% sensitivity 67% specificity	(Troese et al., 2016)
3D segregated co-culture of Episkin Realskin and MUTZ-LC	IL-12, IL-9, VEGF, IFN-ɣ extracellular release	3 chemicals: 2 sensitisers (pro/pre-haptens)	91% accuracy 91% sensitivity 92% specificity	(Lee et al., 2014)
3D segregated co-culture of VitroDerm RhE and THP-1 cells	CD54/CD86 cell markers	12 chemicals: 7 sensitisers (1 pre/pro-hapten)	Comparison to LLNA 100% accuracy 100% sensitivity 100% specificity	(Thélu et al., 2019b)
3D sandwich VG-KDF-Skin tri-culture	IL-4 extracellular release	14 chemicals: 9 sensitisers	Comparison to LLNA 93% accuracy 89% sensitivity 100% specificity	(Uchino et al., 2011)