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CONCISE COMMUNICATION

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On the relevance of an in vitro reconstructed human epidermis model for drug screening in atopic dermatitis

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Abstract

Recent advances in the development of human-based in vitro models offer new tools for drug screening and mechanistic investigations of new therapeutic agents. However, there is a lack of evidence that disease models respond favourably to potential drug candidates. Atopic dermatitis (AD) is a very common disease associated with an altered skin barrier and chronic inflammation. Here, we demonstrate that the AD-like features of a reconstructed human epidermis (RHE) model treated with Th2 cytokines are reversed in the presence of molecules known to have a beneficial effect on damaged skin as a result of modulating various signalling cascades including the Liver X Receptors and JAK/STAT pathways. This work shows that standardized and reproducible RHE are relevant models for therapeutic research assessing new drug candidates aiming to restore epidermal integrity in an inflammatory environment.

KEYWORDS
JAK/STAT, LXR, preclinical, skin model, therapeutic

1 | BACKGROUND

Skin barrier defects are very common and are often associated with inflammation. Although most conditions are benign, certain diseases like atopic dermatitis (AD) may have considerable debilitating effects. Although extensive progress has been made in our understanding of the pathogenesis of AD, the barrier dysfunction linked to a complex inflammatory environment remains poorly understood. Most importantly, current therapies usually aim to decrease cutaneous inflammation and alleviate pruritus, but fail to correct the underlying barrier defects driving the process.[1,2] To meet this need, intense research is being performed to develop specific strategies to restore epidermal integrity. In this search for new treatments, the need for efficient in vitro assays has become crucial.

A recent review by Löwa et al.[3] discusses the current limitations and advances of reconstructed human epidermis (RHE) in AD research, reminding us of the options available to generate disease models, using patient-derived cells, through modulation of disease-associated genes, by co-cultivation with pathogens or by addition of disease-associated stimuli such as cytokines. Several aspects are discussed, such as the study of interactions between different cell types and the role played by immune cells. RHE facilitate the investigation of keratinocyte-specific cellular responses and can help to estimate the influence of distinct inflammatory cytokines. They are considerably less complex than in vivo environments,[3] and we agree that research efforts need to be intensified to further mimic disease patterns in vitro and to allow the study of pathophysiological aspects in a more complex setting. However, increasing the complexity of the models will necessarily accentuate other problems, namely lack of comparability, and may produce contradictory results.[3] In the context of drug screening and mechanistic investigations, RHE models benefit from a certain balance between complexity, standardization, cost and biological relevance. Through the use of multiple specific models, it is possible to target anti-inflammatory properties with one model, while another focuses on the identification of active substances which can restore epidermal homoeostasis and the barrier function in an inflammatory environment. With this last goal in mind, we fully echo the final conclusion presented by Löwa et al.: that the key point today is to validate these models by presenting adequate controls.

2 | QUESTIONS ADDRESSED

While in vitro RHE models mimicking disease conditions have frequently been presented in the literature,[4,5] in most cases their ability to show reversible phenotypes remain to be validated. The aim of this study was to develop a compromised RHE mimicking typical molecular features of AD and to assess its relevance by monitoring the tissue response to molecules known to have beneficial effects on AD-damaged skin in patients. As a proof of concept, we selected two benchmark pathways: modulation of the Liver X Receptors (LXR) and JAK/STAT signalling. LXR agonists induce anti-inflammatory activity but also restore skin barrier function in vivo through direct effects on keratinocytes, hence they present therapeutic potential for AD treatment.[6–8] Given the relevance of the JAK/STAT pathway in the pathogenesis of AD,[9–12] we also used an inhibitor of the STAT6 transcription factor and verified that it counteracted the detrimental signalling induced by the Th2 cytokines.

3 | EXPERIMENTAL DESIGN

RHE tissues were produced as described[13] and grown according to the manufacturer's instructions (StratiCELL, Isnes, Belgium). To reproduce the morphological and functional aspects of AD, RHE were exposed to Th2 cytokines IL-4, IL-13, IL-25, as previously described.[14] Keratinocytes from AD biopsies were also used to reconstruct human epidermis without Th2 cytokine treatment. Biopsies were collected by Dermatest (Germany), in accordance with the Declaration of Helsinki Principles and the study protocol was approved by the Institutional Ethics Committee. Cytokines were purchased from PeproTech (London, UK), the LXR agonist GW3965[6,8,15] and the STAT6 inhibitor AS1517499[10,16] from Sigma (Overijse, Belgium). Doses were selected based on literature data,[15] and lack of toxicity was confirmed by MTS assay (Figure S1) and histological study (Figure 1). The tissue response in the presence and absence of benchmark inhibitors was compared at the morphological (histology) and gene expression levels (qPCR assays). Detailed methods for all assays are described elsewhere.[17] The toluidine blue assay is described in the legend to Figure S4. Primary antibodies for carbonic anhydrase 2 (CA2) were purchased from Sigma (HPA001550), antibodies for filaggrin (FLG) and loricrin (LOR) were from Abcam (ab81468, ab24722). The details of TaqMan assays (Thermo Fisher, Asse, Belgium) are provided in Table S1.

4 | RESULTS AND DISCUSSION

We first evaluated the ability of the RHE model to reproduce the epidermal features of AD when exposed to Th2 cytokines. As expected, we observed typical characteristics of AD, such as a widening of intercellular spaces (spongiosis) and deregulated expression of

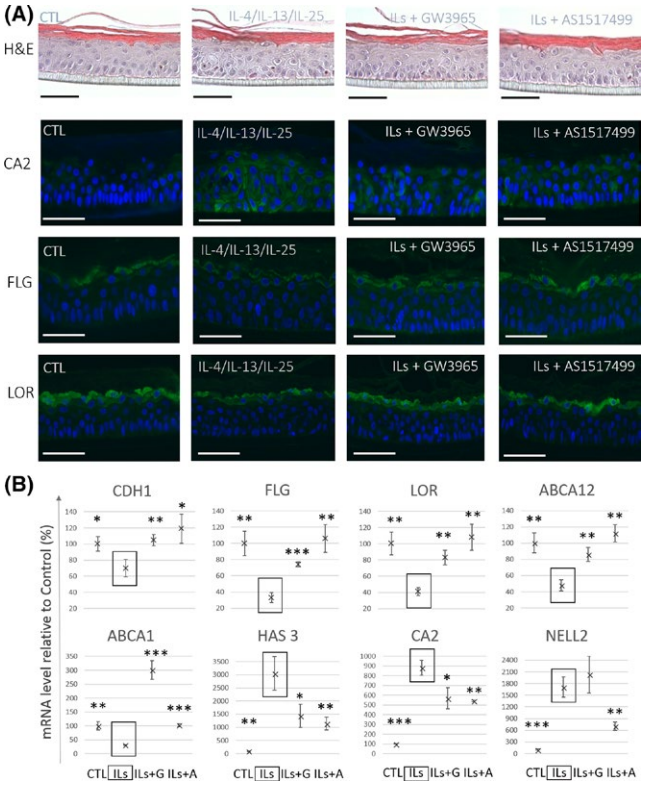

FIGURE 1 Exposure of RHE tissues to Th2 cytokines induces changes in expression patterns for proteins and genes associated with AD. Patterns are restored to near-normal in the presence or absence of an LXR agonist or a STAT6 inhibitor. A, Histology and immunofluorescence staining of RHE tissues after 48 h exposure to IL-4 (50 ng/mL), IL-13 (50 ng/mL) and IL-25 (20 ng/mL) in the culture medium, in the presence of the LXR agonist GW3965 (5 µmol/L), the STAT6 inhibitor AS1517499 (5 µmol/L), or control (DMSO 0.05%). Scale bar: 50 µm. Representative pictures of triplicates. B, Real-time qPCR analysis of mRNA expression levels. Expression levels were normalized by the delta-delta CT method (housekeeping gene: B2M), and are shown as percentages relative to the levels detected in untreated control (mean ± SD of triplicates). Student's *t* test was used to compare the different conditions to the sample treated only with Th2 cytokines (the framed condition on the graphs). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ILs: cytokine cocktail (Il-4, IL-13, IL-25); H&E, Haematoxylin and Eosin staining; CA2, Carbonic anhydrase II; FLG, Filaggrin; LOR, Loricrin; CDH1, Cadherin 1; ABCA1/12, ATP-binding cassette transporter 1/12; NELL2, Neural EGFL-Like 2; HAS3: Hyaluronan Synthase 3; STAT: Signal Transducer and Activator of Transcription

biomarkers at the protein and/or mRNA levels, including a decrease in filaggrin, loricrin and epithelial cadherin, along with an increase in CA2, neural EGFL-Like protein 2 and hyaluronic acid synthase 3, HAS3 (Figures 1 and S2).

As we had selected an LXR agonist to challenge the model, we assessed the impact of the Th2 treatment on the expression levels of two genes directly targeted by these transcription factors: ATP-binding cassette transporter family A members 1 and 12 (ABCA1 and ABCA12, respectively). ABCA12 facilitates the delivery of lipids to lamellar bodies in keratinocytes and therefore plays a crucial role

in maintaining the lipidic epidermal permeability barrier,[18] whereas ABCA1 mediates cholesterol efflux from cells and contributes significantly to regulating cholesterol levels.[19] A significant decrease in ABCA1 and ABCA12 mRNA levels was observed following exposure to the cytokine cocktail (Figure 1).

These results corroborate previous studies where RHE treated with Th2 cytokines reproduced epidermal features of AD, particularly at the gene and protein expression levels. These AD-like characteristics were also in line with previous observations on RHE reconstructed from keratinocytes collected from AD-damaged skin from patients (Figure S3), even though one model requires the application of exogenous Th2 cytokines while the other does not. However, patient-derived samples are not suitable for use in standardized assays due to their inherent variability and the difficulty obtaining them. Nevertheless, the fact that both models led to similar observations reinforces the significance of the Th2 model for in vitro testing and screening, providing that it responds favourably to treatment.

To verify the reversibility of the AD-like characteristics induced by the Th2 cocktail, we assessed the efficacy of the benchmark treatments selected. Both treatments reversed the Th2 cytokine effects, triggering increased expression of FLG and LOR, while also inducing expression of the LXR target genes ABCA12 and ABCA1 (Figure 1). A toluidine blue permeability assay revealed that the increased permeability induced by exposure to the cytokines was restricted when the reference treatments were applied (Figure S4). These results corroborate the changes in morphology observed in the tissues, and the expression levels of the tested biomarkers.

Interestingly, the increase of HAS3 mRNA was significantly reduced in the presence of the tested references. This observation is particularly interesting given the correlation observed with intercellular oedema in RHE from AD patients, associated with decreased filaggrin expression (Figure S3). These results also corroborate those from a previous study showing an imbalance of hyaluronan synthases 1 and 3 in AD conditions.[20]

The respective roles of STAT3 and STAT6 in pathways downstream of the Th2-induced AD have been discussed previously,[21] but are somewhat controversial. Our data confirmed that a STAT6 inhibitor can impede the detrimental signalling cascade induced by Th2 cytokines in this model. Our study therefore substantiates prior observations that STAT6 is involved in regulating keratinocyte differentiation,[11,22] and supports the hypothesis that JAK-STAT inhibitors may be clinically relevant in AD.

Despite the potential of LXR agonists to treat AD, their mechanisms of action within the epidermis are not yet fully understood. Therefore, we pursued our study focusing on the efficacy of the LXR agonist by extending our gene expression assessments. Based on previous transcriptomic data and information from the literature, we selected a list of genes for which expression was modulated by Th2 cytokines and potentially controlled by LXR. The results (Figure 2 and Table S1) clearly show that many genes involved in maintaining the skin barrier, lipid metabolism and transport are under-expressed, whereas genes involved in inflammation are over-expressed. Most significantly in the context of this study, treatment with the LXR agonist reverses the modulation of expression of these genes, restoring a profile similar to the initial homoeostasis.

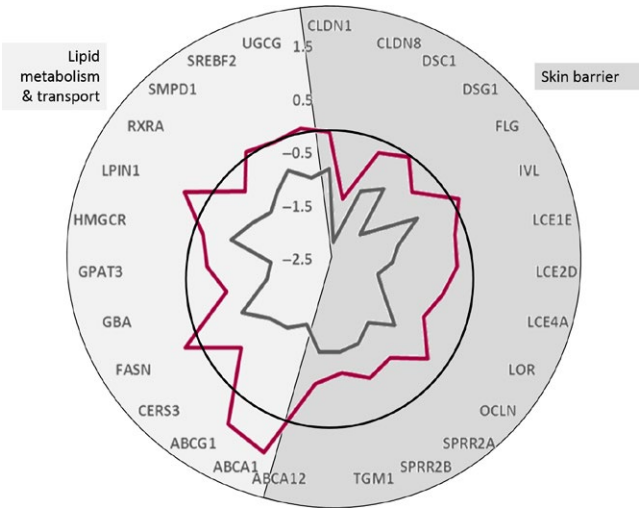

FIGURE 2 Gene expression signature of the AD-like RHE model following exposure to Th2 cytokines, in the presence of DMSO 0.05% or the LXR agonist GW3965. The radar plot shows, on a base-2 log scale, the proportional magnitude of expression changes (means from triplicate experiments) compared to control for 13 genes involved in lipid metabolism and transport and 14 genes involved in the barrier function. The black circle surrounding the centre corresponds to the baseline control level 0 (ie, fold change = 1), whereas the edge of the figure equals 2 (FC = 4) and the centre equals −2.5 (FC = −5.7). The grey line indicates the fold-changes for expression in the presence of Th2 cytokines; the purple line corresponds to levels of expression in the presence of both the LXR agonist and cytokines

5 | CONCLUSION

3D in vitro skin models range from simple RHE to complex full-skin equivalents integrating immune cells or microfluidic platforms. Complexity is relevant when studying cell-type interactions, particularly interactions with the immune system.[23–25] However, complex models may present biological variations and higher production costs. Thus, depending on the research needs, RHE offer a good compromise between complexity and biological relevance. Our results demonstrate the relevance of an RHE model treated with Th2 cytokines for the study of AD-like features that can be reversed by potential therapies. This work shows that standardized and reproducible RHE are relevant models for therapeutic research assessing novel compounds targeting epidermal features in these immune-free models.

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CONFLICT OF INTEREST

The authors are employees of StratiCELL SA; conflict of interests may occur with any structure which could act as a business competitor or that may interfere with the activities of the company.

AUTHOR CONTRIBUTIONS

RH and MS developed the study and designed the experiments. RH wrote the paper. CB performed the experiments. All authors analysed the data, read, contributed to, and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Absence of toxicity of the LXR agonist and STAT6 inhibitor at the tested concentrations. MTS assays (#G3581) were performed on RHE treated for 48 h with GW3965 (GW) or AS1517499 (AS), according to the manufacturer's instructions (Promega, Leiden, Netherlands). Absorbances values were normalized to the values of the untreated controls (DMSO 0.05%) and are shown as percentages (mean + SD of triplicates)

Figure S2 Relative quantification of protein abundance in RHE tissues after exposure to Th2 cytokines in presence or absence of an LXR agonist or a STAT6 inhibitor. Experimental details can be found in the legend to Figure 1. QWin 3 (Leica) analysis software was used to quantify specific labeling, using the pixel as surface unit (1 pixel = 1 μm^2). The labeling intensity was determined for each image and normalized relative to the total area of the tissue (excluding the stratum corneum)

Figure S3 Changes in protein and gene expression levels for markers associated with AD in RHE tissues reconstructed from keratinocytes collected from AD-damaged skin from patients (lower arm and inner leg). The intercellular edema observed on histological sections of the reconstructed epidermal tissues was arbitrarily graded as follows: 1 = patchy separation of cells; 2 = diffuse separation with apparent disruption of intercellular bridges. All groups include donors of both sexes, biopsies from both anatomical sites, and cover a range of ages (from 22 to 47 years old). Undamaged skin samples from a group of healthy donors were included for comparison. A) Histology and immunofluorescence staining of RHE tissues after 14 days of culture at the air-liquid interface. Representative images from at least three biological replicates for each grade. Scale bar: 50 μm. B) Real-time qPCR analysis of mRNA expression levels normalized by the delta-delta CT method (housekeeping gene: B2M) and shown as percentages of the levels relative to the untreated control (mean ± SD, n = 3). Student's *t* test was used to compare results from the different conditions to those obtained with the healthy tissue (from healthy donor) as well as between the two grades of intercellular edema *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; H&E: Haematoxylin and Eosin staining;

CA2: Carbonic anhydrase II; FLG: Filaggrin; LOR: Loricrin; CDH1: Cadherin 1; ABCA1/12: ATP-binding cassette transporter 1/12; NELL2: Neural EGFL-Like 2; HAS3: Hyaluronan Synthase 3

Figure S4 Epidermal permeability barrier measured by toluidine blue assay. Toluidine blue dye was applied topically for 4 h. RHE were then rinsed with PBS and transferred to wells containing 500 µL isopropanol to solubilize dye. After 16 h incubation, the isopropanol fraction was collected and its optical density was measured at 595 nm. The relative concentrations of dye that passed through the skin barrier and were solubilized in the isopropanol are shown (mean + SD from triplicates, optical density values correspond to values after background subtraction and normalization relative to control). Student's *t* test was used to compare the different conditions to the condition treated only with Th2 cytokines (the framed condition on the graphs). *: $P < 0.05$.

Table S1 Changes in gene expression levels for markers associated with both AD and the LXR pathway. Genes are classed in 3 groups depending on their main cellular functions: skin barrier, chemotaxis & inflammation, and lipid metabolism & transport. Levels are expressed as relative quantities (RQ) for treated conditions and the untreated control, after normalization relative to the B2M housekeeping gene using the ddCt method. RQ Max and Min values are the upper and lower limits of the 95% Confidence Interval based on the Student's *t*-distribution with an alpha error level of 0.05.

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