Effective *in vitro* 3D skin models and gene expression assays to study epidermal barrier resilience after Th-2 and Th-17 driven inflammation

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ABSTRACT

One of the main functions of our skin is to protect ourselves from the environment. This natural shield relies largely on the quality of the epidermal barrier, which is regularly under considerable stress. Assaults come from the environment and include, a.o. UV, urban pollution, household cleaning products, organic solvents, etc. They also arise from within through the effects of psychological stress, diet and inflammation. Overall, the majority of the population may be at risk towards skin barrier disruption, and most skin types beneficiate from cosmetic products that increase the resilience of the skin to enhance a restore the epidermal barrier.

In response to the need for evaluating beneficial compounds and formulations, we developed a panel of 3D *in vitro* human reconstructed epidermis (RHE) models that efficiently reproduce sensitive skin features and the associated morphological and functional aspects of a disrupted epidermal barrier.

Here we focus on T-cell mediated inflammatory conditions like eczema or psoriasis driven by either Th2 or Th17 cytokines, with characteristic features of defective barrier properties as filaggrin and loricrin downregulation and dyslipidemia.

We show the relevance and full characterisation of the Th2 inflammation model though functional tests and a full genome expression assay (human transcriptome expression array). Data identified key genes involved not only in epidermal differentiation and inflammatory response of the keratinocytes, but also in other cellular processes and functions associated with lipid homeostasis and itching. Newly identified biomarkers were validated by qPCR and used to design a specific TaqMan Low Density Array (TLDA) to study in a more targeted approach at reasonable costs the expression of 96 genes involved in barrier resilience towards Th2 stimulation.

The relevance of both the Th2 and Th17 RHE models to screen for beneficial effects of test compounds has been confirmed by assessing tissue response after treatment with different benchmarks selected for their known properties to recover the epidermal barrier, targeting lipid metabolism, keratinocyte differentiation and inflammation.

Our data should encourage therapeutic research to use standardized and reproducible RHE models to assess new drug candidates targeting sensitive skin and inflammatory skin diseases.

INTRODUCTION

An intact skin barrier is required to protect the human body from water loss and to prevent the penetration of allergens and pathogens into the skin and underlying tissues. Skin barrier defects are very common, and often associated with a complex inflammatory environment. Current therapies focus at decreasing cutaneous inflammation and alleviating pruritus but do not correct the underlying barrier defects that drive the process (Harris and Cooper, 2017). Therefore, intense research is performed to develop specific strategies to restore the epidermal integrity. In this search for new treatments, the need for efficient *in vitro* assays has become crucial.

Among other human-based *in vitro* models, human reconstructed epidermis (RHE) facilitates the analysis of keratinocyte specific cell responses and can help to estimate the influence of distinct inflammatory cytokines (De Vuyst et al., 2018). Despite the lack of complexity from single reconstructed tissues compared to the *in vivo* environments (Speeckaert et al., 2016), RHE models benefit from a sense of balance between complexity, standardization, cost, and biological relevance in the context of drug screening and mechanistic investigations of new therapeutics. While *in vitro* RHE models mimicking pathologic conditions have flourished in the literature (De Vuyst et al., 2016; Huet et al., 2018), most of those models remain to be validated regarding their ability to show reversible phenotypes upon treatments.

Atopic dermatitis (AD) is one of the most common disease associated with altered skin barrier and chronic inflammation. AD has a strong Th2 component associated with IL-4 and IL-13 over-production by Th2-lymphocytes in the acute phase of the disease. The disease is characterized by a biphasic inflammation and a switch to a Th1 environment is commonly reported during chronic phases of AD (Brandt and Sivaprasad, 2011). Nevertheless, the situation is probably more complicated as small increases in Th1 and Th17 are also found in acute lesions while a progressive activation of Th2 and Th22 cells would also characterize the chronic phase of the disease (Gittler et al., 2012). In addition, specific subtypes of atopic dermatitis in pediatric patients or from Asian origin, have a prominent Th17 component (Guttman-Yassky and Krueger, 2017).

The Th17 pathway associated with IL-17 activation was initially shown as a psoriasis feature. Activation of Th22 T-cells and Th1 pathways are observed in psoriasis, as in AD. However, it is now clear that antagonism of IL-17 is sufficient to reverse cellular and molecular disease features, so the most current disease model considers that the main driver of psoriasis is the Th17 axis. These data confirm that if psoriasis and AD are clearly separable diseases using clinical criteria, there are many overlaps and similarities underlying immune activation and alterations in tissue structure and function. (Guttman-Yassky and Krueger, 2017; Guttman-Yassky et al., 2018).

In this study, we developed 2 compromised RHE mimicking the inflammatory context of AD and psoriasis through Th2 and Th17 cytokines and assessed the relevance of those models by addressing the tissue response to molecules known for their beneficial effects on lesional skin.

METHODOLOGY

Chemicals

The IL-4,13,17,25 cytokines were purchased from PeproTech (London, UK), the TNF-α from R&D Systems / Bio-Techne (Oxon, UK), the LXR agonist GW3965 and the STAT6 inhibitor AS1517499 from Sigma (Overijse, Belgium) and the IKK inhibitor PS1145 from Cayman Chemical (Ann Arbor, Michigan, USA).

Tissue culture

Normal human epidermal keratinocytes (NHEKs) were purchased from Lonza, Verviers, Belgium. All cultures were maintained in culture media in a humid atmosphere at 37 °C with 5 % CO2 according to the manufacturer's instructions. Reconstituted human epidermis (RHEs) were produced by StratiCELL (Isnes, Belgium) and maintained 14 days at the air-liquid interface. In order to reproduce the morphological and functional aspects of AD, RHE were exposed to a Th2 cytokines cocktail made of IL-4 (50ng/ml), IL-13 (50ng/ml), and IL-25 (20ng/ml) for 48H as previously described (De Vuyst et al., 2016). The LXR agonist GW3965 (0.625 µM) and the STAT6 inhibitor AS1517499 (5 µM) were used as positive markers and incubated along with the cytokines. Keratinocytes from AD biopsies have also been used for reconstruction of human epidermis without treatment with Th2 cytokines. These biopsies were collected in accordance with the Declaration of Helsinki Principles and approved by the Ethics Committees.

In order to reproduce the morphological and functional aspects of the Th-17 driven inflammation, RHE were exposed to IL-17 (100ng/ml) and TNF-α (1 ng/ml) for 48H. The LXR agonist GW3965 (10 µM) and the IKK inhibitor PS1145 (25 µM) were used as benchmark and incubated along with cytokines.

Dose selection was based on the literature (Cho et al., 2012; Schmuth et al., 2004) and confirmed by absence of toxicity as observed by MTS assay and histological H&E staining.

IL-19 Quantification

After 48h of cytokines exposition, NHEKs and RHE-Th17 culture supernatants were collected and used for ELISA quantification of interleukin-19 (R&D Systems, Oxon, UK). The methods followed the supplier's specifications based on standard curves.

Immunohistochemistry

RHE samples fixed in formaldehyde were dehydrated with ethanol and isopropanol before paraffin inclusion and storage. Paraffin-embedded sections were de-paraffined and rehydrated for histological assessment (Eosin and Hematoxylin staining) or immune labeling (fluorescence detection). When needed, antigen unmasking was performed using a citrate buffer (pH = 6). Sections were incubated with primary antibody after blocking in the presence of bovine serum albumin. Slides were mounted with Mowiol medium (Sigma, Overijse, Belgium) and examined with a fluorescence microscope (Leica DM 2000, lens 40x) equipped with a digital camera (Leica DFC420C, Diegem, Belgium). Primary antibodies for CA2, FLG and LOR were purchased from Sigma (HPA001550) and Abcam (ab81468, ab24722). Nuclei were stained with DAPI (Invitrogen).

Individual gene expression analysis using specific TaqMan assays

Gene expression changes were analysed using TaqMan qPCR assays. RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Antwerp, Belgium) according to the supplier's

recommendations. The RNA samples were stored frozen at -80°C. The RNA concentrations were determined by spectrophotometric measurements and the RNA quality was analyzed by capillary electrophoresis (Agilent Bioanalyzer 2100, Diegem, Belgium). After reverse transcription the TaqMan assays were processed as described in the manufacturer's instructions (TaqMan™ Assays, Applied Biosystems/Thermo Fisher, Merelbeke, Belgium). In brief, cDNAs were mixed in 96-well plates with a specific buffer and probes specific to the target genes. Plates were sealed and qPCRs were run by a QuantStudio7 Flex System (Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct (ΔΔCt) method. The references for the TaqMan genes expression assays were purchased from (Applied Biosystems/Thermo Fisher, Merelbeke, Belgium).

Transcriptomic analyses

RNA samples were amplified using the Ribo-SPIA technology, purified with the Agencourt RNA Clean up XP Beads kit (Agencourt – Beckman Coulter Genomics, Suarlée, Belgium), and then fragmented and labeled with biotin using the NuGEN Encore Biotin Module (NuGEN, Leek, The Netherlands). Hybridization was carried out on Human Gene 2.0 ST DNA chips (Affymetrix/Thermo Fisher, Merelbeke, Belgium) according to the protocol defined by Affymetrix. Intensity measurements were scanned with the GeneChip Scanner 3000 (Affymetrix). Raw data processing was carried out using R (v3.1.1) (Ihaka R, Gentleman R., 1996) and the oligo package (v1.30.0) of the BioConductor project (v3.0) (Gentleman et al., 2004). The latest version of the libraries provided by Affymetrix built on version 19 of the human genome (UCSC Human genome 19) and the RMA method described by (Irizarry et al., 2003) were used to guide and perform pre-processing and sequence annotation. Individual statistical analysis of the genes/transcripts was carried out using the Moderated t method implemented in the R Limma 3.22.1 package (Smyth, 2004). Gene annotation and definition of gene clusters for over-representation analysis using the hypergeometric test method were carried out with our proprietary "StratiCELL Skin Knowledge Database".

TaqMan arrays

The TaqMan arrays were processed as described by the manufacturer's instructions (Micro Fluidic Card Getting Started Guide, *Applied Biosystems)*. In brief, cDNAs were mixed with a specific buffer (TaqMan Fast Advanced Master Mix, 4444557, *Applied Biosystems*) before being injected into the arrays and dispersed into the wells by centrifugation. Arrays were sealed and qPCRs were run using the Q*uantstudio7* Real-Time PCR System (*Applied Biosystems*) and its software (*QuantStudio real time PCR Software v1.3.* software, *Applied Biosystems*). Threshold cycles (Ct) were obtained for each gene. Results files were exported from the qPCR device and analyzed using the *DataAssist* Software (v3.01, *Applied Biosystems*) designed to perform relative quantification of gene expression using the comparative Ct (ΔΔCt) method, through a combination of statistical analysis. The Ct values were normalized to the Ct of a housekeeping gene present on the array (β2-microglobulin; B2M). The maximum Ct cut-off value was fixed at 36 cycles.

Statistical analyses

Experimental data were analyzed for statistical significance using Student t tests. $p \leq 0.05$ was considered significant (*), $p \leq 0.01$ very significant (**) and $p \leq 0.001$ highly significant (***). Normal distribution was assumed from prior empirical data using similar models and assays.

RESULTS AND DISCUSSION

In vitro validation of AD biomarkers from *ex vivo* keratinocytes

In a first step, we reconstructed RHE with keratinocytes collected from lesioned skin from AD patients. These RHE allowed the *in vitro* observation of typical AD characteristics, such as a widening of intercellular spaces (spongiosis)**.** We also validated in the RHE model the appearance of well-documented AD biomarkers at the protein and/or mRNA levels, including a decrease in filaggrin (FLG), loricrin (LOR), epithelial cadherin (CDH1), ATP-binding cassette transporter 12 (ABCA 12) along with an increase in carbonic anhydrase II (CA2), neural EGFL-like protein 2 (NELL2), and hyaluronic acid synthase 3 (HAS3) **(Figure 1)**. Nevertheless, keratinocytes collected from lesional skin from AD patients are not suitable for routine standardized assays since they present more variability and are difficult to obtain.

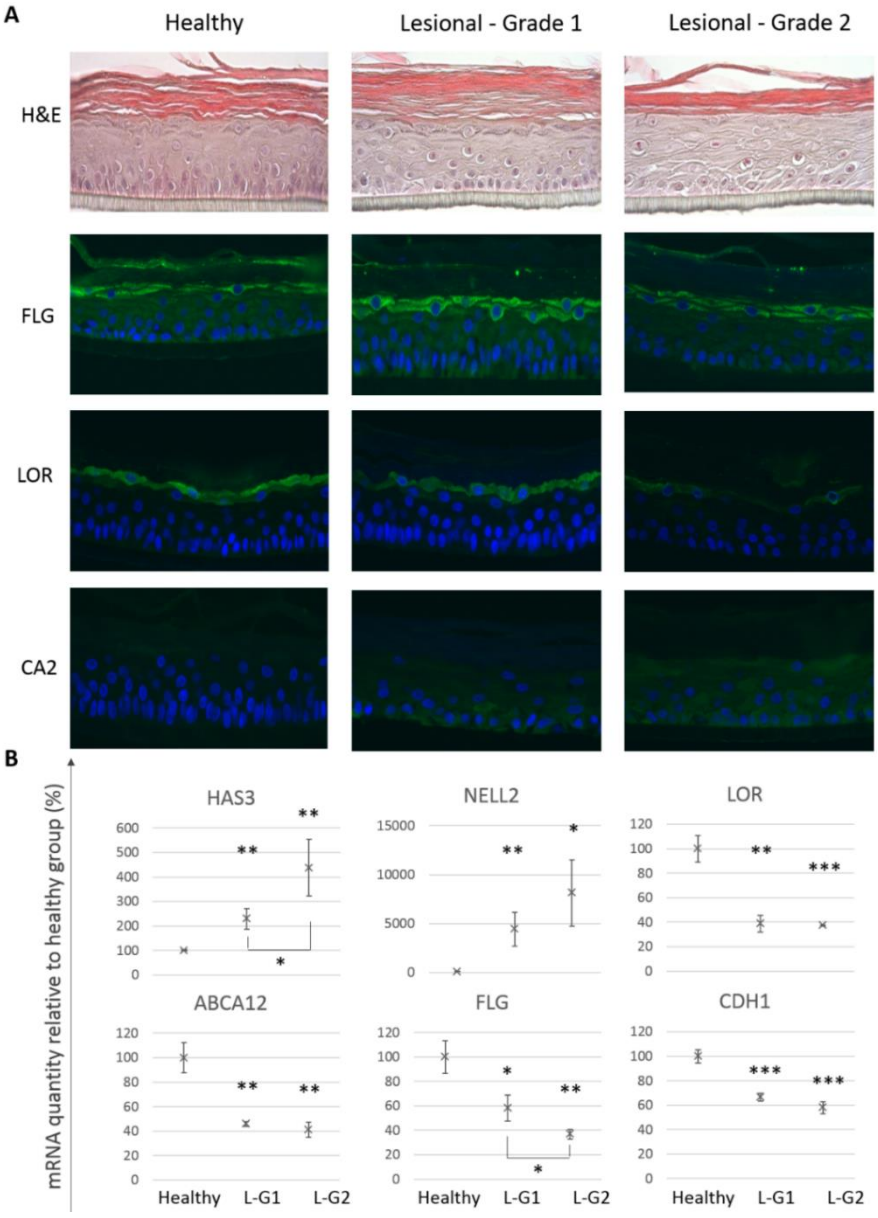


Figure 1. Protein and gene expression changes of markers associated with AD in RHE tissues reconstructed from keratinocytes collected from lesioned skin from AD patients. The intercellular edema was graded semi

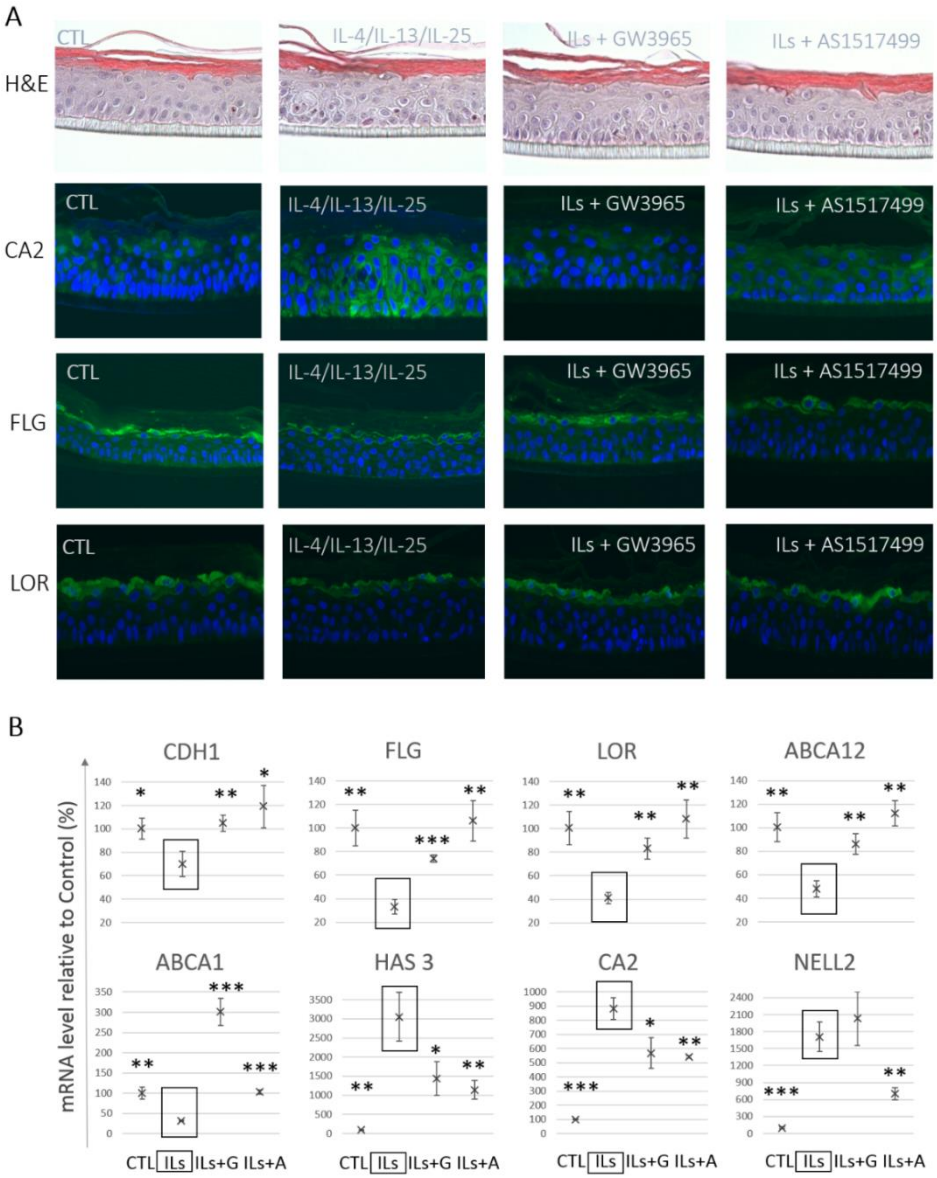
quantitatively: healthy = none; 1 = patchy separation of cells; 2 = diffuse separation with apparent disruption of intercellular bridges. **A)** Histology and immunofluorescence staining of the RHE tissues after 14 days of culture at the air-liquid interface. Representative illustration of at least 3 biological replicates for each grade. **B)** mRNA expression levels evaluated by real-time qPCR using TaqMan assays. Expression levels are normalized through the ΔΔCT method (housekeeping gene: B2M) and showed as percentages of the levels in the untreated control (mean +/- SD from at least 3 biological replicates for each grade). Student's *t* tests have been done to compare the different conditions to the normal tissue (from healthy donor) as well as between the two grades of the pathology *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; H&E: Hematoxylin and Eosin staining; CA2: Carbonic anhydrase II; FLG: Filaggrin; LOR: Loricrin; CDH1: Cadherin 1; ABCA12: ATP-binding cassette transporter 12; NELL2: Neural EGFL Like 2; HAS3: Hyaluronan Synthase 3.

Full characterization of the Th2-inflammation model

We then evaluated the ability of reconstituted epidermis from NHEK cells to reproduce the epidermal features of AD when exposed to a Th2 cytokines cocktail made of IL-4, IL-13, and IL-25. The AD-like phenotype was strongly in line with previous observations on RHE reconstructed with keratinocytes collected from lesional skin from AD patients. We observed typical deregulations of AD biomarkers at the protein and/or mRNA levels, including a decrease in FLG, LOR, CDH1, along with an increase in CA2, NELL2, and HAS3 **(Figure 2)**. The data demonstrate the relevance of RHE models treated with Th2 cytokines to reproduce the epidermal features of AD.

In order to use the Th2 model for testing and screening purposes, we verified that it can respond favorably to treatment. We then assessed the efficacy of 2 selected reference molecules *i.e.* an LXR agonist and a STAT6 inhibitor, to reduce AD biomarkers. In addition to the above markers, we tested two direct target genes of the LXR transcription factor, *i.e.* the ATP-binding cassette (ABC) transporter family A members 1 and 12. ABCA12 facilitates the delivery of lipids to lamellar bodies in keratinocytes and is therefore crucial for the epidermal lipid permeability barrier (Egawa and Kabashima, 2016). ABCA1 mediates cholesterol efflux from cells and plays a major role in regulating cholesterol levels (Schmuth et al., 2008). We observed a significant decrease of both ABCA1 and ABCA12 mRNA levels in the RHE exposed to the cytokines cocktail **(Figure 2)**.

Both the LXR agonist (GW 3965) and the STAT6 inhibitor (AS1517499) were able to restore the epidermis integrity, especially through an increased expression of FLG and LOR, along with induced expression of the LXR target genes ABCA12 and ABCA1 **(Figure 2)**. Of note, the expression of hyaluronic acid synthase 3 mRNA was significantly inhibited in presence of either of these references. The decrease of HAS3 expression is particularly interesting knowing that we observed a correlation between its expression level and the grade of intercellular edema in RHE from AD patients, accompanied by a decreased expression in Filaggrin **(Figure 1)**. These results also corroborate a previous study showing that AD pathological conditions are associated with an imbalance of hyaluronan synthases 1 and 3 (Malaisse et al., 2014). Interestingly, the respective roles of STAT3 and STAT6 in downstream pathways of the Th2-associated atopic dermatitis have been discussed in previous studies (Amano et al., 2015) and are somewhat controverted. The data confirmed that a STAT6 inhibitor is able to hinder the detrimental signaling cascade of Th2 cytokines such as IL-4 and IL-13 on this model. Our study therefore substantiates prior observations demonstrating that STAT6 is involved in the regulation of keratinocyte differentiation (Kim et al., 2008; Sehra et al., 2010) and reinforces the statement that JAK-STAT inhibitors may be of clinical relevance in AD.



<u>Figure 2.</u> Protein and gene expression changes of markers associated with AD in RHE tissues after exposure to Th2 cytokines in presence or absence of an LXR agonist or a STAT6 inhibitor. A) Histology and immunofluorescence staining of the RHE tissues after 48h treatment with IL-4 (50ng/ml), IL-13 (50ng/ml), and IL-25 (20ng/ml) in presence or absence of the LXR agonist GW3965 (5OM) or the STAT6 inhibitor AS1517499 (5OM). **B)** mRNA expression levels evaluated by real-time qPCR using TaqMan assays. Expression levels are normalized through the ΔΔCT method (housekeeping gene: B2M) and showed as percentages of the levels in the untreated control (mean +/- SD of triplicates). Student's *t* tests have been done to compare the different conditions to the treated condition with Th2 cytokines only (the framed condition on the graphs). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ILs: cocktail of above-mentioned cytokines; H&E: Hematoxylin 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.

A full genome expression assay (human transcriptome array) was realized to identify additional biomarkers of the Th2 stimulation of RHE. This assay allows the observation of deregulated genes from 6 major AD-related pathways: lipid metabolism, barrier function, pruritus, chemotaxis, spongiosis and cell activation (**Figure 3**).

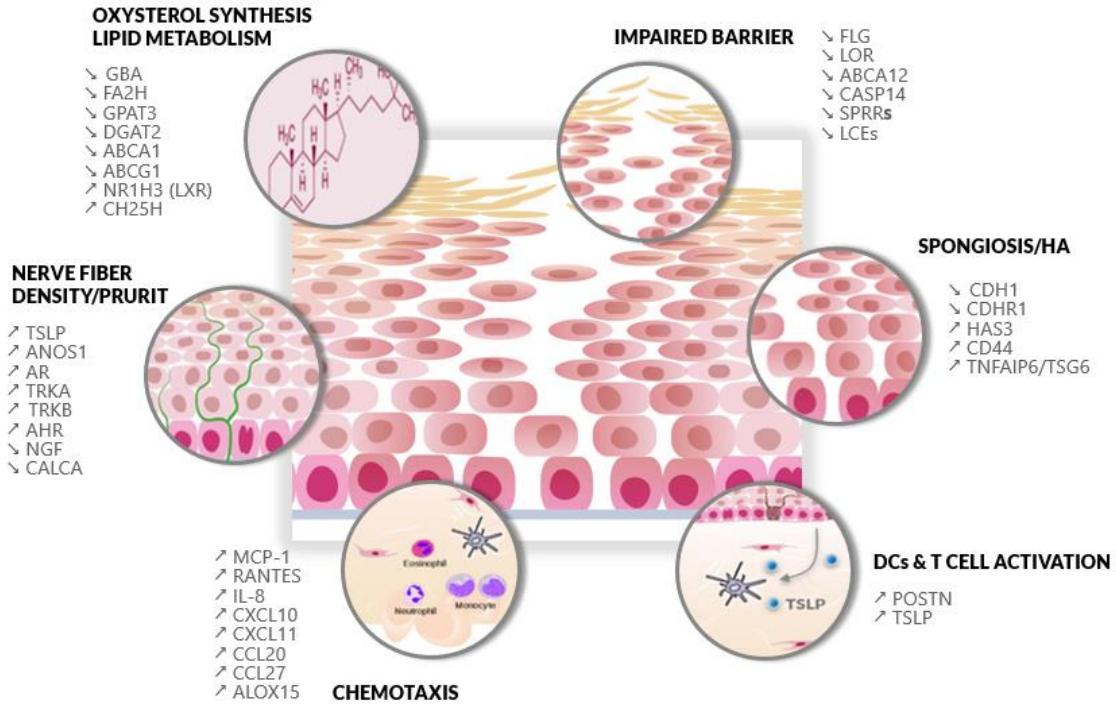


Figure 3. Transcriptomic signature of the RHE-Th2 skin model (2.0 Affymetrix): key observations. All mentioned genes were significant and q-PCR-verified. ↗: upregulated in RHE-Th2 model, ↘: downregulated in RHE-Th2 model.

Based on this full genome expression assay and the literature, we design a specific TaqMan Low Density Array (TLDA) to study in a targeted approach the expression of 96 genes of which expressions were modulated by the Th2 cytokines. This TLDA was then used to fine-tune the gene expression changes from the 3D RHE model exposed to Th2 inflammatory cytokines. The results are illustrated in **Figure 4**, and clearly show that many genes involved in skin barrier, lipid metabolism and transport, axon guidance and inflammation were dysregulated.

Indeed, genes encoding main components of the epidermal cornified envelope Loricrin (*LOR*) and Involucrin (*IVL*), as well as, late cornified envelope proteins (*LCE1E,2D,4A*) were downregulated in our Th2-model assessing the impair skin barrier. The tight junction genes *CLDN1* (Claudin-1) and *OCLN* (Occludin) and *DSC1* gene (Desmocollin-1) were also down-regulated reflecting alteration of epidermal cohesion.

Dermatitis is characterized by a larger number of epidermal nerve fibers and by the production of neuropeptides and neurotrophins that are closely related to the development of the skin inflammatory response and pruritus (Smolyannikova et al., 2015). Dysregulations of *TRPV1, NGF, SEMA3A* and *ARTN* are likely to favor skin hyper-reactivity.

In addition, we observed a decrease of the expression of genes involved in the biosynthesis of ceramides, *i.e.*, Glucosylceramide synthase (*UGG*), Glucosylceramidase (*GA*), Sphingomyelin Phosphodiesterase (*SMPD1*), Ceramide synthase 3 (*CERS3*), cholesterol metabolism (HMG-CoA reductase) and triglyceride synthesis (Glycerol-3-phosphate acyltransferase-3, *GPAT*). The lipid transport is also impacted, as demonstrated by a decrease of ceramide and cholesterol transporters (ATP-binding cassette (*ABCA*) transporters 1 and 12). These observations are in accordance with the observed decrease of EOS ceramides (N-acylated sphingolipids) and other ceramides with very long–chain fatty acids in skin of AD patients (Janssens et al., 2012; van Smeden et al., 2014). Nevertheless, little is known about what drives lipid changes in AD skin (Elias, 2014).

Then, we tested if our Th-2 model can respond favorably to "therapeutic" treatment and selected, as a case study, an activator of the LXR pathway, GW3965. We observed that GW3965 is able to reverse most modulations of gene expression and triggers a return to homeostasis (**Figure 4**, pink dots).

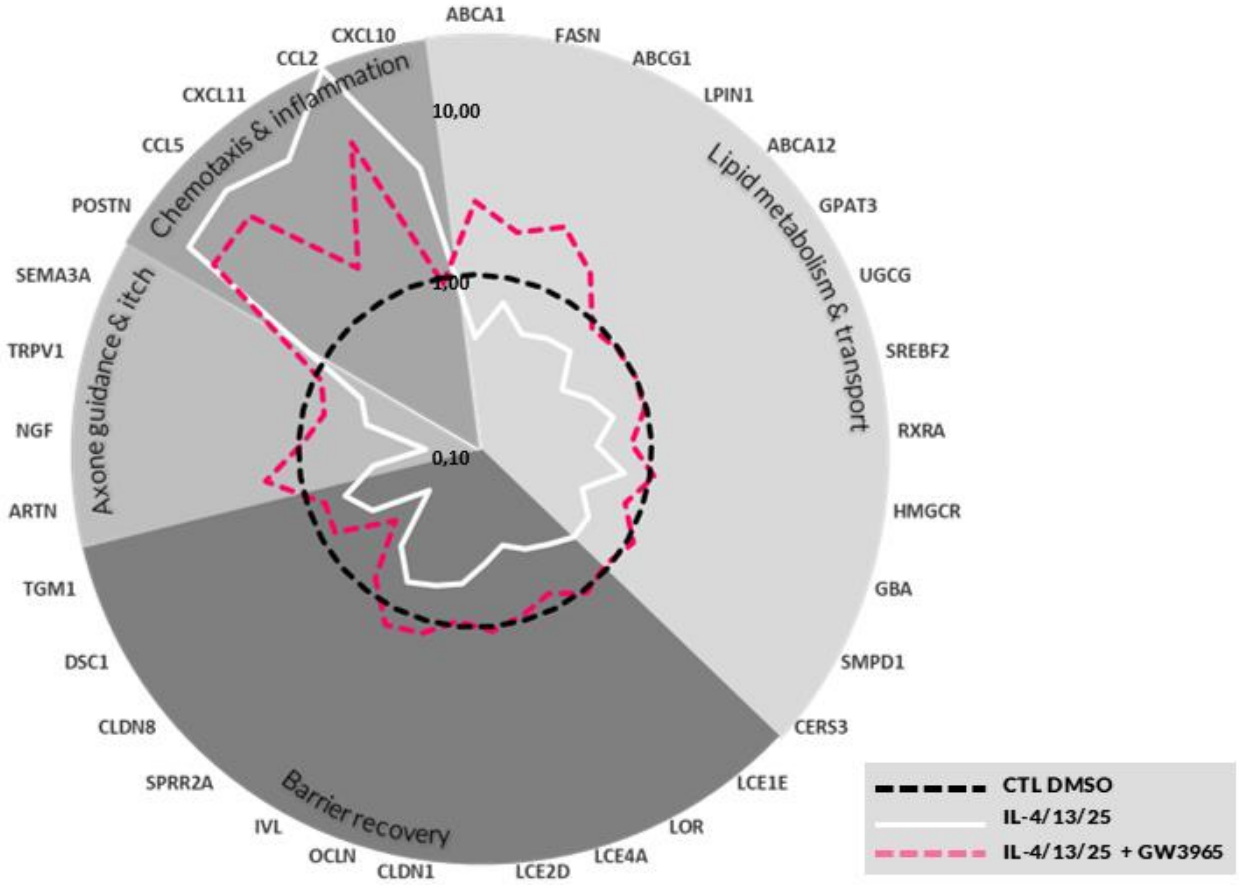


Figure 4. Radar plot of the gene expression signature of the RHE-Th2 model and beneficial effects of the LXR agonist GW3965. The radar chart shows in a base-2 log scale the proportional magnitude of expression changes (means of triplicates) compared with control for 13 genes involved in lipid metabolism and transport, 11 genes involved in the barrier function, 4 genes involved in axon guidance and itch and 5 genes involved in inflammation. The black circle at the center is the baseline control level 0 (i.e. fold change = 1). The white line shows the fold changes of expression in presence of the Th2 cytokines while the pink line shows the levels of expression 1 in presence of both the LXR agonist and the cytokines.

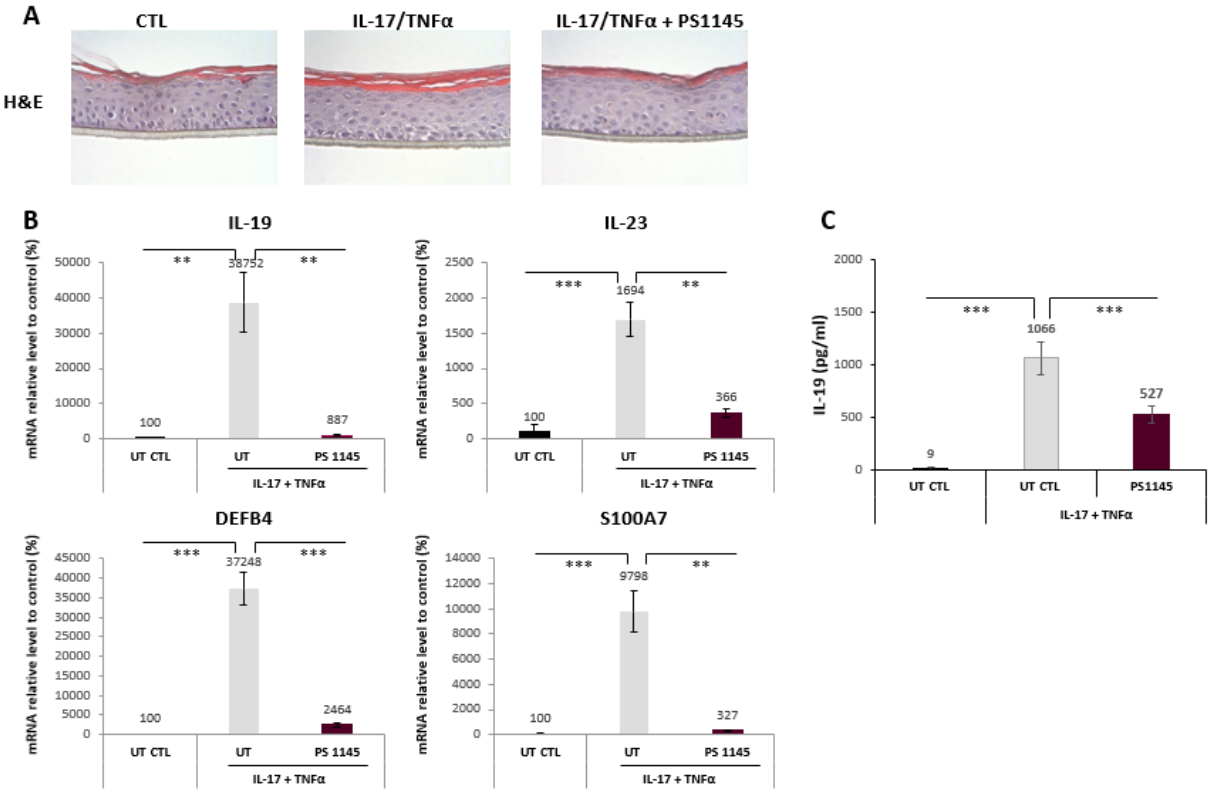
Functional characterization of the Th17-inflammation model

The Th-17 axis demonstrates a growing importance in inflammatory skin diseases (Guttman-Yassky et al., 2018; Smits et al., 2017). In addition to its well-known involvement in psoriasis, the Th-17 axis is also implicated in other skin disease such as AD, vitiligo, acne lesions, *etc.* (Speeckaert et al., 2016). As a major transcription factor to induce IL-17 responsive genes, C/CAATenhancer- binding proteins-b (C/EBP-b) is preferentially expressed in differentiated keratinocytes (Chiricozzi et al., 2014), we then assume that the RHE model would be relevant to study tissue response to IL-17/TNFα stimulation (RHE-Th17 model).

As already reported from *in vitro* model mimicking psoriasis (Smits et al., 2017), epidermal morphology showed hyperplasia and thickening of the *stratum corneum*. Hyperplasia, associated with STAT3 activation, is induced indirectly by IL-17 though increased production of IL-19 and/or IL-36 in epidermal keratinocytes (Kim and Krueger, 2017; Sa et al., 2007). This thickening is partially restored when RHE are exposed to the IKK inhibitor, PS1145 **(Figure 5A)**.

We evaluated the ability of RHEs to respond to IL-17 and TNFα. Of note, IL-17 synergizes with TNF-α resulting in the induction of many key psoriasis-related genes in keratinocyte. TNF-α is also an upstream activator of TIP-DCs (TNF-α and iNOS-producing DCs) for synthesis of IL-23 (Zaba et al., 2007). Data demonstrated an increase of psoriasis biomarkers (Gläser et al., 2005; de Jongh et al., 2005; Kim and Krueger, 2017) at the mRNA levels, including *IL-19, IL-23, DEFB4* and *S100A7* **(Figure 5B)**. IL-19 levels in RHE supernatants was also increased **(Figure 5C)**.

In order to use this model for *in vitro* testing and screening, we tested, as for the Th2-model, if the RHE-Th17 can respond favorably to an inhibition of the IL-17 pathway. We, therefore, tested the efficacy of an IKK inhibitor (PS1145) to reduce psoriasis markers. This inhibitor, targeting the NFkB pathway, is known to restore the expression of the 4 above targets as well as the level of IL-19 in the culture supernatants **(Figure 5B, C)**.



<u>Figure 5.</u> Gene expression changes of biomarkers associated with psoriasis in RHE tissues upon exposure to IL-17 (100ng/ml) and TNFα (1ng/ml) and inhibition by the IKK inhibitor PS1145 (25µM). A) HE staining of the RHE tissues after 48h treatment. **B)** mRNA expression levels evaluated by real-time qPCR using TaqMan assays. Expression levels are normalized through the ΔΔCT method (housekeeping gene: B2M) and showed as percentages of the levels in the untreated control (mean +/- SD of triplicates). **C)** IL-19 levels in RHEs supernatants after 48h of treatment. IL-19 levels evaluated by ELISA. The diagrams present the means of measures in supernatants obtained from 4 independent cultures, as well as the standard deviation.
Student's t tests have been done to compare Untreated control (UT CTL, dark) to the treated condition with IL-17 + TNFα (grey) and the PS1145 treated condition (red) with IL-17 + TNFα only (grey). *: p<0.05; **: p<0.01; ***: p<0.001.

CONCLUSION AND PERSPECTIVES

In summary, we demonstrate the relevance of 2 *in vitro* skin inflammatory models based on either Th-2 or Th-17 stimulation. Application of Th2 cytokines on reconstituted epidermis recapitulates atopic dermatitis while IL-17/TNFα is more likely to mimic psoriasis, as well as, some AD-subtypes. Stimulation of the Th-2 or the Th-17 axis induced known features of AD and psoriasis, as well as disease specific biomarkers. The usefulness of both Th-2 and Th-17 models as screening tools for beneficial/therapeutic compounds were validated using the LXR activator GW3965 and the IKK inhibitor PS1145. Well designed, model specific, TLDA arrays provide a highly reliable tool to gain mechanistic insights as demonstrated with the LXR agonists GW3965.

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