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From the morphologic to the transcriptomic characterization of a compromised 3D *in vitro* model mimicking atopic dermatitis

Running title: Atopic dermatitis and 3D *in vitro* model

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Bulleted Statements:

What's already known about this topic?

- 3D models mimicking atopic dermatitis focus mainly on the filaggrin defect.
- Only two recent studies use Th2-cytokines to induce atopic-like features in reconstructed epidermis.

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What does this study add?

- Full characterization of a reconstructed epidermis mimicking the inflammatory context of atopic dermatitis, from the morphological to the transcriptomic level.

- Creation of a screening tool for selecting natural molecules to treat atopic dermatitis.

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease in which the skin barrier function is disrupted. In this AD environment, pro-inflammatory cytokines are up-regulated, promoting a vicious circle of inflammation. Although several 3-dimensional (3D) *in vitro* models mimicking AD have been published, no study presents a fully characterized and controlled model of AD-related inflammation. In this paper, we aim to develop and characterize - from the morphological to the molecular level - a compromised reconstructed epidermis (RE) mimicking AD-related inflammation *in vitro*. For this purpose, normal human keratinocytes (NHK) were used to generate RE, treated or not with an inflammatory cocktail (Poly I:C, TNF- α , IL-4 and IL-13). Our results show that the inflammatory cocktail induces some modifications observed in AD patients: (1) it leads to spongiosis; (2) it alters early and terminal differentiation proteins; (3) it increases TSLP and IL-8 secretion by keratinocytes and (4) it results in a specific gene expression pattern. These results demonstrate that the inflammatory context contributes to the morphological, functional and transcriptomic changes observed in AD skins. As a result, this compromised RE model shares some characteristics with those found in AD skin and thus can be used as a relevant tool for screening formulations and drugs for the treatment of AD.

Introduction

Atopic Dermatitis (AD) is the most common inflammatory disease of the skin, affecting up to 3% of adults and 25% of children.^{1,2} This disease is characterized by a broad spectrum of clinical manifestations including dry skin, red patches, itching, rash and swelling, whose course is cyclic with alternating crisis and lull periods.

All these symptoms are related to the complex AD pathogenesis, mainly associated with disruption of the skin barrier function in lesional and non-lesional areas, due to low synthesis of differentiation proteins such as cytokeratin 10 (CK10), involucrin (IVL), loricrin (LOR) and filaggrin (FLG).^{3,4} As a consequence, AD is also linked to colonization of lesions by bacteria, allergens sensitization and inflammatory process that initiates and perpetuates the disease.^{5,6}

The etiology of AD is complex since it involves genes mutations and environmental factors.⁷ The highest known genetic risk factor is FLG-null mutation. Since this protein aggregates keratin in cornified cell envelopes and enables the compaction of squames and integrity of the SC⁸⁻¹¹, its deficiency, in combination with abnormalities in both lipids chain length¹²⁻¹⁴ and other important

proteins, leads to a functional epidermal barrier defect, followed by increased water loss, antigen sensitization and finally inflammation^{15–20}.

AD skin is therefore primarily characterized by the over-expression of pro-inflammatory molecules such as IL-4, IL-8, IL-13 and TSLP (Thymic Stromal LymphoProtein).^{21,22} IL-4 and IL-13 are Th2-cytokines,^{23,24} IL-8 is an inflammatory cytokine classically secreted by immune cells after antigen sensitization²⁵ and TSLP is an epithelial cell-derived IL-7-like cytokine induced by antigen sensitization, immune response induced-Th2 cytokines or TLR ligation.^{26,27}

In a vicious circle, this inflammatory response modulates FLG synthesis since keratinocytes differentiated in presence of Th2-cytokines exhibit significantly reduced FLG gene expression.²⁸ Atopic inflammatory response is therefore a central factor contributing to the skin barrier function defect in AD.^{29,30}

In order to understand the mechanisms of skin pathologies and to screen potential therapeutic molecules by substituting animal models^{31,32}, research used to employ tissue engineering and generated *in vitro* models with dominant characteristics of the disease, either by harvesting cells directly from patients biopsies, by using genetic modifications or by culturing cells with appropriate cytokines.^{33–35}

In the context of AD, the majority of published cutaneous 3-dimensional (3D) *in vitro* models focused on the lack of FLG.^{36,37} Only two recent studies have shown the ability of Th2 cytokines, either alone or in combination with TNF- α , to induce AD-like features in reconstructed epidermis (RE).^{38,39} However, AD-like models were only analyzed at the morphological and functional levels and no *in vitro* study presented a transcriptomic characterization of an inflammatory model.

As AD pathogenesis causes both macroscopic and molecular changes and inflammation appears central in this process, the aim of our study was to set up a compromised RE mimicking the inflammatory context of AD and to perform its phenotypic characterization from the morphologic to the transcriptomic level. To determine the relevance of this model, its characteristics were compared to those involved in the physiopathology of AD skins.

Our results show that the use of inflammatory cocktail induces some modifications in the 3D model such as changes in epidermal morphology, altered differentiation process, inflammatory cytokine secretion and specific transcriptomic variations as clinically observed in AD patients.

Materials and Methods

Cell isolation and culture

Normal human keratinocytes (NHK) were isolated from human foreskin, collected from healthy donors undergoing medical surgery. The skin was collected after written informed consent from the donors and institutional approval.

Keratinocytes were cultured in a monolayer with the CnT 57 medium (CELLnTEC) supplemented with 2% penicillin/streptomycin (Invitrogen) until 80% confluent. The keratinocytes were harvested by trypsin digestion. First passage keratinocytes were used to generate RE models (REs).

Generation of the 3D RE models

REs were obtained by seeding 0.2×10^6 NHK into culture inserts (Millipore). Cells were cultured submerged in complete CnT 57 medium (CELLnTEC) for 3 days and in CnT-02-3D Prime medium (CELLnTEC) for 1 day. The cultures were then raised to the air-liquid interface and grown for additional 10 days. During this emersion step, the culture medium was replaced every 2 days.

On day 14 of reconstruction, the inflammatory cocktail (Poly I:C at 10 μ g/ml, Sigma; TNF- α at 10 ng/ml, Life Technologies; IL-4 and IL-13 at 50 ng/ml each, R&D)(40) was supplemented (compromised RE) or not (normal RE) into the culture medium until harvest at day 16.

3D RE models were then used for the following experiments and supernatants were collected for ELISA screening.

Hematoxylin-Eosin (HE) staining and ImmunoHistoFluorescence (IHF)

For both staining procedures, harvested REs were fixed in 4% paraformaldehyde (Carlo Erba), dehydrated and embedded in paraffin. 4 μ m sections were then cut with a microtome (Leica), deparaffinized and rehydrated.

HE staining was realized with hematoxylin and eosin (2 mg/ml and 4 mg/ml, respectively; Dako). IHF analysis of FLG, LOR and IVL were realized after primary antibody incubation for 35 minutes at room temperature (SC-66192 Santa Cruz ; anti- PRB-145P, Eurogentec and I9018, Sigma respectively) followed by secondary antibody incubation, using the same protocol (A11006 or A11001, Invitrogen). Nuclei were stained with DAPI (D3571, Invitrogen).

Staining was visualized with an IX-70 microscope (Olympus) coupled to an image analysis system (NIS-Elements AR, Nikon). Quantitative analysis of the thickness of epidermis (living layers) and of the synthesis of FLG, LOR and IVL (arbitrary unit (AU) proportional to fluorescence intensity) were carried out with Matlab® software (version R2012b The Mathworks).

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ELISA

Collected supernatants were stored at -20°C until use. TSLP and IL-8 secretion were quantified using the Human TSLP Quantikine ELISA Kit and Human CXCL8/IL-8 DuoSet Kit respectively (R&D systems), according to the manufacturer's instructions.

RNA isolation, complementary DNA (cDNA) synthesis and real-time quantitative PCR

REs were recovered and total RNA was extracted using the RNeasy Kit (Qiagen). Quantification and quality assessment of the isolated RNA samples were performed and verified using a NanoDrop spectrophotometer 2000c (ThermoFisher) and an Agilent Bioanalyzer2100 (Agilent Technologies) according to the manufacturer's instructions.

cDNA were synthesized using Improp II-RT (Promega), according to the manufacturer's instructions. Real-time PCR was conducted with LightCycler 480 DNA SYBRGreen Master reaction mix (Roche Diagnostics) and analyzed with LightCycler480 (Roche Diagnostics).

Data were first normalized to the mean of the reference genes (RPS27A, HPRT1 and GUSB), i.e. for each sample and each target gene, $\Delta Ct = [Ct \text{ target gene} - \text{mean } Ct (RPS27A, HPRT1, GUSB)]$ was calculated (Supp Fig. 1). Means of the differences between ΔCt values obtained for compromised RE and normal RE for each donor ($\Delta\Delta Ct$) were then calculated, as well as their 95% confidence intervals (CI). Average expression fold-changes ($2^{-\Delta\Delta Ct}$) are reported on the Figures, together with 95% CI derived from the CI of $\Delta\Delta Ct$.

All the primers used in this study are listed in table 1.

Transcriptomic analysis by microarray

After extraction, mRNA (100 ng) from normal and compromised REs were amplified and labeled with Cyanine 3 using the One-color Low Input Quick Amp Labeling kit (Agilent Technologies) following the manufacturer's protocol. Cy3-labeled cRNA (600 ng) were fragmented and hybridized to SurePrint G3 Human GE 8x60k microarrays (Agilent Technologies). Fluorescence signals were detected using an Agilent SureScan Microarrays scanner and image files were processed with Feature Extraction Software (Agilent Technologies).

All the analyses were performed using the Agi4x44PreProcess package implemented in Bioconductor, an open source software for the analysis of genomic data rooted in the statistical computing environment R. After background correction and normalization between arrays using the quantile method, genes for which the background-corrected signal intensities were not greater than 2.6 standard deviations above the average background in at least 25% of the samples were assumed not to be expressed in the RE and were excluded from further analysis. Differential expression analyses

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of processed data were then performed using the limma package that fits a linear model to the expression data of each gene. An empirical Bayes method was used to borrow information across genes, making the analyses stable even for experiments with small number of arrays.⁴¹ Gene expressions in normal and compromised RE were compared by moderated t-statistics. They were considered as different when the p-value after adjustment for multiple testing (using the Benjamini and Hochberg method⁴²) was less than 0.05 and the expression fold-change between the 2 conditions was at least 1.5.

Analysis of biological processes (pathways and gene ontology) in which these genes are involved was carried out with DAVID bioinformatics resources (Database for Annotation, Visualization, and Integrated Discovery).⁴³ All data are MIAME compliant, and the raw data have been deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession No. GSE65003).

Statistical analysis

Differences between means values of the different variables (thickness of living layers, FLG or LOR staining, TSLP and IL-8 secretion by keratinocytes, and - Δ Ct values) measured in at least 4 normal RE and 4 compromised RE were tested by Student's t-tests, using the StatgraphicsTM Centurion version XV software.

Results

Generation of a compromised RE model

In order to generate a model of AD-related inflammation, we developed a RE mimicking the inflammatory context of AD. To produce this model, NHK were seeded onto inserts and cultured for 14 days following 2 stages: immersion and emersion steps. Once reconstructed, the epidermis was either untreated and used as control (normal RE) or treated with an inflammatory cocktail to mimic the AD inflammatory context (compromised RE).

In order to reproduce the AD inflammatory context as closely as possible, we selected both molecules that constitute the inflammatory cocktail and their individual concentrations according to the literature. In order to induce the highest inflammatory profile while maintaining epidermal reconstruction, we determined the dilution of the whole inflammatory cocktail in the culture medium following a dose-dependent study (Supp. Fig. 1). The results validated the following combination: Poly I:C at 10 μ g/ml, TNF- α at 10 ng/ml, IL-4 and IL-13 at 50 ng/ml each.

Epidermal reconstruction and differentiation are altered in compromised RE

First, to validate the reconstruction as a mimicking model of AD, we examined the morphology of REs supplemented with inflammatory cocktail, by HE staining. Images quantification (mean of 6 areas per RE) showed that the whole thickness of compromised REs (mean of $90 \mu\text{m} \pm 6 \mu\text{m}$, $n=7$) is comparable with that of normal REs (mean of $92 \mu\text{m} \pm 10 \mu\text{m}$, $n=7$) and present all epidermal strata. However, the living layers of compromised REs are less compact and thin with spongiosis within the lower layers (Fig. 1a, upper line).

Next, the influence of this cocktail on epidermal differentiation-protein synthesis was determined by IHF staining. In normal and compromised REs, FLG is expressed in the stratum granulosum (SG) and the SC. Compromised RE models, however, exhibited reduced FLG staining, compared to normal REs (25%, $P<0.05$; Fig. 1a, second line): 33 ± 14 vs. 44 ± 12 AU of FLG, respectively. LOR was expressed in all supra-basal layers in control REs whereas only deeper layers of compromised REs expressed LOR and to a smaller extent (Fig. 1a, third line). Images quantification (mean of 6 areas per RE) showed a reduction of 57% ($P<0.05$) of the synthesis of LOR in compromised REs, compared to normal REs: 20 ± 8 vs. 48 ± 8 AU of LOR, respectively. IVL was expressed in all suprabasal strata of the control REs whereas it is only stained in upper layers of compromised REs. Images quantification revealed that IVL synthesis was also reduced by 28% ($P<0.05$; Fig. 1a, lower line) in compromised REs in comparison to normal REs: 57 ± 22 vs. 82 ± 21 AU of IVL, respectively.

These results were confirmed at the transcript level. Indeed, real-time quantitative PCR showed that the inflammatory cocktail significantly reduces mRNA expression of FLG and LOR by 68% and 57% respectively, when compared with normal RE (Fig. 1b).

Basal TSLP and IL-8 secretions by keratinocytes are increased in compromised RE

In order to ensure that compromised RE is related to the inflammatory context observed in AD patients, we analyzed undernatants TSLP and IL-8 levels by ELISA. As shown in Fig. 2a, supplementation with the inflammatory cocktail significantly enhanced TSLP and IL-8 secretions by keratinocytes when compared to normal REs (122 vs. 1 pg/ml and 510 vs. 23 pg/ml respectively; $P<0.05$).

Transcriptomic profile is modified in compromised RE

The overall response of keratinocytes to inflammatory treatment was analyzed using Agilent technology, covering almost all the human transcriptome. A heat map was created, using the 100 genes with the highest variances across samples among the 20,000 genes expressed in the RE (Fig. 3a). Both rows (genes) and columns (arrays) in this heat map were clustered. Interestingly, the expression of these 100 genes allows clustering the arrays into two groups, one with normal RE samples, and the other with compromised RE samples, confirming that the majority of expressed genes are differentially regulated in these two models. Moderated t-statistics identified 2,086 genes

significantly down-regulated and 1,730 genes significantly up-regulated more than 1.5-fold in compromised RE, after adjustment for multiple testing.

To complete the phenotypic characterization of our model, we analyzed the biological processes in which these genes are involved. We found four pathways over-represented in this set of 3,816 genes - *i.e.* cell adhesion (Supp table 1), cell migration (Supp table 2), inflammation and epidermal cell differentiation - and focused on the two latter. Interestingly, among the 52 genes remaining, 26 are regulated in a similar manner between AD and our compromised RE (Fig. 3b).

Expression of 19 of these genes was validated by real-time quantitative PCR. As shown on Fig. 3c, expression of the inflammation-associated genes IL8, CAII, NELL2, TLR2, STAT2, SPINK5, PLAUR, and IL1a was significantly up-regulated in compromised RE, compared with normal RE. In addition, the expression of the differentiation-associated genes TGM1, S100A8, S100A9, KLK7, AQP3, and TNC was also significantly up-regulated in compromised RE, whereas the expression of the differentiation-associated genes FLG, LOR, KRT1, KRT10, and ITAG2 was significantly down-regulated (Fig. 3d). These expression changes are similar to those already reported in AD patients.

Discussion

In the present study, we generated and characterized a compromised *in vitro* 3D model enabling controlled induction of AD-associated features and gene expression profile after adding relevant pro-inflammatory molecules (TNF- α , IL-4, IL-13 and Poly I:C).

These molecules and their individual concentrations were selected according to the literature. Both IL-4 and IL-13 Th2-cytokines are known to participate in the pathogenesis of AD(24,44) and the clinical effect of blocking them in AD has been proven in clinical trials.⁴⁵ Moreover, recent studies have shown that a Th2 environment, supplemented or not with TNF- α , influences epidermal morphogenesis as found in AD.^{38,39} Poly I:C, a TLR3 ligand mimicking viral double strand RNA, is known to induce a vicious inflammatory cycle involving TSLP secretion by human keratinocytes.^{27,40,46,47}

In order to obtain the highest inflammatory profile while maintaining viable epidermal reconstruction, we selected the dilution of our inflammatory cocktail after a dose-dependent study. The results showed that after addition of the selected pro-inflammatory mixture concentration, REs expressed both microscopic and transcriptomic characteristic changes of AD skin.

Firstly, treatment of REs with the inflammatory cocktail altered epidermal morphology and led to spongiosis, similarly to observations in other *in vitro* AD-like models and in AD patients.^{37,48} This morphological characteristic is consistent with the cytokines used since IL-4 and IL-13 are known to induce spongiosis in epidermal models.^{38,39,49}

Secondly, the inflammatory cocktail delayed the synthesis and the localization of barrier proteins such as FLG, IVL and LOR, with a pattern similar to that observed in other AD-like *in vitro* models^{36,50} and AD patients.⁵¹ Since these proteins are normally localized in the terminally differentiated layers of the epidermis, these results prove that the inflammatory context alters the epidermal differentiation process.⁵²

Thirdly, keratinocytes of compromised REs secrete higher IL-8 and TSLP level compared to normal REs, as observed in AD patients.⁵³⁻⁵⁵ Interestingly, although our compromised RE model is devoid of any immune cells, IL-8 secretion is increased, confirming the suitability of this 3D model to mimic AD *in vitro*. This model requires further refinement to include elements of the immune system together with exposure to stimuli such as bacteria and allergens, which have a functional relevance in the pathogenesis of AD.^{56,57} Conversely, the levels of TSLP produced were higher than those reported in a previous work using a combination of Th2 cytokines and TNF- α on an epidermal model.³⁸ We suggest that the Poly I:C used in our inflammatory cocktail, *via* its TLR-3 ligation on keratinocytes, may mimic an immune response and contribute to these differences.

In turn, TSLP induces a Th2 inflammatory response *via* a vicious cycle, underlying the course of AD and atopic march.^{53,57} A recent study highlighted the relationship between TSLP variations and the persistence of cutaneous symptoms of AD, suggesting that TSLP may be a potential therapeutic target for the treatment of AD.⁵⁸ In this context, our compromised RE model secreting high levels of TSLP may be an interesting tool for this application.

Finally, we characterized our model at the transcriptomic level, in order to determine its molecular relevance to mimic AD skin. The *in vitro* reproduction of gene expression modulations involved in the pathogenesis of AD represents a significant challenge because of the complexity of environmental mechanisms in patients with this disease. This explains why up to the present time, only clinical studies have investigated the transcriptome of these AD skins.^{59,60} Comparatively to that observed between AD and healthy subjects, our transcriptomic analysis revealed a clear difference in the expression of 26 genes between compromised and normal RE models, including key markers of AD.^{51,59,61-68} Genes that are differentially regulated between the compromised RE and AD skin are listed in supplementary table 3. A recent genome-wide association study has identified a total of 19 loci associated with AD *in vivo*, gathering seven biological processes.⁶⁹ Interestingly, when compared to our results of real-time quantitative PCR, four biological processes overlap: (1) epidermal barrier function with FLG and LOR down-regulation; (2) innate immunity with TLR-2 up-regulation; (3) IL-1 family with IL-1 up-regulation and (4) inflammatory response with IL-8 up-regulation of gene expression.^{69,70}

In conclusion, our combination of four inflammatory molecules enables the creation of a compromised RE model presenting many AD-like characteristics, such as thinner epidermis, abnormal differentiation, higher secretion of pro-inflammatory molecules by keratinocytes and specific gene expression pattern.⁷¹ These results demonstrate that the inflammatory context

contributes, at least in part, to the morphological, functional and transcriptomic changes observed in AD skins.

While two inflamed RE models mimicking AD-like features have already been described in the literature^{38,39}, this study is the first *in vitro* AD-model characterized from the microscopic to the transcriptomic levels. Hence our model could become the basis of a relevant tool for screening potential therapeutic molecules to treat AD.⁷²⁻⁷⁵

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Figure legends

Figure 1: Inflammatory cocktail alters morphology and epidermal protein expression in reconstructed epidermis (RE).

(a) Sections of REs cultured in the presence or absence of inflammatory cocktail. x20 magnification. Overall morphology was assessed with hematoxylin-eosin (HE) staining (upper line) and immunohistofluorescence (IHF) analysis was performed for filaggrin (FLG), loricrin (LOR) and involucrin (IVL) (from second to lower lines respectively). For each condition, one representative image out of six is shown. (b) FLG, LOR and IVL mRNA levels were measured by quantitative PCR. Average fold changes in treated versus normal RE are given with their 95 % confidence intervals (CI). Differences between means of $-\Delta\text{Ct}$ values in normal and compromised RE were tested in six samples by paired Student's t-tests. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ns, no significant.

Figure 2: Inflammatory cocktail induces the secretion of inflammatory molecules by REs.

(a) TSLP and (b) IL-8 secretion by keratinocytes were assessed in normal and compromised REs undernats. Results are the mean $\pm$ SD of six different REs tested. *, $p < 0.05$, according to Student's t-test.

Figure 3: Inflammatory cocktail modulates transcriptomic profile of REs.

(a) Heat map obtained with the 100 genes with the highest variances across the 8 RE samples. Both rows (genes) and columns (arrays) in this heat map were clustered. There appears to be 2 clusters, one with normal RE samples, and the other with compromised RE samples. Expression levels are shown in red (high) and green (low). (b) List of the twenty six genes regulated in a similar manner between atopic dermatitis (AD)^{51,59,61-68} and our compromised RE. +: over-expressed genes; -: under-expressed genes; bold: key markers of AD. mRNA levels of genes involved in inflammation (c) and differentiation (d) were measured by quantitative PCR. Average fold changes in treated versus normal RE are given with their 95 % confidence intervals (CI). Differences between means of $-\Delta Ct$ values in normal and compromised RE were tested in at least four samples by paired Student's t-tests. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$.

Supplementary Figure 1: Ct of the three selected reference genes did not change after RE inflammatory cocktail treatment

Ct of the three reference genes are represented for each normal (circle shape) and compromised (square shape) RE tested by RT-QPCR. Means of Ct for each condition are represented by a horizontal bar. ns, no significant.

Supplementary Figure 2: Inflammatory cocktail dose-dependent study

(a) List of the pro-inflammatory molecules and their concentrations used for each dilution of the whole inflammatory cocktail. (b) Sections of REs cultured in the absence or the presence of different dilutions of inflammatory cocktail. x20 magnification. Overall morphology was assessed with hematoxylin-eosin (HE) staining. (c) TSLP secretion by keratinocytes was assessed in REs cultured in the absence or the presence of different dilutions of inflammatory cocktail. (d) TSLP secretion by keratinocytes was assessed in REs cultured in the presence of each of the pro-inflammatory molecules from the cocktail separately. Results are the mean $\pm$ SD of three different REs tested. *, $p < 0.01$; **, $p \leq 0.005$; according to Student's t-test.

Table 1: List of primers used in this study.

Biological Processes	Target Genes	Forward sequence (5'-3')	Reverse sequence (5'-3')
Inflammation	IL1a	ACTCAATTGTATGTGACTGCCCAAG	TGGTCTCACTACCTGTGATGGTTT
	IL8	GCTCTCTTGGCAGCCTTCC	CAGAGCTCTCTTCCATCAGAAAGCT
	CAI1	CGGGCCTTCAGAAAGTTGTTGAT	ATTCAGGAAGGAGGCCACG
	NELL2	ATGCCAATTTCAAGGACGAACCTAC	ACAGCCTGAACTCTCAACAAGTTT
	TLR2	TTGTTTCTTACAGTGAGCGGGATGC	AAGTTCTCCAGCTCCTGGACCATA
	PLAUR	CCTGAGCTATCGGACTGGCTTGA	AGGTAACGGCTTCGGGAATAGG
	SPINK5	TGATGCTGATGGCAAATCGTACAAC	TCCTTCCCTGATTCTGATCCAGTCC
Differentiation	FLG	GGCAAATCCTGAAGAATCCAGA	GCTTGAGCCAACTGAATACCATC
	LOR	GGAGTTGGAGGTGTTTTCCA	AGCTCAATGGCTTCTTCCAG
	TGM1	CCTGGAGCACCTGAACCATGATT	ATCAAAGCCCGAGGGCAGAT
	KRT1	CAATCTCGTTGGATTGCGAACTGA	CTCTGCATTTGTCCGCTTGTTGA
	KRT10	ACAGTCCCAACTGGCCTTGA	GAGAGCTGCACACAGTAGCG
	S100A8	CCGTCTACAGGGATGACCTGAAG	ACTGCACCATCAGTGTTGATATCCA
	S100A9	CTGAACCAGGGGAATCAAAGAGC	GTGTCCAGGTCCCTCCATGATGTG
	KLK7	TTCCATGCTGTGCGCTGGC	CCTCTGCACACCAACGGTCC
	AQP3	CCCTGGATCAAGCTGCCCATCTA	GGCGAAGTGCCAGATTGCAT
	ITAG2	GACCTCACAAACACATTGCGAGC	ACTTTCGTAGCACTTCGTGCGC
Reference Genes	TNC	GTCATCTCCTACACAGGCGAGAAAG	CGAGGTGCGTCAGAGCATACT
	RPS27A	AGAAAGAAGGTTAAGCTGGCTGTCC	GCACTCTCGACGAAGGCGACTA
	HPRT1	CTTTGCTTTCTTGGTCAGGC	GGTCCTTTTACCAGCAAGCT
	GUSB	GAGCCTGCGTCCCACCTAGAA	GTCCAAGGATTGTTGTGAGCGA

Supplementary table 1: List of genes modulated in compromised RE and involved in cell-adhesion pathway.

Supplementary table 2: List of genes modulated in compromised RE and involved in inflammation pathway.

Supplementary table 3: List of inflammation and differentiation genes that are not regulated in the compromised RE model as they are in atopic skin.

Keywords: atopic dermatitis, *in vitro* 3D models, inflammation, transcriptomic analysis

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; SC, *stratum corneum*; TSLP, thymic stromal lymphoprotein; CK10, cytokeratin 10; LOR, loricrin; IVL, involucrin; 3D, 3-dimensional; NHK, normal human keratinocytes; RE, reconstructed epidermis; HE, hematoxylin-eosin; IHF, immunohistofluorescence ; cDNA, complementarity DNA ; SG, *stratum granulosum*; AU, arbitrary unit

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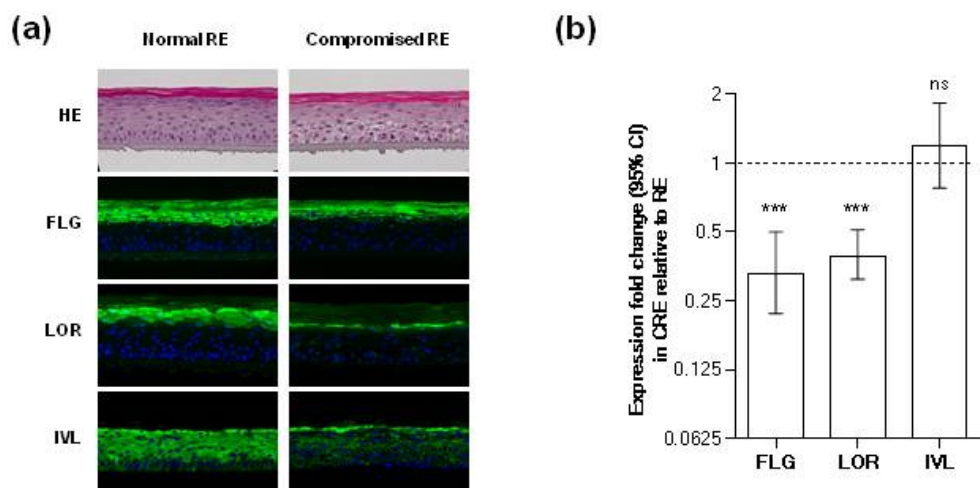


Fig. 1
Epidermal reconstruction and differentiation

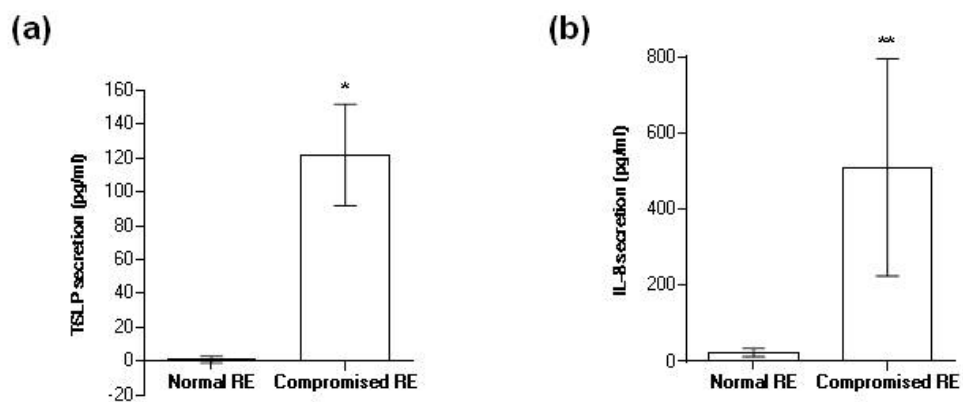


Fig. 2
Inflammation

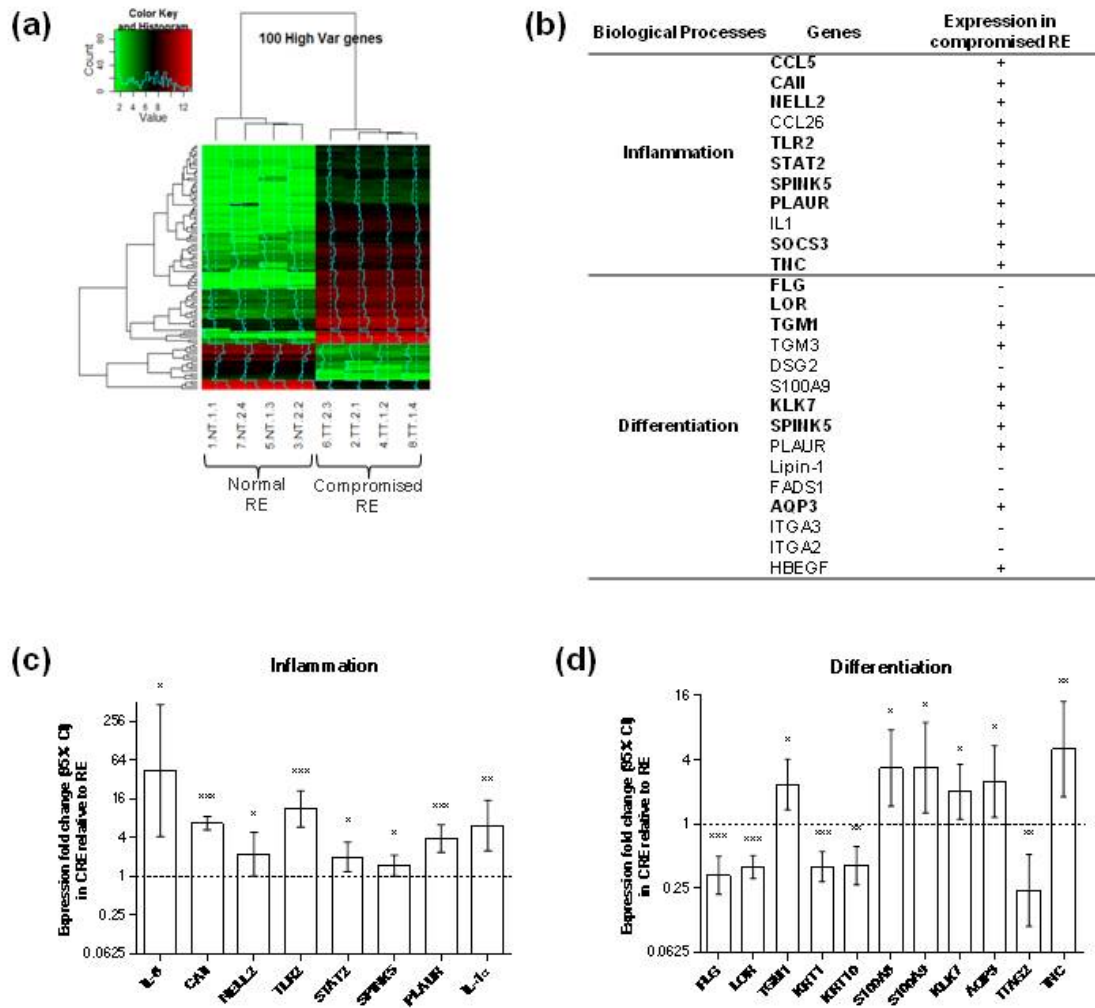
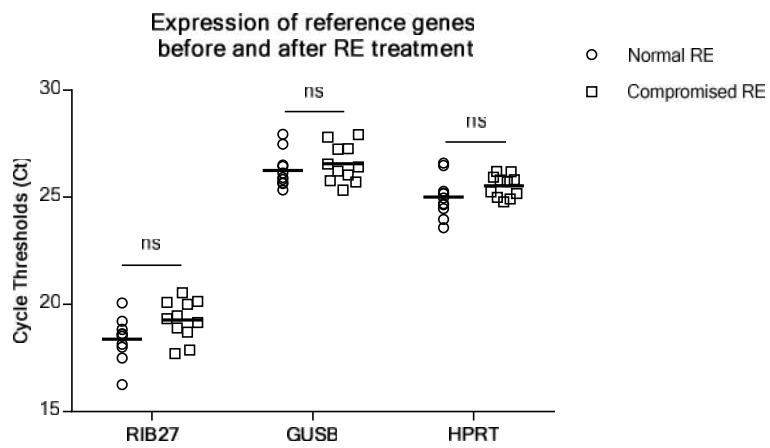
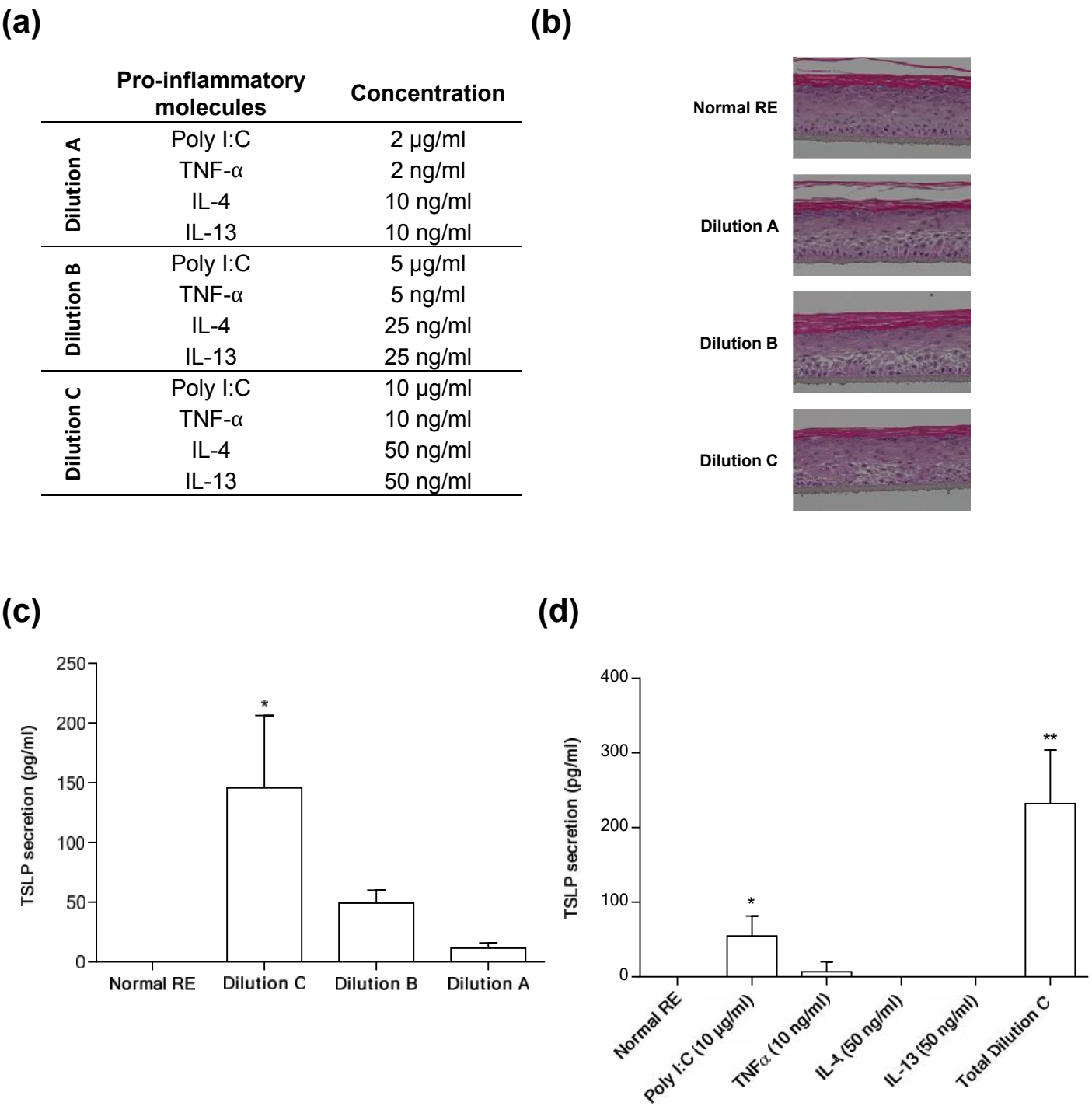


Fig. 3
Transcriptomic Profile



Supp Fig. 1
Ct of the three selected reference genes did not change after RE inflammatory cocktail treatment



Supp Fig. 2
Inflammatory cocktail dose-dependent study