

Methyl- β -cyclodextrin treatment combined to incubation with interleukin-4 reproduces major features of atopic dermatitis in a 3D-culture model

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Abstract Atopic dermatitis (AD) skin is characterized by over-expression of interleukin (IL)-4, IL-13 and IL-25. When methyl- β -cyclodextrin (M β CD) treatment preceded exposure to these interleukins, combination of both treatments was found to mimic hallmarks of AD in vitro, such as barrier weakening, histological alterations and typical signaling responses in a reconstructed human epidermis (RHE). However, the respective role of each IL and whether any of them is critical when combined with M β CD treatment was unknown. Therefore, this work aimed to distinguish RHE responses after exposure to M β CD and each one of the three IL reported to mimic typical features of AD. IL-4 incubation preceded by M β CD was found responsible for altered histology, as well as for barrier alterations, evidenced by electrical resistance and dye permeation measurements. This combination further decreased loricrin (LOR) immunoreactivity, whereas mainly IL-25, combined to M β CD treatment, was able to downregulate filaggrin (FLG) mRNA level. Carbonic anhydrase II (CA2) and hyaluronan synthase 3 (HAS3), two other markers up-regulated in AD, were also induced when M β CD treatment was followed by IL-4, whilst the

expression of neural epidermal growth factor-like 2 (NELL2) was up-regulated by paired IL-4 and IL-13. In conclusion, multiple features of AD were found in this in vitro model mainly when treatment of RHE by IL-4 was conducted after preliminary M β CD incubation.

Keywords Reconstructed human epidermis · Atopic dermatitis · Interleukin-4 and methyl- β -cyclodextrin

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin condition linked to Th2 cell type immune response [4, 13, 23]. Over-expression of pro-inflammatory cytokines such as interleukin (IL)-4 and IL-13 (Th2-cytokines) is indeed observed in AD patients [10, 13, 24, 28, 31, 33], but other studies demonstrate that IL-25 produced by dendritic cells may also be involved, both to induce Th2 response and to inhibit filaggrin synthesis, directly affecting function of skin barrier in AD patients [13].

Recently, our laboratory has shown that plasma membrane cholesterol depletion using methyl-beta-cyclodextrin (M β CD) in reconstructed human epidermis (RHE) followed by exposure to IL-4, IL-13 and IL-25 can mimic hallmarks of AD. This includes barrier weakening, as well as typical histological and signaling alterations [7]. However, in those trials for modeling epidermal AD lesions in vitro, the respective role of each IL and the consequences due to combination of M β CD treatment with each of them remained incompletely understood. Thus, in order to further complete this previous study, the present work aimed to understand responses given to M β CD treatment by RHE exposed subsequently to single or combined cytokines in order to set up an AD-like model.

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Material and methods

Cell culture and production of RHE

Normal adult human skin was obtained from abdominoplasties (Dr. B. Bienfait, Clinique St. Luc, Namur-Bouge, Belgium) and keratinocytes were cultured as described [5, 8, 27]. This study was conducted according to the Declaration of Helsinki Principles and approved by the Ethics Committees. To produce RHE, keratinocytes were cultured at high density submerged, with Epilife® medium (Cascade Biologics, Paisley, UK), inside polycarbonate porous inserts (0.4 µm diameter pore size; Merck-Millipore, Overijse, Belgium) before being raised to the air-liquid interface the next day. Medium was then changed every two days. On the 11th day, RHE were first pretreated or not for 2 h with 7.5 mM methyl-beta-cyclodextrin (MβCD; Sigma-Aldrich, Diegem, Belgium). MβCD was solubilized in the culture medium. Then, RHE were incubated for 48 h with 50 ng/ml IL-4 or IL-13, or with 20 ng/ml IL-25 (Peprotech, London, UK), alone or as a mixture of the three cytokines (IL mix), as previously described [7].

Morphology and indirect immunofluorescence microscopy

Paraffin sections of RHE (6 µm) were stained with hemalun and erythrosine (H&E) for morphological analysis. For carbonic anhydrase II (CA2) and loricrin (LOR), immunofluorescence detection was realized using antibodies from Sigma-Aldrich, Diegem, Belgium diluted 1:500 for CA2 and from Abcam, Cambridge, UK diluted 1:100 for LOR. Antigen retrieval was performed in pH 6.0 citrate buffer for 20 min at 100 °C. Tissue samples were incubated for 1 h with Alexa 488-conjugated anti-rabbit IgG (Life technologies, Ledeborg, Belgium). Secondary antibodies were diluted 1:100 and images obtained using epifluorescence microscopy. Negative controls were prepared using secondary antibodies (data not shown). For CA2, intensity of the detection signal was measured using the image analysis software provided with the Olympus BX63 microscope (Olympus, Berchem, Belgium).

Trans-epithelial electrical resistance (TEER) measurements and permeability to Lucifer yellow

TEER measurements through RHE were performed using Millicell-Electrical Resistance System (Merck-Millipore, Overijse, Belgium). Analysis of the RHE permeability to Lucifer yellow (LY) fluorescent dye (Sigma-Aldrich, Diegem, Belgium) was performed using 150 µl of the fluorescent dye at 1 mM (diluted in PBS) and applied on top of

the RHE for 6 h at 37 °C. Medium was then collected and fluorescence was measured (λ_{ex} : 485 nm; λ_{em} : 535 nm).

Analysis of mRNA expression by RT-qPCR

Total RNA was extracted from RHE using NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed into cDNA using the SuperScript II RNase H-reverse transcriptase kit (Invitrogen, Merelbeke, Belgium). The amplification was performed using the FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) in the Light Cycler® 96 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). Genes were normalized to the reference gene RPLP0 [22], whereas primers (Eurogentec, Liège, Belgium) were used at a concentration of 300 nM in accordance with previously described sequences and reaction conditions [7]. Sequences of primers used to perform the RT-qPCR can be found in table S1. Data were analyzed using the $\Delta\Delta\text{Cq}$ quantitative method and 95% confidence intervals were calculated after two-way analysis of variance [6].

Results

MβCD treatment followed by exposure to IL-4 in RHE induces the strongest morphological alterations and weakening of barrier function in this model

To appreciate the role played by each individual component used as a mixture in a recently described in vitro model developed to mimic the pathological conditions usually encountered in AD, RHE were treated by incubation for 2 h with MβCD or kept untreated. Incubation with MβCD is performed to challenge keratinocytes by depleting cholesterol from their plasma membrane. In several previous studies performed in our laboratory, we have demonstrated that in keratinocytes, either cultured as monolayers [9, 14] or within RHE [6, 7], treatment with MβCD results in significant cholesterol depletion. This was proven by biochemical measurements of cholesterol concentration and/or performing filipin staining to detect and localize cholesterol in frozen sections [6, 7]. After MβCD treatment, RHE was incubated for 48 h with IL-4, IL-13, IL-25, or with a mixture of these interleukins. Figure 1a illustrates that histology of RHE is mostly affected by combination of MβCD and IL-4 incubation, producing alterations almost similar to those seen after incubation with mixed IL. A strong influence is also observed when MβCD incubation of the RHE followed by IL-13 is performed. Conversely, incubation of RHE with IL-25 does not result in noticeable histological alterations, both following MβCD treatment or not. It appears as if incubation with IL-4 reduces the *stratum corneum*'s thickness. Furthermore, when

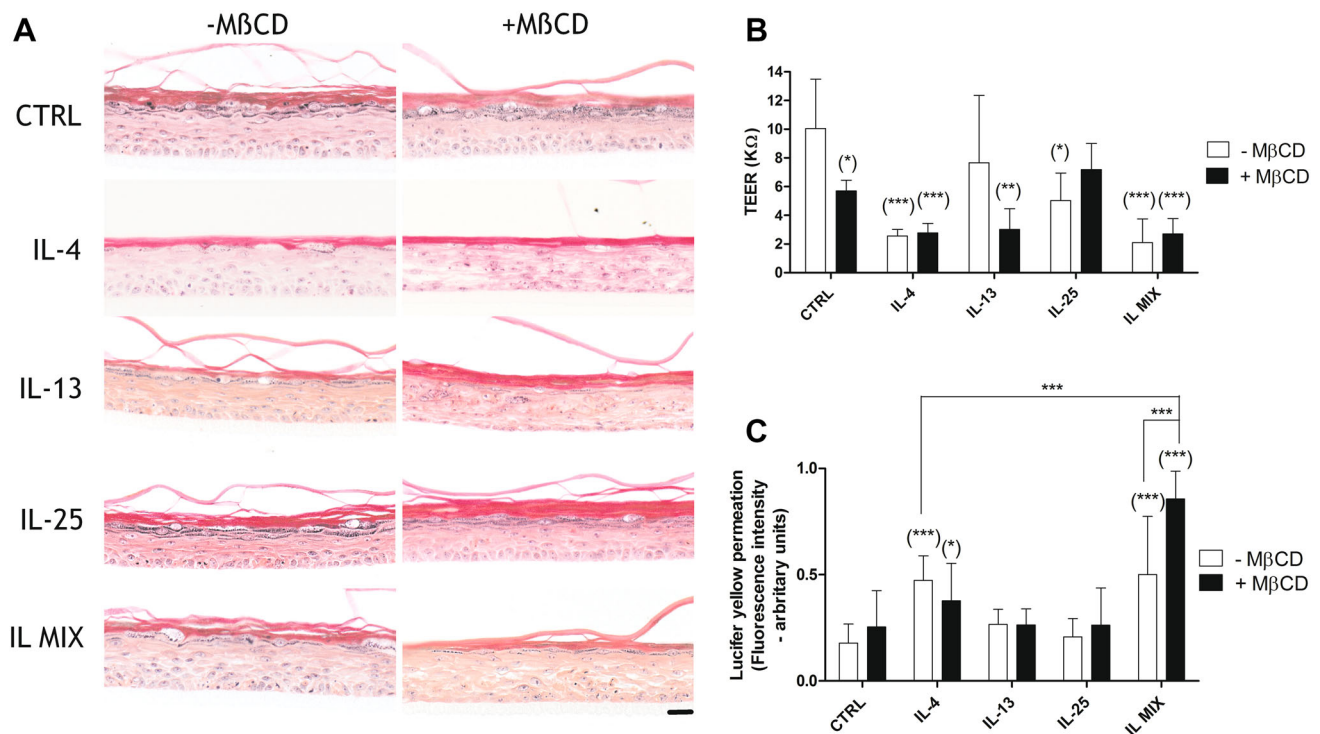


Fig. 1 Morphological and barrier function alterations after MβCD treatment and use of Th2-related cytokines. Reconstructed human epidermis (RHE) was pretreated with 7.5 mM MβCD for 2 h, or was left untreated. Then, RHE was cultured with IL-4 (50 ng/ml), IL-13 (50 ng/ml) and IL-25 (20 ng/ml), alone or in a mixture of the three cytokines (IL MIX), for the next 48 h. **a** Histological sections of RHE are representative of three independent experiments using keratinocytes from three different patients to prepare the RHE. Scale bar 20 μm. **b** Trans-epidermal electrical resistance (TEER)

measurements performed on RHE ($n = 3$). **c** Permeability to Lucifer yellow fluorescent dye. **b, c** Three independent experiments were performed. Data represent mean $\pm$ SD. Two-way ANOVA was performed, followed by Bonferroni tests. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Each averaged measurement is compared with the one measured in control conditions (–MβCD) when the statistical significance is indicated between parentheses; otherwise measurements are compared with the linked condition

MβCD is combined with IL-4 or IL-13, the granular layer is strongly altered and nearly absent.

Alterations in barrier function can be evaluated by TEER measurements (Fig. 1b). TEER mainly decreases when RHE is treated with IL-4. In addition, significant contributions of IL-13 and IL-25 were also observed, but without any additive reductions (Fig. 1b). Reductions in TEER were found independent of eventual previous challenge of keratinocytes by MβCD treatment. Conversely, studies of permeability of RHE to the fluorescent LY dye (Fig. 1c) indicate that incubation with IL-4 has most prominent consequences, which are emphasized by the combination of MβCD with the IL MIX, as previously reported [7].

MβCD treatment enhances overexpression of carbonic anhydrase II (CA2) in RHE subsequently treated with IL-4 or IL-13

CA2 expression was analyzed in the treated RHE using immunofluorescent labeling (Fig. 2 + quantification in suppl. figure S1), as well as by RT-qPCR to assess CA2 relative mRNA expression (Fig. 3d). CA2 mainly appears

overexpressed when RHE was incubated with IL-4, especially after MβCD or with IL-13, but weak increase after incubation with IL-25 is also suggested by the immunofluorescent data (Fig. 2). When looking at the transcript relative levels, both IL-4 and IL-13 indicate significant induction of CA2 gene expression. This induction was even further increased if preceded by MβCD treatment (Fig. 3d).

MβCD treatment reduces loricrin (LOR) expression in RHE treated with IL-4

Results for LOR expression indicate no significant variation at the mRNA level but once MβCD incubation is followed by treatment of RHE with IL-4, strong and reproducible loss in LOR expression at the protein level can be observed through immunofluorescent labeling in this model (Fig. 2).

MβCD treatment in RHE, as well as incubation with IL-4 regulates critical gene expression

FLG is an epidermal differentiation gene whose expression is usually downregulated in lesional AD, in a quite similar way to

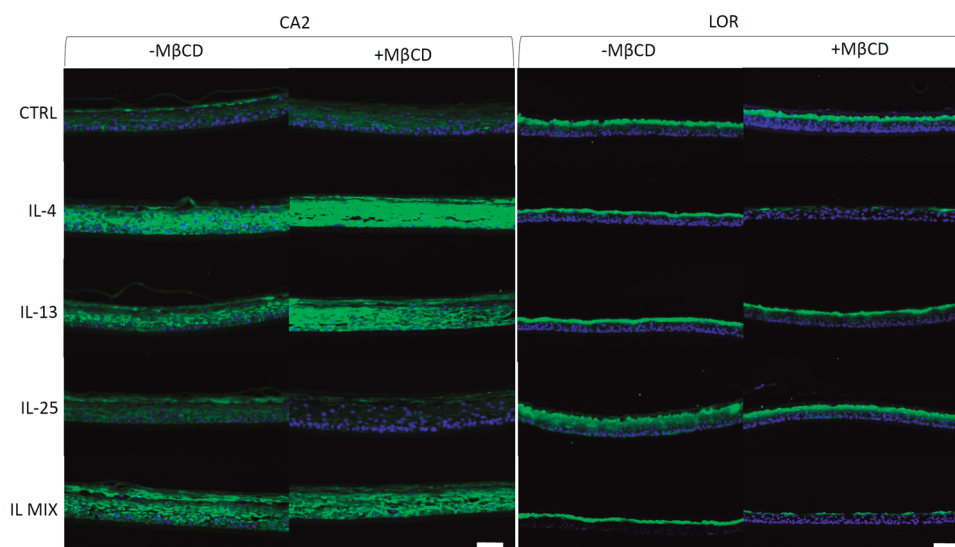


Fig. 2 Immunostaining of AD markers in histological sections of treated RHE. Reconstructed human epidermis (RHE) was pretreated or not for 2 h with M β CD (7.5 mM), then cultured for 48 h with IL-4 (50 ng/mL), IL-13 (50 ng/mL) and IL-25 (20 ng/mL), alone or in a mixture of all cytokines (IL MIX), then analyzed by immunofluorescence. *Left panel* immunofluorescent labeling of carbonic anhydrase

II (CA2). Scale bar 50 μ m. *Right panel* immunofluorescence analysis of loricrin (LOR). Scale bar 100 μ m. Nuclei were stained in blue (Hoechst 33258). Images are representative of three independent experiments using keratinocytes from three different donors

LOR [18, 26, 32]. After treatments with M β CD for 2 h and interleukins for 48 h, FLG-1 and FLG-2 mRNA expression levels are significantly reduced when the treatment is followed by exposure to IL-25 (Fig. 3a, b). Whereas CA2 mRNA expression is mostly increased by IL-4 after M β CD treatment, neural epidermal growth factor-like 2 (NELL2) mRNA level is increased by incubation with IL-4 or IL-13, independently of M β CD treatment. No additive effect is observed though when both IL are present in the mixed IL treatment (Fig. 3d, e).

Data illustrated in Fig. 3f indicate that the increase in hyaluronic acid (HA) synthase 3 (HAS3) relative mRNA level mainly occurs due the combination of incubation with M β CD and IL-4, although the IL MIX alone produces a significant induction.

Finally in the conditions studied herein, mRNA expression levels for thymic stromal lymphopoietin (TSLP) or heparin-binding EGF-like growth factor (HB-EGF) were not significantly affected unlike reported after shorter incubations periods following M β CD incubation [7].

Discussion and conclusion

It was previously shown that M β CD treatment in keratinocytes within RHE [7] and in combination with exposure to IL-4, IL-13 and IL-25 was adding features typical of AD to an in vitro epidermal model of this disease [7]. The present study was devoted at identifying individual roles of IL in creating such features in combination with M β CD treatment.

Th2-related cytokines alter morphology in RHE and prior incubation of the RHE with M β CD further enhances these alterations [7]. Epidermal spongiosis (one of the main characteristics of eczematous lesions) can be induced by IL-4 and IL-13 in skin equivalents [3, 15]. Spongiosis is further increased if previous M β CD incubation is performed in RHE [7]. Here, we demonstrate that M β CD contributes with IL-4 to create spongiosis in RHE, while simultaneously decreasing thickness of *stratum corneum* and inducing hypogranulosis, two other epidermal hallmarks of AD. Accordingly, when studying whether the epidermal barrier is functional in RHE, analyzed after incubation with IL-4, our results indicated that this cytokine produces the strongest alterations, together in TEER measured through the tissue, as well as in RHE permeability to LY.

In addition, IL-13 treatment combined to M β CD, or IL-25 alone, also significantly reduce TEER. According to the studies of LY permeation, maximal alterations are observed in barrier function when IL MIX was applied after M β CD treatment. This indicates potential additive effect of the IL mix when plasma membrane lipid microdomains of keratinocytes were previously disrupted. In the literature, IL-4 and IL-13, but not IL-25, have been shown responsible for barrier disruption in human airway epithelium, with maximal effect attributed to IL-4 [29].

LOR localizes inside granular keratinocytes in normal epidermis, but decreased signals are observed in lesional and non-lesional AD skin, as well as in RHE exposed to IL-4 and IL-13 [18]. Immunofluorescent detection of LOR in this study demonstrated important decrease only when M β CD treatment was performed before incubating RHE

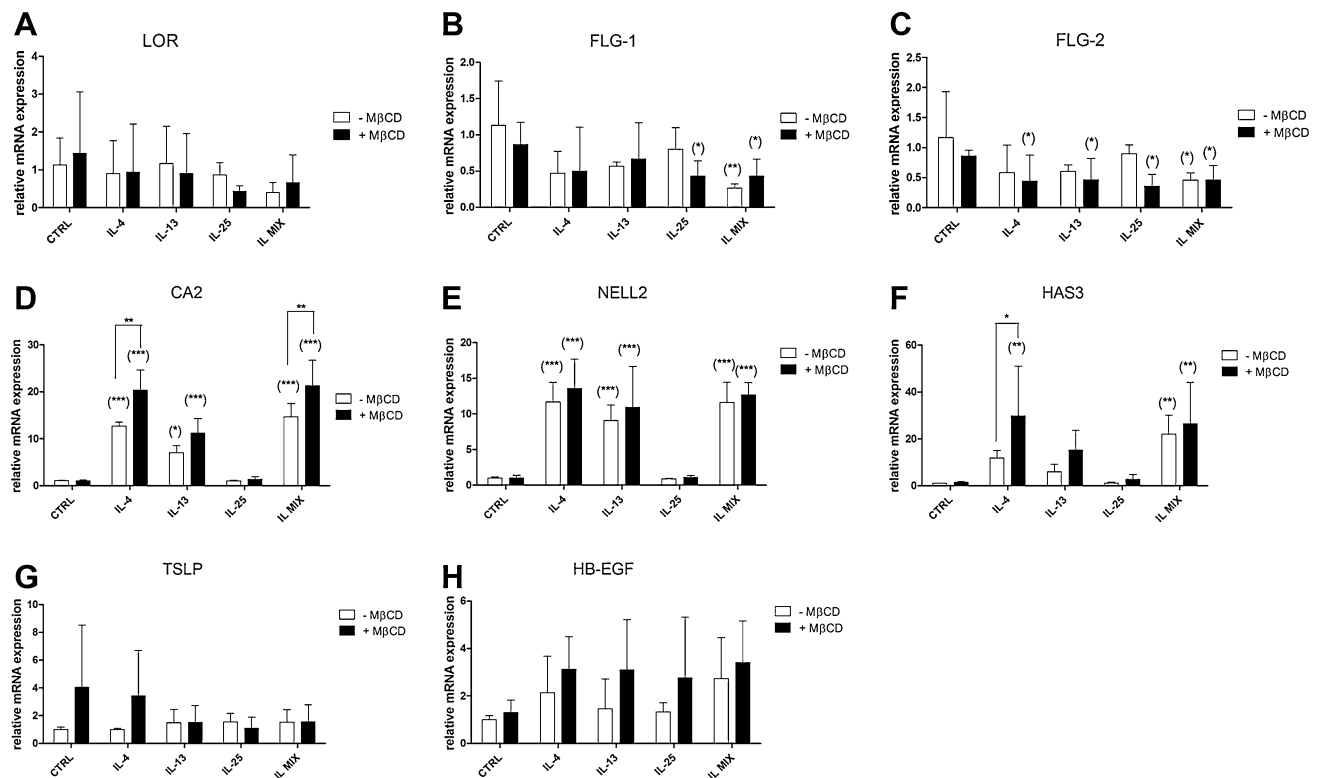


Fig. 3 Transcriptional study of genes commonly regulated in AD. mRNA expression was evaluated by RT-qPCR in RHE treated or not for 2 h with MβCD (7.5 mM), then cultured for 48 h with IL-4 (50 ng/mL), IL-13 (50 ng/mL) and IL-25 (20 ng/mL), alone or in a mixture of all cytokines (IL MIX). **a** Loricrin (LOR); **b** filaggrin-1 (FLG-1); **c** filaggrin-2 (FLG-2); **d** carbonic anhydrase II (CA2); **e** neural epidermal growth factor-like 2 (NELL2); **f** hyaluronan synthase 3 (HAS3); **g** thymic stromal lymphopoietin (TSLP) and

h heparin-binding EGF-like growth factor (HB-EGF) mRNA relative expression levels. Three independent experiments were performed. Error bars represent 95% confidence intervals. Two-way ANOVA was performed, followed by Bonferroni tests. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Each averaged measurement is compared with the one measured in control conditions (–MβCD) when the statistical significance is indicated between parentheses; otherwise measurements are compared with the linked condition

with IL-4 (Fig. 2; [7]). However, this loss in protein expression is not concomitant with a decrease at the mRNA level (Fig. 3a), suggesting either that an effect on transcript level should be analyzed earlier than after 48 h or that the reduced protein level of LOR might result from translational or post-translational regulation.

A lack of FLG expression is connected with disturbances in epidermal differentiation, alterations in barrier lipid composition and organization, reduction in natural moisturizing factors and increase in epidermal permeability [11]. Several studies have already illustrated reduced filaggrin expression as a consequence of keratinocyte exposure to Th2-cytokines [7, 12, 13, 17, 30], acting on both FLG-1 and -2 expression levels. Current data suggest that these cytokines require previous MβCD treatment to significantly reduce FLG-1 and FLG-2 mRNA levels. However, when these cytokines were used individually, no significant decrease in FLG-1 or FLG-2 expression levels could be found, unlike the observations performed after exposure to IL MIX (Fig. 3b, c).

Gene expression for CA2 and NELL2 is upregulated in AD, although no pathophysiological role has been assigned yet [15, 16], and both genes are upregulated in this model [7]. However, data presented herein suggest that the treatment with MβCD prior to the incubation of RHE with IL-4 induces a stronger upregulation of CA2 gene expression. Regarding regulation of NELL2, Th2-cytokines (IL-4 and IL-13) exhibit potential roles but without any additive effect when combined, or after MβCD treatment.

HA synthesis is increased in inflammatory skin diseases and previous studies have shown that HAS3, an enzyme responsible for epidermal HA synthesis, is upregulated in AD lesional skin, as well as in culture when MβCD is combined with exposure of RHE to IL MIX [7, 20, 25]. Skin of AD patients also expresses HB-EGF which in turn can modulate HAS1 and HAS3 gene expression [20]. In addition, studies performed on RHE revealed that HB-EGF dose-dependently raises HAS3 expression [20]. In this study, no significant increase in HB-EGF expression was observed, whilst HAS3 expression was mainly induced by

incubation with IL-4 after M β CD. Expression levels for HB-EGF were reported as increased in keratinocytes solely during the first hours that follow M β CD treatment, but no more 48 h later [6, 9, 21].

The absence of variation in TSLP gene expression is likely a similar phenomenon. Indeed, whereas M β CD incubation significantly increases TSLP expression levels for a few hours [7], it has been reported that IL-4 and IL-13 cannot stimulate TSLP expression by themselves, but rather require combined exposure to TNF α or IL-1 α to reach significant induction [2].

Finally, cholesterol controls cell signaling in keratinocytes [19, 21]. Current data and previous works [6, 7, 20, 21] concur to assess that lipid microdomains influence epidermal signaling, thereby contributing with IL-4 to alter the phenotype of keratinocytes, potentially in all epidermal layers. Both IL-4 and IL-13 act on keratinocytes via the IL-4R α /IL-13R α 1 receptor [1]. Furthermore, this receptor is found upregulated in chronic inflammatory diseases like AD and psoriasis [34]. Interestingly, IL-13R α 2, IL-13R α 1, IL-4R α and IL17RA (subunit of the IL25 receptor) receptors are all upregulated in keratinocytes after M β CD treatment [21], indicating that cholesterol may also regulate cell signaling through these receptors, perhaps due to localization inside lipid microdomains.

In conclusion, IL-4 and IL-13 induce rather similar response profiles by RHE in most tested conditions, but their combination does not produce stronger effects in this model. This may be explained by signaling through common receptors. A significant contribution of IL-25 after M β CD treatment to reduce FLG expression was reported here with simultaneous decreased TEER. However, major consequences mimicking hallmarks of AD in this RHE model mainly resulted from incubation of epidermal keratinocytes with IL-4 after M β CD treatment.

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Compliance with ethical standards

The authors state no conflict of interest. This study was conducted according to the Declaration of Helsinki Principles and approved by the Ethics Committees. Financial support was provided by FRFC 2.4.522.10F Grant to YP and FAPESP (Grant # 2015/23938-0) to TDNP.

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SUPPLEMENTARY INFORMATIONS

Methyl- β -cyclodextrin treatment combined with interleukin-4 reproduces major features of atopic dermatitis in a 3D-culture model

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	Forward (5' – 3')	Reverse (5' – 3')
RPLP0	ATCAACGGGTACAAACGAGTC	CAGATGGATCAGCCAAGAAGG
FLG	GGGCACTGAAAGGCAAAAAG	CACCATAATCATAATCTGCACTACCA
LOR	TCATGATGCTACCCGAGGTTTG	CAGAACTAGATGCAGCCGGAGA
HAS3	GCCCTCGGCGATTCTG	TGGATCCAGCACAGTGTCTAGA
CAII	AACAATGGTCATGCTTTCAACG	TGTCCATCAAGTGAACCCAG
NELL2	TAAGGGTATAATGCAAGATGTCCAATT	AGATCTGGGCACTGAGCAATAAA
TSLP	CGCGTCGCTCGCCAAAGAAA	TGAAGCGACGCCACAATCCTTG
HB-EGF	TGGCCCTCCACTCCTCATC	GGGTCACAGAACCATCCTAGC
FLG2	ACCAGGTTCACTTAAACTTGCA	ATGACATCCACTGTGTCTGGATC

TABLE S1: Oligonucleotides used to perform the RT-qPCR

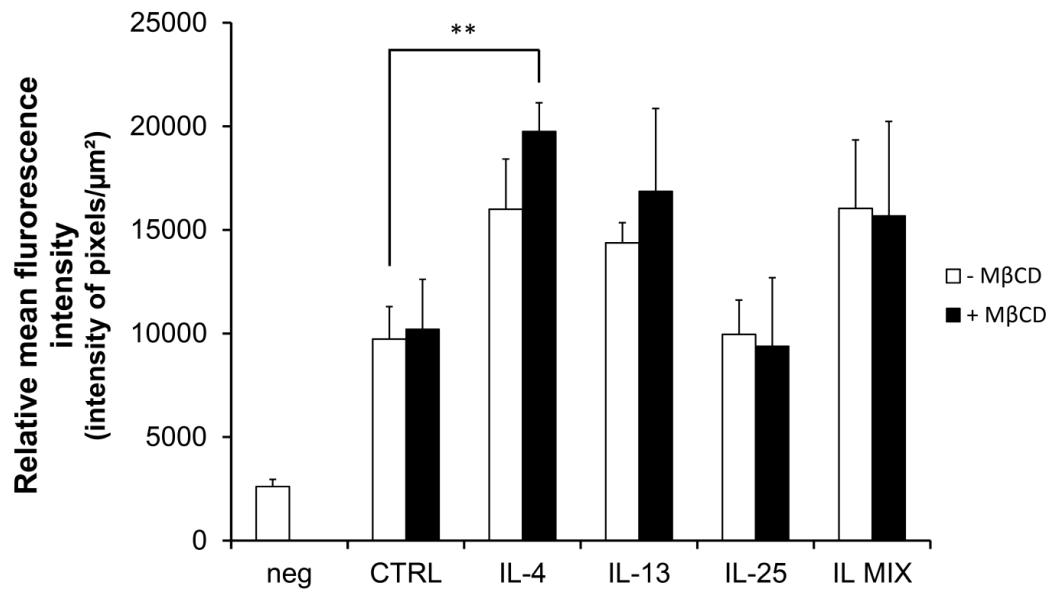


Figure S1: Measurement of CA2 labelling intensity. Reconstructed human epidermis were pretreated (+MβCD) or not (-MβCD) for 2 hours with MβCD, then cultured for 48 hours with IL-4, IL-13 and IL-25, alone or in a mixture containing all cytokines (IL MIX), then analyzed by immunofluorescence for carbonic anhydrase II (CA2). Data are representative of three independent experiments using keratinocytes from three different donors. Negative controls (neg) were performed using secondary antibodies only. Intensity of the detection signal was measured using the image analysis software provided with the Olympus BX63 microscope. Intensity of pixels was divided by the area (μm²) of each epidermis. Means and SD were then calculated and ANOVA 1RM was performed (** p<0.01).