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MβCD concurs with IL-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis

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Abbreviations: AD, atopic dermatitis; TSLP, thymic stromal lymphopoietin; RHE, reconstructed human epidermis; MβCD, methyl-β-cyclodextrin; IL, interleukin; TEER, trans-epidermal electrical resistance; FLG, filaggrin; LOR, loricrin; CA2, carbonic anhydrase II; NELL2, neural epidermal growth factor-like 2; HAS, hyaluronic acid synthase; HA, hyaluronic acid.

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BACKGROUND
Reconstructed human epidermis (RHE) mimic normal human \textit{in vivo} epidermis in terms of histology, distribution of differentiation markers, and barrier functionality (1).

A typical transcriptional profile and the activation of signalling pathways reminiscent of atopic dermatitis (AD) lesional skin can be obtained in RHE upon incubation with methyl-β-cyclodextrin (MβCD) (2, 3), a molecule that extracts cholesterol from plasma membranes, thereby disrupting lipid microdomains. However, barrier function and morphology remain unaltered in those conditions, requiring further refinement of the model.

QUESTION ADDRESSED
Because of the crucial role played by Th2 immune response in AD, a mixture of interleukins linked to this Th2 response (IL-4, IL-13 and IL-25) was used in addition to MβCD, in an attempt to induce most of the epidermal AD features in RHE. Ultimately, a valid RHE model of acute AD would allow studying the epidermal component of pathogenesis.

EXPERIMENTAL DESIGN
RHE were incubated for two hours with MβCD in order to induce cholesterol depletion (Figure S1), then for 48 hours with the interleukin mix and compared to control tissues, and to tissues incubated with either MβCD or interleukins.

RESULTS
When allowed to recover in fresh culture medium after incubation with MβCD for 2 hours, RHE revealed no obvious histological alteration when compared with untreated RHE (Figure 1a). Conversely, RHE treated with IL-4, IL-13 and IL-25 for 48 hours displayed intercellular space widening similar to spongiosis (already reported for IL-4 and IL-13 (4-6)) and hypogranulosis, two histological hallmarks of lesional AD skin (Figure S2). These morphological alterations were enhanced when keratinocytes were challenged by MβCD before being incubated with the three interleukins (Figure 1a).

Barrier function, weakened in AD, was studied using two assays: measurement of trans-epithelial electrical resistance (TEER) and assessment of permeability to the fluorescent dye lucifer yellow (LY) through the RHE. A significant decrease in TEER was observed in RHE incubated with the interleukins, and worsened upon membrane cholesterol depletion (Figure 1b). Accordingly, permeability of RHE towards LY was significantly increased after combined treatments, whereas cholesterol depletion alone or incubation with interleukins only were insufficient to elicit an effect (Figure 1c).

Then, expression of atopic dermatitis markers was investigated. Gene expression of filaggrin (FLG) and loricrin (LOR), epidermal differentiation genes usually downregulated in lesional AD, was analyzed through RT-qPCR (Figure 2a). FLG and LOR exhibited reduced mRNA levels in RHE incubated with IL-4, -13 and -25, as already reported in the literature regardless of the different concentrations and timings used (5). This decrease was exacerbated and became significant when keratinocytes were challenged by cholesterol depletion before incubation with the interleukins. Simultaneously, relative expression levels of carbonic anhydrase II (CA2) and neural epidermal growth factor-like 2 (NELL2), two genes upregulated in AD (7), were significantly enhanced in RHE in response to incubation with interleukins (Figure 2a).

Immunohistological analysis confirmed decreased expression of LOR and increased expression of CA2 in AD lesions (Figure S3a). Similar changes were observed in RHE treated with interleukins and became even more evident in tissues previously treated with MβCD (Figure S3b).
Expression levels for hyaluronan synthase 3 (HAS3) were found elevated in RHE exposed to interleukins and further increased after combined treatments (Figure 2a), in agreement with the upregulation observed in AD (4, 8). Though, no significant changes were found with respect to HAS1 expression levels (data not shown), unlike data collected from AD lesions (8).

Fluorescent detection of hyaluronic acid (HA) revealed increased staining of intercellular spaces between keratinocytes in AD lesions (Figure S4a). HA was also more strongly detected in RHE incubated with interleukins, particularly after previous challenging by MβCD (Figure S4b). Accordingly, increased HA concentrations were measured in culture medium of treated RHE (Figure S4c).

TSLP is a cytokine, upregulated in AD, which contributes to Th2 immune response activation, promotes itch in skin and the ‘atopic march’ in general (9). However, despite significant induction in response to two hours of MβCD (Figure S1c), mRNA expression levels for TSLP were no longer above baseline 48 hours later (Figure 2a). However, in the meantime, over the 6 hours period following cholesterol depletion (Figure 2b), data illustrate that interleukins and MβCD concur to significantly enhance TSLP expression in challenged RHE.

CONCLUSIONS
This study illustrates that challenging RHE through cholesterol depletion, or by incubation with IL-4, IL-13 and IL-25, results in multiple epidermal alterations. But interestingly, a combination of those two treatments has additive effects, allowing mimicking an AD-like epidermal phenotype in vitro in the absence of immune cells. Indeed, morphological alterations such as tissue spongiosis and hypogranulosis, alterations in mRNA expression levels and histological localizations of typical AD and differentiation markers, modulations of epidermal HA synthesis, and epidermal barrier weakening represent hallmarks of AD epidermis.

In vivo, such alterations become responsible for activation of the immune system because they promote penetration of pathogens or allergens, thereby creating some vicious circle likely responsible for AD lesions development (Figure 2c).

Most probably, challenging of keratinocytes by MβCD models alterations in cell signalling through disorganization of specific lipid microdomains in this cell type (3). Interestingly, gene expression levels for IL-13Rα2, IL-13Rα1, IL-4Rα and IL-17RA (subunit of IL-25 receptor) were upregulated in keratinocytes incubated with MβCD (3). This indicates that cholesterol-containing lipid microdomains could regulate signalling through these receptors and could therefore explain the additive effects observed in this study.

In conclusion, this study confirms that membrane cholesterol depletion in keratinocytes concurs with Th2-related cytokines to elicit an AD-like phenotype. The present model could be used in order to study other features encountered in AD epidermis, but also evaluate compounds intending to relieve, prevent AD lesions or restore keratinocyte functions.

ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST
The authors state no conflict of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. Morphology and barrier efficiency in treated RHE.

(a) Histological sections of RHE treated for 2 hours with MβCD followed by 48 hours of recovery (MβCD 2h + recovery 48h) or treated for 48 hours with IL-4, IL-13 and IL-25 (IL 48h), or a combination of both treatments (MβCD 2h + IL 48h), compared to non-treated RHE (Ctrl 48h). Images are representative of three independent cultures. Scale bar=50µm.

Analysis of barrier function, by (b) trans-epidermal electrical resistance (TEER) or (c) fluorescence quantification of lucifer yellow permeation in the culture medium, of RHE treated for 2 hours with MβCD (MβCD2h), followed or not by 48h incubation with interleukins 4, 13 and 25 (IL 48h). Data represent measurements performed on three independent cultures (error bars = SEM, one-way RM ANOVA and *P<0.05, **P<0.01).

Figure 2. Transcriptional regulation of atopic dermatitis markers in treated RHE and hypothetical simplified model for AD-like pathogenesis.

(a) RT-qPCR analysis of the expression of filaggrin (FLG), loricrin (LOR), thymic stromal lymphopoietin (TSLP), carbonic anhydrase II (CA2), neural epidermal growth factor-like 2 (NELL2) and hyaluronan synthase 3 (HAS3) were performed on total RNA extracted from RHE treated with MβCD (MβCD2h)
and/or interleukins 4, 13 and 25 (IL48h). Levels of mRNA expression in control conditions were arbitrarily fixed at 1. Error bars represent 95% confidence intervals (n=3 independent cultures, one-way RM ANOVA, *P<0.05, **P<0.01, ***P <0.001).

(b) TSLP mRNA expression was quantified by RT-qPCR in RHE incubated for 2 hours with MβCD, followed or not by incubation with IL-4, IL-13 and IL-25 for 2h, 4h or 6h. Values are expressed relative to control condition (no MβCD + no IL). Error bars represent 95% confidence intervals (n=3 independent cultures, two-way RM ANOVA, *P<0.05, **P<0.01, ***P <0.001)

(d) Plasma membrane cholesterol depletion with MβCD in RHE challenges keratinocytes which in turn produce TSLP, a Th2 response-activating cytokine. Addition of Th2-related interleukins in the culture medium intends to mimic consequences of the activation of immune cells in vivo. Response of challenged RHE includes morphological alterations as well as weakening of the epidermal barrier. In vivo, activation of the immune system resulting from altered barrier function, would maintain the vicious circle observed in AD.
MβCD concurs with IL-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis

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Figure S1 Effect of incubation with MβCD for 2 hours on RHE analysed at day 11 of tissue reconstruction.

(a) Localization of cholesterol. Frozen sections of RHE were stained with filipin and observed under a fluorescent microscope using UV wavelength as excitation light (DAPI filters). RHE were left untreated (MβCD -) or treated with 7.5mM MβCD for two hours (MβCD +) followed by different recovery periods (0h, 2h, 4h, 6h and 24h). White dotted lines delineate the polycarbonate filter (scale bar = 50 μm). (b) Cholesterol was quantified in RHE left untreated (ctrl 0h and ctrl 24h) or in RHE incubated with 7.5mM MβCD for two hours before different recovery periods (0h, 2h, 4h, 6h and 24h). (c) RT-qPCR analysis of TSLP expression in RHE, incubated or not with MβCD during 1h or 2h. The values are relative to the control conditions (Ctrl 1h and Ctrl 2h). (d) RT-qPCR analysis of heparin-binding EGF-like growth factor (HB-EGF), IL-8 and plasminogen activator urokinase receptor (PLAUR) mRNA expression levels in RHE during the recovery period after 2 hours of MβCD treatment. Error bars represent 95% confidence intervals (n=3, two-way repeated-measures analysis of variance, **P<0.01).
Figure S2. Morphology of healthy and lesional AD skin biopsies.

Histological analysis sections of healthy and lesional AD skin biopsies. After fixation, biopsies were embedded in paraffin before staining with haematoxylin and eosin. Scale bars=50µm.
Figure S3. Labeling of atopic dermatitis-associated markers.

Immunoperoxidase-labelling of carbonic anhydrase 2 (CA2) and hematoxylin counterstaining as well as immunofluorescence labelling of loricrin (LOR) in green and nuclei in blue (Hoechst 33258).

(a) Healthy, non-lesional and lesional atopic dermatitis skin sections. (b) RHE treated for 2 hours with MβCD followed (MβCD 2h + IL 48h) or not (MβCD 2h + recovery 48 h) by 48h of incubation in the presence of IL-4, -13 and -25 (IL 48h) were processed for histology and analyzed for CA2 and LOR. Results shown are representative of three independent experiments. Scale bars= 50µm.
Figure S4. Hyaluronic acid (HA) staining and concentration in the culture medium.

(a) Healthy, non-lesional and lesional atopic dermatitis skin biopsies or (b) reconstructed human epidermis (RHE) were processed for histology and immunostaining of hyaluronic acid (HA) was realised using a biotinylated hyaluronic acid (HA)-binding protein staining (green). Hoechst 33258 (blue) was used for nuclei staining. RHE were treated with a combination of 2 hours of treatment with MβCD followed by 48h of interleukins 4, 13 and 25 treatment at the 11th day of reconstruction (MβCD 2h + IL 48h). These RHE were compared to RHE treated with MβCD during 2 hours followed by incubation in fresh culture medium (MβCD 2h + recovery 48 h) or treated during 48 hours with interleukins only (IL 48h) as well as untreated RHE (ctrl 48h). Results shown are representative of three independent experiments. (c) Culture media of the last 48 hours of culture were harvested and released HA concentrations were measured. Data are shown as means ± SD (n=3, one-way repeated-measures analysis of variance, *P<0.05).
MATERIALS AND METHODS

Skin biopsies and reconstruction of human epidermis

Healthy adult human skin samples were obtained from abdominoplasties (kindly provided by Dr. B. Bienfait, Clinique St. Luc, Namur-Bouge, Belgium) and keratinocytes were cultured as described (De Vuyst et al., 2014; Frankart et al., 2012; Poumay et al., 2004). After 11 days of culture, RHE were treated for 2 hours with 7.5 mM MβCD (Sigma-Aldrich, Diegem, Belgium). A treatment for two hours with MβCD was selected because it induced a significant increase in expression of thymic stromal lymphopoietin (TSLP), a well-known actor in AD (Figure S1c), without alteration of RHE viability (data not shown). Transient extraction of cholesterol as well as activation of known target genes by MβCD was evaluated (Figure S1) confirming previous data (De Vuyst et al., 2015).

After MβCD treatment, RHE were rinsed before culture for the next 48 hours in the presence of respectively 50 ng/ml IL-4, 50 ng/ml IL-13, and 20 ng/ml IL-25 (Peprotech, London, UK). The choice for IL-4 and IL-13 was first based on experiments already published showing elevated expression levels of these cytokines in lesional AD skin (Howell et al., 2009; Tazawa et al., 2004). Further interesting, these interleukins could induce a significant decrease of filaggrin expression in primary human keratinocytes (Howell et al., 2009; Pellerin et al., 2013). Furthermore, other in vitro models of AD also used cytokine mix and notably IL-4 and IL-13 (Bernard et al., 2012; Danso et al., 2014; Kamsteeg et al., 2011). IL-25 was added for different reasons. First, because increased levels of this cytokine and its receptor were detected in the epidermis of AD patients (Akhtar et al., 2015; Hvid et al., 2011; Wang et al., 2007). Besides this reason, IL-25 would play a dual role in AD, promoting type 2 cell immunity (Salimi et al., 2013; Wang et al., 2007), but also inhibiting filaggrin (FLG) expression in keratinocytes (Hvid et al., 2011; Pellerin et al., 2013), thereby affecting skin barrier in AD patients. Therefore, acting both on immune and epidermal cells, IL-25 may provide, in collaboration with TSLP, some explanation for the link observed between inflammation and barrier disruption in AD lesions (Deleuran et al., 2012). Furthermore, in the skin of AD patients, expression of IL-25 and its receptor was increased after exposure to allergens (Corrigan et al., 2011). For all these reasons, we thought it could be of interest as a potentially therapeutic target and could therefore be added in our model contribute to the development of the pathogenesis, and partly explain the vicious circle observed in AD.

AD biopsies were collected from lesional or surrounding non-lesional skin of patients suffering from longstanding disease.

This study was conducted according to the Declaration of Helsinki Principles and approved by the Ethics Committees.

Morphological analysis and labelling

RHE or biopsies were embedded in paraffin. Sections were stained with haematoxylin-eosin-(saffron). Images were obtained by light microscopy using Olympus BX63 microscope. For CA2 immunodetection, antigen retrieval was performed by heat-induction. Anti-rabbit CA2 from Sigma-Aldrich (Diegem, Belgium) was diluted 1:500, anti-rabbit LOR from Abcam (Cambridge, UK) 1:100 and biotinylated HA-binding complex (generously provided by Prof. M. Tammi) was diluted 1:50. Tissue samples were incubated for one hour with anti-rabbit HRP secondary antibody (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) diluted 1:100 for the CAII staining or with Alexa 488 conjugated anti-rabbit IgG (Life technologies, New York, USA) also diluted 1:100 for the LOR staining or with fluorescein-labelled streptavidin (Vector Laboratories, Peterborough, UK) for the HA labelling.
TEER measurements and permeability to lucifer yellow

TEER measurements were performed using Millicell electrical resistance system from Merck-Millipore (Overijse, Belgium). The fluorescent dye lucifer yellow was obtained from Sigma-Aldrich (Diegem, Belgium) and applied topically at a concentration of 1 mM in PBS on the RHE for 6 hours at 37°C. Afterwards, the medium was collected for fluorescence measurement ($\lambda_{ex}$: 485nm; $\lambda_{em}$: 535nm).

Analysis of mRNA expression by RT-qPCR

Total RNA was extracted from RHE using RNeasy kit (Qiagen, Hilden, Germany), reverse transcribed using the Super Script II RNase H-reverse transcriptase kit (Invitrogen, Merelbeke, Belgium) and amplified with the FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) in the Light Cycler® 96 Real-Time PCR system (Roche, Diagnostics, Mannheim, Germany). Results were normalized to the RPLP0 reference gene (Minner and Poumay, 2009; Mound et al., 2015). Primers (Eurogentec, Liège, Belgium) were used at a concentration of 300 nM. Sequences are available in table S1. Data were analyzed using the $\Delta\Delta$Cq quantitative method and 95% confidence intervals were calculated after one-way RM ANOVA followed by Tukey tests or two-way RM ANOVA followed by Bonferroni correction using SigmaStat 3.0.

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<th>TABLE S1: Oligonucleotides used to perform the RT-qPCR</th>
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Filipin staining

Epidermis were embedded in OCT (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and cryopreserved at -80°C. Frozen sections of RHE were realised and fixed for 30 min with 4% paraformaldehyde at room temperature. After a washing step in PBS, sections were incubated with 50 µg/ml of filipin from Sigma-Aldrich (Diegem, Belgium) for 30 min at room temperature. After a washing step, sections were mounted using Mowiol (Molecular Probes, Ghent, Belgium) and filipin was visualised by epifluorescence using Olympus AX70 microscope with a UV filter. Pictures were taken using uniform conditions (quickest exposure times to avoid the rapid bleaching of filipin).

Cholesterol assay

Cellular cholesterol was extracted and purified from RHE by a procedure derived from the protocol of Selvais et al (Selvais et al., 2011). After lysis of RHE in chloroform, washing steps were realised using successively methanol, NaCl and CaCl₂/methanol. After centrifugation, the organic phase was collected, Triton X-100/acetone was added and samples were evaporated before being solubilised in demineralised water. Cholesterol was assayed using the Amplex Red Cholesterol Assay kit from Molecular probes (Ghent, Belgium) according to the manufacturer’s protocol.
HA quantification

HA was quantified in culture media using Hyaluronan DuoSet kit (R&D systems, Abingdon, UK).


