

TNF- α and Th2 Cytokines Induce Atopic Dermatitis–Like Features on Epidermal Differentiation Proteins and Stratum Corneum Lipids in Human Skin Equivalents

Mogbekeloluwa O. Danso¹, Vincent van Drongelen^{1,2}, Aat Mulder², Jeltje van Esch¹, Hannah Scott¹, Jeroen van Smeden¹, Abdoelwaheb El Ghalbzouri² and Joke A. Bouwstra¹

Atopic dermatitis (AD) is a chronic inflammatory skin disease in which the skin barrier function is disrupted. In this inflammatory AD environment, cytokines are upregulated, but the cytokine effect on the AD skin barrier is not fully understood. We aimed to investigate the influence of Th2 (IL-4, IL-13, IL-31) and pro-inflammatory (tumor necrosis factor alpha (TNF- α)) cytokines on epidermal morphogenesis, proliferation, differentiation, and stratum corneum lipid properties. For this purpose, we used the Leiden epidermal model (LEM) in which the medium was supplemented with these cytokines. Our results show that IL-4, IL-13, IL-31, and TNF- α induce spongiosis, augment TSLP secretion by keratinocytes, and alter early and terminal differentiation-protein expression in LEMs. TNF- α alone or in combination with Th2 cytokines decreases the level of long chain free fatty acids (FFAs) and ester linked ω -hydroxy (EO) ceramides, consequently affecting the lipid organization. IL-31 increases long chain FFAs in LEMs but decreases relative abundance of EO ceramides. These findings clearly show that supplementation with TNF- α and Th2 cytokines influence epidermal morphogenesis and barrier function. As a result, these LEMs show similar characteristics as found in AD skin and can be used as an excellent tool for screening formulations and drugs for the treatment of AD.

Journal of Investigative Dermatology (2014) **134**, 1941–1950; doi:10.1038/jid.2014.83; published online 27 March 2014

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with clinical manifestations of erythema, dryness, and pruritus (Leung and Bieber 2003; Cork *et al.*, 2006). The epidermal barrier is disrupted in both lesional and non-lesional AD skin and characterized by the presence of Th1, Th2 and Th22 immune responses (Neis *et al.*, 2006; Gittler *et al.*, 2012). The

interaction between cytokines and the skin barrier is not yet fully understood. Therefore, this study aims to determine whether cytokines influence lipid and protein synthesis in the epidermis and as a consequence affect skin barrier lipid composition and organization.

A fully differentiated epidermis consists of a proliferating stratum basale (SB), differentiating stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). In the basal layer, keratinocytes synthesize proteins such as keratin 5 and 14, while differentiating keratinocytes synthesize proteins such as keratin 10 (K10), filaggrin, involucrin and loricrin. The latter three proteins are associated with the cornified envelope, a surrounding layer of the corneocytes, located in the SC (Steven *et al.*, 1990; Ishida-Yamamoto *et al.*, 1993). Filaggrin especially plays an important role in AD because the occurrence of AD is strongly associated with mutations in the filaggrin gene encoding the barrier protein filaggrin (Palmer *et al.*, 2006; Rodríguez *et al.*, 2008; Brown and McLean 2009; Akiyama 2010). The skin barrier lipids, which are located within the SC also play a role in AD as changes in their chain length distribution correlate with an impaired skin barrier (Janssens *et al.*, 2012). The SC contains a highly organized lipid matrix located in the intercellular spaces between the corneocytes. The organization and composition

¹Department of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands and ²Department of Dermatology, Leiden University Medical Centre, Leiden, The Netherlands

Correspondence: Joke A. Bouwstra, Department of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Einsteinweg 55, 2333 CC Leiden, The Netherlands. E-mail: bouwstra@lacdr.leidenuniv.nl

Abbreviations: CER, ceramides; CHOL, cholesterol; E, ester-linked fatty acids; FFA, free fatty acid; FTIR, Fourier transformed infrared spectroscopy; HPTLC, high performance thin layer chromatography; K10, keratin 10; LC/MS, liquid chromatography/mass spectrometry; LEM, Leiden epidermal model; LPP, long periodicity phase; MUFA, unsaturated free fatty acid; O, ω -hydroxy fatty acids; SAXD, small angle x-ray diffraction; SB, stratum basale; SC, stratum corneum; SFA, saturated free fatty acid; SG, stratum granulosum; SS, stratum spinosum; Th1, T helper type-1; Th2, T helper type-2; TSLP, thymic stromal lymphopoietin

Received 19 June 2013; revised 23 January 2014; accepted 24 January 2014; accepted article preview online 11 February 2014; published online 27 March 2014

of the SC lipid matrix is crucial for a competent skin barrier (Elias *et al.*, 2003; Feingold 2007). The main lipid classes in human SC are ceramides (CER), free fatty acids (FFAs) and cholesterol. Twelve CER subclasses have been identified in human SC with broad chain length distributions observed with FFA and CERs (Wertz *et al.*, 1985; Masukawa *et al.*, 2009; van Smeden *et al.*, 2011). SC lipids form ordered crystalline lamellar phases with repeat distances of 13 nm (long periodicity phase, LPP) and 6 nm (short periodicity phase, SPP) (Bouwstra *et al.*, 1991; Supplementary Figure S1 online). Within the lamellae, the lipids form mainly, an orthorhombic (dense) lateral packing in human SC, while a fraction of lipids are organized in a hexagonal (less dense) lateral packing (Gay *et al.*, 1994; Pilgram *et al.*, 2001; Supplementary Figure S1 online). The CER subclass distribution of AD skin varies from healthy skin (Imokawa *et al.*, 1991; Yamamoto *et al.*, 1991; Di Nardo *et al.*, 1998; Bleck *et al.*, 1999; Jungersted *et al.*, 2010). In addition, AD skin shows i) a decrease in very long chain CERs, ii) reduction in long chain FFAs level and iii) increased level of unsaturated FFAs (Ishikawa *et al.*, 2010; Janssens *et al.*, 2012; van Smeden *et al.*, 2014). These changes are more prominent in lesional than in non-lesional AD skin and correlates with a less dense SC lipid lateral packing and reduced skin barrier function (van Smeden *et al.*, 2014).

It is hypothesized that a disrupted barrier in AD makes the skin vulnerable to penetration of allergens/irritants through the skin, causing a Th2 immune response in the epidermis. Keratinocytes respond to this by secreting thymic stromal lymphopoietin (TSLP). TSLP activates dendritic cells (Watanabe *et al.*, 2004) which further enhances the immune response by proliferation and differentiation of CD4⁺ T-cells. TSLP is also considered to be a link between AD and the development of asthma/atopic march (Zhu *et al.*, 2011).

The aim of this study was to investigate the direct role cytokines play in the development of pathophysiological changes occurring in AD skin focusing on both lipids and epidermal differentiation proteins. Therefore, we used 3-dimensional (3D) tissue engineered human skin equivalents, namely the Leiden epidermal model (LEM), since it harbors a fully differentiated epidermis (El Ghalbzouri *et al.*, 2008). The culture medium was supplemented with IL-4, IL-13, IL-31 (Th2 cytokines) and TNF- α (pro-inflammatory cytokine) singly or a combination during generation of the LEMs. If the supplemented LEMs mimic several features of AD skin, the *in vitro* skin models can be used to examine pharmacologic interventions for AD. This is due to the need for *in vitro* skin models that mimic important features of AD skin due to large variations in skin architecture between the commonly used mice skin and human skin and limitations in the number of drugs that can be tested at a time during a clinical study.

Our results show that cytokines contribute to the changes in epidermal morphology, differentiation and skin barrier lipids observed in AD. This demonstrates that the LEM is an excellent model for studying the pharmacological intervention of topical formulations for the treatment of AD in *in vitro* settings.

RESULTS

Cytokines influence epidermal organization and differentiation

First, we examined the morphology of LEMs supplemented with cytokines. In control LEMs, all epidermal strata are present. LEMs supplemented with IL-4, IL-13 or IL-31 also show the presence of all epidermal strata. However, spongiotic lesions i.e. inter-cellular oedema of keratinocytes, is present within the lower layers of the epidermis. LEMs supplemented with TNF- α only, show spongiosis and flatter suprabasal keratinocytes than the control. In LEMs stimulated with Th2 cytokines, with or without TNF- α , spongiosis and a comparable thickness of the viable epidermis was observed as with single supplementations (Figure 1a).

Next, the influence of these cytokines on epidermal differentiation-protein expression was examined by immunohistochemical (IHC) staining. K10 is expressed in all suprabasal layers in control LEMs (Figure 1a). However, in LEMs with single supplementations of Th2 cytokines or TNF- α , the SB and sections of the lower SS do not express K10. The combination of Th2 cytokines with or without TNF- α , show also this delayed K10 expression (Figure 1a). In control and cytokine stimulated LEMs, filaggrin is expressed in the SG and the interface between the SG and the SC. However, all cytokine stimulated LEMs show reduced filaggrin staining than control LEMs (Figure 1a). qPCR and western blotting analysis also show that these cytokines significantly reduce mRNA and protein expression of filaggrin (Supplementary Figure S2 online, Figure 1b respectively). The expression of loricrin was observed in the SG in both control and cytokine treated LEMs but cytokine stimulated LEMs show discontinuous or patchy expression of loricrin compared with control LEMs (Figure 1a). Th2 cytokines and TNF- α reduced loricrin mRNA expression (38–68%), however, not significantly (Supplementary Figure S2 online). Loricrin protein expression was only clearly reduced with IL-13 or a combination of Th2 cytokines. This is different from the effect of cytokines on filaggrin expression.

Cytokines increase basal cell proliferation and TSLP secretion of keratinocytes

After analyzing the expression of epidermal differentiation proteins in cytokine supplemented LEMs, we further examined basal keratinocyte proliferation by Ki67 staining. Compared with control LEMs, basal cell proliferation increased significantly with the supplementation of IL-4, IL-31 or TNF- α , whilst IL-13 did not affect basal cell proliferation (Figure 2a). The Th2 cytokine mixture with or without TNF- α significantly increased basal keratinocyte proliferation compared with control LEMs. LEMs with increased basal proliferation also show delayed K10 expression and reduced filaggrin expression except for supplementation of IL-13.

Supplementation of single cytokines or a combination enhanced significantly medium TSLP levels ($P < 0.05$; Figure 2b) compared with the control. There was no significant difference in TSLP levels between supplementation of single cytokines and a combination of only Th2 cytokines ($P > 0.05$ – 0.95). In addition, TSLP secretion was not significantly different in LEMs when comparing the mixture of only Th2 cytokines, with the mixture of Th2 cytokines and TNF- α ($P = 0.27$).

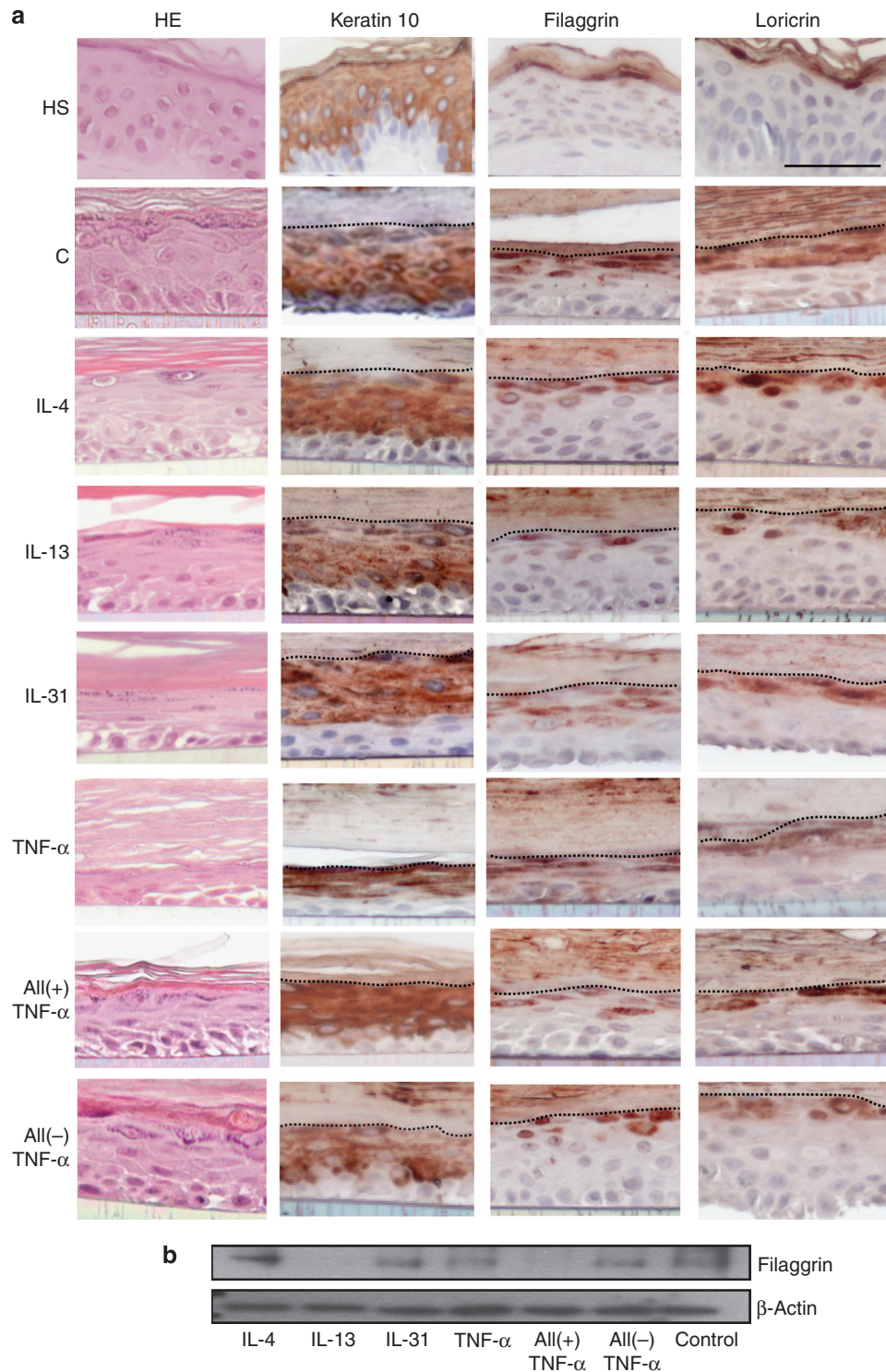


Figure 1. TNF- α and Th2 cytokines alter morphology and epidermal protein expression in Leiden epidermal models (LEMs). (a) Cross sections of LEMs cultured in the presence or absence of cytokines. 20x Magnification; scale bar = 50 μ m. A black dotted line demarcates the viable epidermis from the stratum corneum. Overall morphology was assessed with hematoxylin and eosin (HE) staining and immunohistochemical analysis was performed for K10, filaggrin, and loricrin. Staining of the stratum corneum is considered background/non-specific. (b) Filaggrin western blot of cytokine stimulated and control LEMs (HS, human skin; C, control; All(+)TNF- α , combination of IL-4, IL-13, IL-31, and TNF- α ; All(-)TNF- α , combination of IL-4, IL-13, IL-31). Th2, T helper type-2; TNF- α , tumor necrosis factor alpha.

TNF- α and IL-31 affect SC lipid composition in LEMs

After profiling the effect of these cytokines on epidermal morphology and protein expression, we focused on the SC

lipid barrier properties. The main SC lipid subclasses CER, cholesterol and FFAs are present in the LEM irrespective of cytokine addition (Figure 2c). In addition, the total amount of

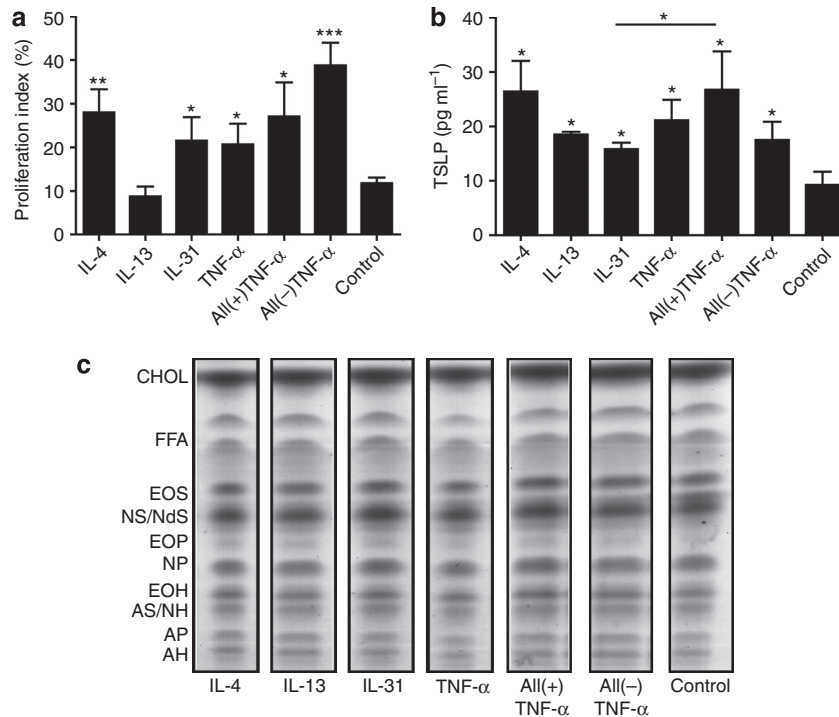


Figure 2. Basal cell proliferation, TSLP secretion, and SC lipid composition (HPTLC) of cytokine supplemented Leiden epidermal models (LEMs). (a) Proliferation index (PI) in LEMs supplemented with cytokines singly or in combination. The PI was determined as the percentage of Ki67 positive nuclei compared to total basal cells. (b) TSLP secretion by LEMs analyzed by ELISA. (c) SC lipid composition analysed by HPTLC of cytokine supplemented LEMs individually or in combination. The data represent the mean and standard deviation of three independent experiments. Statistical significance is indicated from Student's *t*-test between the control and other culture condition groups (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). All(+)/TNF- α , combination of IL-4, IL-13, IL-31, and TNF- α ; All(-)/TNF- α , combination of IL-4, IL-13, IL-31. HPTLC, high performance thin layer chromatography; SC, stratum corneum; TSLP, thymic stromal lymphopoietin. See Supplementary Table S3 online for CER nomenclature according to Motta *et al.* (1993).

inter-cellular SC lipids did not change with cytokine stimulation (Supplementary Table S4 online). TNF- α singly or in combination with Th2 cytokines significantly increased the percentage of CERs and reduced cholesterol levels in LEMs (Supplementary Table S4 online). We further analyzed the lipids with liquid chromatography coupled to mass spectrometry (LC-MS), examining CER subclasses and chain length distribution of saturated FFAs (SFA) and mono-unsaturated FFAs (MUFA). We focused on the even FFA chain lengths since the odd chain lengths in total contributed <5% of the total FFA content.

No significant changes were induced in the total SFA and MUFA with cytokine treatment (data not shown). However, when focusing on SFA chain length distribution in LEMs stimulated with either TNF- α or the combination of Th2 cytokines with TNF- α , we observe an interesting trend where the relative abundance of SFA ≥ 20 carbon atoms (C20:0) is reduced compared with control LEMs. Although the individual FFA chain lengths were not significantly decreased, this trend in the reduction of the relative abundance of SFA $\geq 20:0$ is significant ($P < 0.01$; Figure 3a). The presence of IL-31 in the culture medium increased the relative abundance of SFA $\geq 20:0$ compared with control LEMs. Although the changes in individual chain lengths are minor, the trend in the persistent increase in SFA $\geq 20:0$ is significant ($P < 0.05$; Figure 3c, Supplementary Figure S5 online, absolute values in

Supplementary Table S5 online). There was no significant difference in the levels of MUFA in LEMs treated with IL-31, TNF- α or combination of Th2 cytokines and TNF- α , compared with control LEMs (Figure 3b and d). IL-4, IL-13 and the combination of Th2 cytokines did not affect the chain length distribution of SFA and MUFA (Supplementary Figure S4 online, absolute values in Supplementary Table S4 and S6 online). The mRNA and protein expression of the enzyme elongase 1 (ELOVL1) which is involved in the production of long chain FFAs was analyzed by qPCR and immunofluorescence respectively. The mRNA abundance in LEMs treated with TNF- α and IL-31 was approximately two times lower than the control LEMs although this reduction was not statistically significant (Supplementary Figure S6a online). However, ELOVL1 mRNA levels with the combination of Th2 cytokines and TNF- α was slightly higher compared to control LEMs (Supplementary Figure S6a online). ELOVL1 protein expression is present in the entire epidermis with the highest expression in the SG and upper SS in the control and IL-31 supplemented LEMs. This high expression of ELOVL1 at the SG and upper SS is absent with TNF- α singly or in combination with Th2 cytokines compared with the control (Figure 3e).

The LC-MS CER profile shows 12 CER subclasses in control, IL-31 and TNF- α supplemented LEMs (Figure 4a,b and c). The four CERs with higher masses in the upper part of the mass spectrogram belong to the EO subclasses (ester linked

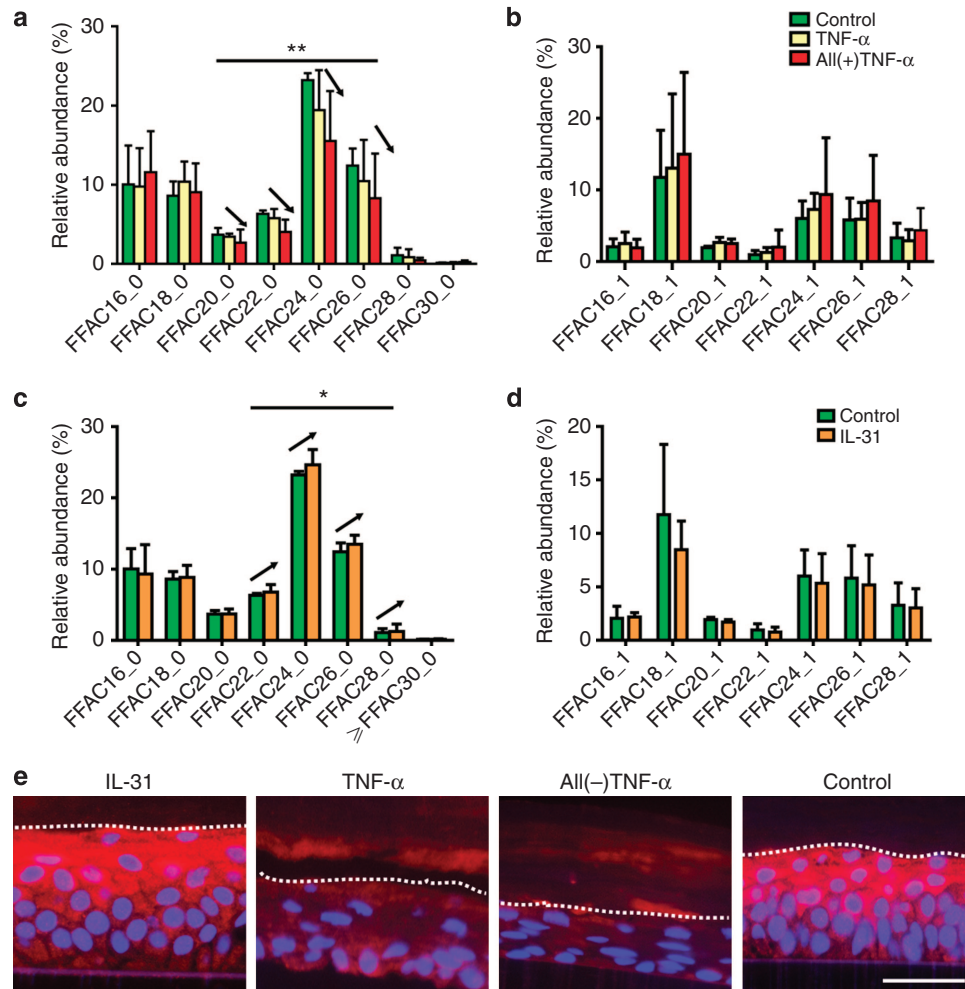


Figure 3. TNF-α reduces and IL-31 increases long chain FFAs. Saturated FFAs chain length distribution in LEM supplemented with (a) TNF-α, a combination of IL-4, IL-13, IL-31, and TNF-α referred to as All(+)TNF-α (c) IL-31. Unsaturated FFAs chain length distribution in LEM supplemented with (b) TNF-α, All(+)TNF-α, and (d) IL-31. (e) Immunofluorescence analysis for ELOVL1, 20x magnification, scale bar = 50 μm. The white dotted line demarcates the viable epidermis from the stratum corneum. The data represent the mean and standard deviation of three independent experiments. Statistical significance is indicated from a two-way ANOVA test between the control and other culture condition groups (* $P \leq 0.05$, ** $P \leq 0.01$). FFAs, free fatty acids; LEM, Leiden epidermal model; TNF-α, tumor necrosis factor alpha.

ω -hydroxy CER subclasses). The α -hydroxy (A) and non-hydroxy (N) CER subclasses have shorter chains and lower masses (non-EO CERs). The total peak area ratios of EO CERs:non-EO CERs was significantly reduced in TNF-α and IL-31 supplemented LEMs compared with the control ($P < 0.05$; Figure 4d, absolute values in Supplementary Table S7 online) demonstrating a lower relative level of EO CERs. Ceramide synthase 3 (CerS3) is responsible for linking the very long acyl chains to the sphingoid base and is particularly involved in the synthesis of EO CERs. A two fold reduction in CerS3 mRNA abundance was observed in LEMs supplemented with IL-31 and TNF-α compared with the control, although the reduction was not statistically significant (Supplementary Figure S6b online). However, LEMs with the combination of Th2 cytokines and TNF-α show similar CerS3 mRNA levels as in the control LEMs (Supplementary Figure S6b online). CerS3 on protein level is expressed in the entire epidermis with the highest expression at the SG and upper SB in the control. The

treatment with IL-31 resulted in a reduction in the expression of CerS3 in suprabasal layers. However, LEMs treated with TNF-α alone or in combination with Th2 cytokines showed only CerS3 expression in the granular layer (Figure 4e).

TNF-α changes SC lipid organization in LEMs

The effect of inflammation on the lipid organization remains unknown, therefore the lamellar organization in SC was examined using small angle X-ray diffraction (SAXD). Diffraction profiles of both control and cytokine supplemented LEM show four diffraction peaks representing the first, second and third order peak of the LPP and cholesterol respectively (referred to as 1, 2, 3 and *, Figure 5a and b). Supplementing LEMs with TNF-α or Th2 cytokines and TNF-α combined, significantly reduced the LPP repeat distance ($P = 0.06$ and $P = 0.02$ respectively; Figure 5c) compared with the control LEMs. All other supplementations did not significantly affect the LPP repeat distance.

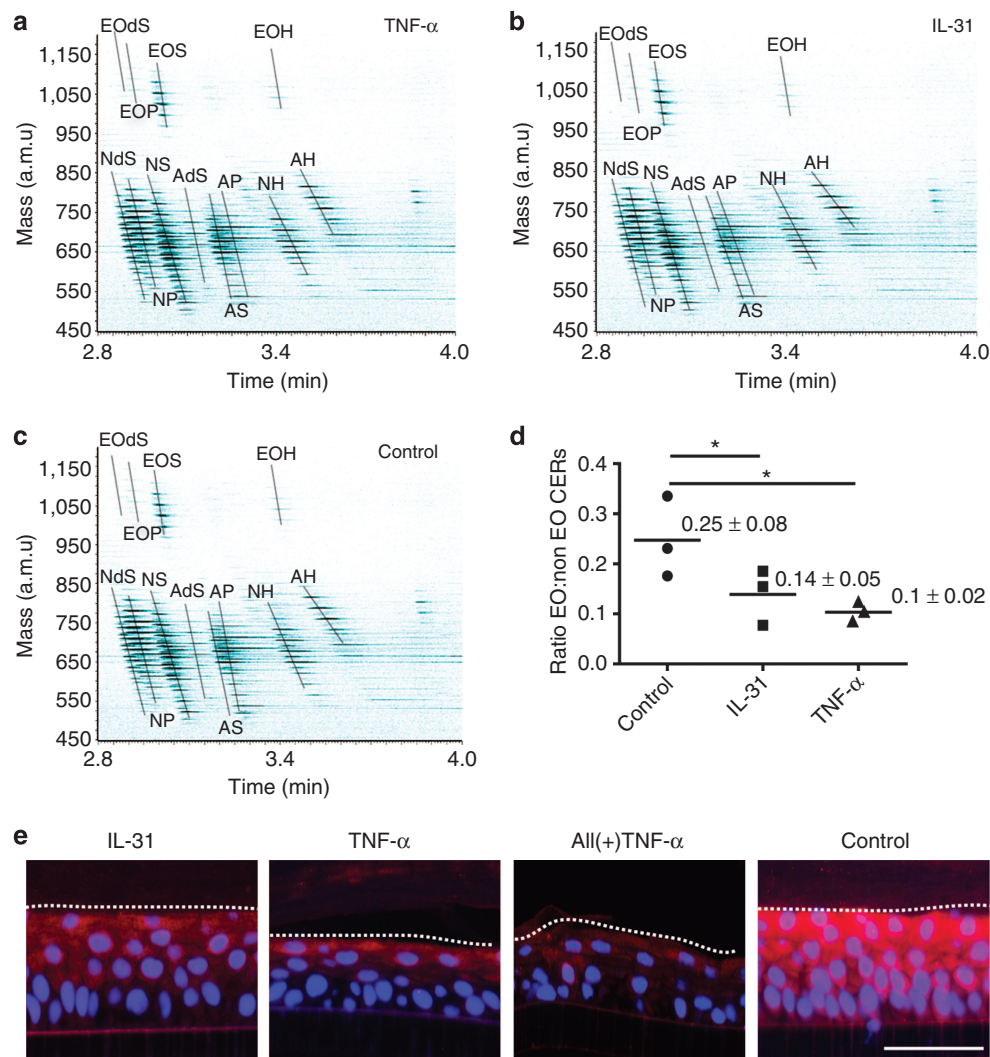


Figure 4. TNF- α and IL-31 reduce the ratio of EO to non-EO CERs in LEMs. Two dimensional multi-mass LC-MS chromatograms of CER subclasses and CER chain length distribution of LEM supplemented with (a) TNF- α , (b) IL-31, or (c) control. The retention time is displayed on the x-axis and the mass on the y-axis. (d) Ratio of very long EO CERs:non-EO CERs in control, IL-31, and TNF- α supplemented LEMs. (e) Immunofluorescence analysis of CerS3, 20x magnification; scale bar = 50 μ m. The white dotted line demarcates the viable epidermis from the stratum corneum. The data represent the ratios from three independent experiments. CERs, ceramides; EO, ester linked ω -hydroxy; LC-MS, liquid chromatography–mass spectrometry; TNF, tumor necrosis factor alpha.

Using FTIR, the lateral organization of the SC lipids was examined as a function of temperature from the CH₂ rocking vibrations of the FTIR spectrum. In an orthorhombic (dense) lipid packing, the CH₂ rocking region consists of vibrations at 719 and 730 cm⁻¹, whilst a hexagonal (less dense) lateral organization results in a vibration at 719 cm⁻¹. All LEMs show a strong vibration band at 719 cm⁻¹ and a weak band at 730 cm⁻¹ between 0 and 20 °C. This demonstrates that below 20 °C the lipids mainly form a hexagonal lateral packing and small orthorhombic domains. Above 20 °C, a single peak is observed at 719 cm⁻¹ showing only hexagonal lateral packing (Supplementary Figure S3a, b online).

The CH₂ stretching vibration provides information about the conformational disordering of the lipids. Crystalline packing results in low conformational disorder and symmetric

stretching frequencies below 2,850 cm⁻¹. High conformational disorder is observed in a liquid phase with symmetric stretching frequencies $\geq$ 2,852 cm⁻¹. The midpoint temperature of the transition (MTT) from crystalline to liquid phase was determined as described by Thakoersing *et al.*, 2012 (Supplementary Figure S3c online). The addition of TNF- α significantly reduced the MTT ($P=0.03$). The CH₂ stretching band position at 32 °C did not statistically differ from the control after the various supplementations (Supplementary Figure S3d online).

DISCUSSION

In the present study we showed the effect of Th2 cytokines and TNF- α on epidermal and SC lipid characteristics. As the concentrations of IL-4, IL-13, IL-31 and TNF- α in AD skin

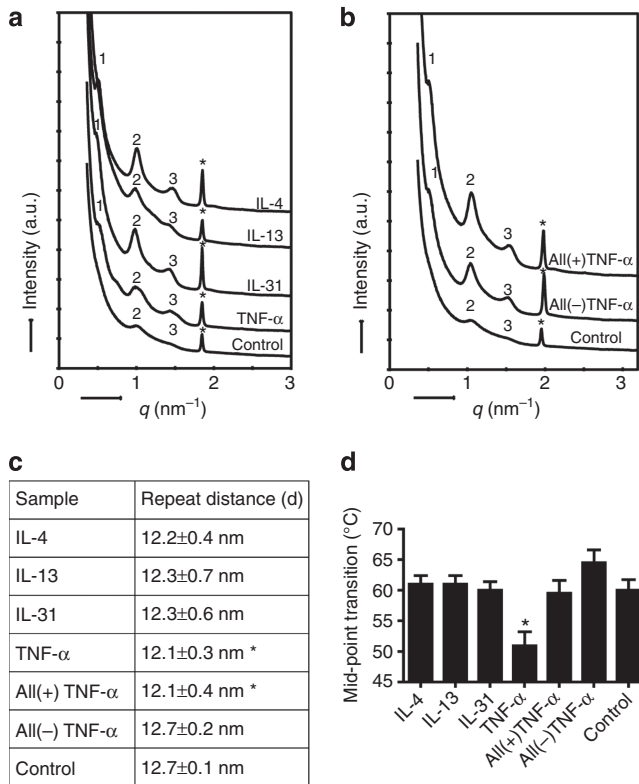


Figure 5. TNF-α reduces the repeat distance of the LPP and the lipid mid-transition temperature in LEMs. (a) The X-ray curve of control, single cytokine supplemented LEMs, and (b) LEMs supplemented with a combination of cytokines. (c) Repeat distance of the LPP for each culture condition. (d) The mid-transition temperature (MTT) from the CH₂ stretching position of the FTIR spectrum. MTT is calculated as the average of the wavenumber at the beginning and end of the transition (see Supplementary Figure S3c online). The MTT is therefore the temperature at this calculated wave number. The data represent the mean and standard deviation of 2-3 samples (**P*≤0.05). All(+)TNF-α, combination of IL-4, IL-13, IL-31 and TNF-α; All(-)TNF-α, combination of IL-4, IL-13, IL-31. FTIR, Fourier transformed infrared spectroscopy; LEMs, Leiden epidermal models; LPP, long periodicity phase; TNF-α, tumor necrosis factor alpha.

remain unknown, the cytokine concentrations added to the LEMs was based on a dose dependent study (data not shown). We selected the cytokine concentrations that induced the highest TSLP levels whilst maintaining viable keratinocytes. We show that Th2 cytokines and TNF-α induce both, epidermal and SC lipid changes characteristic of AD skin. These conclusions are based on the following findings.

Firstly, TNF-α singly or in combination with Th2 cytokines induces modulation in barrier lipid properties, observed in AD lesional skin. The abundance of FFAs ≥C20:0 was observed to be reduced in lesional skin compared to healthy skin (van Smeden *et al.*, 2014). TNF-α singly or in combination with Th2 cytokines caused a coordinated reduction in the relative abundance of saturated FFAs ≥C20:0. This effect on FFA chain length distribution is larger with a combination of Th2 cytokines and TNF-α than TNF-α alone. ELOVL1 plays a role in the synthesis of long chain FFAs (Cameron *et al.*, 2007; Ohno *et al.*, 2010). Our results show that TNF-α alone or in combination with Th2 cytokines reduce ELOVL1

protein expression and this change may consequently affect epidermal permeability (Cameron *et al.*, 2007). This reduction of ELOVL1 expression on protein level was not observed on mRNA level with TNF-α in combination with Th2 cytokines. Since mRNA levels only predict 40% of protein abundance (de Sousa *et al.*, 2009), we suggest that this cytokine combination may mainly influence translational and/or post-translational mechanisms of ELOVL1 rather than the transcription process.

A reduced level of EO CER subclasses and lower average CER chain length is observed in AD skin which strongly correlates to SC barrier function (Ishikawa *et al.*, 2010; Janusova *et al.*, 2011; Janssens *et al.*, 2012). The mRNA expression of CerS3 was not significantly altered with TNF-α. However, we observe reduced CerS3 protein expression with IL-31 and TNF-α followed by a reduction in the ratio of EO CER:non-EO CER levels. As a result, the effect of IL-31 and TNF-α on CerS3 expression and CER composition may therefore be involved in CER changes in AD. Furthermore, IFN-γ has also been suggested to decrease long chain CER NS via down regulation of ELOVL1 and CerS3 mRNA expression in a 3 dimensional epidermal model, suggesting its involvement in CER synthesis in AD and psoriasis (Tawada *et al.*, 2013). As LEMs do not desquamate, the lipid composition of the LEMs is a combination of the entire culture period. This includes the first 10 days of culture without supplementation of cytokines. Therefore, the effects observed in this *in vitro* model may be an underestimation of the *in vivo* situation. Sawada *et al.*, 2012 reports that TNF-α increases and IL-4 decreases the total CER levels in epidermal equivalents. We observe an increase in CER levels with TNF-α but no effect with IL-4. These differences may arise from differences in the experimental set-up. In the studies of Sawada *et al.*, cytokines were added to the cultures after they were fully generated, however, in the present study, the cytokines were added immediately after a stratified epidermis has been produced. The lipid synthesis may therefore differ based on the time point of IL-4 stimulation. Hatano *et al.*, 2005 using human epidermal sheets showed that TNF-α alone does not affect the CER/protein ratio after 3 days of treatment, which is a much shorter period as used in the present study.

Secondly, TNF-α alone or combined with Th2 cytokines significantly reduces the repeat distance of the LPP and TNF-α alone reduces the MTT in comparison to control. The changes in lipid organization can easily be explained by a reduced chain length of the lipids for the following reasons i) shorter lipid chains result in lower number of carbon atoms bridging the lamellae leading to a shorter LPP and ii) a reduction in saturated long chain FFAs is known to reduce the MTT (Groen *et al.*, 2011), contributing to a higher trans epidermal water loss (van Smeden *et al.*, 2014).

Thirdly, we show that TNF-α and Th2 cytokines significantly augment TSLP secretion compared to the control LEMs, as observed in AD (Lee *et al.*, 2010). Previous studies show that the combination of Th2 cytokines and TNF-α induces TSLP production in full thickness human skin explants, however, TNF-α alone has no effect on TSLP levels in their model system (Bogiatzi *et al.*, 2007). The levels of TSLP produced are

also higher than observed in our study with LEMs. We suggest that i) the presence of dendritic cells in full thickness skin and ii) a direct effect from the presence of fibroblasts since they have been shown to express TSLP mRNA *in vitro* (Soumelis *et al.*, 2002) may contribute to these differences. Therefore, LEMs may provide a good model to examine the direct influence of cytokines on keratinocytes.

Fourthly, stimulation of LEMs with IL-4, IL-13, IL-31 and TNF- α induce spongiosis in LEMs similarly as observed in AD (Soter 1989). We also show that IL-31 influences epidermal cohesion in addition to its known effects in regulating keratinocyte differentiation and pruritus in AD (Takaoka *et al.*, 2006; Cornelissen *et al.*, 2012). IL-4 and IL-13 have also been reported as inducers of spongiosis in epidermal models (Ohtani *et al.*, 2009).

Lastly, cytokines examined in this study delay early differentiation and increase proliferation in LEMs in a similar pattern as observed in AD skin. However, no increase in the thickness of the viable epidermis was observed contrasting the *in vivo* situation (Jensen *et al.*, 2004; Sapuntsova *et al.*, 2011). This may result from a high epidermal turnover rate (7 days) in LEMs as compared to 30 days in native human epidermis (Iizuka 1995). In addition, the delayed K10 expression also confirms the proliferation increase, as the undifferentiated cells are escaping to the lower stratum spinosum. Th2 cytokines, in our experimental conditions, less prominently affect the lipids, but rather reduce the expression of barrier proteins, filaggrin and loricrin. We suggest that the Th2 cytokines may affect the permeability barrier by reducing filaggrin and thus reduce NMF levels resulting in increased SC pH. This higher pH can lead to alterations of several enzymes, such as serine proteases and induce alterations in permeability barrier homeostasis, SC integrity and cohesion (Hachem *et al.*, 2003).

We show a clear relationship between the effect of cytokines on proliferation and differentiation. However, these changes in proliferation and differentiation do not reflect in SC lipid composition. This suggests that cytokine induced changes in epidermal differentiation and SC lipid synthesis are influenced by different pathways. We conclude from this study that supplementation of LEMs with cytokines (individually or in synergy) induces several important features of AD skin *in vitro* on lipid and protein level. Therefore, these cytokine supplemented LEMs may serve as an excellent candidate for the testing of prospective drugs and formulations for the treatment of AD.

MATERIALS AND METHODS

Cell culture

Normal human keratinocytes (NHK) were isolated from human adult breast skin donors undergoing cosmetic surgery (Ponec *et al.*, 1997; Supplementary Materials and Methods online). The skin was collected after written informed consent from the donors, institutional approval, and handled according to the Declaration of Helsinki Principles.

Generation of the Leiden epidermal models (LEMs)

LEMs were generated similarly as described by El Ghalbzouri *et al.*, 2008 with some modifications (Supplementary Materials and Methods online).

Morphology and immunohistochemistry

Harvested LEMs were fixed in 4% paraformaldehyde (Lommerce Pharma, Oss, The Netherlands), dehydrated and embedded in paraffin. 5 μ m sections were cut, deparaffinized and rehydrated through graded ethanol series and stained with hematoxylin (2mg/ml) and eosin (4mg/ml). Immunohistochemical analysis of filaggrin, K10, ki67, loricrin, ELOVL1 and CerS3 is described in Supplementary Materials and Methods online. The primary and secondary antibodies used are listed in Supplementary Table S2 online. The qPCR and western blot analysis is described in the Supplementary Materials and Methods online.

ELISA

The culture medium at the end of the culture period was collected and stored at -20°C till use. TSLP secretion was quantified using a TSLP quantikine protein ELISA kit (R&D systems, Abingdon, UK) according to manufacturer's instructions. Statistical significance was performed with paired Student's *t*-test comparison between the control and other culture condition groups.

SC isolation and lipid extraction

The SC from LEM was isolated by trypsin digestion. Lipids were extracted and pooled from three LEM SC using the modified Bligh and Dyer (1959) procedure (Thakoersing *et al.*, 2012) with the addition of 0.25M KCl to extract polar lipids. All extracts were dried under a stream of nitrogen, reconstituted in chloroform:methanol (2:1) and stored at -20°C . For LC-MS analysis the lipids were dried under a stream of nitrogen and dissolved in chloroform:methanol:heptane (2.5:2.5:95) at 1mg/ml concentration. The SC from LEMs were weighed before and after extraction in order to determine the total intercellular lipid and protein content.

High performance thin layer chromatography (HPTLC)

Extracted lipids were analyzed by HPTLC as described previously (Ponec *et al.*, 2003). The solvent system used for separation is listed in Supplementary Table S2 online. Co-chromatography of a standard lipid mixture was performed to identify the various classes of lipids consisting of cholesterol, palmitic acid, stearic acid, tricosanoic acid, behenic acid, arachidic acid, cerotic acid, lignoceric acid (Sigma) and CER EOH, NS, NP, EOS and AP (Evonik, Germany, according to the terminology of the ceramide nomenclature of Motta *et al.*, 1993 and Masukawa *et al.*, 2008 used throughout this article, Supplementary Table S3 online). The liquid chromatography mass spectrometry analysis is described in the Supplementary Materials and Methods online.

Fourier transformed infrared spectroscopy and small angle X-ray diffraction

For FTIR and SAXD, SC samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution.

FTIR: the hydrated SC was measured as described previously (Caussin *et al.*, 2008; Supplementary Materials and Methods online). The phase transitions of the SC lateral lipid organization was analyzed with a temperature range of 0 and 90°C at a rate of $0.25^{\circ}\text{C}/\text{min}$.

SAXD: measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) at station BM26B. The SC samples were measured as described before (Thakoersing *et al.*, 2012; Supplementary Materials and Methods online).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The authors thank the personnel at the DUBBLE beam line (BM26), ESRF for their support with the X-ray measurements. This research was partly financially supported by Dutch Technology Foundation STW (grant no. 10703) and the C.E.R.I.E.S Research Award received by JAB in 2011. The synthetic CERs were kindly provided by Evonik, Germany.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Akiyama M (2010) FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. *Br J Dermatol* 162: 472–7
- Bleck O, Abeck D, Ring J *et al.* (1999) Two ceramide subfractions detectable in Cer(AS) position by HPTLC in skin surface lipids of non-lesional skin of atopic eczema. *J Invest Dermatol* 113:894–900
- Bogiatzi SI, Fernandez I, Bichet J-C *et al.* (2007) Cutting edge: proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *The Journal of Immunology* 178:3373–7
- Bouwstra J, Gooris GS, Spek vd *et al.* (1991) Structural investigations of human stratum corneum by small-angle X-ray scattering. *J Invest Dermatol* 97:1005–12
- Brown SJ, McLean WHI (2009) Eczema genetics: current state of knowledge and future goals. *J Invest Dermatol* 129:543–52
- Cameron DJ, Tong Z, Yang Z *et al.* (2007) Essential role of Elovl4 in very long chain fatty acid synthesis, skin permeability barrier function, and neonatal survival. *Int J Biol Sci* 3:111–9
- Caussin J, Gooris GS, Janssens M *et al.* (2008) Lipid organization in human and porcine stratum corneum differs widely, while lipid mixtures with porcine ceramides model human stratum corneum lipid organization very closely. *Biochim Biophys Acta* 1778:1472–82
- Cork MJ, Robinson DA, Vasilopoulos Y *et al.* (2006) New perspectives on epidermal barrier dysfunction in atopic dermatitis: Gene-environment interactions. *J Allergy Clin Immunol* 118:3–21
- Cornelissen C, Marquardt Y, Czaja K *et al.* (2012) IL-31 regulates differentiation and filaggrin expression in human organotypic skin models. *J Allergy Clin Immunol* 129:426–33
- de Sousa Abreu R, Penalva LO, Marcotte EM *et al.* (2009) Global signatures of protein and mRNA expression levels. *Molecular BioSystems* 5: 1512–26
- Di Nardo A, Wertz P, Giannetti A *et al.* (1998) Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Dermato-Venereologica* 78:27–30
- El Ghalbzouri A, Siamari R, Willemze R *et al.* (2008) Leiden reconstructed human epidermal model as a tool for the evaluation of the skin corrosion and irritation potential according to the ECVAM guidelines. *Toxicology in Vitro* 22:1311–20
- Elias P, Feingold K, Fluhr J (2003) The skin as an organ of protection. In: Friedberg AZEIM, Wolff K, Austen KF *et al.* (eds), In: *Dermatology in General Medicine*. McGraw Hill: New York, 107–18
- Feingold KR (2007) Thematic review series: Skin Lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res* 48:2531–46
- Gay CL GR, Golden GM, Mak VH *et al.* (1994) Characterization of low-temperature (i.e., <65 degrees C) lipid transitions in human stratum corneum. *J Invest Dermatol* 103:233–9
- Gittler JK, Shemer A, Suárez-Farinas M *et al.* (2012) Progressive activation of TH2/TH22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol* 130:1344–54
- Groen DJ, Poole DS, Gooris GS *et al.* (2011) Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1808:1529–37
- Hachem J-P, Crumrine D, Fluhr J *et al.* (2003) pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *J Invest Dermatol* 121:345–53
- Hatano Y, Terashi H, Arakawa S *et al.* (2005) Interleukin-4 suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier functions induced by tumor necrosis factor- α and interferon- γ in human epidermis. *J Invest Dermatol* 124:786–92
- Iizuka H (1995) Epidermal architecture that depends on turnover time. *J Dermatol Sci* 10:220–3
- Imokawa G, Abe A, Jin K *et al.* (1991) Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol* 96:523–6
- Ishida-Yamamoto A, Hohl D, Roop DR *et al.* (1993) Loricrin immunoreactivity in human skin: localization to specific granules (L-granules) in acrosyringia. *Arch Dermatol Res* 285:491–8
- Ishikawa J, Narita H, Kondo N *et al.* (2010) Changes in the ceramide profile of atopic dermatitis patients. *J Invest Dermatol* 130:2511–4
- Janssens M, van Smeden J, Gooris GS *et al.* (2012) Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J Lipid Res* 53:2755–66
- Janusova B, Zbytovska J, Lorenc P *et al.* (2011) Effect of ceramide acyl chain length on skin permeability and thermotropic phase behavior of model stratum corneum lipid membranes. *Biochim Biophys Acta* 1811: 129–37
- Jensen J-M, Folster-Holst R, Baranowsky A *et al.* (2004) Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. *J Invest Dermatol* 122:1423–31
- Jungsted JM, Scheer H, Mempel M *et al.* (2010) Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 65:911–8
- Lee EB, Kim KW, Hong JY *et al.* (2010) Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. *Pediatric Allergy and Immunology* 21:e457–60
- Leung DYM, Bieber T (2003) Atopic dermatitis. *The Lancet* 361:151–60
- Masukawa Y, Narita H, Sato H *et al.* (2009) Comprehensive quantification of ceramide species in human stratum corneum. *J Lipid Res* 50:1708–19
- Masukawa Y, Narita H, Shimizu E *et al.* (2008) Characterization of overall ceramide species in human stratum corneum. *J Lipid Res* 49: 1466–76
- Motta S, Monti M, Sesana S *et al.* (1993) Ceramide composition of the psoriatic scale. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1182:147–51
- Neis MM, Peters B, Dreuw A *et al.* (2006) Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J Allergy Clin Immunol* 118:930–7
- Ohno Y, Suto S, Yamanaka M *et al.* (2010) ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis. *Proceedings of the National Academy of Sciences* 107:18439–44
- Ohtani T, Memezawa A, Okuyama R *et al.* (2009) Increased hyaluronan production and decreased E-cadherin expression by cytokine-stimulated keratinocytes lead to spongiosis formation. *J Invest Dermatol* 129: 1412–20
- Palmer CNA, Irvine AD, Terron-Kwiatkowski A *et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441–6
- Pilgram GSK, Vissers DCJ, van der Meulen H *et al.* (2001) Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *J Invest Dermatol* 117:710–7
- Ponec M, Weerheim A, Kempenaar J *et al.* (1997) The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J Invest Dermatol* 109:348–55

- Ponec M, Weerheim A, Lankhorst P *et al.* (2003) New acylceramide in native and reconstructed epidermis. *J Invest Dermatol* 120:581–8
- Rodríguez E, Illig T, Weidinger S (2008) Filaggrin loss-of-function mutations and association with allergic diseases. *Pharmacogenomics* 9:399–413
- Sapuntsova SG, Lebed'ko OA, Shchetkina MV *et al.* (2011) Status of free-radical oxidation and proliferation processes in patients with atopic dermatitis and lichen planus. *Bull Exp Biol Med* 150:690–2
- Sawada E, Yoshida N, Sugiura A *et al.* (2012) Th1 cytokines accentuate but Th2 cytokines attenuate ceramide production in the stratum corneum of human epidermal equivalents: An implication for the disrupted barrier mechanism in atopic dermatitis. *J Dermatol Sci* 68:25–35
- Soter NA (1989) Morphology of atopic eczema. *Allergy* 44:16–9
- Soumelis V, Reche PA, Kanzler H *et al.* (2002) Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP. *Nat Immunol* 3:673–80
- Steven AC, Bisher ME, Roop DR *et al.* (1990) Biosynthetic pathways of filaggrin and Loricrin: two major proteins expressed by terminally differentiated epidermal keratinocytes. *J Struct Biol* 104:150–62
- Takaoka A, Arai I, Sugimoto M *et al.* (2006) Involvement of IL-31 on scratching behavior in NC/Nga mice with atopic-like dermatitis. *Exp Dermatol* 15:161–7
- Tawada C, Kanoh H, Nakamura M *et al.* (2013) Interferon- γ decreases ceramides with long-chain fatty acids: possible involvement in atopic dermatitis and psoriasis. *J Invest Dermatol* 134:712–8
- Thakoersing VS, Danso MO, Mulder A *et al.* (2012) Nature versus nurture: does human skin maintain its stratum corneum lipid properties *in vitro*? *Exp Dermatol* 21:865–70
- van Smeden J, Janssens M, Kaye EC *et al.* (2014) The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp Dermatol* 23:45–52
- van Smeden J, Hoppel L, van der Heijden R *et al.* (2011) LC/MS analysis of stratum corneum lipids: ceramide profiling and discovery. *J Lipid Res* 52:1211–21
- Watanabe N, Hanabuchi S, Soumelis V *et al.* (2004) Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4⁺ T cell homeostatic expansion. *Nat Immunol* 5:426–34
- Wertz PW, Miethke MC, Long SA *et al.* (1985) The composition of the ceramides from human stratum corneum and from comedones. *J Invest Dermatol* 84:410–2
- Yamamoto A, Serizawa S, Ito M *et al.* (1991) Stratum corneum lipid abnormalities in atopic dermatitis. *Arch Dermatol Res* 283:219–23
- Zhu Z, Oh M-H, Yu J *et al.* (2011) The Role of TSLP in IL-13-induced atopic march. *Sci Rep* 1:23

SUPPLEMENTARY FIGURES

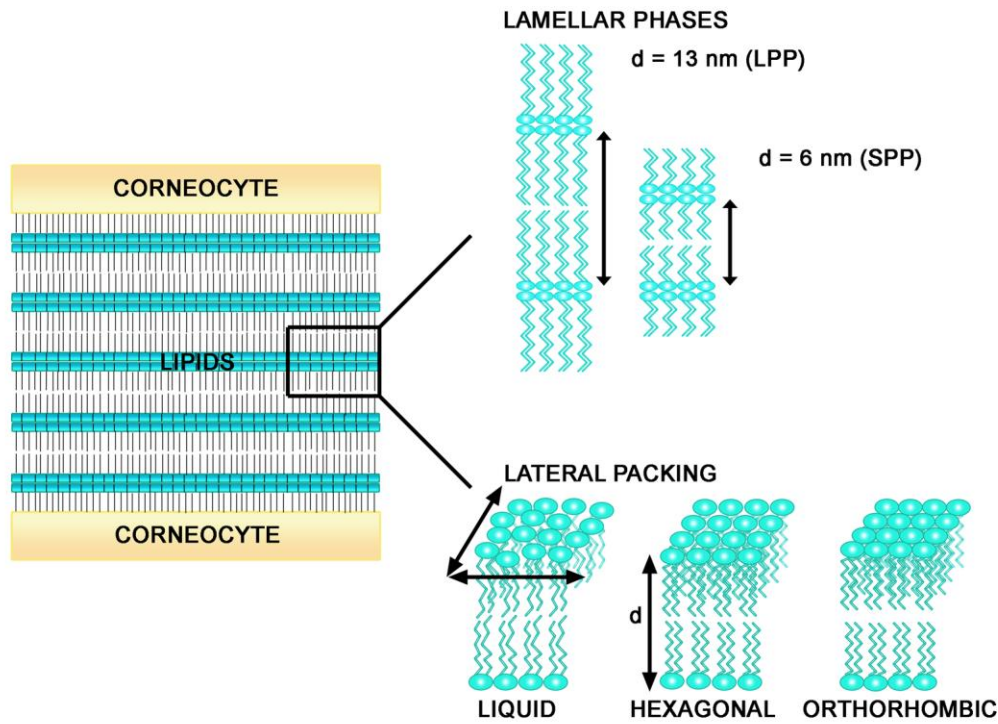


Figure S1: Lateral and lamellar organization in human stratum corneum

The stratum corneum (SC), the outermost part of the epidermis consists of dead cells (corneocytes) embedded in a lipid matrix. The intercellular lipids are organized in layers or lamellae which consists of two lamellar phases. Firstly, the long periodicity phase (LPP) having a repeat distance (d) of 13nm and secondly, the short periodicity phase with a repeat distance of 6nm. The lateral organization is perpendicular to the lamellar organization. The lipids can be arranged in three different patterns: orthorhombic (very dense and ordered), hexagonal (less dense and ordered) and liquid (disordered) organization. Adapted from Thakoersing et al., 2012, Experimental dermatology).

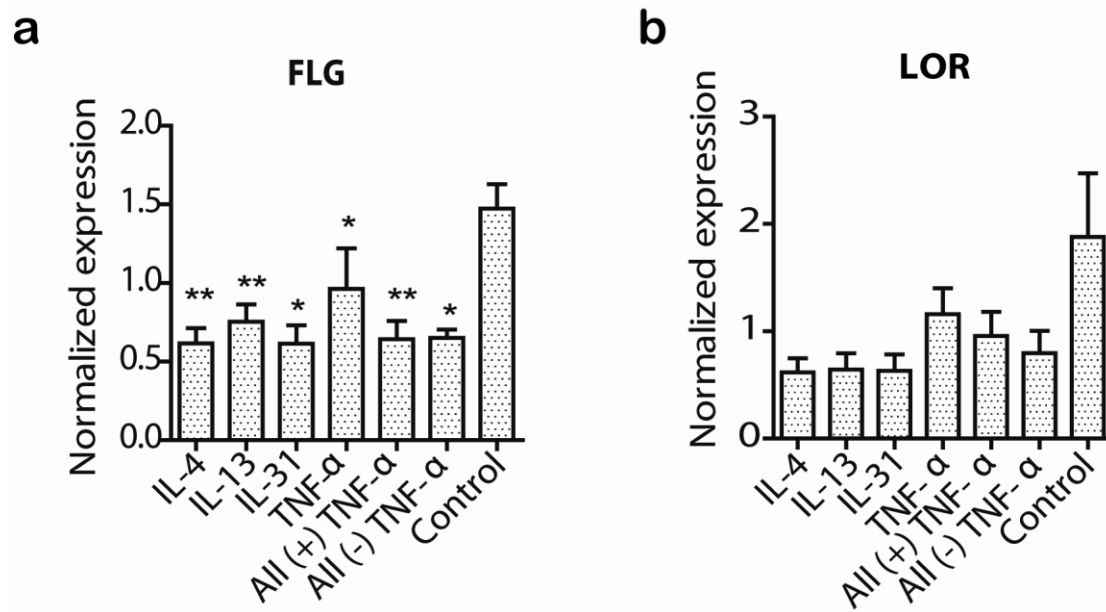


Figure S2: Th2 cytokines and TNF-α down regulate mRNA expression of barrier proteins. Normalized mRNA expression of A) filaggrin and B) loricrin. Statistical significance is indicated from student t-test comparison between the control and other culture condition groups (n=3±SEM, *p ≤ 0.05, **p ≤ 0.01).

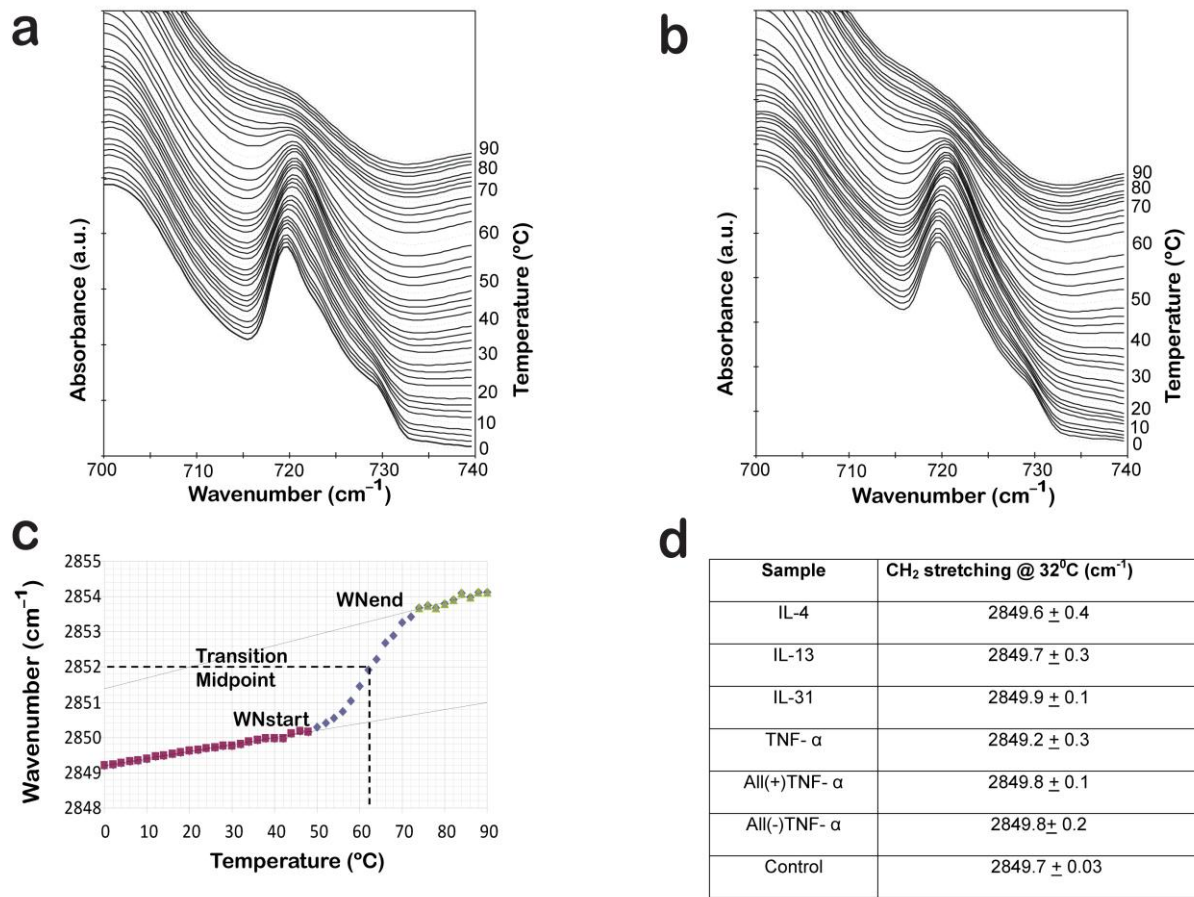


Figure S3: Th2 cytokines and TNF-α do not affect the SC lateral lipid organization in LEM.

The rocking vibrations in the FTIR spectra providing information on the lateral lipid organization in control and cytokine treated conditions is shown as a function of temperature (A and B respectively). At lower temperatures (0°C), the lipids mainly form hexagonal packing (vibrations at 719 cm⁻¹) and a small population of lipids forms orthorhombic domains (additional vibration at 730cm⁻¹). The orthorhombic phase disappears around 20°C in both control and cytokine treated conditions. C) Illustrates the procedure to calculate the midpoint of the crystalline to liquid transition examined by the CH₂ stretching vibrations of the lipids. The midpoint transition temperature is calculated by taking the average of the last wave number at the beginning (WN_{start}) of the transition and the first wave number at the end of the transition (WN_{end}). The midpoint temperature is therefore the temperature at this calculated wave number. D) CH₂ stretching peak position at 32°C derived from the FTIR spectrum. All (+) TNF-α: IL-4/IL-13/IL-31/ TNF-α; All (-)TNF-α: IL-4/IL-13/IL-31.

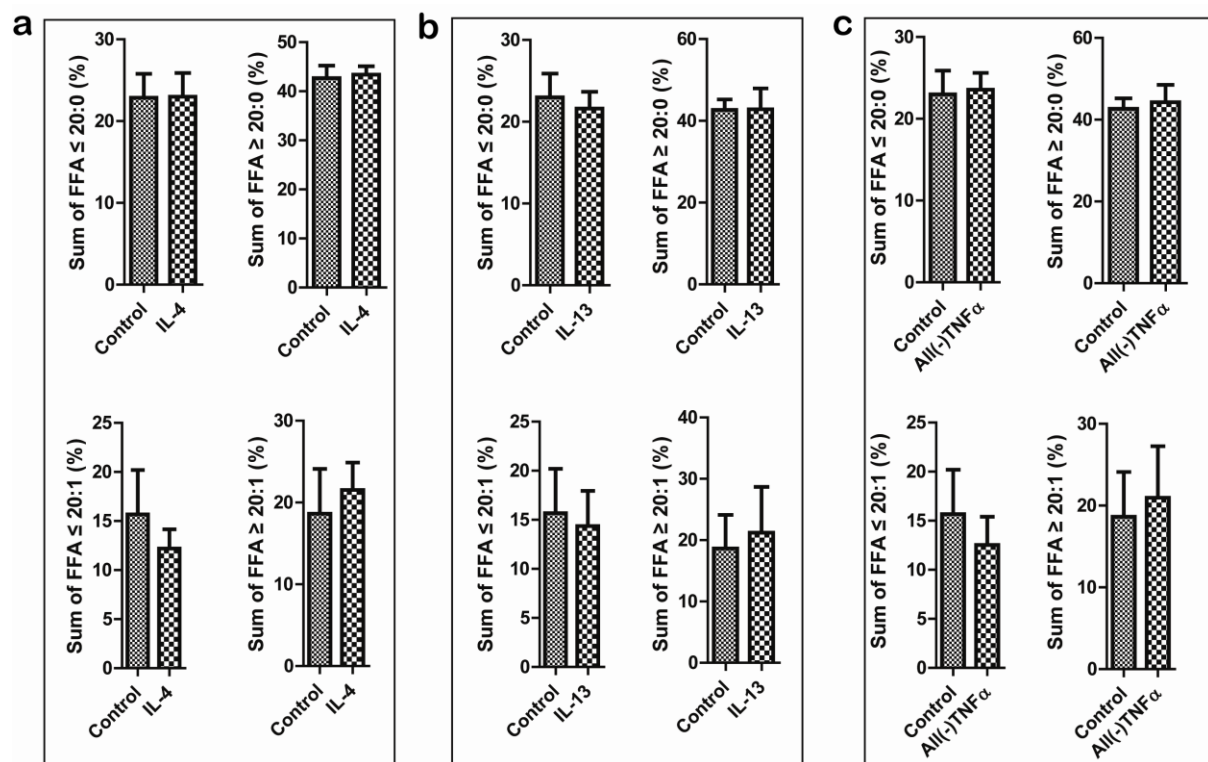


Figure S4: Supplementation of Th2 cytokines singly or in combination does not alter FFA chain length in LEMs. Sum of saturated and mono-unsaturated FFA with chain length $<$ and $\geq$ 20 carbon atoms in LEMs supplemented with A) IL-4 B) IL-13 C) combination of IL-4, IL-13 and IL-31, referred to as All(-) TNF- α and the control. The data represents the mean and standard deviation of 3 independent experiments.

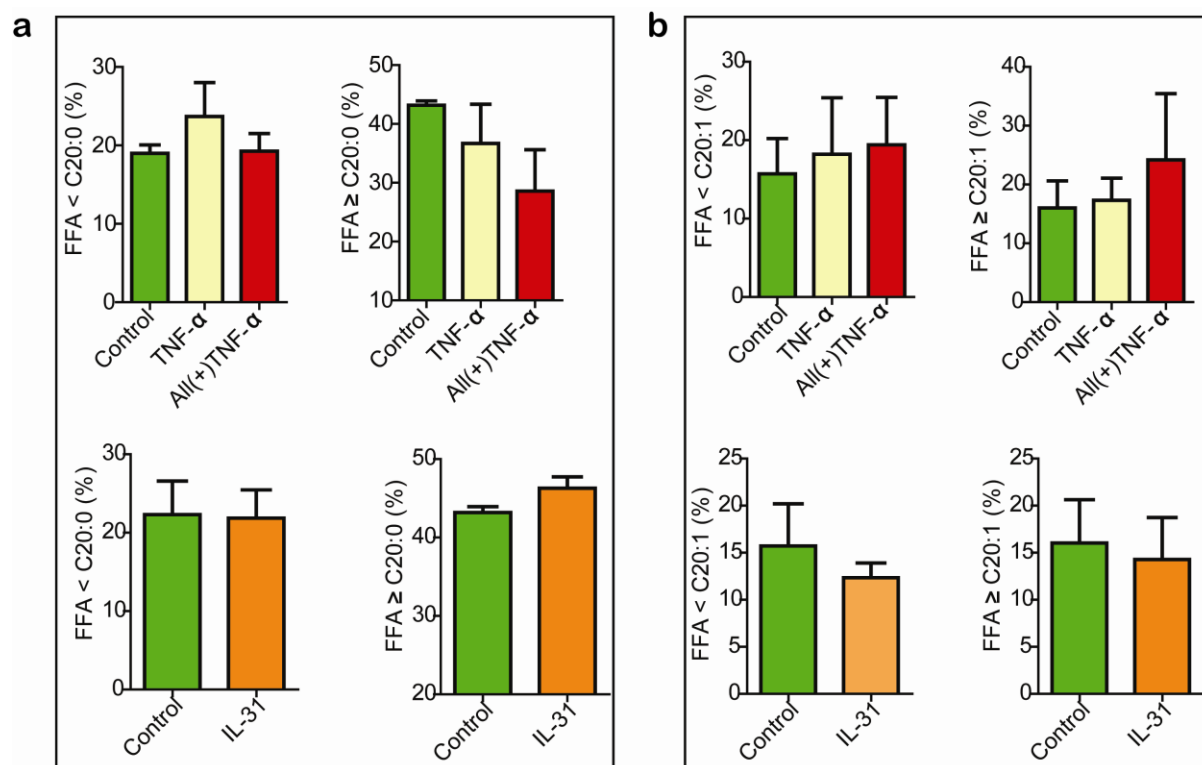


Figure S5: FFA composition in TNF- α and IL-31 stimulated LEMs. Sum of A) saturated and B) mono-unsaturated FFAs with chain lengths < and $\geq$ 20 carbon atoms in LEMs stimulated with TNF- α , combination of IL-4, IL-13, IL-31 and TNF- α referred to as All(+) TNF- α , IL-31 and the control. The data represents the mean and standard deviation of 3 independent experiments.

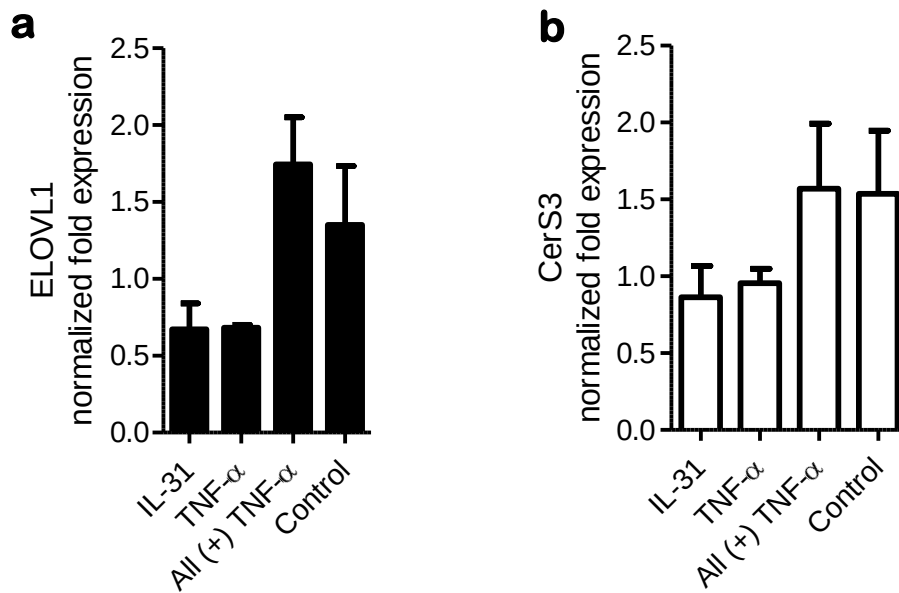


Figure S6: IL-31 and TNF- α influence mRNA expression of ELOVL1 and Cers3 in LEMs.

TNF- α reduces a) ELOVL1 and b) Cers3 mRNA expression in LEMs and IL-31 reduces Cers3 expression compared with the control. Although the reduction in mRNA levels are not statistically significant these findings support the reduction in protein expression observed by immunohistochemistry. The ELOVL1 mRNA expression with All(+)-TNF- α or IL-31 supplementation does not correlate with the protein expression as ELOVL1 protein expression is lower with All(+)-TNF- α compared to control, while in case of IL-31 the ELOVL1 protein expression is similar to the control. As published in literature mRNA abundances only partially predict protein levels since only ~40% of the variation in the concentration of protein can be explained by mRNA abundances (de Sousa et al., 2009). Therefore, the differences between mRNA abundances and protein levels in All(+)-TNF- α may result from changes translational and/or post-translational mechanisms of lipid synthesis proteins rather than the transcription process. In the case of IL-31, the half-life of proteins/mRNA may be an important determining factor that influences protein levels as the mRNA levels may be low but the protein may be stable and translated efficiently. The data represents the mean and standard deviation of three independent experiments measured in duplicate. Statistical significance was determined by a 2-way ANOVA statistical analysis.

SUPPLEMENTARY TABLES

Table S1: Primary and secondary antibodies for immunohistochemical staining

Antibodies	Clone	Dilution	Company
<i>Primary antibodies</i>			
Mouse cytokeratin 10 Ab-2	DE-K10	1:800	Neomarkers, USA
Mouse filaggrin Ab-1	FLG01	1:800	Neomarkers, USA
Rabbit Loricrin	AF62	1:1200	Covance, USA
Rabbit Ki67	SP6	1:800	Neomarkers, USA
Rabbit ELOVL1 (IF)		1:300	Santa Cruz, USA
Rabbit ceramide synthase 3 (IF)		1:75	Sigma Aldrich
<i>Secondary antibodies</i>			
Biotinylated universal antibody, anti-rabbit/mouse IgG			Vector laboratories Burlingame, CA
Rhodamine Red-X (Goat anti-rabbit (IF)		1:300	Jackson immunoresearch Laboratory, USA

IF: immunofluorescence

Table S2: Solvent system used for barrier lipids analysis by thin layer chromatography

Eluent	Composition (v/v)	Distance (mm)
1	Dichloromethane/Ethylacetate/Acetone/Methanol (88:8:4:1)	40
2	Chloroform/Acetone/Methanol (76:8:16)	10
3	Hexane/Chloroform/Acetone/Methanol (6:80:12:2)	70
4	Hexane/Chloroform/Hexyl acetate/Acetone/ Methanol (6:80:0.1:10:4)	95

Table S3: Ceramide nomenclature according to Motta et al., 1993

	Non hydroxy fatty acid (N)	α - hydroxy fatty acid (A)	Esterified ω -hydroxy fatty acid (EO)
Dihydrosphingosine (dS)	NdS	AdS	EOdS
Sphingosine (S)	NS	AS	EOS
Phytosphingosine (P)	NP	AP	EOP
6-hydroxy sphingosine (H)	NH	AH	EOH

Table S4: Total amount of stratum corneum lipids in cytokine stimulated LEMs per dry weight of stratum corneum (mg/mg SC) and percentage distribution of free fatty acids, ceramides and cholesterol.

LEM condition	Amount of SC lipids (mg/mg SC)	Free fatty acids (%)	Cholesterol (%)	Ceramides (%)
IL-4	0.20 $\pm$ 0.04	17.9 $\pm$ 2.4	19.8 $\pm$ 3.4	62.6 $\pm$ 4.7
IL-13	0.22 $\pm$ 0.04	18.3 $\pm$ 1.8	19.6 $\pm$ 4.0	62.2 $\pm$ 5.1
IL-31	0.19 $\pm$ 0.07	20.1 $\pm$ 1.6	21.2 $\pm$ 2.1	58.7 $\pm$ 1.7
TNF- α	0.28 $\pm$ 0.01	16.8 $\pm$ 1.9	20.6 $\pm$ 1.8*	62.6 $\pm$ 0.1*
All(+)TNF- α	0.27 $\pm$ 0.09	18.0 $\pm$ 2.3	20.3 $\pm$ 3.1*	61.7 $\pm$ 2.3*
All(-)TNF- α	0.25 $\pm$ 0.11	17.3 $\pm$ 0.8	21.4 $\pm$ 3.9	61.2 $\pm$ 3.1
Control	0.26 $\pm$ 0.03	18.3 $\pm$ 0.4	23.0 $\pm$ 1.7	58.7 $\pm$ 1.4

SC barrier lipids were fractionated and the bands of the FFA, CER and CHOL were quantified by HPTLC and photodensitometry respectively (sum of FFA, CER and CHOL=100%). The data represents the mean and standard deviation of 3 independent experiments. Statistical significance is indicated from student t-test comparison between the control and other culture condition groups (* $p \leq 0.05$). Cytokine supplementation in LEMs did not significantly alter the total amount of stratum corneum lipids per dry stratum corneum weight. SC: Stratum corneum

Table S5: Absolute amount of saturated FFAs varying in chain length per dry weight of stratum corneum ($\mu\text{g}/\text{mg}$ SC) in cytokine stimulated LEMs (analyzed by LC-MS).

FFA chain length	IL-4	IL-13	IL-31	TNF- α	All(+)TNF- α	All(-)TNF- α	Control
FFAC16:0	3.3 ± 1.1	3.4 ± 1.4	3.6 ± 1.6	4.6 ± 2.3	5.6 ± 2.5	4.2 ± 1.1	4.8 ± 2.3
FFAC18:0	3.4 ± 1.1	3.5 ± 0.5	3.4 ± 0.6	4.9 ± 1.2	4.4 ± 1.8	4.3 ± 0.9	4.1 ± 0.9
FFAC20:0	1.3 ± 0.6	1.5 ± 0.5	1.4 ± 0.3	1.6 ± 0.2	1.3 ± 0.8	1.6 ± 0.8	1.7 ± 0.4
FFAC22:0	2.2 ± 0.5	2.6 ± 0.5	2.6 ± 0.4	2.7 ± 0.5	2.0 ± 0.8	2.8 ± 0.6	3.0 ± 0.2
FFAC24:0	8.4 ± 0.4	9.5 ± 1.9	9.4 ± 0.8	9.2 ± 2.4	7.6 ± 3.1	10.7 ± 1.9	11.0 ± 0.4
FFAC26:0	4.6 ± 0.5	4.8 ± 1.5	5.1 ± 0.5	4.9 ± 2.5	4.0 ± 2.7	5.6 ± 1.6	5.9 ± 1.0
FFAC28:0	0.5 ± 0.4	0.6 ± 0.5	0.5 ± 0.4	0.4 ± 0.5	0.2 ± 0.2	0.4 ± 0.3	0.5 ± 0.4
$\geq$ FFAC30:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0

In LEMs stimulated with either TNF- α or the combination of Th2 cytokines with TNF- α , we observe a trend where the relative abundance of SFA ≥ 20 carbon atoms (C20:0) is reduced compared with control LEMs ($p < 0.05$ and $p < 0.01$ respectively). The data represents the mean and standard deviation of 3 independent experiments. SC: Stratum corneum

Table S6: Absolute amount of unsaturated FFA chain lengths per dry weight of stratum corneum ($\mu\text{g}/\text{mg}$ SC) in cytokine stimulated LEMs (analyzed by LC-MS).

FFA chain length	IL-4	IL-13	IL-31	TNF- α	All(+)TNF- α	All(-)TNF- α	Control
FFAC16:1	0.6 ± 0.2	0.8 ± 0.3	0.8 ± 0.2	1.2 ± 0.8	0.9 ± 0.6	0.8 ± 0.4	1.0 ± 0.5
FFAC18:1	3.0 ± 1.1	4.2 ± 2.3	3.2 ± 1.0	6.1 ± 4.9	7.3 ± 5.6	4.2 ± 2.8	5.6 ± 3.1
FFAC20:1	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	1.3 ± 0.3	1.2 ± 0.3	0.8 ± 0.2	0.9 ± 0.1
FFAC22:1	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	0.6 ± 0.3	1.0 ± 1.2	0.4 ± 0.3	0.4 ± 0.3
FFAC24:1	2.4 ± 0.3	2.6 ± 1.5	2.0 ± 1.1	3.4 ± 1.1	4.5 ± 3.8	2.9 ± 1.9	2.8 ± 1.2
FFAC26:1	2.4 ± 0.5	2.8 ± 1.8	2.0 ± 1.1	2.8 ± 1.1	4.1 ± 3.1	2.6 ± 1.8	2.8 ± 1.4
FFAC28:1	1.4 ± 0.6	1.6 ± 1.1	1.2 ± 0.7	1.4 ± 0.7	2.1 ± 1.5	1.4 ± 1.0	1.6 ± 1.0

There is no significant difference in the levels of MUFA in cytokine treated LEMs compared with control LEMs. The data represents the mean and standard deviation of 3 independent experiments. SC: Stratum corneum.

Table S7: Absolute amount of EO CERs and non-EO CERs in cytokine stimulated LEMs per dry weight of stratum corneum ($\mu\text{g}/\text{mg}$ SC; analyzed by LC-MS).

LEM	EO CERs ($\mu\text{g}/\text{mg}$ SC)	Non-EO CERs ($\mu\text{g}/\text{mg}$ SC)
IL-31	$11.2 \pm 4.2^*$	100.3 ± 4.2
TNF- α	$19.0 \pm 2.8^*$	156.2 ± 2.8
Control	27.6 ± 9.9	125.1 ± 9.9

IL-31 and TNF- α significantly reduce the levels of EO CERs in comparison with the control LEMs. The data represents the mean and standard deviation of 3 independent experiments (* $p < 0.05$). SC: Stratum corneum

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture

The skin was collected after written informed consent from the donors and handled according to the Declaration of Helsinki Principles. Keratinocytes were cultured in a monolayer with the Dermalife K medium complete kit (Lifeline Cell Technology, Walkersville, MD, USA) supplemented with 1% penicillin/streptomycin (Sigma, The Netherlands) until 60-70% confluent. The keratinocytes were harvested by trypsin digestion. First passage keratinocytes were used to generate LEM.

Generation of the Leiden epidermal models (LEMs)

Keratinocytes (0.2×10^6 cells/filter) were seeded into cell culture inserts (Corning Transwell cell culture inserts, membrane diameter 12 mm, pore size $0.4 \mu\text{m}$; Corning Life Sciences, Amsterdam, The Netherlands) and cultured submerged in supplemented Dermalife medium until confluent. Subsequently, the LEMs were submerged for 1 day in CnT-02-3DP5 medium (CellnTec, Bern, Switzerland) and serum free medium containing $0.1\mu\text{M}$ L-carnitine (Sigma), $86\mu\text{M}$ insulin (Sigma), $10\mu\text{M}$ L-serine (Sigma), Isoproterenol $1\mu\text{M}$, 53nM selenious acid (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany), supplemented with $1 \mu\text{M}$ α -tocopherol acetate (Sigma), 25mM ascorbic acid (Sigma) and a lipid mixture of $7 \mu\text{M}$ arachidonic acid (Sigma), $30 \mu\text{M}$ linoleic acid (Sigma) $25 \mu\text{M}$ palmitic acid (Sigma) with $0.024 \mu\text{M}$ bovine serum albumin (Sigma). After this period the cultures were lifted to the air-liquid interface for 7 days. On day 7 of air exposure, IL-4, IL-13, IL-31 and TNF- α (R&D systems, Abingdon, United Kingdom, 30ng/ml , 30ng/ml , 15ng/ml and 3.5ng/ml respectively) or a combination of the interleukins in presence and absence of TNF- α (5ng/ml each) was supplemented to the medium until harvest, for 11 days.

Morphology and Immunohistochemistry

For both staining procedures (with amino-ethylcarbazole and immunofluorescence), the sections were deparaffinised, rehydrated with ethanol series and washed with phosphate-buffered saline (PBS). The sections were incubated in sodium citrate buffer (pH 6) for 30 minutes at 95°C for antigen retrieval. After cooling, the sections were blocked for 20 minutes with normal horse serum and incubated with the primary antibody diluted in 1% PBS/bovine serum albumin (BSA) overnight at 4°C .

Staining procedure with amino-ethylcarbazole: Sections were washed in PBS and incubated with the secondary antibody for 30 minutes, washed with PBS and incubated with ABC reagent for

30 minutes. The sections were further washed with PBS and 0.1M sodium acetate buffer, after which they were incubated for 30 minutes in amino-ethylcarbazole (Sigma) dissolved in sodium acetate buffer (0.02g/100mL) (Sigma) with 0.1% hydrogen peroxide and washed finally with water. Incubation with normal horse serum, ABC reagent and secondary antibody was performed with the RTU vectastain Elite ABC reagent kit (Vector laboratories Burlingame, CA). All sections were counterstained with haematoxylin.

Immunofluorescence staining: Sections were washed in PBS and incubated with the secondary antibody for 30 minutes and again washed with PBS. The sections were mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

RNA isolation, cDNA synthesis and qPCR

The LEMs firstly underwent a proteinase K treatment and then RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer's instructions. cDNA was produced with the iScript cDNA synthesis kit (BioRad, the Netherlands) according manufacturer's instructions. PCR reactions were based on the SYBR Green method (BioRad) with the use of CFX384 system (BioRad). Expression analysis was performed using CFX software using the $\Delta\Delta C_t$ method using the reference gene β -2-microglobulin. Data represent mean and standard deviation of 3 different experiments.

Protein isolation and Western blot

Proteins were isolated from LEMs using Tissue Protein Extraction Reagent (T-PER) which was supplemented with protease inhibitor cocktail and HALTTM phosphatase inhibitors (Thermo Scientific, Etten-Leur, the Netherlands). Protein extracts were loaded onto a 10% SDS-PAGE gel and blotted on a polyvinylidene difluoride membrane (Thermo Scientific). 10% Elk Milk (Campina, The Netherlands) in phosphate-buffered saline-T (0,1% Tween) was used as a blocking buffer. The sample was incubated overnight at 4°C with primary antibody after which they were incubated with the secondary antibody, horseradish peroxidase-conjugated anti-rabbit (Thermo Scientific, 1:2500). Proteins were detected using Supersignal West Femto ECL (Thermo Scientific/Pierce).

Fourier transformed infra-red spectroscopy

SC samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution. A Varian 670-IR FTIR spectrometer (Agilent technologies, Santa Clara, CA), equipped with a broad-band mercury cadmium telluride (MCT) detector, cooled with liquid nitrogen was utilized to acquire spectra. The sample was sandwiched between silver bromide (AgBr)

windows and spectra collected in transmission mode and derived from the addition of 256 scans at 1 cm⁻¹ resolution every 4 minutes (frequency range of 600-4000 cm⁻¹, temperature range of 0 °C and 90°C at a rate of 0.25°C/min). Varian resolutions Pro software (Agilent technologies, the Netherlands) was used to process the spectra.

Small angle X-ray diffraction

All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) at station BM26B. SAXD patterns were detected with a Frelon 2000 CCD detector at room temperature for 10 min using a microfocus of 25µm X 25µm. The X-ray wavelength and sample-to-detector distance were 0.1127nm and 24cm respectively. The samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution. The scattering intensity (I, arbitrary units) was measured as a function of the scattering vector q (in nm⁻¹). Q is defined as $q = (4\pi \sin\theta)/\lambda$, in which θ is the scattering angle and λ is the wavelength. From the positions of a series of equidistant peaks (q_n), the periodicity (d) of a lamellar phase was calculated using the equation $d = 2\pi/q_n$, n being the order of the lamellar phase. At least 3 samples per condition were measured.

Liquid chromatography mass spectrometry (LC-MS)

FFA species: LC-MS analysis of FFA species was performed according to van Smeden et al., submitted¹. Injection volume of extracted lipids (1mg/ml) was set at 10 µl into a LiChroCART Purospher STAR analytical column (55 x 2 mm i.d. Merck, Darmstadt, Germany) under a flow rate of 0.5 ml/min using a binary gradient solvent system of ACN/H₂O (90:10, solvent A) and methanol/heptane (90:10, solvent B). An Alliance 2695 HPLC system (Waters, Milford, MA, USA) was used in reverse phase. The HPLC was connected to a TSQ Quantum Mass Spectrometer (TSQ Quantum, Thermo Finnigan, San Jose, CA) measuring in Atmospheric-pressure chemical ionization (APCI) negative mode (scan range of 200-600 amu) The source heater temperature and heated capillary was set at 450°C and 250°C respectively and the discharge current at 5µA. The analysis was performed using Xcalibur software version 2.0. The ceramide analysis is described in the supplementary materials and methods.

CER species: 1 µl of each lipid sample (40°C) was automatically injected and separated in an analytical normal phase column set at 55°C (ACQUITY HSS Cyano; 21. x 100mm; 1.8µm particle size; Waters, Milford, MA) under a flow rate of 0.8ml/min and using a binary solvent system of heptane (100%, solvent A) and heptane/isopropanol/ethanol (50:25:25, solvent B). An ACQUITY quaternary UPLC system (Waters, Milford, MA) was coupled to a mass spectrometer (Xevo, TQ-S, Waters, Milford, MA) in atmospheric pressure chemical ionization (APCI; ion sabre

II), positive scan mode with a scan range of 350-1200 amu. The temperature of the source heater was set at 625°C, capillary voltage at 3.5kV. The analysis was performed using Target Lynx software version 4.1.