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Activation of TLR3 in keratinocytes increases expression of genes involved in formation of the epidermis, lipid accumulation and epidermal organelles

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Abstract

Injury to the skin, and the subsequent release of non-coding double-stranded RNA from necrotic keratinocytes, has been identified as an endogenous activator of Toll-like receptor 3 (TLR3). Since changes in keratinocyte growth and differentiation follow injury, we hypothesized that TLR3 might trigger some elements of the barrier repair program in keratinocytes. Double-stranded RNA was observed to induce TLR3-dependent increases in human keratinocyte mRNA abundance for ABCA12 (ATP-binding cassette, sub-family A, member 12), glucocerebrosidase, acid sphingomyelinase, and transglutaminase 1. Additionally, treatment with double-stranded RNA resulted in increases in sphingomyelin and morphologic changes including increased epidermal lipid staining by oil-red O and TLR3-dependent increases in lamellar bodies and keratohyalin granules. These observations show that double-stranded RNA can stimulate some events in keratinocytes that are important for skin barrier repair and maintenance.

INTRODUCTION

Rapid recovery of epidermal barrier function following injury prevents water loss and opportunistic infection by infiltrating microbes. Barrier repair after injury involves trafficking of lamellar bodies (LB) to the SC, where they secrete their contents (Menon *et al.*, 1992), and activation of several other genes, including the ATP-binding cassette sub-family A, member 12 (ABCA12), an essential lipid transporter in the epidermis responsible for harlequin ichthyosis (Akiyama *et al.*, 2005) and lamellar ichthyosis type 2 (Lefévre C *et*

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CONFLICT OF INTEREST
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al., 2003), and lipid metabolism enzymes, glucocerebrosidase (GBA) (Holleran *et al.*, 1994) and acid sphingomyelinase (SMPD1) (Jensen *et al.*, 1999). Cholesterol (Menon *et al.*, 1985), free fatty acid (Mao-Quiang *et al.*, 1993; Ottey *et al.*, 1995), and ceramide synthesis all also increase following skin barrier disruption (Holleran *et al.*, 1991; Holleran *et al.,* 1995) and are essential for barrier repair. The mechanisms that regulate the complex events that comprise barrier repair are incompletely defined, though a calcium gradient in the epidermis plays an important role (Lee *et al.*, 1992; Menon *et al.*, 1992). In the present study we sought to test the hypothesis that double-stranded RNA, recently discovered to be an endogenous product produced by epidermal injury following trauma or excess UVB exposure (Lai *et al*., 2009; Bernard *et al*., 2012), might serve as a trigger for expression of genes important to the epidermal barrier repair process.

dsRNA recognition can occur by several mechanisms including binding to Toll-like receptor 3 (TLR3). TLR3 signaling has largely been described as a recognition and response system to combat viral infections (Dunleavy *et al.*, 2010; Kawai and Akira, 2008). Patients who have mutations in TLR3 (Zhang *et al.*, 2007), UNC-93B, an ER membrane protein important for its trafficking to the endosome, (Casrouge *et al.*, 2006), or TIR-domain-containing adapter-inducing interferon-β (TRIF), a key adaptor signaling molecule for TLR3 signaling (Sancho-Shimizu *et al.*, 2011), are more susceptible to herpes simplex virus encephalitis. Thus, the importance of TLR3 in sensing viruses is not disputed, though more recently it has been shown to have an expanded role in a number of epithelial tissues. For example, inflammatory cytokines are induced by RNA released during necrosis in the gut (Cavassani *et al.*, 2008) or damage to the skin (Lai *et al.*, 2009; Bernard *et al.*, 2012). Additionally, recent publications have also demonstrated that TLR3 is important for wound healing, as *Tlr3*$^{-/-}$ mice have a slightly delayed wound healing phenotype (Lin *et al.*, 2011), while Polyinosinic acid:Polycytidylic acid (Poly (I:C)), a double-stranded RNA (dsRNA) analog and ligand for TLR3, can promote wound healing in mice (Lin *et al.*, 2012).

These recent findings suggest that TLR3 may have multiple functions in the skin and may signal the start of barrier repair processes in addition to its role in viral defense. We show herein that TLR3 activation increased expression of genes critical to barrier formation, increased the appearance of epidermal lipids, and increased LB and keratohyalin granules (KHG), important elements of the epithelial barrier repair response. These findings therefore identify TLR3 as a potential regulator of epidermal regeneration following injury.

RESULTS

Gene expression profiling of lipid metabolism and lipid transporter pathways

To identify gene expression pathways in addition to the known inflammatory response associated with TLR3 activation of keratinocytes, we examined the transcriptome of NHEK 24 hours after exposure to the dsRNA Poly(I:C). In response to Poly (I:C), a total of 5542 differentially regulated genes changed by at least 2-fold (2773 upregulated and 2769 downregulated; SAM: triplicate; FDR < 0.01%; delta value = 1.397) (Supplemental Figure S1a). These genes were further analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.*, 2009; Huang *et al.*, 2009). This analysis suggested Poly(I:C) affected a number of pathways involved in lipid metabolism and

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transport. Specifically, changes were observed in the expression of genes in glycosphingolipid biosynthesis, ABC transporters, sphingolipid metabolism, and other lipid biosynthesis/metabolism and inflammatory pathways (Figure 1a). Several specific genes identified by this approach are known to play a role in maintaining or forming the skin barrier, such as: ABCA12 (3.74-fold), GBA (2.02-fold), SMPD1 (2.04-fold), TGM1 (2.40-fold), as well as tumor necrosis factor (TNF) (5.31-fold), interleukin 6 (IL-6) (27.31-fold), and TLR3 (14.58-fold). Involucrin (IVL), loricrin (LOR), keratin 1 (KRT1), keratin 14 (KRT14) and filaggrin (FLG), markers of epidermal differentiation, were not changed significantly (Figure 1b). The results of the microarray showed that genes involved in synthesis of ceramides (UGCG, SPTLC1, SPTLC2), free fatty acids (ACACA and FASN) and cholesterol (FDFT1, HMCCR, and HMGSC1) were not significantly altered other than acetyl CoA carboxylase (ACACA) (0.33-fold).

Poly (I:C) enhances transcript abundance of genes involved in skin barrier formation

To validate the results of the gene expression profile of Poly (I:C)-treated NHEK, we measured a number of genes by real time PCR. mRNA of ABCA12, GBA, SMPD1, and TGM1 were significantly upregulated following treatment with Poly (I:C) (Figure 2a, Table 1). As expected, traditional inflammatory markers TNF, IL-6, and TLR3 were also upregulated (Figure 2b, Table 1). Consistent with microarray data, this treatment did not induce expression of involucrin (IVL), keratin 1 (KRT1), loricrin (LOR), filaggrin (FLG), or keratin 14 (KRT14) (Figure 2c, Table 1). Interestingly, Poly (I:C) treatment significantly induced expression of mRNA for ceramide synthesis enzymes, including serine palmitoyltransferase (SPTLC1 and SPTLC2) and glucosylceramide synthase (UGCG) (Figure 1b, Table 1). Ligands for TLR2,-7, -8, and -9 did not significantly alter expression of these barrier repair genes (Supplemental Figure 2a and 2b). A dose-dependent increase in the mRNA levels of ABCA12, GBA, SMPD1, TNF, and IL-6 was observed following treatment with Poly (I:C), with maximal expression seen after treatment with 0.5 to 1 μg/ml Poly (I:C) (Figure 2d). Increases in transcript abundance for ABCA12, GBA AND SMPD1 was not seen until 24 hours after treatment, while TNF expression increased more rapidly and was maximal at 1hr (Figure 2e). However, the latter effect on gene expression was not due to induction of these cytokines as treatment of NHEK with TNF or IL-6 had no significant effect on the induction of ABCA12, GBA, or SMPD1 mRNA levels (Figure 2e).

TLR3 activation is required for dsRNA-induced changes in gene expression

To determine if the increases in ABCA12, GBA, SMPD1, and TGM1 mRNA after Poly (I:C) treatment were dependent on TLR3 activation, we used siRNA to knockdown TLR3 in NHEK. When TLR3 was significantly decreased in keratinocytes, Poly (I:C) failed to induce a significant increase in mRNA for the barrier repair genes ABCA12, GBA, SMPD1, TGM1 and TNF (Figure 3a). Since TLR3 signaling is dependent on proper acidification and maturation of endosomes (Matsushima *et al.,* 2004), we used Bafilomycin A1 (BafA1), a specific inhibitor of the V-type ATPase required for acidification of endosomes and lysosomes, to inhibit TLR3 signaling. BafA1 blocked Poly (I:C)-induced increases in ABCA12, GBA, and SMPD1 mRNA as well as increases in mRNA of the inflammatory cytokines TNF and IL-6 (Figure 3b). Similar effects on gene expression were seen when TRIF, a key signaling molecule downstream of TLR3, was knocked down (Supplementary

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Figure S4). Unlike silencing of TLR3 or TRIF, knocking down MAVS, a key signaling molecule for RIG-I-like receptors that recognizes cytosolic dsRNA, had no significant effect on Poly (I:C)-induced expression of ABCA12, GBA, SMPD1, TGM1 (Supplemental Figure S5). Although TLR3 activation was important for Poly (I:C)-induced increases in UGCG mRNA, alterations in mRNA for several lipid synthesis genes was largely independent of TLR3 (Figure 3c).

Activation of TLR3 alters epidermal lipid content

To determine whether increases in ABCA12, GBA, and SMPD1 transcripts were paralleled by changes in epidermal lipid composition, we first stained for the presence of lipid in NHEK grown in differentiating conditions. A large increase in oil red O-staining bodies was seen when NHEK were exposed to Poly (I:C) for 72 hours (Figure 4a). A significantly higher expression of ABCA12 and GBA was also seen upon Poly (I:C) treatment under these conditions (Supplemental Figure 3a and 3b). Lipids were then quantified by measuring the amount of oil red O dye that was retained after staining and normalizing this to total protein. Poly (I:C) treatment induced significant increases in lipids stained by oil red O at days 1, 2 and 3 (Figure 4b). Next, 3-dimensional skin constructs were exposed to Poly (I:C) to determine the response of stratified and differentiated keratinocytes, that model the epidermis but are not influenced by the presence of resident or recruited leukocytes that would be present in vivo. In these 3-D skin constructs, the stratum corneum (SC) of Poly (I:C)-treated samples stained more strongly for oil red O compared to control samples (Figure 4c).

To measure the response of specific lipid components produced by cultured keratinocytes, total lipids were isolated from NHEK after Poly (I:C) treatment and resolved using high performance thin layer chromatography. Sphingomyelin was significantly increased following Poly (I:C) treatment and this increase was blocked following siRNA silencing of TLR3 (Figure 4d). Glucosylceramide levels were significantly decreased after Poly (I:C) treatment although this change was independent of TLR3 (Figure 4e). Ceramides increased following Poly (I:C) treatment, though this increase was not abolished by knock-down of TLR3 (Figure 4f). Cholesterol levels were not significantly altered after Poly (I:C) treatment in either control or TLR3 knockdown keratinocytes (Figure 4g).

TLR3 activation increases the quantity of lamellar bodies and keratohyalin granules in the epidermis

Since we observed increases in the staining of lipids following exposure to dsRNA, we next sought to determine if other morphological changes in keratinocytes could be observed by electron microscopy. To assess this, we quantitated the number of lamellar bodies (LB) and keratohyalin granules (KHG) in the upper stratum granulosum in 3-D epidermal skin constructs. Skin constructs treated with Poly (I:C) had significantly more LB and KHG (Figure 5a and 5b). The observed increases in LB and KHG were dependent on TLR3, as skin constructs generated from TLR3-knockdown NHEK failed to exhibit significant increases in LB and KHG when exposed to Poly (I:C) (Figure 5c).

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DISCUSSION

TLR activation is classically considered to result in pro-inflammatory responses. In this study, we demonstrate that TLR3 activation of keratinocytes also leads to changes in expression of some genes in keratinocytes that are associated with epidermal structure. An increase in transcript abundance of ABCA12, GBA, SMPD1, and TGM1 occurred in a TLR3-dependent manner. This response was followed by increases in epidermal lipid accumulation as well as increases in the abundance of LB and KHG in epidermal equivalents. These observations are consistent with recent observations that dsRNA is released by damaged cells and can serve as a damage-associated molecular pattern (DAMP). Thus, we now show that skin epithelial cells initiate some of the events associated with barrier repair after recognition of dsRNA.

The protective properties of the skin barrier reside in the SC and are heavily dependent on the lipid-rich lamellar membranes surrounding differentiated keratinocytes (Feingold and Denda, 2012; Feingold, 2007; Holleran *et al*., 1991). Previous studies have characterized the barrier repair response, delineating the increase in epidermal lipid synthesis and metabolism in the skin (Holleran *et al.*, 1991; Feingold, 1991; Menon *et al.*, 1985) and secretion of LB following barrier disruption (Menon *et al*., 1992). Because our current observations show TLR3 activation is accompanied by increases in mRNA encoding genes involved in epidermal formation, accumulation of epidermal lipids, and formation of epidermal organelles, we provide evidence that TLR3 may be a previously unknown mechanism by which keratinocytes detect epidermal injury and initiate some of the steps involved in formation of a functional skin barrier. However, since mice lacking TLR3 appear to develop normally, this recognition system is not critical to normal development. Furthermore, although many Poly (I:C) induced changes in lipid composition and quantity were observed, not all of these changes were TLR3 dependent. It is important to keep in mind that we did not observe a global upregulation in lipid or differentiation markers. In contrast, we observed that dsRNA can induce TLR3-dependent changes only in specific elements involved in the process of repair. How these responses combine into the complete barrier repair program remain to be determined.

A number of receptors are known to recognize and respond to dsRNA (Yu and Levine, 2011), making it important to determine whether TLR3 activation was required for the gene expression changes seen in response to dsRNA. Using both RNA silencing and pharmacological inhibition, we demonstrated that increases in skin barrier genes in response to Poly (I:C) were dependent on TLR3. Since the Poly (I:C)-induced changes in mRNA of these barrier genes were almost completely abrogated when TLR3 activation was silenced, our data suggest that TLR3 activation is required for the observed changes. These data do not rule out the contribution of cytoplasmic sensors of dsRNA that exist in the cell, including RIG-I, MDA5, PKR, and NLRP3, although the failure of MAVS knockdown to partially inhibit the Poly (I:C) response argues against a role for cytoplasmic RNA recognition. Thus, the relevance of these sensors in keratinocytes is yet to be clearly defined.

As changes in gene expression are not seen until 24 hours after Poly (I:C) treatment, it is possible that the change in transcription of ABCA12, GBA, SMPD1 and TGM1 is not a

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direct downstream transcriptional event of TLR3 activation, but rather an autocrine or paracrine effect dependent on synthesis of intermediate genes. TNF and IL-6 are produced following TLR3 activation and have been shown to improve the skin barrier (Jensen *et al*., 1999; Wang *et al*., 2004). Therefore, we also examined the direct effects of these cytokines on induction of the barrier genes of interest. Since no changes in gene expression of ABCA12, GBA, or SMPD1 were observed with TNF or IL-6 treatment of keratinocytes under conditions similar to those where Poly (I:C) did induce these cytokines, it is unlikely that these cytokines acting alone are responsible for the observed effects. Future work will seek to better understand the factors responsible for transcriptional regulation of ABCA12, GBA, SMPD1, and TGM1 by TLR3, with specific interest in determining if these genes represent an immediate canonical response to TLR3 or are activated in response to stimulation of intermediate factors that may function in an autocrine manner.

Poly (I:C) recognition by TLR3 in keratinocytes appears to be functionally relevant. Sphingolipids, such as ceramides and its precursors sphingomyelin and glucosylceramides, are essential for the formation and maintenance of the skin barrier (Holleran *et al*., 1991). Poly (I:C)-treated keratinocytes displayed a rapid appearance of lipid-containing vesicles and an increase in ceramides. More intense lipid staining in the stratum corneum was also observed in 3-D skin constructs treated with Poly (I:C). Additionally, sphingomyelin levels were increased by Poly (I:C) treatment in a TLR3-dependent manner. In contrast, levels of glucosylceramides decreased following Poly (I:C) treatment, but not in a TLR3-dependent manner. These observations confirmed the importance of TLR3 activation in perturbing epidermal lipid levels but suggest other pathways influenced by the addition of Poly(I:C) also contribute to the response of some lipids. It remains to be determined whether Poly (I:C) treatment of NHEK can stimulate *de* novo lipid synthesis. As our mRNA data of a number of lipid metabolism genes show that dsRNA can alter these transcripts, it is possible that the enzyme activity could also be altered, but this remains to be explored. As ORO-positive vesicles most likely contain a mix of nonpolar lipids, we do not believe that the TLR3-dependent increases in sphingomyelin are being detected in those experiments, rather other lipid species that have yet to be identified. Future studies will hopefully elucidate whether *de novo* lipid synthesis is occurring and which additional lipid species are increased in a TLR3-dependent manner.

To further investigate functional changes in keratinocytes following Poly (I:C) treatment, we examined ultrastructural changes in keratinocytes within 3-D skin constructs. Treatment of keratinocytes with Poly (I:C) yielded a higher amount of both LB and KHG in the granular layer of the epidermis. Although LB have been found to be depleted in the granular layer following barrier disruption due to their rapid trafficking and secretion of barrier components (Menon *et al.,* 1992), they are rapidly regenerated to aid in future barrier repair as well as proper differentiation and barrier formation. We speculate that TLR3 activation by endogenous dsRNA could be an initiation event that leads to downstream effects of epidermal lipid production, trafficking, and metabolism. Increases in KHG also provide further evidence that Poly (I:C) treatment could promote barrier formation or repair as KHG also contain essential barrier components of the stratum corneum including profilaggrin and LOR. Though we do not see increases in transcripts of FLG and LOR after Poly (I:C)

treatment, the increased presence of KHG provides evidence suggesting that dsRNA can influence barrier formation.

We believe that this work identifies a key recognition event that could trigger some elements of skin barrier formation during the process of repair. How this recognition event is propagated after TLR3 activation remains to be determined. These results however, could be utilized in the treatment of certain dermatological diseases such as atopic dermatitis (AD). For years it has been known that AD patients have significantly decreased ceramide levels in the stratum corneum (Melnik *et* al., 1988; Yamamoto *et* al., 1991). Although some recent studies have shown AD to be associated with *FLG* mutations (Palmer *et al*., 2006), a number of reports cite and characterize cases of AD that are independent of *FLG* mutations (Jasaka *et al*., 2011) and demonstrate that AD patients have abnormal ceramide profiles and lamellar lipid organization (Ishikawa *et al*., 2010; Janssens *et al*., 2011). From our research, it could be speculated that this deficiency of ceramides in the stratum corneum of AD patients could be treated by pharmacological activation of pathways downstream of TLR3, thus leading to increases in ceramides. Of course, many unwanted inflammatory side effects may result from TLR3 activation, so it will be important to determine specifically which pathways downstream of TLR3 are involved in the increase of epidermal lipids. By examining these downstream pathways, we may also discover more about the regulatory events involved in ceramide biosynthesis and metabolism that could be affected in AD patients. Future studies will involve identifying and characterizing these downstream pathways of TLR3 activation relevant to barrier repair.

MATERIALS & METHODS

Cell Culture and Stimuli

NHEK were obtained from Cascade Biologics/Invitrogen. (catalog number: C-001-5C, Portland, OR), and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM Ca^{2+} and 1 × EpiLife Defined Growth Supplement (EDGS, Cascade Biologics/Invitrogen) at 37 °C under standard tissue culture conditions. All cultures were maintained for up to eight passages in this media with the addition of 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 250 ng ml^{-1} amphotericin B. Cells at 60–80% confluence were treated with Bafilomycin A1 (5 nM; Sigma), poly(I:C) (1 μg ml^{-1}; Invivogen), IL-6 (10 ng ml^{-1}; R&D Systems), or tumor necrosis factor-α (50 ng ml^{-1}; Chemicon, Temecula, CA) in 12-well flat bottom plates (Corning Incorporated Life Sciences, Lowell, MA) for up to 24 hours. After cell stimulation, RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was stored at –80°C.

For visualization or analysis of lipids, cells were switched to advanced stage differentiation media (DMEM:F-12 (2:1), 10% FBS(Invitrogen),400 ng ml^{-1} hydrocortisone (Sigma), 10 ug ml^{-1} human recombinant insulin (Sigma), and freshly made 50 ug ml^{-1} ascorbic acid (Sigma)) for 24 to 72 hours during stimulation. Media was changed every other day and freshly prepared and filter sterilized Vitamin C was added.

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Quantitative real-time PCR

Total RNA was extracted from cultured keratinocytes using TRIzol® Reagent (Invitrogen, Carlsbad, CA) and 1 ug RNA was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Pre-developed Taqman® Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to evaluate mRNA transcript levels of ABCA12, GBA, SMPD1, TGM1, TNF, IL6, KRT1, KRT14, IVL, FLG, LOR, and TLR3. GAPDH mRNA transcript levels were evaluated using a VIC-CATCCATGACAACTTTGGTA-MGB probe with primers 5′ CTTAGCACCCCTGGCCAAG-3′ and 5′-TGGTCATGAGTCCTTCCACG-3′. All analyses were performed in triplicate and representative of three to five independent cell stimulation experiments that were analyzed in an ABI Prism 7000 Sequence Detection System. Fold induction relative to GAPDH was calculated using the C_t method. Results were considered to be significant if $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ABCA12	ATP-binding cassette, sub-family A, member 12
AD	atopic dermatitis
dsRNA	double-stranded RNA
FLG	filaggrin
GBA	glucocerebrosidase
IL-6	interleukin 6
IVL	involucrin
KHG	keratohyalin granules
LB	lamellar bodies
LOR	loricrin
NHEK	Normal human epidermal keratinocytes
Poly (I:C)	Polyinosinic acid: polycytidylic acid
SC	stratum corneum
SMPD1	acid sphingomyelinase

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TGM1	transglutaminase 1
TLR3	Toll-like receptor 3
TNF	Tumor necrosis factor

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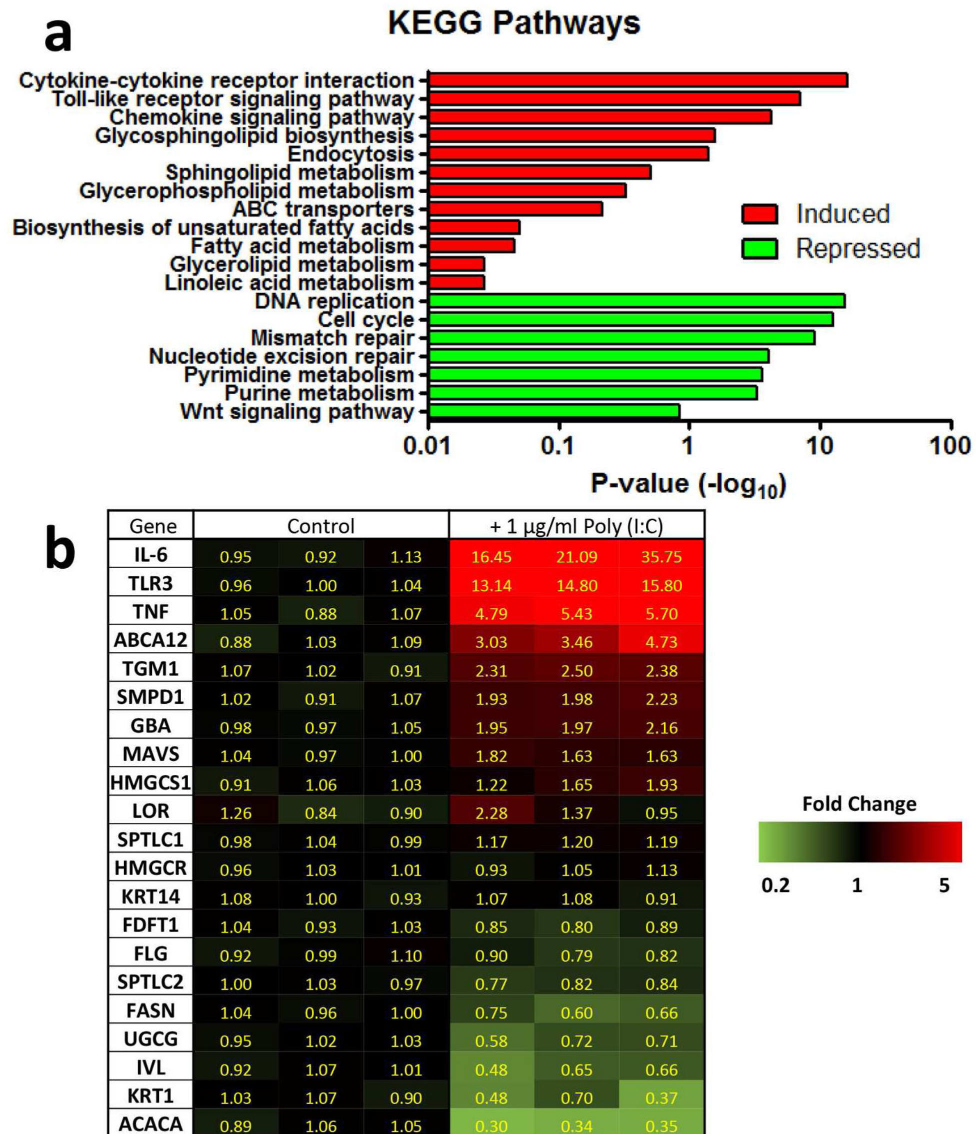

Gene	Control			+ 1 μg/ml Poly (I:C)		
IL-6	0.95	0.92	1.13	16.45	21.09	35.75
TLR3	0.96	1.00	1.04	13.14	14.80	15.80
TNF	1.05	0.88	1.07	4.79	5.43	5.70
ABCA12	0.88	1.03	1.09	3.03	3.46	4.73
TGM1	1.07	1.02	0.91	2.31	2.50	2.38
SMPD1	1.02	0.91	1.07	1.93	1.98	2.23
GBA	0.98	0.97	1.05	1.95	1.97	2.16
MAVS	1.04	0.97	1.00	1.82	1.63	1.63
HMGCS1	0.91	1.06	1.03	1.22	1.65	1.93
LOR	1.26	0.84	0.90	2.28	1.37	0.95
SPTLC1	0.98	1.04	0.99	1.17	1.20	1.19
HMGCR	0.96	1.03	1.01	0.93	1.05	1.13
KRT14	1.08	1.00	0.93	1.07	1.08	0.91
FDFT1	1.04	0.93	1.03	0.85	0.80	0.89
FLG	0.92	0.99	1.10	0.90	0.79	0.82
SPTLC2	1.00	1.03	0.97	0.77	0.82	0.84
FASN	1.04	0.96	1.00	0.75	0.60	0.66
UGCG	0.95	1.02	1.03	0.58	0.72	0.71
IVL	0.92	1.07	1.01	0.48	0.65	0.66
KRT1	1.03	1.07	0.90	0.48	0.70	0.37
ACACA	0.89	1.06	1.05	0.30	0.34	0.35

Figure 1. Gene expression profiling of NHEK identifies upregulation of genes involved in lipid biosynthesis, metabolism, and transporter pathways following treatment with dsRNA

(a) Significantly changed genes analyzed using DAVID to identify significant pathways (EASE = 1.0). (b) Genes involved in barrier formation with a significant change as identified by SAM

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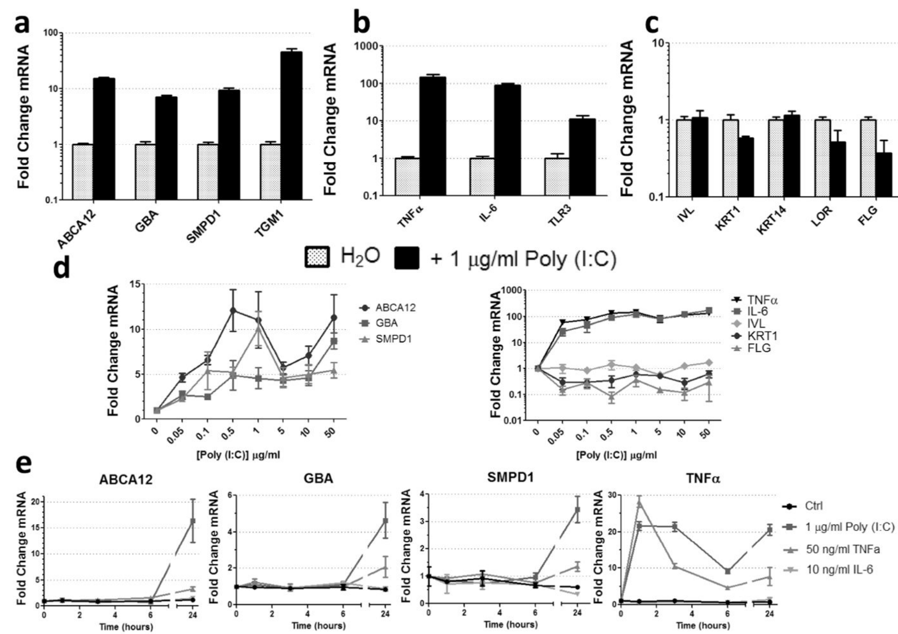

Figure 2. Poly (I:C) enhances transcript abundance of genes involved in skin barrier formation

NHEK were cultured in the presence of 1 μg/ml Poly (I:C) for 24 h. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative and normalized to GAPDH expression for a number of (a) barrier genes, (b) inflammatory cytokines, and (c) keratinocyte differentiation markers. (d) NHEK were cultured with various doses of Poly (I:C) for 24 h. (e) NHEK were incubated with 1 μg/ml Poly (I:C), 50 ng/ml TNF, or 10 ng/ml IL-6 for 0, 1, 3, 6, or 24. h. Data are mean ± SEM, n = 3, and are representative of at least three independent experiments. *$P < 0.05$. Two tailed T-test.

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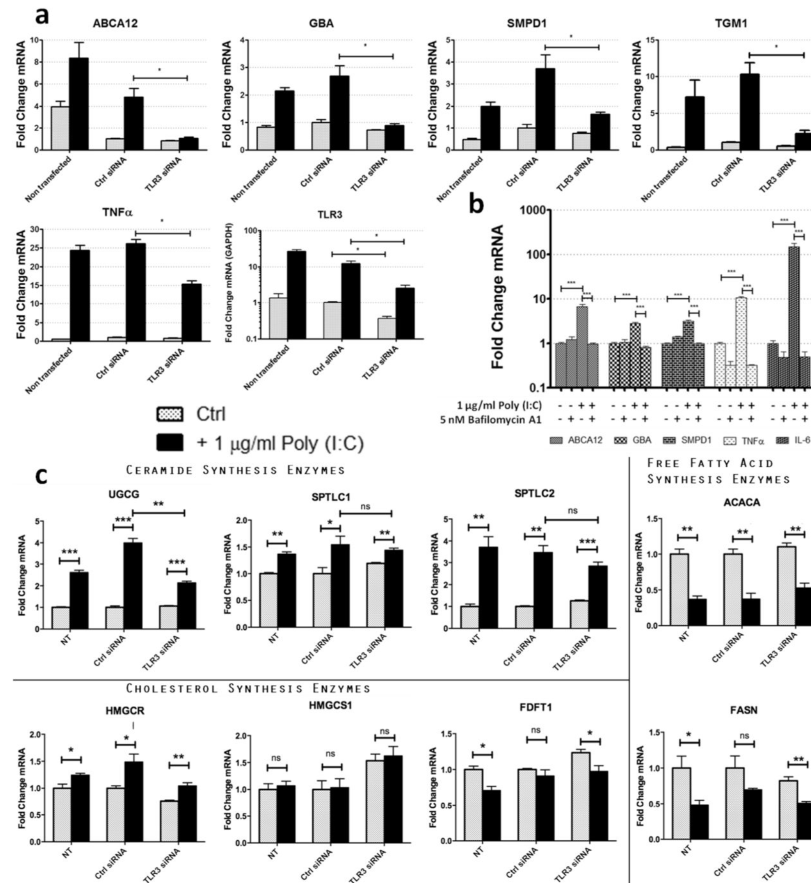

Figure 3. TLR3 activation is required for dsRNA-induced changes in gene expression

(a) TLR3 was silenced in NHEK for 48 h before treatment with 1 μg/ml Poly (I:C) for 24 h. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative and normalized to GAPDH expression. **P* < 0.05. Two tailed T-test. (b) NHEK were treated with 5 nM Bafilomycin A1 for 1 h prior to treatment with 1 μg/ml Poly (I:C) for 24 h. ****P* < 0.001. One-way ANOVA. (**c**) TLR3 was silenced in NHEK for 48 h before treatment with 1 μg/ml Poly (I:C) for 24 h. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative to and normalized to GAPDH expression. **P* < 0.05. Two tailed T-test. Data are mean ±SEM, n = 3, and are representative of at least three independent experiments.

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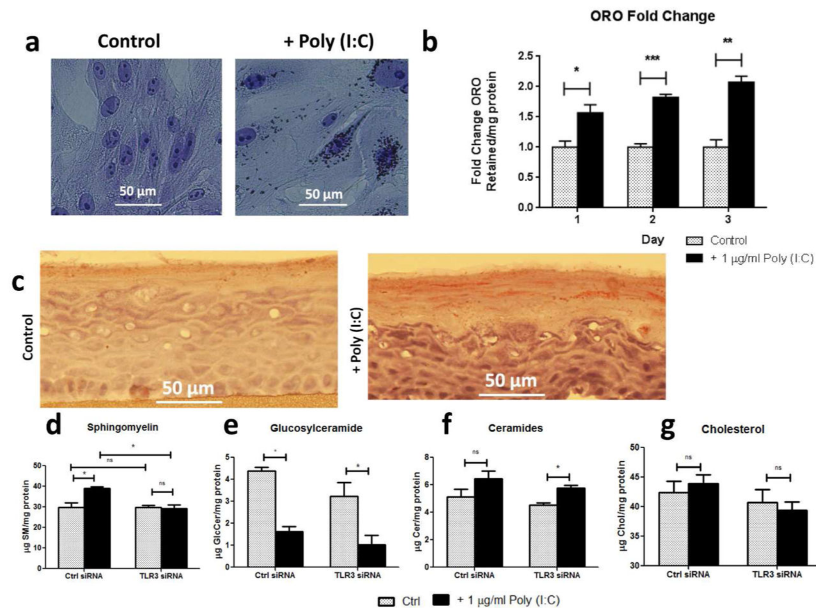

Figure 4. Activation of TLR3 alters epidermal lipid content

(a) NHEK were treated for 72 h with 10 µg/ml Poly (I:C), stained with Oil Red O and counterstained with Hematoxylin. Scale bar = 50 µm. (b) NHEK were treated with 1 µg/ml Poly (I:C) for 1, 2, or 3 days, then stained with Oil Red O. (c) 3-D tissue constructs were treated with 1 µg/ml Poly (I:C) for 72 h. Samples were stained with Oil Red O and counterstained with Hematoxylin. Scale bar = 50 µm. (d–g) NHEK were treated with 1 µg/ml Poly (I:C) for 24 h following siRNA knockdown of TLR3. (d) Sphingomyelin, (e) glucosylceramide (f) ceramides, and (g) cholesterol were quantified using HPTLC relative to total protein. *$P < 0.05$. Two-tailed T-test. Data are mean ±SEM, n =3, and are representative of at least three independent experiments.

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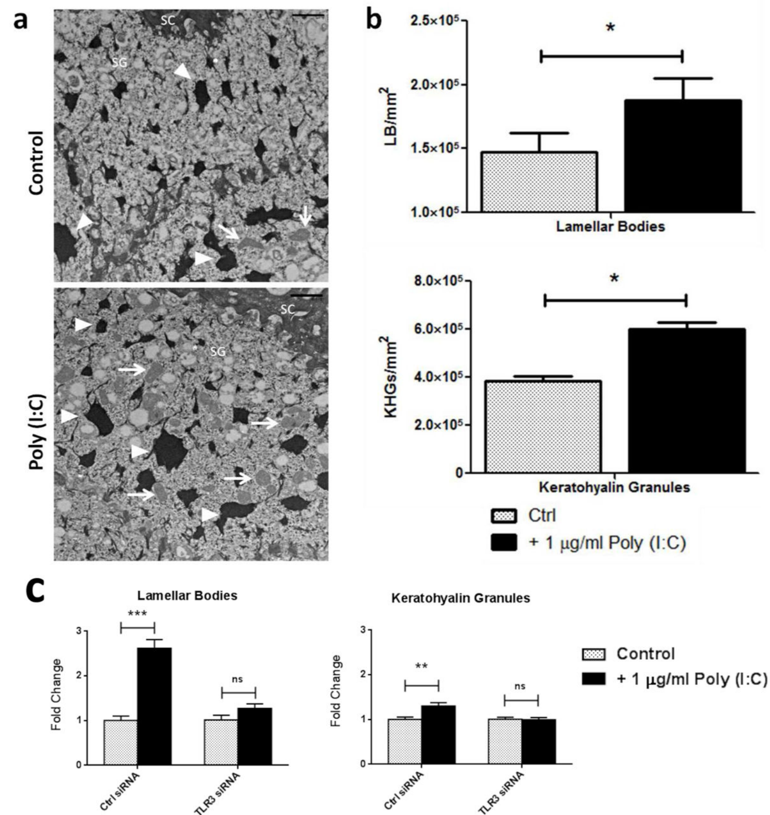

Figure 5. TLR3 activation increases the quantity of LB and KHG in the epidermis

(a) Transmission electron microscopy of 3-D skin construct treated with 1 μg/ml Poly IC for 72 h. Arrow = LB. Arrowhead= KHG. Scale bar = 1 μm. SC = stratum corneum. SG = stratum granulosum. (b) Quantification of LB and KHG. $*P < 0.05$; One-tailed T-test. Control (n = 54), 1 μg/ml Poly (I:C) (n = 47). Data are mean ± SEM. (c) Quantification of LB and KHG in TLR3 knockdown NHEK. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. One tailed T-Test. Control siRNA Control (n=54), Control siRNA + 1 μg/ml Poly (I:C) (n = 51), TLR3 siRNA Control (n = 55), TLR3 siRNA + 1 μg/ml Poly (I:C) (n = 54). Data are mean ± SEM.

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Table 1

Poly (I:C)-induced gene expression changes

Gene name	Fold Change (realtime PCR)	+/− SD	T-Test	Fold Change (microarray)	SAM
ABCA12	22.68	5.551	**	3.74	+
GBA	10.84	1.243	***	2.03	+
SMPD1	9.48	1.143	***	2.05	+
TGM1	30.65	4.231	***	2.40	+
TNF	23.07	0.564	***	5.31	+
IL-6	41.99	2.480	***	27.31	+
TLR3	69.66	9.100	***	14.58	+
MAVS	1.40	0.309	ns	1.70	−
KRT1	1.33	1.727	ns	0.52	−
KRT14	0.74	0.012	***	1.02	−
IVL	0.79	0.064	ns	0.60	−
LOR	1.43	1.216	ns	1.53	−
FLG	0.83	1.224	ns	0.84	−
SPTLC1	1.36	0.071	**	1.19	−
SPTLC2	3.72	0.834	**	0.81	−
UGCG	2.61	0.174	***	0.67	−
ACACA	0.37	0.079	**	0.33	+
FASN	0.48	0.113	*	0.67	−
HMGCR	1.24	0.057	*	1.04	−
HMGCS1	1.07	0.143	ns	1.60	−
FDFT1	0.71	0.096	*	0.85	−

Data in table represents real-time PCR and microarray fold change data from NHEK treated with 1 μg/ml Poly (I:C) versus control, water-treated NHEK.

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.001$.

Student's T-Test. Data are mean of triplicate samples and representative of at least three independent experiments for real-time PCR. Data are mean of triplicate samples and analyzed for significance with SAM (fold change >2, FDR < 0.01%; delta value = 1.397).

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