Interleukin-1 alpha blockade prevents hyperkeratosis in an in vitro model of lamellar ichthyosis

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INTRODUCTION

Epidermal barrier function is a prerequisite of ex utero terrestrial existence. Williams was the first to liken the barrier function-conferring cornified layer of the epidermis to bricks and mortar (1). Defects in either the cornified layer of bricks or the non-polar lipid mortar are the cause of a group of diseases called the ichthyoses, so-called, because of the fish-like scaling which is an aberrant homeostatic response to chronic barrier dysfunction [reviewed in (2)].

Infants with a rare severe form of congenital barrier abnormality, lamellar ichthyosis (LI) present at birth with a collodion membrane covering the skin of the infant. The condition is associated with a low risk of perinatal mortality rate due to insensible water loss, renal failure and temperature dysregulation (3). Although most infants survive, they have an abnormal skin throughout life, covered in thick dark scales, and are at risk of a wide spectrum of complications, including skin infections.

Around 40% of the cases of LI are caused by function abolishing or reducing mutations in the keratinocyte transglutaminase (TGM1) (2,4). TGM1 is an enzyme involved both in the covalent cross-linking of the glutamine and lysine residues of cornified envelope proteins, presumably providing physical strength to the cornified envelope [reviewed in (5)], as well as cross-linking of ceramides to the surface of the cornified layer to further augment barrier function (6). The most severe cases of LI are caused by nonsense and missense mutations resulting in the absence of gene or loss of function (4). Although the genetic basis for this family of diseases is on the whole well established, there have been no investigations on the mechanisms of response to the barrier dysfunction that results in scaling.
The only direct evidence for the mechanism of scaling in LI comes from experiments in which skin from the Tgm1 and Alox12b knockout mice is grafted onto immunocompromised mice. Although both mouse knockouts die very soon after birth due to severe barrier dysfunction caused by insensible water loss, after grafting, barrier function is recovered by a combination of hyperproliferation, hyperkeratinization and overproduction of lipids (7–9).

The current treatments concentrate on the reduction of scaling, either by the use of keratolytics such as urea to soften the scales (10), or the long-term use of retinoids, which although successful in reducing hyperkeratosis (11) is problematic as they would have to be used long term, requiring careful monitoring of bone density (12), and in addition they can cause erosion of the skin surface and skin thinning which itself can further reduce epidermal barrier function (13). Physical approaches are normally used in conjunction with these therapies. This involves the scrubbing away of scales. Emollients are then used to augment barrier function after these treatments (3). A significant improvement would be a treatment that can reduce hyperkeratosis and scaling without reducing the overproduction of lipid. This would lessen the amount of bathing and scrubbing required; reduce the frequency of emollient application, as well as reduce the reliance on retinoid-based therapies.

To determine the keratinocyte-specific mechanisms leading to the scaling phenotype, we knocked down expression of the transglutaminase-1 gene in a rat epidermal keratinocyte (REK) model (14) that is able to create barrier-competent skin equivalents highly similar to normal human epidermis (15). Three-dimensional organotypic cultures expressing siRNA to rat Tgm1 displayed hyperproliferation, hyperkeratinization and increased synthesis of non-polar lipids. An array analysis of siRNA expressing keratinocytes revealed up-regulation of interleukin-1 alpha (IL1A) expression, and an increase in IL1A was seen in all patients with either LI or ARCI. Treatment of siRNA expressing organotypics with IL-1 receptor antagonist (IL1RA) prevented hyperkeratosis without affecting levels of non-polar lipid synthesis, suggesting a novel therapy for the scaly skin seen in LI.

RESULTS

siRNA knockdown of Tgm1 leads to altered expression of cornified envelope components, hyperkeratosis and increased neutral lipid synthesis in organotypic culture

To determine the downstream signalling effects of loss of transglutaminase-1 function in LI, we used an REK model in which expressed a plasmid that expressed shRNAs to rat Tgm1. We tested knockdown by western blot and determined that siRNA2 produced a nearly 90% knockdown by densitometry analysis (Fig. 1A), approximately equivalent to the reduction in Tgm1 cross-linking activity in mutant Tgm1 from individuals with bathing suit ichthyosis at 37°C (16), suggesting that we would see a phenotypic effect in organotypic culture.

Large, Tgm1-positive cells were present in REKs in culture, but not in the siRNA expressing cells (Fig. 1B). When these cells were grown in organotypic culture on a de-epidermalized dermal scaffold, they showed a reduction in cell surface Tgm1 staining in the granular layer (arrowheads, Fig. 1C) and consistent with the terminal differentiation and cornification defects reported in LI, the culture was highly hyperkeratotic compared with scrambled controls, with a highly thickened cornified layer (Fig. 1D). This hyperkeratosis correlated with hyperproliferation in the basal layer of the epidermis, as shown by the increase in PCNA-positive nuclei in the basal layer of the epidermis (Fig. 1E and F). As epidermal lipid expression is altered in patients with LI, we examined the expression of non-polar lipids in the siRNA expressing organotypic culture, and determined that compared to control organotypics, there was a marked increase in Nile red positivity throughout the organotypic culture and non-polar lipid in the cornified layer (Fig. 1G and H).

We analysed the siRNA expressing keratinocytes by western blot and determined that keratin 1 expression increased while the expression of involucrin and interestingly keratin 10 was decreased (Fig. 2A). Overall expression of loricin was significantly reduced. Loricin is cross-linked to a dimeric form by Tgm1 (17,18; dimer, Fig. 2A). Therefore, the presence of dimeric loricin is a proxy for Tgm1 cross-linking activity. siRNA expressing cells had significantly less dimeric loricin expressed as a percentage of total loricin (26% versus 93% in controls, P = 0.01, Fig. 2B), suggesting a significant reduction in cross-linking activity in the siRNA expressing cells. We confirmed the reduction in cross-linking in an organotypic culture by monodansyl cadaverine incorporation. There was a significant reduction in cadaverine fluorescence in siRNA-expressing organotypic cultures (Fig. 2C). Immunofluorescent analysis of the same terminal differentiation markers was performed in organotypic cultures (Fig. 2D). Consistent with the western analysis, there was a marked increase in keratin 1 expression and decrease in keratin 10 expression. Involucrin staining was decreased in the siRNA expressing organotypic cultures. Expression of keratin 6, a marker of the activated epidermal phenotype was also increased in the Tgm1 siRNA expressing organotypic culture. Loricin expression was slightly increased, probably reflecting an increase in monomeric, antibody accessible loricin (c.f. Fig. 2A and B).

Gene expression analysis reveals differences in fatty acid synthesis pathways and the expression of pro-inflammatory cytokines caused by loss of Tgm1

To examine expression differences in cornified envelope components as well as downstream signalling pathways in the Tgm1 siRNA expressing cells, we performed a microarray analysis of these cells, comparing them with scrambled controls. In total, 277 genes were differentially regulated in the Tgm1 null cells (Fig. 3A and Supplementary Material, Table S1). Keratin 1 was the only markedly up-regulated keratinocyte differentiation gene, and IL1A was the most highly up-regulated gene in the siRNA expressing cells.

To determine potential signalling pathways that were altered by the loss of Tgm1, we analysed the differentially expressed genes using an unsupervised approach that clustered the differentially expressed genes according to Gene Ontology (Supplementary Material, Fig. S1). This approach indicated that the differentially expressed genes were enriched in
components of the fatty acid synthesis pathway, and in genes involved in the inflammatory/stress response. We confirmed some of these findings of the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis by using the Ingenuity Pathway Analysis suite. Differentially expressed genes were enriched in genes involved either with fatty acid synthesis or acute response signalling, and also it confirmed that retinoid acid receptor signalling-related genes were enriched in the data set (Fig.3A).

This unsupervised approach created scale-free networks, enriched for differentially expressed genes in the Tgm1 siRNA expressing cells. One network incorporated the stress–response signalling pathways with the most prominent node being NFκB (Fig.3B and C), a principle signalling molecule downstream of IL1A. As retinoic acid signalling was also part of this network, we tested the hypothesis that IL1A expression was reduced with retinoic acid treatment. Treatment with 5 μM all-trans retinoic acid, caused a marked reduction in intracellular IL1A in untransfected REKs (Fig.3D).

The IL1A pathway is up-regulated in Tgm1 null cells and organotypic cultures

We performed real-time polymerase chain reaction (PCR) analysis on IL1A and related components of the IL-1 pathway. We confirmed the marked (>30-fold) up-regulation of IL1A, and modest up-regulation in expression of its cognate receptor IL1R1. There were also significant reductions in both IL1RA (not detected by array hybridization) and IL1RL1, both inhibitors of IL-1 signalling (Fig. 4A). This suggested that there was an overall increase in IL1A-specific signalling in the Tgm1 siRNA expressing cells. We detected up-regulation of IL1A in the medium by western blot, a significant increase in secreted IL1A by enzyme-linked immunosorbent assay (ELISA; Fig. 4B and C), and a concomitant depletion of the intracellular pool of IL1A (Fig. 4B). Furthermore, we detected up-regulation of IL1A in the upper epidermal layers of siRNA expressing organotypic cultures (Fig. 4D).

IL1A principally signals via nuclear translocation of the NFκB transcription complex, resulting from degradation of IkappaB. By western blot, there was no significant change in the levels of either total NFκB (p65) or phosphorylated NFκB. However, there was a marked down-regulation of IkappaBalpha, suggesting that in Tgm1 siRNA expressing cells, NFκB would be translocated to the nucleus (Fig. 4B).

To confirm this possibility, we looked for nuclear p65 in organotypic culture, indicative of NFκB activity (Fig. 4E). There was clear NFκB expression in the nucleus of keratinocytes in the Tgm1 siRNA organotypic, which was absent in the scrambled control organotypic, providing further evidence that NFκB signalling was increased in the siRNA expressing organotypic. As there was also an increase in overall NFκB intensity in the siRNA expressing organotypic, we performed image analysis...
and determined that there was a significant increase in the percentage of total NFκB in the nucleus of siRNA expressing organotypic cultures (Fig. 4F).

Expression of IL1A and keratin-1 is increased in LI patient skin

To determine whether the changes we observed in our model were relevant to patients with LI, we took scrapings from one control individual and nine individuals with different forms of ichthyosis, and examined the expression of IL1A, keratin 1 and loricrin compared with the control (Fig. 5A and B). While the patient with ichthyosis vulgaris had no change in the levels of IL1A, all the other patients showed a significant 5–7-fold increase in IL1A expression, with the largest increase in expression being observed in the individuals with ‘classical’ LI, strongly suggesting that not only is the over expression of IL1A potentially an important diagnostic criterion but also that the up-regulation of IL1A may be responsible for the hyperkeratotic phenotype in these individuals. Dimeric loricrin species (asterisks, Fig. 5A) were observed in two of the four LI patients, suggesting that their disease may be caused by mutation in a gene other than TGM1. Overexpression of keratin 1 was seen in both the LI and related patients and the patient with ichthyosis vulgaris, suggesting that the up-regulation of keratin 1 may be more widely associated with hyperkeratosis, unlinked to severe barrier dysfunction. The patient treated with long-term retinoids showed a marked reduction in keratin 1 when compared with other patients.

Treatment of normal keratinocytes and organotypic cultures with IL1A leads to hyperkeratosis without an increased lipid synthesis

To test whether the up-regulation of IL1A was a principle driver of hyperkeratosis in the Tgm1 siRNA expressing cells, we treated REK organotypic cultures with 2.5 ng/ml IL1A every 48 h for 10 days. The treated organotypics were hyperkeratotic (Fig. 6A), but there was no change in non-polar lipid synthesis in the IL1A-treated organotypics by Oil Red O, and no change in lipid synthesis by Nile red staining (Fig. 6B and C), and expression of the fatty acid desaturase 2 (FADS2), markedly down-regulated in the siRNA expressing cells, was actually slightly up-regulated (Fig. 6D and F). There was no increase in nuclear PCNA staining in the treated organotypics (data not shown). By immunofluorescence, there was a slight increase in keratin 1 expression and a significant decrease in keratin 10. Involucrin expression was significantly reduced. Consistent with the data in the Tgm1 siRNA expressing organotypic culture, there was no apparent change in loricrin expression (Fig. 6E and F). Taken together, these data suggested that the mechanism of hyperkeratosis in the REK LI model was driven, at least in part, by the up-regulation of IL1A, and that, consistent with our array analysis, another pathway was responsible for the increase in non-polar lipid synthesis.

Treatment of Tgm1 null keratinocytes with IL1RA restored expression of some cornified envelope components to near normal levels

To test whether the up-regulation of IL1A was necessary to cause the hyperkeratosis observed in the Tgm1 siRNA expressing cells, we treated cells with increasing concentrations of IL1RA (Fig. 7A), a soluble competitive inhibitor of the IL-1
receptor that competes with IL1A for binding. Treatment with IL1RA decreased the expression of keratin 1 and increased the expression of involucrin and keratin 10. It also reduced the expression of full-length loricrin; however, this was not due to a restoration of cross-linking of the protein (arrowheads, monomer and dimer, respectively, Fig. 7A). Also treatment with IL1RA had no effect on the levels of FADS2 in the Tgm1 siRNA expressing cells (Fig. 7A). The optimal IL1RA concentration for the restoration of differentiation marker expression was determined to be between 0.5 and 2.5 ng/ml (Fig. 7A). Treatment with IL1RA caused a dose-dependent reduction in the phosphorylation of NFκB, and a reduction in total NFκB but no increase in the expression of IkappaB, indicating that the treatment was effective at inhibiting NFκB signalling at these concentrations.

Treatment with IL1RA reduced hyperkeratosis in Tgm1 siRNA expressing organotypic cultures

As it was clear that treatment with IL1RA at the concentration described above altered both the expression of keratinocyte differentiation markers and reduced NFκB signalling, we tested whether treatment at these concentrations was able to reduce the hyperkeratosis seen in the Tgm1 siRNA expressing cells. Treatment with 2.5 ng/ml IL1RA had no effect on the cornified layer of control (scrambled) organotypics (data not shown). However, treatment with 0.5 and 2.5 ng/ml IL1RA caused a dose-dependent reduction in the thickness of the cornified layer (Fig. 8A). Expression of keratin 6 was unaltered in organotypic culture, suggesting that the epidermis was still ‘activated’. Consistent with this, the levels of PCNA staining were not reduced (Supplementary Material, Fig. S3A and B). IL1RA treatment was effective in the organotypic cultures, as the nuclear NFκB seen in the Tgm1 siRNA expressing culture (arrowheads, Supplementary Material, Fig. S3A) was replaced with cytoplasmic staining in the treated cultures, and there was a reduction in nuclear p65 as a result of IL1RA treatment (Supplementary Material, Fig. S3A and C).

There were reductions in the expression of keratin 1 and loricrin back to the levels in the scrambled control organotypics and the expression of involucrin and keratin 10 was similarly increased. Examination of non-polar lipid by Oil Red O staining showed no reduction as a result of IL1RA treatment (Fig. 8B). Consistent with this, the expression of FADS2 was not increased (Fig. 8B). Taken together these data provide further evidence for IL1A signalling being responsible for the hyperkeratosis caused by aberrant expression of keratinocyte differentiation and cornified envelope-associated proteins, but is not responsible for the increase in non-polar lipids in the disease model. Therefore, IL1RA could potentially be used as a therapeutic to reduce the scaling in ichthyosis, while maintaining sufficient epidermal barrier function, by maintaining increased non-polar lipid synthesis.

DISCUSSION

A novel in vitro model of LI recapitulates many of the clinical characteristics of the disease

We report a novel in vitro model of LI by organotypic culture of Tgm1 siRNA expressing REKs. The model was valid in that it exhibited many features of the human disease. The model showed increased expression of IL1A, which we also observed in patient skin scraping samples, further validating our
approach. Increased expression of IL1A in organotypic culture resulted in hyperkeratosis, but not increased non-polar lipid expression. This hyperkeratosis was reduced in a dose-dependent fashion by treatment of Tgm1 siRNA expressing organotypic cultures with IL1RA. This decrease in hyperkeratosis and lack of change in non-polar lipids, which would maintain barrier function in the presence of a reduced cornified layer, makes IL-1 signalling blockade an attractive novel therapy for the autosomal recessive ichthyoses.

We chose the REK line (14) for two reasons. First, there had been reported problems culturing LI patient cells long term (19), and secondly we have previously been able to grow genetically altered organotypic cultures that have a barrier defect, and then subsequently recover, becoming hyperkeratotic (15). Our model displayed not only hyperkeratosis and hyperproliferation, reported previously as a response to barrier dysfunction in the Tgm1 null mouse by Ref. (8), but also defective cross-linking of loricrin (17,18,20) and defective dansyl cadaverine incorporation. Finally, we report an increase in non-polar lipids by the relatively crude Nile red and Oil-red O stains, consistent with previous reports of the importance of fatty acids in this group of conditions (19). On the basis of these criteria, even though the line is not human derived, the Tgm1 siRNA expressing cells and organotypic cultures more than adequately model the human disease.

Loss of Tgm1 causes specific changes in epidermal differentiation markers and lipid synthesis pathways

Our initial analysis of epidermal differentiation markers indicated an increase in keratin 1, loricrin and keratin 6 and a decrease in involucrin, suggesting an altered composition of the hyperkeratotoc cornified envelope, as described previously (20). Increase in keratin 1 has not been formally reported
before in LI. However, we observed this in the cultured cells, the organotypic culture and in patient samples. Keratin 6 up-regulation has been observed by flow cytometry in a range of ichthyotic skin diseases, including LI (21), consistent with keratin 6 being a marker of the ‘activated phenotype’ (22,23). Reduction of involucrin and keratin 10 in LI has previously been reported by flow cytometry (21), and more relevant to this study by immunohistochemistry (24). Why these changes in epidermal differentiation marker expression occur, in particular the apparent opposing expression differences of the suprabasal keratin partners 1 and 10, is not clear from this work. However, the fact that keratin 1 is up-regulated suggests that there may well be another partner for this keratin in hyperkeratotic epidermis, potentially an over-expressed minor keratin, such as Krt80, which was up-regulated in siRNA expressing cells (data not shown). Loss of involucrin has not yet been reported in any models of hyperkeratotic or ichthyotic skin disease. We speculate, therefore, that these specific changes together may comprise a ‘signature’ of hyperkeratosis in ichthyosis, and this could be confirmed by further analysis of patient scales.

From the gene expression analysis we determined two key pathways that were altered in the Tgm1 siRNA expressing cells, one involving lipid synthesis and desaturation of fatty

Figure 6. IL1A treatment causes hyperkeratosis but no change in neutral lipids. (A) Histology indicated hyperkeratosis in the IL1A-treated organotypic cultures. (B) Oil Red O staining was unchanged in treated cultures. (C) Nile red staining was unchanged in treated cultures. (D) Immunofluorescent staining of FADS2 in treated cultures. (E) Immunofluorescent analysis of keratin 1, keratin 10, loricrin and involucrin. (F) Image analysis of intensity of staining (*P < 0.05, **P < 0.005, n.s. not significant, n = 3, two-tailed t-test). DAPI was used as a nuclear counterstain. The dotted line indicates the dermo-epidermal junction. Bar 50 μm (A–E).
down-regulation of a cluster of lipids (29,30), as is its cognate receptor (31), and the soluble IL1A is constitutively expressed in the upper epidermis levels correlated to clinical severity, the first time that an ARCI, regardless of the genetic mutation, and that the evidence that IL1A has keratinocyte-specific functions. Further research is warranted on the transcription regulation of these genes during hyperkeratosis, and what the specific down-regulation of these particular genes would do to the composition of the lipids in the cornified layer.

**IL1A over-expression occurs in all lamellar ichthyoses tested: mechanisms of IL1A control in response to barrier dysfunction**

Over-expression of IL1A occurred in a setting without immune cells, which adds further weight to the growing evidence that IL1A has keratinocyte-specific functions. Furthermore, an increase in IL1A from patient skin scrapings suggests that the up-regulation of this cytokine is common to all ARCI, regardless of the genetic mutation, and that the levels correlated to clinical severity, the first time that an increase in this cytokine has been reported in the disease. IL1A is constitutively expressed in the upper epidermis (29,30), as is its cognate receptor (31), and the soluble decoy receptor IL1RA (32,33). Normally, in response to barrier disruption, trauma and in normal keratinocyte homeostasis, the expression of IL1A and IL1RA are tightly controlled and linked (34), and in transgenic models that over express either in the epidermis, there is counter-regulation of the other (35).

That we see over expression of IL1A and down-regulation of IL1RA in the Tgm1 siRNA expressing cells is surprising, and it is intriguing to speculate what the mechanism is. As it is common to all patients with LI or ARCI it is unlikely to be a Tgm1 mediated phenomenon. There are many mechanisms that up-regulate both IL1A and IL1RA, however recent data suggests that the PPAR transcription factors, already strongly implicated in epidermal barrier function (36), stimulate synthesis of IL1RA, a direct transcriptional target in response to IL-1 increase (37). Although we see no change in PPAR gene expression in our gene expression analysis, it doesn’t preclude some block of function of PPAR leading to an imbalance between IL1RA and IL1A. This would be consistent with finding that topical addition of PPAR alpha activators restore tissue homeostasis in hyperkeratotic epidermis (38) but the loss of PPAR alpha or beta in the epidermis causes no effects to normal homeostasis. How IL1A causes hyperkeratosis is a subject that requires further study, one possibility is a reduction in desquamation.

NFkappaB activation is the downstream effect of IL1A up-regulation and we see this in our model. Knockout of IkappaBalpaha, which we see in the Tgm1 siRNA expressing cells, causes hyperplasia and apparent hyperkeratosis in mouse epidermis (39), and the same authors show in the same paper that epidermal knockout of RelA (p65) causes an increase in keratin 10, which we observed when NFkB signalling was inhibited by addition of IL1RA. Reduction in both NFkB phosphorylation and expression was seen in response to IL1RA treatment. One possible explanation for this is that the dephosphorylation of p65 at Ser468 causes a ubiquitin-dependent degradation of the protein (40,41). This, however, also suggests that IL1RA in this context is not working to suppress the induced canonical IL-1A signalling, as we do not observe a restoration in IkBa expression. This may be due to differential activation and inhibition of the type I and type II IL-1 receptor resulting from competition between IL1A and IL1RA as well as other soluble inhibitors that were not investigated (42). Further analysis of the components of the IL1A/NFkB pathway present in keratinocytes is therefore important to determine which forms of IL1A signalling blockade could potentially work as therapies for LI.

Up-regulation of NFkappaB has been reported in a number of mouse models with hyperkeratosis. Overexpression of Dsg2 in differentiating keratinocytes and ablation of p120 catenin (43,44), both cause hyperproliferation, hyperkeratosis and epidermal barrier defects, consistent with our model that IL1A transduces signals via NFkappaB, leading to hyperkeratosis in response to an epidermal barrier defect.

**The potential for IL1RA as a novel therapeutic agent for LI**

Retinoid therapies reduce scaling in patients with LI but have potential serious side-effects, and although there are conflicting reports about the changes in bone density with long-term use (45–47), retinoids are reported to reduce epidermal barrier function in rodents (13). One reported effect of retinoids is the reduction in keratin 1 expression (48) which we see with IL1RA treatment. The other is the effect on
epidermal fatty acid synthesis (49), which we do not observe with IL1RA treatment. Finally, topical retinoid treatment has been reported to reduce IL1A expression in both reconstructed human epidermis and the rhino mouse (50). These data are consistent with our discovery of a signalling nexus involving NFkappaB and retinoic acid signalling and our finding that retinoic acid reduced IL1A expression in REKs. This may suggest that the effects of retinoic acid in reducing hyperkeratosis are principally due to the reduction of IL1A and subsequent inhibition of NFkappaB signalling. It is tempting, therefore, to speculate that IL1RA treatment could replicate the reduction of scaling seen with retinoid treatment, without the associated loss in barrier lipids, and that use of IL1RA either alone or in combination with retinoids would reduce any potential deleterious side-effects of retinoid use.

MATERIALS AND METHODS

Ethical consent statement

Skin scraping samples were obtained with ethical consent from families attending the dermatology outpatient clinic at Great Ormond Street Hospital for Children, London.
siRNA knockdown of Tgm1, REK culture and organotypic culture

siRNA knockdown of expression of Rat Tgm1 was achieved by transfection of commercially available plasmids (SAbiosciences) by normal lipofectamine approaches (Invitrogen), expressing specific shRNA sequences targeting rat Tgm1. We used two different sequences (called siRNA2 and siRNA3) with insert sequences of ACACTGCGCCAGACATTTGTT and TACCCGTACTGTCACTCAACTT, respectively. Only siRNA2 produced a successful knockdown of Tgm1 expression (Fig. 1) and hence was used for further studies. Transfected cells were grown under G418 selection for a minimum of 2 weeks prior to experiments. Cells were routinely cultured in DMEM (Sigma) + 10% foetal calf serum (Invitrogen). Organotypics were cultured on de-epidermalized dermis (DED). Briefly, REKs were plated on the upper side of a DED. When confluent, the DED was raised to the air–liquid interface and cultured for another 10 days. The organotypic culture was either embedded in OCT for frozen sections, or paraffin embedded.

IL1A and IL1RA treatment of cells in culture and organotypic culture

REKs were treated daily for 3 days with either rat recombinant IL1A (Abd serotec) at 2.5 ng/ml, or human recombinant IL1RA (IL1RN, Abnova) at 0.5 and 2.5 ng/ml. Organotypic cultures were treated with the same IL1RA concentrations, but every 2 days for the 10 days of the culture being lifted.

Array hybridization and analysis of gene expression pathways

An amount of 0.1 mg of RNA was extracted (RNeasy kit, Qia-gen, Valencia, Spain), and poly-A + RNA was selected using the Oligotex system (Qiagen). Second-strand cDNA was synthesized using the Superscript II kit (Invitrogen, Carlsbad, NM, USA) after the RNA was annealed with a T7 promoter–poly-T primer (Genset, Evry, France). Biotin-labelled cRNA was made from this cDNA (Enzo Diagnostics, Farmingdale, NY, USA). The whole probe was hybridized to the RG230 exon array rat genome chip (Affymetrix, Santa Clara, CA, USA) according to the manufacturers’ specifications. Three scrambled hybridizations and three siRNA hybridizations allowed six crosswise comparisons, with the scrambled control cells being the baseline in all analyses. Genespring (Agilent, Santa Clara, CA, USA) was used to compare the resulting CEL files. Genes that were tagged as present and increased in all six analyses with a P-value of ≤0.05 by Mann–Whitney analysis, a P-value of <0.05 after Benjamini–Hochberg False Discovery Rate correction and ≥2-fold altered in expression were regarded as differentially expressed.

Supervised analysis of gene expression pathways was performed by inputting gene lists into the DAVID analysis program functional annotation clustering (http://david.abcc.ncifcrf.gov/) (51) and looking for gene ontology pathways that were over represented in the list of genes differentially expressed in Tgm1 siRNA expressing cells. Unsupervised creation of scale-free networks of protein and genetic interactions was performed by inputting the same list of differentially expressed genes into the Ingenuity pathway analysis suite (Agilent).

Confirmation of differential expression and analysis of IL1 signalling related genes by RT-PCR

Real-time PCR was performed on cDNA from RNA extracted for the expression analysis above. Primers were as follows:

- Il1a-F: TCGGGAGGAGACGACTCTAA
- Il1a-R: GAAAGCTGCGGATGTGAAGT
- Il1ra-F: GCTGAAGGGTGTTCCAAAAA
- Il1ra-R: GACAGCAAGAGGGACAGAG
- Il1rl1-F: AAGTCCTTTGTCCGTACCC
- Il1rl1-R: F: GACAGCAAGAGGGACAGACC
- Il1rl-F: GCTGAAGGGTGTTCCAAAAA
- Beta-actin-F: CAACCTTCTTGCAAGCTCCTC
- Beta-actin-R: CAACCTTCTTGCAAGCTCCTC

Threshold cycle values were read off a dilution curve of the cDNA to provide relative quantities and data were normalized using the beta-actin values.

Immunofluorescent analyses

Immunofluorescent analysis was performed either on frozen sections of the organotypics fixed for 2 min in 4% paraformaldehyde in phosphate-buffered solution (PBS), or cultured REKs were simultaneously fixed in 4% paraformaldehyde in PBS/0.2% triton-X 100. The following primary antibodies were used at the following concentrations. Rabbit anti TGase1 (Santa Cruz Biotechnologies, 1/50), Rabbit anti keratin 1 (Covance, 1/200), Rabbit anti keratin 10 (Covance, 1/200) Rabbit anti Involutin (Covance, 1/100), Rabbit anti Loricrin (Covance, 1/100), Rabbit anti NFkappaB (p65), (Auto-gen Bioclear, 1/10), Rabbit anti FADS2 (Sigma, 1/20), Rabbit anti PCNA (Santa Cruz Biotechnologies, 1/50). Primary antibodies were detected using Alexa 488-conjugated goat anti mouse and anti rabbit (Invitrogen, 1/500). Cells were counterstained with TRITC conjugated phalloidin (Sigma). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for. Images were taken with a Nikon Eclipse E600 microscope with either ×20 (NA 0.4) or ×40 (NA 1.40) objectives, using a Coolsnap digital camera (MediaCybernetics, Bethesda, MD, USA), with the ImagePro 6.0 software (MediaCybernetics). Image analysis was performed using the ImageJ software suite.

Analysis of non-polar lipid by Oil Red O staining and Nile red staining

Frozen sections were fixed in 1% neutral-buffered formalin for 5 min, washed in deionized water and incubated in 60% isopropanol for 5 min. Sections were stained with filtered Oil Red O working solution, prepared immediately before use by making a 6:4 mixture of stock (0.5% Oil Red O in 99% isopropanol) and deionized water. Sections were transferred to 60% isopropanol, washed in deionized water and counterstained using haematoxylin prior to light microscopic
analysis. For Nile red staining, sections were mounted in 0.5 μg/ml Nile red (Sigma) prior to fluorescent microscopy.

**Analysis of cross-linking by monodansyl cadaverine incorporation**

Monodansyl cadaverine [MDC (Sigma)] incorporation was performed on freshly cut frozen sections. Briefly, dried sections were incubated in 1% BSA in 0.1 M Tris, pH 7.5, for 30 min, prior to incubation for 2 h in substrate solution (add 10 μl of 10 mM MDC plus 25 μl of 200 mM CaCl₂ to 965 μl 0.1 M Tris, pH 7.5). Negative control replaced the 200 mM CaCl₂ with 200 mM EDTA. MDC autofluorescence was detected by fluorescence microscopy using the DAPI filter, and intensity levels were quantified using the ImageJ program.

**Western blotting of cells and patient scale**

Keratinocyte protein lysates were prepared by boiling keratinocytes in a denaturing sodium dodecyl sulphate (SDS) buffer (2% 2-mercaptoethanol, 2% SDS, 10 mM Tris, pH 7.5) for 10 min.

Scale from LI patients and cornified envelopes from a normal control and ichthyosis vulgaris patient were obtained by scraping with a razor blade. Typically 20 mg dry weight of scale or cornified envelopes was obtained equating to 100,000 cornified envelopes in controls. Twenty-five to fifty microgram soluble protein was obtained from these preparations by lysis in a non-denaturing SDS buffer (2% SDS, 10 mM Tris, pH 7.5). In all cases, protein was quantified using the bicinchoninic acid assay (Sigma).

Lysates were separated on 7.5–10% SDS/polyacrylamide gels and transferred onto nitrocellulose filters (Hybond C, Amersham). Primary antibodies and concentrations were as follows: Rabbit anti-TGase1 (1/100), Rabbit anti-keratin 1 (1/1000), Rabbit anti-involvecin (1/1000), Rabbit anti-FADD (1/200), Rabbit anti-IRF5 (p56) (1/500), Rabbit anti-Src563NFkappaB (Cell Signalling Technologies, 1/500). Rabbit anti-IkappaBeta (Cell Signalling Technologies, 1/500). Rabbit anti-loricrin (1/1000) was raised to the C-terminus, which does not participate in gamma-glutamyl cross-linking reactions (17). Primary antibody incubations were in TBST [100 mM Tris–HCl, 0.2 mM NaCl, 0.1% Tween-20 (v/v)] containing either 5% bovine serum albumin (Sigma, Gillingham, UK) or 5% skimmed milk powder either overnight at 4°C or for 1–2 h at room temperature, while secondary antibody incubations were in 5% skimmed milk powder for 1 h at room temperature. The following concentrations were used; swine anti-rabbit-HRP (DakoCytomation) 1:3000; rabbit anti-mouse HRP (DakoCytomation) 1:2000. Protein was visualized using the ECL plus kit (Amersham). Densitometry was performed using the ImageJ software.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None cleared.

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**REFERENCES**
