Fetal inhibition of inflammation improves disease phenotypes in harlequin ichthyosis

Denny L. Cottle¹,†, Gloria M. A. Ursino¹,†, Sally Chi Ieng Ip¹, Lynelle K. Jones¹, Tia Ditommaso¹, Douglas F. Hacking⁴,⁹, Niamh E. Mangan⁵, Natalie A. Mellett⁷, Katya J. Henley³, Dmitri Sviridov⁷, Claudia A. Nold-Petry⁶, Marcel F. Nold⁶, Peter J. Meikle⁷, Benjamin T. Kile³,⁸ and Ian M. Smyth¹,²,*

¹Department of Biochemistry and Molecular Biology, ²Department of Anatomy and Developmental Biology, Monash University, Wellington Road, Clayton, VIC 3800, Australia, ³Walter and Eliza Hall Institute, 1G Royal Parade, Parkville, VIC 3052, Australia, ⁴Department of Anaesthetics, Saint Vincent’s Hospital Melbourne, 41 Victoria Parade, Fitzroy, VIC 3065, Australia, ⁵Centre for Innate Immunity and Infectious Diseases, ⁶The Ritchie Centre, MIMR-PHI Institute of Medical Research, 27-31 Wright Street, Clayton, VIC 3168, Australia, ⁷Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, VIC 3004, Australia, ⁸Department of Medical Biology, University of Melbourne, Parkville, VIC 3010, Australia and ⁹Department of Paediatric Intensive Care, The Royal Children's Hospital, Melbourne, VIC, Australia

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Harlequin ichthyosis (HI) is a severe skin disease which leads to neonatal death in ~50% of cases. It is the result of mutations in ABCA12, a protein that transports lipids required to establish the protective skin barrier needed after birth. To better understand the life-threatening newborn HI phenotype, we analysed the developing epidermis for consequences of lipid dysregulation in mouse models. We observed a pro-inflammatory signature which was characterized by chemokine upregulation in embryonic skin which is distinct from that seen in other types of ichthyosis. Inflammation also persisted in grafted HI skin. To examine the contribution of inflammation to disease development, we overexpressed interleukin-37b to globally suppress fetal inflammation, observing considerable improvements in keratinocyte differentiation. These studies highlight inflammation as an unexpected contributor to HI disease development in utero, and suggest that inhibiting inflammation may reduce disease severity.

INTRODUCTION

The ichthyoses are a family of >20 congenital diseases characterized by the development of a thick hyperkeratotic epidermis (1). The most severe form of this disease spectrum is Harlequin ichthyosis (HI) (OMIM #242500) which is caused by mutations in ABCA12 (2,3), a putative lipid transport protein of the ATP-binding cassette (ABC) family. Gene targeting and ENU mutagenesis approaches have generated three different mouse models of the disease. These have helped to establish that loss of ABCA12 causes defective extracellular lipid trafficking and profound intracellular lipid accumulation within keratinocytes (4–6). In particular lamellar bodies, the small specialized intracellular organelles responsible for lipid transport in keratinocytes, lack lipid cargos in HI epidermis. Thus, the characteristic extracellular lipid lamellae which surround the cornified cells upon terminal differentiation are variably disrupted or absent, leading to a profound defect in the barrier function of the skin. Loss of cutaneous barrier function presents particular challenges to the neonate, particularly through dehydration and infection. As a result, the survival of newborn HI individuals is only ~50%, although their disease upon delivery is already extreme (7). For those patients who do survive beyond birth, a modest improvement in disease phenotypes is observed, although a lifetime regime of frequent bathing, removal of scales and frequent application of emollient oils is required to manage the disorder (8). In mouse models of HI neonatal mortality is fully penetrant, but grafted fetal skin exhibits an analogous self-improvement which has been attributed to better keratinocyte differentiation (9). Retinoid therapy is the main treatment for HI neonates (7), as they typically promote keratinocyte differentiation and shedding. However, there are a number of undesirable side-effects that limit

†To whom correspondence should be addressed. Tel: +61 399029119; Fax: +61 399052500; Email: ian.smyth@monash.edu
*To whom correspondence should be addressed. Tel: +61 399029119; Fax: +61 399052500; Email: ian.smyth@monash.edu
†The authors wish it to be known that, in their opinion, these first authors should be regarded as joint First Authors.
their long-term use (10). Their effectiveness as an HI treatment is also under review amid debate about whether the perceived improvement in disease is a function of retinoid activity or the product of better disease management (8).

In skin diseases such as dermatitis and psoriasis, where compromised barrier function plays a role in the development of hyperkeratotic phenotypes, inflammatory immune responses are thought to be important for the progression of disease (11). Keratinocytes themselves express a range of cytokines which act to recruit components of the innate immune system (12). In the context of the ichthyoses, a limited number of studies have suggested a role for inflammatory dysregulation in disease development. This is particularly the case in lamellar ichthyosis (LI; caused by mutations in TGM3) wherein interleukin 1 alpha (IL-1α) receptor antagonists have been shown to improve epidermal differentiation in in vitro models (10). Similarly, transgenic overexpression of the pro-inflammatory transcription factor NRF2 was found to phenocopy LI through a mechanism involving the response to the presence of reactive oxygen species in the epidermis (13).

The availability of HI mouse models provides an opportunity to characterize and delineate the molecular pathways impacted by Abca12 mutation. In particular, they facilitate the investigation of the processes underlying the development of in vivo and in utero phenotypes which contribute to the high incidence of neonatal death in HI patients. Using these models, we have profiled profound defects in keratinocyte differentiation and an underlying and unexpected upregulation of a number of pro-inflammatory chemokines and cytokines. Transgenic expression of the broad spectrum inflammatory suppressor IL-37b largely ameliorated inflammation in utero, effecting a considerable improvement in the defects ordinarily apparent in keratinocyte differentiation in HI. Our studies indicate that the low rates of survival in HI patients at birth result in part from an inflammatory phenotype in the skin which contributes to aberrant keratinocyte differentiation; combined with lipid defects in barrier function. We show that ameliorating these inflammatory processes results in a considerable improvement in keratinocyte differentiation and thus lowers disease severity in models of this disease.

RESULTS

Microarray analysis of Abca12<sup>lx12/lx12</sup> embryonic epidermis

We first profiled the temporal development of keratinocyte differentiation defects in the embryonic skin of the Abca12<sup>lx12/lx12</sup> knockout mouse and found epidermal thickening and hyperkeratosis associated with the induction of wounding and hyperproliferative keratin 6 (K6) from E17.5 (Fig. 1A and B). To profile the global gene expression changes associated with these late embryonic phenotypes, microarray analysis was conducted on E17.5 embryonic epidermis (but not dermis) collected from the dorsal back skin of Abca12<sup>lx12/lx12</sup> and littermate controls. Although previous studies have examined gene expression in HI keratinocytes (9), these were only performed on cultured cells. In Abca12<sup>lx12/lx12</sup> embryos, we found 216 differentially regulated genes (from n = 178 upregulated and n = 70 downregulated probes; changed by >1.6-fold of P < 0.05 statistical significance). One class of upregulated genes were those typically associated with hyperproliferation and wounding (Fig. 1C). These included the epithelial mitogen Epigen (Epgn), an EGF family protein, which promotes proliferation in keratinocytes (14), and the wound healing and hyperproliferative Keratin 6 and K16 (15). Consistent with the latter genes, we observed increased expression of Repetin, a filagrin-like intermediate filament-associated protein which is believed to function in the aggregation of K16 filaments (16). We also observed upregulation of several small proline rich proteins which act as precursor proteins in the formation of the cornified envelope (17) and as reactive oxygen species scavengers in the skin (18) (Fig. 1C). These altered gene profiles highlight hyperkeratotic phenotypes but we also observed a second cohort of gene expression changes which suggest that the program of keratinocyte differentiation is impaired, particularly with respect to the process of terminal differentiation. These changes include downregulation of members of the late cornified envelope (Lee) gene family (19), extracellular matrix 1 (Ecm1) (which controls terminal cell differentiation) (20) and alcohol dehydrogenase 1 (Adh1) (linked to retinoic acid metabolism which strongly promotes keratinocyte differentiation) (21,22) (Fig. 1D). Taken together, these observations support the hypothesis that terminal differentiation of keratinocytes in the developing HI epidermis is defective.

We subjected our list of differentially expressed transcripts to gene ontology (GO) analysis using GOSat (23) (Supplementary Material, Fig. S1A). As expected, genes encompassed by the terms GO:0031424 (keratinization) and GO:0001333 (cornified envelope) were enriched (P < 0.009 and P < 0.003, respectively); however, the most significantly altered genes were those related to immune/inflammatory changes. While a basic skin barrier initially forms in utero; to permit rapid fetal growth the fully cornified epidermal barrier to which Abca12 contributes is not completed until just before birth (24). Thus, these in utero inflammatory changes were particularly unexpected as they were triggered before complete barrier maturation or the need for barrier competency. Enriched GO terms included GO:0002376 [immune system processes (P < 1.37e<sup>-07</sup>)], GO:0006955 [immune responses (P < 1.22e<sup>-05</sup>)], GO:0009605 [response to external stimulus (P < 1.28e<sup>-05</sup>)], GO:0008009 [chemokine activity (P < 3e<sup>-05</sup>)], GO:0042379 [chemokine receptor binding (P < 3e<sup>-05</sup>)], GO:0006954 [inflammatory response (P < 0.0008)], GO:0006935/GO:0042330 [chemotaxis/taxis (P < 0.0008)] and GO:0009611 [response to wounding (P < 0.0002)]. A sample of genes associated with this innate immune response, inflammation, chemokine and cytokine signature are shown in (Fig. 1E and F). These include Lysozymes (Lyz<sup>z</sup> and Lyz<sup>z2</sup>) which are anti-bacterial glycoside hydrolase enzymes integral to the innate immune system of the skin (25), genes regulating inflammatory IL-1 receptor and Nfkb signalling (Il-1rn, Nfkbia and Nfkbiz), many targets suggestive of a type 1 interferon response and a large number of chemokines of the Ccl and Cxcl gene families and the chemokine response associated genes S100a8 and S100a9 (Fig. 1E and F).

Chemokine up-regulation and immune recruitment

IL-1α and IL-1 receptor signalling have been identified as key players in other related inflammatory skin pathologies (10,26), so we investigated their activity in HI. However, we did not
observe elevation of IL-1α mRNA in Abca12 knockout embryonic epidermis (samples at E17.5), despite the sample integrity being validated by downregulation of Abca12 mRNA (Supplementary Material, Fig. S1B and C). Instead, we did confirm the upregulation of IL-1RN mRNA [encoding the endogenous IL-1 receptor antagonist (IL-1RA)] and Nfkbia (encoding the NFκB inhibitor, IκBa), consistent with our array results (Supplementary Material, Fig. S1D and E). These changes are qualitatively different from the activation of the classic IL-1 inflammatory pathway identified in in vitro rat models of LI (10) and suggest that the inflammatory circuits activated as a consequence of ABCA12 dysfunction are distinct from LI (and hence are unlikely to be a simple product of defective barrier function). In assessing other potential pro-inflammatory mechanisms, we were struck by the upregulation of a number of chemokines, in particular Ccl7, Ccl9, Ccl11, Cxcl1, Cxcl12 and Cxcl16. These changes reflect the results of the GO analysis [chemokine activity (P < 3e−05), chemokine receptor binding (P < 3e−05)]. We confirmed upregulation of Ccl9, Ccl11, Cxcl1 and Cxcl16 by qRT-PCR (Fig. 2A–D) and/or by western blot analysis (Fig. 2E–H) in Abca12lox12/lox12 skin relative to wild-type mice and examined the protein expression of CCL9, CCL11 and CXCL16 by immunofluorescence. CCL9 was predominantly detected in K14-expressing cells with a perinuclear to nuclear distribution and increased numbers of K14/CCL9 expressing cells were noted in Abca12lox12/lox12 embryonic skin (Fig. 2I).
CXCL16 expression was increased throughout the epidermis but especially in the more differentiated epidermal layers of Abca12<sup>lx12/lx12</sup> mutants (excluding the cornified envelope) (Fig. 2K). We were unable to detect CXCL1 expression by immunofluorescence using available antibodies. Given that CCL9 recruits monocytes (27), CCL11 recruits all granulocytes (28) (Fig. 2L).
Including eosinophils, neutrophils and basophils (28) and both CXCL1 and CXCL16 are chemo-attractants for neutrophils (29,30) we examined immune cell recruitment to either the dermis or epidermis of fetal skin. No significant changes were observed in the epidermal fraction; however, the percentage of granulocytes (CD11b\(^{+}\)Gr1\(^{+}\)) and inflammatory monocytes (CD11b\(^{+}\)Ly6c\(^{+}\)) present in the dermis were significantly elevated (Fig. 2L–O), indicating increased chemokine activity.

**Grafted HI model**

Mouse models of HI are uniformly characterized by perinatal lethality (4–6), making it difficult to assess whether the inflammatory changes that we observed in the fetus are maintained or modulated after birth. We therefore attempted to graft E17.5 skin from Abca12\(^{el12/el12}\) embryos onto Nude mice (Supplementary Material, Fig. S2A and B) but in our hands, these grafts were not viable (Supplementary Material, Fig. S2C). As an alternative, we employed the ENU mutagenesis derived strain Abca12\(^{lx12/lx12}\), which carries a missense mutation in one of the ABCA12’s transmembrane domains. In contrast to Abca12\(^{el12/el12}\) grafts, those from Abca12\(^{lx12/lx12}\) embryos were 100% viable after 14 days (Fig. 3A and B; Supplementary Material, Fig. S2C), suggesting that this might be a hypomorphic allele. These grafts demonstrated all the typical properties of HI previously observed, including loss of lipid in the stratum corneum (stained via Nile Red, Fig. 3C), loss of lipid in lamellar bodies (Fig. 3D) and premature expression of differentiation markers alongside K14 (Fig. 3E; Supplementary Material, Fig. S2F–H). Close comparison of Abca12\(^{el12/el12}\) embryonic skin at E17.5 and after 14 days after grafting, highlighted some important changes in Abca12\(^{el12/el12}\) grafted skin. While embryonic el12 skin has initial subtle differences to lx12 [lack of K6, normal proliferation (4)], these are lost upon grafting (Supplementary Material, Fig. S2D and E) suggesting that el12 represents a hypomorphic allele (albeit a very severe one) that requires slightly more time to manifest its full HI phenotype. Premature expression of filaggrin (FIL), involucrin and loricrin (LOR) also increased in grafts (Supplementary Material, Fig. S2F–H, see arrowheads). Importantly, we noted improvements in graft skin that in basal K10 was not detected (Fig. 3E, see arrowheads) and FIL expression was detected in the cornified envelope (Supplementary Material, Fig. S2F). These changes are consistent with the self-improvement of HI keratinocyte differentiation previously reported (9) and with the moderate reduction in disease severity in HI patients in the weeks and months following birth.

We next examined if the chemokine signature identified in embryonic Abca12\(^{el12/el12}\) skin was also apparent in embryonic and grafted Abca12\(^{el12/el12}\) skin. CCL9, CCL11 and CXCL16 were indeed detected with increased expression in embryonic Abca12\(^{el12/el12}\) skin and CCL9 and CXCL16 remained elevated in HI grafts (Fig. 3G–I, compare brackets). However, we observed a reduction in granular layer CCL11 expression (range and intensity) when comparing Abca12\(^{el12/el12}\) 14dpg grafts with fetal skin (Fig. 3H, compare brackets). These observations indicate that this embryonic inflammatory signature is a feature of different HI mouse models and that at least some elements of the pro-inflammatory response precipitated by loss of Abca12 persist in adult HI.

**Suppression of inflammatory cytokines via cross with IL-37b transgenic mice**

We next sought to examine the impact of these changes on the development of disease phenotypes that may contribute to neonatal lethality. Because the inflammatory response we observed was both fetal and pleiotropic (making drug selection and delivery difficult) we chose to modulate inflammation via a genetic approach. This was achieved by crossing the Abca12\(^{lx12}\) loss-of-function allele with mice constitutively overexpressing IL-37b. This enigmatic cytokine has been shown to possess a remarkable capacity to suppress inflammatory phenotypes (32). The transgenic mouse strain we employed uses a CMV promotor to drive low levels of ectopic IL-37b expression which has been shown to lower plasma expression of pro-inflammatory cytokines (IL-6 and IL-1β) and chemokines (including CCL2, CCL5, CCL11, CXCL1, CXCL2, CXCL9 and CXCL10) and which protects against LPS-induced endotoxic shock and colitis (31,32). The full mechanism of action for IL-37b is currently unknown; however, it is thought to act in part through Smad3 to limit signalling downstream of a number of activated receptors that respond to a variety of pro-inflammatory stimuli (32). To confirm the efficacy of IL-37b expression in repressing pro-inflammatory chemokines in our model, we analysed the protein levels of CCL9, CCL11, CXCL1 and CXCL16 in samples derived from E18.5 embryos. By western blot, mice carrying the IL-37b transgene exhibited reduced levels of CCL9, CCL11 and CXCL16 (but not CCL11) in whole skin (Fig. 4A–D). Downregulation of CCL9 and CXCL16 was also confirmed by immunohistochemistry (Fig. 4E–G). These results demonstrate that IL-37b expression is able to repress at least a subset of the pro-inflammatory chemokines upregulated in HI.

**Modulating inflammation alters lipid profile but fails to rescue barrier function defects**

Having shown we could dampen the inflammatory response, we first examined whether this translated into an improvement in skin barrier function. We assessed this using an ‘outside-in’ toluidine blue dye assay and found that expression of IL-37b on the background of ABCA12 loss of function was unable to prevent overt defects in the skin’s capacity to exclude dye (Fig. 5A). To examine whether a more subtle rescue was effected, we also undertook trans-epidermal water loss (TEWL) assays to assess ‘inside-out’ loss of barrier function, using samples of skin taken from embryos just prior to birth. We confirmed the persistent loss of barrier function and showed no improvements (Fig. 5B). These results were not surprising, given that the central lipid dysregulation defect caused by loss of ABCA12 is unlikely to be corrected simply by altering inflammation. However, we did wish to determine whether the inflammatory response in HI embryos could feedback and impact lipid profiles in any way. To this end, we finely analysed the profile of >400 lipid species in E18.5 epidermis using mass spectrometry. We saw dramatic changes in several lipid classes between control and HI embryos, but notably observed subtle changes in the lipid profile between Abca12\(^{lx12/lx12}\) and Abca12\(^{lx12/lx12}\)IL-37b+ embryos, with increases in dihydroceramides, ceramides, acylceramides and sphingomyelin (Fig. 5C;
Supplementary Material, Fig. S3A). While these lipids did not normalize to WT levels (nor did we expect them to, given the overarching primary lipid transport defect), this general increase in the ceramide production pathway is consistent with the elevated ceramide levels observed upon self-improvement in grafting models (9). Although these changes are subtle, they do indicate that the inflammatory response initiated in keratinocytes lacking ABCA12, influences pathways which regulate lipid homeostasis in the organ.

In utero suppression of inflammatory chemokines promotes improvement of keratinocyte differentiation

Although barrier function defects remain in IL-37b rescue animals, we wished to determine whether expression of IL-37b was able to correct any other HI skin features we had characterized prior to birth. Double transgenic offspring still demonstrated the epidermal thickening evident in HI (Fig. 6A and B); however, the distribution of cells within the various

Figure 3. Grafted HI model. (A) E17.5 embryonic skin from e112/e112 HI mice and control siblings were grafted onto the backs of Nide mice and analysed 14 days post graft (14 dpg) (n = 5–9). (B) H&E stained section of 14 dpg skins. (C) Nile red lipid staining of the 14 dpg epidermis with stratum corneum (SC) indicated by bracket. (D) Transmission electron micrograph (TEM) of 14 dpg stratum corneum with lamellar bodies indicated by arrowheads. (E–I) E17.5 and 14 dpg skin sections, immunostained as indicated (n = 3). Arrowheads indicate basal keratinocytes expressing K10. Brackets show expression/distribution of indicated chemokine for comparison. Scalebars are 100 µm (B and C) and 50 µm (E–I), unless otherwise indicated (as in TEM).
layers of the epidermis was considerably improved. In double transgenic embryos, the expansion of the K14$^+$ layer was reduced relative to Abca12$^{lx12/lx12}$ embryos, as detected and quantified by IHC (Fig. 6C; Supplementary Material, Fig. S3B), western blot and densitometry (Fig. 6D). Importantly, the proliferation of keratinocytes did not change appreciably as a result of IL-37b expression on an Abca12$^{lx12/lx12}$ background (Fig. 6E and F) nor were changes in apoptosis a significant feature (Supplementary Material, Fig. S3E), indicating that the reduction in K14$^+$ keratinocytes is most likely a function of improved differentiation. To investigate this possibility further, we measured expression of other markers of epidermal differentiation and wounding. The expression of the wounding keratin, K6, was considerably reduced by expression of IL-37b (Fig. 6G and H). While K10 protein expression levels were unchanged (Supplementary Material, Fig. S3C), there was also improvement by way of a reduction in aberrant co-expression of basal (K14) and spinous (K10) layer markers (Fig. 7A and B). The distribution of FLG also shifted from the granular layer towards the cornified envelope as a consequence of suppressed inflammation (Fig. 7C); however, the expression and processing of the protein remained unchanged (Fig. 7D). Similarly, LOR expression in the granular layer was more compact (Fig. 7E and G), despite no change in total levels of protein expression (Fig. 7F). Finally, we measured the thickness of the cornified envelope and observed an increase in thickness in Abca12$^{lx12/lx12}$; IL-37b$^+$ embryos relative to Abca12$^{lx12/lx12}$ skin (Fig. 7H). These observations clearly demonstrate that suppressing inflammatory responses in an HI model is able to considerably correct the defects caused by loss of ABCA12 function through a significant normalization of the keratinocyte differentiation program (Fig. 7I).
DISCUSSION

HI presents as a severe disease from birth, and neonatal lethality associated with the condition is very high. By using mouse models of this disorder, we have profiled a significant increase in pro-inflammatory signalling within the fetal epidermis. That this occurs in utero means that it does so without the considerable influence of dehydration and infection which characterizes the organ after birth. Our localization studies and the array itself (which was undertaken only on the epidermis) instead suggest that keratinocyte-derived expression of pro-inflammatory chemokines is an intrinsic feature of HI. This raises the question as to what is driving this pre-natal response in the skin? We propose that this is a consequence of the very considerable impact of each of these different proteins on keratinocyte differentiation and disease aetiology remains to be determined; however, several of these proteins have already been linked to inflammatory skin disease. CXCL1 (GROα) is a chemoattractant for neutrophils and is enriched in the upper epidermal layers in the skin diseases verruca vulgaris, psoriasis, keratoacanthoma and squamous cell carcinoma (29). CXCL1 is also upregulated during chronic skin inflammation triggered by epidermal

levels of ceramide species in extracts of keratinocytes. While we did not see Pparβ/δ mRNA levels reduce upon IL-37b expression, PPARβ/δ activity can be reduced by inflammatory inhibitors such as aspirin (38) and it will be interesting to examine if IL-37b impacts PPARβ/δ activity or downstream inflammatory mediators. Similarly, links are emerging between the production of chemokines and cell differentiation. One such example is Caspase-14, whose expression is downregulated in response to LPS-induced inflammatory signals (39) and which encodes a protease critically required for FLG processing during keratinocyte differentiation and barrier formation (40). Although the precise links between inflammation and differentiation require further elucidation, the results of this study support a model in which the accumulation of ceramides, their derivatives and/or other dysregulated lipid species elicit the production of chemokines in keratinocytes prior to birth which then impacts their differentiation. This model therefore splits the HI phenotype into two distinct driver pathways, the barrier dysfunction caused by loss of extracellular lipids in the stratum corneum, and inflammatory responses due to lipid dysregulation (Fig. 7J).

The involvement of defective lipid transport in HI compared with other forms of ichthyosis which derive from ‘structural’ epidermal defects (such as TGM3 mutations in LI) might explain the differences we note in the type of inflammatory response elicited (10,26) (Supplementary Material, Fig. S1B–E). Notably, we demonstrate an increase in signalling pathways mediated by members of the CCL/CXCL chemokine families. The specific impact of each of these different proteins on keratinocyte differentiation and disease aetiology remains to be determined; however, several of these proteins have already been linked to inflammatory skin disease. CXCL1 (GROα) is a chemoattractant for neutrophils and is enriched in the upper epidermal layers in the skin diseases verruca vulgaris, psoriasis, keratoacanthoma and squamous cell carcinoma (29). CXCL1 is also upregulated during chronic skin inflammation triggered by epidermal

Figure 5. Assessment of barrier function. (A) Toluidine Blue dye exclusion analysis was performed on a litter of intact E18.5 embryos. (B) TEWL assays were performed on E18.5 back skin with skins weighed for water loss every half an hour over 5 h (n = 5–8). (C) Lipid analysis of E18.5 epidermis. Control samples (pool of WT and lx12/+) are defined as 1 and HI samples are shown relative to this baseline. Errorbars represent SEM.
deletion of BLIMP1, a transcriptional repressor expressed in cells of the granular layer (41). Similarly, CXCL16 is upregulated in psoriasis (42) and has anti-microbial activity (43). CCL11 expression by differentiating keratinocytes recruits granulocytes including eosinophils, neutrophils and basophils to the site of infection or inflammation (28) and over expression is associated with chronic atopic dermatitis (44).

A key question is to what extent anti-inflammatories might present a potential therapeutic for ameliorating disease severity? We have addressed this question using a pleiotropic genetic approach, through the expression of the anti-inflammatory molecule, IL-37b. This led to a significant decrease in the expression of many of the chemokines we had previously shown were upregulated as a consequence of ABCA12 mutation, and to a number of significant improvements in disease phenotype. Principal among these was a correction in keratinocyte differentiation defects; namely a reduction in basal layer thickness, a decrease in the expression of wounding-associated keratins, a correction in the level of co-expression of keratins that are normally sequentially activated during differentiation and a reduction in abnormal thickening of the granular layer that occurs as a consequence of failed compaction. Taken together, these findings indicate that inflammatory signals which arise from loss of ABCA12 function are central to the defective keratinocyte differentiation observed in HI. Quantitatively dissecting the individual (or combinatorial) effects of these different chemokines will be important in assessing the mechanism(s) underlying the correction of these defects.

There has been considerable recent interest in the emerging role that anti-inflammatory cytokines such as IL-10, IL-27, IL-35 and IL-37 play in moderating immune responses. The ability of these proteins to limit inflammatory reactions in a number of disease contexts suggests considerable promise as a new type of therapeutic approach by which to treat a number of different diseases. The work presented here is one of only a handful of studies that have employed IL-37b in vivo to modulate...
an inflammatory response or to study a disease process. In particular, it suggests that IL-37b might be employed effectively as a novel anti-inflammatory therapeutic for the treatment of several skin diseases. We particularly note that IL37b is considerably upregulated in dermatitis, in which the epidermal skin barrier is compromised (45) as well as in other conditions such as colitis where barrier function in the colonic epithelium is disrupted (31). IL-37b has also been shown to ameliorate inflammation in psoriasis (46). While suppressing the inflammatory response in HI does not improve the intrinsic defects in barrier function of the skin nor prevent lipid accumulation (as we expected, given that the central failure in lipid transport remains), we did observe changes in lipid levels which were mediated by expression of IL-37b. These changes suggest communication between inflammation and lipid homeostasis. In the absence of a therapy to directly correct ABCA12 mutation, our findings indicate that a beneficial strategy to increase HI neonate survival might therefore involve a two-pronged approach (Fig. 7J). First, a lipid emollient would be required to create an artificial external skin barrier to address the loss of water-proofing extracellular lipids in the stratum corneum. Secondly, as we demonstrate in this report, anti-inflammatory

Figure 7. In utero suppression of inflammatory cytokines promotes signs of keratinocyte self-improvement of differentiation continued. (A) K14/K10 immunostaining in E18.5 embryonic skin (n = 3–8). Note: the aberrant K14/K10 co-expressing cells in yellow, as quantified in (B) (n = 3). (C) and (E) Immunostaining as indicated in E18.5 embryonic skin (n = 3). Arrowheads show aberrant K14/FLG co-expressing keratinocytes. (D) Western blot analysis of filaggrin (FLG) protein expression, and (F) LOR protein expression standardized to loading control actin, in Wild type, IL-37b+, lx12/lx12 HI and lx12/lx12; IL-37b+ HI whole skin protein extracts (n = 4). (G) The thickness of the granular epidermal layer was quantified in cross sections of LOR stained skin across the four genotypes indicated (n = 3). (H) The thickness of the cornified layer was quantified in cross sections of H&E stained skin across the four genotypes indicated (n = 4). (I) Summary of epidermal layer redistribution observed. (J) Summary model showing outcome of in utero suppression of inflammation. Errorbars represent SEM. Scalebars are 50 μm.
therapies may then serve to correct defects in keratinocyte differentiation that occur as a consequence toxic lipid dysregulation.

MATERIALS AND METHODS

Mouse strains

Abca12<sup>tm1Lex</sup> mice have been previously described (4). We obtained mouse strain ‘Abca12<sup>tm1Lex</sup>’ NIH-0129 by Lexicon Pharmaceuticals, The Woodlands, TX, USA genetics and refer to them as Abca12<sup>lx12</sup> mice in this study. These mice have a puromycin selection cassette-mediated exon 8 disruption and recapitulate the features of HI skin to other Abca12 mutant strains (4–6). Transgenic IL-37b mice have been described previously (32) and were crossed with Abca12<sup>lx12</sup> mice. All animal procedures complied with standards set under Australian guidelines for animal welfare and experiments were subject to Monash University animal welfare ethics review panels. At least three mice have been analysed for all conditions unless otherwise stated.

Histological analysis and microscopy

Embryonic skins were excised from E17.5 to E18.5 embryos and fixed for 2 h in 4% paraformaldehyde at room temperature, while grafts were fixed overnight at 4°C. Tissues were paraffin imbedded and sectioned at 4–7 μm. Antigen retrieval was performed in Citrate buffer pH 6 using rice/pressure cookers, or using an automated (Dako) PT link, with EnVISION FLEX buffer (Dako, Glostrup, Denmark). Antibody staining was performed as described elsewhere (47), and cover slips mounted using MolWiol (Calbiochem, Merck Millipore, Billerica, MA, USA). Nile Red staining was performed as described previously (48). Imaging was using an Olympus fluorescent microscope (Monash University, Clayton, Victoria, Australia). Transmission electron microscopy was performed as described previously (4).

Antibodies and stains


RNA and microarray analysis

Epidermal peels from E17.5 lx12 and wild-type embryos were prepared by dermal separation using 5 mM EDTA for 1 h at 37°C. RNA extraction was performed with RNAqueous, RNA purification kit (Ambion, Life Technologies, Carlsbad, CA, USA). RNA integrity was determined using agarose gel electrophoresis and by the Agilent Bioanalyser 2100 NanoChip protocol. A total of 500 ng was labelled using the Illumina Total Prep RNA amplification kit (ILL791, Ambion, Life Technologies, Carlsbad, CA, USA). A total of 1.5 μg of labelled cRNA was prepared for hybridization to the Sentrix MouseWG6v2 BeadChip, processed and analysed according to Illumina protocols. Expression values were analysed using GeneSpring GX 12.5–6 software. Genes were normalized to the all sample average. Gene changes between Abca12 lx12/lx12 and control littersmates were filtered for absent flags, limited to fold changes of at least 1.6-fold or greater, and the resulting gene list further screened for statistical significance using the unpaired t-test with P < 0.05 cut off. Data are available at NCBI’s GEO archive under GSE56125. For qR-PCR, RNA was isolated from whole skin using Trizol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) as described previously (47) except DNAse treatment was performed using Ambion TURBO DNA free DNase (Invitrogen, Life Technologies, Carlsbad, CA, USA). cDNA synthesis was performed using Supercript Vilo (Invitrogen, Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) as described previously (49). Primers were as follows:

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<th>Gene</th>
<th>5′-Forward-3′</th>
<th>5′-Reverse-3′</th>
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<tr>
<td>Abca12</td>
<td>ACGTGTGCATTTCCTCC TCCAAC</td>
<td>TCTCATTCCTCC GACAC</td>
</tr>
<tr>
<td>Ccl9</td>
<td>TCATACTGCCCTCT CTCC</td>
<td>GCCACGGCAGCAA TCTGAAG</td>
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<td>Nkbia</td>
<td>GACCTGTCCTGCA CTTGG</td>
<td>AAGTGGAAGTGAGT CTGGTCD</td>
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</table>
Protein sample, western blot and densitometry analysis

Whole skin pieces were collected to ice-cold RIPA buffer containing protease inhibitors and homogenized. The protein supernatant was collected after centrifugation and stored at −80°C. Twenty-five micrograms of aliquots of protein were prepared in 4× reducing sample buffer (0.25 M Tris–HCl at pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue with a reducing agent, 10% 2-ME) and boiled for 5 min before loading and performing SDS–PAGE and western blotting. Signals were detected using ECL. Densitometry was performed as described previously (50).

Cell isolation and flow cytometry

E18.5 belly skin was placed in 2.5% dispase + 0.125% trypsin in DMEM for 1–1.5 h at 37°C. Samples were vortexed then mechanically peeled with forceps. The dermal peel was returned to dispase/trypsin solution and an equal volume of 0.2% Collagenase IV in DMEM added for 1–1.5 h at 37°C. The epidermal peel was incubated in 0.25% trypsin for 1–1.5 h at 37°C. 1 ml of Foetal Bovine Serum was added and each sample passed through 70 μm cell strainer, and rinsed with 9 ml DMEM. The cell solution was spun at 1100 rpm (Sorvall Heraeus 75006445 rotor), rinsed in 1 ml PBS and pelleted again before resuspension in 300 μl PBS. Isolated cells were fixed by addition of 1 ml pre-warmed 1.5% PFA in PBS for 10 min at room temperature. Fixation was terminated with 1 ml of ice-cold PBS and fixed cells pelleted at 1100 rpm (Sorvall Heraeus 75006445 rotor) for 5 min at 4°C, then washed with ice-cold PBS and pelleted again. The final cell pellet was resuspended in 1 ml ice-cold 90% methanol, added drop wise while swirling. Cells were placed on ice for 20 min then stored at −80°C until use. Flow cytometry of cells was performed as described elsewhere (51) but briefly, cells were resuspended in staining buffer (2% FCS, 0.5 mM EDTA in PBS) and incubated for 2 h on ice, to rehydrate. Fc receptors were blocked using anti-CD16/32 (eBioscience). Cell populations were gated for CD45 and then characterized as: CD11bhiGr1+CD11c−, CD11bhiGr1+CD11c+, CD11b+Gr1−CD11c−, inflammatory monocytes. Gates were determined using fluorescence-minus-one gating and stained spleen cells for control staining due to low cell numbers sample. These were then averaged across at least three independent biological samples per genotype to allow for comparison across the four genotypes used in this study. The data throughout this study were analysed by Student’s t-test.

Determination of lipid levels by mass spectroscopy

Epidermal fractions from E18.5 Lx12 litters were collected and immersed in 0.3 ml of 2.5% dispase/0.125% trypsin in DMEM at 37°C for 1.5 h. Following incubation, the epidermis was mechanically peeled from the dermis and homogenised in 150–200 μl PBS and stored at −80°C. Protein concentration was quantified using a BCA protein assay (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer’s protocol. Lipid analysis was performed as described previously (52), but briefly, involved the analysis of ~400 lipid species of the lipid classes and subclasses: dihydroceramide, ceramide, acylceramide, glucosylceramide, acylglucosylceramide, sphingomyelin, diacylglycerol, triacylglycerol, cholesterol, cholesterol esters, dihexosylceramide, trihexosylceramide, GM3 ganglioside, lysophosphatidylinositol, lysophosphatidylethanolamine, lysophosphatidylglycerol, bismonoacylglycerol phosphate, lysophosphatidylcholine, lysalkylphosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Data are presented as the sum total of each lipid family and output was normalized to the abundant cellular membrane lipid, phosphatidylcholine.

Barrier function assays

TEWL and toluidine blue dye exclusion assay were performed as described previously (4).

Quantification and statistical analyses

All quantification of the thickness of various strata of the epidermis was carried out using ImageJ (Java) software. In all cases, the average of three measurements from three separate areas of each sample was taken, giving a total of nine measurements per sample. These were then averaged across at least three independent biological samples per genotype to allow for comparison across the four genotypes used in this study. The data throughout this study were analysed by Student’s t-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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