

# Imiquimod-Induced Interleukin-1 $\alpha$ Stimulation Improves Barrier Homeostasis in Aged Murine Epidermis

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**In response to acute disruption of the permeability barrier of aged mammals there is a diminished capacity for barrier recovery, analogous to other aged organs when stressed. Acute barrier disruption increases levels of epidermal cytokines, and cytokines are known regulators of keratinocyte mitogenesis, as well as lipid synthesis in extracutaneous tissues. Underlying the sluggish barrier recovery in aged skin are diminished mRNA and protein levels for the interleukin-1 cytokine family, and its receptors. To further elucidate the role of the interleukin-1 family of cytokines in the barrier repair response, cytokine production was stimulated in aged murine skin with topical imiquimod application. Imiquimod accelerated barrier recovery after acute insults to aged and young skin. These functional results correlated temporally with increased interleukin-1 $\alpha$  production in the epidermis following topical imiquimod administration to murine skin. Furthermore, intracutaneous injections of interleukin-1 $\alpha$  accelerated barrier recovery in aged mice. Finally, we showed that interleukin-1 $\alpha$  added to cultured human keratinocytes stimulates epidermal lipid synthesis. These studies provide further evidence for the role of reduced interleukin-1 $\alpha$  signaling in the decline of permeability barrier function in aged skin, and point to the potential use of cytokine augmentation in barrier dysfunction of the aged.**

Key words: epidermis/aging/interleukin-1/imiquimod/permeability barrier/mouse.

J Invest Dermatol 122:330–336, 2004

Mammalian epidermal permeability barrier, which allows survival in a terrestrial environment, is dependent upon robust levels of epidermal lipid synthesis. The epidermal lipid synthetic apparatus is highly active and relatively autonomous from circulating influences (Feingold *et al*, 1983; Mommaas-Kienhuis *et al*, 1987; Williams *et al*, 1987). Yet, epidermal lipid synthesis is regulatable by changes in permeability barrier function: perturbation of the barrier results in increased synthesis of all three key lipids (cholesterol, fatty acids, and ceramides) (Menon *et al*, 1985; Feingold *et al*, 1986; Elias and Menon, 1991; Holleran *et al*, 1991; Mao-Qiang *et al*, 1993). These changes in synthesis can be attributed to antecedent increases in the activities and mRNA levels of their rate-limiting enzymes (Proksch *et al*, 1990; Holleran *et al*, 1991; Jackson *et al*, 1992; Harris *et al*, 1997).

Whereas barrier function appears normal in aged epidermis under basal, nonstressed conditions, when aged epidermis is stressed, it reveals decreased stratum corneum (SC) integrity and a diminished capacity for barrier recovery, analogous to other organs in the aged when stressed (Ghadially *et al*, 1995); and superimposition of photoaging on chronologic aging imposes an additional stress, with a further decline in barrier function (Reed *et al*, 1997). The functional abnormality in aged epidermis can be explained by structural abnormalities in the SC intercellular

lamellar bilayers, attributable to decreased delivery of lipids to the SC interstices.

As in other aged tissues (Eisenberg *et al*, 1969), the structural abnormality can be explained, in turn, by a lipid abnormality in aged epidermis. In addition to a global decrease in SC lipid content and synthesis, there is a profound decrease in cholesterol synthesis (Ghadially *et al*, 1996). The significance of the cholesterol biosynthetic abnormality is shown by (1) the ability of topical cholesterol alone to improve barrier homeostasis in aged skin (Ghadially *et al*, 1996); and (2) the requirement for cholesterol as the dominant lipid species, when mixtures of the three key SC lipids are employed together to improve barrier function in human and murine skin (Zettersten *et al*, 1997).

More recently, we have assessed the potential role of defective cytokine signaling as the basis of the barrier abnormality in aged epidermis. Acute disruption of the barrier by either acetone treatment or tape stripping, as well as chronic barrier disruption induced by feeding an essential fatty acid deficient diet, increase the epidermal mRNA levels of interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and granulocyte-macrophage colony stimulating factor in young mice, whereas neither IL-6 nor interferon (IFN) levels change (Wood *et al*, 1992). Consistent with these findings, in human skin semiquantitative PCR studies have shown an increase in mRNA for TNF $\alpha$ , as well as other cytokines/growth factors, following barrier perturbation (Nickoloff and Naidu, 1994). We showed recently that the levels of IL-1 $\alpha$  decrease progressively in aged murine epidermis, as shown both by immunohistochemistry and RNase protection assays (Ye *et al*, 2002). Furthermore,

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Abbreviation: IL-1ra, interleukin 1 receptor antagonist; IL-1 $\alpha$ , interleukin-1 $\alpha$ ; IFN, interferon; TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

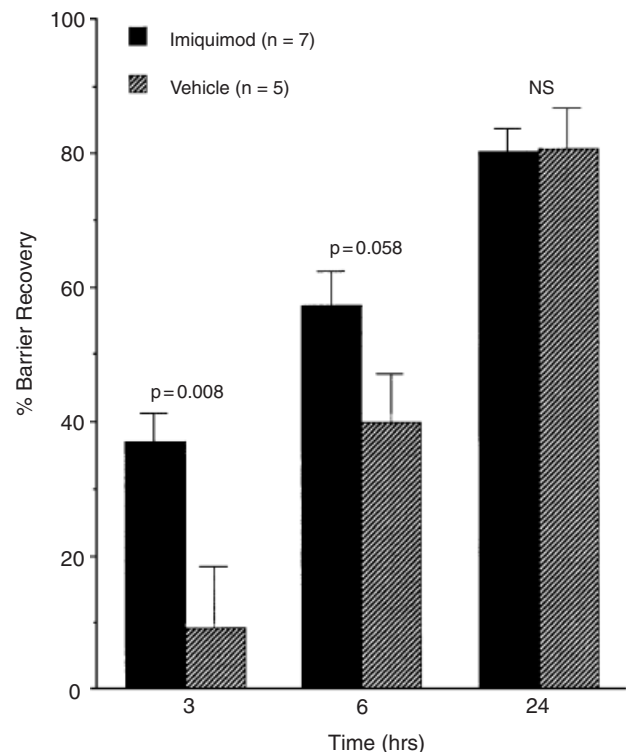
aged epidermis modulates both IL-1 $\alpha$  production and IL-1 receptor expression abnormally following barrier perturbation (Ye *et al*, 2002). These results suggest that cytokines could signal homeostatic repair responses, and that these responses may be abnormal in aged skin.

Cytokines alter lipid metabolism in liver and other tissues. For example, systemic administration of TNF $\alpha$ , IL-1 $\alpha$ , IL-6, and IFN $\alpha$  increase *de novo* fatty acid and cholesterol synthesis in liver (Feingold *et al*, 1989; Grunfeld *et al*, 1990). Therefore, the barrier abnormality in aged epidermis could be due to abnormal cytokine/growth factor signaling of epidermal lipid synthesis. This hypothesis is supported by recent studies in transgenic animals. Although young transgenic animals with single knockouts of the IL-1 $\alpha$  functional (type 1) receptor, TNF functional p55 receptor, or amphiregulin do not display abnormalities in barrier homeostasis (Man *et al*, 1999), crossbred double knockout animals reportedly display abnormal barrier recovery after acute insults (Jensen *et al*, 1998).<sup>1</sup> Moreover, administration of exogenous TNF $\alpha$  reportedly accelerates barrier recovery in normal animals (Jensen *et al*, 1999). More pertinent for aged skin, we demonstrated recently that (1) aged (22–27 mo) mice with a knockout of the IL-1 receptor type 1 ( $n = 14$ ) show a significant delay in barrier recovery *versus* their aged, wild-type counterparts, and (2) that the barrier defect appears earlier in knockout mice than in age-matched, wild-type controls (Ye *et al*, 2002) or in several other strains of tested mice. Together, these results further support the hypothesis that abnormal IL-1 signaling could account for barrier abnormality in aged animals.

Imiquimod (Aldara, 3M Pharmaceuticals) is an immunomodulating agent that has been shown to stimulate increased IL-1 $\alpha$ , TNF $\alpha$ , and IFN $\gamma$  production in cultured human keratinocytes (Kono *et al*, 1994; Fujisawa *et al*, 1996). Moreover, in a recent study of murine skin, IL-6, IL-10, IFN $\gamma$ , and TNF $\alpha$  expression also increased following a single topical application of imiquimod (Miller *et al*, 1998). Therefore, in these studies we assessed the effects of cytokine stimulation on barrier homeostasis in aged *versus* young murine skin, and determined whether correction of defective cytokine levels in the aged epidermis correlates with amelioration of the aged barrier abnormality. We then determined whether the amelioration of barrier homeostasis could be attributed to IL-1 $\alpha$  stimulation of lipid synthesis.

## Results

**Topical applications of imiquimod improve permeability barrier homeostasis in aged murine skin** We first assessed whether topical treatment with imiquimod (10%) altered the barrier abnormality in aged animals. Imiquimod ( $n = 7$ ) *versus* vehicle ( $n = 5$ ) was applied topically once daily to aged mouse skin for 7 d prior to acute barrier abrogation. Although humans occasionally experience skin irritation with imiquimod we did not see clinical or histologic evidence of this in the mice. Barrier recovery kinetics were



**Figure 1**

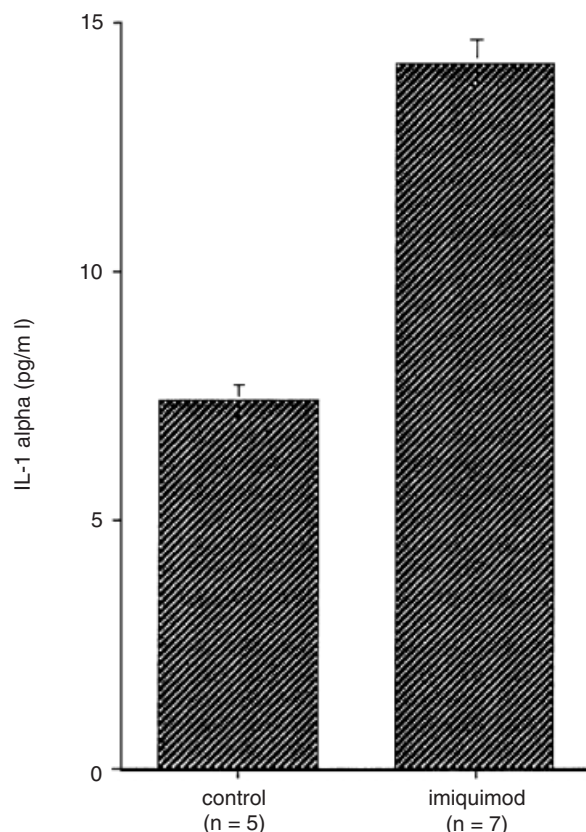
**Topical applications of imiquimod improve permeability barrier recovery in aged skin.** Barrier recovery in control *versus* treated sites revealed that imiquimod ( $n = 7$ ), a stimulator of endogenous keratinocyte cytokine production (including IL-1 $\alpha$ , see Fig 4 below), improves barrier recovery significantly *versus* vehicle ( $n = 5$ ) (37% *versus* 9% recovery at 3 h,  $p < 0.01$ ) when applied topically to aged murine skin twice daily for 1 wk prior to acute barrier perturbation.

significantly accelerated in imiquimod- *versus* vehicle-treated skin at early time points (Fig 1;  $p < 0.01$  at 3 h). These results show that imiquimod pretreatment accelerates the early stages of barrier recovery in aged animals.

**IL-1 $\alpha$ , but not IL1ra, protein increases after imiquimod applications to aged animals** We next measured IL-1 $\alpha$  and IL-1ra protein levels in aged epidermis, following 7 d of daily topical imiquimod (10%) *versus* vehicle application (as above), using a quantitative ELISA for IL-1 $\alpha$  and western blotting for IL-1ra. The improvement in barrier recovery at 7 d (see above) was paralleled by a 2-fold increase in IL-1 $\alpha$  protein levels in the epidermis of imiquimod- *versus* vehicle-treated mice (Fig 2;  $p < 0.0005$ ). In contrast, there was no significant change in IL-1ra levels by western immunoblotting at the same time point (data not shown). These results show that the improvement in barrier homeostasis that occurs in aged animals treated with imiquimod treatment correlates with increased IL-1 $\alpha$  levels in the epidermis.

**Administration of intracutaneous IL-1 $\alpha$  improves barrier function in aged animals** To determine directly whether *in vivo* administration of IL-1 $\alpha$  improves barrier function, IL-1 $\alpha$  50 ng in 50  $\mu$ L PBS *versus* the vehicle alone was injected intracutaneously into the flanks of aged and young BALB/c mice (25 mo old ( $n = 6$ ) *versus* 6–12 wk old ( $n = 6$ )). The dose of IL-1 $\alpha$  (50 ng) was chosen because it is comparable to those employed in prior studies in rodents (Granstein *et al*,

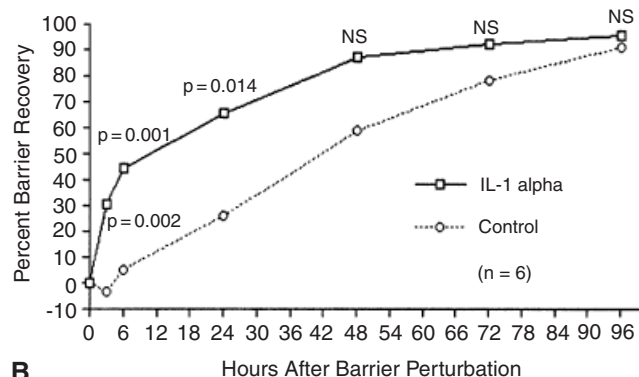
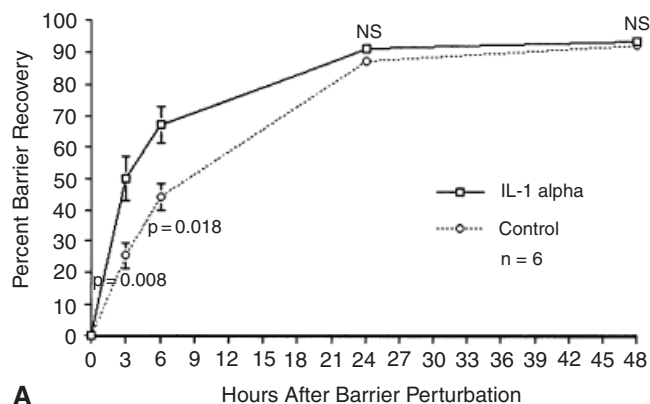
<sup>1</sup>Jensen J, Kupper T, Proksch E: IL-1 $\alpha$ /IL-1 $\alpha$  receptor over-expression and knockout constructs in permeability barrier repair of transgenic mice. *J Invest Dermatol* 110:499, 1998 (abstr)

**Figure 2**

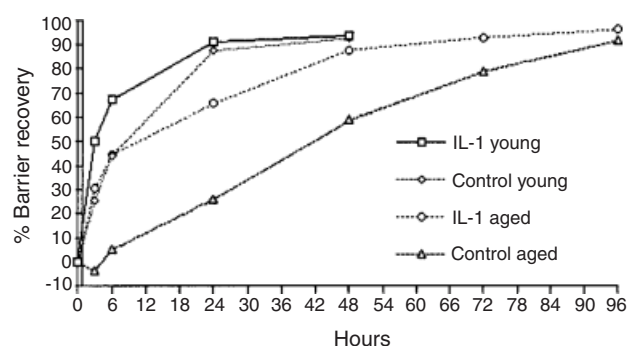
The improvement in barrier recovery with topical imiquimod is associated with increased epidermal IL-1 $\alpha$  expression. Imiquimod, applied topically to aged murine skin twice daily for 1 wk prior to barrier perturbation, doubled epidermal IL-1 $\alpha$  protein levels by ELISA ( $p < 0.0005$ ), but did not change IL-1 $\alpha$  levels by western blotting (not shown).

1986). Because of possible systemic effects of the intracutaneous IL-1 $\alpha$ , separate mice were used for IL-1 $\alpha$ -treated and vehicle-treated groups. The barrier was perturbed by tape stripping immediately following the injection, and barrier recovery rates were compared as above. Barrier recovery kinetics accelerated significantly in both IL-1 $\alpha$ -treated young and aged mice versus vehicle-treated controls, but the extent of improvement was greater in aged animals, remaining significant to at least 24 h post-IL-1 $\alpha$  administration (Fig 3a, b). These results show that exogenous IL-1 $\alpha$  accelerates epidermal permeability barrier recovery in both aged and young mice, with more significant improvement in aged skin.

**IL-1 $\alpha$  treatment normalizes lamellar bilayer structure in aged epidermis** Prior studies have shown that the barrier abnormality in aged mice is associated with reduced numbers of lamellar bodies, decreased secreted lamellar body contents, and patchy reductions in extracellular lamellar bilayers (Ghadially *et al*, 1995). As the most marked acceleration in barrier recovery is apparent 3–6 h after IL-1 $\alpha$  administration, we compared the lamellar body secretory system and SC morphology at these time points. As seen in Fig 4, IL-1 $\alpha$  treatment increased the density of lamellar bodies in the cytosol of cells in the outer stratum granulosum, as well as the amounts of secreted organelle

**Figure 3**

Administration of intracutaneous IL-1 $\alpha$  improves barrier function in young and aged mice. IL-1 $\alpha$  versus vehicle alone was injected intracutaneously into the flanks of young (A) and aged (B) BALB/c mice (6–12 wk old ( $n = 6$ ) versus 25 mo old ( $n = 6$ )) 5 min prior to barrier perturbation. In both young and aged mice, the IL-1 $\alpha$ -treated mice versus vehicle-treated mice exhibited a significant improvement in barrier recovery, although the improvement in young mice was not as pronounced as in the aged.

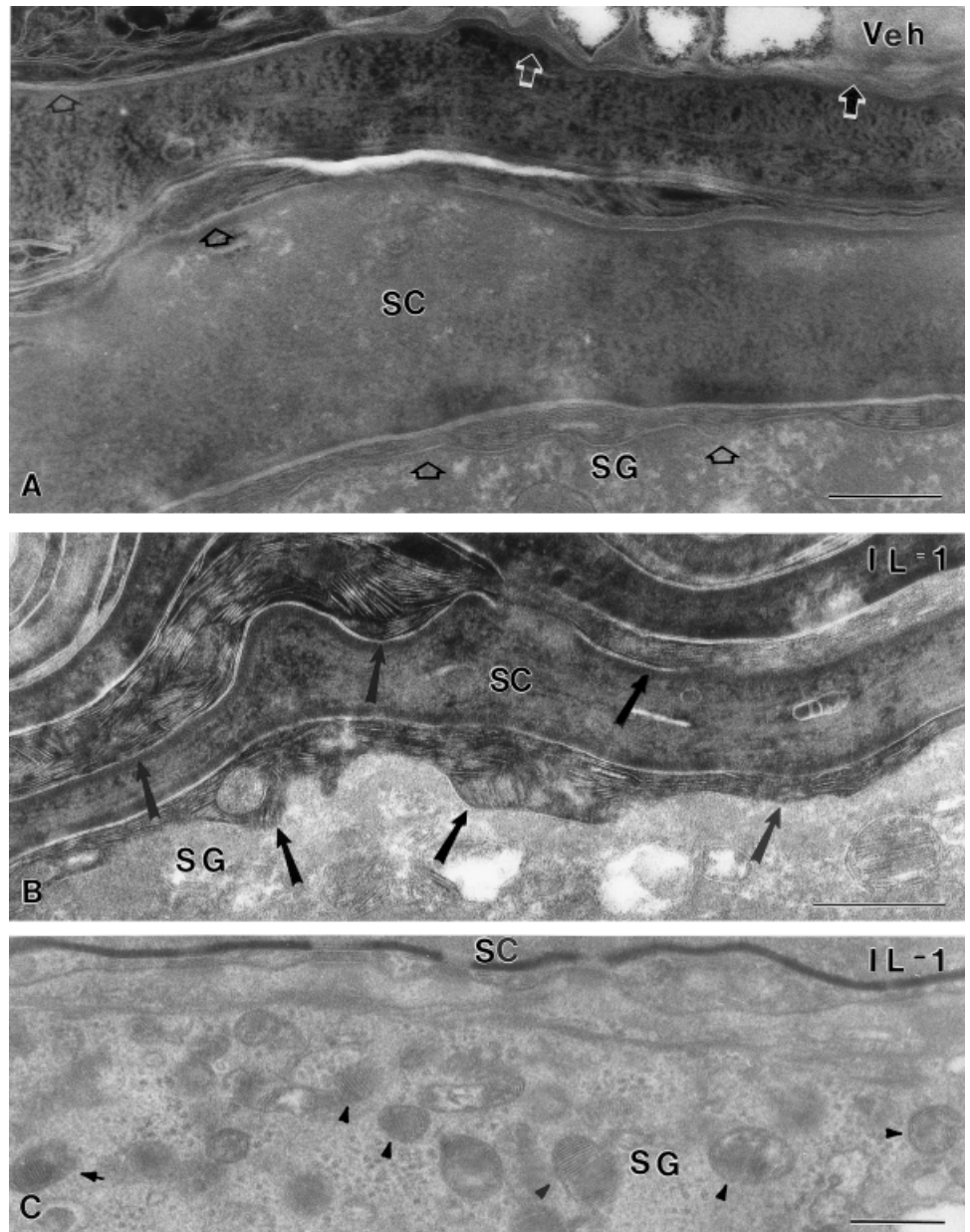
**Figure 4**

Comparison of results in Fig 3(A), (B). Although no statistical analysis is possible as these are separate experiments, it can be seen that following imiquimod application aged barrier recovery is improved, such that, especially at early time points, barrier recovery in imiquimod-treated aged skin is comparable to that of untreated young skin.

contents at the stratum granulosum–SC interface. Moreover, whereas vehicle-treated, aged epidermis displayed little or no return of extracellular lamellar bilayers, IL-1 $\alpha$ -treated samples displayed bilayer quantities comparable to young murine skin at comparable time points after barrier

**Figure 5**

**IL-1 $\alpha$  treatment augments lamellar body formation and secretory response in aged murine skin:** (A) vehicle treated (Veh); (B), (C) IL-1 $\alpha$  treated. Three hours after acute barrier disruption, IL-1 $\alpha$ -treated sites reveal enhanced secreted material (B, arrows) at the stratum granulosum–SC interface in comparison to secreted material in vehicle-treated sites (A, open arrows). (C) The number of lamellar bodies (arrowheads) in the cytosol of outer stratum granulosum cells is greatly increased in IL-1 $\alpha$ -treated versus vehicle-treated epidermis (not shown; see Ghadially *et al*, 1995). (A), (B) Ruthenium tetroxide postfixation; (C) osmium tetroxide postfixation. Magnification bar: (A)–(C) 0.25  $\mu$ m.



disruption (Fig 5; see also Ghadially *et al*, 1995). Finally, the number of lamellar bodies was increased in IL-1 $\alpha$ -treated samples (Fig 5; see also Ghadially *et al*, 1995). Together, these results demonstrate that the IL-1 $\alpha$ -stimulated acceleration of barrier recovery is paralleled by a normalization of the lamellar body secretory system and its resultant bilayers in aged epidermis.

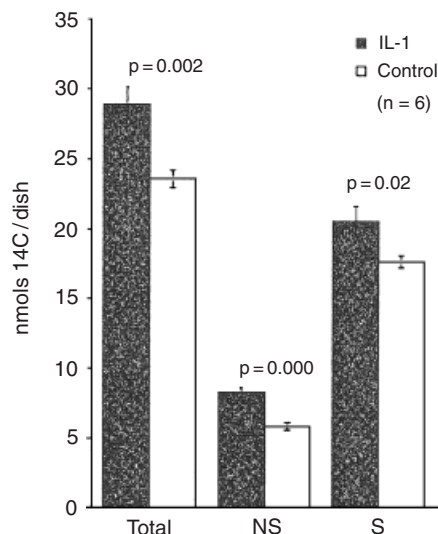
**IL-1 $\alpha$  increases lipid synthesis in cultured human keratinocytes** Because of the limited availability of aged mice, we performed further mechanistic studies in cultured keratinocytes. To determine whether the IL-1 $\alpha$ -stimulated increase in lamellar body secretion reflects increased lipid synthesis, we next assessed whether exogenous IL-1 $\alpha$  directly stimulates keratinocyte lipid production in cultured human keratinocytes. IL-1 $\alpha$  (10 ng per mL) or vehicle alone was added to second passage, cultured human keratinocytes ( $n=6$ ), grown under serum- and growth-factor-free conditions for the prior 24 h. After 24 h total lipid synthesis

increased by approximately 25% in IL-1 $\alpha$ -treated cultured human keratinocytes ( $p=0.002$ ). Synthesis of both non-saponifiable and saponifiable lipids was increased significantly (41%,  $p=0.00$ , and 16%,  $p=0.02$ , respectively) (Fig 6). These results show that exogenous IL-1 $\alpha$  directly stimulates lipid synthesis in cultured keratinocytes, correlating with both increased lamellar body production and barrier recovery in cytokine-treated, aged tissues.

## Discussion

Whereas permeability barrier function is normal under basal, nonstressed conditions, aged epidermis displays delayed barrier recovery kinetics when it is subjected to stress, analogous to the diminished reparative capacity of other organs in the aged (Ghadially *et al*, 1995). Furthermore, superimposition of photoaging produces an additional delay in barrier recovery (Reed *et al*, 1997). The basis





**Figure 6**  
**Basis for improved barrier: IL-1 $\alpha$  added to cultured human keratinocytes results in increased synthesis of barrier lipids.** Addition of 10 ng per mL IL-1 $\alpha$  to cultured human keratinocytes ( $n=6$ ) resulted in a 23% increase in total lipid synthesis ( $p=0.002$ ). Although both nonsaponifiable (NS) and saponifiable (S) lipids increased significantly (41%,  $p=0$ , and 16%,  $p=0.02$ , respectively), the most dramatic increase was in NS lipids (primarily cholesterol).

for the barrier abnormality in aged epidermis is a decrease in the quantities of SC extracellular bilayers, attributable to decreased secreted lipid at the SC–stratum granulosum interface, and a global decrease in SC lipid content (Ghadially *et al*, 1996). The metabolic basis for the structural/functional abnormality is a decrease in the synthesis of cholesterol, fatty acids, and ceramides, which are destined for lamellar bilayer formation (Ghadially *et al*, 1996). The most profound alterations occur in cholesterol synthesis, however, as well as in 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity. These studies suggest that the barrier abnormalities in aged epidermis can be attributed to a failure to synthesize and deliver sufficient lamellar-body-derived lipids to the SC interstices. As topically applied, physiologic lipids incorporate directly into nascent lamellar bodies, bypassing the synthetic apparatus, they have the potential of ameliorating the barrier abnormality in aged epidermis. Indeed, a single topical application of cholesterol alone, or an equimolar mixture of the three key SC lipid classes, improves barrier recovery in aged murine skin, and applications of cholesterol-dominant mixtures of these three lipids further normalize barrier recovery in aged murine and human skin (Zettersten *et al*, 1997).

In recent studies, we showed that expression of the IL-1 family of proteins and its receptors is abnormal in aging epidermis (Ye *et al*, 2002). Prior work has also shown that IL-1 $\alpha$  levels decrease in primary keratinocyte cultures of aged human epidermis (Sauder *et al*, 1988), and that mRNA levels for IL-1 $\alpha$  reportedly decrease in aged murine epidermis (Sauder *et al*, 1989). In contrast, IL-1 $\alpha$  mRNA increases in primary cultures from aged human epidermis, with a lesser increase in keratinocytes from photoaged human epidermis (Garmyn *et al*, 1992; Gilchrist *et al*, 1994). Based upon this work, we assessed whether modulation of IL-1 $\alpha$  levels, either by administration of immunomodulatory

agents or by direct administration of exogenous IL-1 $\alpha$  would improve barrier recovery. Barrier recovery was improved with imiquimod administration to aged murine skin. As imiquimod produces alterations in multiple cytokine pathways, we next proceeded to show that imiquimod administration increases IL-1 $\alpha$  levels in aged epidermis.

Indeed, imiquimod administration did increase IL-1 $\alpha$  2-fold, and IL-1 $\alpha$  was most effective at improving barrier recovery in aged skin, such that the rate of barrier recovery approached that of young control skin. IL-1 $\alpha$  was also able to increase significantly the barrier recovery in young skin, although not as dramatically as in aged skin. This would be expected because aged skin is known to start out with a deficit in IL-1 $\alpha$  and barrier recovery (Ye *et al*, 2002).

Barrier recovery of the epidermis is associated with an increase in lipid synthesis of barrier associated lipids. IL-1 $\alpha$  administration resulted in increased lipid synthesis in cultured human keratinocytes. The greatest increase was in the more profoundly defective lipid (in human epidermis), cholesterol.

Cytokines alter lipid metabolism in liver and other tissues. For example, systemic TNF $\alpha$ , IL-1 $\alpha$ , IL-6, and IFN $\alpha$  increase *de novo* fatty acid and cholesterol synthesis in liver by increasing hepatic levels of citrate, an allosteric activator of acetyl coenzyme A carboxylase, a key enzyme in fatty acid synthesis. More recently, it was shown that TNF and IL-1 $\alpha$  stimulate ceramide synthesis, and serine palmitoyl transferase mRNA and enzyme levels, in hepatocytes. Most importantly, it was shown that IL-1 $\alpha$  stimulates cholesterol synthesis and 3-hydroxy-3-methylglutaryl-coenzyme A reductase in hepatocytes. Although there are no studies of IL-1 $\alpha$  regulation of SREBP, an interleukin-1 $\beta$  converting enzyme-related cysteine protease that cleaves SREBP between the leucine zipper and transmembrane domains has previously been purified. Also, TNF and IL-6 have been shown to activate SREBP. These findings suggest that cytokines could regulate lipid synthesis through activation of SREBP.

In summary, we have shown that immunomodulation of IL-1 $\alpha$  levels improves barrier function in aged epidermis. The ability of imiquimod applied topically, and IL-1 $\alpha$  administered intracutaneously, to improve barrier recovery in the epidermis of aged mice, and the ability of cytokines to increase lipid synthesis in keratinocytes, provide further evidence for the role of cytokine alterations in the decline in aged barrier function, and suggest a new form of therapy for aged skin.

## Materials and Methods

**Animals** Outbred hairless mice CrL:SKH1 (hr/hr) BR (Charles River Laboratories, Wilmington, MA) comprised the murine model. The aged mice were 18–24 mo old. The young mice were 6–10 wk old. Aged mice were checked regularly for microbial diseases and tumors. Mice that showed evidence of either systemic illness or tumor development were not studied. Institutional approval was obtained prior to all studies.

**Barrier disruption and tissue harvesting** Barrier abrogation in all experiments was achieved by sequential applications of cellophane tape (six to eight times). The procedure was stopped when the transepidermal water loss reached 6–10 mg per cm<sup>2</sup> per h as

measured with an electrolytic water analyzer (Meeco, Warrington, PA). Control mice were untreated. It has been found previously that untreated mice, as well as those treated with sterile endotoxin-free saline, exhibit identical baseline levels of epidermal cytokines (Wood *et al*, 1992). Samples for ELISA and western blot studies were obtained immediately prior and 3 h following barrier disruption. Skin excised from the treated area (entire torso) was placed epidermis side downward on plastic Petri dishes. Subcutaneous fat was removed with a scalpel, and skin was then floated epidermis upward on 10 mL of 10 mM ethylenediamine tetraacetic acid (EDTA) in Ca, Mg-free phosphate-buffered saline (PBS). Incubations were continued at 37°C for 35 min in order to separate the epidermis from the dermis (Menon *et al*, 1985; Grubauer *et al*, 1987). Epidermis was scraped off the underlying dermis with a scalpel and kept at -70°C until proteins were isolated (see below).

**Imiquimod applications** In studies on barrier homeostasis, we assessed whether topical treatment with 10% imiquimod in propyleneglycol:propanol (7:3, vol/vol) (3M Pharmaceuticals, St Paul, MN) improved the aged barrier abnormality. Imiquimod 10% ( $n = 7$ ) *versus* propylene glycol:propanol vehicle ( $n = 5$ ) was applied once daily topically to aged (18–24 mo) mouse skin for 4–7 d prior to barrier abrogation. Barrier recovery kinetics were then assessed, and ELISA and western blot studies were performed on samples obtained just prior to barrier abrogation (see below).

**IL-1 $\alpha$  administration** To determine whether cytokine administration improves barrier function *in vivo*, IL-1 $\alpha$  (50 ng in 50  $\mu$ L PBS) or PBS (50  $\mu$ L) alone was injected intradermally into the flanks of aged (25 mo,  $n = 6$ ) and young (8–12 wk,  $n = 6$ ) BALB/c mice (Granstein *et al*, 1986; Enk *et al*, 1993; Lee *et al*, 1994). Mice were shaved 3–4 d prior to barrier perturbation. The barrier was perturbed immediately after and barrier recovery was monitored. Samples were taken 3 h post barrier perturbation for electron microscopy (see below). For studies in cultured human keratinocytes IL-1 $\alpha$  (10 ng per mL) or vehicle alone was added to the culture medium for 3 h before harvesting for lipid synthesis studies (see below). Results were then assessed using a Student's unpaired *t* test.

**ELISA** IL-1 $\alpha$  protein concentrations were quantitated by ELISA, using the Quantikine Mouse IL-1 $\alpha$  Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. All samples were assayed in duplicate. 2.5 ng of protein was used for each sample well. ELISA results were analyzed on an Emetic microplate reader (Molecular Devices, Sunnyvale, CA). Results were compared in imiquimod-treated *versus* vehicle-treated epidermis using an unpaired Student's *t* test.

**Western blotting** The levels of murine IL-1 receptor antagonist (IL-1ra) protein in mouse epidermis was analyzed by Western immune blotting. The frozen epidermis, obtained from one mouse flank, was homogenized on ice in a polytron (Kinematica, Littau/Lucerne, Switzerland), for 30 s, in 3 mL of 2  $\times$  sample loading buffer (100 mM Tris Cl, 200 mM dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol, 0.1%  $\beta$ -mercaptoethanol, 0.2% bromophenol blue). 25  $\mu$ g of each sample in 1  $\times$  sample loading buffer was loaded onto a 15% sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA). After electrophoresis one gel was used for transfer onto nitrocellulose membrane (Bio-Rad, Hercules, CA) (Burnette, 1981), and another parallel gel was used for staining with Coomassie Blue to check for sample loading equivalence. Immunoblotting was performed with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Primary antibody was goat antimouse IL-1ra (R&D Systems) used at 0.1  $\mu$ g per mL. The secondary antibody was horseradish-peroxidase-conjugated affinity purified mouse antigoat IgG (H + L) (Jackson ImmunoResearch Laboratories, Western Grove, PA) used at 0.04  $\mu$ g per mL. The band of IL-1ra on Kodak BIOMAX MR film was scanned by a densitometer (E-C Apparatus, St Petersburg, FL).

**Electron microscopy** Biopsy specimens from aged mice 3 h after barrier disruption, IL-1 $\alpha$  treated *versus* vehicle treated, were processed for electron microscopy. Samples were cut in pieces 1 mm<sup>3</sup> or smaller and fixed overnight at 4°C in 2% glutaraldehyde, 2% paraformaldehyde with 0.06% calcium chloride in 0.1 mol per L sodium cacodylate buffer, pH 7.3. Specimens were then washed in 0.1 mol per L sodium cacodylate buffer before further processing. Tissue sections were placed in either (1) 0.2% ruthenium tetroxide (Polysciences, Warrington, PA) with 0.5% potassium ferrocyanide in 0.1 mol per L sodium cacodylate, pH 7.4, at room temperature in the dark for 0.5 h, or (2) 1% osmium tetroxide with potassium ferrocyanide (1.5%) in 0.1 mol per L sodium cacodylate at room temperature in the dark for 1 h. After rinsing in cacodylate buffer, tissue samples were dehydrated in a graded ethanol series and subsequently embedded in a low viscosity epoxy resin containing DER 736 and Epon 812. Thin sections were examined both unstained and/or after staining with uranyl acetate and lead citrate in a Zeiss 10 A electron microscope.

**Lipid synthesis** Neonatal human keratinocytes were obtained in accordance with Declaration of Helsinki Guidelines; they were grown in KGM (Clonetics) with 0.07 mM CaCl<sub>2</sub> to 90% confluence and then changed to KGM with 1.2 mM CaCl<sub>2</sub> for 24 h until 100% confluent. Subsequently, they were changed to KBM (no growth factors) with 1.2 mM CaCl<sub>2</sub> for 18 h before experimentation. Medium was then changed to fresh KBM + 1.2 mM CaCl<sub>2</sub> + 1 mM sodium acetate  $\pm$  IL-1 $\alpha$  10 ng per mL; the cultures were incubated for 90 min, and then <sup>14</sup>C-acetate (American Radio Chemicals, 56.7 mCi per mmol) was added (8.385  $\mu$ Ci per mL). Cultures were incubated for 2 h at 37°C and then harvested; and saponifiable lipids were extracted separately and counted on a Beckman LS 1800 liquid scintillation counter.

Full-thickness skin was collected, the fat was scraped away, the total surface area recorded, and the skin was floated in KBM without CaCl<sub>2</sub>, plus 10 mM EDTA, 1 mM sodium acetate, and 16.88  $\mu$ Ci per mL <sup>14</sup>C-acetate. Full-thickness skin samples were incubated 2 h at 37°C, rinsed with distilled H<sub>2</sub>O, and the epidermis was peeled off and saponified as above. Total, saponified, and nonsaponified lipids were then assessed in IL-1 $\alpha$  *versus* control samples, using an unpaired Student's *t* test.

DOI: 10.1046/j.0022-202X.2004.22203.x

Manuscript received November 19, 2002; revised August 22, 2003; accepted for publication September 15, 2003

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## References

- Burnette WN: 'Western blotting': Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Ann Biochem* 112:195–203, 1981
- Eisenberg S, Stein Y, Stein O: Phospholipases in arterial tissues: IV. Regulation of phospholipid composition in the normal human aorta with age. *J Clin Invest* 48:2320–2329, 1969
- Elias PM, Menon GK: Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1–23, 1991
- Enk AH, Angeloni VL, Udey MC, Katz SI: An essential role for Langerhans cell-derived IL-1 $\beta$  in the initiation of primary immune responses in skin. *J Immunol* 150:3698–3704, 1993
- Feingold KR, Brown BE, Lear SR, Moser AH, Elias PM: Localization of *de novo* sterologenesis in mammalian skin. *J Invest Dermatol* 81:365–369, 1983
- Feingold KR, Brown BE, Lear SR, Moser AH, Elias PM: The effect of essential fatty acid deficiency on cutaneous sterol synthesis. *J Invest Dermatol* 87:588–591, 1986

- Feingold KR, Soued M, Serio MK, Moser AH, Dinarello CA, Grunfeld C: Multiple cytokines stimulate hepatic lipid synthesis *in vivo*. *Endocrinology* 125:267–274, 1989
- Fujisawa H, Shivji GM, Kondo S, Wang B, Tomai MA, Miller RL, Sauder DN: Effect of a novel topical immunomodulator, S-28463, on keratinocyte cytokine gene expression and production. *J Interferon Cytokine Res* 16:555–559, 1996
- Garmyn M, Yaar M, Boileau N, Backendorf C, Gilchrist BA: Effect of aging and habitual sun exposure on the genetic response of cultured human keratinocytes to solar-simulated irradiation. *J Invest Dermatol* 99:743–748, 1992
- Ghadially R, Brown BE, Sequeira-Martin SM, Feingold KR, Elias PM: The aged epidermal permeability barrier. Structural, functional, and lipid biochemical abnormalities in humans and a senescent murine model. *J Clin Invest* 95:2281–2290, 1995
- Ghadially R, Brown BE, Hanley K, Reed JT, Feingold KR, Elias PM: Decreased epidermal lipid synthesis accounts for altered barrier function in chronologically aged murine epidermis. *J Invest Dermatol* 106:1064–1069, 1996
- Gilchrist BA, Garmyn M, Yaar M: Aging and photoaging affect gene expression in cultured human keratinocytes. *Arch Dermatol* 130:82–86, 1994
- Granstein RD, Margolis R, Mizel SB, Sauder DN: *In vivo* inflammatory activity of epidermal cell-derived thymocyte activating factor and recombinant interleukin 1 in the mouse. *J Clin Invest* 77:1020–1027, 1986
- Grubauer G, Feingold KR, Elias PM: The relationship of epidermal lipogenesis to cutaneous barrier function. *J Lipid Res* 28:746–752, 1987
- Grunfeld C, Soued M, Adi S, Moser AH, Dinarello CA, Feingold KR: Evidence for two classes of cytokines that stimulate hepatic lipogenesis: Relationships among tumor necrosis factor, interleukin-1 and interferon- $\alpha$ . *Endocrinology* 127:46–54, 1990
- Harris IR, Farrell AM, Grunfeld C, Holleran WM, Elias PM, Feingold KR: Permeability barrier disruption coordinately regulates mRNA levels for key enzymes of cholesterol, fatty acid, and ceramide synthesis in the epidermis. *J Invest Dermatol* 109:783–787, 1997
- Holleran WM, Feingold KR, Mao-Qiang M, Gao WN, Lee JM, Elias PM: Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151–1158, 1991
- Jackson SM, Wood LC, Lauer S, Taylor JM, Cooper AD, Elias PM, Feingold KR: Effect of cutaneous permeability barrier disruption on HMG-CoA reductase, LDL receptor, and apolipoprotein E mRNA levels in the epidermis of hairless mice. *J Lipid Res* 33:107–114, 1992
- Jensen J, Schutze MS, Forl M, Kronke M, Proksch E: Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J Clin Invest* 104:1761–1770, 1999
- Kono T, Kondo S, Pastore S, Shivji GM, Tomai MA, McKenzie RC, Sauder DN: Effects of a novel topical immunomodulator, imiquimod, on keratinocyte cytokine gene expression. *Lymphokine Cytokine Res* 13:71–76, 1994
- Lee WY, Lockniskar MF, Fischer SM: Interleukin-1 $\alpha$  mediates phorbol ester-induced inflammation and epidermal hyperplasia. *FASEB J* 8:1081–1087, 1994
- Man MQ, Wood L, Elias PM, Feingold KR: Cutaneous barrier repair and pathophysiology following barrier disruption in IL-1 and TNF type I receptor deficient mice. *Exp Dermatol* 8:261–266, 1999
- Mao-Qiang M, Elias PM, Feingold KR: Fatty acids are required for epidermal permeability barrier function. *J Clin Invest* 92:791–798, 1993
- Menon GK, Feingold KR, Moser AH, Brown BE, Elias PM: *De novo* sterogenesis in the skin. II. Regulation by cutaneous barrier requirements. *J Lipid Res* 26:418–427, 1985
- Miller R, Tomai M, Arany I, *et al*: Cytokine induction in hairless mouse and human skin by topical immune response modifier, imiquimod. *J Invest Dermatol* 110:680, 1998
- Mommaas-Kienhuis AM, Grayson S, Wijsman MC, Vermeer BJ, Elias PM: Low density lipoprotein receptor expression on keratinocytes in normal and psoriatic epidermis. *J Invest Dermatol* 89:513–517, 1987
- Nickoloff BJ, Naidu Y: Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol* 30:535–546, 1994
- Proksch E, Elias PM, Feingold KR: Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in murine epidermis. Modulation of enzyme content and activation state by barrier requirements. *J Clin Invest* 85:874–882, 1990
- Reed JT, Elias PM, Ghadially R: Integrity and permeability barrier function of photoaged human epidermis. *Arch Dermatol* 133:395–396, 1997
- Sauder DN, Monick MM, Hunningshake GW: Epidermal cell-driven thymocyte activating factor (ETAf) is a potent T-cell chemoattractant. *J Invest Dermatol* 85:431–433, 1988
- Sauder DN, Ponnappan U, Cinader B: Effect of age on cutaneous interleukin 1 expression. *Immunol Lett* 20:111–114, 1989
- Williams ML, Mommaas-Kienhuis AM, Rutherford SL, Grayson S, Vermeer BJ, Elias PM: Free sterol metabolism and low density lipoprotein receptor expression as differentiation markers of cultured human keratinocytes. *J Cellular Physiol* 132:428–440, 1987
- Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR: Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90:482–487, 1992
- Ye J, Garg A, Calhoun C, Feingold KR, Elias PM, Ghadially R: Alterations in cytokine regulation in aged epidermis: Implications for permeability barrier homeostasis and inflammation. I. IL-1 gene family. *Exp Dermatol* 11:209–216, 2002
- Zettersten EM, Ghadially R, Feingold KR, Crumrine D, Elias PM: Optimal ratios of topical stratum corneum lipids improve barrier recovery in chronologically aged skin. *J Am Acad Dermatol* 37 (3 Part 1):403–408, 1997