# IL-1α Stimulation Restores Epidermal Permeability and Antimicrobial Barriers Compromised by Topical Tacrolimus

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In a previous study, we showed that barrier recovery was delayed after acute barrier disruption in the skin treated with topical calcineurin inhibitors. Tacrolimus decreases lipid synthesis and the expressions of antimicrobial peptide (AMP) and IL-1α in the epidermis. IL-1α is an important cytokine for improving barrier function, lamellar body (LB) production, and lipid synthesis in keratinocytes (KCs). We aimed to evaluate whether IL-1α stimulation could restore the barrier dysfunction observed in tacrolimus-treated skin. Topical imiquimod, an IL-1α inducer, restored the epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment in human ($n=15$) and murine ($n=10$) skins, and improved stratum corneum integrity by restoring corneodosmosomes in murine skin ($n=6$). Imiquimod co-applied on the epidermis resulted in an increase in the production of LB and three major lipid synthesis-related enzymes, and in the expressions of mBD3, CRAMP, and IL-1α ($n=5$). Furthermore, intracutaneous injection of IL-1α restored permeability barrier recovery ($n=6$). In IL-1 type 1 receptor knockout mice, topical imiquimod was unable to restore permeability barrier recovery after tacrolimus treatment ($n=21$). In conclusion, IL-1α stimulation induced positive effects on epidermal permeability and antimicrobial barrier functions in tacrolimus-treated skin. These positive effects were mediated by an increase in epidermal lipid synthesis, LB production, and AMP expression.

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## INTRODUCTION

Tacrolimus, an inhibitor of phosphatase calcineurin, has recently been advanced as an effective and safe topical therapy for atopic dermatitis (Boguniewicz *et al.*, 1998; Bos, 2002). Tacrolimus is the first topical immune suppressant that is not a derivative of hydrocortisone, the key component in dermatological treatment for nearly 50 years (Nghiem *et al.*, 2002). Tacrolimus does not cause glucocorticoid-related side effects such as skin atrophy and telangiectasiae. However, tacrolimus does not prevent viral skin infections such as eczema herpeticum, which can be life-threatening (Paller *et al.*, 2001), even though it decreases the incidence of bacterial skin infections compared with other treatments (Pournaras *et al.*, 2001; Ashcroft *et al.*, 2005). We previously showed that tacrolimus negatively affected skin barrier function, including permeability and antimicrobial functions, which are mediated by the downregulation of epidermal lipid synthesis, lamellar body (LB) secretion, and the epidermal expressions of mBD3 and CRAMP, two major epidermal antimicrobial peptides (AMPs). Topical tacrolimus also suppressed the expression of IL-1α, suggesting that its action includes a mechanism acting on skin barrier function (Kim *et al.*, 2010).

IL-1α is a proinflammatory cytokine that has an important role in inflammatory diseases of the skin, including bacterial infections, bullous diseases, and UV damage (Yano *et al.*, 2008). IL-1α serves as a signal for undamaged keratinocytes (KCs) to be activated. Activated KCs become migratory and hyperproliferate to produce growth factors and cytokines that regulate inflammatory and wound healing processes. When added to cultured human KCs, IL-1α stimulates the synthesis of epidermal lipids, the expression of CCL20, and the production of a potent bacteriostatic agent. Imiquimod-induced IL-1α stimulation improves barrier homeostasis in aged murine epidermis (Barland *et al.*, 2004). As IL-1α production and the epidermal permeability barrier are closely linked, we hypothesized that decreased IL-1α production observed in tacrolimus-treated skin could be attributed to abnormal skin barrier function. Therefore, IL-1α stimulation could induce positive effects on epidermal permeability and antimicrobial barrier function in tacrolimus-treated skin.

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*Abbreviations: AMP, antimicrobial peptide; CD, corneodesmosomes EM, electron microscopy; KC, keratinocyte; KO, knockout; LB, lamellar body; SC, stratum corneum; TCI, topical calcineurin inhibitor*

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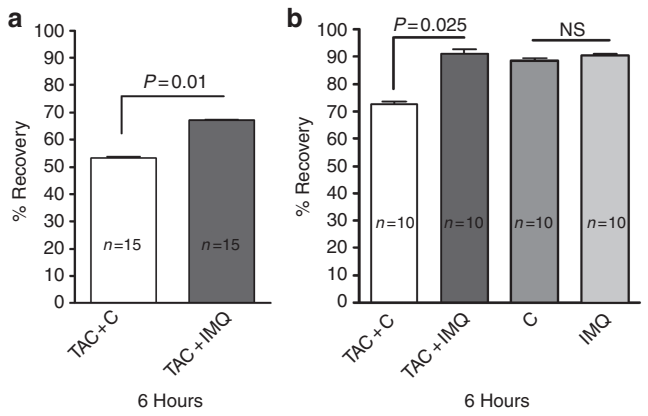


**Figure 1. Topical imiquimod restored epidermal permeability barrier delayed by tacrolimus treatment in human and murine skin.** In humans, topical tacrolimus was applied on both forearms twice a day for 5 days. Immediately after acute barrier disruption, imiquimod was applied on one forearm, and the control on the other forearm. Barrier recovery rates were measured after 6 hours. Imiquimod restored the delayed barrier recovery induced by tacrolimus in human skin ($n=15$) (**a**). In the animal study, one group consisted of flanks of mice treated with tacrolimus twice daily and then imiquimod once daily on one flank with a control cream on the other flank for 4 days. The other group was treated the same way minus tacrolimus application. As seen in humans, the barrier recovery rates improved in tacrolimus and imiquimod-treated skin ($n=10$) (**b**). The numbers represent mean ± SEM. C, control; IMQ, imiquimod; NS, not significant; TAC, tacrolimus.

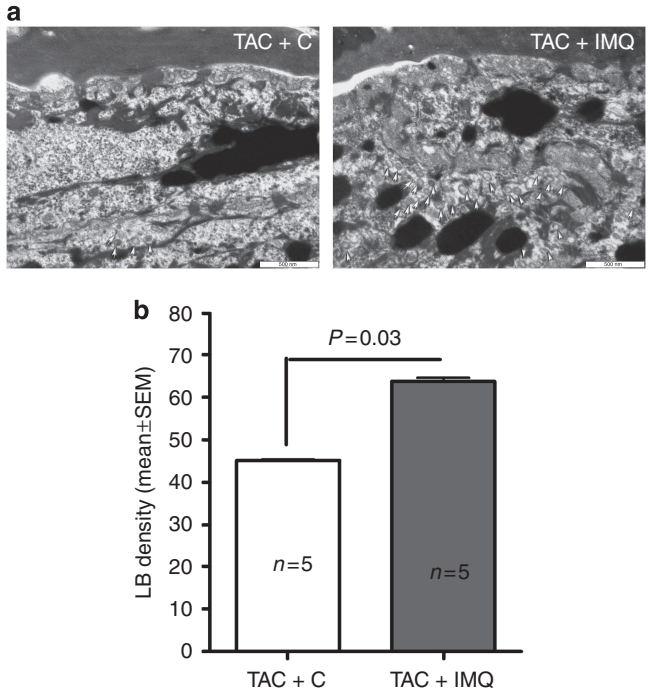


**Figure 2. Topical imiquimod increased the density and content of lamellar bodies (LBs) in tacrolimus-treated murine skin.** Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for 4 days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod and processed for electron microscopy (EM) to analyze LB concentration. Epidermis treated with imiquimod shows an increased number (density) of LBs (white arrows) in comparison to the control (**a**). Quantitative analysis of randomly obtained and coded EM pictures showed a significant increase in LB density in imiquimod-treated murine skin (**b**). The numbers represent mean ± SEM ($n=5$ in each group). C, control; IMQ, imiquimod; TAC, tacrolimus. Bar = 2 μm.

## RESULTS

### Topical imiquimod restored epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment in human and murine skin

Our previous report showed that tacrolimus disrupts epidermal permeability barrier homeostasis and decreases IL-1α in murine epidermis (Kim *et al.*, 2010). In this study, we first assessed whether topical imiquimod, an IL-1α activator, restored permeability barrier function in tacrolimus-treated skin. Imiquimod restored permeability barrier recovery in human skin (Figure 1a). We next assessed the effects of imiquimod on tacrolimus-treated murine skin. Imiquimod significantly restored permeability barrier recovery. However, imiquimod did not affect barrier recovery in control mice that had not been treated with tacrolimus (Figure 1b).

### Topical imiquimod stimulated epidermal lipid production that had been decreased by tacrolimus treatment in murine skin

Using an LB counting and lipid synthesis-related enzyme assay, we examined whether imiquimod reversed tacrolimus-induced barrier abnormalities by promoting epidermal lipid production. Murine epidermis treated with imiquimod exhibited an increased number (density) of LBs in comparison to control sites treated with an inactive cream (Figure 2a). Quantitative analyses of randomly obtained and coded electron microscopy (EM) pictures by a blinded investigator also indicated a significant increase in LB density in imiquimod-treated murine skin (Figure 2b).

We examined whether the imiquimod-induced increase in LB production was, in turn, attributed to activate epidermal lipid synthesis. The activities of rate-limiting enzymes for three key epidermal lipids, such as cholesterol, ceramides, and free fatty acids, that mediate barrier function are normally high in epidermal KCs (Proksch *et al.*, 1993). In our previous study, we observed that three key enzymes required for epidermal lipid synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase, serine-palmitoyl transferase, and fatty acid synthase, decreased after tacrolimus treatment compared with controls (Kim *et al.*, 2010). As a result, the mRNA expression of 3-hydroxy-3-methylglutaryl-CoA reductase, serine-palmitoyl transferase, and fatty acid synthase was measured and we confirmed that the mRNA levels for these three key enzymes increased after imiquimod treatment (Figure 3).

### Topical imiquimod improved SC integrity by restoring corneodosmosomes that had been decreased by tacrolimus treatment in murine skin

Intercorneocyte adhesion, which is mediated largely by corneodesmosomes (CD), a unique intercellular junction modified from epidermal desmosomes (Serre *et al.*, 1991; Haftek *et al.*, 1998), is important not only for SC integrity, but also for the maintenance of the epidermal permeability barrier. CD density was measured in the lower SC by quantitative EM analysis using a previously described method

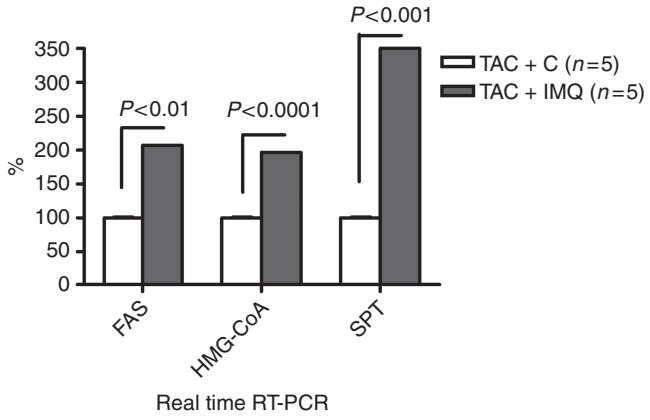


**Figure 3. Topical imiquimod increased epidermal lipid synthesis-related enzymes in tacrolimus-treated murine skin.** Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for 4 days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod and assayed with quantitative reverse transcription PCR to assess the mRNA levels of epidermal lipid synthesis-related enzymes. mRNA levels in murine epidermis treated with imiquimod increased compared with the control. The numbers represent mean ± SEM. FAS, fatty acid synthases; HMG-CoA, 3-hidroxy-3-methylglutaryl-CoA reducatase; SPT, serine-palmitoyl transferase. For each group, $n = 5$. C, control; IMQ, imiquimod; TAC, tacrolimus.

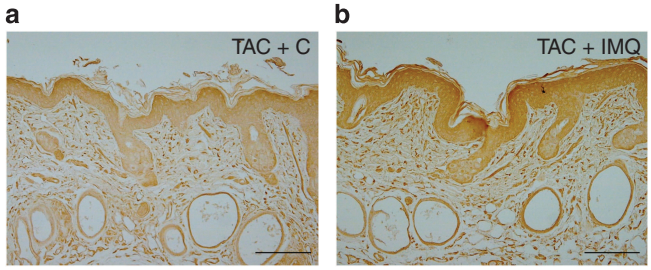


**Figure 4. Topical imiquimod increased the epidermal expression of IL-1α decreased by tacrolimus in murine skin.** Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for 4 days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod. Biopsy specimens for the IL-1α immunohistochemical stain were taken from imiquimod or control sites. Imiquimod-treated skin (**a**) showed more intense IL-1α expression in immunohistochemical stain compared with the control (**b**). C, control; IMQ, imiquimod; TAC, tacrolimus. Bar = 100 μm.

(Choi *et al.*, 2005). We observed that CD density increased in the imiquimod-treated group compared with the control group (Supplementary Figure S1 online), indicating that imiquimod improved SC integrity in tacrolimus-treated skin by restoring CD density.

### Topical imiquimod augmented the epidermal expression of IL-1α that had been diminished by tacrolimus treatment in murine skin

IL-1α is an important cytokine for improving permeability barrier function, LB structure, and lipid synthesis in human KCs (Barland *et al.*, 2004). In our previous study, we observed that topical calcineurin inhibitors (TCIs) suppressed the epidermal expression of IL-1α (Kim *et al.*, 2010). Based on these findings, we measured IL-1α expression using immunohistochemical staining in murine skin co-applied with imiquimod, which means that tacrolimus was applied and followed by imiquimod. Skin sites co-applied with imiquimod showed a much greater expression of IL-1α than the control sites (Figure 4), suggesting that the positive effect of imiquimod on the permeability barrier of tacrolimus-treated epidermis possibly resulted from the augmentation of IL-1α.

### Intracutaneous IL-1α injection restored epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment in murine skin

To further assess the importance of IL-1α on tacrolimus-induced barrier disruption, we compared the barrier recovery rates between intracutaneous IL-1α and vehicle injection in tacrolimus-treated mice and controls.

Barrier recovery kinetics accelerated significantly in IL-1α and tacrolimus-treated mice. This indicated that IL-1α corrected the abnormalities induced by tacrolimus. However, the barrier recovery rate was not restored significantly in normal IL-1α level mice injected with IL-1α alone (Figure 5a). This result revealed an additional clue that IL-1α has an important role in restoring barrier function impaired by tacrolimus treatment.

### In transgenic IL-1 receptor knockout (KO) mice, topical imiquimod did not restore epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment

To assess whether IL-1α signaling has a key role in permeability barrier abnormality induced by tacrolimus, permeability barrier recovery rates were compared between IL-1 type 1 receptor KO mice and wild-type mice. IL-1 receptor type 1 KO mice exhibited no significant difference in barrier recovery between imiquimod and control cream-treated sites. In contrast, wild-type mice demonstrated that imiquimod restored permeability barrier recovery delayed by treatment with topical tacrolimus (Figure 5b). These findings emphasize the role of IL-1α stimulation in restoring barrier function impaired by tacrolimus treatment.

### Topical imiquimod restored the expression of mBD3 and CRAMP, two major epiderml AMPs that were decreased by tacrolimus treatment in murine skin

The epidermal expressions of mBD3 and CRAMP changed according to permeability barrier status because of their colocalization in the LB (Oren *et al.*, 2003; Braff *et al.*, 2005; Elias and Choi, 2005). As TCIs suppressed the epidermal expression of mBD3 and CRAMP (Kim *et al.*, 2010), we assessed whether the expression of mBD3 and CRAMP was recovered on tacrolimus-treated murine epidermis with the co-application of imiquimod. In immunohistochemical staining, imiquimod co-applied skin showed more intense mBD3 and CRAMP expression compared with the control skin (Figure 6a and b). The mRNA levels of mBD3 and CRAMP of the tacrolimus-treated murine epidermis with the co-application of imiquimod also increased compared with the control skin (Figure 6c).

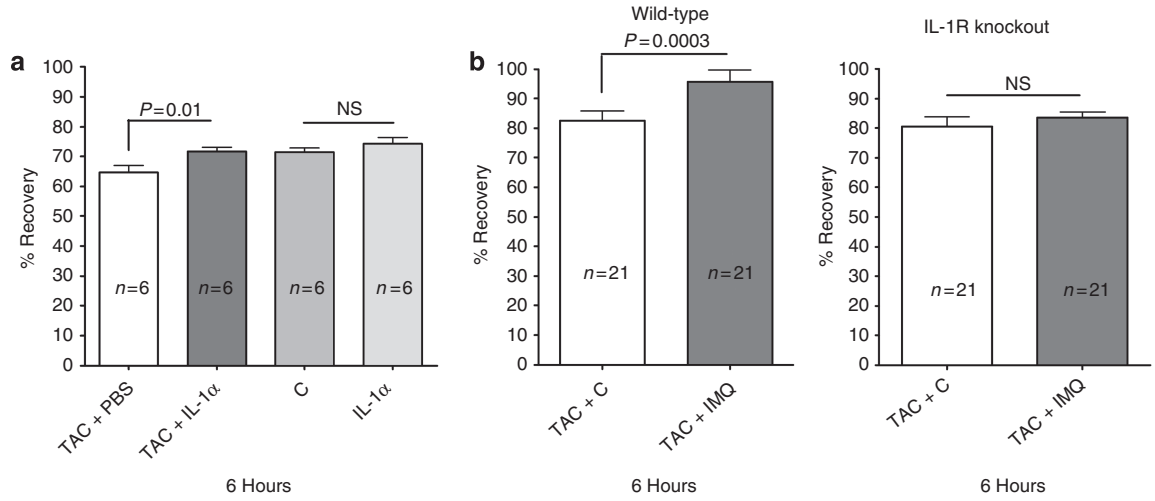


**Figure 5. IL-1α injection and IL-1 receptor knockout (KO) mouse model support evidence that IL-1α signaling mediated the permeability barrier homeostasis inhibited by tacrolimus.** IL-1α and the vehicle (phosphate-buffered saline, PBS) were injected intracutaneously into the flanks of tacrolimus-treated mice at 5 minutes before tape stripping. In addition, the vehicle was injected intracutaneously into petrolatum-treated mice as a normal control. IL-1α-injected mice exhibited an improvement in barrier recovery compared with the vehicle ($n=6$) (**a**). Both flanks of IL-1R type 1 KO and wild-type mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for 4 days. The barrier recovery rates were measured 6 hours after tape stripping. Imiquimod restored permeability barrier recovery in wild-type mice ($n=21$). IL-1R type 1 KO mice exhibited no significant difference in permeability barrier homeostasis between imiquimod and the control ($n=21$) (**b**). The numbers represent mean ± SEM. C, control; IMQ, imiquimod; NS, not significant; TAC, tacrolimus.

## DISCUSSION

TCIs such as tacrolimus and pimecrolimus are topical immune suppressants that have fewer side effects than topical glucocorticoids, which frequently cause hypothalamic-pituitary-adrenal axis suppression, skin atrophy, telangiectasiae, and secondary skin infection (Ellison *et al.*, 2000). TCIs are widely used to treat not only atopic dermatitis, but also other dermatoses including vitiligo and psoriasis. TCIs decrease the incidence of bacterial skin infections such as *Staphylococcous aureus* compared with vehicle (Reitamo *et al.*, 2000; Pournaras *et al.*, 2001; Nghiem *et al.*, 2002; Ashcroft *et al.*, 2005), but do not prevent viral skin infections including potentially life-threatening cases of eczema herpeticum (Lubbe *et al.*, 2000; Paller *et al.*, 2001). We have recently shown that topical tacrolimus treatment negatively impacts epidermal permeability barrier function and AMP expression in normal skin (Kim *et al.*, 2010). Tacrolimus-treated epidermis exhibits delayed barrier recovery in both human and murine skin. Tacrolimus decreases epidermal lipid production, as evidenced by fewer LBs and the reduced activity of lipid synthesis-related enzymes. Tacrolimus also suppresses the expressions of mBD3, CRAMP, and IL-1α, suggesting a mechanism for its negative impact on skin barrier function (Kim *et al.*, 2010).

Homey *et al.* (1998) demonstrated that topical tacrolimus treatment suppresses cytokine and co-stimulatory molecule expression in epidermal cells. By analyzing its immunosuppressive action mechanisms *in vivo,* they demonstrated that topical tacrolimus suppresses mRNA expression of both primary (IL-1 and tumor necrosis factor-α) and secondary (GM-CSF and MIP-2) epidermal cytokines during the early and late stages of primary contact hypersensitivity responses in mice (Homey *et al.*, 1998). Injuries to the epidermis stimulate the secretion of IL-1, IL-6, tumor necrosis factor, and other cytokines, which have a crucial role in signaling the repair response after barrier disruption (Wood *et al.*, 1992). Cytokine treatment after barrier disruption accelerates barrier repair, perhaps by enhancing epidermal lipid synthesis and the production of LB. Animal models knocked out for cytokines or their receptors displayed delayed barrier repair compared with the wild-type models (Jensen *et al.*, 1999; Man *et al.*, 1999). Imiquimod, a nucleoside analog of the imidazoquinoline family, has major biological effects through agonistic activity on toll-like receptors 7 and 8, and consecutively, activation of NF-κB, which enhances the induction of proinflammatory cytokines, such as IL-1α, IL-6, and tumor necrosis factor-α, with other mediators activating antigen-presenting cells along with other components of innate immunity. It also stimulates a profound T helper-weighted cellular response (Schon and Schon, 2007).

Based on these results, we first assessed whether the activation of IL-1α by an application of topical imiquimod could restore barrier recovery downregulated by tacrolimus. We demonstrated that barrier recovery was restored with an application of 2.5% imiquimod cream to the tacrolimus-treated human and murine skins compared with a control cream (Cetaphil, Galderma, Biot, France). Topical imiquimod was unable to potentiate barrier recovery in the normal control. This result demonstrates that the compensation of IL-1α levels decreased by topical tacrolimus restores barrier homeostasis downregulated by tacrolimus treatment. However, topical imiquimod had no effect on normal skin having a normal level of IL-1α. The other cytokine levels including IL-6 and tumor necrosis factor-α induced by imiquimod treatment were not checked within this study. We found IL-1α suppression in tacrolimus-treated skin in our previous study

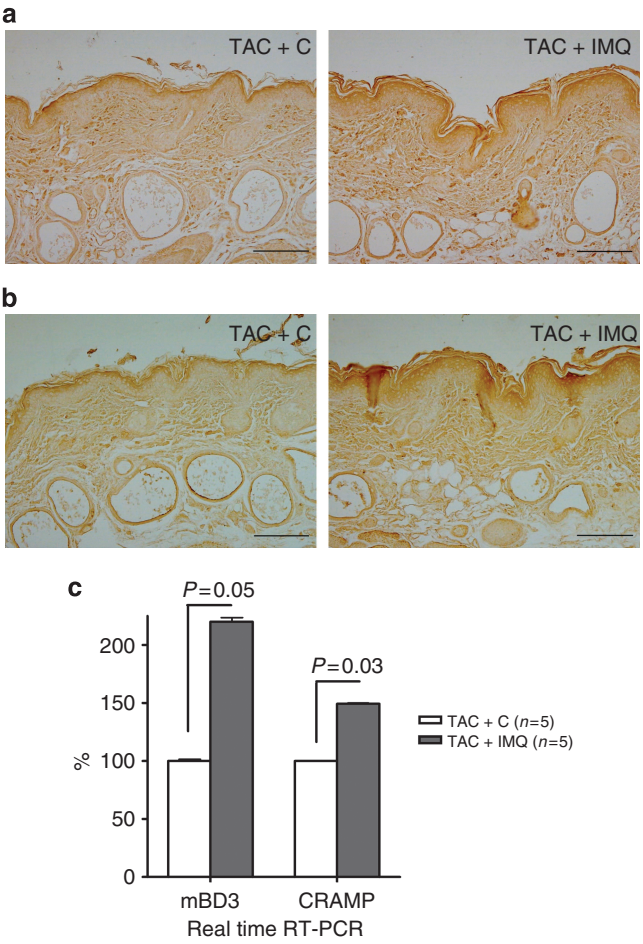


**Figure 6. Imiquimod restored the expression of mBD3 and CRAMP that was decreased by tacrolimus in murine epidermis.** Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for 4 days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod. Biopsy specimens taken from imiquimod or the control groups were processed with immunohistochemical staining for mBD3 and CRAMP, and assayed for real-time reverse transcription PCR to assess mRNA levels in the epidermis. Imiquimod-treated skin showed more intense staining for mBD3 and CRAMP expression compared with the control (**a**: mBD3, **b**: CRAMP). mRNA levels for mBD3 and CRAMP in imiquimod-treated murine epidermis increased compared with the control ($n=5$) (**c**). The numbers represent mean ± SEM. C, control; IMQ, imiquimod; TAC, tacrolimus. Bar = 100 μm.

and focused on IL-1α stimulation for the recovery of impaired barrier homeostasis in tacrolimus-treated skin (Kim *et al.*, 2010). The role of other cytokines except IL-1 in tacrolimus-treated skin would be revealed in the future.

In our preliminary study, we observed no differences in barrier recovery between normal mice skin and cetaphil-applied mice skin. Cetaphil cream improved stratum corneum (SC) hydration, but did not affect transepidermal water loss (Draelos, 2008). We used 2.5% imiquimod cream instead of 5% imiquimod cream because adverse reactions including erythema and mild weeping were seen in 5% imiquimod-treated mice. The role of IL-1α signaling in tacrolimus-induced permeability barrier dysfunction was assessed by the intracutaneous injection of IL-1α. We observed that intracutaneously injected IL-1α significantly improved barrier recovery in tacrolimus-treated murine skin when compared with controls. Barland *et al.* (2004) previously reported similar results that topical imiquimod accelerated barrier recovery after acute insults to aged BALB/c mice skin. These results were correlated with increased IL-1α production in the epidermis following topical imiquimod administration. Intracutaneous injection of IL-1α also accelerated barrier recovery in aged mice. The improvement in barrier recovery in young mice was not as pronounced as it was in aged mice (Barland *et al.*, 2004). The definite importance of IL-1α signaling for barrier homeostasis diminished by TCIs was supported by the observation that transgenic mice with KO of the IL-1 receptor type 1 demonstrated no significant difference in barrier recovery rate between imiquimod and control cream-treated skin, whereas the permeability barrier recovery rate was improved in imiquimod-treated skin compared with the control in wild-type mice. Therefore, we concluded that imiquimod improved barrier homeostasis affected by tacrolimus, which was derived from increased IL-1α levels in the epidermis.

IL-1 is a proinflammatory and immunomodulatory cytokine that has a key role in inflammatory diseases of the skin. In KCs, IL-1α is stored intracellularly, but can be quickly released in the case of epidermal infection or injury. Released IL-1 serves as a paracrine signal to fibroblast and endothelial cells and guides the chemotaxis of lymphocytes toward the site of injury (Dinarello and Wolff, 1993; Yano *et al.*, 2008). IL-1 also serves as an autocrine signal to surrounding, undamaged KCs, stimulating them to become activated. Activated KCs are migratory, hyperproliferative, and produce growth factors and cytokines that function in inflammatory and wound healing processes (Freedberg *et al.*, 2001). Barrier recovery of the epidermis is linked with an increase of lipid synthesis and the increased production of potentially regulatory cytokines including IL-1α. IL-1α administration results in increased lipid synthesis in cultured human KCs (Barland *et al.*, 2004). Aged mice with KOs of the IL-1α receptor type I develop more profound barrier deficits than age-matched wild-type mice (Ye *et al.*, 2002). In the present study, we demonstrated that topical imiquimod applied to tacrolimus-treated skin increased the expression of IL-1α and induced epidermal lipid production via lipid synthesis-related enzymes, which in turn enhanced LB production. These findings may indicate that IL-1α has a key role in barrier abnormalities caused by topical tacrolimus treatment, and that the stimulation of IL-1α in tacrolimus-treated epidermis induces positive effects on the skin barrier.

IL-1α is also related to antimicrobial functions (Liu *et al.*, 2002; Wehkamp *et al.*, 2006). There are several antimicrobial genes induced by IL-1α. β-Defensins, the most important defensins for host protection against microbes, display a broad spectrum of antimicrobial activity and are most effectively induced by IL-1α in KCs (Liu *et al.*, 2002). Yano *et al.* (2008) observed the induction of β-defensin expression and lipocalin-2 protein with a bacteriostatic function in IL-1α-treated KCs. All of these genes are induced by IL-1α in

KCs, implying a correlation of the antimicrobial effects and IL-1α (Yano *et al.*, 2008). We found that imiquimod upregulated the mRNA levels of mBD3 and CRAMP in the epidermis, suggesting that imiquimod-induced IL-1α has an antimicrobial role in tacrolimus-treated skin through the induction of AMP.

In conclusion, we have shown that the stimulation of IL-1α improves skin barrier function that was compromised by tacrolimus treatment. Our results show that topical imiquimod and intracutaneous IL-1α administration improve epidermal permeability and antimicrobial barrier previously compromised by tacrolimus treatment. This provides a definitive evidence for the role of IL-1α in their decline by tacrolimus treatment, and suggests the selective IL-1α inducers as therapeutic agents. The positive effects that we observed can be expected if patients with chronic inflammatory dermatoses, including atopic dermatitis, who require long-term use of TCIs receive IL-1α augmentation to protect the skin barrier.

## MATERIALS AND METHODS

### Human study

***Imiquimod application and functional study.*** For the human study, eight volunteers (20–50 years old, five males and three females) without skin disease were recruited. This study was conducted according to the Declaration of Helsinki Principles. The medical ethical committee of Institutional Review Board of Yonsei University Wonju College of Medicine approved all described studies. All participants granted written informed consent. Subjects applied 0.03% tacrolimus cream (Protopic, Fujisawa Healthcare, Deerfield, IL) on both sides of the volar surface of the forearms twice daily for 5 days. After 24 hours of the final application, 2.5% imiquimod cream, which was made by mixing 5% imiquimod cream (Aldara, 3M Health Care, St Paul, MN) with Cetaphil cream (Galderma), was applied on one forearm, and plain Cetaphil cream was applied to the other forearm as a control cream immediately after tape stripping (TS). Basal transepidermal water loss and barrier recovery rate values were measured 6 hours after acute barrier disruption by TS using Tewameter TM 210 (Courage and Khazaka, Cologne, Germany; Grubauer *et al.*, 1989; Feingold *et al.*, 1990; Pinnagoda *et al.*, 1990; Holleran *et al.*, 1991; Rogiers *et al.*, 2001). The baseline value of normal human skin is $7.2 \pm 0.48\,g\,m^{-2}$ per hour. The measurement conditions at room temperature ranged between 20 and 23 °C with a relative humidity between 55 and 58%.

### Animal study

Female hairless mice (Skh1/Hr) were housed in the animal laboratory of Yonsei University Wonju College of Medicine. Transgenic animals knocked out for the IL-1α functional (Type 1) receptor and wild-type age-matched controls were purchased from Jackson Laboratory (Bar Harbor, ME). Yonsei University Wonju Campus Institutional Animal Care and Use Committee approved this animal experiment.

***Imiquimod application.*** In the animal study, we subdivided the mice into two groups. One group represented mice treated with the combination of tacrolimus and imiquimod on one flank, and tacrolimus alone on the other flank. The other group of mice was treated with only imiquimod on one flank and a control on the other flank. In the former group, both flanks of the hairless mice were treated with 0.03% tacrolimus (Protopic) twice daily and then 2.5% imiquimod once daily on one flank with a control (Cetaphil) on the other flank for 4 days. In the latter group, only 2.5% imiquimod was applied once daily on one flank and a control (Cetaphil) on the other flank for four days. After 24 hours of the last application, basal transepidermal water loss and SC integrity, which was determined by transepidermal water loss after stripping with D-Squame tape (CuDerm, Dallas, TX), were measured. The barrier recovery rate was determined 6 hours after TS. Skin specimens were taken from all the hairless mice and processed by EM, immunohistochemical staining of IL-1α, mBD3 and CRAMP, real-time reverse transcription PCR for mRNAs of mBD3, CRAMP, and epidermal lipid synthesis-related enzymes.

***IL-1α intracutaneous administration.*** Flanks of hairless mice were treated with topical 0.03% tacrolimus or petrolatum twice daily for 4 days. After 24 hours of the final application, IL-1α (Sigma-Aldrich, St Louis, MO; 50 ng in 100 μl of phosphate-buffered saline; $n = 6$) or phosphate-buffered saline (100 μl; $n = 6$) was injected intracutaneously into the flank of the mice. The barrier was perturbed 5 minutes after IL-1α or phosphate-buffered saline injection and the barrier recovery rate was measured after 6 hours The dose of IL-1α (50 ng) was chosen because it is comparable to previous studies in aged mice (Barland *et al.*, 2004).

***EM and quantitative analysis.*** Samples for EM were processed using 2% aqueous osmium tetroxide postfixation, as described previously. In order to exclude subjective bias in these morphological studies, we quantitated both CD length and LB number in EM pictures using a previously described objective method (Choi *et al.*, 2005). Five EM pictures taken at the same magnification (20,000 ×) were analyzed and compared between the 2.5% imiquimod cream and control cream-treated groups.

***Assay for epidermal lipid synthesis-related rate-limiting enzymes.*** To evaluate the effect of imiquimod on epidermal lipid synthesis in tacrolimus-treated skin, full-thickness murine skin samples were obtained from mice. For the quantitative analysis of 3-hydroxy-3-methylglutaryl-CoA reductase, serine-palmitoyl transferase, and fatty acid synthase activity, respective mRNAs were measured using real-time reverse transcription PCR.

### Real-time reverse transcription PCR

***Isolation of the epidermis.*** Skin samples that were excised from the treated area were immediately placed with the epidermis side down on Petri dishes. Subcutaneous fat was removed with a scalpel, and then the skin samples were placed epidermis side up in 10 ml of 10 mM EDTA in phosphate-buffered saline, at an incubation of 37 °C for 35 minutes in order to separate the epidermis from the dermis. The epidermis was finally scraped off with a scalpel and total RNA was extracted (Wood *et al.*, 1994).

***Total RNA preparation and complementary DNA synthesis.*** Total RNA was extracted using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Gibco BRL, Grand Island, NY). RNA concentration was determined by a UV spectrometer at 260 nm. Aliquots (1.0 μg) of RNA from each sample were reverse

transcribed using Moloney murine leukemia virus reverse transcriptase (MML-V RTase, Promega, San Luis Obispo, CA). Briefly, RNA samples were incubated at 80 °C for 5 minutes with molecular biology grade water. After incubation on ice, primer extension and reverse transcription were performed by adding 1 × RT-buffer, 2 mM deoxynucleotide triphosphates (dNTPs), 0.2 pM random hexamer primer (Promega), and MML-V RTase (2.5 U $\mu l^{-1}$) in 20 µl reaction volumes. Samples were then incubated at 42 °C for 45 minutes before storage at −20 °C.

***Quantitative PCR analysis of gene expression.*** The expression of specific mRNAs was quantified using a Rotor-Gene 3,000 (Corbett Life Science, Sydney, Australia). Briefly, 10 µl PCR reactions were setup containing Quantitect probe PCR Master mix (Qiagen, Hilden, Germany) in a 2 × solution, 8 mM manganese chloride, 200 µM deoxynucleotide triphosphates (dNTPs), 1.25 U HotstartTaq polymerase, and 0.5 pM $\mu l^{-1}$ each of probes and primers. About 60 ng of complementary DNA were used per reaction. All reactions used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, provided as an optimized control probe labeled with TAMRA (Operon Biotechnologies, Cologne, Germany), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. All fluorogenic probes for gene of interest were labeled with 6-carboxyfluorescein (6-FAM). Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and used to determine ΔCt values (Ct of target geneCt of housekeeping gene) as raw data for gene expression. Fold change in gene expression was determined by subtracting ΔCt values for imiquimod-treated samples from their respective control cream-treated samples. The resulting ΔCt values were then used to calculate fold change in gene expression as $2^{\Delta\Delta Ct}$. All reactions were performed in triplicate and the results are expressed as the mean of values from three separate experiments. Samples were amplified using primers and probes under the following conditions: 95 °C for 15 minutes followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

***Primers and probes for PCR.*** Primer and probe sequences for real-time reverse transcription PCR analyses are given in Supplementary Table S1 online.

***Immunohistochemical staining.*** Skin specimens were fixed in 10% formalin solution and embedded in paraffin. Sections of 5 µm thickness were cut and stained with primary antibodies for IL-1α (SantaCruz, Santa Cruz, CA), mBD3 (SantaCruz), and CRAMP (SantaCruz). Briefly, after de-paraffinization, the sections were rehydrated sequentially with 100, 90, and 70% ethanol and incubated for 5 minutes in 3% $H_2O_2$ in Tris-buffered saline to inactivate endogenous peroxidases. Samples were then blocked for 10 minutes with blocking serum solution (DAKO, Carpinteria, CA) and incubated overnight at 4 °C with a primary antibody. After several washes in Tris-buffered saline, samples were incubated for 30 minutes with a secondary biotinylated antibody. The antigen was visualized with the avidin–biotin complex system (Vector, Burlingame, CA), according to the manufacturer's instructions, by using 3,3′-diaminobenzidine tetrahydrochloride as the substrate. Samples were examined under a light microscope.

### Statistical analyses

All data are expressed as mean ± SEM. Statistical analyses were performed using paired and unpaired Student's *t*-tests. $P < 0.05$ was considered statistically significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## SUPPLEMENTARY MATERIAL

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

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