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Research Article

Dermatology

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Severe dermatitis with loss of epidermal Langerhans cells in human and mouse zinc deficiency

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Zinc deficiency can be an inherited disorder, in which case it is known as acrodermatitis enteropathica (AE), or an acquired disorder caused by low dietary intake of zinc. Even though zinc deficiency diminishes cellular and humoral immunity, patients develop immunostimulating skin inflammation. Here, we have demonstrated that despite diminished allergic contact dermatitis in mice fed a zinc-deficient (ZD) diet, irritant contact dermatitis (ICD) in these mice was more severe and prolonged than that in controls. Further, histological examination of ICD lesions in ZD mice revealed subcorneal vacuolization and epidermal pallor, histological features of AE. Consistent with the fact that ATP release from chemically injured keratinocytes serves as a causative mediator of ICD, we found that the severe ICD response in ZD mice was attenuated by local injection of soluble nucleoside triphosphate diphosphohydrolase. In addition, skin tissue from ZD mice with ICD showed increased levels of ATP, as did cultured wild-type keratinocytes treated with chemical irritants and the zinc-chelating reagent TPEN. Interestingly, numbers of epidermal Langerhans cells (LCs), which play a protective role against ATP-mediated inflammatory signals, were decreased in ZD mice as well as samples from ZD patients. These findings suggest that upon exposure to irritants, aberrant ATP release from keratinocytes and impaired LC-dependent hydrolysis of nucleotides may be important in the pathogenesis of AE.

Introduction

Zinc (Zn) is a trace element essential for cell growth, development, and differentiation and is involved in maintaining the structure and function of over 300 different enzymes (1, 2). More than 2,000 transcription factors regulating gene expression require Zn for their structural integrity and binding to DNA (3). Recent studies revealed that Zn acts as an intracellular second messenger for transducing extracellular stimuli into intracellular signaling events in monocytes, DCs, and mast cells (4–7).

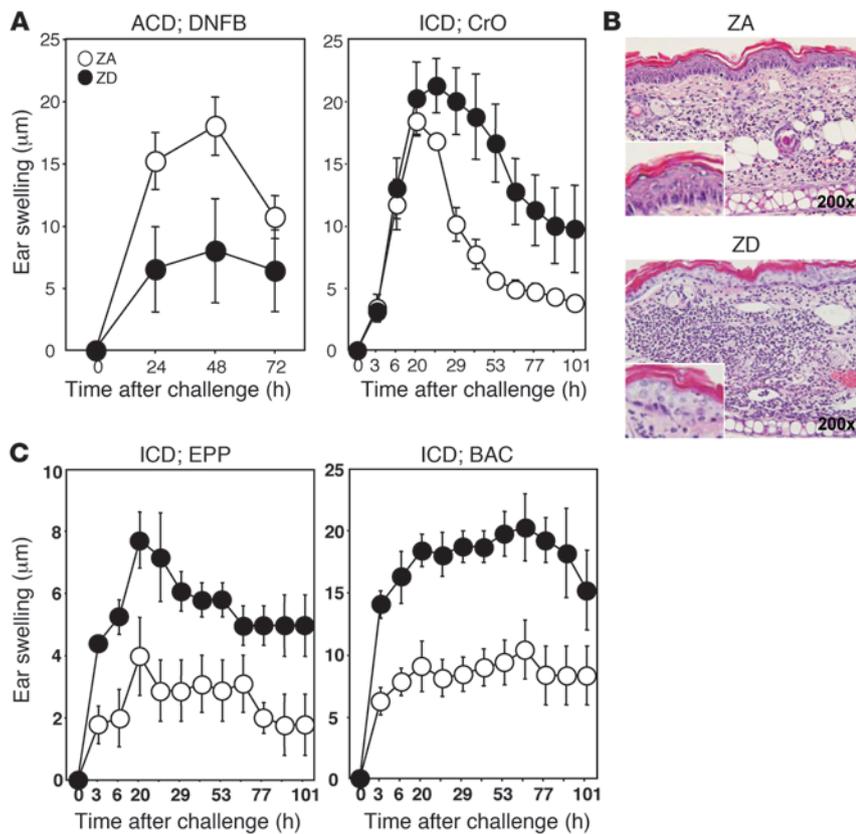
Zn deficiencies can be divided into 2 groups – a congenital form, called acrodermatitis enteropathica (AE; OMIM 201100), and the acquired forms (8). Recently, mutations in SLC39A4 have been identified as being responsible for congenital AE (9–11). SLC39A4 encodes ZIP4 Zn transporter, which is involved in Zn uptake via transporting Zn into the cytoplasm in intestine (9, 10). Congenital AE occurs worldwide, with an estimated incidence of 1 per 500,000 children, while it has been estimated that more than 2×10^9 people have a nutritional deficiency for Zn in developing countries (3, 8). It is even estimated that a considerable proportion of the Western population is at risk of marginal Zn deficiency (12, 13). Conditional Zn deficiencies also occur in many diseases and abnormal conditions, including malabsorption syndrome, chronic liver and renal diseases, sickle cell disease, excessive intake of alcohol, malignancies, and other chronic debilitating conditions (1, 3, 8).

The clinical manifestations of inherited and acquired Zn deficiency include growth retardation, diarrhea, alopecia, and characteristic skin lesions on acral, periorificial, and anogenital areas. Since Zn is indispensable for an adequate immunological response to all pathogens (14), the most serious complication observed in Zn deficiency is repeated infections due to impaired immune function. Indeed, several studies using animal models of Zn deficiency have confirmed that decreased levels of Zn induce thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated immune responses (14, 15). Zn deficiency affects many aspects of immune function, including a shift of the Th cell response to a Th2 predominance, reduced antibody formation, reduced killing activity by NK cells and lower levels of phagocytosis and intracellular killing in granulocytes, monocytes, and macrophages (14–18). Zn also influences the production of chemokines and proinflammatory cytokines like TNF- α , IL-1 β , and IL-6 (19–22).

The effects of Zn deficiency are particularly obvious in the skin and are seen as erythematous rashes, scaly plaques, and ulcers on acral and periorificial areas. Paradoxically, despite the impaired immune function in Zn deficiency, patients with hereditary and acquired AE present with immunostimulating skin inflammation, known as “acrodermatitis.” It remains unclear which cellular processes induce this characteristic skin inflammation and account for the cutaneous pathological features of Zn deficiency (8). Here we investigated the mechanisms by which Zn deficiency induces dermatitis in AE using dietary Zn-deficient (ZD) mice.

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Figure 1**

ZD mice demonstrate severe ICD with histological degeneration of keratinocytes. (A and C) ACD responses to DNFB and ICD responses to CrO, EPP, or BAC were induced in ZA (white circles) and ZD (black circles) mice, as described in Methods. Ear thickness was evaluated at the indicated time points. The data shown are ($n = 5$) the swelling responses (mean \pm SD). (B) H&E staining of ear samples collected from ZA or ZD mice 24 hours after topical application of CrO. Original magnification: $\times 200$; $\times 400$ (insets). Data are representative of 3 independent experiments.

Results

Dietary Zn deficiency causes severe and prolonged irritant contact dermatitis with the histological features of AE. Symptoms of Zn deficiency in animals are similar across different species. Zn deficiency causes a well-characterized nutritional-immunological syndrome in mice (14–18, 23, 24), whereby young adults quickly manifest symptoms within 4 to 5 weeks of feeding a ZD diet containing 0.5 mg Zn/kg or more (23, 24). It is of note that, although ZD mice at approximately 70% to 80% of the control group weight exhibited visible cutaneous symptoms, such as alopecia and parakeratosis, they did not present with the characteristic inflammatory dermatitis of patients with AE (refs. 23, 24, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58618DS1). Because AE in humans is typically seen on areas subject to repeated contact, we investigated allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) in dietary ZD mice. Five-week-old BALB/c mice were fed a ZD or Zn-adequate (ZA) diet, and at 10 weeks of age, ACD in response to dinitrofluorobenzene (DNFB) and ICD in response to croton oil (CrO) were quantified. Consistent with previous findings (24), ZD mice showed markedly decreased ear swelling responses to DNFB compared with those of ZA mice (Figure 1A). This was probably due to immunodeficiency in ZD mice, as previously reported (14, 15). Surprisingly, in contrast, the ear swelling response to CrO in ZD mice was significantly increased and prolonged compared with that of ZA mice (Figure 1A and Supplemental Figure 2). ICD caused by other skin irritant chemicals, benzalkonium chloride (BAC) and ethyl phenylpropionate (EPP), was also tested, with similar results (Figure 1C and Supplemental Figure 2). Histological examination of ICD lesions in ZD mice revealed parakeratosis and cytoplasmic pallor, subcorneal vacuolization, and ballooning

degeneration of keratinocytes and leukocyte infiltration (Figure 1B). These signs are histological features of cutaneous AE lesions in humans. No such degeneration of keratinocytes was observed in ICD lesions in ZA mice or ACD lesions in either ZA or ZD mice (Figure 1B and data not shown). These findings suggest that ICD, but not ACD, responses in ZD mice mimic the characteristic cutaneous manifestations observed in AE and can thus be considered an appropriate model for human disease.

Zn deficiency increases ATP release from keratinocytes in response to irritants. Accumulating evidence suggests that different environmental stimuli (e.g., chemical irritants) trigger adenosine 5'-triphosphate (ATP) release from keratinocytes via nonlytic mechanisms and also, more frequently, as a consequence of cell damage or acute cell death (25–27). Once released, ATP activates a family of plasma membrane receptors known as purinergic (P2) receptors. Because ATP released from chemically injured keratinocytes has been shown to cause ICD (28), we next compared the amount of ATP that was released from ZD or ZA mouse skin tissue after CrO application in ex vivo organ culture. Skin tissue obtained from ZD mice 4, 6, or 20 hours after CrO application released significantly greater amounts of ATP than tissues from ZA mice (Figure 2A). We next examined the effect of the Zn-chelating reagent TPEN on exogenous ATP release from Pam-212 keratinocytes cultured in vitro. Consistent with previous findings (26, 28), Pam-212 keratinocytes rapidly released ATP after exposure to CrO (Figure 2B). The addition of TPEN to the culture significantly augmented release of ATP in response to CrO, whereas TPEN alone failed to increase ATP release (Figure 2, B and D). The TPEN-mediated increase of ATP secretion was prevented by the addition of ZnSO₄ in a dose-dependent manner (Figure 2C). Furthermore, TPEN also significantly increased the secretion of ATP in response to other skin

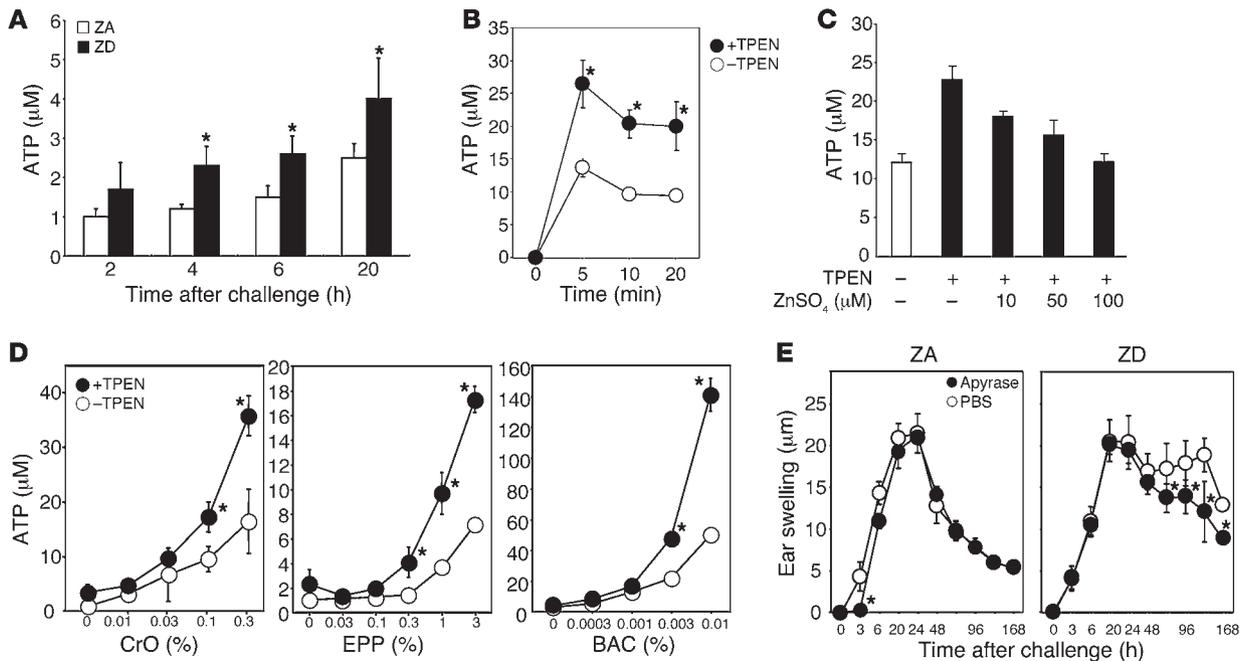


Figure 2 Zn deficiency increases ATP release by keratinocytes in response to irritant chemicals. (A) 1% CrO was painted on the ears of ZA (white bars) and ZD (black bars) mice ($n = 5$), and skin samples were taken at the indicated time points. ATP secretion from skin organ cultures was quantified. (B–D) Pam-212 keratinocytes were treated with (B and C) 0.3% CrO or (D) titrated concentrations of CrO, EPP, or BAC in the presence (black circles/bars) or absence (white circles/bars) of 2 μM TPEN. ATP in the culture supernatants was quantified at (B) the indicated time points or (C and D) 10 minutes after stimulation. (C) Cells were cultured with 10–100 μM ZnSO_4 . * $P < 0.05$, compared with cells that were not treated with TPEN. (E) ICD responses to CrO were induced, as described in Figure 1. ZA and ZD mice ($n = 5$) received local injections of potato apyrase on the right ear (black circles) or PBS alone on the left ear (white circles) before and after CrO application to both ears. The data shown are the swelling responses (mean \pm SD). * $P < 0.05$, between the apyrase and PBS treatments. Data are representative of 3 independent experiments.

irritant chemicals (herein, EPP and BAC) (Figure 2D). Together, these findings clearly indicate that Zn deficiency and irritant chemicals synergistically increase ATP release from keratinocytes.

Although various types of cells release ATP, the mechanism underlying the release of ATP is controversial (29). Several studies have shown that the release of ATP is reduced by carbenoxolone (CBX), suggesting the involvement of connexin or pannexin membrane channels in the release. In addition, inhibition of ATP release by vesicular ATPase inhibitors or intracellular Ca^{2+} deprivation was also reported, suggesting that the mechanisms of ATP release could include exocytosis. As shown in Supplemental Figure 3, CBX significantly decreased ATP release from CrO-treated keratinocytes, and, more importantly, it dramatically inhibited TPEN-mediated increase of ATP secretion. By contrast, BAPTA-AM, a membrane-permeable, strong Ca^{2+} -chelator, did not affect the CrO-evoked ATP release from keratinocytes. These results clearly indicate that membrane channel-mediated diffusible mechanisms, but not Ca^{2+} -dependent exocytosis, play a key role in the release of ATP from keratinocytes treated with CrO.

Previous studies have shown that hydrolysis of nucleotides by s.c. injections of soluble ecto-nucleoside triphosphate diphosphohydrolase (NTPDase; soluble potato apyrase) diminishes early inflammation in ICD (28). As shown in Figure 2E, we obtained similar results in ZA mice. In contrast, injections of apyrase before and after CrO application in ZD mice significantly decreased the late ICD response (Figure 2E). This suggests that aberrant ATP release from epidermal keratinocytes in response to

irritant chemicals contributes, at least in part, to the prolonged ICD response in ZD mice.

Aberrant chemokine gene expression in ZD keratinocytes. In general, there are very few differences in the histological, immunohistochemical, and electron microscopy findings in ACD and ICD (30). However, ICD is thought to be mediated via T cell-independent innate immunity, because the ear swelling response to CrO is similar for both athymic and normal mice and neutrophils are the predominant cells in ICD (31). Recent work has shown that different patterns of inflammatory chemokines may distinguish between ACD and ICD (32). To explore such mechanisms for increased ICD in Zn deficiency, we first analyzed gene expression profiles for 44 chemokines in epidermal sheets obtained from ZA and ZD mice 24 hours after vehicle or CrO exposure. Profound differences in chemokine gene expression were observed (Supplemental Figure 4). However, *Cxcl1* was markedly induced by CrO in both ZA and ZD mice, suggesting a possible association between this chemokine and neutrophil recruitment. Because CXCL1 and CXCL2 are known to mediate neutrophil influx into tissues (33), we next quantified their mRNA expression in epidermal sheets obtained from ZA and ZD mice after vehicle or CrO application in vivo. Quantitative real-time PCR demonstrated that Zn deficiency resulted in a significant increase of *Cxcl1* mRNA 4 and 24 hours after CrO exposure and *Cxcl2* mRNA at 4 hours (Figure 3, A and B). The same enhancing effects of Zn deficiency for the chemokine mRNA expression were observed when mice were treated with BAC (Supplemental Figure 5A). In addition, in vitro exposure of Pam-

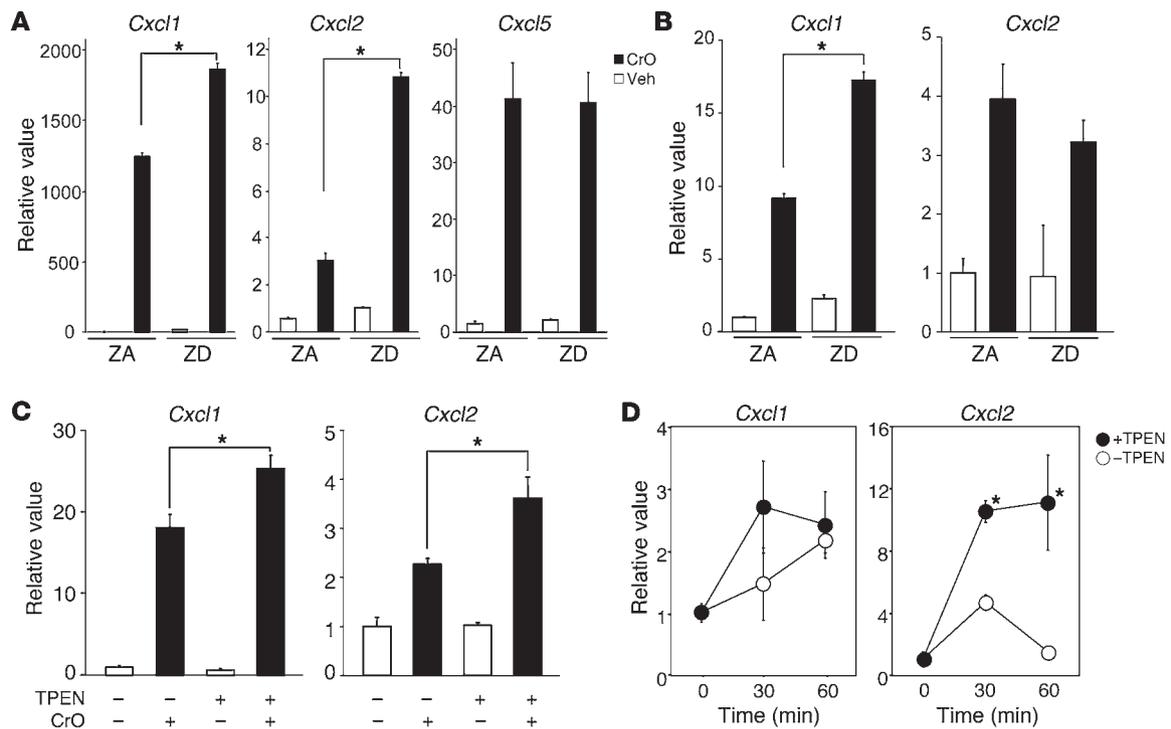


Figure 3

Zn deficiency increases *Cxcl1* and *Cxcl2* gene expression in keratinocytes after treatment with CrO. (A and B) 1% CrO (black bars) or vehicle alone (white bars) was painted on the ears of ZA and ZD mice ($n = 5$). Epithelial sheets were obtained from the ears at (A) 4 hours or (B) 24 hours, and total RNA was extracted. (C) Pam-212 keratinocytes were cultured in the presence or absence of 2 μ M TPEN. Total RNA was extracted 4 hours after 0.3% CrO (black bars) or vehicle (white bars) exposure. (D) Pam-212 keratinocytes were treated with 100 μ M ATP γ S in the presence (black circles) or absence (white circles) of 2 μ M TPEN. Total RNA was extracted 4 hours after ATP γ S exposure. Quantitative real-time RT-PCR analysis of *Cxcl1* and *Cxcl2* was performed. *Cxcl5* was examined as control in A. mRNA expression was normalized to *Gapdh*. The fold induction (mean \pm SD) was calculated from the normalized mRNA expression by CrO- or ATP-stimulated keratinocytes relative to nonstimulated ZA keratinocytes. * $P < 0.05$, compared with cells that were from ZA mice or not treated with TPEN. Data are representative of 3 independent experiments.

212 keratinocytes to CrO rapidly induced *Cxcl1* and *Cxcl2* mRNA (Figure 3C). When TPEN was added to the cultures, significant further augmentation of CrO-induced chemokine mRNA accumulation was observed, which was not seen with TPEN alone (Figure 3C). Similar results were obtained in BAC-treated keratinocytes (Supplemental Figure 5B). Furthermore, consistent with recent findings (34), exogenous ATP γ S induced *Cxcl1* and *Cxcl2* mRNA expression by ZA Pam-212 keratinocytes (Figure 3D). Together, our results suggest that Zn deficiency indirectly augments *Cxcl1* and *Cxcl2* gene expression in CrO-stimulated keratinocytes via increased ATP release, as observed in Figure 2. Interestingly, Zn deficiency further augmented ATP γ S-induced *Cxcl2*, but not *Cxcl1*, mRNA expression (Figure 3D).

Loss of epidermal Langerhans cells in Zn deficiency. Langerhans cells (LCs) are a long-lived subset of tissue DCs that reside in the epidermis. LCs acquire skin antigens and then migrate to skin-draining LNs in both inflammatory and steady-state conditions (35). Recently, it has been shown that CD39, which is expressed exclusively by LCs in the epidermal compartment, is responsible for ecto-NTPDase activity in LCs (28). Because the P2-receptor signaling pathway is negatively regulated by NTPDase-dependent hydrolysis of ATP, LC-associated CD39 plays a protective role against ATP-mediated inflammatory signals by hydrolyzing extracellular nucle-

otides released by keratinocytes in ICD responses (28). Therefore, we next examined the number, distribution, and morphology of epidermal LCs in Zn deficiency. Although epidermal cells prepared from the ear skin of ZA mice contained a normal contingent of LCs (I-A^{d+} CD11c⁺ cells), in mice fed the ZD diet, the number of epidermal LCs decreased with time on the diet, and few LCs were observed after 6 weeks (Figure 4, A and B). Immunofluorescence microscopy confirmed the loss of LCs from epidermal sheets prepared from mice fed a ZD diet for 6 weeks (Figure 4C). In contrast, the frequency and morphology of dendritic epidermal T cells (DETCs; Thy-1⁺ cells), which are skin-resident $\gamma\delta$ T cells expressing a monoclonal T cell receptor containing V γ 3 and V δ 1 determinants, in ZD mice was normal even after 6 weeks (Figure 4, C and D). It is of note that the proportions of I-A^{d+} CD11c⁺ cells in axillary and inguinal lymphoid nodes, including migratory DCs (e.g., LCs) and resident lymphoid DCs, from ZD mice were comparable in number to those of ZA mice (Figure 4A). The possibility that Zn deficiency downregulates the expression of cell surface markers on epidermal LCs was considered unlikely, because treatment of epidermal sheets with TPEN (2 μ M) for 4 days failed to decrease surface expression of I-A^d and CD11c on murine epidermal LCs (data not shown).

Loss of epidermal LCs in patients with AE. We next examined the number, distribution, and morphology of epidermal LCs in human skin

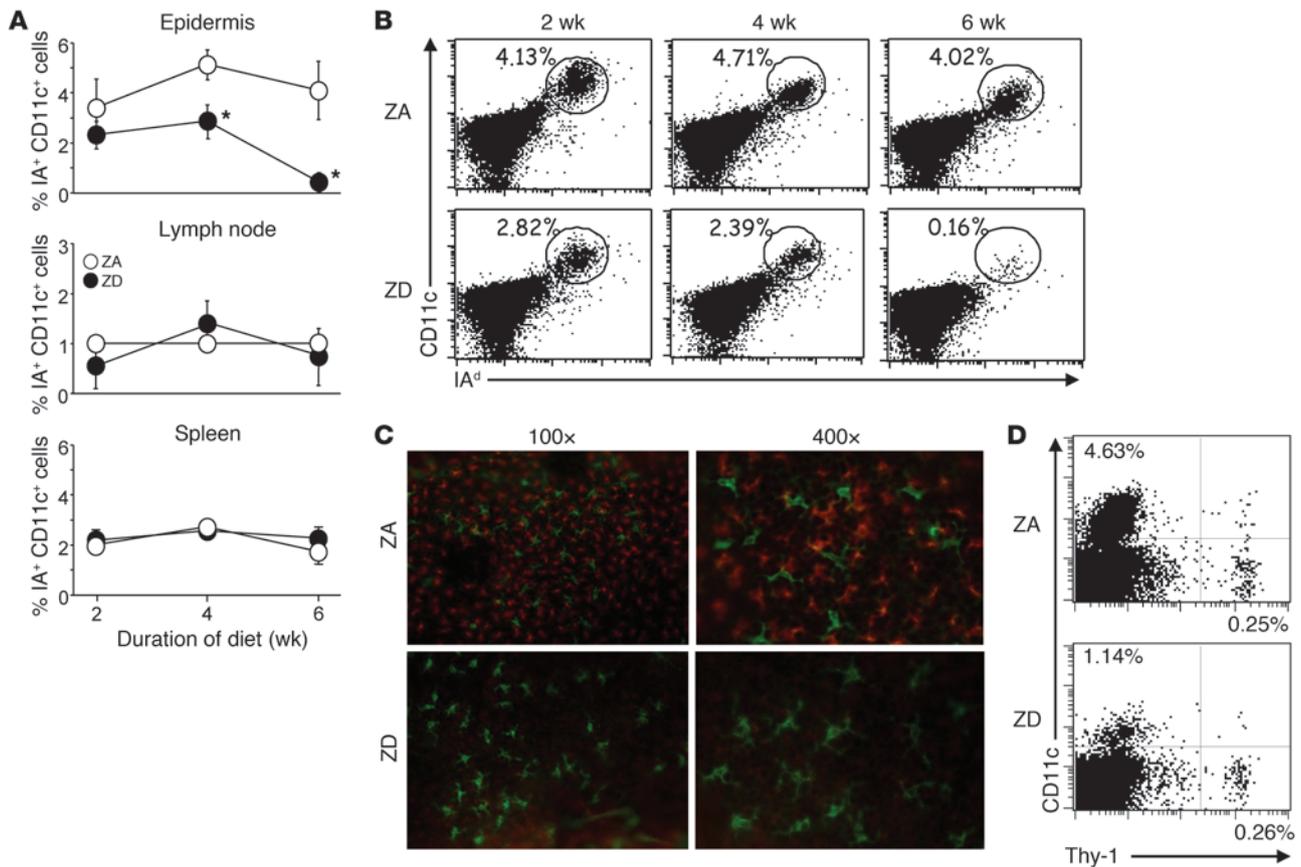


Figure 4 Loss of epidermal LCs in Zn deficiency. (A) Cell suspensions of epidermis, axillary, and inguinal lymph nodes and spleens were prepared from mice fed ZA (white circles) or ZD (black circles) diets for the indicated time and stained for I-A and CD11c antigens. The percentages of I-A and CD11c double-positive cells were assessed within each live-gated cell population. Results are the mean \pm SD ($n = 3$). * $P < 0.05$, compared with mice fed the ZA diet. A representative FACS analysis of live-gated epidermal cell suspensions from mice fed ZA or ZD diets for 6 weeks and stained for (B) I-A and CD11c or (D) Thy-1 and CD11c antigens. Numbers indicate the percentages of cells in the (B) circles or (D) gate. (C) Immunofluorescence of epidermal whole mounts stained for I-A (red) and Thy-1 (green) from mice fed ZA or ZD diets for 6 weeks. Original magnification, $\times 100$ (left panels); $\times 400$ (right panels). Data are representative of 3 independent experiments.

samples obtained from inflammatory lesions of patients, including those with atopic dermatitis, psoriasis vulgaris, and hereditary and acquired AE, by immunofluorescence staining analyses. Langerin⁺ LCs were found to be regularly scattered throughout the epidermis of control specimens but were absent from the epidermis of patients with AE (Figure 5A). HLA-DR antigens were also rarely expressed in the epidermis of AE specimens but were present on the surface of dermal mononuclear cells in the same specimens (Figure 5B). Interestingly, skin biopsy specimens obtained from the same lesions in a patient with hereditary AE after 6 months of oral Zn supplementation revealed recolonization of the epidermis with LCs, accompanied by marked clinical improvement (Figure 5C). We also examined immunohistochemical staining with formalin-fixed skin samples obtained from patients with AE. As shown in Supplemental Figure 6A, langerin⁺ LCs were absent from the epidermis of the patients with AE. Quantitation of langerin⁺ cells in the epidermis ($n = 3$) revealed that the number of LCs in the lesional epidermis of AE specimens was significantly decreased as compared with that in normal skin (the mean positive cell per field \pm SD was 16.7 ± 2.8 in normal skin and 0.4 ± 0.3 in AE skin; $P < 0.05$). Similarly, the number of HLA-DR⁺ cells in the lesional

epidermis of AE specimens was also significantly decreased (the mean positive cell per field \pm SD was 10.4 ± 0.6 in normal skin and 3.7 ± 2.7 in AE skin; $P < 0.05$). Taken together with the findings in ZD mice (Figure 4), these findings indicate that Zn deficiency results in depletion of epidermal LCs.

Decreased steady-state production of TGF- β 1 in cutaneous, but not mucosal, epidermis in Zn deficiency. Although the mechanisms that regulate LC homeostasis in the steady state are yet to be fully elucidated, previous studies demonstrated that LC development requires TGF- β 1 (35). Mice lacking TGF- β 1 possess no LCs, owing to either a failure in LC differentiation, survival, or both (35, 36). Therefore, we next assessed the cutaneous expression of TGF- β 1 in Zn deficiency. Mucosal TGF- β 1 levels in the large and small intestine in ZD mice were comparable to those in ZA mice (Figure 6B), whereas the skin tissue obtained from ZD mice contained significantly lower amounts of TGF- β 1 than that from ZA mice (Figure 6A). To detect the TGF- β activity in the skin samples, MFB-F11 reporter cells were used in a bioassay (Figure 6C and ref. 37). Secreted alkaline phosphatase (SEAP) activity was not detected without activation of the samples by HCl, suggesting that murine skin contains TGF- β protein in a latent form. However, after activation, skin samples

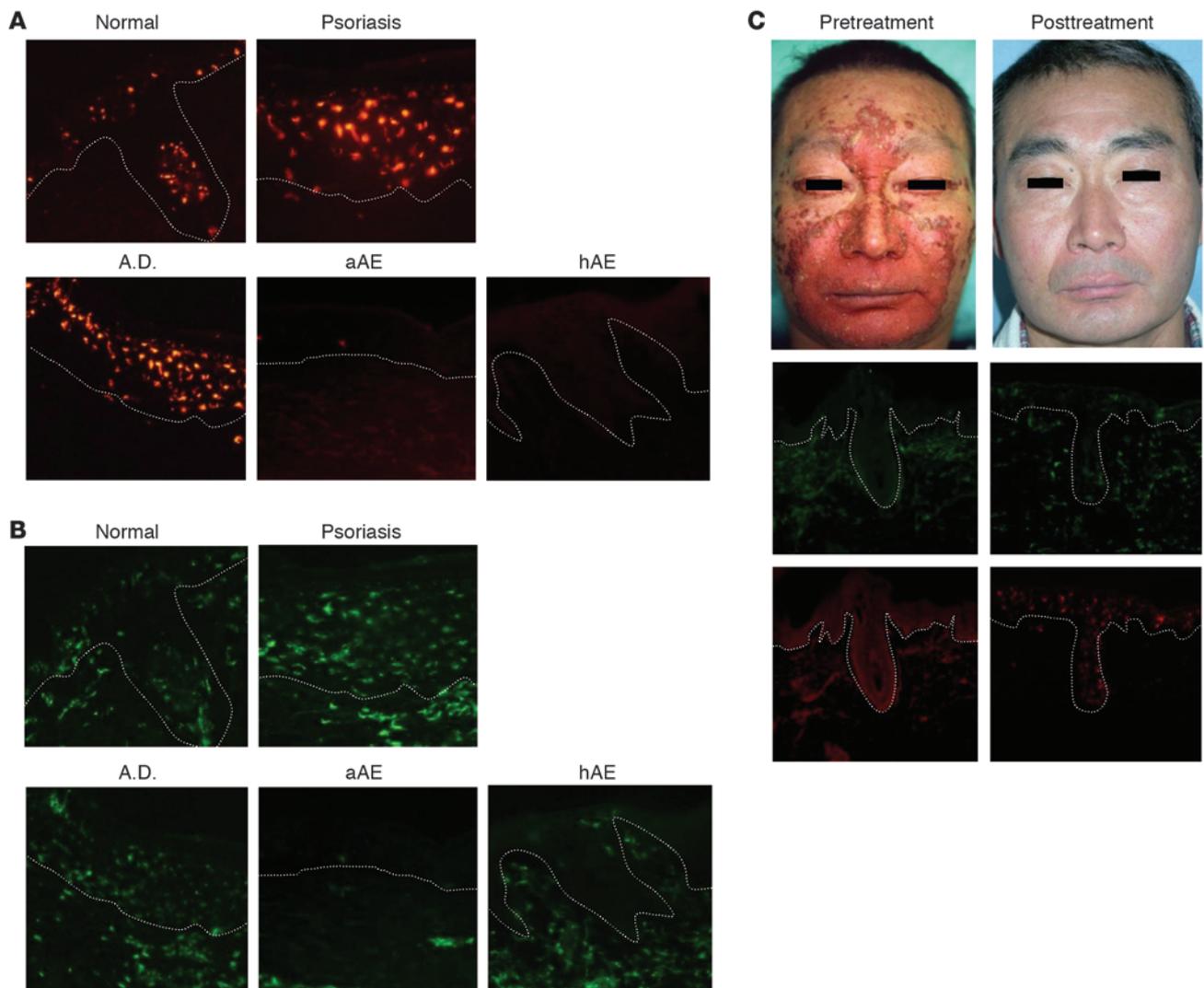


Figure 5 Loss of epidermal LCs in patients with AE. **(A and B)** Immunohistochemical staining for HLA-DR (green) and langerin (red) in the erythematous lesions of patients with psoriasis vulgaris (psoriasis), atopic dermatitis (A.D.), hereditary AE (hAE), and acquired AE (aAE) or normal skin. Original magnification, $\times 200$. **(C)** Clinical appearance and immunohistochemical staining of a patient with hereditary AE before and after treatment by oral Zn supplement. White dotted lines denote the epidermis/dermis interface. Original magnification, $\times 200$.

obtained from ZD mice were found to mediate significantly less SEAP activity than those from ZA mice (Figure 6C). In addition, we found that *Tgfb1* mRNA levels in epidermal sheets from ZD mice were significantly lower than those from ZA mice (Figure 6D). These results suggest that Zn deficiency decreases epidermal TGF- $\beta 1$ expression in the steady state, and this might, at least in part, be associated with the reduction of epidermal LCs in Zn deficiency.

Because recent studies have shown that TGF- $\beta 1$ has antiapoptosis effects in LCs or DCs (38, 39), we next assessed for apoptosis in epidermal LCs freshly isolated from ZA and ZD mice. As shown in Supplemental Figure 7A, the incidence of annexin V⁺ cells in LCs from mice fed ZD diets for 3 weeks was slightly, but significantly, higher than that from mice fed ZA diets. We next performed in vitro experiments to obtain additional evidence for the hypothesis that Zn deficiency directly induces apoptosis of LCs. After in vitro culture of epidermal cell suspensions obtained from ZA mice with

varying concentrations of TPEN for 48 hours, 5 μ M TPEN significantly increased the incidence of annexin V⁺ LCs (Supplemental Figure 7B). Similarly, we also found a significant increase of apoptosis in human monocyte-derived LCs cultured with TPEN at a concentration of 5 μ M (Supplemental Figure 7C). Notably, consistent with a previous finding in HaCaT keratinocytes (40), Pam-212 keratinocytes cultured with 5 μ M TPEN for 48 hours demonstrated no changes in cell viability and growth (data not shown). These results suggest that, in addition to the reduced TGF- $\beta 1$ in epidermis, severe Zn deficiency may directly induce apoptosis in LCs, resulting in the loss of LC networks in ZD mice.

Enhanced ICD in the absence of LCs. At present, there are 2 types of LC ablation murine models. The first LC ablation model uses transgenic langerin⁻ diphtheria toxin A (DTA) mice (41), which constitutively lack LCs. The second uses mice that express EGFP fused with a diphtheria toxin receptor (DTR) under the control

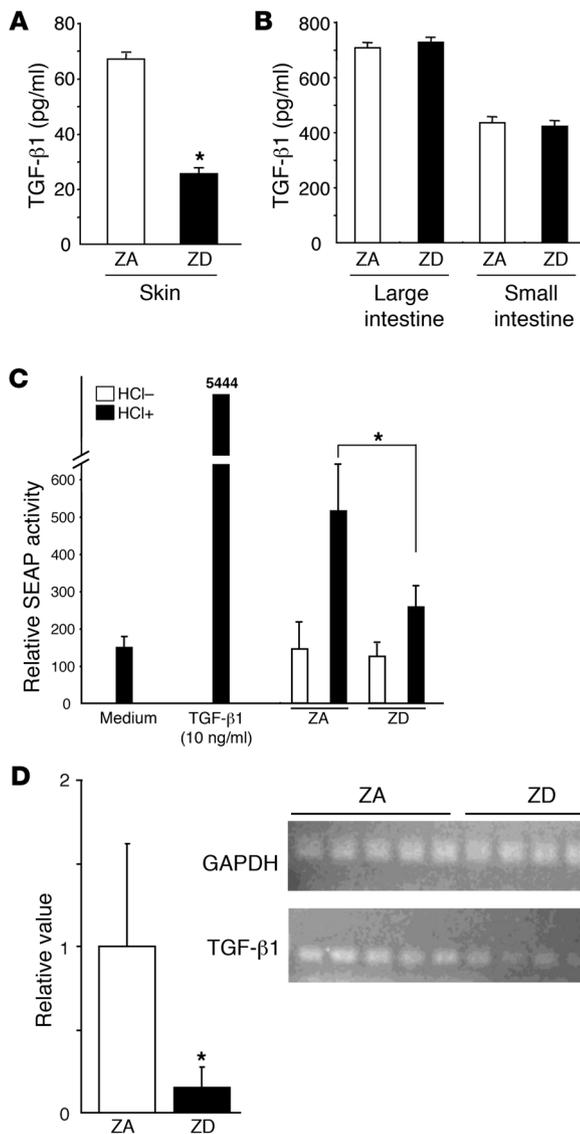


Figure 6

Decreased TGF-β1 production in the skin of ZD mice. (A and B) Samples of (A) skin tissue or (B) mucosal tissue were taken from the backs of mice fed a ZA (white bars) or ZD (black bars) diet for 5 weeks and cultured for 24 hours (n = 5). TGF-β1 secretion in the ex vivo skin or mucosa organ cultures was assessed by ELISA assays. (C) SEAP activity of the skin samples measured using MFB-F11 cells with or without HCl in the culture system. (D) Quantitative real-time RT-PCR for the detection of *Tgfβ1* and *Gapdh* mRNAs in epidermis from the ears of mice fed a ZA (white bar) or ZD (black bar) diet for 5 weeks. The fold induction was calculated from normalized mRNA expression by the epidermis of ZD mice relative to that of ZA mice. Results are the mean ± SD (n = 5). *P < 0.05, compared with mice fed the ZA diet. Data are representative of several experiments with similar results.

ogy of the inflammatory skin manifestation in AE remains unclear. Nearly pathognomonic in the histopathologic examination of AE is the presence of “necrosis,” a term describing cytoplasmic pallor, vacuolization, ballooning degeneration, and subsequent confluent necrosis of keratinocytes within the epidermis (8). As several in vitro studies have shown that Zn deficiency can induce cell death in HaCaT keratinocytes via apoptosis or necrosis (40, 45, 46), one of the mechanisms of epithelial damage observed in AE lesions might be by increased death of keratinocytes. However, the characteristic inflammatory dermatitis in clinical AE is seen only in the acral areas, suggesting the participation of additional cellular processes, which could account for the restricted localization of the dermatitis and the severe cutaneous inflammation in immunodeficient AE patients.

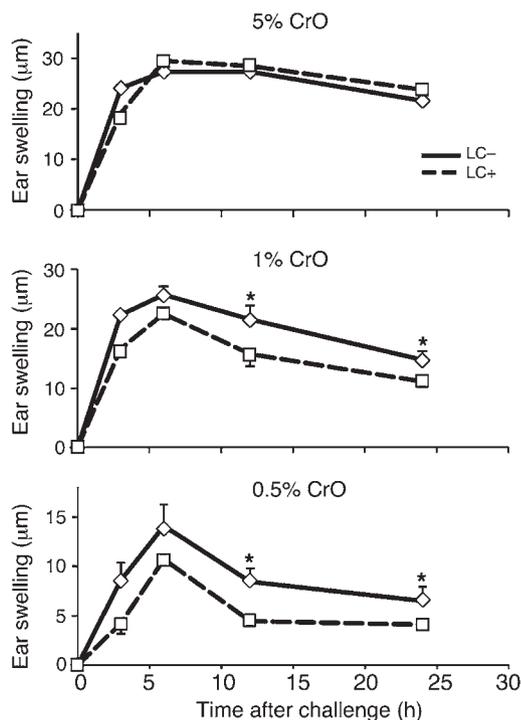
Our data, which we believe to be novel, reveal that irritants, but not haptens, can cause severe skin inflammation in immunodeficient ZD mice, providing a biologic basis for understanding the pathogenesis of AE. We found that ICD responses in ZD mice exhibited similar histological features similar to those of the characteristic dermatitis in clinical AE, highlighting the possibility that the acral, periorificial, and anogenital dermatitis in AE might be caused by contact with different irritants in daily life, such as chemicals, foods, urine, and feces, etc. We have addressed the mechanisms responsible for the severe and prolonged ICD in ZD mice and have identified several abnormalities which we believe to be novel, including aberrant chemokine gene expression and ATP release by keratinocytes in response to irritant chemicals. These 2 abnormalities are closely associated, because extracellular ATP induces keratinocyte *Cxcl1* and *Cxcl2* gene expression, major chemoattractants for neutrophils (Figure 3D and refs. 33, 34). Together with the insight that neutrophils are the pathogenic cells in ICD (31), our results suggest that ATP plays a key role in the pathogenesis of severe and prolonged ICD in Zn deficiency. This notion is supported by the finding that local injection of apyrase significantly diminished the ICD response in ZD mice (Figure 2E). Our in vitro assay revealed that membrane channel-mediated diffusible mechanisms play a pivotal role in the Zn deficiency-induced increased ATP release from irritant-stimulated keratinocytes (Supplemental Figure 3). However, because chemical irritants also trigger ATP release from keratinocytes as a consequence of cell damage or acute cell death (26, 28), Zn deficiency and irritants may synergistically induce keratinocyte cell damage, as shown in Figure 1B, leading to increased ATP release in vivo.

We found that ex vivo ATP release from CrO-treated skin was increased in Zn deficiency (Figure 2A). This observation is in line

of the langerin promoter, which are known as langerin-EGFP-DTR mice (42) and langerin-DTR mice (43). After i.p. injection of diphtheria toxin (DT) into langerin-EGFP-DTR mice, LCs do not repopulate for at least 4 weeks, while the number of langerin⁺ dermal DCs in the skin recover to the basal level about 7 days after depletion by DT (44). Langerin-EGFP-DTR mice 14 days after treatment with DT are used as a model to deplete LCs alone. Fourteen days after treatment with DT, langerin-DTR mice and C57BL/6 (B6) mice were treated with 5%, 1%, and 0.5% CrO on the ears and monitored for ear swelling. As expected, the ear swelling response to 1% and 0.5% CrO in langerin-DTR mice was significantly increased at 12 and 24 hours after application compared with that in corresponding B6 mice (Figure 7). These results suggest that depletion of LCs enhances ICD responses, consistent with our findings that ZD mice lacking epidermal LCs exhibited increased ICD responses to 1% CrO (Figures 1 and 4).

Discussion

Although the skin is commonly involved and is often one of the first organs affected in Zn deficiency, the underlying pathophysiol-

**Figure 7**

Enhanced ICD in the absence of LCs. Fourteen days after treatment with DT, langerin-DTR (LC-) and B6 (LC+) mice were applied with 5% (mean \pm standard error [SE]; $n = 4$ and 6, respectively), 1% (mean \pm SE; $n = 10$ and 16, respectively), and 0.5% (mean \pm SE; $n = 6$ and 10, respectively) CrO on the ears and monitored for ear swelling 3, 6, 12, and 24 hours later. Data are presented as the mean \pm SEM (μm) and are a representative of 3 independent experiments. * $P < 0.05$, between langerin-DTR and corresponding B6 mice.

with our *in vitro* findings that Zn deficiency augments the release of ATP from irritant-stimulated Pam-212 keratinocytes. In addition, the decreased number of epidermal LCs observed in ZD mice (Figure 4) might also contribute to the increased ATP release from skin organ cultures *ex vivo* (Figure 2A) as well as the severe and prolonged ICD responses *in vivo* (Figure 1). Mechanisms include loss of the potent ecto-NTPDase activity of LC-associated CD39, which plays a protective role against ATP-mediated inflammation in ICD responses (28). Taken together, our results suggest that the inflammatory skin manifestations in patients with AE may occur as a result of excessive ICD responses, presumably due to aberrant ATP release upon repeated exposure to various irritants in daily life.

According to our data, Zn deficiency decreases epidermal TGF- β 1 expression and its activity in the steady state. While the signaling via TGF- β receptor has been well defined, the homeostatic regulation of TGF- β is largely obscure. So far, the best-characterized regulator (inducer) of TGF- β expression is TGF- β itself (47), and an autocrine production of TGF- β 1 is generally required for the homeostasis of various cell types, including LCs (48). Therefore, signaling molecules downstream of TGF- β receptor, such as Smad proteins, are thought to be involved in the regulation of their TGF- β production. Since a number of Zn finger transcription factors (e.g., ZNF580) has been reported to be associated with TGF- β signaling (49), Zn deficiency might decrease the autocrine production of TGF- β 1 in LCs and keratinocytes by multiple pathways via those transcription factors.

The present results indicate that Zn deficiency induced the loss of epidermal LCs. Since several lines of evidence have indicated that TGF- β 1 governs LC homeostasis (36, 50), the reduced TGF- β 1 in epidermis may be, at least in part, responsible for the LC disappearance. The other possible mechanism for LC depletion is apoptosis, because several studies have suggested that Zn is an important physiological regulator of apoptosis (51). Indeed, the

incidence of apoptosis in epidermal LCs in ZD mice was increased in the steady state (Supplemental Figure 7A). We also found that murine and human LCs underwent apoptosis when cultured with TPEN at a concentration of 5 μM (Supplemental Figure 7, B and C), while the culture condition did not induce apoptosis in keratinocytes (data not shown), suggesting that LCs are more sensitive to Zn deficiency-induced apoptosis. Taken together with the findings that TGF- β 1 has antiapoptosis effects (38, 39), our results suggest that severe Zn deficiency may induce apoptosis in LCs through synergy between its direct effect and the reduced TGF- β 1 expression, resulting in the loss of LC networks. Additional studies are needed to determine the impact of TGF- β 1 on LC survival *in vivo* and the paramount mechanism underlying LC disappearance in ZD mice and patients with AE.

A previous study has reported that, in transgenic langerin-DTA mice, depletion of LCs does not affect ICD responses induced by 0.5%–2.5% sodium dodecyl sulfate or 5% BAC (41). In our study using langerin-EGFP-DTR mice, however, the ear swelling response to 1% and 0.5% CrO was significantly increased (Figure 7), consistent with the previous findings that LC-associated CD39 plays a protective role against ATP-mediated inflammation in ICD (28). Nevertheless, both langerin-DTR mice and control mice developed a similar degree of ear swelling after application of 5% CrO (Figure 7), as previously observed in transgenic langerin-DTA mice (41). Since CrO induces ATP release from keratinocytes in a dose-dependent manner (Figure 2), it is possible that an excess ATP release in ICD in response to a high dose of CrO *in vivo* might be beyond the capacity of NTPDase-dependent hydrolysis by LCs. Alternatively, differences in irritants or depletion timing might explain the phenotypic differences among the LC ablation models (41, 42). Further detailed analysis under different conditions is needed to reveal the functions and significance of LCs in ICD.

Methods

Animals and diet. Five-week-old female BALB/c mice were purchased from Oriental Yeast Co. Ltd. Mice were maintained under specific pathogen-free conditions throughout this study. ZD diet was purchased from CLEA Japan Inc. The mice were fed a powdered ZD diet or control diet from 5 to 11 weeks of age ($n = 5$ –10 per group). Both diets were of almost the same nutritional quality, differing only in terms of Zn content (Zn, 0.11–0.38 mg/100 g in ZD diet, 6.00 mg/100 g in control diet). Langerin-EGFP-DTR mice on a B6 background (provided by Bernard Malissen, Université de la Méditerranée, Aix-en-Provence, Gap and Marseille, France) express a high-affinity human DTR in the langerin locus, as described previously (44). After *i.p.* injection of 1,000 ng DT in PBS, LCs do not repopulate for at least 4 weeks, while the number of langerin⁺ dermal DCs in the skin recovers to the basal level about 7 days after depletion by DT (44).

Patients. Biopsies were obtained from lesional skin of patients, including those with atopic dermatitis, psoriasis vulgaris, hereditary AE (pretreatment and posttreatment with oral Zn sulfate), and acquired AE. Addition-



ally, control biopsies were obtained from healthy volunteers. The samples were snap frozen in liquid nitrogen and stored at -80°C .

Reagents and antibodies. DNFB, CrO, BAC, EPP, TPEN, CBX, and BAPTA-AM were purchased from Sigma-Aldrich. ZnSO_4 was purchased from Kanto Chemical Inc. FITC-conjugated anti-human HLA-DR, anti-mouse I-A^d, and CD90 (Thy-1) mAbs and PE-conjugated anti-mouse CD11c and I-A^d mAbs were purchased from BD Biosciences. Purified and PE-conjugated anti-human langerin mAbs were purchased from Abcam and R&D Systems, respectively.

ACD and ICD responses. For the induction of chemically induced allergic skin inflammation, mice were topically treated with 20 μl 0.5% DNFB dissolved in acetone/olive oil (4:1), which was painted onto the shaved abdomen at days 0 and 1. The ears were then challenged with 10 μl of 0.2% DNFB on the right ear and vehicle alone on the left ear on day 5. For chemically induced irritant skin inflammation, mice received topical application of 1% CrO, 10% BAC, or 30% EPP on the right ear and vehicle alone on the left ear. Swelling responses were quantified (right ear thickness minus left ear thickness) by a third experimenter using a micrometer, as described previously (28). In some experiments, potato apyrase grade VII (0.2 U/ear; Sigma-Aldrich) was injected s.c. into the ear 10 minutes before and 1 hour after CrO painting (28). In experiments with langerin-EGFP-DTR mice, 5%, 1% and 0.5% CrO was painted on the ears.

Quantification of ATP release from the skin and keratinocytes. ATP secretion from skin organ culture was quantified, as previously reported (52). Skin samples were taken from both ears immediately. s.c. fat was removed with a scalpel, and the skin explants were prepared by being cut into 8.0-mm circular pieces. The 2 pieces of skin from the 2 ears were floated with the epidermis side upward in 12-well plates containing 4 ml PBS and incubated on ice for 10 minutes. In some experiments, Pam-212 keratinocytes cultured in DMEM medium containing 10% FCS, 10 mM HEPES, 0.25 $\mu\text{g}/\text{ml}$ gentamicin, and 2 mM L-glutamine were pretreated with 2 μM TPEN for 6 hours and then cultured with PBS with CrO, BAC, or EPP. ATP concentrations in the supernatants were then quantified using the luciferin-luciferase assay.

Preparation of epidermal cell suspensions and epidermal sheets. The ears were removed, mechanically divided into dorsal and ventral cutaneous sheets, and then incubated with a 0.5% solution of trypsin (type XI, Sigma-Aldrich) in PBS for 30 minutes at 37°C to separate the epidermis from the underlying dermis. After removal of the loosened dermis, the epidermal sheets were gently agitated with 0.05% DNase (DN25; Sigma-Aldrich) in PBS for 10 minutes, and the resulting EC suspension was passed through a nylon mesh to remove hair and stratum corneum prior to use. The viability determined by trypan blue exclusion was always more than 90% (53). For immunohistological staining, epidermal sheets were prepared from ear skin by incubation in 0.5 M ammonium thiocyanate (37°C for 20 minutes), fixed in acetone (-20°C for 30 minutes), and rehydrated in PBS (36).

Preparation of monocyte-derived LCs. Monocyte-derived LCs were cultured from PBMCs, as described previously (54). Briefly, monocytes were isolated by depletion of magnetically labeled nonmonocytes (Monocyte Isolation Kit II; Miltenyi Biotec) from plastic-adherent PBMCs obtained from healthy blood donors. Monocytes were cultured with 1,000 U/ml recombinant human GM-CSF (R&D Systems), 1,000 U/ml recombinant human IL-4 (R&D Systems), and 10 ng/ml human platelet-derived TGF- β 1 (R&D Systems) for 7 days.

Flow cytometry. Single-cell suspensions (5×10^5) of epidermal sheets, inguinal and axillary lymph nodes, and spleens obtained from ZA and ZD mice (5 mice per group) were stained for LCs with FITC-conjugated anti-I-A^d and PE-conjugated anti-CD11c mAbs or for DETCs with FITC-conjugated anti-Thy-1 mAb for 30 minutes at 4°C . Live/dead discrimination was performed using propidium iodide (Sigma-Aldrich). After washing,

samples were analyzed on a FACSCalibur (BD Biosciences). Annexin V-FITC and propidium iodide staining was carried out according to the instructions of BD Pharmingen.

Histological examination. Tissue specimens from the ears from ZA or ZD mice were surgically removed immediately after determination of ear swelling, embedded in OCT compound (Tissue-tek), frozen in liquid nitrogen, and stored at -80°C until use. Cryostat sections were fixed in absolute acetone and stained with H&E. For immunohistochemical staining, murine epidermal sheets were stained for LCs and DETCs with PE-conjugated anti-I-A^d mAb or FITC-conjugated anti-Thy-1 mAb, washed, and analyzed by fluorescence microscopy. For human skin samples, cryostat sections were double stained with FITC-conjugated anti-HLA-DR and PE-conjugated anti-langerin mAbs, washed, and analyzed by fluorescence microscopy. For formalin-fixed skin samples, they were stained with anti-HLA-DR and anti-langerin mAbs, incubated with biotinylated goat anti-mouse immunoglobulins, and then incubated with streptavidin-peroxidase. Reactions were developed with aminoethylcarbazole. Sections were incubated with a DAKO LSAB Kit, HRP (DakoCytomation) and then counterstained with Mayer's hematoxylin. Positive (HLA-DR and langerin) cells were counted at high magnification ($\times 400$) in 4 different fields, and the average number of positive cells per field was calculated for each sample.

DNA microarray analysis. RNA was harvested using TRIzol (Invitrogen) from epidermal sheets obtained from ZA and ZD mice 24 hours after vehicle or CrO exposure. Preparation of cRNA and hybridization of probe arrays (U133.2.plus) was performed according to the manufacturer's instructions (Affymetrix). Data were analyzed according to the MIAME rule. The average μ and SD σ values of ZA and ZD mouse distributions are $(-0.049, 1.35)$ and $(-0.041, 1.34)$, respectively. The accession numbers for each chemokine gene are as follows (GenBank; <http://www.ncbi.nlm.nih.gov/genbank>): AF065933.1 (CCL2), AF128218.1 (CCL4), AF128196.1 (CCL9), U50712.1 (CCL12), NM_011332.1 (CCL17), AF099052.1 (CCL20), NM_009138.1 (CCL25), NM_020279.1 (CCL28), NM_008176.1 (CXCL1), NM_009140.1 (CXCL2), NM_009141.1 (CXCL5), NM_008599.1 (CXCL9), NM_021274.1 (CXCL10), and AF252873.1 (CXCL14).

Quantitative real-time PCR analysis. Murine epidermal sheets were homogenized in liquid nitrogen using a Mikro-Dismembrator U (Braun Biotech). Total RNA was extracted from Pam-212 keratinocytes or homogenized epidermis using ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript system (Invitrogen Life Technologies). Subsequently, relative mRNA expression was determined by real-time PCR using an ABI PRISM 5500 Sequence Detection System (Applied Biosystems) with SYBR Green I Dye (Qiagen). Primers corresponding to mouse chemokines, TGF- β , and G3PDH were designed by TAKARA BIO INC. Ct numbers were derived from the exponential phase of PCR amplification. Fold differences in the expression of gene x in the cell populations y and z were derived by the formula 2^k , where $k = (\text{Ct}_x - \text{Ct}_{\text{G3PDH}})_y - (\text{Ct}_x - \text{Ct}_{\text{G3PDH}})_z$.

Measurement of TGF- β production and its activity in mucosa and skin. To measure TGF- β production, skin and mucosa samples were taken from the trunk and the large or small intestine of ZA and ZD mice. The pieces of cutaneous ($1.0 \times 1.0 \text{ cm}^2$) or mucosal (100 mg) tissues were cultured with the epithelial side upward in 24-well plates containing 2 ml RPMI medium (Invitrogen), with 10% FCS, 10 mM HEPES, 0.25 $\mu\text{g}/\text{ml}$ gentamicin, and 2 mM L-glutamine for 24 hours. TGF- β production in culture supernatants was measured by ELISA (R&D Systems). In general, TGF- β is secreted in a latent complex in which TGF- β homodimers are noncovalently associated with homodimers of the propeptide called the latency-associated peptide (LAP), and the release of TGF- β from its LAP is required for binding of TGF- β to the cellular receptors (55). To determine TGF- β activity, we used a bioassay using MFB-F11 cells stably transfected with the reporter plasmid containing 12 CAGA boxes (Smad-binding element) fused to a SEAP



reporter gene (56). Briefly, MFB-F11 cells (4×10^4 cells/well) in 96-well flat-bottom tissue culture plates were incubated in 50 μ l serum-free DMEM for 2 hours, and then skin culture supernatants or TGF- β (10 pg/ml) were added in a 50 μ l volume in the presence or absence of 10 μ M HTS466284. After 24 hours of incubation, SEAP activity in the culture supernatants was measured using Gene Light 55 (Microtec).

Statistics. Significant differences between experimental groups were analyzed by Student's *t* test (1 tailed). *P* values less than 0.05 were considered significant.

Study approval. The murine studies were conducted with the approval of and in accordance with the Guidelines for Animal Experiments of the University of Yamanashi. The human study protocol was approved by the institutional review boards of the University Hospital (University of Yamanashi), and informed consent was obtained from all subjects.

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