EPF occurs in young adults of 20-30 years old and the male/female ratio is 4.8:1 in Japanese literature[6]. The most commonly affected lesion of classic EPF is the face (85%), however, the eruptions can be seen on the back, arms and the chest. Of great interest is that the eruption can be found on palms and soles in 22% of the patients[7]. One may infer that the term "folliculitis" seems to be inappropriate since 6% of the patients start only with palmoplantar pustular lesions, though 66% of them first appear on the face[8]. Although some papers proposed to use the term of sterile eosinophilic pustulosis[9] or eosinophilic pustular dermatosis[10], Prof. Ofuji insisted that the name of the disease should represent the characteristic features of the typical eruption, and thus the name of EPF should be kept irrespective of the palmoplantar lesions (personal communication). In his own review article, Prof. Ofuji mentioned that he personally had an impression that the classic EPF seemed to be more commonly observed in patients with a past history of acne[11].

Pathophysiology

At present, nobody can answer the questions why eosinophils are attracted in hair follicles, why peripheral number of eosinophils is increased, why the skin lesions flare up periodically, why pustules are observed in palmoplantar area or why EPF is frequently found in patients with HIV infection. However, the possible pathomechanism of EPF has gradually been elucidated in recent days.

As for the mechanism of eosinophilic accumulation to hair follicles, it has been reported that eosinophilic chemotactic factor was found in skin surface lipid products[12], which seems to be a reasonable explanation because most of the EPF lesions are distributed in seborrheic region of the patients with the past history of acne.

Since the clinical features of EPF resemble dermatophyte and/or *Malassezia* folliculitis, it is speculated that some unknown reactions may occur to these infectious microorganisms, though the pustules in EPF are definitely sterile. A favorable response to antifungal therapy in some cases may support this concept[13].

Elicitation of follicular inflammation by eosinophils can be attributable to eosinophilic cationic protein[14] and nitric oxide[15], which may provide a novel treatment tool for EPF by regulating these inflammatory factors.

Peripheral eosinophilia can partly be explained by micro environmental change of cytokines such as IL-5, because peripheral eosinophilia was observed in EPF patients with myelodysplastic syndrome[16] and drug-induced EPF[17]. The periodical flare up of EPF lesions may be partly due to the fluctuation of cytokine levels in view of interferon studies[18,19].

Most of the immunosuppression-associated EPF is seen in patients with AIDS, which shows both common and different features of classic EPF. It is reported that eotaxin-1 and Th2 cytokines play a crucial role in eosinophil recruitment and inflammation leading to the tissue injury of hair follicles[20]. Since immunosuppression-associated EPF can be found in patients with other hematologic malignancy such as lymphoma and leukemia, some common immunological impairment induced by whatever stimuli may be involved in the pathogenesis of EPF. In immunosuppression-associated EPF, superficial fungal infections are suspected to participate[21,22] because EPF displays similarities with dermatophyte folliculitis, however, no direct evidence supports this concept.

Infancy-associated EPF which is usually found in the scalp of children and shows good clinical response to corticosteroids[23-25], may be somewhat different from adult EPF but a variety of skin diseases such as scabies, insect bites and linear IgA-dermatosis though histopathology bears a close resemblance[26].

Clinical manifestations and laboratory findings

Classic EPF occurs predominantly in middle-aged men, which affects face, trunk and arms. Characteristic features of classic EPF are pruritic follicular papules and sterile pustules which enlarge gradually making a well demarcated area (Fig. 2a). There is a central healing tendency leaving slightly scaly pigmentation (Fig. 2b). On face, slightly elevated maculoerythematous indurations with scattered papules and pustules are occasionally seen, which subside

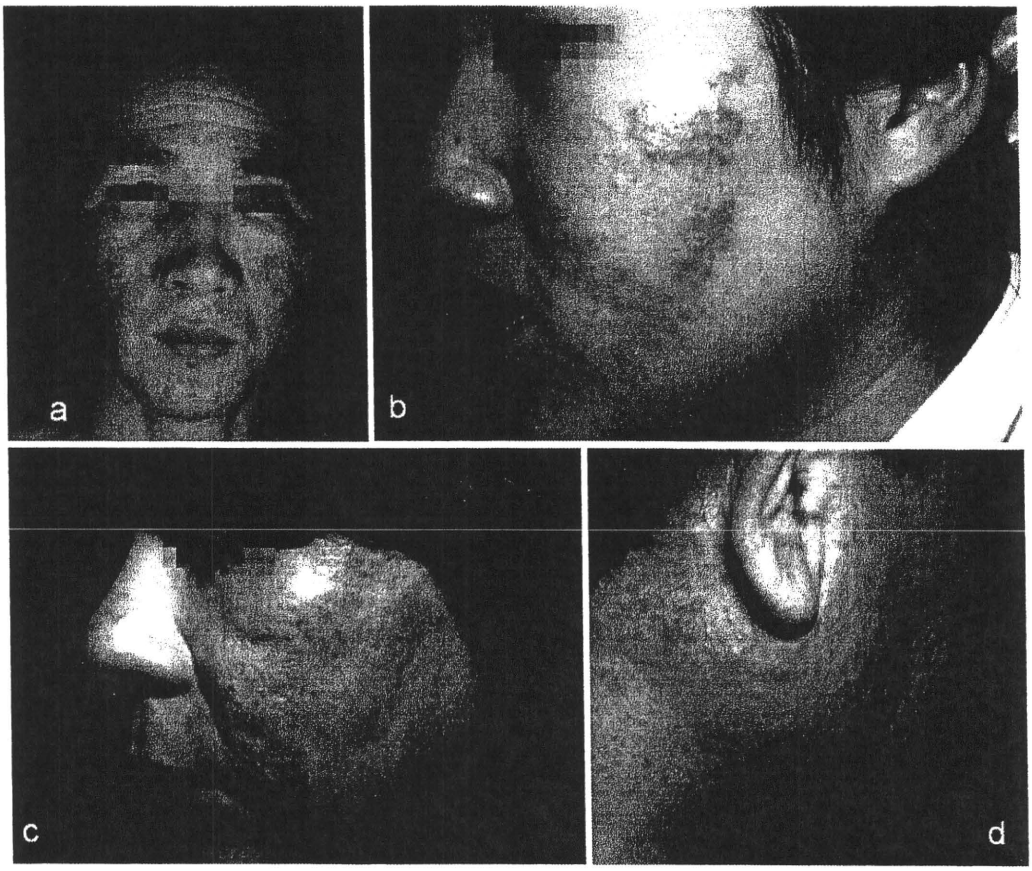

Fig. 2. Clinical features of eosinophilic pustular folliculitis. (a) Characteristic features of classic EPF are pruritic follicular papules and sterile pustules which enlarge gradually making a well demarcated area. (b) There is a central healing tendency leaving slightly scaly pigmentation. (c, d) On face, slightly elevated maculoerythematous indurations with scattered papules and pustules are occasionally seen, which subside spontaneously in due course but flare up periodically resulting in a chronic course.

spontaneously in due course but flare up periodically resulting in a chronic course (Fig. 2c, d). When palmoplantar regions are involved, symmetrical erythema and pustules resembling palmoplantar pustulosis are observed with hypertrophic scaling. Over half patients complain of itching. Usually there is no prodrome or systemic symptoms noticed. Differential diagnosis of classic EPF includes inflammatory acne, rosacea, tinea corporis, pustular psoriasis, subcorneal pustular dermatosis and seborrheic dermatitis.

Mild to moderate eosinophilia is occasionally observed with elevated IgE level. Since EPF is occasionally accompanied with HIV infections and other immunosuppressive conditions such as hematologic malignancies, these complications should be carefully checked.

Histopathology

Immunosuppression-associated EPF and infancy-associated EPF are indistinguishable histologically from classic EPF regardless of their different clinical features. The histopathology of typical EPF is characterized by a dense inflammatory infiltrate of mononuclear cells and eosinophils around hair follicles and sebaceous glands (Fig. 3a). In early phase papular eruption, spongiosis of the outer root sheath of the follicles presumably due to the destruction mediated by eosinophils is observed (Fig. 3b).

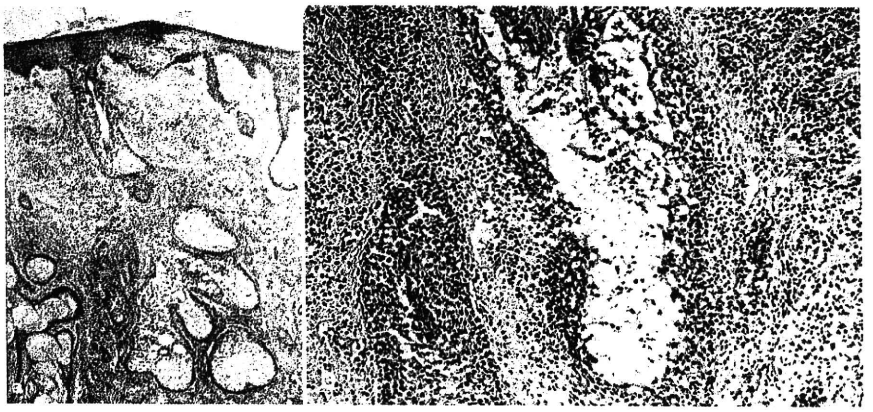

Fig. 3. (a) The histopathology of typical EPF is characterized by a dense inflammatory infiltrate of mononuclear cells and eosinophils around hair follicles and sebaceous glands. (b) In early phase papular eruption, spongiosis of the outer root sheath of the follicles presumably due to the destruction mediated by eosinophils is observed.

In advanced stage, eosinophilic infiltration extends to the whole hair follicles leading to the pustular formation. Mucinous degeneration of the sebaceous gland and outer root sheath may be seen. Moderate increases of tryptase-positive and chymase-negative mucous type mast cells are observed which might play some role in the pathogenesis[27]. Electron microscopic observation revealed that T cells may be involved in the pathomechanism of EPF[28]. As for the infancy-associated EPF, it is speculated that this type of EPF is not a distinctive inflammatory disease of the skin but a variety of different diseases, and the term "eosinophilic folliculitis" is better defined as a histopathologic pattern for infancy-associated EPF[29].

Treatment

Various treatments have been proposed for EPF, however, the endpoint of the treatment should be to control the disease with mild side effects, since EPF shows a chronic course with wax and wane. Topical corticosteroids might be the first choice of the drug, but the clinical effect is sometimes limited. Among other treatment options such as phototherapy[30], systemic steroids, dapsone[31], metronidazole[32], minocycline and retinoids, oral indomethacin seems to be the most promising choice of the treatment[33,34]. Topical indomethacin is also effective in some cases[35]. The mechanism of action by which indomethacin works on EPF still remains unclear, however, indomethacin either suppresses the production of cyclooxygenase-dependent eosinophilic chemotactic factor or alters the cytokine balance leading to the inhibition of prostanoids synthesis[18].

Recently, in addition to indomethacin, other treatments have been applied for EPF[36]. Oral cyclosporine[37] and topical tacrolimus[38] may be beneficial choices when patients have been resistant to previous conventional treatments. Tacrolimus may act against EPF presumably through the inhibition of several proinflammatory cytokines by T cells as well as the prevention of the cytokine release from mast cells[39].

Prognosis of EPF is relatively poor resulting in chronic course with the remission and relapsing for years in many patients.

Conclusion

EPF was first described by an Asian dermatologist as a noninfectious inflammatory skin disease characterized by eosinophilic infiltration mainly of hair follicles, which revealed later as a globally important skin disease because immunosuppression-associated EPF was recognized as an HIV-related skin disease. EPF may be induced by whatever

antigenic stimuli and some unknown immunological reaction patterns with eosinophil participation mainly in the hair follicles may be involved. Development of a new treatment may reveal not only the pathomechanism of EPF but also some hidden multifactorial immune responses in skin diseases mediated by cytokines and chemokines for eosinophils.

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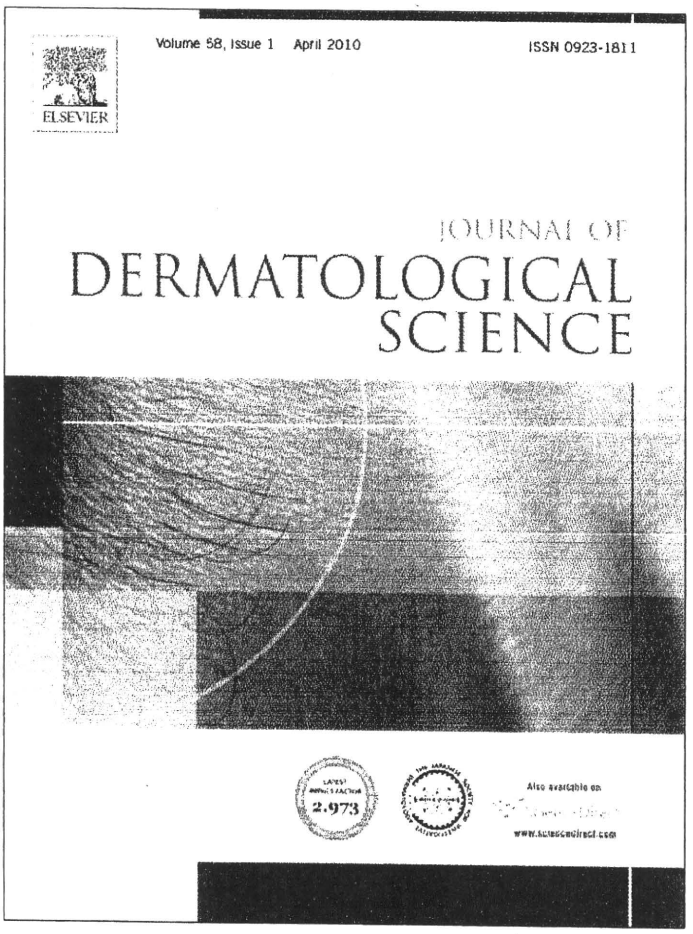

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Invited review article

Extrinsic and intrinsic types of atopic dermatitis

Yoshiki Tokura *

Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

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ABSTRACT

Atopic dermatitis (AD) can be categorized into the extrinsic and intrinsic types. Extrinsic or allergic AD shows high total serum IgE levels and the presence of specific IgE for environmental and food allergens, whereas intrinsic or non-allergic AD exhibits normal total IgE values and the absence of specific IgE. While extrinsic AD is the classical type with high prevalence, the incidence of intrinsic AD is approximately 20% with female predominance. The clinical features of intrinsic AD include relative late onset, milder severity, and Dennie-Morgan folds, but no ichthyosis vulgris or palmar hyperlinearity. The skin barrier is perturbed in the extrinsic, but not intrinsic type. Filaggrin gene mutations are not a feature of intrinsic AD. The intrinsic type is immunologically characterized by the lower expression of interleukin (IL)-4, IL-5, and IL-13, and the higher expression of interferon-γ. It is suggested that intrinsic AD patients are not sensitized with protein allergens, which induce Th2 responses, but with other antigens, and metals might be one of the candidates of such antigens.

Contents

* Tel.: +81 93 691 7445; fax: +81 93 691 0907.
E-mail addresses: tokura@med.uoeh-u.ac.jp, jsid@mbox.med.uoeh-u.ac.jp.

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1. History of extrinsic and intrinsic atopic dermatitis (AD)

AD is a clinically defined, chronic-intermittent, genetically predisposed, eczematous dermatitis that starts at infancy or early childhood. Although a large number of clinical, laboratory and experimental studies have been performed, the pathophysiology of AD remains to be elucidated, because AD has a variety of aspects in the causes and pathogenesis.

The clinical phenotype of AD has been classified into the extrinsic and intrinsic types [1]. Historically, this dichotomy was first used for asthma. The terminology of extrinsic or allergic asthma was first introduced by Rackeman in 1947 and referred to the triggering role of allergens in asthma. By symmetry, he described intrinsic or non-allergic asthma as a disease characterized by later onset in life, female predominance, higher degree of severity, and more frequent association with nasosinusal polyposis. As intrinsic asthmatic patients was not improved by conventional treatments, this author considered intrinsic asthma to be caused by a non-allergic, unknown phenomenon [2].

In AD, the extrinsic and intrinsic types began to be adopted in the late 1980s [3]. They are also called the allergic (or classical) and non-allergic types. Since there is still no sufficient consensus whether the intrinsic type is a distinct entity, some researchers denominate it atopiform dermatitis [4]. However, the classification into the extrinsic and intrinsic AD has been widely used especially since the millennium. Recently, various kinds of clinical studies have been performed under this dichotomy in many countries, including Germany [1,5,6], Netherland [4], Hungary [7], Italy [8,9] and other European countries, and Asian countries such as Korea [10,11], and Japan [12].

2. Definition

Extrinsic AD and intrinsic AD are defined according to IgE-mediated sensitization, namely the presence or absence of specific IgE for environmental allergens and food allergens [11,12,13]. According to the EAACI nomenclature task force, the term "atopic eczema/dermatitis syndrome (AEDS)" can be used to cover the different subtypes of AD. In this nomenclature, the intrinsic type is termed non-allergic AEDS, which shows normal IgE levels, no specific IgE, no association with respiratory diseases (bronchial asthma or allergic rhinitis), and negative skin-prick tests to common aeroallergens or food allergens [14]. Since total serum IgE values are significantly associated with the allergen-specific IgE status [15], total IgE can be regarded as a clinically useful parameter to differentiate between the extrinsic and intrinsic types in both adults [5,12] and children [15]. The reported mean values of total serum IgE in the intrinsic type are from 22.2 to 134 kU/l, or alternatively, IgE values less than 150 or 200 kU/l have been used for an indication of intrinsic AD [16]. Our study of Japanese patients also showed that the mean value of total serum IgE was 110.5 kU/l (11–219 kU/l) [12].

Among specific IgE antibodies, infantile AD patients are more allergic to food [11], while environmental antigens are common in adults. It should be careful that some allergens may not be useful to discriminate the two types. For example, IgE to *Malassezia sympodialis* was found in patients with the intrinsic type as well as the extrinsic type [17].

3. Prevalence of intrinsic AD

3.1. Incidence

Since extrinsic AD is the prototype of AD, its prevalence is well known. On the other hand, the frequency of intrinsic AD has been a matter of investigation. Schmid et al. [16] summarized the twelve reports that has been published from 1990 to 2000 and documented the clinical features of extrinsic and intrinsic AD. According to their review paper, the frequency of intrinsic AD was 10–45%. More recently, the incidence of extrinsic AD and intrinsic AD were reported as follows: 73% *vs* 27% [18] and 63% *vs* 37% [15] in German children, 88% *vs* 12% in Hungarian adults [7], 78.2% *vs* 21.8% in Dutch patients from 13 to 37 years of age [4], and approximately 80% *vs* 20% in Korean [19]. These data are in accordance with the empirical knowledge that about 20% of AD patients show normal IgE levels and lack of sensitization towards environmental allergens. Intrinsic AD is seen in various countries, but the prevalence may depend on local areas, as it was reported that intrinsic AD was higher in incidence in East Germany than West Germany, although the exact reason remains unclear [6].

3.2. Female predominance

The female predominance in intrinsic AD is well known and has been observed by a number of studies [1,4,16,20]. Our observation disclosed that 76.5% of AD patients were female [12]. More extremely, the 14 intrinsic AD patients enrolled in a study by another group were all female [20].

3.3. Adults and children

Several reports on the prevalence may provide an implication that the intrinsic type is seen at higher frequencies in children than adults [15]. A Korean group of AD investigators showed that the intrinsic type is more prevalent in infancy, and even the third group of the indeterminate type between the intrinsic and extrinsic ones can be identified in this younger generation [11]. A prospective birth cohort study followed for 5 years by a German group demonstrated that one third of child AD was the intrinsic one, and more common in female [21]. Another German group indicated the low prevalence of the intrinsic AD among adult patients [5]. They showed 6.9% patients fulfilled the criteria of intrinsic AD, and after follow-up, the incidence was declined to 5.4% because some patients developed respiratory allergies or IgE-mediated sensitizations. Taken together these observations, it is likely that the intrinsic type is more prevalent in children than adults.

4. Clinical features

The skin manifestations of the two types of AD are indistinguishable. As shown in Figs. 1–3, patients with the intrinsic type share the features with those with extrinsic type. However, Brenninkmeijer et al extensively studied the clinical features of intrinsic AD [4] and found that the Dennie-Morgan fold is significantly more present in the intrinsic type (Fig. 2). The later onset of disease and milder

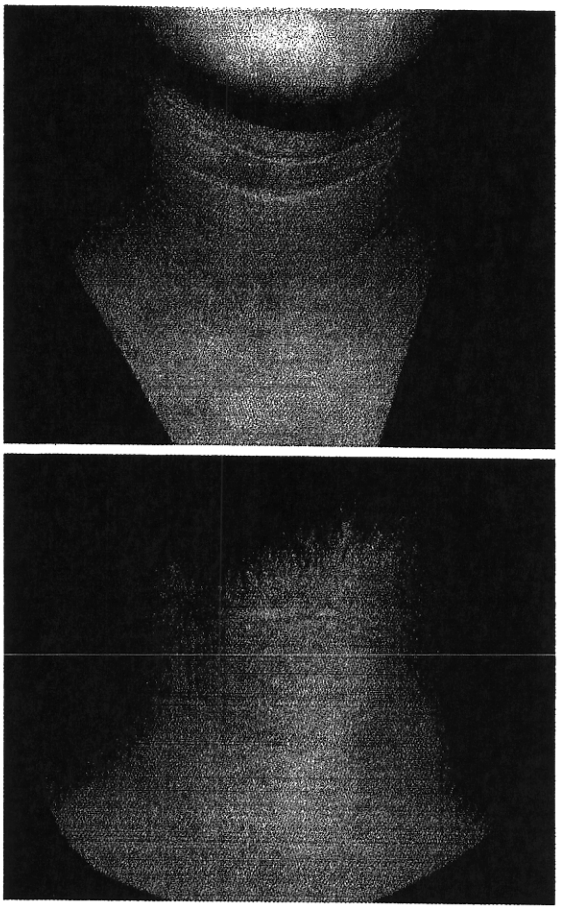

Fig. 1. Intrinsic AD. A 25-year-old female, with total serum IgE, 69 kU/l; and blood eosinophils, 10%. A lichenified eruption on the neck and upper chest (top) and nuchal area (bottom).

disease severity are also characteristics of intrinsic AD. The features that are negatively associated with intrinsic AD include personal or family history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, non-specific hand or foot eczema, and influence of emotional or environmental factors [4]. As mentioned below, some of these non-associated features are considered to stem from the lack of barrier disruption and/or filaggrin gene mutations in intrinsic AD (Table 1).

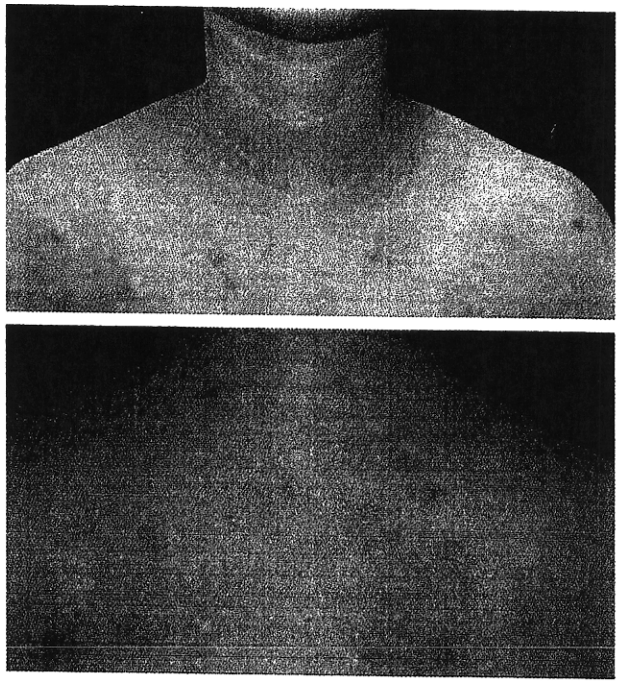

Fig. 3. Intrinsic AD. A 29-year-old female, with total serum IgE, 43 kU/l; and blood eosinophils, 11%. A lichenified eruption on the neck and upper chest (top) and upper back with scratches (bottom).

5. Skin barrier function

5.1. Barrier function of stratum corneum

The barrier function is usually assessed by transepidermal water loss (TEWL) and skin surface hydration (capacitance). The extrinsic AD patients showed increased TEWL and lower skin surface hydration, whereas the intrinsic patients showed no significant differences in TEWL or skin surface hydration as compared to control [19]. On the antecubital fossae, both types of AD patients showed higher TEWL and decreased capacitance. We examined the skin surface hydration and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between the extrinsic and intrinsic types [12]. The level of skin surface hydration was significantly lower in extrinsic AD than in normal control subjects. On the other hand, there was

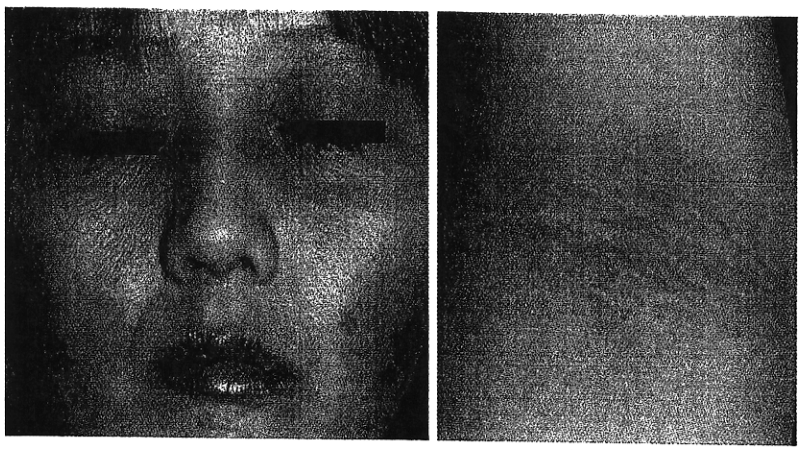

Fig. 2. Intrinsic AD. A 32-year-old female, with total serum IgE, 226 kU/l; and blood eosinophils, 18%. A lichenified eruption with Dennie-Morgan folds on the lower eyelids and pigmentation on the lips (left); and a pigmented and chronic lesion on the antecubital fossa (right).

Table 1
Characteristics of intrinsic AD.

1. Definition
Normal total serum IgE values (mean total serum IgE, 22.2–134 kU/l [1])
Absence of specific IgE for environmental allergens and food allergens
2. Incidence
Percentage of intrinsic AD in total AD: 10–45% [1], 27% [2], 37% [3], 12% [4], 21.8% [5], 20% [6]
Female predominance (collectively 70–80%) [1,5,7,8]
3. Clinical features
Dennie-Morgan fold [5]
No ichthyosis vulgris or palmar hyperlinearity [5]
No non-specific hand or foot eczema [5]
Lower colonization of *Staphylococcus aureus* [9]
Relative late onset
Milder severity
4. Skin barrier
Normal barrier function [6,10]
No filaggrin mutation (presence of filaggrin mutations in extrinsic AD [24,25])
5. Immunological features
Lower expression of IL-4, IL-5, and IL-13 [11]
Higher expression of IFN-γ [12]
6. Contact allergens
High prevalence of metal allergy [39,40]

no significant difference in the hydration level between intrinsic AD and healthy control. The extrinsic type tended to be lower than the intrinsic type at both sites. Thus, the skin barrier function was impaired in extrinsic AD and preserved in intrinsic AD.

5.2. Pruritus perception and barrier function

The skin perception threshold of electric current stimuli is one of the indices of itch. We found that the electric current perception threshold was significantly correlated with the skin surface hydration and inversely with TEWL in intrinsic AD patients as well as healthy individuals. In contrast, extrinsic AD patients did not exhibit such a correlation. Therefore, intrinsic AD patients retain the normal barrier function and sensory reactivity to external pruritic stimuli [14].

5.3. Presence and lack of filaggrin mutations in extrinsic and intrinsic AD, respectively

The recent identification of loss-of-function mutations in filaggrin as a widely replicated major risk factor for eczema sheds new light on the mechanisms of AD [22].

These mutations also represent a strong genetic predisposing factor for atopic eczema, asthma and allergies in various countries [23]. Profilaggrin is the major component of the keratohyalin granules within epidermal granular cells. During epidermal terminal differentiation, the profilaggrin polyprotein is dephosphorylated and rapidly cleaved by serine proteases to form monomeric filaggrin, which is further degradated into natural moisturising factor. Recent human genetic studies strongly suggest that perturbation of skin barrier function as a result of reduction or complete loss of filaggrin expression leads to enhanced percutaneous transfer of allergens. Filaggrin is therefore in the frontline of defense, and protects the body from the entry of foreign environmental substances that can otherwise trigger aberrant immune responses. The association of the filaggrin mutations in particular with the extrinsic type of AD was observed [24,25]. Furthermore, filaggrin mutations are significantly associated with palmar hyperlinearity in patients with AD, which represents a shared feature of AD and ichthyosis vulgaris. This is in accordance with the finding that palmar hyperlinearity is negatively associated in the intrinsic type [4]. In our preliminary study, we found that typical cases of intrinsic AD had no mutation in filaggrin, whereas some of the extrinsic patients possessed filaggrin mutations. Although future studies are necessary, it is expected that barrier disruption, as represented by filaggrin gene mutations, is associated with extrinsic AD.

6. Immunological characteristics of circulating T cells and cytokines/chemokines

6.1. Systemic Th1/Th2 immunological state

AD is well known as a Th2-polarized disease. However, there have been reported some differences in systemic cytokine polarization between the two types of AD. As expected with elevation of total serum IgE, extrinsic AD patients show high levels of Th2 cytokines, IL-4, IL-5 and IL-13, and intrinsic AD is linked with much lower levels of IL-4 and IL-13 [8]. Along with the elevation of IL-5 [26,27], eosinophil counts [11] and eosinophil cationic protein levels [18] are increased in the extrinsic type of AD. On the other hand, there was a report demonstrating that both extrinsic and intrinsic patients showed increased production of IL-5 and IL-13 [28]. In that study, however, when peripheral blood mononuclear cells were stimulated with anti-CD3 antibody, extrinsic AD patients had a decreased capacity to produce IFN-γ and GM-CSF as compared to the intrinsic AD [28]. Accordingly, we found, in our preliminary study, that there was no significant difference in the percentages of IL-4$^+$ or IL-17$^+$ T cells between the extrinsic and intrinsic types, but that of IFN-γ$^+$ T cells was higher in the intrinsic type than the extrinsic type. Thus, there are some variations in these results of Th1 and Th2 cytokines, perhaps depending on the evaluation systems, i.e., measurements of cytokine protein amounts in either *in vivo* sera or *in vitro* culture supernatants, mRNA expression by lymphocytes, and intracellular cytokine staining in T cells. However, all the data can be interpreted to indicate that the extrinsic pathogenetic factors mount a Th2-skewing action, and that the intrinsic type shows less Th2-skewing state or relative overproduction of Th1 cytokine IFN-γ.

6.2. Chemokines and others

With regard to chemokines, patients of both types showed high serum amounts of CCL17/TARC and CCL22/MDC and high peripheral blood mononuclear cell expression of CCL17 and CCL22 at comparable levels [29]. Therefore, no difference was observed in the promoted production of chemokines attractive to Th2 cells. The blood levels of soluble receptors derived from lymphocytes correlate to the activity in various diseases. There is no significant difference in the elevated amounts of sCD23, sCD25, and sCD30 between the two types [30].

7. Immunological characteristics of skin lesions

7.1. T cells and cytokines

In skin lesions, CD4$^+$ T cells, CD8$^+$ T cells, and Langerhans cells are comparably increased in both extrinsic and intrinsic AD, but eosinophils infiltrate in the dermis more markedly in the extrinsic than the intrinsic type, and the extrinsic type exhibits more prominent deposition of eosinophil granular protein and higher staining for eotaxin [10,31]. Although the levels of mRNA expression for IL-5, IL-13, and IL-1β are higher in both types of AD patients than non-atopic subjects, extrinsic AD shows even higher levels than intrinsic AD [31]. The expression of IFN-γ, IL-12, and GM-CSF, IL-4, and IL-10 are elevated in both types without

differences between the extrinsic and intrinsic AD [31]. Thus, tissue eosinophilia and IL-5 expression may be a characteristic of the extrinsic type.

7.2. Dendritic cells (DC) and Langerhans cells (LC)

As to epidermal DC, the extrinsic type is characterized by a significantly high level of the expression of IgE high-affinity receptor (FCεR) on the $CD1a^+$ epidermal DC compared to the intrinsic type [1,32]. When the high-affinity/low-affinity expression ratio is used as a disease marker for AD, the values for intrinsic AD fall below the diagnostic cut-off level, suggesting that intrinsic AD can be distinguished by phenotyping of epidermal DC [1,32]. In accordance with these data from the lesional skin, the surface expression of the high- and low-affinity receptor for IgE and the IL-4Rα chain are significantly elevated in monocytes from patients with the extrinsic type [2]. As described below, it is possible that epidermal LC in the barrier-disrupted skin produce high amounts of Th2 and eosinophil chemokines, further suggesting that LC are activated in the extrinsic type.

8. Relationship between barrier status and skin immune reactions

8.1. Epidermal cytokine production in barrier-disrupted skin

The skin immune status is closely associated with the disordered condition of skin barrier (Fig. 4). Studies using a mouse model of contact hypersensitivity (CHS) have shown that CHS responses to hapten are increased when a hapten is applied to the barrier-damaged skin [33]. Barrier disruption of the skin is experimentally performed by extraction of epidermal lipids with acetone or removal of corneocytes by tape stripping. Both procedures can induce elevated CHS responses. Not only increased permeability of hapten through the epidermis but also altered immune functions of epidermal cells potentiate T-cell activation in acute barrier disruption [33]. Such augmentation of immune reactivity may be critical to elimination of environmental noxious agents that penetrate easily into the barrier-disrupted epidermis, and it is also closely related to the mechanism underlying extrinsic AD.

8.2. Epidermal chemokine production in barrier-disrupted skin

Regarding epidermal chemokines of the barrier-disrupted skin, the mRNA expression levels of Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil chemoattractant (CCL5) are high in the epidermal cells from BALB/c mice. In particular, we found that CCL17, CCL22 and CCL5 were remarkably elevated in BALB/c mice [34]. Tape stripping induced dermal infiltration of eosinophils in BALB/c mice, and the late-phase reaction was increased with infiltration of Th2 cells as well as eosinophils, when challenged *via* the tape-stripped skin. Notably, Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22) are derived mainly from keratinocytes and LC, respectively [35]. In this notion, one of the crucial actions of IFN-γ is upregulation of keratinocyte production of Th1 chemokines and downregulation of LC production of Th2 chemokines. Therefore, the barrier damage likely induces the infiltrates of Th2 cells and eosinophils in extrinsic AD, but their infiltrates are inhibited by IFN-γ in intrinsic AD.

8.3. Implications for the difference between extrinsic and intrinsic AD

The above findings suggest that Th2 and eosinophil responses and resultant late-phase reaction are prone to take place in the skin with damaged barrier by the modulated function of LC. This may provide the mechanism of Th2-polarized immunophenotype of the extrinsic AD. On the contrary, LC are not stimulated to produce Th2 chemokines in the intrinsic type because of the presence of normal stratum corneum. Protein antigens penetrating the damaged barrier further induce the Th2-shifted response in the extrinsic AD, while non-protein antigens exert the Th1 response as well in the intrinsic AD (Fig. 4).

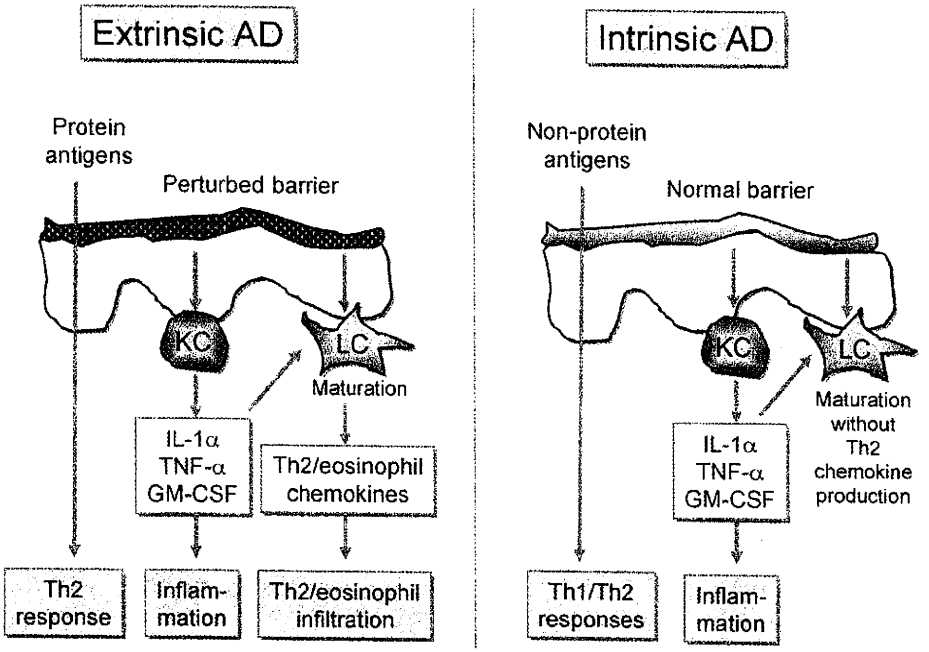

Fig. 4. Comparison between extrinsic and intrinsic AD in relation to the barrier and immune states.
Protein and non-protein antigens are causative in the extrinsic and intrinsic types, respectively. In both types, antigen application to the skin stimulates keratinocytes to produce cytokines, including IL-1α, TNF-α, and GM-CSF, which induce maturation of Langerhans cells (LC). In the perturbed skin of extrinsic AD, LC can produce CCL17/TARC, CCL22/MDC, and CCL5/RANTES, which promote infiltration of Th2 cells and eosinophils. On the other hand, LC of the intrinsic AD do not elaborate those chemokines.

It has been reported that Th2 cytokine IL-4 suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier functions, which further aggravates the barrier [36]. This 'outside-to-inside, back to outside' paradigm [37] is applicable for the pathogenesis of extrinsic AD. A more recent observation suggests that neutralization of the normally acidic stratum corneum has deleterious consequences for permeability barrier homeostasis and stratum corneum integrity/cohesion attributable to serine proteases activation leading to deactivation/degradation of lipid-processing enzymes and corneodesmosomes [38]. Hyperacidification improves permeability barrier homeostasis, attributable to increased activities of two key membrane-localized, ceramide-generating hydrolytic enzymes, which correlate with accelerated extracellular maturation of stratum corneum lamellar membranes. Thus, the surface pH may be another important factor to differentiate between the extrinsic and intrinsic types.

9. Patch tests and metal allergy

9.1. Patch tests for mite antigens

An Italian group performed patch test with house dust mites at a concentration of 20% in petrolatum in the extrinsic and intrinsic types of adult male AD patients [9]. The patch test was positive in 47.4% of extrinsic AD and in 66.6% of intrinsic AD, and in 12.2% of healthy subjects [9]. Since that extrinsic AD patients usually have high levels of IgE specific for mites, the authors wondered the reason why the patch test was highly positive in the intrinsic AD. However, patch tests can reflect mostly the T-cell mediated contact sensitivity, and the IgE-high extrinsic nature does not promote the patch test reactions. Rather, given that IFN-γ is produced at a higher level in the intrinsic type than the extrinsic type, the higher frequency of positive reaction in the intrinsic type seems to be reasonable.

9.2. Patch tests for metals

It is known in patients with AD that the most frequent contact allergens are metals [39]. In 137 atopic children, 19.3% patients were positive to metals [39]. In 1965, Shanon reported that patients with metal allergy occasionally exhibit a skin manifestation indistinguishable from AD under the name of "pseudo-atopic dermatitis" [40,41], and chrome is the causative in their report [40]. Some patients with AD were improved by intake of metal-free diet and elimination of metals [41]. We found that patients with intrinsic AD showed positive patch tests to cobalt, chrome, and/or nickel at a higher percentage than extrinsic one, suggesting that systemic metal allergy is one of the potential causes of intrinsic AD. With regard to metals, our tentative observation with sweat demonstrated that a high incidence of sweat allergy in AD and a therapeutic effect of desensitization with sweat in the patients. Since sweat contains high concentrations of metals, this finding might be related to the pathogenetic role of metals in intrinsic AD.

10. Skin infections

Both extrinsic and intrinsic AD patients suffer from recurrent bacteria and viral infections [42]. A higher colonization of *Staphylococcus aureus* was observed in the extrinsic (71%) *vs* the intrinsic children (49%) [43]. The expression of human β-defensin-3, an anti-microbial peptide, is decreased in both types of AD as compared to normal skin and psoriatic skin [42]. Therefore, skin infection with microorganisms, in particular *S. aureus*, may be severer in the extrinsic type because of barrier perturbation, but it remains unclear whether or not the defense responses are different between the types.

11. Neurotrophins and neuropeptides

Neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are increased in both extrinsic and intrinsic AD, suggesting a similar pathophysiologic background implicating a neuroimmune network [27]. However, there is a significant correlation between BDNF and SCORAD only in the intrinsic type [27]. Maternal NGF levels were significantly higher in patients with both extrinsic and intrinsic AD than controls [30]. It is an issue to be elucidated whether these neurotrophines or neuropeptides such as substance P are different between the two types.

12. Animal models for intrinsic AD

A non-IgE-associated AD model was regarded as a mode of human intrinsic AD [44]. In an animal model of AD, IL-18 contributes the spontaneous development of AD-like skin lesions independently of IgE [45]. When the skin barrier was destroyed in mice and protein A from *S. aureus* was topically applied to the skin, the mice developed AD lesions with dermal infiltration of eosinophils and mast cells and showed an increase in serum levels of IL-18, but not IgE [46]. In this model, IL-18 might be important for the development of infection-associated AD by induction of IL-3 from IFN-γ- and IL-13-producing "super" Th1 cells. Since the intrinsic AD shows high levels of IFN-γ-producing cells [28] and normal levels of IgE, this mouse model resembles intrinsic AD and suggests that some intrinsic AD patients may be related to infection.

13. Conclusions

The causes and mechanisms of intrinsic AD remain unfully elucidated. However, as compared to extrinsic AD, the intrinsic type can be characterized by normal barrier function [12] and IFN-γ-producing potency [28]. These findings suggest that the intrinsic AD patients are not sensitized with protein allergens, which induce Th2 responses, but with other antigens. Metals might be one of the candidates as antigens [40].

Some dermatologists are still skeptical whether the distinction between the intrinsic and extrinsic types of AD is really meaningful. If the contactants and pathophysiology of intrinsic AD are clarified, we might eliminate the term of "intrinsic" from the whole spectrum of AD. However, we have been unable to elucidate them to date. Furthermore, as shown in Figs. 1–3, the clinical appearance of intrinsic AD resembles that of extrinsic AD, expect for a small group of intrinsic patients. In this context, it is reasonable to use "intrinsic" in clinical dermatology.

The extrinsic nature may be changed as the patients grow. Therefore, the classification into the extrinsic and intrinsic types is necessary at each stage of life, i.e., infancy, childhood, teenage, and adult for the allergological management of patients as to allergen avoidance, second allergy prevention, and immunotherapy [14]. However, the risk of an "atopy march" is significantly lower in children with the intrinsic type [14].

A German study demonstrated that the intrinsic type was associated with early daycare attendance [21]. In relation to the feasibility of AD in individuals, early daycare attendance is known as a factor related to the hygiene hypothesis as well as the number of older siblings of individuals. Therefore, intrinsic AD is different from extrinsic AD, whose development is depressed by Th1-inducing environmental factors. Again, it appears that the intrinsic type is not related to the pure Th2 dominant immunological state. Future studies on the intrinsic type of AD may clarify the pathophysiology of not only intrinsic AD, but also dermatitis of

unknown cause that have been called atopiform dermatitis [4] or pseudo-atopic dermatitis [40].

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Yoshiki Tokura (M.D., Ph.D.) is Professor and Chairman at Department of Dermatology in University of Occupational and Environmental Health, Japan. He serves as the president of The Japanese Society for Investigative Dermatology (JSID) since 2008.
He received his MD degree from Hamamatsu University School of Medicine, Japan in 1982. In 1989 he served as Visiting Researcher at Department of Dermatology, Yale University School of Medicine, USA. In 2000 he was promoted to Associate Professor in the Dermatology Department of Hamamatsu University School of Medicine. He moved to University of Occupational and Environmental Health as the Dermatology Professor in 2002. His current research is focused on Immunology/Allergology, Photobiology, Cutaneous lymphomas and Occupational dermatology.

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The Mandatory Role of IL-10–Producing and OX40 Ligand-Expressing Mature Langerhans Cells in Local UVB-Induced Immunosuppression

Ryutaro Yoshiki, Kenji Kabashima, Jun-ichi Sakabe, Kazunari Sugita, Toshinori Bito, Motonobu Nakamura, Bernard Malissen and Yoshiki Tokura

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The Mandatory Role of IL-10–Producing and OX40 Ligand-Expressing Mature Langerhans Cells in Local UVB-Induced Immunosuppression

Ryutaro Yoshiki,* Kenji Kabashima,† Jun-ichi Sakabe,* Kazunari Sugita,* Toshinori Bito,* Motonobu Nakamura,* Bernard Malissen,‡ and Yoshiki Tokura*

The mechanism underlying the local UVB-induced immunosuppression is a central issue to be clarified in photoimmunology. There have been reported a considerable number of cells and factors that participate in the sensitization phase-dependent suppression, including Langerhans cells (LCs), regulatory T cells, IL-10, and TNF-α. The recent important finding that LC-depleted mice rather exhibit enhanced contact hypersensitivity responses urged us to re-evaluate the role of LCs along with dermal dendritic cells (dDCs) in the mechanism of UVB-induced immunosuppression. We studied the surface expression of OX40 ligand (OX40L) and the intracellular expression of IL-10 in LCs and dDCs from UVB-irradiated (300 mJ/cm^2) skin of BALB/c mice and those migrating to the regional lymph nodes from UVB-irradiated, hapten-painted mice. In epidermal and dermal cell suspensions prepared from the UVB-irradiated skin, LCs expressed OX40L as well as CD86 and produced IL-10 at a higher level than Langerin$^-$ dDCs. The UVB-induced immunosuppression was attenuated by the administration of IL-10–neutralizing or OX40L-blocking Abs. In mice whose UVB-irradiated, hapten-painted skin was dissected 1 d after hapten application, the contact hypersensitivity response was restored, because this treatment allowed dDCs but not LCs to migrate to the draining lymph nodes. Moreover, LC-depleted mice by using Langerin-diphtheria toxin receptor–knocked-in mice showed impaired UVB-induced immunosuppression. These results suggest that IL-10–producing and OX40L-expressing LCs in the UVB-exposed skin are mandatory for the induction of Ag-specific regulatory T cells. *The Journal of Immunology*, **2010, 184: 5670–5677.**

Ultraviolet radiation is one of the significant environmental factors affecting humans or other animals. It is well known that UV, in particular the middle wavelength range (290–320 nm, UVB), can be hazardous to human skin by acutely evoking sunburn and epidermal cell death and by chronically inducing skin cancers and skin aging (1–4). UVB radiation also exerts an immunomodulating effect on cutaneous contact hypersensitivity (CHS) by affecting various skin-constituent cells and factors (5). Preirradiation of sensitizing area with low-dose UVB suppresses the development of CHS to hapten in mice (6). In addition to the failure to generate hapten sensitization, mice develop tolerance, because animals treated in this way cannot be resensitized with the same hapten at a later time point. The UVB-induced immunosuppression appears to be hapten-specific, because the sensitization with other nonrelated haptens is not affected (6). Moreover, this hapten-specific immunosuppression can be transferable, as an injection of lymph node cells or splenocytes from UVB-tolerized mice into naive mice inhibits the sensitization with the relevant hapten in the recipients (7). It was once considered that the UVB-induced immunosuppression was mediated by hapten-specific suppressor T cells (5, 8, 9). Now, this suppressor T cell is renamed regulatory T cell (Treg) (10–12). Therefore, a suppressive signal that causes UVB-induced tolerance is hypothesized to exist in the draining lymph node (DLN) of UVB-irradiated skin, where Tregs are induced and suppress the generation or function of effector T cells. However, it remains unclear how the suppressive signal is transmitted from the skin to the DLNs. On one hand, Tregs act in part through the induction of IL-10 production (13). On the other hand, IL-10 is a key cytokine to induce Tregs, and keratinocytes have been considered to be the source of IL-10. However, all the mechanisms underlying UVB-induced immunosuppression are not attributed to keratinocyte-derived IL-10, because human keratinocytes are incapable of producing IL-10 (14). More fundamentally, keratinocytes are unable to migrate to the DLN. Therefore, alternative cells with a migrating ability are likely responsible for mediation of suppressive signals.

Recent studies have revealed the involvement of OX40 (CD134) and its ligand (OX40L) in T cell–APC interaction (15–18). OX40 is expressed on activated CD4$^+$ T cells and on certain populations of CD8$^+$ T cells (15–17, 19), whereas OX40L is expressed on APCs, such as activated B cells (20), dendritic cells (DCs) (21, 22), microglia (23), and endothelial cells (24). Ligation of OX40L on human DCs enhances their maturation and production of cytokines (22), and blockade of OX40L during naive T cell–DC interaction suppresses the development of IL-4–producing T cells (25). It is thus suggested that OX40 and OX40L play an important role in the interaction of DCs with T cells to induce, in particular, Th2 cells. Moreover, CD4$^+$CD25$^+$ Tregs express OX40 at a high level compared with CD4$^+$CD25$^-$ T cells (26, 27). Considering that UVB-induced suppressor T cells were historically identified

*Department of Dermatology, University of Occupational and Environmental Health, Fukuoka; †Department of Dermatology, Kyoto University, Kyoto, Japan; and ‡Centre d'Immunologie de Marseille-Luminy, Institute National de la Santé et de la Recherche Médicale U631, Centre National de la Recherche Scientifique Unité Mixte de Recherche 6102, Université de la Méditerrannée, Marseille Cedex 07, France

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Address correspondence and reprint requests to Dr. Ryutaro Yoshiki, University of Occupational and Environmental Health, 1-1, Iseigaoka, Yahatanishi-ku, Kitakyushu, Fukuoka 807-8555 Japan. E-mail: yockey@med.uoeh-u.ac.jp

Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal dendritic cell; DLN, draining lymph node; DNFB, dinitrofluorobenzene; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LC, Langerhans cell; OX40L, OX40 ligand; RANKL, RANK ligand; Treg, regulatory T cell.

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as Th2 cells (28), these findings provide an implication that OX40–OX40L interaction participates in the development of UVB-mediated $CD4^+CD25^+$ Tregs.

Langerhans cells (LCs) are capable of migrating from the epidermis into the DLNs on sensitization (29). Several investigator groups have suggested that LCs are responsible for induction of Tregs (30, 31), but the mechanism underlying the Treg induction by DCs in the UVB-irradiated skin remains unclear in major parts. Recent immunological studies have demonstrated that there are dermal DCs (dDCs), including $Langerin^+$ dDCs and $Langerin^-$ dDCs in the murine skin (32–36). This raises the possibility that not only LCs but also dDCs have an ability to induce Tregs by UVB irradiation of the skin.

In this study, we demonstrate that UVB irradiation of the skin leads to IL-10 production and OX40L expression by LCs. Our study using Langerin-diphtheria toxin receptor (DTR)–knocked-in mice shows that the IL-10–producing and OX40L-expressing LCs play a mandatory role in the induction of Tregs.

Materials and Methods

Animals and reagents

Six- to 10-wk-old BALB/c female mice were purchased from Kyudo (Kumamoto, Japan). Mice were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. Langerin-DTR–knocked-in mice was generated (37). To deplete $Langerin^+$ cells, mice were injected i.p. with diphtheria toxin (DT) (100 ng each; Sigma-Aldrich, St. Louis, MO). Protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health, Fukuoka, Japan.

Contact hypersensitivity

Mice were sensitized with dinitrofluorobenzene (DNFB) by applying 50 μl 0.5% DNFB in acetone:olive oil (4:1) to the shaved abdomen on day 0. On day 5, 20 μl 0.2% DNFB was applied to both ears for elicitation. Ear swelling was measured with a micrometer 24 h postelicitation.

UVB irradiation

The shaved abdomen was exposed to UV with a bank of four UVB lamps (Toshiba FL 20S, Toshiba Medical Supply, Tokyo, Japan) (5, 8, 9) that emit most of their energy within the UVB range (290–320 nm), with an emission peak at 313 nm. The irradiance was measured with a UVR-305/365 digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan). Mice were exposed to 300 mJ/cm^2 UV on the shaved abdomen on day −2 presensitization (day 0). Although BALB/c mice are usually not very susceptible to UVB, we found that a single exposure of BALB/c mice to UVB at 300 mJ/cm^2 induces UVB immunosuppression with an elevated percentage of $Foxp3^+$ $CD25^+$ cells in the DLN cells. Because a single irradiation of the skin to UVB and following FITC paining are convenient for the study of DC migration to the DLN, we used this protocol and strain of mice in this study. The ears of mice were protected from radiation with opaque foil.

Culture medium

RPMI 1640 (Life Technologies, Grand Island, NY) was supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies).

Preparation of whole skin suspensions

Skin sheets from mouse abdomen were floated in 0.2% trypsin in PBS ([pH 7.4]; Sigma-Aldrich) for 30 min at 37°C as described previously (38). The epidermis was separated from the dermis with forceps in PBS supplemented with 10% FCS. Both epidermis and dermis were minced and incubated for 1 h at 37°C in PBS with collagenase II (Sigma-Aldrich). The obtained cells were filtered through a 40-μm filter.

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated mAbs and analyzed with an FACSCanto flow cytometer (BD Biosciences, San Diego, CA) and FlowJo software (Tree Star, Ashland, OR). The expression of cell surface or intracellular molecules and intracytoplasmic cytokines were analyzed using the following Abs: Alexa Fluor 488-conjugated antiepithelial cell adhesion molecule (EpCAM; Biolegend, San Diego, CA); PE-conjugated anti-OX40 ligand (OX40L), anti-CD86, anti-RANK, anti-rat IgG; APC-conjugated anti-MHC class II Ab; biotin-conjugated anti-mouse CD207 (Langerin), anti–IL-10 Ab, and anti-rat IgG; and PE-Cy7–conjugated streptoavidine (eBioscience, San Diego, CA). Intracytoplasmic IL-10 and Langerin was detected in permeabilized cell suspensions using a BD Cytofix/Cytoperm Plus Kit (BD Biosciences).

Cutaneous DC migration into DLNs

Mice were painted on the clipped abdomen with 200 μl 2% FITC (Sigma-Aldrich), and axillar and inguinal lymph nodes were taken 24 h later. Single-cell suspensions were prepared and subjected to flow cytometric analysis.

Apoptosis analysis

Twenty-four hours after UVB irradiation (300 mJ/cm^2), whole skin suspensions were stained with APC-Cy-7–conjugated anti-MHC class II or PE-conjugated EpCAM and FITC-conjugated CD103 (BD Biosciences) for 30 min and stained with Alexa Flour 647-conjugated Annexin V (Invitrogen, Carlsbad, CA) and 7-aminoactinomycin D (7-AAD; BD Biosciences), according to the manufacturer's protocol. Apoptosis in keratinocytes or DCs was analyzed by FACSCanto (BD Biosciences) using FlowJo software (Tree Star) as previously described (39).

In vitro promotion of LC IL-10 production by RANK ligand and its blockade with neutralizing Ab against RANK

Freshly isolated epidermal cell suspensions (5×10^5/well) were cultured with or without 1 μg/ml rRANK ligand (RANKL) (R&D Systems, Minneapolis, MN) for 24 h. For RANK-neutralizing assay, 1 μg/ml anti-RANK Ab or isotype-matched control Ab (R&D Systems) was added to the culture 3 h before the addition of rRANKL. Intracellular IL-10 of LCs was measured by FACS.

In vivo neutralization of IL-10 and blocking of OX40L

Mice received i.p. injections of 25 μg anti-mouse IL-10 Ab (R&D Systems) or 10 μg anti-mouse OX40L Ab (Biolegend) for 4 consecutive days (on days 1–4) after UVB irradiation (on day −2) and DNFB sensitization (on day 0). They were challenged with DNFB on day 5, and the ear swelling responses were measured. For control, mice received the same volume of PBS and were sensitized and challenged with DNFB.

Statistical analysis

All data were statistically analyzed using the Student t test. A p value of <0.05 was considered to be significant. Bar graphs were presented as mean ± SD of the mean value.

Results

$Langerin^+$ dDCs are decreased in number and become apoptotic in UVB-irradiated skin

It is a long-held concept that LCs play a critical role in CHS, as they serve as APCs and migrate to the DLNs (40). However, recent immunological studies have demonstrated that not only LCs but also $Langerin^+$ dDCs and $Langerin^-$ dDCs exist in the skin and may differentially function as APCs. We first investigated the numerical change of LCs, $Langerin^+$ dDCs, and $Langerin^-$ dDCs in UVB-irradiated skin. Whole skin suspensions were prepared from the UVB-irradiated and nonirradiated skin as control 24 h after UVB exposure and analyzed by flow cytometry. Using anti-MHC class II, anti-CD11c, anti-Langerin (CD207), and EpCAM Abs, skin-resident DCs were clearly sorted out of the suspensions (Fig. 1*A*, 1*B*). As assessed by the percentage analysis, the populations of LCs ($Langerin^+$ $EpCAM^+$) and $Langerin^-$ dDCs ($Langerin^-$ $EpCAM^-$) showed no substantial change after UVB irradiation (Fig. 1*C* versus 1*D*), although UVB-irradiated skin-derived DCs had a slightly broader MHC class II expression (Fig. 1*B*). However, the percentage of $Langerin^+$ dDCs was dramatically decreased in UVB-irradiated skin (Fig. 1*C* versus 1*D*). When the absolute number of each LC/DC subset per skin specimen was calculated, UVB irradiation reduced dramatically the number of $Langerin^+$ dDCs and moderately that of LCs and did not affect that of $Langerin^-$ dDCs

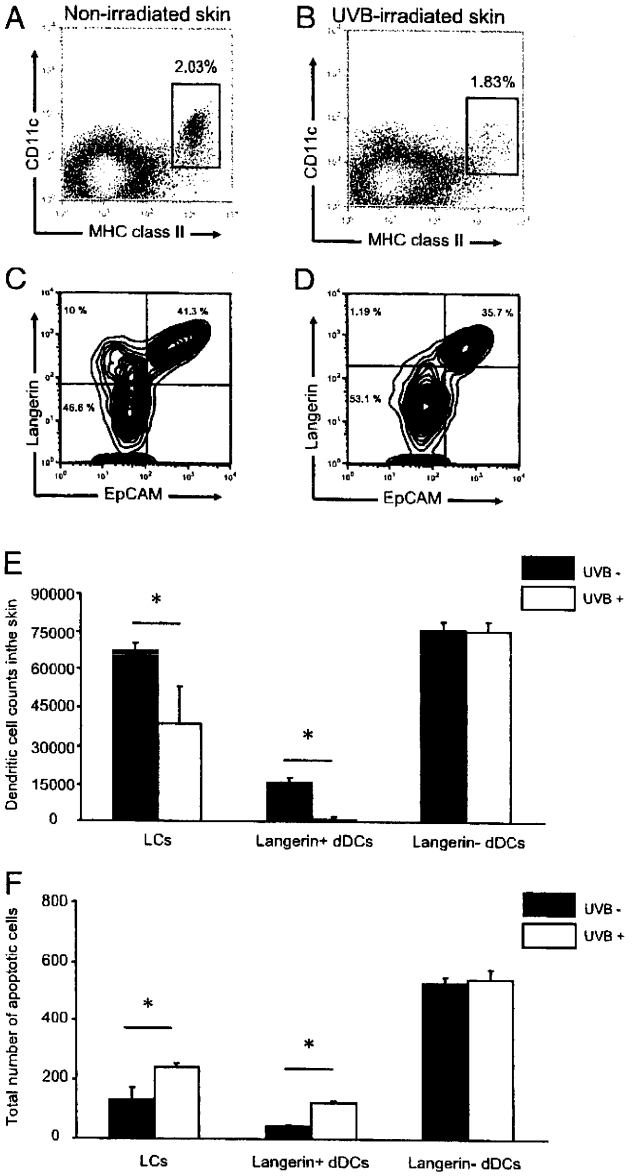

FIGURE 1. Numerical alterations of cutaneous DCs after UVB irradiation of the skin. Single-cell suspensions were stained with APC-conjugated anti-MHC class II and APC-Cy7-conjugated CD11c Abs and subjected to flow cytometric analysis. *A*, Nonirradiated skin. *B*, UVB-irradiated skin. *C*, With the use of anti-EpCAM and anti-Langerin Abs, DCs from nonirradiated skin were clearly sorted out into the three categories: LCs (Langerin$^+$ EpCAM$^+$), Langerin$^+$ dDCs (Langerin$^+$ EpCAM$^-$), and Langerin$^-$ dDCs (Langerin$^-$ EpCAM$^-$). *D*, In UVB-irradiated skin, Langerin$^+$ dDCs were diminished. *E*, Total cell number of each DC subsets. *F*, Apoptotic cell number of LCs and Langerin$^+$ dDCs as assessed by flow cytometric analysis (7-AAD$^-$ and Annexin$^+$). $*p < 0.05$.

(Fig. 1*E*), confirming the decreased number of Langerin$^+$ dDCs in the UVB-irradiated skin. We analyzed apoptosis of LCs and DCs in the UVB-irradiated mice. Six hours after UVB irradiation, we assessed apoptotic cells by flow cytometry and defined them as Annexin V$^+$ and 7-AAD$^-$ cells. Langerin$^+$ dDCs and LCs became apoptotic after UVB irradiation (Fig. 1*F*). There was no selectivity for UVB-induced apoptosis in these two subsets, but when they were compared in the apoptotic cell percentage, Langerin$^+$ DCs were more sensitive to UVB. In contrast to these cells, Langerin$^-$ DCs were resistant to UVB.

LCs but not Langerin$^+$ dDCs migrate from UVB-irradiated skin to DLNs

We examined the numbers and migration timings of LCs, Langerin$^+$ dDCs, and Langerin$^-$ dDCs in the DLNs after FITC application of UVB-irradiated or nonirradiated skin. UVB-irradiated (day −2) and nonirradiated control mice were painted with FITC (day 0). On days 1–4, single-cell suspensions were prepared from the DLNs and stained with anti-CD11c, anti-EpCAM, and anti-Langerin Abs. By flow cytometry, LC subsets (Langerin$^+$ EpCAM$^+$) and Langerin$^+$ dDC subsets (Langerin$^+$ EpCAM$^-$) of CD11c$^+$ FITC$^+$ cells were detected in the DLNs. LCs were gradually increased in number in both UVB-irradiated and nonirradiated groups (Fig. 2*A*). In contrast, the number of Langerin$^+$ dDCs peaked on day 3 in nonirradiated mice, but their number in UVB-irradiated mice was very low (Fig. 2*B*). The number of Langerin$^-$ dDCs in UVB-nonirradiated skin was increased until day 2 and gradually declined, whereas that in UVB-irradiated skin peaked on day 1 and rapidly decreased (Fig. 2*C*). Thus, UVB irradiation allowed LCs and Langerin$^-$ dDCs to migrate into the DLNs, but Langerin$^+$ dDCs in the irradiated skin did not migrate to the DLNs. There was no significant difference in the number of FITC$^-$ DCs of each subset (data not shown). Therefore, the numerical reduction of Langerin$^+$ dDCs in the UVB-irradiated skin did not result from their emigration from the skin. It is assumed that when a hapten is applied to the UVB-preirradiated skin, there are few Langerin$^+$ dDCs capable of migrating to the DLNs and priming Tregs or effector T cells.

UVB upregulates LC maturation and promotes IL-10 production and OX40L expression

It has long been thought that LCs represent one of the most likely targets for UVB in immunosuppression because of their location in the skin and their importance as APCs. Recent studies using LC-depleted mice have shown that LCs are dispensable for CHS (37) and rather downregulate the CHS response (41). In this line of thinking, dDCs may play an essential role for the development of CHS (42). To address the regulatory functions of UVB-irradiated DC populations, we examined the expression of intracellular IL-10 and surface OX40L as well as CD86 in LCs and Langerin$^-$ dDCs.

Epidermal suspensions were prepared from UVB-irradiated and nonirradiated skin and subjected to flow cytometric analysis. Compared to the nonirradiated control skin, LCs from UVB-irradiated skin showed high expression levels of CD86, OX40L, and intracellular IL-10 (Fig. 3*A*). However, such elevations were not observed in Langerin$^-$ dDCs. This suggests that UVB irradiation upregulates the maturation (CD86 expression) of LCs and promotes the production of IL-10 and the expression of OX40L by LCs, but Langerin$^-$ dDCs are not susceptible to UVB.

To examine these IL-10–producing and OX40L-expressing mature LCs in the UVB-irradiated skin retain the ability to migrate to the DLNs and to serve as APCs, FITC, which is not only a hapten but also a cell trafficking marker, was applied to the UVB-irradiated skin 24 h postirradiation. The migrating LCs were identified as the FITC$^+$CD11c$^+$EpCAM$^+$Langerin$^+$ cell fraction, whereas the migrating Langerin$^-$ dDCs were determined as the FITC$^+$CD11c$^+$ EpCAM$^-$Langerin$^-$ cell fraction. The IL-10–producing and OX40L-expressing LCs from UVB-irradiated skin migrated to the DLNs as compared with the nonirradiated skin (Fig. 3*B*), suggesting that LCs of UVB-irradiated skin can induce Treg or Th2 cells in the lymph nodes.

IL-10 production by LCs is mediated by RANKL from UVB-irradiated apoptotic keratinocytes

It has recently been reported that LCs express RANK, and UVB irradiation upregulates cutaneous RANKL, which modulates the

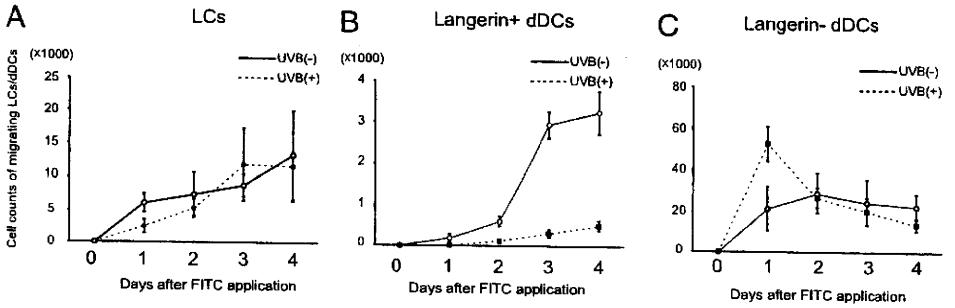

FIGURE 2. Numbers of LCs, Langerin$^+$ dDCs, and Langerin$^-$ dDCs in DLNs after FITC application to UVB-irradiated or nonirradiated skin. Mice were irradiated with UVB (300 mJ/cm^2) on day −2 or nonirradiated and sensitized with FITC on day 0. On days 1–4, DLNs were collected and stained for CD11c, Langerin, and EpCAM. We gated on the FITC$^+$CD11c$^+$ population and counted the EpCAM$^+$ Langerin$^+$ (LCs), EpCAM$^-$ Langerin$^+$ (Langerin$^+$ dDCs), and EpCAM$^-$ Langerin$^-$ (Langerin$^-$ dDCs) cells. *A*, The number of LCs was gradually increased after FITC application in the UVB-irradiated and nonirradiated skin. *B*, The number of Langerin$^+$ dDCs was increased sharply at day 3 in nonirradiated mice but not increased in UVB-irradiated mice. *C*, The number of Langerin$^-$ dDCs peaked at day 1 in the UVB-irradiated mice.

functions of DCs to induce Tregs (43). We have previously reported that when rRANKL was added to LC culture, the RANKL-exposed LCs produce a high amount of IL-10 (44). In contrast, UVB radiation is known to induce apoptosis of epidermal cells. To examine whether epidermal keratinocytes produce RANKL upon UVB exposure in relation to the apoptotic state, epidermal suspensions were prepared from the UVB-irradiated skin 24 h post-exposure and stained to see apoptosis and RANKL expression. By flow cytometry (Fig. 4*A*), keratinocytes were divided into live (Fig. 4*Aa*; 7-AAD$^-$, Annexin$^-$), apoptotic (Fig. 4*Ab*; 7-AAD$^-$, Annexin$^+$), and dead (Fig. 4*Ac*; 7-AAD$^+$, Annexin$^+$) populations. The apoptotic keratinocyte expressed RANKL at a higher degree than did the live and dead keratinocytes (Fig. 4*B*). Thus, UVB-irradiated apoptotic keratinocytes are capable of producing RANKL and subsequently stimulate LCs to produce IL-10 (44). Next, we performed a RANK-blocking study. The production of IL-10 by LCs was promoted by the addition of rRANKL to the culture of epidermal cells, and this increased IL-10 production was blocked by the further addition of an anti-RANK Ab, whereas an isotype-matched control Ab did not suppress IL-10 production (Fig. 4*C*). These results suggest that RANKL from UVB-irradiated keratinocytes mediates IL-10 production by LCs (Fig. 4*C*).

IL-10 neutralization or OX40L blockade abrogates UVB-induced immunosuppression in vivo

It is likely that the production of IL-10 and the expression of OX40L in LCs contribute to the UVB suppression of CHS. To test the significance of IL-10 and OX40L in the suppression, UVB-preirradiated mice (on day −2) were injected i.p. with anti–IL-10 or anti-OX40L Ab for 4 consecutive days (days 0–3), whereas mice were sensitized (day 0) and challenged (day 5) with DNFB. Preirradiation of sensitizing sites to UVB suppressed CHS in mice (Fig. 5). The administration of anti–IL-10 Ab completely restored the CHS response. In contrast, UVB-induced CHS suppression was partially but significantly abrogated by anti-OX40L Ab. We cannot negate the possibility that not only LCs but also other cells are the targets of this blocking procedure, but it seems that IL-10 is profoundly involved in UVB-induced suppression, and OX40L expression is required for the full-blown suppression of CHS.

CHS is successfully induced by dissection of UVB-irradiated and hapten-applied skin at early phase of sensitization

To determine whether LCs and dDCs serve as inducers of Tregs, a skin dissection study was performed for prevention of LC migration at the sensitizing phase. Mice were sensitized with FITC on day 0. When the sensitized skin was dissected on day 1, the total number of migrating LCs was significantly decreased particularly in mice preirradiated with UVB before FITC application (Fig. 6*A*). We therefore examined the CHS response to FITC in mice whose UVB-irradiated and hapten-applied skin was dissected on day 1. This treatment is considered to allow Langerin$^{+/-}$ dDCs to migrate to the DLNs, but most LCs cannot emigrate there. Mice receiving dissection of the sensitizing site did not exhibit UVB-induced immunosuppression of CHS compared with the nondissected and

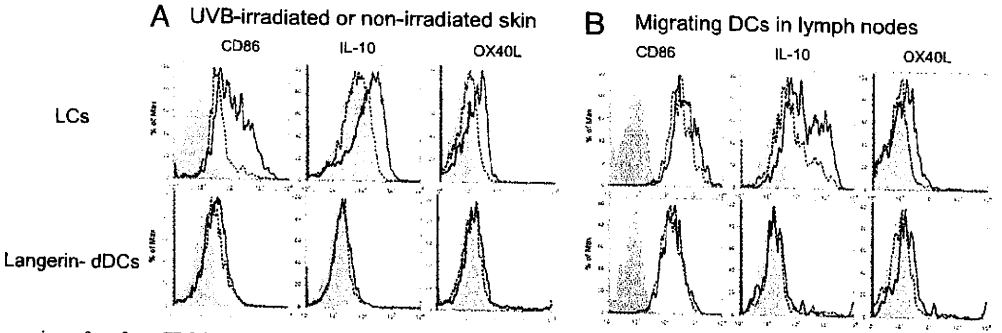

FIGURE 3. Expression of surface CD86, intracellular IL-10, and surface OX40L in LCs and Langerin$^-$ dDCs from the skin and DLNs. *A*, Epidermal cell suspensions were obtained from UVB-irradiated skin 24 h after UVB exposure or nonirradiated skin. Solid line, UVB-irradiated skin; dotted line, nonirradiated skin; and closed shadow, isotype-matched control. *B*, Cell suspensions were obtained from the DLNs of mice receiving UVB irradiation (day −2) and FITC painting (day 0) or mice receiving FITC painting without UVB irradiation. Lymph nodes were taken on day 1, and migrating LCs were identified as FITC$^+$CD11c$^+$EpCAM$^+$Langerin$^+$ cells and migrating Langerin$^-$ dDCs as FITC$^+$CD11c$^+$EpCAM$^-$Langerin$^-$ cells. Solid line, UVB-irradiated mice; dotted line, nonirradiated mice; and closed shadow, isotype-matched control.

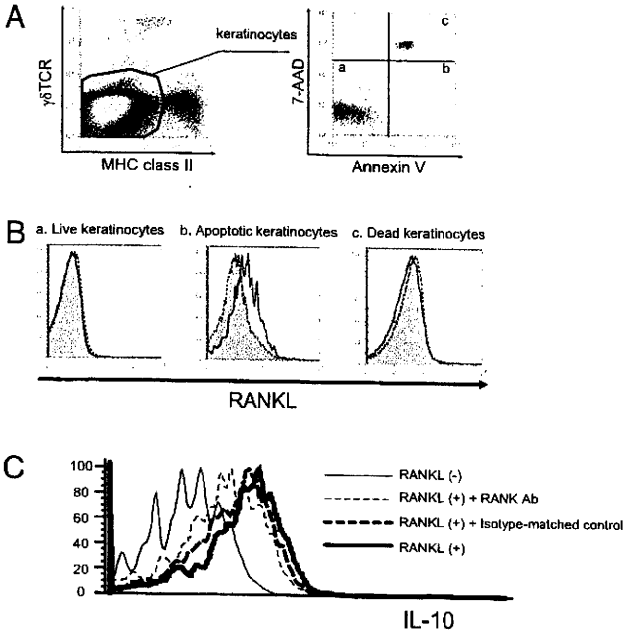

FIGURE 4. RANKL expression in apoptotic keratinocytes from UVB-irradiated skin and RANKL promotion of LC IL-10 production. *A*, Epidermal cell suspensions were obtained from UVB-irradiated (300 mJ/cm^2) skin 24 h postirradiation. Keratinocytes were identified as the MHC class II$^-$ and γδTCR$^-$ fraction by flow cytometry. *a*, Live keratinocyte; *b*, apoptotic keratinocyte, *c*; dead keratinocytes. *B*, Apoptotic keratinocytes expressed RANKL at a higher degree than live and dead keratinocytes. Solid line, UVB-irradiated skin; dotted line, nonirradiated skin; and closed shadow, isotype-matched control. *C*, Epidermal cell suspensions were cultured with or without rRANKL for 24 h. Intracellular IL-10 was measured by FACS. IL-10 production in LC was increased by the addition of rRANKL, and the increased IL-10 production was reduced by the further addition of anti-RANK Ab.

UVB-irradiated mice (Fig. 6*B*). The data suggest that migration of LCs, but not Langerin$^{+/-}$ dDCs, to the lymph nodes is required for UVB-induced CHS suppression.

LC-depleted mice do not exhibit UVB-induced immunosuppression

To discriminate the function of LCs from that of dDCs more clearly, LCs were depleted with DT in Langerin-DTR–knocked-in mice. LCs were completely ablated from the epidermis within 24 h postinjection of DT (Fig. 7*A*, 7*B*). We then addressed the role of LCs in the UVB-induced suppression of CHS. The LC-depleted mice were irradiated with UVB on shaved skin (day −2), painted with DNFB on the same site (day 0), and challenged with DNFB on the ears (day 5). The magnitude of the hapten-specific challenge response was measured 24 h later. As compared with UVB-irradiated non-DT control mice, LC-depleted and UVB-irradiated mice developed a markedly high CHS response (Fig. 7*D*).

We also investigated whether Langerin$^+$ dDCs are involved in the UVB-induced immunosuppression. It has been reported that Langerin$^+$ dDCs recolonize 5 d or less after DT injection (32). Ten days after DT injection, when LCs are still absent in the epidermis but dDC are present (Fig. 7*A* versus 7*C*), mice were preirradiated with UVB and sensitized and elicited with DNFB. Compared to the control mice, LC-depleted but Langerin$^+$ dDC-bearing mice did not show UVB-induced immunosuppression (Fig. 7*E*). Therefore, it is most likely that LCs induce the UVB-induced immunosuppression, but neither Langerin$^+$ nor Langerin$^-$ dDCs have the ability to mediate the suppression.

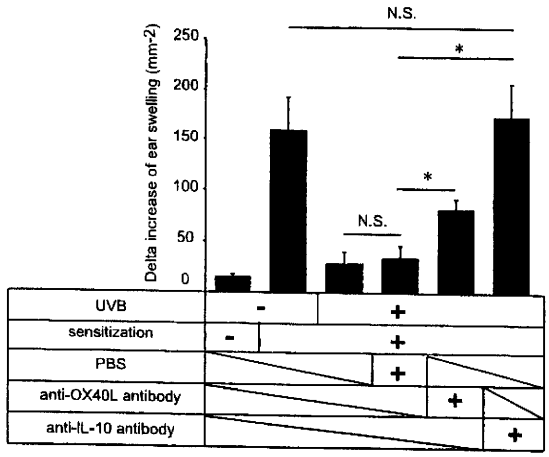

FIGURE 5. Effects of administration of IL-10–neutralizing and OX40L-blocking Abs. Mice were irradiated with UVB (300 mJ/cm^2) on day −2, sensitized with DNFB on day 0, and challenged with DNFB on day 5. IL-10–neutralizing Ab (25 μg per mouse), OX40-blocking Ab (10 μg per mouse), or PBS (for control) was injected i.p. on days 0–3. Positive control mice were sensitized and challenged, and negative control mice were challenged without sensitization. $*p < 0.05$.

Discussion

This study addressed the immunological mechanism underlying the impaired sensitization through UVB-irradiated skin. We found that the UVB-induced immunosuppression of CHS is mediated by IL-10–producing, OX40L-expressing, and CD86 highly expressing mature LCs, which are induced by exposure to RANKL released from UVB-irradiated, apoptotic keratinocytes. The mandatory role of LCs for the UVB-induced suppression was confirmed by the two types of studies, the dissection of sensitizing site and the use of LC-depleted mice. In addition, the recently identified Langerin$^+$ dDCs as well as Langerin$^-$ dDCs (36) seem to play no suppressive role.

Many studies have shown that IL-10 is an essential cytokine in depression of CHS (45–47). The administration of rIL-10 suppresses CHS and induces Ag-specific tolerance (48). IL-10 has also been reported to be a key cytokine in the mechanism of UVB-induced tolerance, as anti–IL-10 Ab treatment before UVB exposure prevents UVB-induced tolerance (49). The neutralizing study using anti–IL-10 Ab further confirmed that IL-10 is essential for the UVB-induced immunosuppression of CHS. Concerning the source of IL-10, a number of studies have demonstrated keratinocytes to be the producer. However, our present study showed that IL-10 is efficiently produced by LCs when the skin is exposed to UVB. The earlier studies on the production of IL-10 by keratinocytes were performed by determining IL-10 mRNA induction and IL-10 protein release in murine keratinocytes shortly postirradiation with UVB (50) or poststimulation with hapten coupling (51). Because cultured keratinocytes were used in those studies, the conclusion may not correctly reflect the in vivo UVB exposure to the skin. In addition, the mechanism of human UVB-induced immunosuppression cannot be explained with the finding obtained from murine keratinocytes. Whereas murine keratinocytes are capable of releasing IL-10 (50, 52), human keratinocytes are an unlikely source of IL-10 following in vivo UVB exposure, as they express little mRNA for IL-10 and secrete no IL-10 protein (14). We have previously reported that IL-10–producing LCs in the grafted skin have a crucial role in the induction of Ag-specific Tregs (44). Together with the present finding, it is suggested that the LCs that migrate from the skin to the DLNs are the important source of IL-10 under the condition of UVB irradiation or skin grafting. Such a finding of

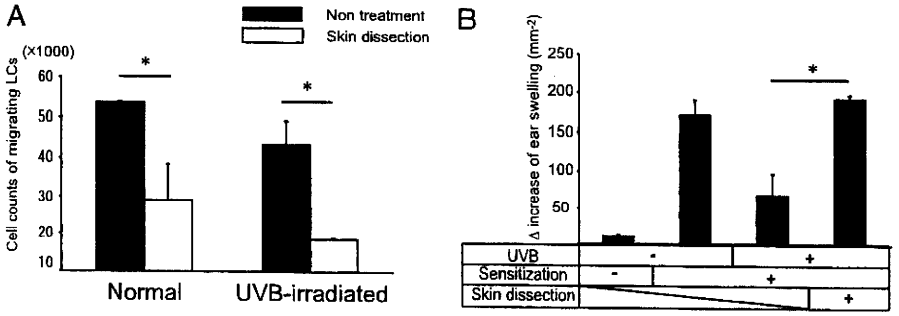

FIGURE 6. Effect of dissection of UVB-irradiated and/or hapten-applied skin on CHS. *A*, The number of LCs migrating to the lymph nodes on day 5 in mice receiving skin dissection on day 1. The LC counts were significantly decreased in mice receiving skin dissection (white bar) compared with non-dissected mice (black bar). *B*, Mice were irradiated with UVB (300 mJ/cm^2) on day −2, sensitized with DNFB on day 0, and challenged with DNFB on day 5. On day 1, a group of mice were skin-dissected. Positive control mice were sensitized and challenged, and negative control mice were challenged without sensitization. $*p < 0.05$.

DC production of IL-10 has also been reported in pulmonary DCs critical for the induction of tolerance (53).

A group of investigators have found that RANKL, which is expressed in keratinocytes of the UVB-irradiated skin, regulates Treg numbers via activation of DCs (43). In another line of studies, i.v. injection of photopheresis-induced apoptotic cells inhibited an immune response to hapten, and this was caused by CD11c$^+$ cells that induce Ag-specific Tregs (54). Likewise, Tregs have been shown to be generated following APC engagement of apoptotic cells (55). Thus, ingestion of apoptotic cells is not merely a scavenging event but also an active process of immune tolerance induction. Teleologically, this process has been described as one of the peripheral tolerance mechanisms (56). We have previously shown that when LCs are exposed to RANKL, they produce IL-10 (44). In this report, we found that apoptotic keratinocytes express RANKL at a higher degree than live keratinocytes and dead keratinocytes. Besides the phagocytosis of apoptotic cells by APCs, RANKL is another tolerogenic signal from apoptotic cells, and the resultant change of DCs to regulatory cells is one of the mechanisms by which apoptosis is related to tolerance.

The blockade of OX40–OX40L interaction by neutralizing OX40 Ab ameliorates experimental allergic encephalomyelitis and experimental colitis, which are Th1-mediated inflammatory diseases (23, 57). OX40 signaling is thus required for the optimal evolution of the Th2 response (58). Moreover, OX40 signaling is involved in the generation of Tregs, and the delivery of OX40 signals can override Treg activity (59). In our data, the blockade of OX40–OX40L interactions partially abrogated the UVB-induced immunosuppression in a comparison with IL-10 neutralization, suggesting that OX40–OX40L interaction is partially responsible for the Treg induction.

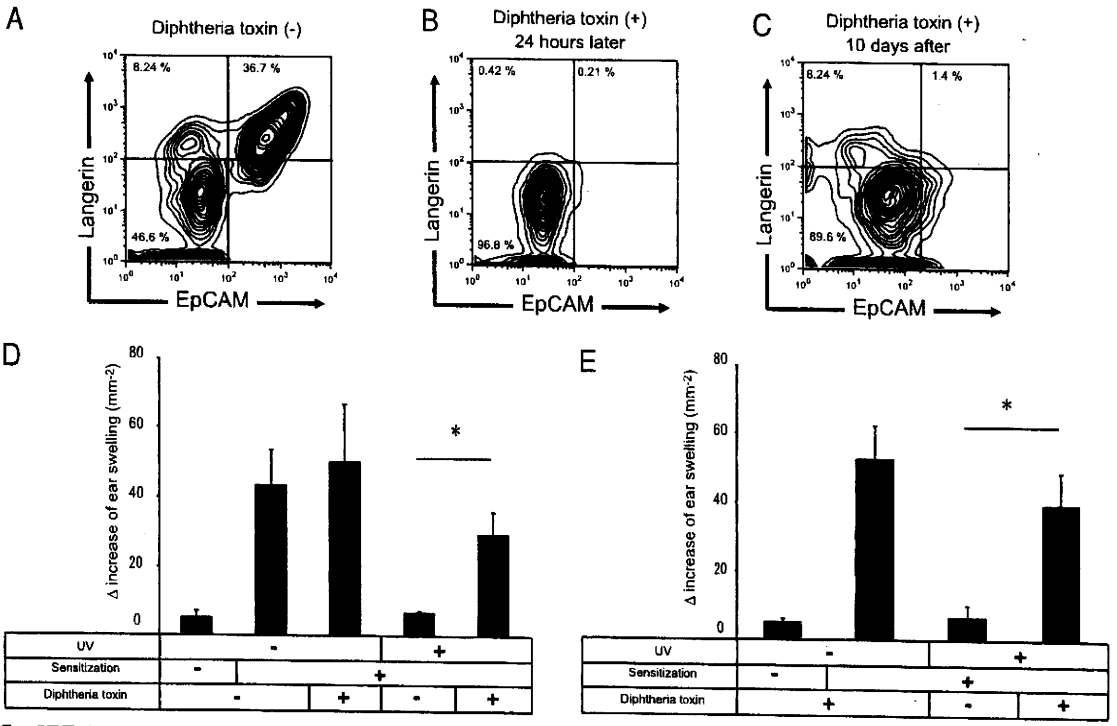

FIGURE 7. UVB-induced immunosuppression in LC-depleted mice. LCs (EpCAM$^+$ Langerin$^+$ cells) and Langerin$^+$ dDCs (EpCAM$^-$ Langerin$^+$ cells) were depleted by DT (100 ng per mouse) in Langerin-DTR–knocked-in mice, Langerin$^+$ dDCs repopulated 10 d later. *A*, Nonirradiated skin. *B*, Twenty-four hours after DT injection. *C*, Ten days after DT injection. *D*, LCs were depleted in Langerin-DTR–knocked-in mice by DT (day −3) before UVB irradiation (day −2). They were sensitized (day 0) and challenged (day 5) with DNFB. *E*, LCs were depleted in Langerin-DTR–knocked-in mice by DT 10 d (day −12) before UVB irradiation (day −2). They were sensitized (day 0) and challenged (day 5) with DNFB. $*p < 0.05$.