T cells increased further as the disease progressed. Therefore, it is likely that the exhausted tumor-killing T cells are elevated in number in parallel with the elevation of its ligand on the tumor cell. This dual alternation strongly suggests the clinical importance of PD-L1 expression on melanoma cells. Our findings provide evidence of the clinical relevance of PD-L1 expression. When PD-L1 is highly expressed on tumor cells in biopsy or excised specimens from patients with melanoma, more careful follow-up and management may be required because of the predicted poor prognosis. Although treatments for melanoma are performed on the basis of the stage of this neoplasm,[29] the PD-L1 expression level may be an additional informative item for the consideration of treatments. Furthermore, it is possible that patients who have high PD-L1 expression are refractory to immunotherapies because of their "exhausted" tumoricidal T cells. For example, immunotherapy using tumor-antigenic peptides induces CTLs against tumor cells; however, when CTLs express PD-1, they may be less functional. Likewise, therapy with tumor antigen-specific MoAbs by antibody-dependent cellular cytotoxicity[2] may be ineffective in patients who have high PD-L1 expression.

Malignant melanoma is an immunogenic[1] but immunosuppressive[7] tumor. Our current finding that PD-L1 expression is correlated with tumor proliferation and patient survival indicates the immunosuppressive aspect of melanoma. Many groups of investigators have reported that blockade of the PD-1/PD-L1 interaction promotes tumor immunity.[35-37] Conversely, there remains a problem regarding PD-1/PD-L1 blockade because of the possible expansion of poorly immunogenic cells.[38] Further studies may be required to clarify the therapeutic effect of PD-1/PD-L1 blockade in malignant melanoma models and clinical trials.

CONFLICT OF INTEREST DISCLOSURES

Supported in part by the program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

REFERENCES

1. Walden P. Therapeutic vaccination for the treatment of malignant melanoma. *Recent Results Cancer Res.* 2007;176:219-227.
2. Slingluff CL Jr, Chianese-Bullock KA, Bullock TN, et al. Immunity to melanoma antigens: from self-tolerance to immunotherapy. *Adv Immunol.* 2006;90:243-295.
3. Lizee G, Radvanyi LG, Overwijk WW, Hwu P. Immunosuppression in melanoma immunotherapy: potential opportunities for intervention. *Clin Cancer Res.* 2006;12(7 pt 2):2359s-2365s.
4. Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer.* 1996;77:1303-1310.
5. Benlalam H, Labarriere N, Linard B, et al. Comprehensive analysis of the frequency of recognition of melanoma-associated antigen (MAA) by CD8 melanoma infiltrating lymphocytes (TIL): implications for immunotherapy. *Eur J Immunol.* 2001;31:2007-2015.
6. Letsch A, Keilholz U, Schadendorf D, et al. High frequencies of circulating melanoma-reactive CD8+ T cells in patients with advanced melanoma. *Int J Cancer.* 2000;87:659-664.
7. Nemunaitis J, Fong T, Shabe P, Martineau D, Ando D. Comparison of serum interleukin-10 (IL-10) levels between normal volunteers and patients with advanced melanoma. *Cancer Invest.* 2001;19:239-247.
8. Baumgartner J, Wilson C, Palmer B, Richter D, Banerjee A, McCarter M. Melanoma induces immunosuppression by up-regulating FOXP3(+) regulatory T cells. *J Surg Res.* 2007;141:72-77.
9. Miracco C, Mourmouras V, Biagioli M, et al. Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. *Oncol Rep.* 2007;18:1115-1122.
10. Mourmouras V, Fimiani M, Rubegni P, et al. Evaluation of tumour-infiltrating CD4+CD25+FOXP3+ regulatory T cells in human cutaneous benign and atypical naevi, melanomas and melanoma metastases. *Br J Dermatol.* 2007;157:531-539.
11. Strauss L, Bergmann C, Szczepanski MJ, Lang S, Kirkwood JM, Whiteside TL. Expression of ICOS on human melanoma-infiltrating CD4+CD25highFoxp3+ T regulatory cells: implications and impact on tumor-mediated immune suppression. *J Immunol.* 2008;180:2967-2980.
12. McCarter MD, Baumgartner J, Escobar GA, et al. Immunosuppressive dendritic and regulatory T cells are upregulated in melanoma patients. *Ann Surg Oncol.* 2007;14:2854-2860.
13. Torisu-Itakura H, Lee JH, Huynh Y, Ye X, Essner R, Morton DL. Monocyte-derived IL-10 expression predicts prognosis of stage IV melanoma patients. *J Immunother.* 2007;30:831-838.
14. Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer.* 1997;73:309-316.
15. Greenwald R, Freeman G, Sharpe A. The B7 family revisited. *Annu Rev Immunol.* 2005;23:515-548.
16. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol.* 2006;27:195-201.
17. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med.* 2006;203:883-895.
18. Tseng SY, Otsuji M, Gorski K, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med.* 2001;193:839-846.
19. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* 2002;8:793-800.
20. Yang W, Chen PW, Li H, Alizadeh H, Niederkorn JY. PD-L1: PD-1 interaction contributes to the functional

suppression of T-cell responses to human uveal melanoma cells in vitro. *Invest Ophthalmol Vis Sci.* 2008;49:2518-2525.
21. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia.* 2006;8:190-198.
22. Hamanishi J, Mandai M, Iwasaki M, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A.* 2007;104:3360-3365.
23. Thompson RH, Kwon ED. Significance of B7-H1 overexpression in kidney cancer. *Clin Genitourin Cancer.* 2006; 5:206-211.
24. Nomi T, Sho M, Akahori T, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res.* 2007;13:2151-2157.
25. Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res.* 2005;11:2947-2953.
26. Inman BA, Sebo TJ, Frigola X, et al. PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. *Cancer.* 2007;109:1499-1505.
27. Shimauchi T, Kabashima K, Nakashima D, et al. Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma. *Int J Cancer.* 2007;121:2585-2590.
28. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A.* 2002;99:12293-12297.
29. Balch CM, Buzaid AC, Soong SJ, et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol.* 2001;19:3635-3648.
30. Chen Q, Daniel V, Maher DW, Hersey P. Production of IL-10 by melanoma cells: examination of its role in immunosuppression mediated by melanoma. *Int J Cancer.* 1994;56:755-760.
31. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol.* 2004;173:1444-1453.
32. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci U S A.* 2008;105:9331-9336.
33. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature.* 2006;439:682-687.
34. Wong RM, Scotland RR, Lau RL, et al. Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. *Int Immunol.* 2007;19:1223-1234.
35. Blank C, Kuball J, Voelkl S, et al. Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro. *Int J Cancer.* 2006;119:317-327.
36. He YF, Zhang GM, Wang XH, et al. Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine. *J Immunol.* 2004;173:4919-4928.
37. Strome SE, Dong H, Tamura H, et al. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res.* 2003;63:6501-6505.
38. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol.* 2005;17:133-144.

BJD
British Journal of Dermatology

Comparison of skin barrier function and sensory nerve electric current perception threshold between IgE-high extrinsic and IgE-normal intrinsic types of atopic dermatitis

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Summary

Background Two types of atopic dermatitis (AD) have been proposed, with different pathophysiological mechanisms underlying this seemingly heterogeneous disorder. The extrinsic type shows high IgE levels presumably as a consequence of skin barrier damage and feasible allergen permeation, whereas the intrinsic type exhibits normal IgE levels and is not mediated by allergen-specific IgE.

Objectives To investigate the relationship between pruritus perception threshold and skin barrier function of patients with AD in a comparison between the extrinsic and intrinsic types.

Methods Enrolled in this study were 32 patients with extrinsic AD, 17 with intrinsic AD and 24 healthy individuals. The barrier function of the stratum corneum was assessed by skin surface hydration and transepidermal water loss (TEWL), and pruritus perception was evaluated by the electric current perception threshold (CPT) of sensory nerves upon neuroselective transcutaneous electric stimulation.

Results Skin surface hydration was significantly lower and TEWL was significantly higher in extrinsic AD than intrinsic AD or normal controls. Although there was no statistically significant difference in CPT among extrinsic AD, intrinsic AD and normal controls, CPT was significantly correlated with skin surface hydration and inversely with TEWL in intrinsic AD and normal controls, but not extrinsic AD. Finally, CPT was correlated with the visual analogue scale of itch in the non-lesional skin of patients with extrinsic but not intrinsic AD.

Conclusions Patients with extrinsic AD have an impaired barrier, which increases the pre-existing pruritus but rather decreases sensitivity to external stimuli. In contrast, patients with intrinsic AD retain a normal barrier function and sensory reactivity to external pruritic stimuli.

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Accepted for publication
24 July 2009

Key words
electric current perception threshold, extrinsic atopic dermatitis, intrinsic atopic dermatitis, skin barrier function

Conflicts of interest
None declared.

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DOI 10.1111/j.1365-2133.2009.09440.x

Atopic dermatitis (AD) is a chronic inflammatory skin disease with complicated pathophysiological mechanisms and causative agents. Two subtypes of AD have been proposed: extrinsic AD and intrinsic AD. The extrinsic type is the IgE-mediated common form of AD and is associated with respiratory allergies, such as rhinitis and asthma, and high serum levels of IgE.[1–3] In contrast, intrinsic AD is characterized by the absence of allergen-specific IgE and thus shows normal total IgE levels, although this newly introduced concept is still controversial among academic dermatologists.[1–3] Approximately 20%[4] or fewer[5] patients are estimated as having intrinsic AD. Its characteristics include female predominance, absence of atopic diseases, later onset of disease, and milder disease severity.[3–6] A history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, and hand and/or foot eczema are significantly less present in the intrinsic type, but Dennie-Morgan fold is positively associated with intrinsic AD.[3]

Several studies have suggested differences in various aspects of pathophysiology between extrinsic and intrinsic AD.

Increased transepidermal water loss (TEWL) and reduced skin surface hydration are hallmarks of atopic skin, and there are some differences in these values between the two types of AD.[4] Immunologically, surface expression of the high- and low-affinity receptor for IgE and of the interleukin (IL)-4Rα chain is elevated in monocytes from patients with extrinsic AD, but serum levels of IL-13 are significantly increased in patients with intrinsic AD.[7] Skin lesions of extrinsic AD show high levels of chemokines such as CCL18.[8] Expression of neurotrophins is increased comparably in both types.[6]

The stratum corneum of the epidermis, consisting of more than 10 layers of corneocytes and intercellular lipids, serves as the skin barrier.[9] In extrinsic AD, impairment of the skin barrier may be the primary condition which facilitates permeation of environmental allergens and leads to immunological responses such as elevation of allergen-specific IgE.[1] A recent finding of filaggrin gene mutations in a high percentage of patients with AD,[10,11] together with an older finding of ceramide reduction in the stratum corneum,[12,13] have further suggested the presence of skin barrier damage in extrinsic AD. On the other hand, intrinsic AD shows normal or mildly elevated serum IgE, in striking contrast to extrinsic AD.[14] The mechanisms underlying intrinsic AD remain unclear and more speculative than those underlying extrinsic AD.[2,6,8,15]

Patients with AD are well known to be sensitive to irritation from the environment due to the impaired skin barrier function. Given that the extrinsic and intrinsic types are different from each other in the skin barrier condition, each type might respond to external stimuli in a different manner. However, little is known regarding the difference in sensitivity to irritants and in elicitability of pruritus between the two types. It appears that most previous studies on sensitivity were performed in patients with extrinsic AD because of its higher incidence.

There are several reported methods to assess the threshold for the itch sensation to various environmental stimuli.[16–18] Local administration of histamine, either by needle injection or by iontophoresis, is one of the most common procedures for this purpose.[19] Electrically evoked itch is another useful method with the use of a neuroselective transcutaneous electrical stimulator, Neurometer™ CPT/C (Neurotron Inc., Baltimore, MD, U.S.A.), in a noninvasive fashion. Evaluation of electric current perception threshold (CPT) quantifies the sensory threshold to electric stimulation of the sensory nerves.[20,21] This device has been used mainly by neurologists to demonstrate abnormalities in a variety of neuropathic conditions. It does not measure the sensation only to histamine, but the device directly excites large- and small-diameter sensory nerve fibres in a differentiating fashion, independent of local factors such as skin thickness, temperature and substances involved in the induction of pruritus.[20,22] The CPT for 250- and 5-Hz frequency current emitted by the Neurometer CPT/C has been reported to enable quantification of the sensory threshold of Aδ- and C-fibres, respectively, that are thought to transmit the itch sensation from the skin. Therefore, this instrument allows us to investigate the elicitability of pruritus in patients with AD. Kobayashi *et al.*[23] have reported that patients with AD showed lower CPT than healthy controls, and CPT was inversely correlated with TEWL after tape stripping in normal subjects.

To address the differences in the mechanisms between extrinsic and intrinsic AD, we measured CPT in patients with AD, together with measurements of stratum corneum function. Our results show that there are prominent differences in the relationship of CPT with the skin barrier function between the two types.

Materials and methods

Participants

Patients over 18 years of age from our department were included in this study. Forty-nine patients with AD (25 men and 24 women), diagnosed in accordance with the Hanifin and Rajka classification,[24] and 24 healthy controls were enrolled in this study. The distribution of skin symptoms in all patients was characterized for adult AD. The hands, shoulders, neck, flexures and face were the predilection sites, while the extremities were less involved. Patients who had total serum IgE levels < 220 U mL^{-1} (normal range for Japanese subjects) were classified as having intrinsic AD, and those with levels > 400 U mL^{-1} were classified as having extrinsic AD. There was no patient with IgE levels between 220 and 400 U mL^{-1}. IgE RAST for *Dermatophagoides pteronyssinus* was measured in 20 patients. The disease activity was assessed by SCORAD (severity scoring of AD). Patient details are listed in Table 1. All participants provided written informed consent, and the institutional review board approved this study.

Electric current perception threshold and stratum corneum function

C-fibres are sensory nerves conducting itch and pain. Transcutaneous electric current with 5-Hz sine wave stimulates C-fibres, as assessed by the active action potentials of rat dorsal root ganglia.[21,25] Depending on the body surface site, 5-Hz electric current induces itch and/or pain. In addition, transcutaneous 250-Hz current has also been known to stimulate Aδ-fibres, thus inducing itch.[25] In this direct stimulation of nerve fibres with transcutaneous electric current, the condition of the stratum corneum possibly modifies the perception by affecting the current or other factors.

We measured CPT, skin surface hydration and TEWL at the nonlesional flexor forearm, the nonlesional lower leg, and at lesional skin on the trunk or extremities. When the patients had skin lesions on the flexor forearms or lower legs we avoided these regions and chose clinically normal areas on the volar skin as the sites to perform measurements on nonlesional skin. The patients did not apply any ointment or cream to the examined sites for at least 2 days before the measurements. Concerning the inflammatory state at the clinically normal sites tested, we have previously demonstrated

Table 1 Patients and healthy individuals enrolled in this study

	Extrinsic AD	Intrinsic AD	Healthy controls
Number of subjects	32 (21 men and 11 women)	17 (four men and 13 women)	24 (nine men and 15 women)
Age (years), mean ± SD (range)	30·0 ± 8·1 (19–51)	33·0 ± 10·4 (18–57)	28·9 ± 3·8 (23–37)
IgE (U mL^{-1}), mean ± SD (range)	5034·8 ± 7538·0 (436–30 000)	110·5 ± 66·8 (11–219)	–
SCORAD, mean ± SD (range)	41·8 ± 19·0 (4·6–84·5)	27·1 ± 20·6 (3·5–73)	–
VAS (nonlesional forearm), mean ± SD	30·9 ± 20·6	15·8 ± 22·1	–
VAS (nonlesional lower leg), mean ± SD	36·0 ± 24·9	20·4 ± 28·5	–
VAS (lesional skin), mean ± SD	55·3 ± 28·3	47·3 ± 36·0	–

AD, atopic dermatitis; VAS, visual analogue scale.

that clinically normal-appearing skin of patients with AD has no histological evidence of inflammation.[26] As control, we measured CPT, skin surface hydration and TEWL on the mid-flexor forearm and lower leg in healthy individuals. CPT was measured by using the Neurometer CPT/C as described previously.[23] Skin surface hydration was evaluated by capacitance using the Corneometer CM825 (Courage & Khazaka Electronic GmbH, Cologne, Germany) and was expressed as arbitrary units.[27] TEWL was measured by detecting the evaporated water using the VapoMeter SWL-2 (Delfin Technologies Ltd, Kuopio, Finland).

Visual analogue scale

All patients rated current itching on a 100-mm visual analogue scale (VAS)[28] at the following sites: nonlesional forearm, nonlesional lower leg and lesional skin.

Statistical analyses

Data were expressed as mean ± SD and assessed for statistical significance. We used Student's t-test to compare skin surface hydration, TEWL and CPT. A linear regression analysis was performed for correlations between the skin surface hydration or VAS and CPT, using Peason's correlation coefficient. For all tests, $P < 0{\cdot}05$ was considered statistically significant.

Results

Impaired skin barrier function in extrinsic but not intrinsic atopic dermatitis

Patients were classified as having extrinsic or intrinsic AD by means of IgE level (> 400 and < 220 U mL^{-1}, respectively). IgE RAST was scored by index values 0–6 according to the manufacturer's criteria (BML, Tokyo, Japan). An index value > 3 to *D. pteronyssinus* was obtained in 11 of 12 (92%) patients with extrinsic AD, but in only one of eight (12·5%) patients with intrinsic AD. Moreover, 67% of the patients with extrinsic AD showed a RAST score index value of 6, and none of the patients with intrinsic AD showed this highest score. As summarized in Table 1, more patients had extrinsic AD than intrinsic AD, and women predominated in the intrinsic type, as previously reported.[3–5] No significant difference was noted in age between the two types. There was a tendency that SCORAD and VAS at the three test sites were higher in extrinsic than intrinsic AD, as reported previously.[3–5]

As extrinsic AD is caused by external allergens invading through the damaged skin barrier, we initially examined the skin surface hydration (capacitance) and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between extrinsic and intrinsic AD. Skin surface hydration was significantly lower in extrinsic AD than in normal control subjects (Fig. 1). There was no significant difference in the hydration level between intrinsic AD and healthy controls. Extrinsic AD tended to show lower values than intrinsic AD at both sites. TEWL, another assessment of the barrier function, was statistically significantly higher in extrinsic AD than intrinsic AD and normal controls at the nonlesional forearm (Fig. 1). Thus, the skin barrier function was impaired in extrinsic AD and preserved in intrinsic AD, validating this clinical dichotomy.

Correlation between disease severity and pruritus in both extrinsic and intrinsic atopic dermatitis

In advance of analysing CPT, we also examined the correlation between the itch levels and SCORAD in the two types of AD. In both types, VAS scores on the lesional skin were correlated with SCORAD (Fig. 2), suggesting that both types of AD are associated with disease severity-dependent pruritus.

Significant correlation between electric current perception threshold (CPT) and skin surface hydration and between CPT and transepidermal water loss in intrinsic atopic dermatitis as well as in normal individuals

CPT for 5- and 250-Hz current stimuli was measured in patients with AD and normal volunteers. In all experiments, the results from 5- and 250-Hz current stimuli were virtually the same. Figure 3 shows the mean ± SD CPT in each group, and there was no significant difference between the groups in the nonlesional or lesional skin.

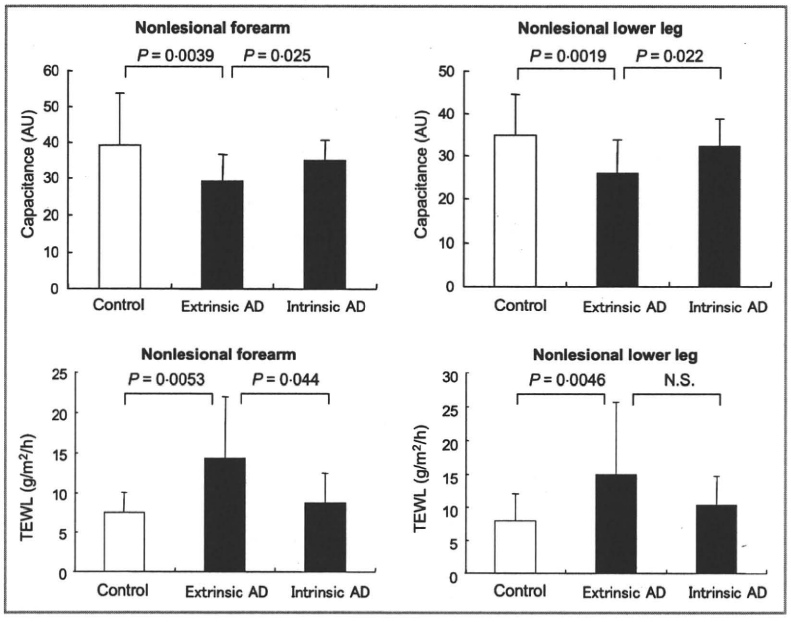

Fig 1. Skin surface hydration on nonlesional forearm and lower leg in extrinsic and intrinsic atopic dermatitis (AD) and healthy controls. Skin surface hydration is represented by capacitance in arbitrary units (AU). Results are shown as mean ± SD. N.S., not significant.

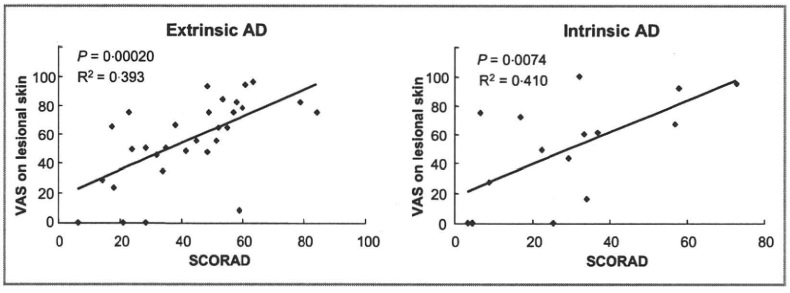

Fig 2. Relationship between SCORAD and visual analogue scale (VAS) score in extrinsic and intrinsic atopic dermatitis (AD).

When CPT was analysed in relation to skin surface hydration, an interesting finding was obtained. In normal subjects (control), CPT was significantly correlated with skin surface hydration (Fig. 4), suggesting that the water-poor cornified layer has a property to evoke pruritus in response to external stimuli. Similarly, the lesional skin of patients with intrinsic AD showed such a significant correlation between CPT and skin surface hydration. However, there was no correlation in extrinsic AD, as large individual variations of CPT were seen in the patients with extrinsic AD and low levels of skin surface hydration.

As to the relation of CPT to TEWL, there was no significant correlation between these two parameters in the lesional skin of patients with AD. However, CPT of nonlesional forearm, as assessed by 250-Hz sensitivity, was inversely correlated with TEWL in intrinsic AD as well as in controls (Fig. 5). The results suggest that intrinsic AD is associated with a normal stratum corneum and no excess elicitability of externally stimulated pruritus, while extrinsic AD does not show such a regular, surface hydration-related irritant perception.

Different electric current perception threshold levels in relation to pre-existing pruritus between extrinsic and intrinsic atopic dermatitis

It is possible that CPT is affected by the itch state in patients with AD. We therefore investigated the relationship between CPT and the pre-existing pruritus assessed by VAS. In the lesional skin of both types of AD there was no correlation between CPT and VAS (data not shown). In the nonlesional lower leg, however, CPT was significantly correlated with VAS in extrinsic but not intrinsic AD (Fig. 6), suggesting that the pre-existing pruritus rather downmodulates the sensitivity to external stimuli in extrinsic AD. The nonlesional forearm exhibited the same tendency but without statistical significance. Thus, the pruritic normal-appearing skin seems to be insensitive to further itchy stimuli in extrinsic AD.

Discussion

The precise concept of intrinsic AD in comparison with extrinsic AD has been a matter of controversy. Extrinsic AD seems

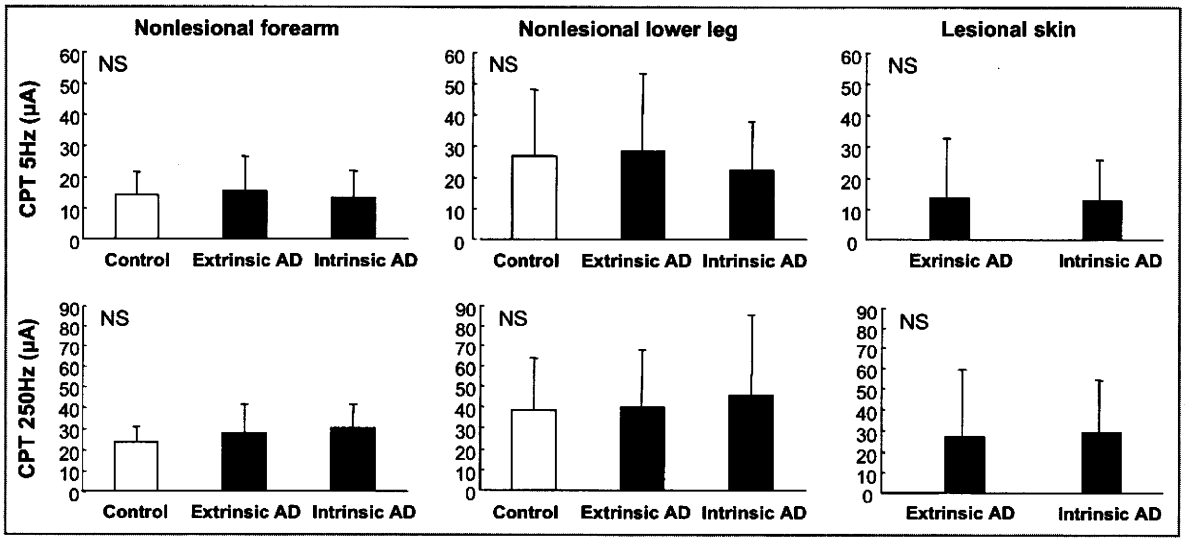

Fig 3. Electric current perception threshold (CPT) on nonlesional forearm or lower leg and lesional skin in extrinsic and intrinsic atopic dermatitis (AD). Results are shown as mean ± SD. N.S., not significant.

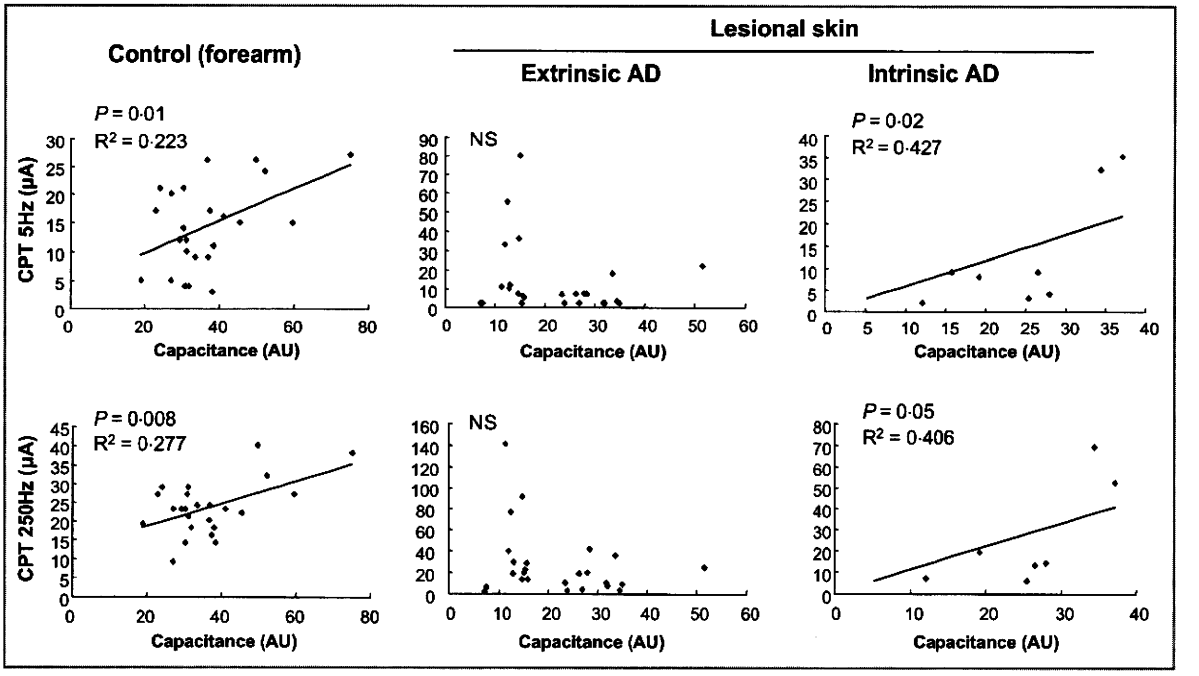

Fig 4. Relationship between skin surface hydration, represented by capacitance in arbitrary units (AU), and electric current perception threshold (CPT) in lesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and in nonlesional forearm skin of healthy controls. NS, not significant.

to be induced by sequential events, including impairment of stratum corneum, permeation of external substances, exposure of immunocompetent cells to the allergens, and T cell and IgE responses to the antigenic determinants.[1] Growing evidence has supported this mechanism underlying the extrinsic type. The recent finding that filaggrin gene mutations are a predisposing factor for AD has clearly demonstrated the presence of barrier impairment in patients with AD.[10,11] On the other hand, the pathophysiology of intrinsic AD remains obscure, and it may be difficult for clinicians to differentiate the two

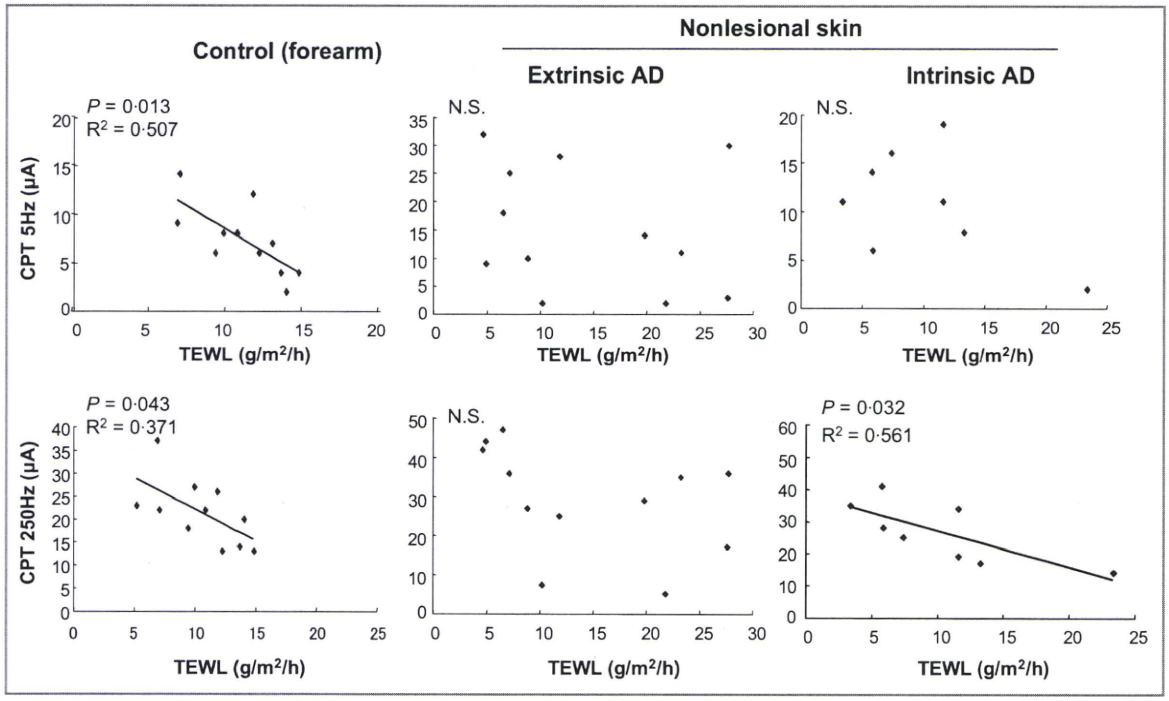

Fig 5. Relationship between transepidermal water loss (TEWL) and electric current perception threshold (CPT) in nonlesional forearm skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and healthy control skin. NS, not significant.

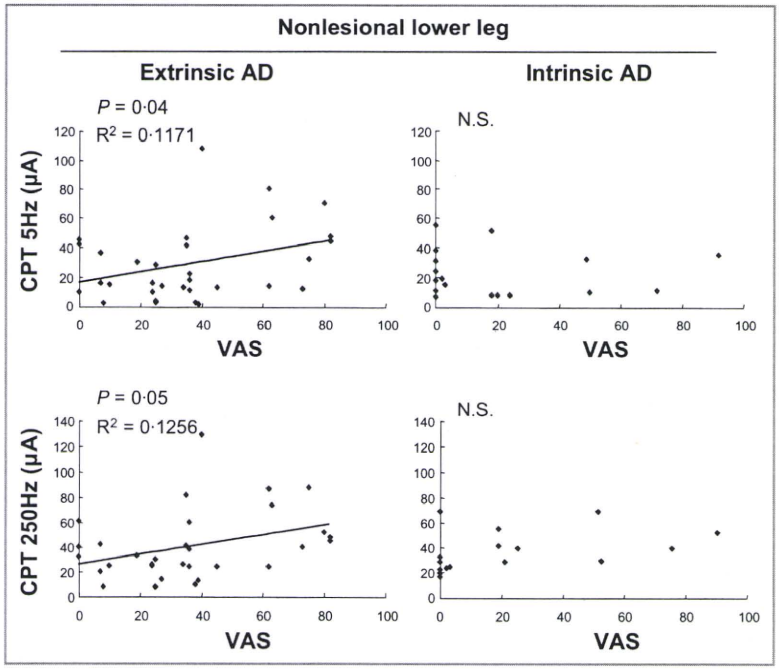

Fig 6. Relationship between visual analogue scale (VAS) score and electric current perception threshold (CPT) on nonlesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD). NS, not significant.

types accurately. Only one clear way to discriminate the two types is the serum levels of IgE,[29] but its precise cutoff value has not been determined.

In this study, we tentatively divided the patients with AD into two groups by IgE levels of > 400 and < 220 U mL^{-1}, because the normal range in Japanese individuals is < 220 U mL^{-1}. This division was confirmed by a high percentage and high scores of positive RAST to *D. pteronyssinus* in extrinsic AD and a low percentage in intrinsic AD. We found that more of our patients had intrinsic than extrinsic AD, and that women were more likely than men to have intrinsic AD, as already reported in previous studies.[5] In contrast to

extrinsic AD, intrinsic AD is thought to show a normal skin barrier function. We validated this general concept by measuring skin surface hydration and TEWL, and found no significant difference in these values between patients with intrinsic AD and normal individuals, while the patients with extrinsic AD had lower surface hydration levels and higher TEWL levels than the normal subjects.

C-fibres (unmyelinated fibres) are sensory nerves conducting pruritus. A transcutaneous electric current at 5 Hz can stimulate C-fibres.[25] Alternating current stimulus at 250 Hz activates Aδ-fibres (small myelinated fibres), which may also participate in itch. The condition of the stratum corneum may modify the perception by affecting the current or other factors. In our study, low levels of hydration of the stratum corneum reduced CPT. This suggests that the itch perception to external stimuli is promoted in skin with low hydration.

The difference in the barrier function between the extrinsic and intrinsic types raised the possibility that the elicitability of pruritus differs between them. In normal individuals, CPT and skin surface hydration or TEWL were correlated with each other, suggesting that the barrier-damaged skin is sensitive to external irritants. In normal individuals, Kobayashi *et al.*[23] have reported that CPT is inversely correlated with TEWL levels after tape stripping, providing further evidence that barrier damage leads to elicitability of sensation. In our study, the correlation between CPT and skin surface hydration and the inverse correlation between CPT and TEWL were also found in patients with intrinsic AD, suggesting that intrinsic AD shows a normal skin barrier and elicitability of sensation to external stimuli. In contrast, the patients with extrinsic AD showed different elicitability with individual variations presumably due to the low surface hydration. Kobayashi *et al.*[23] have also shown that the skin of patients with AD is not extremely sensitive as compared with that of normal individuals to the electric stimulation of their Aδ- and C-fibres. Their patients seem to include those with both extrinsic and intrinsic AD. Our results suggest that some patients with extrinsic AD have high CPT levels despite the impaired barrier function.

Pre-existing pruritus elevated CPT on the nonlesional skin of patients with extrinsic AD, as CPT and VAS were correlated with each other in the nonlesional sites of the extrinsic type. Accordingly, Ikoma *et al.*[30] found that when histamine prick tests are performed in nonlesional skin of patients with AD, itch rating increases more slowly and is significantly lower than in controls.[30] Our unexpected finding was not observed in intrinsic AD. It is possible that in the already itchy skin of extrinsic AD, Aδ- and C-fibres are in a stimulated state, resulting in insensitivity to external irritants, while the steady-state interaction between the barrier and sensory fibres might be kept in intrinsic AD. The end of sensory fibres in the skin of extrinsic AD seems to be continuously stimulated by the damaged stratum corneum, leading to the elevated CPT.

Our study suggests that the two types of AD are different from each other in the mode of elicitability of pruritus, because of the different skin barrier states between them. Furthermore, it was recently found that IgE autoantibodies can target keratinocytes in AD; this might promote barrier damage and modify resultant itch elicitability in extrinsic AD.[31] As the response of sensory nerves to irritants appears to be intact in intrinsic AD, the mechanisms of pruritus underlying this type of AD are an important issue to be elucidated.

Acknowledgments

This work is supported by Grants-in Aid for Science Research from the Ministry of Health, Labour, and Welfare of Japan.

References

1 Pónyai G, Hidvégi B, Németh I *et al.* Contact and aeroallergens in adulthood atopic dermatitis. *J Eur Acad Dermatol Venereol* 2008; **22**:1346–55.
2 Wang IJ, Hsieh WS, Guo YL *et al.* Neuro-mediators as predictors of paediatric atopic dermatitis. *Clin Exp Allergy* 2008; **38**:1302–8.
3 Brenninkmeijer EE, Spuls PI, Legierse CM *et al.* Clinical differences between atopic and atopiform dermatitis. *J Am Acad Dermatol* 2008; **58**:407–14.
4 Choi SJ, Song MG, Sung WT *et al.* Comparison of transepidermal water loss, capacitance and pH values in the skin between intrinsic and extrinsic atopic dermatitis patients. *J Korean Med Sci* 2003; **18**:93–6.
5 Fölster-Holst R, Pape M, Buss YL *et al.* Low prevalence of the intrinsic form of atopic dermatitis among adult patients. *Allergy* 2006; **61**:629–32.
6 Paap U, Werfel T, Goltz C *et al.* Circulating levels of brain-derived neurotropic factor correlate with disease severity in the intrinsic type of atopic dermatitis. *Allergy* 2006; **61**:1416–18.
7 Novak N, Kruse S, Kraft S *et al.* Dichotomic nature of atopic dermatitis reflected by combined analysis of monocyte immunophenotyping and single nucleotide polymorphisms of the interleukin-4/interleukin-13 receptor gene: the dichotomy of extrinsic and intrinsic atopic dermatitis. *J Invest Dermatol* 2002; **119**:870–5.
8 Park CO, Lee HJ, Lee JH *et al.* Increased expression of CC chemokine ligand 18 in extrinsic atopic dermatitis patients. *Exp Dermatol* 2008; **17**:24–9.
9 Vielhaber G, Pfeiffer S, Brade L *et al.* Localization of ceramide and glucosylceramide in human epidermis by immunogold electron microscopy. *J Invest Dermatol* 2001; **117**:1126–36.
10 Henderson J, Northstone K, Lee SP *et al.* The burden of disease associated with filaggrin mutations: a population-based, longitudinal birth cohort study. *J Allergy Clin Immunol* 2008; **121**:872–7.
11 Weidinger S, Illig T, Baurecht H *et al.* Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol* 2006; **118**:214–19.
12 Imokawa G, Abe A, Jin K *et al.* Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol* 1991; **96**:523–6.
13 Murata Y, Ogata J, Higaki Y *et al.* Abnormal expression of sphingomyelin acylase in atopic dermatitis: an etiologic factor for ceramide deficiency? *J Invest Dermatol* 1996; **106**:1242–9.
14 Park JH, Choi YL, Namkung JH *et al.* Characteristics of extrinsic vs. intrinsic atopic dermatitis in infancy: correlations with laboratory variables. *Br J Dermatol* 2006; **155**:778–83.
15 Casagrande BF, Flückiger S, Linder MT *et al.* Sensitization to the yeast *Malassezia sympodialis* is specific for extrinsic and intrinsic atopic eczema. *J Invest Dermatol* 2006; **126**:2414–21.
16 Felix R, Shuster S. A new method for the measurement of itch and the response to treatment. *Br J Dermatol* 1975; **93**:303–12.

17 Ebata T, Iwasaki S, Kamide R *et al.* Use of a wrist activity monitor for the measurement of nocturnal scratching in patients with atopic dermatitis. *Br J Dermatol* 2001; **144**:305–9.
18 Wahlgren CF. Measurement of itch. *Semin Dermatol* 1995; **14**:277–84.
19 Heyer G, Hornstein OP, Handwerker HO. Skin reactions and itch sensation induced by epicutaneous histamine application in atopic dermatitis and controls. *J Invest Dermatol* 1989; **93**:492–6.
20 Katims JJ, Naviasky EH, Ng LK *et al.* New screening device for assessment of peripheral neuropathy. *J Occup Med* 1986; **28**:1219–21.
21 Ozawa M, Tsuchiyama K, Gomi R *et al.* Neuroselective transcutaneous electric stimulation reveals body area-specific differences in itch perception. *J Am Acad Dermatol* 2006; **55**:996–1002.
22 Tay B, Wallace MS, Irving G. Quantitative assessment of differential sensory blockade after lumbar epidural lidocaine. *Anesth Analg* 1997; **84**:1071–5.
23 Kobayashi H, Kikuchi K, Tsubono Y *et al.* Measurement of electrical current perception threshold of sensory nerves for pruritus in atopic dermatitis patients and normal individuals with various degrees of mild damage to the stratum corneum. *Dermatology* 2003; **206**:204–11.
24 Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980; **92** (Suppl.):44–7.
25 Koga K, Furue H, Rashid M *et al.* Selective activation of primary afferent fibers evaluated by sine-wave electrical stimulation. *Mol Pain* 2005; **1**:13.
26 Wakita H, Sakamoto T, Tokura Y *et al.* E-selectin and vascular cell adhesion molecule-1 as critical adhesion molecules for infiltration of T lymphocytes and eosinophils in atopic dermatitis. *J Cutan Pathol* 1994; **21**:33–9.
27 Hata M, Tokura Y, Takigawa M *et al.* Assessment of epidermal barrier function by photoacoustic spectrometry in relation to its importance in the pathogenesis of atopic dermatitis. *Lab Invest* 2002; **82**:1451–61.
28 Syed S, Weibel L, Kennedy H *et al.* A pilot study showing pulsed-dye laser treatment improves localized areas of chronic atopic dermatitis. *Clin Exp Dermatol* 2008; **33**:243–8.
29 Flohr C, Johansson SG, Wahlgren CF *et al.* How atopic is atopic dermatitis? *J Allergy Clin Immunol* 2004; **114**:150–8.
30 Ikoma A, Rukwied R, Ständer S *et al.* Neuronal sensitization for histamine-induced itch in lesional skin of patients with atopic dermatitis. *Arch Dermatol* 2003; **139**:1455–8.
31 Altrichter S, Kriehuber E, Moser J *et al.* Serum IgE autoantibodies target keratinocytes in patients with atopic dermatitis. *J Invest Dermatol* 2008; **128**:2232–9.

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

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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

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Received for publication October 7, 2009. Accepted for publication March 17, 2010.

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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.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903254

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

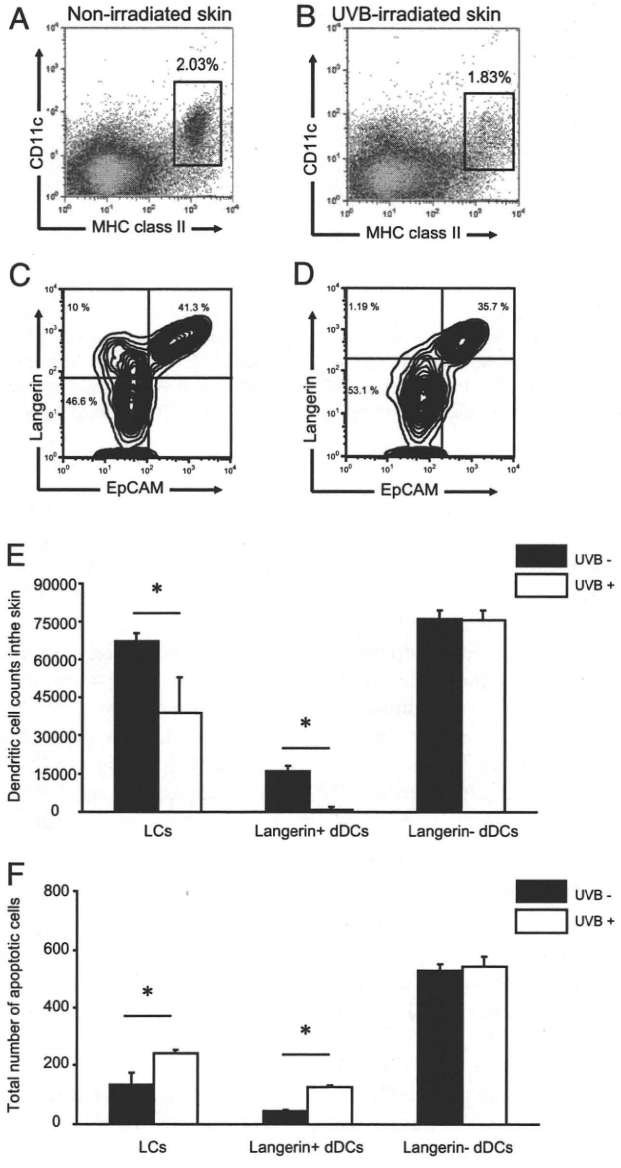

FIGURE 1. Numerical alterations of cutaneous DCs after UVB irradiation of the skin. Single-cell suspensions were stained with APC-conjugated anti-MHC class II and APC-Cy7-conjugated CD11c Abs and subjected to flow cytometric analysis. *A*, Nonirradiated skin. *B*, UVB-irradiated skin. *C*, With the use 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

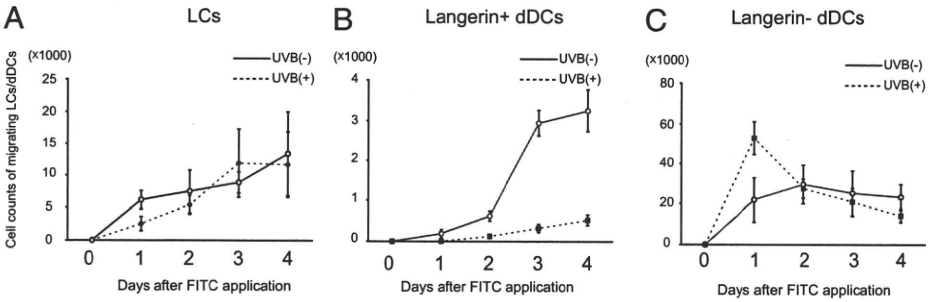

FIGURE 2. Numbers of LCs, Langerin$^+$ dDCs, and Langerin$^-$ dDCs in DLNs after FITC application to UVB-irradiated or nonirradiated skin. Mice were irradiated with UVB (300 mJ/cm^2) on day −2 or nonirradiated and sensitized with FITC on day 0. On days 1–4, DLNs were collected and stained for CD11c, 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

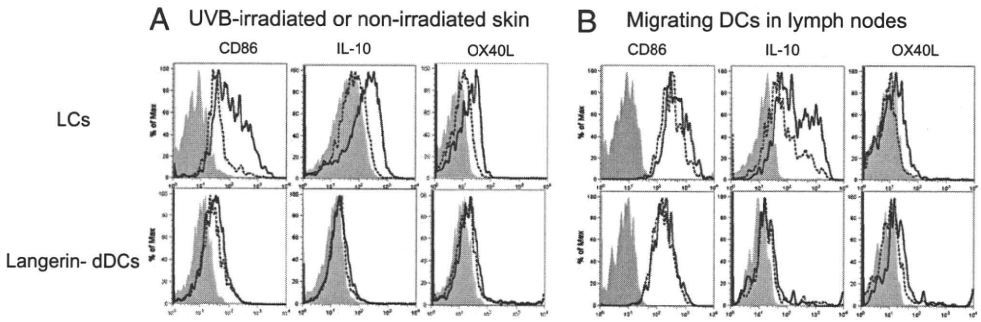

FIGURE 3. Expression of surface CD86, intracellular IL-10, and surface OX40L in LCs and Langerin$^-$ dDCs from the skin and DLNs. *A*, Epidermal cell suspensions were obtained from UVB-irradiated skin 24 h after UVB exposure or nonirradiated skin. Solid line, UVB-irradiated skin; dotted line, nonirradiated 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.

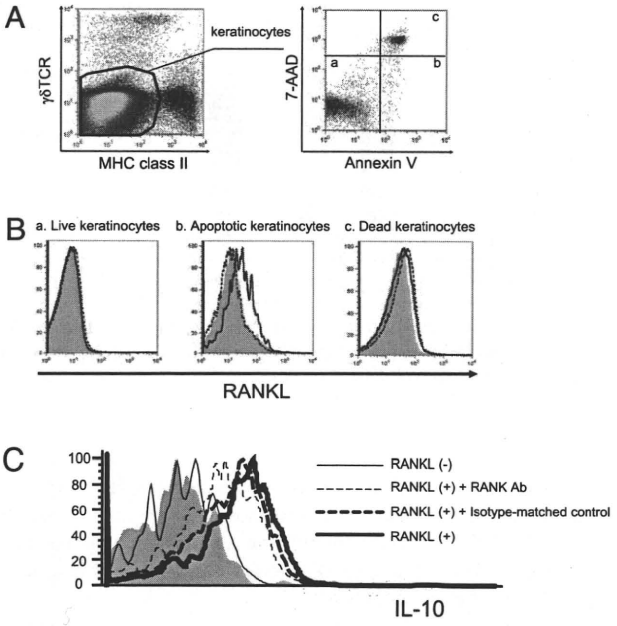

FIGURE 4. RANKL expression in apoptotic keratinocytes from UVB-irradiated skin and RANKL promotion of LC IL-10 production. *A*, Epidermal cell suspensions were obtained from UVB-irradiated (300 mJ/cm^2) skin 24 h postirradiation. Keratinocytes were identified as the MHC class II$^-$ and γδTCR$^-$ fraction 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.

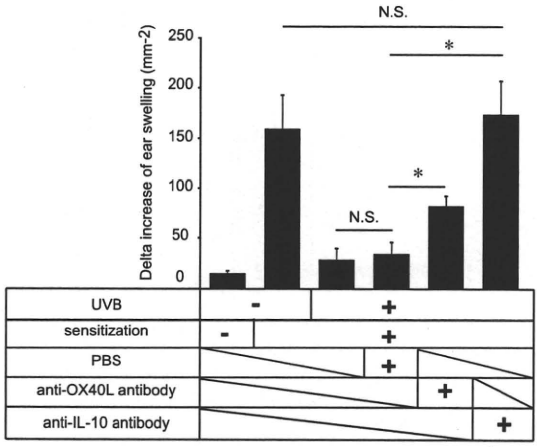

FIGURE 5. Effects of administration of IL-10–neutralizing and OX40L-blocking Abs. Mice were irradiated with UVB (300 mJ/cm^2) on day −2, sensitized with DNFB on day 0, and challenged with DNFB on day 5. IL-10–neutralizing Ab (25 μg per mouse), OX40-blocking Ab (10 μg per mouse), or PBS (for control)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

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.

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

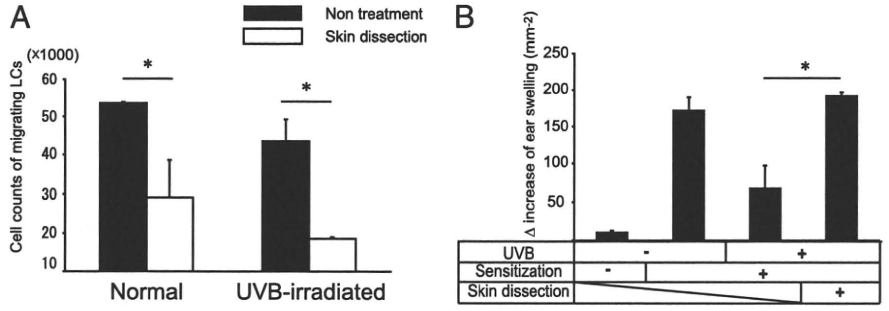

FIGURE 6. Effect of dissection of UVB-irradiated and/or hapten-applied skin on CHS. *A*, The number of LCs migrating to the lymph nodes on day 5 in mice receiving skin dissection on day 1. The LC counts were significantly decreased in mice receiving skin dissection (white bar) compared with non-dissected 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.

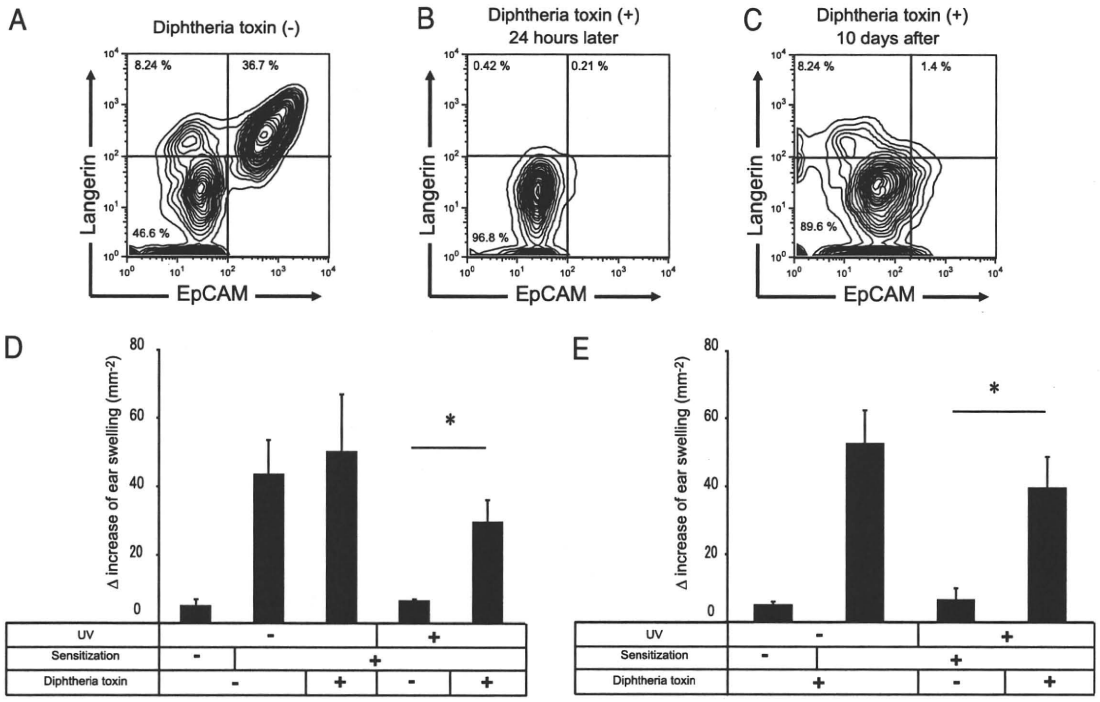

FIGURE 7. UVB-induced immunosuppression in LC-depleted mice. LCs (EpCAM$^+$ Langerin$^+$ cells) and Langerin$^+$ dDCs (EpCAM$^-$ Langerin$^+$ cells) were depleted by DT (100 ng per mouse) in Langerin-DTR–knocked-in mice, Langerin$^+$ dDCs repopulated 10 d later. *A*, Nonirradiated skin. *B*, Twenty-four 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. $*p < 0.05$.

We confirmed the crucial role of LCs in the UVB-induced immunosuppression by two strategies. One is that UVB-irradiated, hapten-painted skin was dissected 1 d after hapten application. By this treatment, a considerable number of dDCs could migrate to the DLNs, but LC migration was inhibited, and as a result, the CHS response was restored. In the other study, LCs were more effectively depleted by DT injection to Langerin-DTR–knocked-in mice, and the UVB-induced suppression was markedly abolished in the mice, clearly demonstrating the necessity of LCs for the suppression. Recently, Wang et al. (60) have reported that LCs play no critical role for the UVB-induced immunosuppression. There are major differences in UVB-irradiation doses and mouse strains between their and our studies. Wang et al. (60) irradiated C57BL/6 with UVB at 45 mJ/cm^2 for 3 consecutive days. We irradiated BALB/c mice with UVB at 300 mJ/cm^2 once. 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 single irradiation of the skin to UVB and following FITC paining is convenient for the study of DC migration to the DLN, we used this protocol and this strain of mice in our study. These differences in the UVB dose and mouse strain possibly give rise to the different results. Alternatively, because their study includes no adoptive transfer experiment, it is unclear whether Tregs are induced in their experimental system. As shown in our study, Langerin$^+$ dDCs become apoptotic after UVB irradiation and cannot migrate to the DLN following hapten application. Given the CHS-inductive role of Langerin$^+$ dermal DCs (37), the abrogation of them by UVB may attenuate the sensitization process of CHS to hapten even without the induction of Tregs. The suppression observed by Wang et al. (60) might be related to this phenomenon. Moreover, the study using the mice deprived of LCs and repopulated with Langerin$^+$ dDCs showed that Langerin$^+$ dDCs are not a requirement for the suppression. Therefore, the UVB immunosuppression in our system is not merely caused by the attenuation of inductive role of Langerin$^+$ dDCs.

The UVB-induced immune tolerance is mediated by Ag-specific Tregs, as the suppression can be adoptively transferred into naive recipients (61). According to the recent observations, UVB-induced Tregs have the CD4$^+$CD25$^+$ phenotype (62), express CTLA-4 (13), and bind to the lectin dectin-2 (61). The Tregs are modulated by IL-10 (46), but also release IL-10 upon antigenic stimulation (13). We have previously reported that cutaneous hypersensitivities to hapten are controlled by Th1 chemokines from keratinocytes and Th2 chemokines from LCs (63). IL-10 released from these LCs at the time of Ag presentation can induce Tregs and also may inhibit the priming of Ag-specific effector T cells. Knowledge of the roles of DCs in immunosuppression may help to further explain the pathways regulating Treg induction and desensitization and provide an insight for immunosuppressive treatments, exemplified by UV therapy for skin diseases.

Disclosures

The authors have no financial conflicts of interest.

References

1. Fisher, G. J., Z. Q. Wang, S. C. Datta, J. Varani, S. Kang, and J. J. Voorhees. 1997. Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.* 337: 1419–1428.
2. Gilchrest, B. A. 1990. Actinic injury. *Annu. Rev. Med.* 41: 199–210.
3. Kraemer, K. H. 1997. Sunlight and skin cancer: another link revealed. *Proc. Natl. Acad. Sci. USA* 94: 11–14.
4. Kulms, D., and T. Schwarz. 2000. Molecular mechanisms of UV-induced apoptosis. *Photodermatol. Photoimmunol. Photomed.* 16: 195–201.
5. Tokura, Y. 1992. Mechanisms of local, low-dose UVB-induced immunosuppression in contact hypersensitivity. *J. Dermatol.* 19: 923–931.
6. Toews, G. B., P. R. Bergstresser, and J. W. Streilein. 1980. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* 124: 445–453.
7. Elmets, C. A., P. R. Bergstresser, R. E. Tigelaar, P. J. Wood, and J. W. Streilein. 1983. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* 158: 781–794.
8. Satoh, T., Y. Tokura, Y. Satoh, and M. Takigawa. 1990. Ultraviolet-induced suppressor T cells and factor(s) in murine contact photosensitivity. III. Mode of action of T-cell-suppressor factor(s) and interaction with cytokines. *Cell. Immunol.* 131: 120–131.
9. Tokura, Y., Y. Miyachi, M. Takigawa, and M. Yamada. 1987. Ultraviolet-induced suppressor T cells and factor(s) in murine contact photosensitivity. I. Biological and immunochemical characterization of factor(s) extracted from suppressor T cells. *Cell. Immunol.* 110: 305–320.
10. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
11. McHugh, R. S., and E. M. Shevach. 2002. The role of suppressor T cells in regulation of immune responses. *J. Allergy Clin. Immunol.* 110: 693–702.
12. Roncarolo, M. G., R. Bacchetta, C. Bordignon, S. Narula, and M. K. Levings. 2001. Type 1 T regulatory cells. *Immunol. Rev.* 182: 68–79.
13. Schwarz, A., S. Beissert, K. Grosse-Heitmeyer, M. Gunzer, J. A. Bluestone, S. Grabbe, and T. Schwarz. 2000. Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J. Immunol.* 165: 1824–1831.
14. Kang, K., C. Hammerberg, L. Meunier, and K. D. Cooper. 1994. CD11b+ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J. Immunol.* 153: 5256–5264.
15. Baum, P. R., R. B. Gayle, III, F. Ramsdell, S. Srinivasan, R. A. Sorensen, M. L. Watson, M. F. Seldin, E. Baker, G. R. Sutherland, K. N. Clifford, et al. 1994. Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J.* 13: 3992–4001.
16. Godfrey, W. R., F. F. Fagnoni, M. A. Harara, D. Buck, and E. G. Engleman. 1994. Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. *J. Exp. Med.* 180: 757–762.
17. Mallett, S., S. Fossum, and A. N. Barclay. 1990. Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor. *EMBO J.* 9: 1063–1068.
18. Miura, S., K. Ohtani, N. Numata, M. Niki, K. Ohbo, Y. Ina, T. Gojobori, Y. Tanaka, H. Tozawa, M. Nakamura, et al. 1991. Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. *Mol. Cell. Biol.* 11: 1313–1325.
19. Paterson, D. J., W. A. Jefferies, J. R. Green, M. R. Brandon, P. Corthesy, M. Puklavec, and A. F. Williams. 1987. Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol. Immunol.* 24: 1281–1290.
20. Stüber, E., M. Neurath, D. Calderhead, H. P. Fell, and W. Strober. 1995. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* 2: 507–521.
21. Brocker, T., A. Gulbranson-Judge, S. Flynn, M. Riedinger, C. Raykundalia, and P. Lane. 1999. CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. *Eur. J. Immunol.* 29: 1610–1616.
22. Ohshima, Y., Y. Tanaka, H. Tozawa, Y. Takahashi, C. Maliszewski, and G. Delespesse. 1997. Expression and function of OX40 ligand on human dendritic cells. *J. Immunol.* 159: 3838–3848.
23. Weinberg, A. D., K. W. Wegmann, C. Funatake, and R. H. Whitham. 1999. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J. Immunol.* 162: 1818–1826.
24. Imura, A., T. Hori, K. Imada, T. Ishikawa, Y. Tanaka, M. Maeda, S. Imamura, and T. Uchiyama. 1996. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J. Exp. Med.* 183: 2185–2195.
25. Ohshima, Y., L. P. Yang, T. Uchiyama, Y. Tanaka, P. Baum, M. Sergerie, P. Hermann, and G. Delespesse. 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood* 92: 3338–3345.
26. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat. Immunol.* 3: 33–41.
27. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
28. Yagi, H., Y. Tokura, H. Wakita, F. Furukawa, and M. Takigawa. 1996. TCRV beta 7+ Th2 cells mediate UVB-induced suppression of murine contact photosensitivity by releasing IL-10. *J. Immunol.* 156: 1824–1831.
29. Kabashima, K., T. Murata, H. Tanaka, T. Matsuoka, D. Sakata, N. Yoshida, K. Katagiri, T. Kinashi, T. Tanaka, M. Miyasaka, et al. 2003. Thromboxane A2 modulates interaction of dendritic cells and T cells and regulates acquired immunity. *Nat. Immunol.* 4: 694–701.
30. Probst, H. C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek. 2005. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat. Immunol.* 6: 280–286.

31. Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
32. Ginhoux, F., M. P. Collin, M. Bogunovic, M. Abel, M. Leboeuf, J. Helft, J. Ochando, A. Kissenpfennig, B. Malissen, M. Grisotto, et al. 2007. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J. Exp. Med.* 204: 3133–3146.
33. Kaplan, D. H., A. Kissenpfennig, and B. E. Clausen. 2008. Insights into Langerhans cell function from Langerhans cell ablation models. *Eur. J. Immunol.* 38: 2369–2376.
34. Nagao, K., F. Ginhoux, W. W. Leitner, S. Motegi, C. L. Bennett, B. E. Clausen, M. Merad, and M. C. Udey. 2009. Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc. Natl. Acad. Sci. USA* 106: 3312–3317.
35. Poulin, L. F., S. Henri, B. de Bovis, E. Devilard, A. Kissenpfennig, and B. Malissen. 2007. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J. Exp. Med.* 204: 3119–3131.
36. Wang, L., L. S. Bursch, A. Kissenpfennig, B. Malissen, S. C. Jameson, and K. A. Hogquist. 2008. Langerin expressing cells promote skin immune responses under defined conditions. *J. Immunol.* 180: 4722–4727.
37. Kissenpfennig, A., S. Henri, B. Dubois, C. Laplace-Builhé, P. Perrin, N. Romani, C. H. Tripp, P. Douillard, L. Leserman, D. Kaiserlian, et al. 2005. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22: 643–654.
38. Tokura, Y., H. Yagi, H. Hashizume, J. Yagi, F. Furukawa, and M. Takigawa. 1994. Accessory cell ability of Langerhans cells for superantigen is resistant to ultraviolet-B light. *Photochem. Photobiol.* 60: 147–153.
39. Goldszmid, R. S., J. Idoyaga, A. I. Bravo, R. Steinman, J. Mordoh, and R. Wainstok. 2003. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. *J. Immunol.* 171: 5940–5947.
40. Romani, N., S. Holzmann, C. H. Tripp, F. Koch, and P. Stoitzner. 2003. Langerhans cells - dendritic cells of the epidermis. *APMIS* 111: 725–740.
41. Kaplan, D. H., M. C. Jenison, S. Saeland, W. D. Shlomchik, and M. J. Shlomchik. 2005. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 23: 611–620.
42. Fukunaga, A., N. M. Khaskhely, C. S. Sreevidya, S. N. Byrne, and S. E. Ullrich. 2008. Dermal dendritic cells, and not Langerhans cells, play an essential role in inducing an immune response. *J. Immunol.* 180: 3057–3064.
43. Loser, K., A. Mehling, S. Loeser, J. Apelt, A. Kuhn, S. Grabbe, T. Schwarz, J. M. Penninger, and S. Beissert. 2006. Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. *Nat. Med.* 12: 1372–1379.
44. Yoshiki, R., K. Kabashima, K. Sugita, K. Atarashi, T. Shimauchi, and Y. Tokura. 2009. IL-10-producing Langerhans cells and regulatory T cells are responsible for depressed contact hypersensitivity in grafted skin. *J. Invest. Dermatol.* 129: 705–713.
45. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.
46. Ghoreishi, M., and J. P. Dutz. 2006. Tolerance induction by transcutaneous immunization through ultraviolet-irradiated skin is transferable through CD4+ CD25+ T regulatory cells and is dependent on host-derived IL-10. *J. Immunol.* 176: 2635–2644.
47. Girolomoni, G., P. Gisondi, C. Ottaviani, and A. Cavani. 2004. Immunoregulation of allergic contact dermatitis. *J. Dermatol.* 31: 264–270.
48. Enk, A. H., J. Saloga, D. Becker, M. Mohamadzadeh, and J. Knop. 1994. Induction of hapten-specific tolerance by interleukin 10 in vivo. *J. Exp. Med.* 179: 1397–1402.
49. Niizeki, H., and J. W. Streilein. 1997. Hapten-specific tolerance induced by acute, low-dose ultraviolet B radiation of skin is mediated via interleukin-10. *J. Invest. Dermatol.* 109: 25–30.
50. Rivas, J. M., and S. E. Ullrich. 1992. Systemic suppression of delayed-type hypersensitivity by supernatants from UV-irradiated keratinocytes. An essential role for keratinocyte-derived IL-10. *J. Immunol.* 149: 3865–3871.
51. Enk, A. H., and S. I. Katz. 1992. Identification and induction of keratinocyte-derived IL-10. *J. Immunol.* 149: 92–95.
52. Ullrich, S. E. 1994. Mechanism involved in the systemic suppression of antigen-presenting cell function by UV irradiation. Keratinocyte-derived IL-10 modulates antigen-presenting cell function of splenic adherent cells. *J. Immunol.* 152: 3410–3416.
53. Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2: 725–731.
54. Maeda, A., A. Schwarz, K. Kernebeck, N. Gross, Y. Aragane, D. Peritt, and T. Schwarz. 2005. Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. *J. Immunol.* 174: 5968–5976.
55. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2: 965–975.
56. Steinman, R. M., D. Hawiger, K. Liu, L. Bonifaz, D. Bonnyay, K. Mahnke, T. Iyoda, J. Ravetch, M. Dhodapkar, K. Inaba, and M. Nussenzweig. 2003. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann. N. Y. Acad. Sci.* 987: 15–25.
57. Higgins, L. M., S. A. McDonald, N. Whittle, N. Crockett, J. G. Shields, and T. T. MacDonald. 1999. Regulation of T cell activation in vitro and in vivo by targeting the OX40-OX40 ligand interaction: amelioration of ongoing inflammatory bowel disease with an OX40-IgG fusion protein, but not with an OX40 ligand-IgG fusion protein. *J. Immunol.* 162: 486–493.
58. MacPhee, I. A., H. Yagita, and D. B. Oliveira. 2006. Blockade of OX40-ligand after initial triggering of the T helper 2 response inhibits mercuric chloride-induced autoimmunity. *Immunology* 117: 402–408.
59. Takeda, I., S. Ine, N. Killeen, L. C. Ndhlovu, K. Murata, S. Satomi, K. Sugamura, and N. Ishii. 2004. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. *J. Immunol.* 172: 3580–3589.
60. Wang, L., S. C. Jameson, and K. A. Hogquist. 2009. Epidermal Langerhans cells are not required for UV-induced immunosuppression. *J. Immunol.* 183: 5548–5553.
61. Aragane, Y., A. Maeda, A. Schwarz, T. Tezuka, K. Ariizumi, and T. Schwarz. 2003. Involvement of dectin-2 in ultraviolet radiation-induced tolerance. *J. Immunol.* 171: 3801–3807.
62. Schwarz, A., A. Maeda, M. K. Wild, K. Kernebeck, N. Gross, Y. Aragane, S. Beissert, D. Vestweber, and T. Schwarz. 2004. Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J. Immunol.* 172: 1036–1043.
63. Mori, T., K. Kabashima, R. Yoshiki, K. Sugita, N. Shiraishi, A. Onoue, E. Kuroda, M. Kobayashi, U. Yamashita, and Y. Tokura. 2008. Cutaneous hypersensitivities to hapten are controlled by IFN-gamma-upregulated keratinocyte Th1 chemokines and IFN-gamma-downregulated langerhans cell Th2 chemokines. *J. Invest. Dermatol.* 128: 1719–1727.

The American Journal of Pathology, Vol. 177, No. 4, October 2010

DOI: 10.2353/ajpath.2010.100119

Immunopathology and Infectious Diseases

FTY720 Regulates Bone Marrow Egress of Eosinophils and Modulates Late-Phase Skin Reaction in Mice

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Eosinophilia in the blood and skin is frequently observed in patients with certain inflammatory skin diseases, such as atopic dermatitis. However, the mechanism underlying eosinophil circulation and the role of eosinophils in cutaneous immune responses remain unclear. In repeated hapten application-induced cutaneous responses in BALB/c mice, the administration of FTY720 before the last challenge decreased the number of skin-infiltrating eosinophils and reduced the late-phase reaction. A similar reduction of the late-phase reaction was observed by a sphingosine-1-phosphate G protein-coupled receptor (S1P1)-selective agonist, SEW2871. We monitored numerous alterations of eosinophils in the blood, spleen, bone marrow, and lymph nodes of interleukin-5 transgenic mice, used as an eosinophilia model, following FTY720 administration. The number of circulating eosinophils was significantly decreased after treatment with FTY720, and eosinophils accumulated in the bone marrow. In addition, eosinophils expressed S1P1, S1P3, and S1P4 mRNAs, and their chemotactic response to S1P was abolished by FTY720 as well as by SEW2871. These findings suggest that FTY720 affects the number of eosinophils in both the blood and skin by inhibiting the egress of eosinophils from the bone marrow and thus downmodulating the late-phase reaction. *(Am J Pathol 2010, 177:1881–1887; DOI: 10.2353/ajpath.2010.100119)*

Eosinophils are thought to be involved in the pathogenesis of several skin disorders. For example, atopic dermatitis is characterized by an infiltrate of inflammatory cells, mainly consisting of $CD4^+$ memory T cells, but distinct eosinophils constitute the inflammatory infiltrate in conjunction with T cells.[1] The trafficking of eosinophils into inflammatory sites is regulated by a number of cytokines such as Th2 cell-derived interleukin (IL)-4, IL-5, and IL-13, chemokines such as regulated on activation normal T cell expressed and secreted/CCL5, and the eotaxins/CCL11 and adhesion molecules.[2–6]

Although accumulating studies have provided a significant insight on the association of cytokines and chemokines with cutaneous tissue eosinophilia, little information exists on eosinophil localization and functional role of cutaneous eosinophils.[7] To study eosinophil kinetics and recruitment, IL-5 transgenic mice have been established and used as an eosinophilia model.[8] In our preliminary study, when IL-5 transgenic mice were repeatedly sensitized to hapten and subsequently challenged with the same antigen, mice developed a late-phase cutaneous reaction and tissue eosinophilia, suggesting that eosinophils may be involved in the establishment of cutaneous late-phase reactions. Therefore, we speculated that the migration of eosinophils from lymphoid organs is a crucial step in the eosinophil localization to the skin, and this migration could be targeted to prevent the development of cutaneous diseases.

Sphingosine-1-phosphate (S1P) is a biologically active metabolite of plasma-membrane sphingolipids that is essential for immune-cell trafficking.[9] The immune functions of S1P result from the engagement of five subtypes of G protein-coupled receptors, S1P1–S1P5. Initial studies for evaluating the roles of S1P were performed with the compound FTY720. S1P1 expression on T cells determines the retention of naïve T cells, but not memory T cells, in

Supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Ministry of Health, Labor, and Welfare of Japan.

Accepted for publication June 24, 2010.

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lymph nodes and their exit into the lymph in response to the S1P gradient,[10,11] thereby modulating immune responses *in vivo*.[12] Moreover, recent studies have identified that S1P is required for natural killer cell egress from lymph nodes and bone marrow.[13] These findings raise a possibility that S1P and S1P receptors may function in the egress of lymphoid organs of eosinophils. However, the mechanism of migration of eosinophils from the lymphoid organs into the peripheries remains unelucidated.

In this study, we investigated the modulatory effect of FTY720 on eosinophil and late-phase reaction in mice. We evaluated the migration and trafficking of eosinophils between the systemic circulation and the skin. FTY720 inhibited eosinophil infiltration into the skin and attenuated the late-phase reaction of the skin. Our results propose a novel therapeutic approach of FTY720 on eosinophil localization and trafficking.

Materials and Methods

Animals and Reagents

Female C57BL/6 (B6), BALB/c, and New Zealand White (NZW) mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were conducted on 8-week-old mice. IL-5 transgenic mice were obtained from RIKEN BioResource Center (Tsukuba, Japan). These mice were maintained on a 12-hour light/dark cycle under specific pathogen-free conditions. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health. 2,4,6-Trinitrochlorobenzene (TNCB) was obtained from Tokyo Kasei (Tokyo, Japan).

For treatment with FTY720 (Cayman Chemical, Ann Arbor, MI), mice were injected i.p. with 3.0 mg/kg FTY720 or with an equivalent volume of normal saline as a control at the indicated time points. Alternatively, mice were administered with S1P1 selective agonist SEW2871 (Calbiochem, San Diego, CA) before the final TNCB challenge by gavage because of its low solubility in aqueous solution. SEW2871 was dissolved in 10% dimethyl sulfoxide and diluted with 25% Tween 20 as described previously.[14] The dosage of 20 mg/kg was chosen based on the previous study showing a consistent lymphocytopenia up to 12 hours.[14]

Flow Cytometry

For flow cytometry, cells were plated at a density of 1×10^6 cells/well in 96-well U-bottomed plates (Falcon; BD Biosciences, San Jose, CA). Cells were stained for 20 minutes on ice with monoclonal antibodies (mAbs) in 25 μl of PBS containing 2% FCS, 1 mmol/L EDTA, and 0.1% NaN_3 and washed twice with 200 μl of this buffer. Data were collected on a FACSCanto system (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA). The mAbs used were as follows: fluorescein isothiocyanate-conjugated anti-CD4 mAb, phosphatidylethanolamine-conjugated anti-Siglec-F mAb, phosphatidylethanolamine-Cy5-conjugated anti-CD8 mAb, antigen-presenting cell-conjugated anti-B220 mAb (all from BD Biosciences), and phosphatidylethanolamine-Cy7-conjugated anti-Gr-1 mAb (eBioscience, San Diego, CA). Eosinophils were detected as Siglec-F^+Gr-1^{int+} population.[15,16] The number of cells was determined by flow cytometry using Flow-Count Fluorospheres (Beckman Coulter, Fullerton, CA).[17]

Inflammatory Skin Disease Model and Histology

BALB/c mice were sensitized through the application of 50 μl of 3% TNCB in 4:1 acetone/olive oil to their shaved abdomens on day 0. They were challenged on both sides of each ear with 20 μl of 0.2% TNCB in 1:9 acetone/olive oil on day 5 to evaluate contact hypersensitivity. To evaluate the inflammatory skin disease, mice sensitized in the same way 5 days before the first elicitation (day 0) were repeatedly challenged on the originally elicited ears with 20 μl of 0.2% TNCB in 1:9 acetone/olive oil from days 8 to 29 at 3-day intervals as outlined in a previous report, with some modifications.[18] On day 30, they were then challenged on both sides of each ear with 20 μl of 0.2%TNCB in 1:9 acetone/olive oil. Ear thickness change was calculated as follows: (ear thickness after final challenge) − (ear thickness before final challenge). FTY720 was applied by i.p. injection (3 mg/kg body weight) 24 hours before final TNCB challenge.

At 48 hours after the challenge with hapten, the ears of the mice were excised for histology and fixed in 10% formaldehyde. Five-micrometer-thick sections were prepared and stained with H&E. Papanicolaou staining was used for eosinophil evaluation.

5- (and 6-)Carboxyfluorescein Diacetate Succinimidyl Ester Cell Labeling and Tracking

The axillary and inguinal lymph node cells, splenocytes, blood cells, and bone marrow cells from IL-5 transgenic mice were prepared, and single-cell suspensions were labeled with 6.5 μmol/L 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as outlined in a previous report.[6] Twenty-four hours before cell transfer, wild-type NZW mice were injected i.p. with 3 mg/kg FTY720 or normal saline. The cells resuspended in RPMI 1640 containing 0.2% FCS were injected i.v. in NZW mice (2×10^7 cells/mouse). Twelve hours after the cell transfer, the number of fluorescent cells in the bone marrow was enumerated by flow cytometry.

Effect of IL-5 and FTY720 on Levels of Circulating and Bone Marrow Eosinophils

B6 mice were administered with or without FTY720, and 24 hours later, the mice were injected i.v. with 100 μl of PBS containing IL-5 (500 pmol/kg) or PBS alone according to methods outlined in a previous study, with some modifications.[19] Blood and bilateral femurs were taken 1 hour after i.v. injection of IL-5, and the number of eosinophils was determined by flow cytometry.