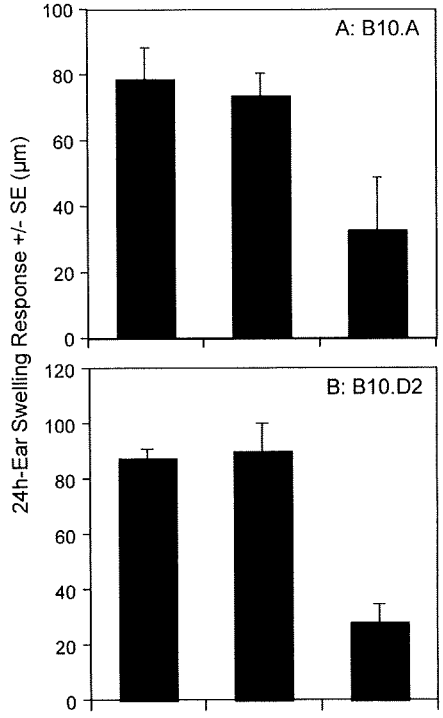


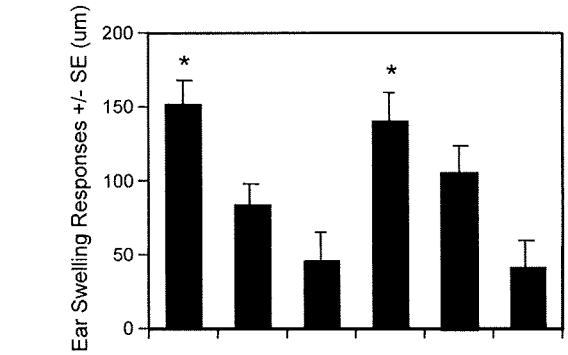
group	a	b	c
LPS (μg)	25	-	-
DNFB	+	+	-
challenge	+	+	+

Fig. 2. LPS fails to augment the induction of CH in B10.A and B10.D2 mice. Panels of mice of B10.A (A) and B10.D2 (B) received i.d. injections of 25 μg LPS (a) and PBS (b), respectively. Within 30 min, the injected sites were painted with 185 μg DNFB. After 5 days, the ears were challenged with DNFB. Unsensitized animals were also challenged and used as negative controls (c). Ear swelling responses at 24 h are presented as mean ± S.E.M. (μm).

LPS augments CH in MyD88-KO mice, as demonstrated in wild-type mice. We concluded that the effect of LPS upon CH is independent of the MyD88 pathway.

3.4. LPS augments the allo-stimulatory ability of the draining LN cells

We next determined whether LPS could alter the allo-stimulatory ability of the draining LN cells. In this experiment, we used C57BL/6 mice as stimulators and BALB/c mice as responders. LPS (25 μg/mouse) or PBS was injected (i.d.) into the mice. Within 30 min, 185 μg DNFB was applied on the cutaneous surface of the injected sites. LN cells were obtained after 5 days. In some experiments, DC population was enriched, X-irradiated, and cocultured with allogeneic splenocytes (2×10^5/well) from BALB/c for 5 days. The LN cells were cultured alone as the negative control. Cell proliferation was measured by [^{3}H]-thymidine uptake. The results of a representative experiment are shown in Fig. 4. LN cells from the LPS-treated mice showed significantly enhanced ability to stimulate allogeneic splenocytes (Fig. 4A). However, the ability of the DC-enriched LN cells from the LPS-treated mice to stimulate allogeneic splenocytes was comparable with that in the PBS-treated mice (Fig. 4B). It is concluded that LPS does not enhance the antigen-presenting function of migratory DCs from skin when hapten is applied on the LPS-injected sites.



	KO			wild		
group	a	b	c	d	e	f
LPS	+	-	-	+	-	-
DNFB(185μg)	+	+	-	+	+	-
DNFB(15μg)	+	+	+	+	+	+

Fig. 3. LPS augments CH in MyD88-KO mice. Panels of MyD88-KO (a–c) and wild-type mice (B6 background; d–f) were injected intradermally with 25 μg LPS (a and d) or PBS (b and e). Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the ears were challenged with 15 μg DNFB. Unsensitized animals were also challenged and used as negative controls (c and f). Ear swelling responses at 24 h are presented as mean ± S.E.M. (μm). $^*P < 0.05$ versus PBS (positive control).

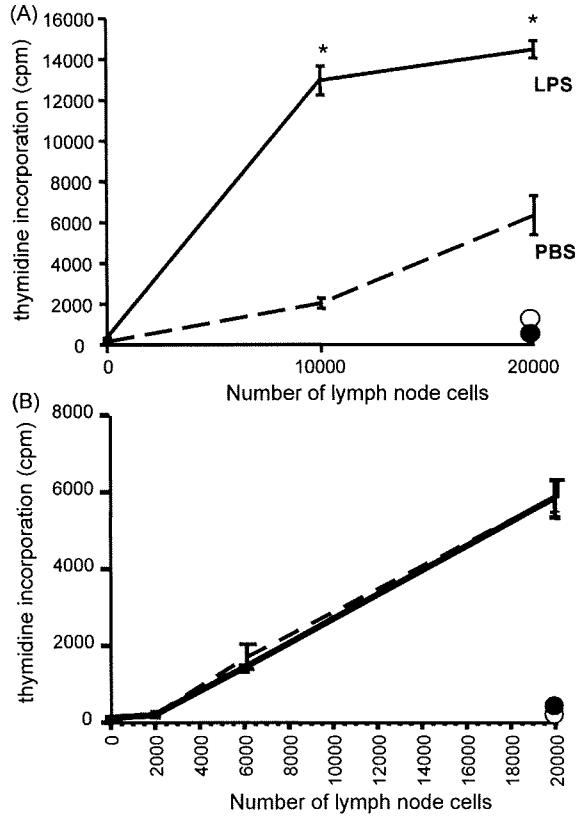


Fig. 4. LPS augments the allo-stimulatory ability of the draining LN cells. Mice received i.d. injection of LPS (25 μg/mouse) or PBS alone. Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the LN cells, the DC-enriched LN cells and splenocytes were prepared. Allogeneic splenocytes as responders were co-cultured with X-irradiated LN cells (Fig. 4A) or DC-enriched LN cells (Fig. 4B) as stimulators for 5 days. Data are expressed as the mean cpm of triplicate cultures ± S.E.M. $^*P < 0.05$ versus PBS (positive control). Empty and solid circles indicate thymidine incorporation of stimulator cells in the LPS-treated group and the PBS-treated group, respectively.

3.5. LPS alters the density and morphology of epidermal DCs

We next examined whether LPS can alter the density and morphology of the epidermal DCs and Langerhans cells (LCs). Panels of mice belonging to 3 different inbred strains received i.d. injections of LPS (25 μg) or PBS alone as the control group. The skins of these mice were excised 2 h later. LCs in these epidermal sheets were stained with anti-I-A^k or I-A^d mAb and then with FITC-tagged goat anti-mouse Ab and evaluated under an epifluorescence microscope. The results of a representative experiment are presented in Table 2. The number of I-A+ epidermal cells was clearly reduced in the skin excised from the LPS-injected sites in HeN and BALB/c mice. In contrast, the number of I-A+ epidermal cells in the LPS-injected HeJ mice was similar to that of I-A+ epidermal cells in the PBS-injected control mice. We conclude that the intracutaneously injected LPS can reduce the density of epidermal I-A+ cells. The lack of an effect of LPS on epidermal DCs in the TLR4-deficient HeJ mice indicates that the effect of LPS is TLR4-dependent.

The changes in the epidermal I-A+ cells after i.d. injections of LPS included not only a reduction in the density of these cells but also an extensive alteration in the morphology of HeN (Fig. 5A) and BALB/c (Fig. 5C) mice. In mice in which i.d. injections of LPS were administered, the I-A+ cells no longer displayed the dendrites, and the cell bodies appeared plump and round, rather than slender, which is the morphology of I-A+ cells after PBS injection (Fig. 5D and F). The morphology of I-A+ cells in C3H/HeJ mice (Fig. 5B), however, was similar to that of I-A+ cells in the PBS-injected control mice (Fig. 5E); this indicates that TLR4 signaling mediates the morphological alteration of epidermal DCs by LPS injections. Therefore, LPS appears to have two histologically distinct effects on epidermal DCs: reduction in the number of I-A+ cells and loss of dendrites.

3.6. Anti-TNF-α Abs restore the effects of intracutaneously injected LPS on the density of epidermal DCs

We next examined the possibility that the inhibitory effects of LPS on the density and the morphology of epidermal LCs might be reversed by anti-TNF-α Abs. LPS was injected intradermally into BALB/c mice 6 h after i.p. injections of anti-TNF-α polyclonal Abs (200 μg) or normal goat IgG Abs. The skins of these mice were excised 2 h later. The density of epidermal DCs was assessed by a fluorescence microscope, using anti-I-A^d mAbs (Table 3). Intradermal injections of LPS after i.p. administration of normal goat IgG Abs reduced the number of I-A+ cells. In contrast, i.p. administration of anti-TNF-α Abs restored the number of I-A+ cells in LPS-injected skin, which was comparable with the number of I-A+ cells in PBS-injected skin. This indicates that TNF-α mediates the effect of LPS on the density of epidermal DCs.

However, the morphology of the epidermal I-A+ cells remained more or less plump and round even after anti-TNF-α Ab pretreatment (Fig. 6B) and was almost comparable with the morphology after normal goat IgG Ab pretreatment (Fig. 6A). Although anti-TNF-α Ab pretreatment largely inhibited the reduction in the number of I-A+ cells induced by LPS, it was able to only partially restore the morphology of epidermal cells.

Table 2
LPS alters the density of epidermal I-A^+ cells

Mice	Treatment	Number of I-A+ cells[a]
C3H/HeN	PBS	610.0 ± 15.4
	LPS	525.6 ± 14.2[b]
BALB/c	PBS	599.2 ± 17.4
	LPS	484. ± 15.6[b]
C3H/HeJ	PBS	622.0 ± 15.1
	LPS	631.2 ± 23.2

[a] Mean number of cells ± S.E.M. per mm^2.
[b] $P < 0.05$ versus PBS.

Table 3
Anti-TNF-α antibodies restore the effect of LPS on epidermal dendritic cells

	Number of I-A+ cells[a]
Anti-TNF antibody + LPS	628.0 ± 19.0
Normal goat IgG + LPS	484.8 ± 17.9[b]
PBS	634.8 ± 22.7

[a] Mean number of cells ± S.E.M. per mm^2.
[b] $P < 0.05$ versus PBS.

3.7. LPS alters the ability of antigen-bearing DCs to migrate into draining LNs

We next determined whether LPS could alter the ability of antigen-bearing DCs to migrate into draining LNs. Within 30 min after the i.d. injections of LPS (25 μg/mouse) or PBS, 400 μl of 0.5% FITC was epicutaneously applied on the HeN (I-A^k), HeJ (I-A^k), or BALB/c (I-A^d). Forty-eight hours after applying FITC, draining LNs were collected. A suspension of LN cells was prepared and immunolabeled with PE-conjugated anti-I-A^k, or I-A^d mAb. The data for two-color analysis for $FITC^+$ and I-A+ cells are shown in Fig. 7A. The proportion of I-A+ FITC-bearing cells in the LNs of HeN mice was significantly higher in the LPS-treated mice (0.49%) than in the PBS-treated mice (0.21%). However, those of BALB/c (Fig. 7A) or HeJ (data not shown) were approximately identical between the 2 groups. The expression intensity of I-A in the LPS-treated mice was comparable with that in the PBS-injected mice ($n = 2$ for each group) (Fig. 7B). The proportion of dead cells in the FITC-bearing cells is comparable between the LPS- and PBS-treated groups (data not shown). These data suggest that LPS alters the ability of antigen-bearing DCs to migrate into draining LNs. There was no significant difference in the proportion of I-A+ FITC-bearing cells in the TLR4-deficient HeJ mice, indicating that the effect of LPS on the ability of antigen-bearing DCs to migrate to draining LNs is TLR4-dependent.

3.8. TNF-α itself abrogates but in synergy with LPS augments the induction of CH

Because anti-TNF-α antibodies block the migration of epidermal DCs (Table 3), we assessed the role of TNF-α in the induction of CH. As reported previously [15], TNF-α itself impairs CH induction. We tested the dose of TNF-α that is capable of impairing CH induction in different strains of mice. Panels of B6 or BALB/c mice received TNF-α (50 ng), or vehicle (PBS with 0.1% BSA) on the abdominal skin. Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the ears of these mice were challenged with 15 μg DNFB and the ear swelling response was measured after 24 and 48 h. The results of a representative experiment are shown in Fig. 8A. This dose of TNF-α is solely effective on the LPS-susceptible strain, HeN mice, as reported previously [15,16]. Thus, we chose this dose for the subsequent experiment.

We next applied LPS (25 μg), TNF-α (50 ng), or both reagents on the abdominal skin of BALB/c mice. Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the ears of these mice were challenged with 15 μg DNFB, and the ear swelling response was measured after 24 and 48 h. The results of a representative experiment are shown in Fig. 8B. Surprisingly, mice that received both reagents showed enhanced CH (Fig. 8B, group a). In contrast, mice that received either LPS or TNF-α showed

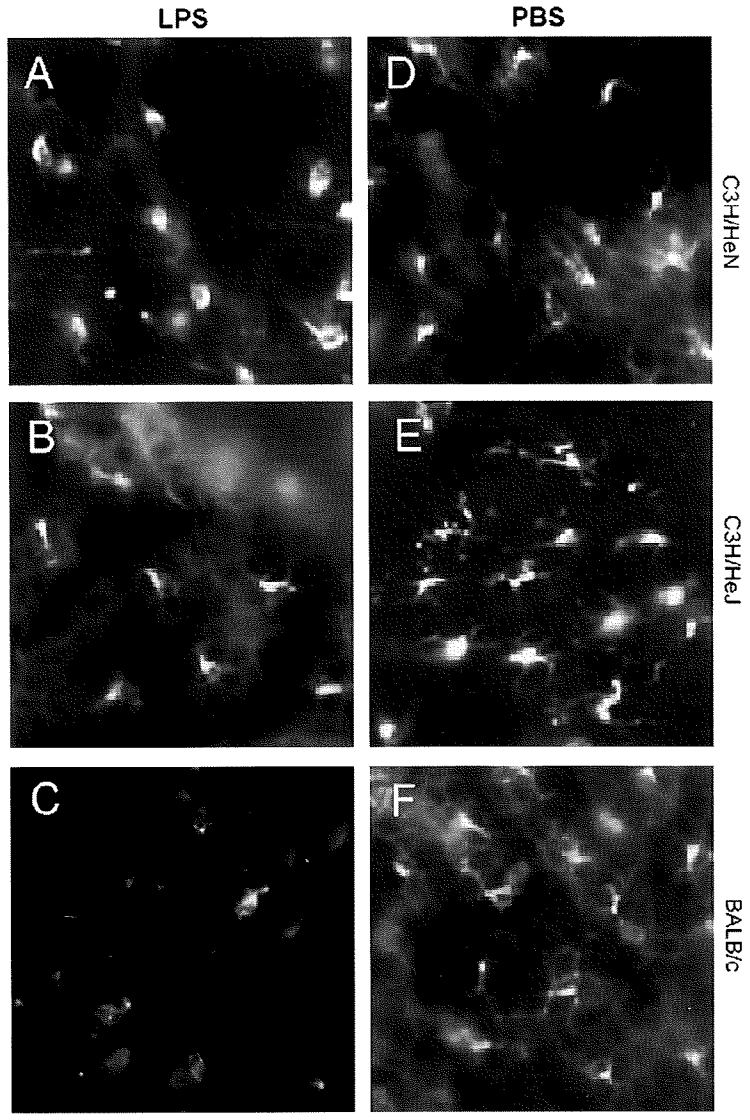


Fig. 5. LPS alters the density and morphology of epidermal DCs. Panels of C3H/HeN (A and D), C3H/HeJ (B and E), and BALB/c (C and F) mice received i.d. injections of LPS (25 μg) (upper row; A–C) or PBS (lower row; D–F). The original magnification: ×200.

comparable to that of the positive control (Fig. 8B, groups b and c). Thus, we concluded that TNF-α is capable of enhancing CH induction.

4. Discussion

The proposed role of TNF-α in CH has been controversial. Mice deficient for p55 TNF-α receptor exhibit an enhanced CH reaction, suggesting an immunosuppressive role of this receptor in CH [17]. TNF-α-deficient mice show a reduced CH reaction. This suggests that TNF-α is necessary for optimal CH and establishes a physiological role of TNF-α in CH [18]. Moreover, TNF-α stimulates murine LCs to migrate from the skin into draining LNs after allergen application [19]. We showed that LPS augmented the induction of CH in TLR4-sufficient HeN mice, but not in TLR4-deficient HeJ mice. Anti-TNF-α Abs prevented the decrease in the density of epidermal DCs induced by intracutaneous injection of LPS, which increases the number of I-A+ cells in the draining LNs in HeN but not in HeJ mice. Furthermore, we showed that TNF-α is capable of enhancing the effect of LPS on CH induction (Fig. 8B). These data indicate that DC migration via TLR4 is TNF-α-dependent and that TNF-α has the ability to modulate the magnitude of CH.

In contrast, LPS could not enhance CH in BALB/c mice despite the presence of TLR4, indicating that BALB/c mice respond only slightly to LPS in an allergic response against DNFB. A study of the genetic effect on the in vivo production of TNF-α showed that TNF-α production is genetically controlled by H-2D, to which the TNF locus is closely linked [20,21]. The quantitative difference in TNF-α produced in response to ultraviolet (UV) B radiation has been reported to account for the phenotypic traits of UVB-susceptibility both in humans and mice [14,22,23]. Acute low-dose protocol of UV exposure of the skin impairs CH induction via the TLR4-dependent pathway [15]. Furthermore, we showed that TNF-α is capable of enhancing the effect of LPS on CH induction (Fig. 8B). These results indicate that the quantitative difference in TNF-α produced via TLR4 in response to the LPS pathway may also be related to the phenotypic traits of LPS responsiveness.

The source of TNF-α production in the induction phase of CH remains unclear. Sugita et al. [24] reported an interesting experiment showing that Langerhans cell (LC) function can be up-regulated indirectly by cytokines that are released by

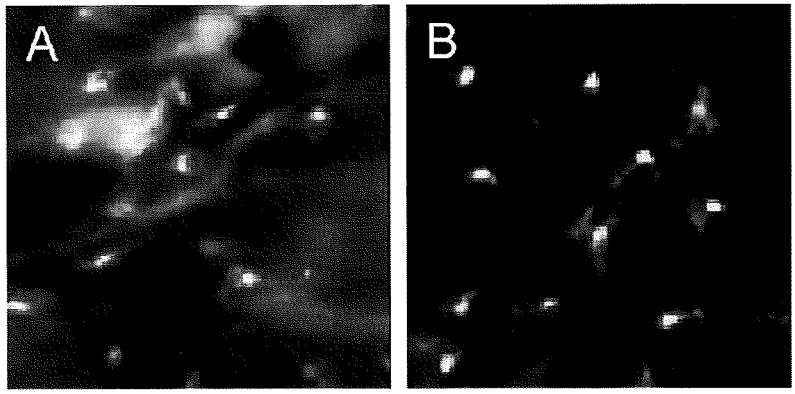


Fig. 6. Anti-TNF-α Abs restores LPS-altered epidermal DCs. BALB/c mice received i.d. injection of LPS 6 h after intraperitoneal injections of normal goat IgG Abs (A) or anti-TNF-α Abs (B). The original magnification is ×200.

keratinocytes stimulated with CpG, a TLR9 ligand. TLR9 ligand was capable of enhancing the hapten-presenting ability of LCs when LC-enriched epidermal cells, but not purified LCs, were used as the LC source; this suggests that bystander keratinocytes play a role in the enhancement of LC function. The addition of a cocktail of neutralizing antibodies against keratinocytes-induced cytokines, including TNF-α, abrogated the CpG-promoted, antigen-presenting ability of LC-enriched epidermal cells.

In this study, another candidate of TNF-α production was dermal mast cells. Marshall et al. [25] reported that LPS enhance LC migration in mast cell-deficient mice, indicating that mast cells are not associated with LPS-dependent enhanced LC migration.

In TNF-α-deficient mice, however, nickel chloride (Ni) concomitant with LPS induced a Ni allergy to a similar degree to that in the respective control mice [26]. In this model, interestingly, Ni + LPS induced a Ni allergy only weakly in IL-1-deficient and

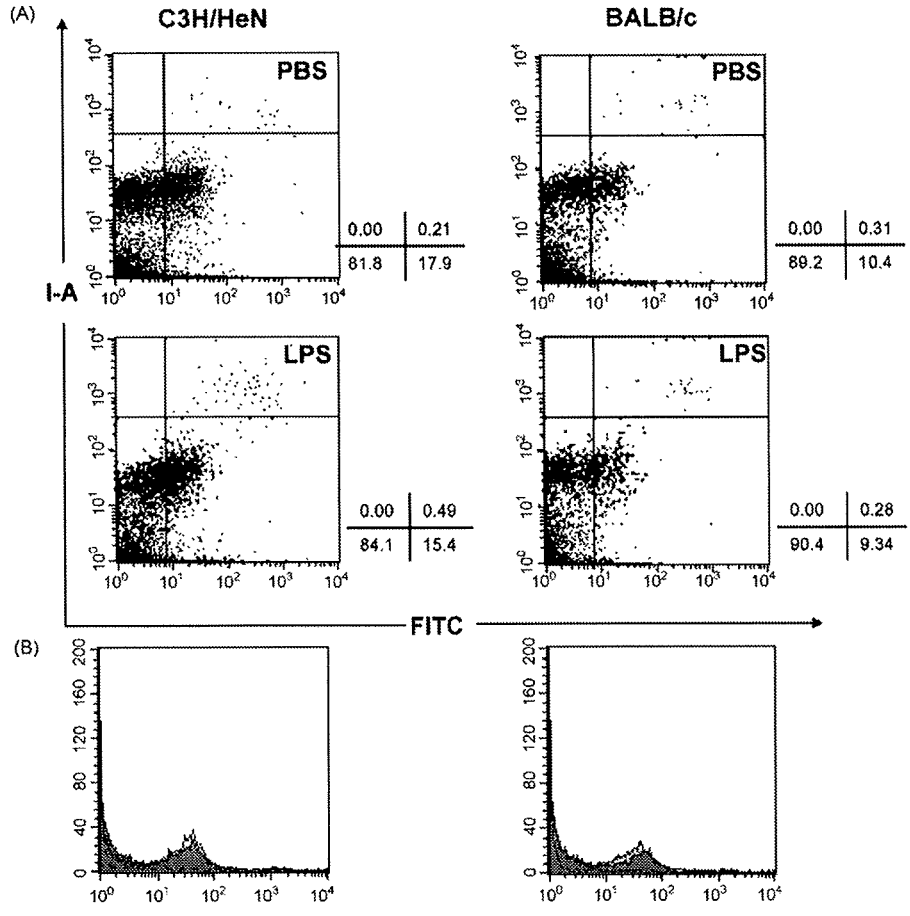
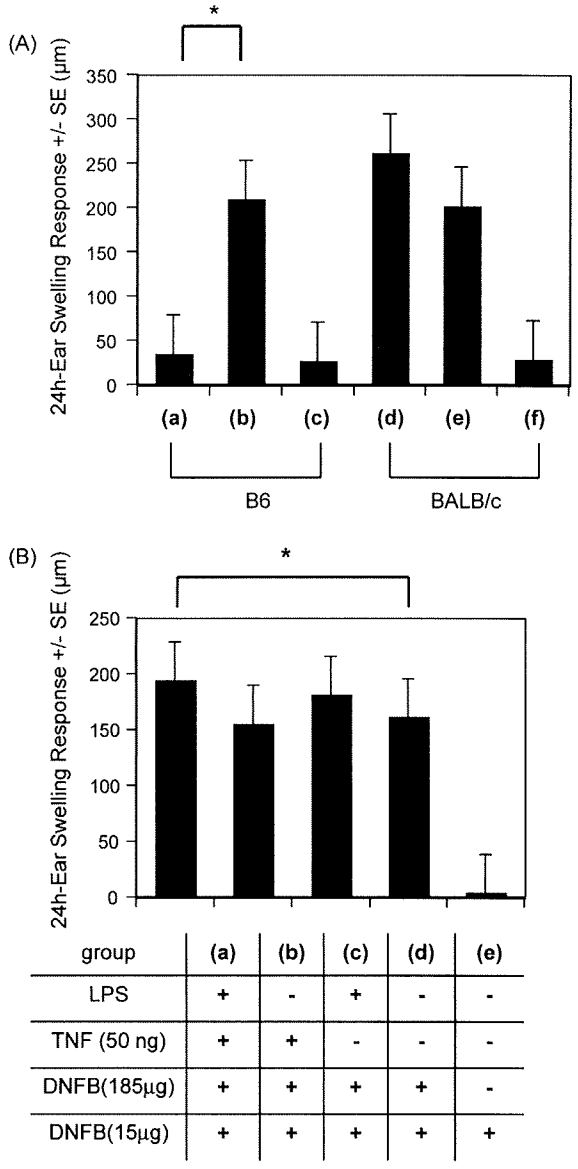


Fig. 7. LPS alters an ability of antigen-bearing DCs to migrate to draining LNs. Panels of mice of C3H/HeN and BALB/c received application of 0.5% FITC (400 μl) after i.d. injection of LPS (25 μg/mouse) or PBS. Twenty-four hour later, the draining LN cells were collected, the DC populations were enriched with Nycoprep, then immunolabeled with PE-conjugated I-A^k monoclonal Ab. A total of 5×10^4 cells were analyzed using FACScaliber for I-A^k+ FITC-bearing cells. Representative data of two-color analysis for FITC+ and I-A^k+ cells were shown (A). The intensity of expression of I-A in LPS-treated mice (solid line) was comparable with PBS-injected mice (gray box) ($n = 2$ for each group). The dotted line indicates the intensity of isotype-matched antibody in LPS-treated mice (B).



group	(a)	(b)	(c)	(d)	(e)
LPS	+	-	+	-	-
TNF (50 ng)	+	+	-	-	-
DNFB(185μg)	+	+	+	+	-
DNFB(15μg)	+	+	+	+	+

Fig. 8. TNF-α itself abrogates in synergy with LPS augments the induction of CH. Panels of B6 (a–c) or BALB/c (d–f) mice received TNF-α (50 ng: a and d), or vehicle (PBS with 0.1% BSA: b and e) on the abdominal skin. Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the ears of these mice were challenged with 15 μg DNFB and the ear swelling response was measured after 24 and 48 h. Unsensitized animals were also challenged and used as negative controls (c and f). Panels of BALB/c mice received vehicle (group d), LPS (25 μg: group c), TNF-α (50 ng: group b), or both reagents (group a) on the abdominal skin of BALB/c mice. Within 30 min, 185 μg DNFB was applied on the injected sites. After 5 days, the ears of these mice were challenged with 15 μg DNFB and the ear swelling response was measured after 24 and 48 h. The results of a representative experiment at 24 h are shown. Ear swelling responses at 24 h are presented as mean ± S.E.M. (μm). $^{*}P < 0.05$ versus positive control.

macrophage-depleted mice but not in mast cell-deficient mice and even in nude (T cell-deficient) mice. The authors suggest that the promotion and augmentation of metal allergies by LPS in mice are dependent on innate immunity [26]. Taken together with our results, the mechanism by which LPS alters the magnitude of CH depends on antigens. They suggested some similarities and dissimilarities between the 2 types of CH responses: metal-induced and classical hapten-induced [26]. Moreover, similar promoting effects of TLR ligands (such as TLR7 and TLR9 ligands) have been reported in models entailing CH induction by the classical haptens, and in these models, TLR ligands enhance the antigen-presenting functions of DCs [24,27–29].

IL-1 is an indispensable cytokine for CH induction. Antonopoulos et al. [30] reported that TNF-α failed to induce LC migration in caspase-1-deficient mice. Caspase-1 is necessary to cleave the proprotein form of IL-1 into the active form of IL-1 protein. Intradermal injection of IL-1 beta (50 ng) but not TNF-α (50 ng) resulted in a similar reduction in epidermal LCs in both wild-type and caspase-1-deficient mice, indicating that, after receiving an appropriate signal, caspase-1-deficient epidermal LCs are capable of migration and that IL-1 and IL-1 signal are downstream of TNF-α to induce LC migration.

We wanted to determine whether MyD88 is required to conduct CH response, because MyD88 is also an adaptor protein of IL-1 receptor [5]. Mice deficient for MyD88 showed a vigorous CH comparable to that in wild-type mice (Fig. 3, groups b and e), indicating that CH response to DNFB is independent of the IL-1-MyD88 pathway. Fig. 3 also shows that LPS can augment CH induction in MyD88-KO mice, indicating that augmented CH by LPS is independent of the (TLR4-) MyD88 pathway. This result is consistent with the fact that caspase-1 activation induced by IL-1 signaling is independent of the MyD88 pathway [3]. We doubt that LPS is capable of enhancing CH induction in IL-1R1-KO mice since no MyD88-independent IL-1 pathway is blocked in these mice.

TLR4 stimulation facilitates the activation of 2 pathways: the MyD88-dependent and Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathways [3,5]. The MyD88-dependent pathway involves the early phase of nuclear factor-κB (NF-κB) activation, which leads to the production of inflammatory cytokines. The TRIF-dependent pathway activates interferon (IFN)-regulatory factor (IRF) 3 and involves the late phase of NF-κB activation followed by late-phase TNF-α production [3,5]. In the present study, we showed that LPS augmented CH in both MyD88-KO mice and wild-type mice and that the effect of LPS upon CH was independent of the MyD88-dependent pathway. Kaisho et al. [6] reported that the TLR4-dependent effects of LPS on the antigen-presenting ability of DCs are mediated mainly via the MyD88-independent pathway. We suspect that the TRIF pathway is associated with LPS with regard to CH induction [3,5]. Further studies are required to address this issue.

We showed that LPS increased the proportion of I-A^k+ FITC-bearing cells in draining LNs 24 h after the hapten painting (Fig. 7). A majority of migratory DCs in the LNs at this time point are derived from the dermis but not from the epidermis [31]. Dermal DCs migrate into the draining LNs earlier than epidermal DCs. We did not observe that LPS increased the proportion of I-A^k+ FITC-bearing cells in draining LNs 48 h after painting (data not shown). These data imply that LPS is capable of enhancing the migratory ability of dermal DCs.

We also showed that LPS failed to increase the proportion of I-A^k+ FITC-bearing cells in draining LNs 24 h after FITC painting in BALB/c mice (Fig. 7). LPS has no effect on CH induction in this strain of mice (Fig. 1C). However, the same dose of LPS did alter the density and morphology of epidermal DCs (Fig. 5). This is consistent with a recent report showing that dermal DCs are indispensable for CH induction [31]. This report also shows that elimination of epidermal DCs does not alter CH response to hapten. Therefore, we conclude that the dose of LPS we chose does alter the density and morphology of epidermal DCs, however, this dose may be insufficient to augment the migration of dermal DCs, leading to no effect on CH response.

A small amount of TNF-α is capable of alteration in the number and morphology of LCs in C3H/HeN mice rather than in BALB/c mice [15]. Simultaneous administration of LPS and TNF-α succeeded in enhancing CH response in BALB/c (Fig. 8B). This

indicates that BALB/c strain needs more amount of TNF-α to enhance conventional CH response.

We showed that LPS responsiveness with regard to CH varies in some strains of mice. The effect of LPS on CH induction is lower in BALB/c mice than in HeN and C57BL/6 mice. All low-responsive strains carry H-2^d or H-2^a background (Table 2).

In studies on humans, a common mutation in TLR4 is associated with the difference in LPS responsiveness and alters the ability of the host to respond to environmental stress [32]. Moreover, in a study on Swedish children, decreased LPS-induced IL-12 and IL-10 responses were found to be associated with TLR4 polymorphism and were independently associated with asthma [33]. In a study conducted on the Japanese, TNF-α gene polymorphism was found to be associated with an increased production of TNF-α protein [34]. These results imply a possibility to set up standard protocol in clinical settings for the application of bacterial components in human vaccines. Moreover, not only antigenicity of environmental factors but also LPS responsiveness as an adjuvant may explain in part the individual difference in the susceptibility to establish CH for daily antigen exposures.

Acknowledgements

We thank Professor Shizuo Akira for kindly providing MyD88-KO mice. We also thank Dr. Kenji Kabashima for technical advices in Flow Cytometry analyses and Dr. Yasuyuki Sumikawa for technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research 16591112 from the Ministry of Education, Culture, Sports, Science, Technology of Japan, and Lydia O'Leary Memorial Foundation (to H. Niizeki), and the Department of Indoor Environmental Medicine, Nara Medical University (to H. Asada).

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Repeated episodes of fixed eruption 3 months after discontinuing pegylated interferon-α-2b plus ribavirin combination therapy in a patient with chronic hepatitis C virus infection

M. Sato, H. Sueki and M. Iijima*

*Division of Dermatology, Showa University Fujigaoka Hospital, Yokohama, Japan; and *Department of Dermatology, Showa University School of Medicine, Tokyo, Japan*

doi:10.1111/j.1365-2230.2009.03560.x

Summary

We report a 73-year-old man who developed repeated episodes of erythematous, bullous plaques beginning 3 months after discontinuation of combination treatment with pegylated interferon (IFN)-α-2b and ribavirin for hepatitis C virus infection. The first episode resolved within a week without treatment, but the lesions recurred about once a month and were associated with high fever. Physical examination found darkly reddish, pigeon-egg-sized erythematous plaques with occasional flaccid blisters, predominantly on the trunk and proximal limbs, lip and penis. Histological examination showed well-demarcated foci of full-thickness epidermal necrosis and exocytosis of lymphoid cells. Pegylated IFN-α2b and ribavirin produced no response in lymphocyte stimulation tests. Systemic prednisolone led to rapid healing of skin lesions at the time of the fifth episode, leaving pigmented macules, but lesions recurred at the same sites within weeks of discontinuation of this treatment. It is uncertain whether this case represented a prolonged drug rash provoked by pegylated IFN-α2b or a fixed eruption in response to another antigen.

A combination of pegylated interferon IFN-α2b and ribavirin is currently recommended for the treatment of chronic hepatitis C virus (HCV) infection, because it yields a better therapeutic response than either drug as monotherapy.[1] However, a high prevalence of adverse skin reactions to this combination, including eczema, prurigo, lichenoid eruption, maculopapular rash, injection-site reactions, and worsening of psoriasis, has been reported.[2,3] Some cases have shown a delay between implementation of this treatment and the occurrence of adverse skin reactions.[4] We report a patient with repeated episodes of fixed eruption 3 months after discontinuing this combination treatment.

Correspondence: Dr Hirohiko Sueki, 1-30, Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan
E-mail: hirsueki@med.showa-u.ac.jp

Conflict of interest: none declared.

Accepted for publication 6 March 2009

Report

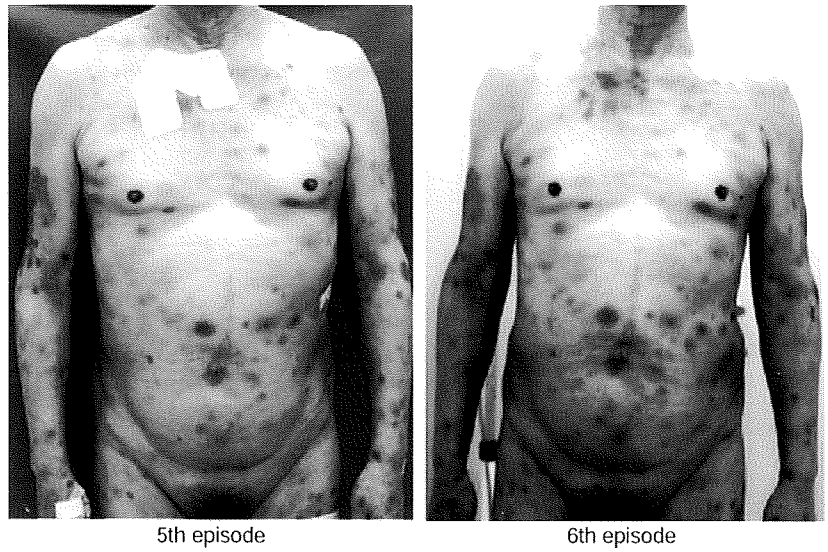
A 73-year-old man presented with a fever and multiple pruritic bullous–erythematous plaques on the trunk and proximal limbs. The patient had a history of HCV infection, and had been receiving amulodipine besilate, doxazosin mesilate, clonidine hydrochloride and ursodesoxycholic acid for several years. Combination treatment with pegylated IFN-α2b and ribavirin for the HCV infection was started in February 2006, but this was discontinued on July 25, 2006, because of worsening transaminase levels. Injection sites were limited to both upper arms, and appreciable injection-site reactions, consisting of redness and swelling with pruritus, were noted during the treatment. In mid October 2006, round pruritic erythematous plaques initially developed on the bilateral femoral areas, distant from injection sits, without further medication or supplements. The skin lesions resolved within a week. These episodes recurred about once every month. The quantity of HCV RNA significantly increased just before

the first episode of the rash, and it remained at a high level for about 11 months until about 3 months after the sixth episode, when the viraemia dissipated. Although most of the lesions recurred at the same spot each time after healing, they gradually increased in number and extended to the trunk. The fourth episode was associated with prominent fever, and the skin lesions finally extended to the arms. The fifth episode developed in mid March 2007, admixing blisters and erosions on the erythematous plaques. The patient was referred to us at the end of March. Examination showed darkly reddish, pigeon-egg-sized round erythematous plaques with occasional flaccid blisters, located predominantly on the trunk and proximal limbs, lower lip and penis (Fig. 1a). Plaques on the upper arms did not correlate with the previous injection site reactions.

A preliminary diagnosis of erythema multiforme major was made. The patient was hospitalized, and a skin-biopsy sample was taken from the left forearm distant from the previous injection-reaction sites. Histological examination found well-demarcated foci of full-thickness epidermal necrosis producing subepidermal bullae, exocytosis of lymphoid cells, and perivascular inflammatory cell infiltration, predominantly of lymphoid cells (Fig. 2). No significant increase in antibodies against herpes simplex virus, human herpesvirus 6, and mycoplasma was evident in paired serum samples. Drug lymphocyte stimulation tests for pegylated IFN-α2b and ribavirin found no reaction to these drugs.

Systemic prednisolone (20 mg/day) for 6 days led to prompt resolution of the skin lesions at the time of the 5th episode, leaving pigmented macules. Seven days after withdrawal of prednisolone, however, a high fever coincided with recurrence of erythematous, blistered plaques coinciding with the sites of the previous lesions. This occurred in the absence of treatment with any systemic medication or supplements (Fig. 1b). After the sixth episode, a diagnosis of fixed drug eruption was made based on the clinical appearance of the lesions, histological findings, and the fact that the lesions recurred at the identical locations at the time of the fifth and sixth episodes. Treatment with systemic prednisolone was started, which again led to prompt resolution of the lesions leaving only residual pigmented macules. Subsequently, the patient was treated with an ordinary IFN-α (not pegylated) alone, and no recurrence of the skin lesions has been noted for over a year (Fig. 3).

The longer serum half-life of pegylated IFN-α-2b results in greater tissue permeation, and a higher incidence of skin reaction at the injection site in patients treated with pegylated IFN-α-2b and ribavirin than in patients treated with standard IFN-α-2b and ribavirin.[5] In a prospective cohort study, adverse skin reactions were more common in the combined IFN/ribavirin group than in the IFN monotherapy group (11/33 vs. 2/35; $P = 0.001$).[2] In the present case, some of the erythematous plaques were distributed on the upper arms corresponding to the sites of IFN injections. This suggests some role of pegylated IFN-α-2b in the pathogenesis of the generalized rash. It remains unexplained why the skin lesions on the arms occurred only after the fourth episode. Only one case of fixed drug eruption in response to combined IFN/ribavirin



5th episode 6th episode

Figure 1 Clinical findings of the fifth and sixth episodes. (a) Darkly reddish, pigeon-egg-sized, round, erythematous plaques with occasional flaccid blisters, located predominantly on the trunk and proximal limbs in the fifth episode. (b) Sixth episode, coinciding with the sites of the previous pigmented spots.

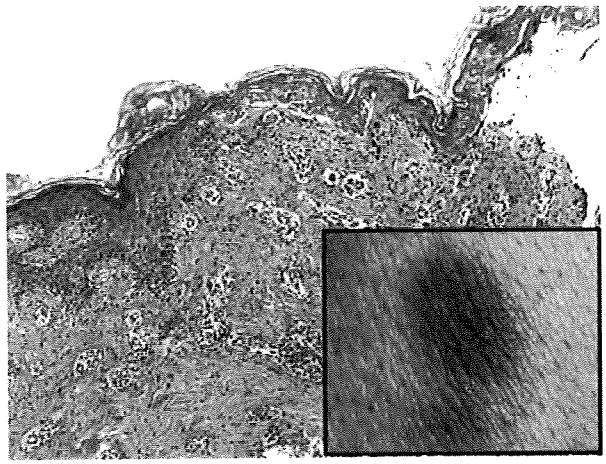


Figure 2 Well-demarcated pan epidermal necrosis, forming subepidermal bullae, exocytosis of lymphoid cells, and perivascular inflammatory cell infiltration (haematoxylin and eosin × 40). Inset shows a close-up view of an erythematous plaque.

therapy has been reported to date.[6] In that reported case, erythematous plaques developed on the forehead and legs, including the injection sites, very soon after the start of the injections, and no recurrence of the spots was seen after cessation of pegylated IFN-α-2b and ribavirin.[6] Delayed onset of adverse skin reaction with this combination therapy has been described before;[4] however, the three-month time lag in the present case casts doubt on the direct participation of pegylated IFN-α-2b. In a recent cohort study of patients undergoing treatment with combined IFN/ribavirin therapy, 6 patients were examined by patch testing and intradermal testing with IFN and ribavirin, and the test results were negative except for one reaction to intradermal IFN-α-2b.[7] In any event, the results of lymphocyte stimulation tests failed to provide definitive evidence that pegylated IFN-α-2b was the cause of this cutaneous reaction.

An alternative interpretation of the present case was that the repeated episodes of erythematous plaques represented a fixed eruption in response to food[8] or viral antigens such as herpes simplex virus and HCV.[9] In the sixth episode, the sites of the erythematous plaques corresponded precisely to those of the fifth episode. Well-demarcated necrosis of the entire epidermis also favoured a diagnosis of fixed eruption rather than epidermal-type erythema multiforme. Intraepidermal CD8+ T cells have been shown to play a major role in the epidermal injury seen in fixed drug eruption.[9] Clinical flare-up of the lesions of a fixed drug eruption after intake of other drugs or nonspecific stimuli may offer an important clue to the nature of ligand/antigen recognition by intraepidermal T cells.[9] Our patient expressly denied taking any additional medications or supplements.

The lesions could result from broad cross-reaction with a variety of exogenous antigens, such as herpes simplex virus and some foods.[9] The occasional association of erythema multiforme with HCV infection has been documented.[10] In that reported case, the

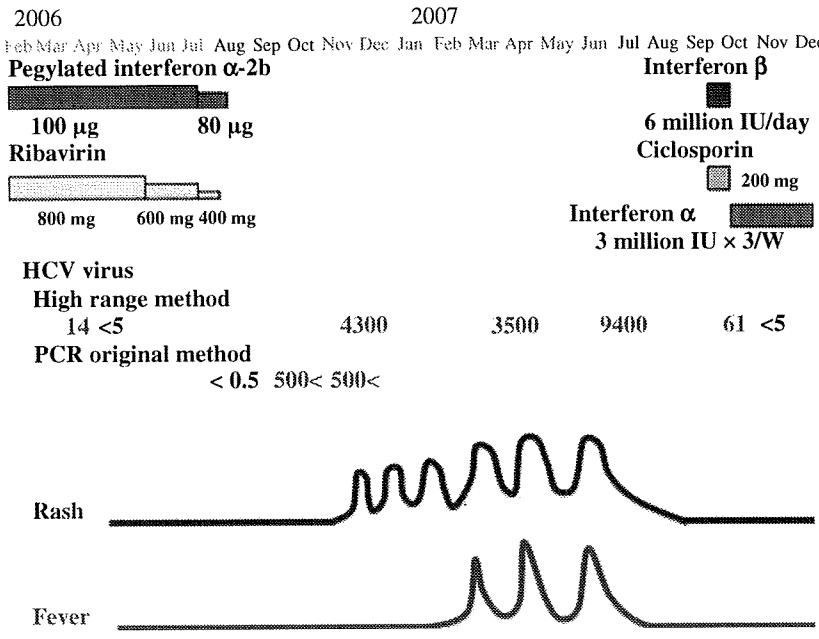


Figure 3 Episodes in this patient developed at the time of increasing hepatitis C viraemia, caused by withdrawal of pegylated interferon-α2b and ribavirin treatment.

concurrent onset of both diseases and extension of erythema multiforme were correlated to higher levels of HCV viremia.[10] Interestingly, the reaction in our patient also started at the time of increasing HCV viraemia, caused by withdrawal of pegylated IFN-α-2b and ribavirin treatment, and recurrent reactions continued in conjunction with a high quantity of HCV-RNA (Fig. 3). This course suggested a temporal relationship between the episodes of fixed eruption and serum HCV-RNA levels. However, this relationship cannot easily explain the relapsing and remitting course of the eruption during the period of high HCV-RNA levels. More common sampling for determination of serum HCV-RNA levels would have been desirable in the present case.

It is still not conclusive whether the present case represented a prolonged drug rash provoked by pegylated IFN-α-2b and ribavirin combination therapy, or a fixed eruption in response to another antigen. We favour the former possibility, although it is not clear exactly what prompted the series of recurrences beginning 3 months after the pegylated IFN-α-2b/ribavirin was discontinued. In any event, this case may provide a novel insight into the relationship between virus infection and drug rash due to IFN.

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Molecular therapies for heritable blistering diseases

Katsuto Tamai[1], Yasufumi Kaneda[1] and Jouni Uitto[2]

[1] Division of Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2 Yamadaoka Suita City, Osaka, 565-0871, Japan
[2] Department of Dermatology and Cutaneous Biology, Jefferson Medical College and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA 19107, USA

Tremendous progress has been made over the past two decades in understanding the molecular genetics of heritable skin diseases. The paradigm for such conditions is epidermolysis bullosa (EB), which comprises a group of heritable blistering disorders caused by mutations in ten genes expressed in the cutaneous basement membrane zone and has high morbidity and mortality. Identification of distinct mutations has improved the diagnosis and subclassification of EB, leading to improvements in disease prognosis, and has provided a basis for prenatal and pre-implantation genetic diagnosis for this disorder. Nevertheless, there is no cure or effective treatment for EB. Here, we review recent exciting developments in the areas of molecular therapies, including gene therapy, protein replacement therapy and bone-marrow-derived stem cell transfer, as potential new avenues to treat EB and other currently intractable heritable skin diseases.

The phenotypic spectrum of heritable skin diseases

Heritable skin diseases comprise a group of disorders with a wide spectrum of phenotypic manifestations [1]. At one end of the spectrum, the skin manifestation can be minor, manifesting, for example, with pigmentary changes, whereas at the other end of the spectrum the cutaneous manifestations can be extremely severe, causing considerable morbidity and mortality. Examples of the latter situation include the most severe forms of epidermolysis bullosa (EB), which manifest with extreme fragility of the skin, often resulting in the early demise of the affected individual within a few days or weeks of birth [2,3]. Considerable progress in understanding the molecular genetics of many of these conditions has been made over the past two decades, with diagnostic and prognostic implications (Box 1).

The cutaneous findings in EB can be associated with extracutaneous manifestations encountered in different subtypes. For example, the patients can have corneal blistering, dental abnormalities (including enamel dysplasia), fragility of the tracheal epithelium, gastrointestinal and urogenital-tract abnormalities, and progressive, late-onset muscular dystrophy. This phenotypic spectrum has led to complex classification schemes riddled with eponyms and there are suggestions that as many as 30 different subtypes of EB exist [4]. Traditionally, however, EB has been divided into three broad categories based on the location of blistering within the cutaneous basement membrane zone (BMZ; see Glossary) [2] (Table 1) and demonstration of abnormalities in the critical attachment complexes: (i) in the simplex forms (EBS), tissue separation occurs within the basal keratinocytes of the epidermis, the outer layer of the skin; (ii) in the classic junctional forms (JEB), tissue separation occurs within the cutaneous basement membrane that separates epidermis from the underlying dermis; (iii) the dystrophic forms of EB (DEB) depict tissue separation below the dermo–epidermal basement membrane within the upper papillary dermis. In addition, the most recent consensus conference on classification of EB [2] has proposed that additional, rare forms of blistering diseases should be considered to be part of the EB spectrum – these include lethal acantholytic EB [5], plakophilin-deficient skin fragility–ectodermal dysplasia syndrome [6–8] (with suprabasal location of cleavage) and Kindler syndrome [9,10] (with mixed location of blistering).

Here, we focus on the classic forms of EB by discussing the genetic basis of different variants of this disease, summarizing the translational implications of the molecular genetics and, finally, highlighting the progress in molecular therapies for this group of currently intractable blistering disorders.

Molecular genetics of EB

The classic forms of EB simplex are inherited in most cases in an autosomal dominant manner owing to dominant-negative mutations in the keratin 5 *(KRT5)* and keratin

Glossary

Attachment complexes at the cutaneous BMZ: ultrastructurally recognizable structures in the skin, crucial for stable association of the epidermis to the underlying dermis; these include: (i) hemidesmosomes (i.e. protein complexes extending from the intracellular milieu of keratinocytes to the lamina lucida and consisting of α6β4 integrin, type XVII collagen and plectin); (ii) anchoring filaments, thread-like structures consisting primarily of laminin 332 and traversing the lamina lucida; and (iii) anchoring fibrils, attachment structures composed of type VII collagen and extending from the lower part of the lamina lucida to the upper papillary dermis. These attachment complexes form a continuum of the network required for physiologic stability of the cutaneous BMZ.
Cutaneous basement membrane zone (BMZ): the interface of the two principal layers of the skin, the epidermis (the outer layer) and the dermis (the underlying inner layer), separated by a dermal–epidermal basement membrane consisting of lamina lucida (upper layer) and lamina densa (lower layer).
Epidermolysis bullosa (EB): a heterogeneous group of heritable disorders (Box 1), characterized by separation of the epidermis and the dermis at the cutaneous BMZ upon trauma of varying degrees. Mutations in the genes encoding components of the attachment complexes can result in weakness of the BMZ and manifest as blisters and erosions, which are characteristic of EB [2,3].

Corresponding author: Uitto, J. (Jouni.Uitto@jefferson.edu).

1471-4914/$ – see front matter doi:10.1016/j.molmed.2009.05.004

Box 1. Examples of recent advances in molecular genetics of heritable skin diseases

Epidermolysis Bullosa (EB)

- A group of heritable blistering disorders with considerable phenotypic variability, with an overall incidence of 1 in 20 000.
- Over ten distinct genes expressed in the cutaneous basement membrane zone harbor mutations underlying different subtypes [2,3] (Table 1).

Ichthyosis

- A heterogeneous group of scaling disorders with highly variable prognosis. The most common variant, ichthyosis vulgaris, with life-long scaling and dryness of the skin, affects 1 in 250 individuals, whereas harlequin ichthyosis, which is frequently lethal perinatally, has an incidence of 1 in 10^6.
- Ichthyosis vulgaris has semi-dominant inheritance owing to mutations in the filaggrin gene; harlequin ichthyosis with autosomal recessive inheritance is caused by mutations in the ABC transporter gene *ABCA12*; the classic autosomal recessive lamellar ichthyosis is caused by mutations in the transglutaminase 1 gene (*TMG1*), although at least five additional gene loci have been identified; the X-linked variant is caused by steroid sulfatase gene (*STS1*) deficiency [41–45].

Keratinization disorders

- A group of disorders manifesting characteristically with palmo-plantar hyperkeratosis, variably associated with hair, nail and tooth abnormalities, owing to mutations in genes encoding desmosomal cell–cell adhesion molecules.
- Mutations in the desmoplakin (*DSP*) and desmoglein 1 (*DSG1*) genes can result in cardiac manifestations, in addition to cutaneous abnormalities [46,47].

Cutaneous mineralization disorders

- The prototypic diseases, pseudoxanthoma elasticum (PXE) and familial tumoral calcinosis (FTC) are characterized by mineral deposits in the dermis, in addition to the retina and arterial blood vessels in PXE and in subcutaneous periarticular tissues in FTC.
- PXE is caused by mutations in the *ABCC6* gene expressed primarily in the liver and is considered to be a metabolic disorder [43,48].
- Two forms of FTC exist: (i) the hyperphosphatemic variants show elevated serum phosphate levels and are due to defects in the genes regulating renal reabsorption of phosphate (*GALNT3, FGF23, KLOTHO*); and (ii) the normophosphatemic variant is caused by mutations in the *SAMD9* gene, a TNF-α responsive gene of unknown function [49,50].

14 *(KRT14)* genes expressed by keratinocytes exclusively in the basal layer of the epidermis. The junctional forms are inherited in an autosomal recessive fashion and mutations in several genes encoding the components of hemidesmosomes and anchoring filaments, crucial attachment complexes at the dermal–epidermal junction, have been disclosed. Most commonly, mutations in junctional forms of EB reside in the *LAMA3*, *LAMB3* and *LAMC2* genes, which encode the subunit polypeptides of the secreted extracellular glycoprotein laminin 332 [11]. The dystrophic, severely scarring forms of EB arise owing to mutations in the *COL7A1* gene, which encodes type VII collagen, the major, if not the exclusive, component of anchoring fibrils [12]. Type VII collagen is synthesized by both epidermal keratinocytes and dermal fibroblasts.

In general, the severity of skin fragility is dependent on the types and combinations of mutations and their consequences at the mRNA and protein levels [3]. For example, complete absence of laminin 332 as a result of null mutations in any of the three subunit polypeptide genes mentioned earlier results in the most severe phenotype (Herlitz junctional EB), with profound fragility of the skin, leading to demise of the affected individuals usually within the first year of life. In addition, mutations in the integrin α6β4 subunit genes (*ITGA6* and *ITGB4*) are frequently associated with congenital pyloric atresia, which necessitates perinatal surgery, whereas mutations in the plectin gene *(PLEC1)* can result both in neonatal skin fragility and late-onset muscular dystrophy. In the dystrophic forms of EB, the diagnostic hallmark is an abnormality in the anchoring fibrils, which can be morphologically altered, reduced in number or entirely absent [12]. Because the anchoring fibrils are crucial for the stable association of the dermo–epidermal basement membrane to the underlying dermis, the severity of DEB frequently reflects the degree of abnormalities in anchoring fibrils so that, for example, the absence of type VII collagen manifests with extreme fragility of the skin. This phenotype presents with accompanying scarring, analogous to a third-degree burn, in the recessively inherited form of dystrophic EB (known as generalized RDEB).

Translational implications of the molecular genetics of EB

Distinct mutations in ten different genes underlying the classic forms of EB have been identified in well over 1000

Table 1. Clinical and genetic heterogeneity of EB[a]

EB subtype[b]	Inheritance	Location of blisters	Mutated genes	Altered or missing proteins
Simplex				
• Classic	AD (AR)	Basal layer of epidermis	*KRT5, KRT14*	Basal keratins
• EB-MD	AR	Basal layer of epidermis	*PLEC1*	Plectin
Junctional				
• Classic	AR	LL	*LAMA3, LAMB3, LAMC2, COL17a1*	Laminin 332, type XVII collagen
• EB-PA	AR	Basal layer–LL interface	*ITGA6, ITGB4, PLEC1*	α6β4 integrin, plectin
Dystrophic				
• Generalized, localized	AD, AR	Sub-lamina densa	*COL7A1*	Type VII collagen

[a]This classification highlights the most common subtypes of EB (i.e. simplex, junctional and dystrophic), which are differentiated by the location of blister formation within the cutaneous BMZ, as determined by ultrastructural analysis and/or immuno-epitope mapping. Additional extremely rare phenotypes with superficial or mixed locations of blistering have been proposed to belong to the spectrum of EB phenotypes (see main text) [2].

[b]Abbreviations: AD, autosomal dominant; AR, autosomal recessive; EB-MD, epidermolysis bullosa with muscular dystrophy; EB-PA, epidermolysis bullosa with pyloric atresia; LL, lamina lucida.

families [11,12] (Table 1) and comparison of the mutation database with the phenotypic manifestations has enabled the establishment of general genotype–phenotype correlations. Traditionally, EB was divided into three broad categories on the basis of the location of blisters in the skin, as determined by electron microscopic examination or epitope mapping of skin biopsies from the affected individuals [2]. Molecular genetics on EB have now improved the accuracy of diagnosis and subclassification, delivering prognostic improvements [3]. Furthermore, identification of mutations has enabled development of DNA-based prenatal testing for families at risk for recurrence of EB [13,14]. Such testing can be performed from chorionic villus sampling (CVS) as early as the tenth week of gestation, thus providing the parents and healthcare provider with information on the fetal genotype during the first trimester of pregnancy. An extension of DNA-based prenatal testing is pre-implantation genetic diagnosis, which has been established for EB and related blistering disorders [15,16]. Furthermore, an application currently under development for DNA-based prenatal diagnosis entails the examination of fetal cells or free fetal DNA in maternal blood for pathogenic mutations [17]. The benefits of this form of non-invasive prenatal diagnosis include avoidance of complications of invasive procedures, such as the small but clearly increased risk of fetal loss in CVS. Furthermore, an analysis of fetal DNA in the maternal circulation could provide information on the fetal genome as early as the fifth week of gestation [18]. In spite of the impressive progress in the molecular diagnostics of EB over the past two decades, the fact remains that there is no specific or effective treatment currently available for this group of blistering disorders. However, as we show here, recent exciting developments of molecular therapies, including gene therapy, protein replacement therapy and cell-based therapies, suggest that the era of treatment of EB and other heritable skin disorders is fast approaching.

Prospects for molecular therapies for heritable skin diseases

As indicated earlier, specific molecular defects have been identified in ten distinct genes in the classic variants of EB. In the recessive forms, the majority of the mutations are premature termination-codon-causing mutations resulting in truncation of the newly synthesized proteins. In the dominantly inherited forms of EB, the majority of the mutations are dominant-negative ones, resulting in synthesis of mutated polypeptides that interfere with their wild-type counterparts during the assembly of proteins crucial for stability of the dermal–epidermal junction.

The feasibility of molecular therapies has recently been addressed using preclinical animal models of human EB [19,20]. Specifically, mouse models recapitulating the clinical, genetic, histopathological and ultrastructural features of the most severe junctional and dystrophic forms of EB have been engineered through targeted ablation of laminin 332 genes (*Lama3*, *Lamc2*) or the type VII collagen gene (*Col7a1*). Several spontaneous mutant animal models for recessively inherited EB have also been identified, including the *Lamb3* mutant mouse with severe cutaneous blistering. In addition, animal models for the dominantly inherited EB simplex have been developed through targeted substitution of crucial amino acid residues in the *keratin 5* gene. Thus, many of these mutant animals serve as excellent model systems to study therapeutic approaches under development for EB.

Gene therapy

Several general strategies have been considered for gene therapy for EB. One of them uses keratinocytes, or isolated

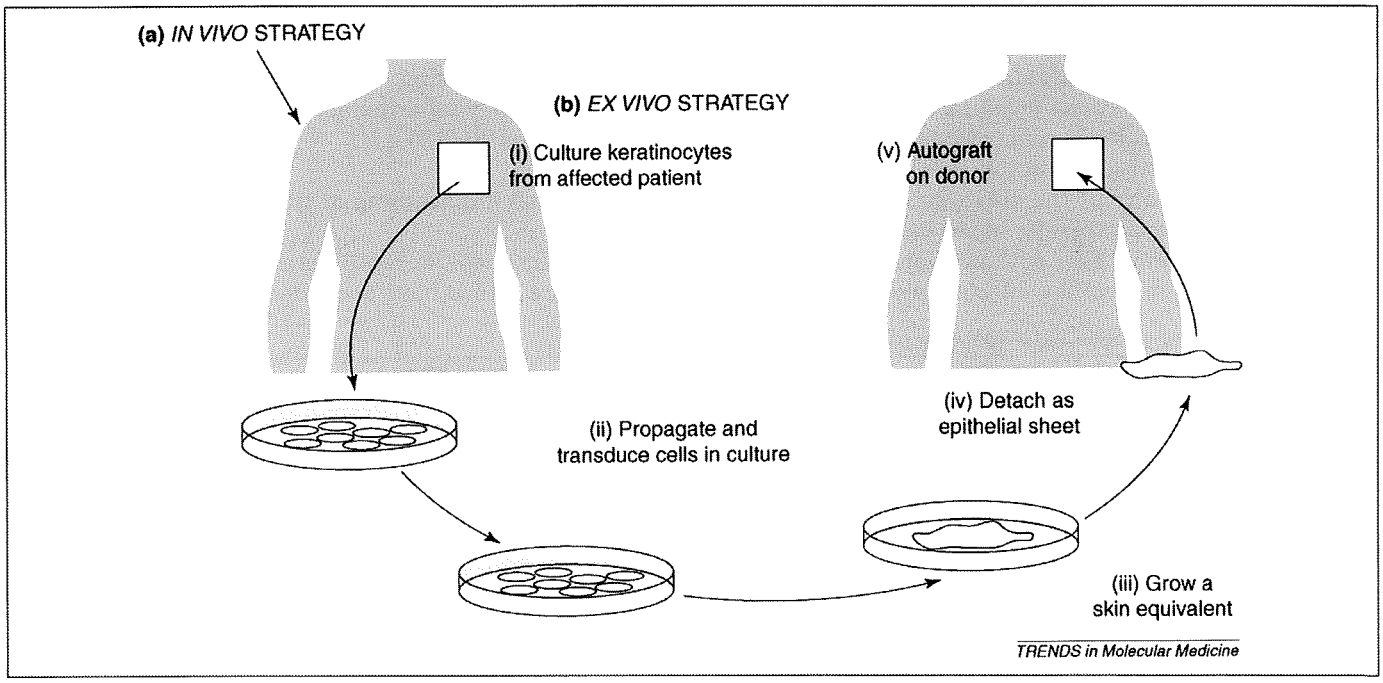

Figure 1. Principles of two primary strategies for cutaneous gene delivery. **(a)** As an *in vivo* strategy, the genes can be directly delivered into the skin by topical application, intracutaneous injections or by biolistic particle bombardment ('gene gun'). **(b)** As an *ex vivo* strategy, target cells such as keratinocytes are removed from the skin and propagated in culture. The cells are then transduced with the vector expressing the transgene and the transgenic cells are selected and grown into epithelial sheets that can then be grafted back to the original donor.

subpopulations with stem cell characteristics, in cultures established from the patient's skin, and these cells are then transduced with a cDNA expression construct in a viral vector. Such gene-corrected keratinocytes are grown into epithelial sheets that are grafted back to the donor skin to the site prepared by removal of the keratinocytes *in situ* harboring the mutations (Figure 1) [21]. The feasibility of this approach has already been demonstrated in an Italian study on a patient with relatively mild junctional EB treated with keratinocyte grafts expressing the wild-type *LAMB3* cDNA [22]. A follow-up examination at five years after the grafting has revealed sustained phenotypic reversal and persistence of the skin graft and continued expression of laminin 332 protein (M. DeLuca, personal communication). Furthermore, there is no evidence of immune challenge to the graft and there are no circulating antibodies to the β3 chain of laminin 332. It should be noted that, in this particular case, one of the underlying mutations (E210K) in the *LAMB3* gene enables a low level of expression of the corresponding protein, and the patient's immune system does not recognize the newly synthesized polypeptide as a neoantigen [22]. Although this *ex vivo* keratinocyte gene therapy approach has been successful in treating a small area of skin (a total of ~500 cm^2) in one patient, this approach has several potential limitations. In particular, a general concern of the DNA-based, viral-vector-driven gene therapy relates to potential carcinogenesis owing to integration of the vector into the genome in a manner that might conceivably activate proto-oncogenes or inactivate tumor-suppressor genes. Nevertheless, application of such keratinocyte gene therapy to additional patients with junctional EB and for patients with the dystrophic subtypes is currently being contemplated in other medical centers and the general applicability of this approach might become evident soon.

Protein replacement therapy

The concept of protein therapeutics for EB envisages administration of recombinant protein to the skin by topical application, local injection or systemic administration to the circulation. The feasibility of this approach is suggested by recent observations in type VII collagen 'knockout' mice injected intradermally with purified human type VII collagen [23]. The untreated *Col7a1*$^{-/-}$ mice, which recapitulate the cardinal features of RDEB, usually die within the first week of life as a result of extreme fragility of the skin and mucous membranes owing to the lack of type VII collagen and anchoring fibrils [24]. Intradermal injections of recombinant human type VII collagen into these mice significantly prolonged their survival and some mice survived as long as 20–25 weeks (Figure 2a). Examination of the treated mice revealed that the injected collagen homed to the cutaneous basement membrane zone in the areas of blistering and resulted in formation of anchoring fibrils and amelioration of the blistering phenotype (Figure 2b). It is of interest that the mice injected with human type VII collagen developed antibodies recognizing this protein but the antibodies did not react with the mouse protein and were not pathogenic when injected into normal mice [23]. Although these observations do not exclude the possibility that treatment of

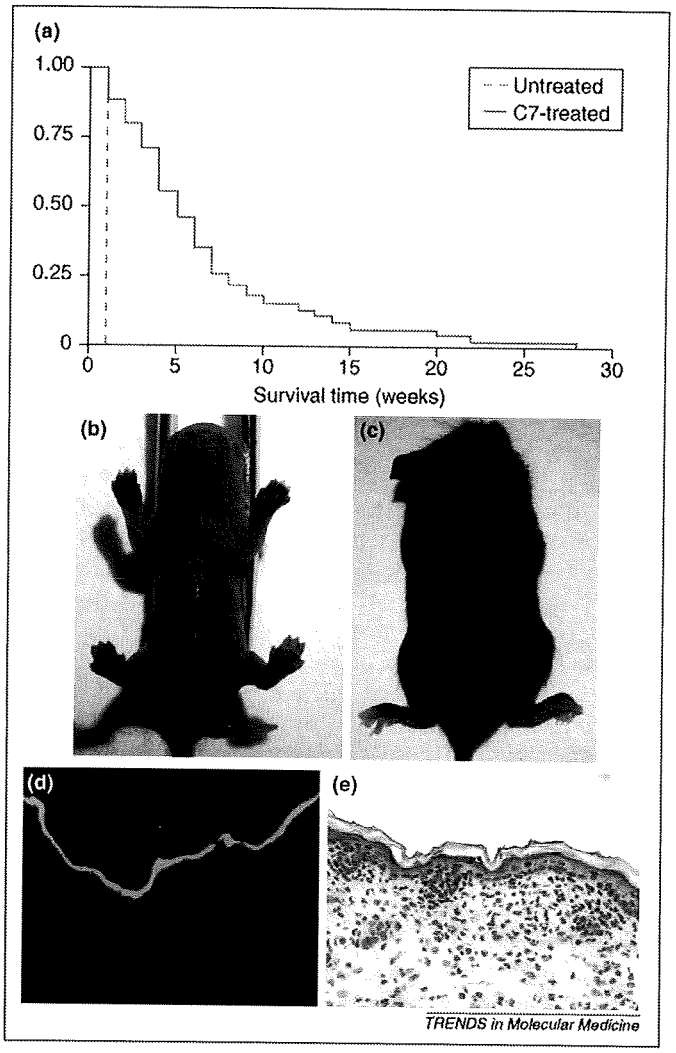

Figure 2. Extended survival of *Col7a1*$^{-/-}$ mice after injection with purified human type VII collagen. **(a)** Kaplan–Meier curves reflect the fact that untreated mice die within the first few days, whereas mice treated with recombinant protein survive up to a further 20–25 weeks. (b–e). A one-day-old *Col7a1*$^{-/-}$ mouse has hemorrhagic blisters on the paws **(b)**; after injections with purified human type VII collagen (C7), the same mouse survived past 22 weeks **(c)**. Immunofluorescence staining with antibody against type VII collagen demonstrates the presence of the injected protein in the skin at the cutaneous basement membrane zone **(d)** and a histopathological analysis of the skin reveals stable association of the epidermis and dermis **(e)**. Figure adapted, with permission, from Ref. [23].

Col7a1$^{-/-}$ mice with the mouse type VII collagen – and, by inference, treatment of RDEB patients with human type VII collagen – would not develop pathogenic antibodies, the antibody formation, if shown to be a problem at all, could be blocked, as demonstrated in the mice by administration of an anti-CD40 ligand monoclonal antibody (MR1) that profoundly depresses antibody production [23]. The latter observation highlights the potential of manipulating the innate and adaptive immune systems in the recipient to reduce the immune responses to neoantigens introduced to the patients as part of the molecular therapies, whether in the form of viral vectors or missing gene products [25]. With such refinements, it is conceivable that protein replacement therapy might become an option for treatment of recessive DEB patients in the future and similar work is currently under way for the development of protein replacement strategies for patients with junctional forms of EB owing to absence of laminin 332 [26].

Cell-based therapies

Recent development of molecular strategies for treatment of EB by cell-based approaches has focused primarily on two different cell types. First, DEB fibroblasts, engineered to overexpress human type VII collagen, have been shown to home to skin wounds and deliver type VII collagen when injected into mice, with subsequent promotion of wound healing [27]. Furthermore, DEB fibroblasts, devoid of type VII collagen expression, were more efficient in producing this protein and forming anchoring fibrils than the corresponding keratinocytes when transduced with type VII collagen cDNA in a retroviral delivery system upon implantation into nude rats [28]. Additional support for the postulate that fibroblast therapy could be feasible for treatment of DEB comes from observations in a hypomorphic mouse that expresses type VII collagen at ~10% of normal levels [20]. These mice display a phenotype closely resembling human DEB, characterized by cutaneous blistering owing to reduced type VII collagen deposition and paucity of anchoring fibrils in the BMZ. Intradermal injections of fibroblasts cultured from wild-type mice to the hypomorphic mice resulted in local depositon of type VII collagen at the dermal-epidermal junction, accompanied with functional improvement of the blistering phenotype. The increase in type VII collagen deposition at three weeks after the introduction of the fibroblasts to the skin was ~3.5 fold over the baseline (i.e. ~25–30% of the wild-type level), suggesting that partial restoration of type VII collagen assembly to anchoring fibrils can be beneficial to patients with EB.

Fibroblast therapy has also been tested by direct injection of these cells to the blistering areas of the skin in patients affected by RDEB. Specifically, autologous or allogeneic fibroblasts were injected intradermally, which resulted in local expression of type VII collagen in the patients' skin and lessening of the tendency to blister [29]. As expected, the autologous cells did not cause major adverse effects and the allogeneic fibroblasts elicited minor inflammation, however, they did not seem to survive in the skin for more than two weeks. Nevertheless, the benefits of cell injections were sustained for at least three months [29]. The mechanism was determined to be sustained cytokine-mediated upregulation of the expression of the mutant type VII collagen gene product in the resident cells in those patients who possessed a residual level of synthetic activity from their mutant alleles, whereas little evidence of the synthesis of normal type VII collagen from the newly introduced cells was noted. Consequently, those patients demonstrating some baseline synthesis of partially functional type VII collagen might benefit from this approach, whereas patients completely lacking type VII collagen gene expression owing to null alleles might not [30]. An associated finding in this study indicated that there seemed to be an improvement in the healing of chronic wounds, a major complication of RDEB. Collectively, injection of cultured fibroblasts from unrelated donors might be useful in improving epidermal–dermal adhesion and in accelerating wound healing in a select subgroup of patients with RDEB.

The cell-based therapy for RDEB has more recently been extended to use bone marrow cell transfer, including stem cells, to the type VII collagen 'knockout' mouse model system. Bone-marrow-derived cells have long been regarded to have a crucial role in the homeostasis of skin, in part through delivery of a variety of inflammatory cells, which are constitutive at low levels in normal skin. More recently, however, it has become clear that the plasticity of bone marrow stem cells enables their differentiation into cell types responsible not only for skin maintenance but also for rebuilding skin structures after injury [31,32]. For example, bone marrow cells expressing green fluorescent protein (GFP) that were transplanted into non-GFP mice revealed trafficking and homing of bone-marrow-derived cells to both wounded and non-wounded skin [33]. Wounding of the skin also stimulated the engraftment of these cells into skin and facilitated their differentiation into cells, such as fibroblasts, aiding in regeneration of damaged tissues. These observations illustrate the potential of bone marrow to serve as a valuable source of stem cells for the skin.

Two recent studies used GFP-expressing mice as the source of bone marrow cells for transplantation of RDEB mice, thus enabling the investigators to trace the donor cells in the skin and other tissues. In one study, various isolated subpopulations of cells within the source bone marrow were tested in *Col7a1*$^{-/-}$ mice by injecting the mice at birth or within a few days of birth, and survival of the mice beyond three weeks was monitored as a robust sign of amelioration of the blistering phenotype [34]. A specific subpopulation of bone-marrow-derived cells, positive for signaling lymphocytic activation molecule (SLAM/SLAMF1) family receptor (CD150^{+}/CD48^{-}), extended the survival of some animals beyond three weeks. The surviving animals also showed evidence of engraftment of the GFP-positive donor cells in the skin, production of type VII collagen and healing of skin blisters.

Another study has demonstrated successful engraftment of GFP-positive bone marrow cells in the skin after embryonic bone marrow cell transfer [35] (Figure 3). These cells also showed evidence of differentiation towards fibroblastic phenotypes and expression profiles, including deposition of type VII collagen. The embryonic bone marrow cell transfer also ameliorated the severity of the dystrophic EB phenotype at birth and the treated mice had an extended survival of up to several weeks (Figure 3a). An intriguing observation in this study was that the mice subjected to embryonic bone marrow cell transfer became tolerant to GFP and subsequent grafting of GFP-expressing skin did not induce the production of antibodies against GFP [35]. This situation enabled survival of the graft in GFP–bone-marrow-treated mice, whereas the graft in control mice was rejected before six weeks (Figure 3b).

Collectively, these preclinical studies attest to the possibility that bone-marrow-derived cells can serve as a source of dermal cells, such as fibroblasts, for regeneration of damaged skin in heritable skin diseases.

Clinical perspective

The preclinical studies using *Col7a1*$^{-/-}$ mice as an animal model for EB constitute a 'proof-of-principle' in support of the possibility that allogeneic hematopoietic stem cell transplantation, either from bone marrow or from umbili-

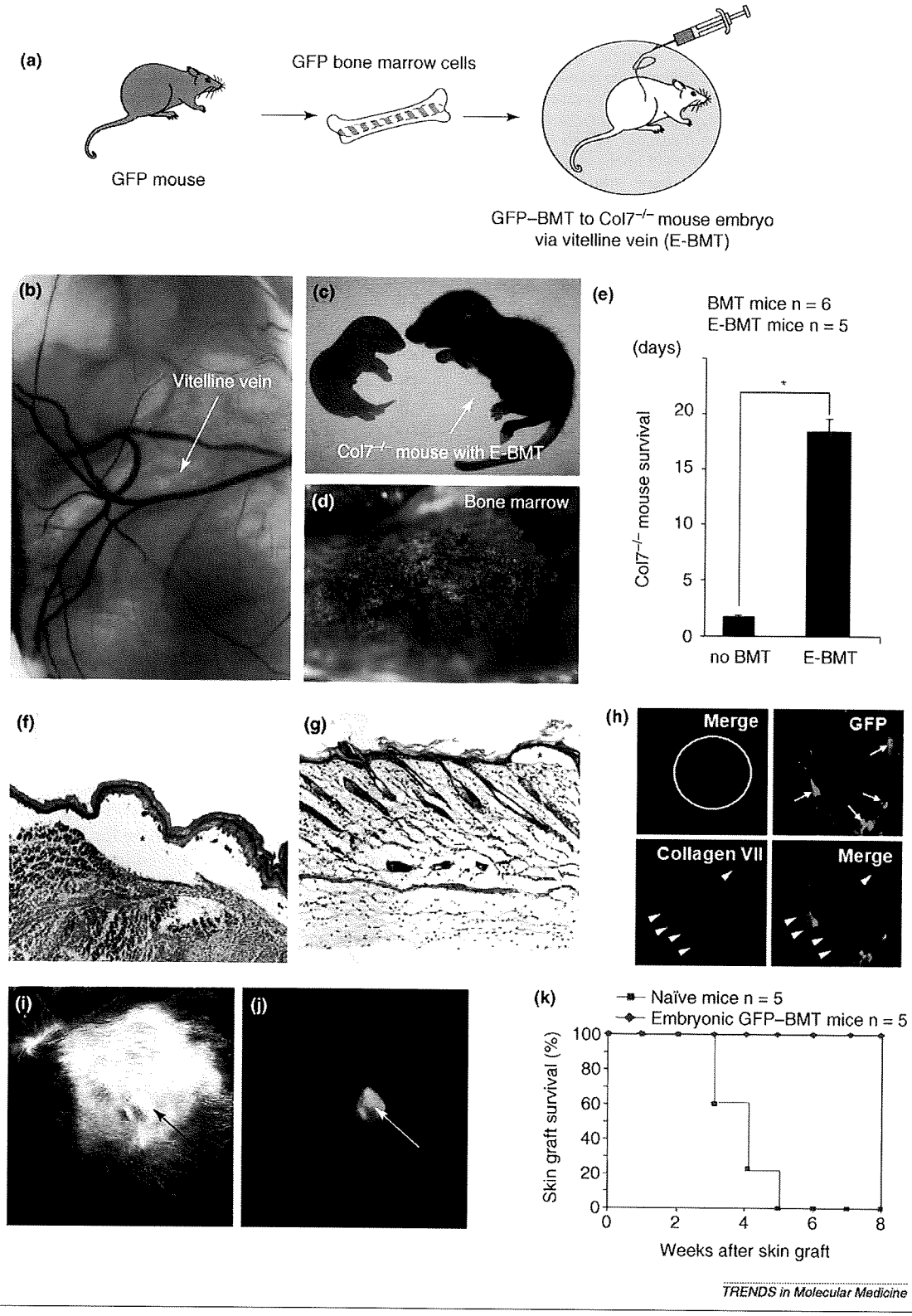

Figure 3. Embryonic bone-marrow transfer (E-BMT) into fetal circulation ameliorates recessive dystrophic epidermolysis bullosa (RDEB) by providing fibroblasts to the skin and by inducing tolerance. **(a,b)** Transgenic mice expressing GFP were used as donors of bone marrow cells that were injected into the fetus through the vitelline vein of the mother carrying *Col7a1*$^{-/-}$ pups. **(c,e)** The untreated *Col7a1*$^{-/-}$ mice died within the first few days postpartum, whereas those mice that received embryonic bone marrow transfer survived for up to three weeks. **(d)** The E-BMT-treated mice demonstrated bone marrow chimerism, as detected by GFP fluorescence. Examination of the skin revealed the presence of GFP-positive (green) fibroblastic cells that demonstrated expression of type VII collagen (red); these cells were frequently observed in close proximity to the hair follicles **(h)**. Histopathology of the newborn *Col7a1*$^{-/-}$ mice demonstrated ready separation of epidermis from the underlying dermis **(f)**, whereas the corresponding mice treated with E-BMT showed the presence of microblisters only **(g)** (asterisks indicate blisters). Induction of tolerance to GFP by E-BMT (i–k). Skin from GFP-positive transgenic mice was engrafted onto seven-week-old mice with or without prior treatment by embryonic GFP-bone marrow transfer; skin grafts were visualized either under normal (black arrow) **(i)** or under fluorescent (white arrow) **(j)** light. The grafts placed on naïve mice without treatment were rejected within five weeks of grafting, whereas grafts placed on mice treated previously with E-BMT persisted beyond eight weeks of grafting **(k)**. Thus, tolerization of the embryonic mice by bone marrow transfer offers an avenue to prevent antibody formation after treatment at a later age. Figure adapted, with permission, from Ref. [35].

Box 2. Outstanding questions

- What are the most suitable preclinical models for testing of molecular therapies for epidermolysis bullosa? Do transgenic mice appropriately recapitulate the features of the human disease?
- What are the most beneficial approaches of molecular therapies for treatment of heritable blistering diseases? Which strategies have the lowest risk–benefit ratio in different forms of the disease?
- If there is a risk of carcinogenesis in gene therapy, owing to potential random integration of viral constructs, can close surveillance enable timely removal of the skin tissue undergoing malignant transformation?
- Does the treatment of patients with RDEB with human recombinant type VII collagen result in formation of antibodies against type VII collagen and, if so, are these antibodies pathogenic?
- Can the immunosuppressive preparation of patients serving as recipients of bone marrow transfer be modified to lessen the morbidity and avoid the mortality associated with standard bone marrow transfer procedures?
- What is the potential role and feasibility of embryonic bone marrow transfer in human pregnancies carrying an affected fetus?
- What are the immune barriers to successful introduction of viral vectors, transgenes and autologous cells to the patients' skin? Can manipulation of innate and adaptive immune systems prevent or dampen the immune response?
- What are the efficiencies of exogenously introduced proteins for assembling into the supramolecular organizations of the skin, and what is the half-life of such components in the skin?
- What is the capacity of good manufacturing practice (GMP) production to generate type VII collagen or other proteins for lifelong treatment of affected individuals?
- Are the approaches contemplated so far for treatment of autosomal recessive diseases also applicable to autosomal dominant variants of EB? In particular, will strategies such as silencing of the mutant allele by siRNA, RNA transplicing or antisense oligomer technology counteract diseases owing to dominant-negative mutations?
- What are the best approaches for the treatment of extracutaneous manifestations frequently associated with cutaneous findings encountered in patients with epidermolysis bullosa?
- What are the costs of the different strategies for molecular therapies?

cal cord blood, could be an option for treatment of human RDEB. Indeed, in 2007, a one-year-old male with severe RDEB was infused with cells derived from the bone marrow of an HLA-matched older sibling donor, after standard myeloablative preparation [36]. Subsequent examination of the skin by immunofluorescence and electron microscopy has documented sustained expression of type VII collagen and assembly of anchoring fibrils, with gradual decrease in blister formation [36]. Three additional patients have entered similar trials and the overall outcome of these interventions is still pending. However, one of the patients died of cardiomyopathy during the myeloablative conditioning before bone marrow infusion and another patient died from complications after mismatched umbilical cord blood transfer [36]. Thus, optimistically, these early observations suggest that transfer of bone marrow stem cell populations could provide a means to correct the basement membrane defect in patients with RDEB and perhaps in other genetic skin diseases characterized by compromised integrity of the skin. At the same time, it should be noted that intense myeloablative conditioning routinely used for preparation of recipients for bone marrow transplant is associated with considerable morbidity and even mortality owing to susceptibility to infections, cytokine storm and graft-versus-host disease. For these reasons, strategies for reduced-intensity conditioning, which can have less complications during the conditioning phase, and non-myeloablative allogeneic stem cell transplantation have been developed both for malignant and non-malignant diseases [37]. In non-malignant conditions, such as RDEB, this strategy could well provide enough immunosuppression to promote engraftment of the stem cells and permit correction of the underlying genetic defect. This kind of reduced-intensity conditioning can be combined with the use of umbilical cord blood as the source of pluripotent stem cells with capacity to differentiate into different lineages, including cutaneous cells [38]. In fact, such reduced intensity conditioning strategies are being contemplated for the treatment of patients with different variants of EB [39]. It is clear, however, that these strategies need to be refined in experimental settings in specialized centers before these modalities can be recommended for the treatment of patient populations at large (Box 2).

As discussed here, several complementary avenues are currently being pursued towards treatment of patients with EB, including gene therapy, protein replacement and cell therapy approaches. It is clear that the information emanating from these studies will be helpful to ongoing efforts to find a cure for other heritable and acquired skin diseases as well. Furthermore, crucial insight into the mechanisms of pathology can be obtained from genetic observations, as for example with revertant mosaicism, a phenomenon noted particularly in the junctional forms of EB [40]. Nevertheless, although most of these studies are still at the preclinical level, some of them, such as bone-marrow-derived stem cell therapy, have already entered the clinical arena. Which of these approaches, if any, will be successful in providing amelioration and perhaps a cure for EB and other heritable skin disorders in the future remains to be proven by carefully controlled clinical trials.

Acknowledgements
We thank Carol Kelly for assistance; Mei Chen and David Woodley provided illustrations and Michele DeLuca provided information. The authors' original work was supported by the Ministry of Health, Labor and Welfare of Japan and by the DHHS, NIH/NIAMS (US).

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