2C). 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 little 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 been long thought that LCs represent one of the most likely target for UVB in the 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 concept, 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 non-irradiated skin and subjected to flow cytometric analysis. Compared to the non-irradiated control skin, LCs from UVB-irradiated skin showed high expression levels of CD86, OX40L, and intracellular IL-10 (Fig. 3A). 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 draining lymph nodes 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 hours after irradiation. The migrating LCs were identified as FITC+CD11c+EpCAM+Langerin+ cell fraction, while the migrating Langerin- dDCs were determined as FITC+CD11c+EpCAM-Langerin- cell fraction. The IL-10-producing and OX40L-expressing LCs from UVB-irradiated skin migrated to the draining lymph nodes as compared to the non-irradiated skin (Fig. 3B), 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 RANK ligand (RANKL) from UVB-irradiated apoptotic keratinocytes.

It has recently been reported that LCs express RANK and UVB irradiation upregulates cutaneous RANKL, which modulates the functions of DCs to induce Tregs (43). We have previously reported that when recombinant RANKL was added to LC culture, the RANKL-exposed LCs produce a high amount of IL-10 (44). On the other hand, 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 hours after exposure and stained to see apoptosis and RANKL expression. By flow cytometry (Fig. 4A), keratinocytes were divided into live (a; 7-AAD-, Anexin-), apoptotic (b; 7-AAD-, Anexin+), and dead (c; 7-AAD+, Anexin+) populations. The apoptotic keratinocyte expressed RANKL at a higher degree than did the live and dead keratinocytes (Fig. 4B). 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 recombinant RANKL to the culture of epidermal cells, and this increased IL-10 production was blocked by the further addition of an anti-RANK antibody (Fig. 4C). These results suggest that RANKL from UVB-irradiated keratinocytes mediates IL-10 production by LCs (Fig. 4C).

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 intraperitoneally with anti-IL-10 or anti-OX40L antibody for 4 consecutive days (days 0-3), while mice were sensitized (day 0) and challenged (day 5) with DNFB. Preirradiation of sensitizing site

to UVB suppressed CHS in mice (Fig. 5). The administration of anti-IL-10 antibody completely restored the CHS response. On the other hand, UVB-induced CHS suppression was partially but significantly abrogated by anti-OX40L antibody. 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. 6A). 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 of LCs cannot emigrate there. Mice receiving dissection of the sensitizing site did not exhibit UVB-induced immunosuppression of CHS compared to the non-dissected and UVB-irradiated mice (Fig. 6B). 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 hours after injection of DT (Fig.7A *vs* B). We then addressed the role of LCs in the UVB-induced suppression of CHS. The LC-depleted mice were irradiated with UVB on the 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 hours later. As compared to UVB-irradiated non-DT control mice, LC-depleted and UVB-irradiated mice developed a markedly high CHS response (Fig. 7D).

We also investigated whether Langerin+ dDCs are involved in the UVB-induced immunosuppression. It has been reported that Langerin+ dDCs recolonize 5 days or less after DT injection (32). Ten days after DT injection, when LCs are still absent in the epidermis but dDC are present (Fig. 7A *vs* C), mice were pre-irradiated with UVB, sensitized and elicited with DNFB. Compared to the control mice, LC-depleted but Langerin+ dDC-bearing mice did not show UVB-induced immunosuppression (Fig. 7E). 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-exressing, 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 recombinant IL-10 suppresses CHS and induces antigen-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 antibody treatment before UVB exposure prevents UVB-induced tolerance (49). The neutralizing study using anti-IL-10 antibody 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 after irradiation with UVB (50) or after stimulation with hapten-coupling (51). Since 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 antigen-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 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, intravenous injection of photopheresis-induced apoptotic cells inhibited an immune response to hapten, and this was caused by CD11c+ cells that induce antigen-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 antibody 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.

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 one day after hapten application. By this treatment, a considerable number of dDCs could migrate to the draining lymph nodes, 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. 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 much susceptible to UVB, we found that 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. Since 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 this strain of mice in our study. These differences in the UVB dose and mouse strain possibly give rise to the different results. Alternatively, since 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* 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 antigen-specific Tregs, as the suppression can be adoptively transferred into naïve 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 antigen presentation can induce Tregs and also may inhibit the priming of antigen-specific effector T cells. Knowledge on 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.

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