Low-Energy Visible Light Irradiation Modulates Immune Responses Induced by Epicutaneous Sensitization with Protein Antigen

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Epicutaneous sensitization has been an important route for protein allergen sensitization in atopic disease. Although the skin is irradiated by sunlight daily, the influence of visible light on epicutaneous sensitization has not been explored. In this study, by using a well-established murine protein-patch model, we show that low-energy visible light (LEVL) irradiation could differentially modulate the predominant Th2 immune response induced by epicutaneous sensitization with protein antigen. When the induced Th2 response was strong, as usually observed in BALB/c mice, LEVL irradiation suppressed the response. In contrast, LEVL irradiation enhanced the weaker Th2 response in C57BL/6 mice. Increased IL-18 and decreased TGF-β expression in draining lymph nodes after LEVL irradiation was observed in BALB/c mice, but not in C57BL/6 mice. LEVL irradiation also enhanced IL-18 expression in skin and reduced the downregulation of CD24 expression on epidermal Langerhans cells in draining lymph nodes of BALB/c mice. Collectively, these results provide evidence for immunomodulatory effects of LEVL irradiation and will help us develop a useful strategy for prevention of allergen sensitization.

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INTRODUCTION

Life on earth is radiated by sunlight daily. Previous studies have focused on the effects of ultraviolet (UV) radiation of sunlight, whereas studies on the effects of visible light have been sparse. UV light elicits many biological effects, such as immunomodulation, skin aging, and carcinogenesis (Ullrich, 2005). The development of a helium-neon laser, which delivers red light at a wavelength of 633 nm, has spurred the discovery of some biological effects of low-energy visible light (LEVL). Clinically, LEVL irradiation has beneficial effects on wound healing and recovery of nerve injury (Lipovsky *et al.*, 2008). Our group has reported that LEVL irradiation induces repigmentation in segmental-type vitiligo (Yu *et al.*, 2003). *In vitro* studies have shown that LEVL irradiation increases the motility of cultured human keratinocytes and stimulates their secretion of IL-1α and IL-8 (Haas *et al.*, 1990; Yu *et al.*, 1996). LEVL also enhances migration, proliferation, and growth factor release of melanocytes (Yu *et al.*, 2003). The underlying mechanisms of bio-modulation by LEVL irradiation are still unclear, although it has been reported that cytochrome *c* oxidase serves as a photoreceptor, and induction of reactive oxygen species and increase in intracellular calcium concentration are involved (Karu, 1999; Lavi *et al.*, 2003).

The prevalence of atopic disease has progressively increased in the past few decades. The interactions of genetic susceptibility to atopy with various environmental protein allergen exposures play a central role in the pathogenesis of atopic diseases (Ngoc *et al.*, 2005). Atopic dermatitis (AD) is often the first manifestation of the atopic triad and is the beginning of the ''atopic march'' (Spergel and Paller, 2003). The route of protein allergen sensitization in AD patients is still unclear. However, compelling clinical evidence suggests that epicutaneous exposure to protein allergens is one of the important sensitization routes for AD (Santamaria Babi *et al.*, 1995; Teraki *et al.*, 2000). In animal models, we and others have demonstrated that epicutaneous sensitization with protein antigens induces a predominant Th2 and a weak Th1 response, which lead to AD-like skin lesions and development of asthma (Wang *et al.*, 1996; Spergel *et al.*, 1998). An epicutaneously induced Th2 immune response requires the production and secretion of IL-10 and IL-13 (Herrick *et al.*, 2003; Laouini *et al.*, 2003). Downregulation of the epicutaneously induced Th2 response has been mediated by cyclooxygenase-2 in an animal model (Laouini *et al.*,

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Abbreviations: AD, atopic dermatitis; LC, Langerhans cell; LEVL, low-energy visible light; UV, ultraviolet

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2005). As human skin is irradiated daily by visible light, we investigated the influence of LEVL irradiation on the immune response induced by epicutaneous sensitization with protein antigen.

RESULTS

Low-energy visible light irradiation suppresses the Th2 immune response induced by epicutaneous sensitization with protein antigen in BALB/c mice

The irradiation dose used in previous reports regarding LEVL irradiation was quite variable. Most investigators used 0.1–10 J cm^{-2} as the range of irradiation doses. Hence, we chose 4.8 J cm^{-2} for our experiments (as described in Materials and methods, mice received LEVL irradiation for 20 minutes by LED at 0.04 mW at 10 cm distance). BALB/c mice were divided into three groups. One group received LEVL irradiation immediately before OVA patch application successively for 5 days. Another group of mice received similar treatment but without prior LEVL irradiation. A group that received a PBS patch application served as negative control. The cytokine contents of the supernatants from *in vitro* OVA reactivation cultures were chosen as indicators of Th immune response. IL-5 and IL-13 represent the Th2 immune response, and IFN-γ represents the Th1 immune response. As expected, epicutaneous sensitization with OVA induced a predominant Th2 and a weak Th1 immune response in the sham-irradiated group, as indicated by high IL-5 and IL-13 as well as low IFN-γ contents in the culture supernatants (Figure 1). When the LEVL-irradiated group was compared to the sham-irradiated group, it showed much lower IL-5 and IL-13 contents in the supernatant. However, LEVL irradiation did not modulate Th1 induction, as IFN-γ content from the LEVL-irradiated group was similar to that of the sham-irradiated group (Figure 1). Thus, we conclude that LEVL irradiation has a suppressive effect on the Th2 response induced by epicutaneous sensitization with protein antigen in BALB/c mice without modulating the Th1 response.

LEVL irradiation has differential effects on Th2 immune response induced by epicutaneous sensitization in BALB/c and C57BL/6 mice

BALB/c mice are genetically Th2-prone and have been used as an experimental model for human atopy. By contrast, C57BL/6 mice are Th1-prone and used for human non-atopy. To assess if the suppressive effect of LEVL irradiation on the Th2 immune response observed in BALB/c mice also existed in C57BL/6 mice, similar experiments were performed in C57BL/6 mice. As expected, epicutaneous sensitization with OVA induced less IL-5 and IL-13 production in the OVA reactivation culture from C57BL/6 mice (Figure 2a). However, to our surprise, C57BL/6 mice receiving LEVL irradiation showed higher, not lower, concentrations of IL-5 and IL-13 in the supernatants than did the sham-irradiated group (Figure 2a). The levels of IFN-γ production were similar in the LEVL-irradiated and in the sham-irradiated group. Similar differential effects of LEVL irradiation were observed when BALB/c and C57BL/6 mice were epicutaneously immunized simultaneously (data not shown).

To further explore this dilemma, the TLR-2 agonist, a well-known Th2 enhancer, was employed. As expected, C57BL/6 mice receiving co-administration of OVA plus Pam3CSK4 and sham irradiation showed higher IL-5 and IL-13 contents in their culture supernatants (Figure 2b). However, C57BL/6 mice that received LEVL irradiation and co-administration of OVA plus Pam3CSK4 showed decreased IL-5 and IL-13 contents compared to sham-irradiated mice. The IFN-γ contents were again similar in sham-irradiated and LEVL-irradiated groups of C57BL/6 mice. Taken together, these results demonstrate that LEVL irradiation has differential effects on the Th2 immune response induced by epicutaneous sensitization with protein antigen in BALB/c and C57BL/6 mice.

LEVL irradiation increases IL-18 and decreases TGF-β expression in draining lymph nodes in BALB/c mice but not in C57BL/6 mice

As priming of naive T cells occurs in draining lymph nodes, we next explored the underlying mechanisms of the differential effects of LEVL irradiation by comparing the cytokine expression profiles in draining lymph nodes from both BALB/c and C57BL/6 mice. As shown in Figure 3, the expression of IL-18 was comparable in draining lymph nodes of naive BALB/c and C57BL/6 mice. Three days after epicutaneous sensitization with OVA, IL-18 expression was always lower in the sham-irradiated group of BALB/c mice compared to that of the C57BL/6 mice. However, LEVL irradiation enhanced the IL-18 expression in BALB/c mice but not in C57BL/6 mice. Moreover, 3 days after immunization, the TGF-β expression level in draining lymph nodes (LNs) of the LEVL-irradiated group of BALB/c mice was always lower than in the sham-irradiated group (Figure 3). A similar decrease was not observed in C57BL/6 mice. The change in IL-18 and TGF-β expression is small in amplitude but significant, because we have repeated the experiments at least three times and similar results were always observed. We also checked the IL-18 and TGF-β expression levels in BALB/c and C57BL/6 mice 2 and 4 days after epicutaneous immunization; similar trends with less amplitude were observed (data not shown). The expression of IFN-γ, IL-12(p35), IL-12(p40), IL-4, IL-10, IL-6, IL-1α, IL-1β, TNF, IFN-α, and IFN-β in draining LNs was very low or undetectable and showed no significant differences between LEVL-irradiated and sham-irradiated groups in either BALB/c or C57BL/6 mice (data not shown). Collectively, these data reveal that the differential effects of LEVL irradiation correlate with the expression of IL-18 and TGF-β in draining lymph nodes of BALB/c and C57BL/6 mice.

LEVL irradiation increases IL-18 expression in skin and reduces the downregulation of CD24 expression on epidermal Langerhans cells in draining lymph nodes of BALB/c mice

As LEVL irradiation had modulated the strong Th2 immune response induced by epicutaneous sensitization with protein antigen in BALB/c mice, we explored its effects on the skin of BALB/c mice. First, cytokine expression profiles of skin from LEVL- and sham-irradiated mice were compared. As shown

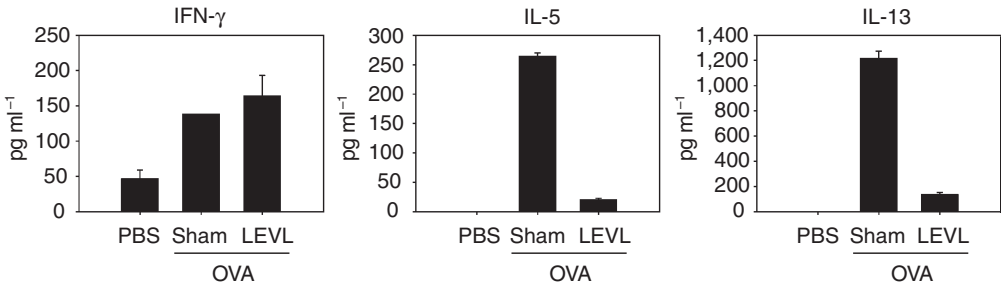

Figure 1. Low-energy visible light (LEVL) irradiation suppresses the epicutaneously induced Th2 immune response in BALB/c mice. Groups of BALB/c mice ($n = 5$) were immunized by patch application with OVA solution or PBS control. For the LEVL group, mice received LEVL irradiation immediately before patch application. Ten days later, draining LNs were obtained and the IFN-γ, IL-5, and IL-13 contents of the supernatants of the *in vitro* OVA reactivation culture of the pooled draining LN cells were determined by ELISA. Net concentrations (concentration in the absence of OVA subtracted from concentration in the presence of OVA) are presented. Three independent experiments were performed with similar results. Data from one representative experiment are shown.

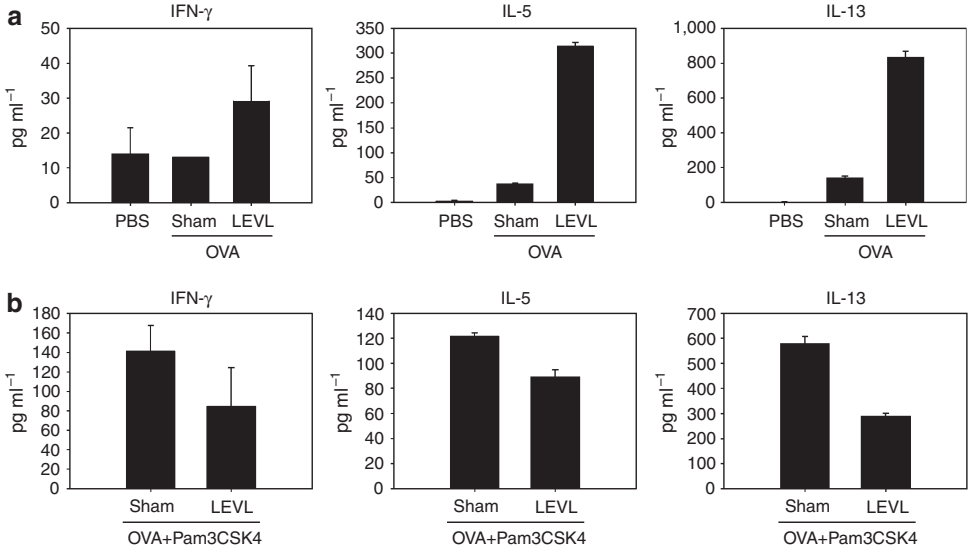

Figure 2. LEVL irradiation has differential effects on strong and weak Th2 immune responses induced by epicutaneous sensitization. Groups of C57BL/6 mice ($n = 5$) were immunized by patch application with OVA solution or PBS control (**a**) or OVA plus Pam3CSK4 (**b**). The LEVL group received irradiation immediately before patch application. Ten days later, draining lymph nodes were obtained and IFN-γ, IL-5, and IL-13 contents of the supernatants of the *in vitro* OVA reactivation culture of the pooled draining LN cells were determined by ELISA. Net concentration is presented. Three independent experiments were performed with similar results. Data from one representative experiment are shown.

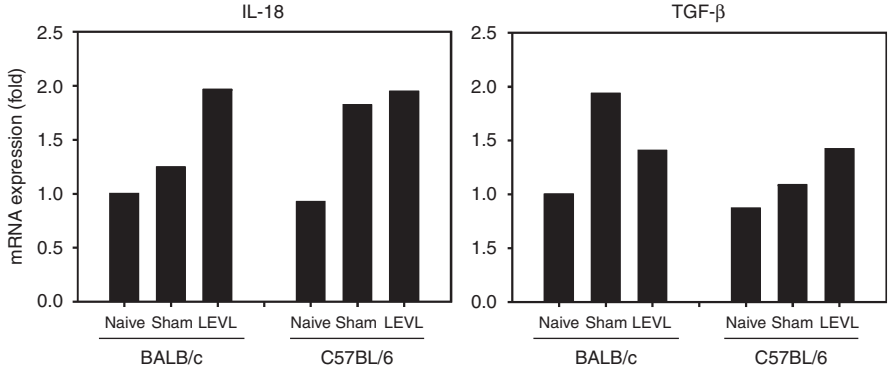

Figure 3. LEVL irradiation induced increased IL-18 and decreased TGF-β expression in draining LNs in BALB/c but not in C57BL/6 mice. Groups of BALB/c and C57BL/6 mice were immunized by patch application with OVA solution either with prior LEVL irradiation (LEVL) or not (sham).Naive mice received no treatment. Draining lymph nodes were obtained 72 hours after the start of immunization. Total RNA extraction, cDNA preparation, and quantitative real-time PCR for various cytokines were performed. The relative cytokine mRNA expression of each group was normalized to its β-actin expression. Results are shown as fold of the expression level of the naive BALB/c group. Similar results were obtained from three independent experiments. Results from one representative experiment are shown.

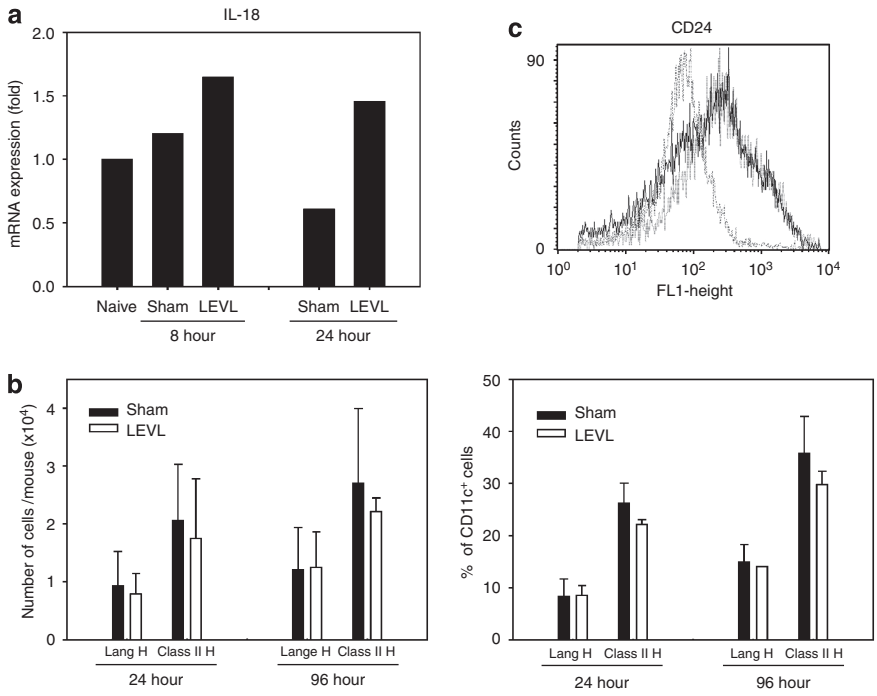

Figure 4. Effect of LEVL irradiation on skin and DCs in draining LNs in BALB/c mice (**a–c**). Groups of BALB/c mice were immunized by patch application with OVA solution either with prior LEVL irradiation (LEVL) or without (sham). Naive mice received no treatment. (**a**) 8 and 24 hours after patch application, skin was obtained and quantitative real-time PCR for various cytokines was performed. Similar results were obtained from three independent experiments. Results from one experiment are shown. (**b**) 24 and 96 hours after patch application, draining LNs were obtained and prepared for positive selection for DCs. Intracellular staining for Langerin and surface staining for MHC class II and CD11c were performed. CD11c$^+$ cells were gated. The results are shown as numbers per mouse as well as ratio in total CD11c$^+$ cells. (**c**) 96 hours after patch application, intracellular staining for Langerin and surface staining for various costimulatory molecules were performed. Langerin^{high} cells were gated. The expression level of CD24 is shown. LEVL group (gray, dashed line), Sham group (black), and isotype control (gray, dotted line).

in Figure 4a, IL-18 expression was detected in skin from naive and sham-irradiated BALB/c mice at 8 and 24 hours after epicutaneous sensitization with OVA. LEVL irradiation increased IL-18 expression in BALB/c skin at 8 and 24 hours post-sensitization. Again, the increased amplitude was small but significant, because repeated experiments always got similar results. The mRNA expression levels of TGF-β, TNF-α, IL-1α, IL-1β, IFN-γ, IL-4, IL-10, IL-12(p35), IL-12 (p40), IL-6, IFN-α, and IFN-β in skin were very low or undetectable and showed no significant differences between LEVL-irradiated and sham-irradiated groups (data not shown).

As IL-18 has been reported to be a key proximal mediator of Langerhans cell (LC) migration, and UV light irradiation has been shown to influence their migration (Antonopoulos *et al.*, 2007), we analyzed the kinetics and characteristics of skin-derived migratory dendritic cells (DCs) in draining lymph nodes. Skin-derived migratory DCs in the draining lymph nodes express the highest level of MHC class II molecule and have been divided into two subsets based on intracellular Langerin expression (Villadangos and Schnorrer, 2007). Langerin^{high} and Langerin$^-$ skin-derived DCs in draining lymph nodes have long been accepted as epidermal LCs and dermal DCs, respectively (Villadangos and Schnorrer, 2007). Recently, several groups reported a subset Langerin^{high} DC population, which resides in the dermis and shows characteristics and migratory kinetics different from those of epidermal LCs (Bursch *et al.*, 2007; Poulin *et al.*, 2007). Dermal Langerin^{high} DCs arrive at draining nodes significantly earlier than epidermal LCs after epicutaneous sensitization (Shklovskaya *et al.*, 2008). The peak influx of dermal Langerin^{high} DCs and epidermal LCs in draining lymph nodes is at 24 and 96 hours post-immunization, respectively. Thus, the cellular influx in the draining LNs was measured at 24 and 96 hours post epicutaneous sensitization with OVA. As shown in Figure 4b, the numbers and percentages of MHC class II^{high} DCs in draining LNs, which represent skin-derived DCs, were comparable in sham-irradiated and LEVL-irradiated groups of BALB/c mice. Moreover, the numbers and percentages of Langerin^{high} DCs in draining LNs 24 and 96 hours post-immunization, which represent dermal Langerin$^+$ DCs and epidermal LCs, respectively, were also similar between the sham-irradiated and the LEVL-irradiated group.

To further examine the LEVL effect on the activation of DCs, the surface expression of costimulatory molecules, including CD80, CD86, CD54, CD24, and CD40, on Langerin^{high} DCs was compared between the sham-irradiated and the LEVL-irradiated group. No significant differences in expression levels of CD80, CD86, CD54, CD24, and CD40 between the sham-irradiated and the LEVL-irradiated group 24 hours after immunization were detected (data not shown). Interestingly, the expression of CD24 on Langerin^{high} DCs in

draining lymph nodes 96 hours after immunization was always lower in sham-irradiated mice (MFI = 392) than in naive mice (MFI = 575) (data not shown). However, the LEVL group always showed reduced CD24 downregulation (MFI = 504) compared with the sham-irradiated group (MFI = 392) (Figure 4c). The expression of CD80, CD86, CD54, and CD40 was similar in sham-irradiated and LEVL-irradiated groups 96 hours post-immunization. Thus, we conclude that LEVL increased IL-18 expression in skin and reduced the downregulation of CD24 expression on epidermal LCs in draining LNs after epicutaneous sensitization with protein antigen.

Table 1. Known effects of IL-18 which might contribute to immunomodulation by low-energy visible light

A potent proinflammatory cytokine in innate immunity
Enhances IL-12-driven Th1 immune response
Stimulates Th2 immune response in the absence of IL-12
Promotes migration of epidermal LC into draining lymph nodes by a TNF-α and IL-1β-dependent mechanism
Upregulates the expression of MHC class II and costimulating molecules on human DCs

DISCUSSION

In this study, we demonstrated that LEVL could modulate a Th2 immune response induced by epicutaneous sensitization with protein antigen. When the induced Th2 immune response was strong, as usually observed in Th2-prone BALB/c mice, LEVL irradiation suppressed it. In contrast, an epicutaneously induced weaker Th2 response usually observed in Th1-prone C57BL/6 mice was enhanced by LEVL radiation. The increased IL-18 and decreased TGF-β expression observed in draining lymph nodes of BALB/c, but not C57BL/6, mice correlated with the differential effects of LEVL irradiation. We also showed that LEVL irradiation increased IL-18 expression in irradiated skin and reduced the downregulation of CD24 expression on epidermal LCs in draining LNs of BALB/c mice. To our knowledge, this effect of LEVL irradiation on protein allergen sensitization of atopic diseases has not been reported previously.

IL-18 is structurally homologous to IL-1 and is also synthesized as a precursor that becomes active after cleavage with caspase-1 (Nakanishi *et al.*, 2001). IL-18 has many known effects that might contribute to immunomodulation by low-energy visible light (summarized in Table 1). IL-18 is a potent proinflammatory cytokine in innate immunity that contributes to many pathophysiological inflammatory conditions. For example, active IL-18 has been demonstrated in patients with Crohn's disease and a mouse colitis model (Kanai *et al.*, 2001). Daily treatment with IL-18 and IL-12 has induced severe colitis and mortality in mice and was independent of T cells (Nakamura *et al.*, 2000). In adaptive immunity, IL-18 is unique in that it produces opposite effects on T cells, depending on the presence or absence of IL-12. IL-18 enhances the IL-12-driven Th1 immune response, whereas it stimulates the Th2 immune response in the absence of IL-12 (Nakanishi *et al.*, 2001).

In our protein-patch model, because we could detect comparable low-levels of IL-12 expression in draining lymph nodes of immunized BALB/c and C57BL/6 mice (data not shown), we suggest the following hypothesis. First, higher IL-18 expression in immunized C57BL/6 mice cooperates with low-level IL-12 to enhance the epicutaneously induced Th1 response, which in turn suppresses the predominant Th2 response. Thus, differential IL-18 expression in draining lymph nodes after immunization may contribute to the high and low intensity of the Th2 response observed in BALB/c and C57BL/6 mice. Second, because LEVL irradiation enhanced IL-18 expression in draining lymph nodes of immunized BALB/c mice, the above-mentioned mechanism may operate to suppress the strong Th2 response induced in BALB/c mice. The weak point in this hypothesis is that we did not detect increased IFN-γ production in culture supernatants from C57BL/6 mice or from the LEVL-irradiated group of BALB/c mice. Our explanation is that the enhancement of the Th1 response is below the detectable limit of the *in vitro* assay, whereas a more sensitive biological effect could reflect the change. Nevertheless, because we could not detect any significant change in the expression levels of all measured cytokines in draining LNs of C57BL/6 mice after LEVL irradiation, the mechanism by which LEVL irradiation enhanced the epicutaneously induced Th2 response in C57BL/6 mice is still elusive. IL-18 has been reported to promote migration of epidermal LCs into draining LNs by a TNF-α- and IL-1β-dependent mechanism (Antonopoulos *et al.*, 2007). However, in our study, we did not detect any significant differences in the kinetics of migration of skin-derived DCs into draining lymph nodes between the sham- and the LEVL-irradiated group of BALB/c mice. One possible reason is the lack of induction of IL-1β and TNF-α after LEVL irradiation, because the effect of IL-18 on migration of epidermal LCs was mediated by these two cytokines. Moreover, IL-18 has been reported to upregulate CD54, CD83, CD86, and MHC class II expression on human DCs in *in vitro* studies (Gutzmer *et al.*, 2003). The discrepancy between our results and the previous report regarding the expression of CD54, CD86, and MHC class II may be attributed to differences between *in vivo* and *in vitro* systems and between different subsets of DCs. The information about CD24 as a costimulatory molecule has been sparse (Enk and Katz, 1994). As epicutaneous sensitization with protein antigen induces a remarkable downregulation of CD24 expression on epidermal LCs in draining LNs, and epidermal LCs have been suggested to mediate the Th2 immune response, reduced downregulation of CD24 expression might contribute to the suppressive effect of LEVL irradiation on the Th2 response. Further studies are needed to clarify this issue.

Immunosuppression induced by UVB irradiation has been intensively investigated for many years. To date, three photoreceptors have been identified: DNA, trans-urocanic acid, and membrane lipid (Ullrich, 2005). Although three photoreceptors contribute to immunosuppression,

UVB-induced DNA damage is considered to be the most critical event. Local and systemic immunosuppression was achieved by modulation of APC function, production of soluble immunomodulatory mediators, and induction of T-regulatory cells (Ullrich, 2005). Thus, a shift in the T-cell differentiation from Th1 to Th2 cells and promotion of the development of AD-like lesions in NC/Nga mice were observed following UVB irradiation (Simon *et al.*, 1990; Mutou *et al.*, 2007). On the other hand, the photoreceptor for LEVL irradiation is cytochrome *c* oxidase in mitochondria. The major events following LEVL irradiation are a change in the redox properties of respiratory chain components and production of reactive oxygen species (Karu, 1999; Lavi *et al.*, 2003). Obviously, owing to totally different photoreceptors and the following events, it is not surprising for UVB and LEVL irradiation to have different immunomodulatory effects. However, a review of the literature found several reports that deserve special attention. First, Teunissen and co-workers reported that after UVB irradiation, two separate subpopulations of LCs were distinguished (Nakagawa *et al.*, 1999). One group was apoptotic and mediated immunosuppression, whereas the second group could immunopotentiate the response by enhancing the upregulation of costimulatory molecules and the production of proinflammatory cytokines. Second, Lee and co-workers reported increased IL-18 production in a human keratinocyte cell line by UVB irradiation, which required reactive oxygen intermediates and AP-1 signaling (Cho *et al.*, 2002). We wonder whether UVB irradiation may also simultaneously induce a weaker immunopotentiating pathway that may be comparable to that induced by LEVL irradiation in addition to its predominant immunosuppressive effect. This issue deserves further clarification.

In summary, we have provided evidence for immunomodulatory effects of LEVL irradiation. As epicutaneous sensitization is an important route for protein allergen sensitization in atopic diseases, these results will help us elucidate the regulation of allergen sensitization and develop a useful strategy to prevent allergen sensitization.

MATERIALS AND METHODS

Mice and reagents

Six- to ten-week-old female BALB/c and C57BL/6 mice were purchased from the animal center of National Taiwan University College of Medicine and kept in a specific-pathogen-free environment. All animal experiments were approved by the animal care committee of the Medical College of National University. OVA (Grade V) and Pam3CSK4 were purchased from Sigma-Aldrich (St Louis, MO) and InvivoGen (Carlsbad, CA), respectively. Capture and biotin-conjugated detecting antibodies for IFN-γ and IL-5 used in the ELISA were from PharMingen (San Diego, CA). Streptavidin-alkaline phosphatase was purchased from Southern Biotechnology (Birmingham, AL). The murine IL-13 ELISA kit purchased from R&D Systems (Minneapolis, MN) was used for determination of the IL-13 content of supernatants. Antibodies used for flow cytometry were all purchased from PharMingen or e-Bioscience (San Diego, CA). Antibody-conjugated microbeads used for isolating cells were all purchased from Miltenyi (Bergisch Gladbach, Germany).

LEVL irradiation and immunization

Mice were immunized as previously described (Wang *et al.*, 2006). Briefly, 20 μl of OVA (100 mg ml^{-1}) was placed on the disc of a Finn chamber (Epitest, Tuusula, Finland). This disk was applied to an area of shaved skin on the back of a mouse. For each course of immunization, freshly prepared patches were applied on days 1–5. The LEVL-irradiated group received anesthesia by intraperitoneal injection, were irradiated for 20 minutes with a visible red (633 nm) light-emitting diode-based system, at 4 mW power at a distance of 10 cm and immediately received the patch. For the sham-irradiated group, mice received a similar treatment but without LEVL irradiation.

Cytokine determination in supernatants of lymph node cell reactivation culture

Ten days after the start of the immunization course, mice were killed, and axillary, subscapular, and inguinal LNs were harvested. Pooled LN cells (1×10^6) were cultured in the presence or absence of 100 μg ml^{-1} OVA. Supernatants were harvested 48 hour later and stored at −80°C. IFN-γ, IL-5, and IL-13 content of supernatants was measured for each by a standard sandwich ELISA. The limit of detection for IL-5 and IL-13 was 10 pg ml^{-1} and for IFN-γ was 50 pg ml^{-1}. OVA-specific cytokine production was calculated by subtraction of cytokine production measured in the absence of OVA from that in the presence of OVA.

Total RNA extraction, cDNA preparation, and quantitative real-time PCR

Skin was obtained 8 and 24 hours after patch immunization. Draining LNs were obtained 72 hours after patch immunization. They were frozen with liquid nitrogen and soaked in 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA). After homogenization, the total RNA was extracted, cDNA was synthesized, and the expression of mRNA of IFN-γ, IFN-α, IFN-β, IL-4, IL-6, IL-10, IL-12 (p35), IL-12 (p40), IL-18, TGF-β, IL-1α, IL-1β, and TNF-α was determined by quantitative real-time PCR according to the manufacturer's instructions. Each sample was analyzed in triplicate. The relative cytokine mRNA expression level of each sample was normalized according to its β-actin expression.

Flow cytometry analysis

Skin-draining LNs (axillary, subscapular, and inguinal) were excised 24 or 96 hours after the start of the immunization course. LN cell suspensions were prepared by digestion with 2.5 mg ml^{-1} collagenase for 30 min at 37°C, and the tissue was ground up and resuspended in HBSS containing 10 mM EDTA for another 5 minutes. The CD11c$^+$ cells were isolated by anti-CD11c microbeads. Cells were stained by various combinations of antibodies (CD11c-APC (HL3), CD24-biotin (M1/69), CD40-biotin (3/23), CD54-biotin (3E2), CD80-biotin (16-10A1), CD86-biotin (PO3), MHC class II-biotin (2G9)) and their isotype controls with or without subsequent staining with streptavidin-FITC. Intracellular staining of anti-Langerin (CD207)-PE was also performed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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