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Homeostasis of Thymus-Derived Foxp3+ Regulatory T Cells Is Controlled by Ultraviolet B Exposure in the Skin

Sayuri Yamazaki,* Akiko Nishioka,* Saori Kasuya,* Naganari Ohkura,† Hiroaki Hemmi,‡ Tsuneyasu Kaisho,†,§ Osamu Taguchi,**†† Shimon Sakaguchi,‡ and Akimichi Morita*

Accumulating evidence shows that immunological tolerance induced by Ag administration together with UVB irradiation is dependent on Foxp3+ CD4+ regulatory T (Treg) cells. However, the mechanisms by which UVB controls Treg cells in the skin are currently unclear. In this study, we have shown that exposure to UVB induced expansion of Treg cells up to 50–60% of the CD4+ T cells in the irradiated skin. The Treg cell expansion in the skin lasted for 2 wk after exposure, which contributed to homeostasis of Treg cells in the periphery later. UVB-expanded Treg cells formed clusters with dendritic cells and proliferated in situ. Furthermore, the expanded Treg cells appeared to derive from neuropilin 1+ thymus-derived Treg (tTreg) cells in the periphery because UVB-expanded Treg cells possessed Treg–specific CpG hypomethylation pattern, as seen in iTreg cells. These results collectively indicate that homeostasis of tTreg cells is controlled by UVB exposure in the skin. UVB therapy may be useful for not only inflammatory skin disorders, but also autoimmune, transplantation, and allergy. The Journal of Immunology, 2014, 193: 5488–5497.

Regulatory Treg cells suppress a variety of immune responses, including autoimmunity, antitumor immunity, transplant rejection, allergy, and microbial immunity (1–3). Two origins of Treg cells have been identified to date, thymus-derived Treg (tTreg) cells and peripherally derived Treg (pTreg) cells (4). Recent studies indicate that homeostasis of pTreg cells is primarily maintained in the intestine by commensal microbes (5–7). The mechanism by which homeostasis of tTreg cells is controlled in the periphery remains unclear.

Immunological skin disorders, such as psoriasis and atopic dermatitis, are clinically treated with UVB therapy. There is accumulating evidence that tolerance induced by UV is mediated by Treg cells (8–10). Suppressive function of peripheral blood Treg cells from psoriasis patients was restored by UV therapy (11). UV-induced receptor activator for NF-κB ligand on keratinocytes stimulates Langerhans cells to expand Treg cells (8), and PG E2-PE receptor subtype 4 signaling plays a role in UV-induced tolerance (10). These works mainly investigated the UVB effect on Treg cells in the draining lymph nodes. How Treg cells in the skin are controlled by UVB is poorly understood.

In this work, we investigated the role of UVB in Treg cell maintenance in the skin. We found vigorous expansion of Foxp3+ Treg cells in the skin after UVB exposure. Treg cells actively proliferated in UVB-exposed skin in situ, which contributed to homeostasis of Treg cells in the periphery later. The expanded Treg cells in the skin expressed cutaneous homing receptors and were able to migrate to other parts of the skin. Expanded Treg cells were mainly identified as neuropilin 1+, indicative of iTreg cells. DNA methylation analysis revealed that expanded Treg cells have acquired the Treg cell–type CpG hypomethylation pattern. Accordingly, we propose that UVB plays a critical role in maintaining homeostasis of tTreg cells in the periphery, especially in the skin.

Materials and Methods

Mice
C57BL/6J (B6) mice were obtained from SLC (Shizuoka, Japan) and maintained in the Nagoya City University Animal Facility under specific pathogen-free conditions. The institutional animal care and use committee of Nagoya City University approved this study (ID: H24-M24), and committee guidelines were followed throughout.

Abs and reagents
The anti-mouse Foxp3 (FIK-16s) staining kit was purchased from e Bioscience (San Diego, CA); anti-MHC class II Ab, anti–P-selectin ligand (P-lig) Ab was from BD Biosciences (San Diego, CA); and anti-neuropilin 1 Ab was from R&D Systems (Minneapolis, MN). Anti-Langerin Ab is from Dendritics (Lyon, France). Other Abs were purchased from BioLegend (San Diego, CA). Anti-CD4 and streptavidin microbeads were from Miltenyi Biotec (Gladbach, Germany). CFSE, Live/Dead fixable aqua, and 5-ethyl-2′-deoxyuridine (EdU) were obtained from Molecular Probes (Eugene, OR). Mitomycin C was from Sigma-Aldrich (St. Louis, MO).

UVB irradiation
UVB was provided by TL20/W12 lamps (Phillips, Eindhoven, The Netherlands), which emit most of their energy within the UVB range (290–320 nm; emission peak 313 nm). Mice were shaved and irradiated with a systemic exposure of 500 mJ/cm², reported to induce tolerance by Treg cells (10). In the experiment of Fig. 1C, mice were also irradiated with 150 mJ/cm² daily.
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The number of Treg cells in the skin peaked on day 7, which continued until day 14 (Fig. 1B). The frequency of Foxp3+ Treg cells in the skin was analyzed. The frequency of Foxp3+ Treg cells in the skin was increased by ∼3–4% on day 7 after UVB exposure (Fig. 1A, 1B). The Treg cell population remained significantly higher in DLN and spleen, but not skin, 3 wk after UVB exposure (Supplemental Fig. 1). The results indicate the expanded Treg cells in skin migrate to DLN and spleen by 3 wk after UVB exposure.

To ascertain the specific contribution of UVB irradiation to Foxp3+ Treg cells, expansion up to 50–60% in the skin, cervical lymph nodes were surgically exposed and irradiated. After 7 d, the frequency of Foxp3+ Treg cells in the UVB-irradiated cervical lymph nodes was increased only by 2–3% compared with sham surgery alone (data not shown), suggesting that UVB exposure of lymph node is unable to expand Treg cells up to 50–60% of CD4+ T cells.

Next we compared the effect of a single UVB exposure of 500 mJ/cm^2 with multiple UVB exposures of 150 mJ/cm^2 daily on consecutive 4 d or a single UVB of 150 mJ/cm^2 (Fig. 1C). We found that multiple UVB exposures of 150 mJ/cm^2 expanded Foxp3+ Treg cells, similarly to a single UVB exposure of 500 mJ/cm^2 (Fig. 1C). A single UVB of 150 mJ/cm^2 did not expand Treg cells (Fig. 1C). Therefore, we used a single UVB exposure of 500 mJ/cm^2 in further experiments.

Exposure to UVB expands skin-homing Foxp3+ Treg cells

It is reported that skin Treg cells express cutaneous homing receptors such as CD103, CCR4, and P-lig (19, 20). To investigate whether UVB expands skin-homing Treg cells, we analyzed the expression of CD103, CCR4, and P-lig on skin Treg cells. We found that UVB induced significant expansion of CD103+ CCR4+, and P-lig+ Treg cells in the skin (Fig. 2A, 2B). To further determine whether expanded Treg cells could migrate to other parts of the skin that were not exposed to UVB, we protected left ear from UVB irradiation. After 7 d, we observed an increased frequency of Foxp3+ Treg cells in the protected ears, similarly to that in the ears exposed to UVB (Fig. 2C, 2D). When we investigated the time points, Treg cells in the UVB-protected skin were still increased after 2 wk (Fig. 2D). These indicate that UVB expands skin-homing Treg cells that can migrate to other parts of the skin.

Foxp3+ Treg cell clusters formed with dendritic cells in the UVB-exposed skin

To determine the location of Treg cell expansion in the skin, we examined frozen sections of the skin from UVB-exposed mice using confocal microscopy. In the skin from nontreated mice, a limited number of Foxp3+ Treg cells were present in the dermis from UVB-exposed mice (Fig. 3A, 3B). Moreover, in UVB-exposed mice, Foxp3+ Treg cells formed clusters with MHC class II+ cells in the dermis (Fig. 3A, Supplemental Fig. 2). The majority of MHC class II+ cells were CD11c+, indicative of dendritic cells (DCs) (Fig. 3C). We also found that Langerin+ DCs were within the MHC class II+ DCs close to Treg cells (Supplemental Fig. 2). In contrast, no clusters of Treg cells and MHC class II+ DCs were present in the skin from nontreated mice (Fig. 3A). These results indicate that Foxp3+ Treg cells have expanded in the dermis after UVB irradiation, supporting a possible role of DCs in Treg cell expansion in the skin (21).

Treg cells proliferate in the skin after UVB exposure

The formation of clusters between DCs and Treg cells in the skin was examined by flow cytometer (Fig. 4A, 4B).
Staining of skin sections additionally revealed that EdU incorporation was detected in Foxp3+ Treg cells in the dermis after UVB exposure (Fig. 4C). Collectively, Treg cells appear to actively proliferate in the skin in situ after UVB exposure. Expansion of Treg cells after UVB exposure may also reflect their influx into the skin. To determine whether T cell influx from peripheral blood or lymphatics was the source of Treg cells in UVB-exposed skin, we adoptively transferred CFSE-labeled CD4+ T cells and assessed whether the transferred CD4+ T cells migrated to the skin after UVB exposure. Compared with DLN and spleen, fewer CFSE+CD4+ T cells were detected in the skin, with or without UVB exposure (Fig. 4D, 4E). Similar results were observed using CD45.1 marker to discriminate between transferred and host-derived T cells (data not shown). These findings indicate that T cell influx from peripheral blood or lymphatics to the UVB-exposed skin is limited and thus not the source of Treg cell expansion in the skin. Based on these results, we conclude that in situ proliferation of Foxp3+ Treg cells is the main source of Treg cell expansion in the skin after UVB exposure.

In situ neuropilin 1+ Treg cells are mainly expanded upon UVB exposure

To determine the origin of proliferating Treg cells in the skin following UVB exposure, we analyzed the expression of neuropilin 1, which is expressed on Treg cells and a marker to distinguish Treg and pTreg cells in mice (22, 23). On day 7 after UVB exposure, we observed significant expansion of neuropilin 1+ Foxp3+ Treg cells in the skin, but not neuropilin 1− Foxp3+ Treg cells (Fig. 5A, 5B). Neuropilin 1+ Foxp3+ Treg cells additionally showed a significant increase in DLN and spleen after UVB exposure (data not shown). These results suggest that the Treg cell population expanded by UVB comprises Treg cells.
The UVB-expanded Foxp3+ CD4+ T cells in the skin possess Treg cell–type hypomethylation pattern

It has been shown that Treg cell development was achieved by the combination of Foxp3 expression and hypomethylation of the Treg cell–representative regions (18). To determine whether UVB-expanded Foxp3+ CD4+ T cells underwent Treg cell–type hypomethylation, we analyzed CpG DNA methylation of Treg-specific sites, Foxp3 intron1, Tnfrii18 exon 5, Ctlad exon2, Ick intron1b, and Ick2 intron3a (18). Foxp3+ CD4+ T cells from skin of UVB-irradiated mice showed Treg cell–type hypomethylation (Fig. 6A), although the number of skin Treg cells in naive mice was too low to analyze. Foxp3+ CD4+ T cells from DLN of UVB-irradiated mice showed Treg cell–type hypomethylation, similar to those from naive mice (Fig. 6B). It is thus likely that UVB mainly triggers expansion of tTreg cells.

The hypomethylation analysis indicates that fully functional Treg cells are expanded by UVB in the skin (18). We performed in vitro suppression assay using DLN CD25+CD4+ T cells from UVB-irradiated mice because the sorted number of skin Treg cells was too low for the suppression assay. We sorted CD25high+CD4+ T cells from naive mice (Fig. 7A). The CD25+CD4+ T cells from UVB-irradiated mice did not contribute to Treg cell expansion in the skin, although UVB might stimulate tTreg cell output from the thymus.

To confirm the above notion, thymectomy was performed 1 wk before UVB irradiation. Thymectomy or sham surgery alone did not increase the frequency of neuropilin 1+ Foxp3+ Treg cells in the skin (Fig. 5B, top). However, thymectomy, in combination with UVB exposure, led to increase of neuropilin 1+ Foxp3+ Treg cells in the skin similar to that observed with sham surgery plus UVB (Fig. 5B, top). These results indicate that the neuropilin 1+ tTreg cells that already present in the periphery have expanded in the skin after UVB exposure.

To determine whether thymus plays a role in Treg cell expansion after UVB exposure, the frequency of Foxp3+ Treg cells among CD4+CD8+ thymocytes was analyzed. We observed a significant increase in Foxp3+ Treg cell frequency within CD4+CD8+ thymocytes on day 7 after UVB exposure, in some mice, although Treg cells were not increased in the thymus (Fig. 5C). To further establish whether the output of new tTreg cells from the thymus contributes to Treg cell expansion after UVB exposure, the expression of CD24/heat-stable Ag (24, 25), a marker of recent thymic emigrants, was investigated. Notably, CD24+ Foxp3+ Treg cells were significantly increased in DLN, not in spleen, after UVB exposure (Fig. 5D, 5E). There were almost no CD24+ Foxp3+ Treg cells in the skin before UVB exposure, and there was no increase after exposure (Fig. 5D, 5E). These results indicate that the increased output of tTreg cells does not directly contribute to Treg cell expansion in the skin, although UVB might stimulate tTreg cell output from the thymus.

Discussion

Our results showed that UVB triggers the expansion of Treg cells up to 50–60% of the CD4+ T cell population in the skin. Treg cell expansion lasted for 2 wk after UVB and contributed to Treg cell increase in skin–DLN and the spleen 3 wk after UVB exposure. The UVB-expanded Treg cells upregulated skin-homing receptors and migrate to nonirradiated skin. UVB-expanded Foxp3+ Treg cells in the skin have Treg cell–type hypomethylation, indicative of fully functional Treg cells (18). These results suggest
that UVB contributes to homeostasis of Treg cells in the skin as well as in peripheral lymphoid organs. Considering that homeostasis of pTreg cells is controlled by intestinal microbes and their metabolites (5–7), it is intriguing that homeostasis of tTreg cells is regulated by UVB in the skin.

FIGURE 3. Foxp3+ Treg cells are increased with clustering DCs in the skin after UVB exposure. (A) As in Fig. 1A, but the back skin of mice was obtained on day 7 after UVB irradiation (UVB+) and stained with MHC class II (green), CD4 (blue), and Foxp3 (red, top) or isotype (red, bottom) for Foxp3. For control, back skin from nonirradiated mice was stained simultaneously (UVB−). The white dotted lines indicate the border between epidermis and dermis. Pictures were obtained at original magnification ×400. Data are representative of four independent experiments. (B) As in (A), but Foxp3+CD4+ cells were counted in three to seven different high power fields (×400) in four separate experiments. The average of each experiment was plotted, whereby each data point represents a separate experiment. Horizontal bars indicate mean values, and vertical bars signify SD. The p values were obtained from the Student t test. (C) As in (A), but the back skin of UVB-irradiated mice was stained with CD11c (red) and MHC class II (green). Images were obtained at original magnification ×400. Data are representative of two independent experiments.

Following exposure to UVB, Treg cells formed clusters with MHC class II$^{high}$ DCs in the dermis (Fig. 3A, Supplemental Fig. 2) and proliferate in situ (Fig. 4A–C). In naive mice, Treg cells preferentially localize to dermis (26). Our results showed that small numbers of Treg cells were detected in the dermis from the
nontreated skin, whereas many Treg cells located in the dermis making clusters with DCs in the UVB-exposed skin (Fig. 3A, Supplemental Fig. 2). MHC class II^high^ DCs may have a role in expanding Treg cells in the skin after UVB exposure, as DCs are critical APCs that induce expansion of tTreg cells as well as pTreg cells from Foxp3^−^ precursors (13–15, 27). MHC class II^high^ DCs

**FIGURE 4.** Foxp3^+^ Treg cells proliferate in situ after UVB exposure. (A) EdU was injected i.p. into nontreated mice (UVB −) or those exposed to UVB 6 d before. The following day, ear skin was examined for EdU by FACS. Plots were gated on CD4^+^ T cells. Data are representative of three independent experiments. (B) As in (A), a summary of three separate experiments is shown, whereby each data point represents a separate experiment. Horizontal bars indicate mean values. (C) As in (A), but back skin from UVB-treated mice was stained with CD4 and Foxp3, and EdU detection was performed by microscopy. Data are representative of three independent experiments. (D) CFSE-labeled CD4^+^ T cells (2.5 × 10^6^) were injected i.v. into B6 mice. The following day, mice were irradiated with UVB or left untreated. After 7 d, DLN, spleen, and skin were analyzed. Plots of gated CD45.2^+^CD4^+^ T cells are shown. Data are representative of three independent experiments. (E) As in (D), but a summary of three independent experiments is shown, whereby each data point represents a separate experiment. Horizontal bars indicate mean values, and vertical bars signify SD. The p values were obtained from the paired t test.
may present self-Ags to Treg cells in the UVB-exposed skin because TCRs of Treg cells skewed to self-reactive (1, 28). Schwarz et al. (29) reported that Langerhans cells (LCs) are required for UVB-induced immune suppression. We observed Langerin + DCs within MHC class II high DCs in the UVB-exposed dermis (Supplemental Fig. 2). They may be Langerin + dermal DCs, but it is also possible that they are transit LCs because some LCs came back to epidermis at day 7 after UVB exposure (Supplemental Fig. 2). It is reported that both LCs and Langerin+ dermal DCs play a role in inducing Foxp3+ Treg cells (30–32). Moreover, Langerin+ dermal DCs may be equivalent to CD8+ DC subsets, which can induce Foxp3+ Treg cells (16, 21, 33). Therefore, it is possible that Langerin+ dermal DCs play a role in expanding Treg cells in the skin after UVB exposure in our system. Further studies are necessary to determine the mechanisms underlying the expansion of Treg cells by DCs in the skin after UVB exposure.

In addition to the role of UVB on tTreg cell homeostasis, we propose that Treg cells recognize self-Ags from UVB-damaged skin and expand to suppress further autoimmune reaction. In support of this hypothesis is prior observation that UV-damaged skin produces self-Ags (34–36). It is recently reported that UV-damaged keratinocytes release self-RNA and induce skin inflammation through TLR-3 signaling (37). We also speculate that UVB expands tTreg cells to repair UVB-damaged skin. Mathis and colleagues (38) recently reported that a special population of Treg cells expands after muscle injury to repair muscle. The muscle

**FIGURE 5.** Neuropilin 1+ tTreg cells existing in the periphery provide the source of Treg cells expanded by UVB. (A) Ear skin was stained with anti-Foxp3 Ab and anti-neuropilin 1 Ab at d 7 after UVB exposure. Isotype control for anti-neuropilin 1 Ab is shown in Supplemental Fig. 3. Plots were gated on live CD45.2+CD4+ T cells. Data are representative of three independent experiments. (B) As in (A), but the percentages of neuropilin 1+ Foxp3+ T cells/CD4+ T cells (top) or neuropilin 1+ Foxp3+ T cells/CD4+ T cells (bottom) in skin from the indicated mice are shown. ATX indicates adult thymectomized mice, and sham indicates sham surgery for thymectomy. A summary of three or more separate experiments, whereby each data point represents a separate experiment. Horizontal bars indicate mean values, and vertical bars indicate SD. (C) Mice were irradiated with UVB, and thymus was analyzed at the indicated time points with flow cytometer. Plots were gated on live CD4+CD8+ thymocytes. Horizontal bars indicate mean values, and vertical bars indicate SD. (D) As in (B), but a summary of three to four independent experiments is shown, whereby each data point represents a separate experiment. Horizontal bars indicate mean values. The p values were obtained from the Student t test.
Treg cells expanded to 40–50% of CD4⁺ T cells for ~1 mo. It is possible that similar mechanisms with muscle Treg cells are expanding Treg cells in the UVB-exposed skin. However, the expansion of Treg cells in UVB-exposed skin should have another mechanism involving DCs because DCs formed clusters with Treg cells in the UVB-exposed skin (Fig. 3A, Supplemental Fig. 2). We showed that UVB-expanded Treg cells mainly comprise neuropilin 1⁺ Treg cells. Neuropilin 1⁺ Treg cells in UVB-exposed skin originate from existing peripheral tTreg cells because neuropilin 1⁺ Treg cells were expanded in the UVB-exposed skin in thymectomized mice (Fig. 5B). It is known that thymus produces Treg cells at day 3 of birth and that tTreg cells go to periphery after day 3 of birth to suppress autoimmune diseases (39–41). Therefore, the expansion of neuropilin 1⁺ Treg cells in the skin from adult thymectomy mice after UVB exposure in Fig. 5B indicates the expansion of already existing tTreg cells in the periphery, not new recruitment from the thymus. Moreover, UVB could stimulate the production of tTreg cells in the thymus (Fig. 5C) and induce recruitment of CD24⁺ tTreg cells from thymus to DLN, but not to skin (Fig. 5D, 5E).

Interestingly, UVB exposure expanded skin Treg cells expressing cutaneous homing receptors such as CD103, CCR4, and P-lig (Fig. 2A, 2B). The expanded Treg cells could migrate to UVB-unexposed skin (Fig. 2C, 2D). Therefore, systemic exposure of UVB may not be necessary to maintain Treg cells. Only a part of skin exposed to UVB is enough to expand Treg cells and maintain immunological self-tolerance. Taken together, UVB expands tTreg cells in the skin in situ, and the expanded Treg cells migrate to other parts of skin as well as DLN and spleen later, which contribute to homeostasis of Treg cells in the periphery.

Importantly, the UVB-expanded Treg cells in the skin showed Treg cell–type hypomethylation as tTreg cells (Fig. 6) (18). Recent
studies indicate that expression of Foxp3 alone is not sufficient for conferring Treg cell phenotype and function and that Foxp3+ T cells require the Treg cell–type hypomethylation pattern to acquire Treg cell–type gene expression, full suppressive activity, and sustained expression of Treg cell function–associated molecules (18, 42, 43). Treg cell–type hypomethylation contributes to the stability and cell lineages of Treg cells and reflects the fully functional Treg cells (44). In addition to Foxp3 expression, the Treg cell–type hypomethylation pattern can be a reliable marker for defining functional Treg cells (18, 44). Thus, possessing both the expression of Foxp3 and the Treg cell–type hypomethylation indicates that the UVB-expanded Foxp3+ T cells in the skin are fully functional Treg cells.

Our observation that UVB contributes to Treg cell homeostasis in the periphery provides a new dimension to UVB therapy. Occasional exposure to UVB could be required for maintenance of Treg cells in the periphery. This may be one of the reasons that sun exposure is associated with a decreased risk of multiple sclerosis (36). Currently, UVB is an effective treatment for immunological skin disorders such as psoriasis and atopic dermatitis. We suggest that the potential roles for UVB in controlling Treg cells might be able to improve future Treg cell therapy for autoimmunity, transplantation, and allergy.

FIGURE 7. Treg cells from UVB-irradiated mice are suppressive in vitro. (A) CD4+ T cells from DLN were stained with anti-CD4 and CD25 Abs for sorting (presort) at 5–7 d after UVB exposure. After sorting, CD25+CD4+ T cells or CD25−CD4+ T cells (postsort) were fixed and further stained with anti-Foxp3 Ab. (B) CD25−CD4+ responder T cells (1 × 10^5) from CD45.1 mice were CFSE labeled and stimulated with spleen APCs (1 × 10^5) and anti-CD3 mAb. To these, the purified CD25+CD4+ T cells or CD25−CD4+ T cells as in (A) were added in graded numbers. After 3 d, CFSE dilution was analyzed with flow cytometry. Cells were stained with anti-CD45.1 and CD4 Abs. Dead cells were gated out by TOPORO-3. Data are representative of three independent experiments.
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Disclosures
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