The Journal of Immunology

Tolerance Induction by Transcutaneous Immunization through Ultraviolet-Irradiated Skin Is Transferable through $CD4^+CD25^+$ T Regulatory Cells and Is Dependent on Host-Derived IL-10

Mehran Ghoreishi and Jan P. Dutz

J Immunol 2006; 176:2635-2644; ;
doi: 10.4049/jimmunol.176.4.2635
http://www.jimmunol.org/content/176/4/2635

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Tolerance Induction by Transcutaneous Immunization through Ultraviolet-Irradiated Skin Is Transferable through $CD4^+CD25^+$ T Regulatory Cells and Is Dependent on Host-Derived IL-10

Mehran Ghoreishi and Jan P. Dutz[1]

UV radiation of the skin impairs immune responses to haptens and to tumor Ags. Transcutaneous immunization (TCI) is an effective method of inducing immune responses to protein and peptide Ag. We explore the effect of UV irradiation on TCI. The generation of Ag-specific CTL to OVA protein, but not class I MHC-restricted OVA peptide, is inhibited by TCI through UV-irradiated skin. Consequently, the induction of protein contact hypersensitivity and in vivo Ag-specific CTL activity following OVA protein immunization is prevented. Application of haptens to UV-exposed skin induces hapten-specific tolerance. We demonstrate that application of protein or class II MHC-restricted OVA peptide to UV-irradiated skin induces transferable Ag-specific tolerance. This tolerance is mediated by $CD4^+CD25^+$ T regulatory (T_{reg}) cells. These Ag-specific T_{reg} cells inhibit the priming of CTL following protein immunization in the presence of CpG adjuvant. IL-10 deficiency is known to prevent hapten-specific tolerance induction. In this study, we demonstrate, using IL-10-deficient mice and adoptive T cell transfer, that IL-10 is required for the direct inhibition of CTL priming following immunization through UV-irradiated skin. However, IL-10 is not required for the induction of T_{reg} cells through UV-irradiated skin as IL-10-deficient T_{reg} cells are able to mediate tolerance. Rather, host-derived IL-10 is required for the function of UV-generated T_{reg} cells. These experiments indicate that protein and peptide TCI through UV-irradiated skin may be used to induce robust Ag-specific tolerance to neo-Ags and that UV-induced T_{reg} cells mediate their effects in part through the modulation of IL-10. *The Journal of Immunology,* **2006, 176: 2635–2644.**

Peripheral immune tolerance is required in the maintenance of immune homeostasis. Peripheral immune tolerance is mediated in part by a subset of $CD4^+CD25^+$ T regulatory (T_{reg})[2] cells (1). This subset of T_{reg} cells has been shown to prevent autoimmune disease and to modulate host reactivity to viral and other neo-Ags (1, 2). There is thus interest in the development of methodologies to reliably expand/induce Ag-specific T_{reg} cells. Existing methodologies that induce Ag-specific T cells with regulatory function include oral (3) or intranasal (4) Ag administration in the absence of inflammatory signals or parenteral Ag administration such as by i.v. (4) or s.c. infusion (5) or directed targeting to dendritic cells (DCs) (6, 7). These methodologies may fail in the presence of inflammatory signals during the "tolerance induction" phase (6, 7).

The skin is an active immune organ that contains a high frequency of DCs and other specialized APCs. Recently, the skin has been used as an organ of immunization and methodologies to induce immune responses through the skin without the use of needles, commonly termed transcutaneous immunization (TCI), are being developed (8). Both peptides and full proteins can be delivered through the skin with the aid of barrier disruption (tape-stripping) and/or adjuvants. Contact-hypersensitivity occurs when haptens, small chemically reactive molecules that also have adjuvant properties, are applied to the skin. Hapten-mediated contact hypersensitivity is abrogated when immunization occurs through skin previously irradiated repeatedly with low doses of UV radiation (9). Furthermore, application of haptens to UV-irradiated skin induces hapten-specific tolerance and further immunization with an identical, but not different, hapten is prevented. UV tolerance to hapten Ags is mediated by transferable T cells (10) that have been characterized as $CD4^+CD25^+$ T_{reg} cells (11). These cells act in part through the induction of IL-10 production (11). However, the origin of the IL-10 and cellular target in vivo is still not clear.

UV irradiation can alter and impair the immune response to subsequent percutaneous immunization (12). The effect of UV on the outcome of TCI with either protein or peptide Ag has not been described. In this study, we describe the effect of TCI through UV-irradiated skin on the priming of Ag-specific $CD8^+$ T cells (CTL). We demonstrate that the skin can efficiently be used for the induction of Ag-specific peripheral tolerance through the induction of $CD4^+CD25^+$ T_{reg} cells. UV-induced T_{reg} cells can be generated in the absence of IL-10 and are functional in the absence of autonomous IL-10 production but require host-derived IL-10 to prevent the priming of Ag-specific CTL.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles Rivers Laboratories. IL-10 knockout (−/−) (13) and OT-I mice were purchased from The Jackson Laboratory. OT-I transgenic mice express a TCR specific to OVA residues 257–264 in the context of H2-K^b (14). For adoptive transfer, all C56BL/6

Department of Medicine and British Colombia Research Institute of Children and Women's Health, University of British Colombia, Vancouver, British Colombia, Canada

Received for publication July 15, 2005. Accepted for publication November 28, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Jan P. Dutz at the current address: The Skin Care Center, 835 West Tenth Avenue, Vancouver, British Columbia, V5Z 4E8, Canada. E-mail address: dutz@interchange.ubc.ca

[2] Abbreviations used in this paper: T_{reg}, T regulatory; DC, dendritic cell; TCI, transcutaneous immunization; LN, lymph node; LC, Langerhans cell.

0022-1767/06/$02.00

mice and OT-I mice were between the ages of 8 and 10 wk and sex-matched mice were used. All mice were housed under specific pathogen-free conditions and all procedures were approved by the Animal Care Committee of the University of British Columbia.

UV radiation

UV radiation was provided by four FS40 TL12 lamps (National Biological Corporation). FS40 lamps emit 3% in the UVC range, 45% UVB, and 52% UVA. The emission peak is at 310 nm (in the UVB range). The irradiance of the source at the center averaged 10 J/m^2/s, as measured by an IL400A radiometer, using an SEL 240 UVB Detector (International Light). Groups of mice were anesthetized with ketamine hydrochloride (Bimeda-MTC) and xylazine (Bayer) and subsequently irradiated on shaved dorsal skin on 4 consecutive days (days 0–3, daily 1200 J/m^2) before immunization. The ears of the mice were protected from radiation by opaque foil.

Peptides and proteins

OVA protein (OVA-V; Sigma-Aldrich) and BSA (Roche Diagnostic Systems) were used as immunogens. The H2-K^b-restricted immunodominant epitope of OVA-SIINFEKL ($OVA_{254-267}$-OVA peptide), the immunodominant I-A^b-restricted OVA peptide ISQAVHAAHAEINEAGR ($OVA_{323-339}$), and the control K^b-restricted peptide (2C-SIYRYYGL) were synthesized at the Nucleic Acid and Peptide Synthesis Facility of the University of British Columbia and purified to over 80% purity.

Adoptive transfer of T cells

OT-I T cells were isolated from the pooled lymph nodes (LNs) and spleen of naive OT-I mice. CD8$^+$ T cells were purified by positive selection using CD8 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Purity was routinely >90%. OT-I CD8$^+$ T cells were labeled with CFSE (Molecular Probes) and 5×10^6 were injected into the lateral tail vein on the second day of UV irradiation. Mice were immunized 24 h after the last irradiation (day 4). Mice were euthanized 3 days (day 7) following immunization and the skin draining LNs were harvested for analysis.

In experiments with adoptive transfer of CD4$^+$ T cells, peripheral draining LNs were pooled and CD4 T cells were positively selected to >95% purity using CD4$^+$ microbeads (Miltenyi Biotec). For adoptive transfer of CD4$^+$CD25$^-$ cells, peripheral draining LN cells were pooled and CD25$^-$ cells were first negatively selected using mouse anti-CD25 PE and PE selection beads (Stem Cell Technologies). Cells were then washed with washing buffer and CD4$^+$CD25$^-$ cells were next positively selected using CD4$^+$ microbeads (Miltenyi Biotec). In separate experiments, CD4$^+$CD25$^+$ T cells were positively purified from skin draining LN cells and splenocytes using microbeads (Miltenyi Biotec) to a purity of >90%. Where indicated, CFSE-labeled OT-1 cells were cotransferred with the donor LNs. Mice were then immunized 24 h after the transfer and skin draining LNs were harvested 3 days later.

Epicutaneous immunization

Protein or peptide immunization was performed as previously described (15, 16) 1 day after the last UV irradiation or sham irradiation. Mice were anesthetized with ketamine and xylazine. Animals were then immunized with OVA (500 μg), $OVA_{254-267}$ (25 μg), or $OVA_{323-339}$ (200 μg) with adjuvant, cholera toxin (25 μg; Sigma-Aldrich) or 500 μg of CpG (oligodeoxynucleotide 1826 5′-TCCATGACGTTCCTGACGTT-3′, prepared by the Oligonucleotide Synthesis Facility of the University of British Columbia) after tape-stripping of the shaved back skin. All immunogens were applied in 50 μl of PBS followed by tape occlusion for 24–48 h. In selected experiments, mice were immunized once and boosted with the same immunogens 1 wk later.

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated Abs and analyzed by four-channel FACS using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences). Anti-mouse fluorescent Abs to CD8$^+$ (clone 53-6.7), CD4$^+$ (clone RM 4-5), IFN-γ (clone MG1.2), CD25 (clone PC61), CD45R/B220 (clone RA3-6B2), CD11c (clone HL3), and CD40 (clone 3/23) were purchased from BD Pharmingen. Ab for Foxp3 (clone FJK-16s) was from eBioscience. Staining was conducted in PBS (Invitrogen Life Technologies) with 2% FCS (Invitrogen Life Technologies).

Peptide-specific transgenic CD8$^+$ T cells were identified using a PE-conjugated K^b-OVA tetramer (Beckman Coulter) after gating for B220$^-$ LN cells or splenocytes. CFSE was used to track OT-I T cell proliferation in the recipient mice. For intracellular cytokine staining, single-cell suspensions from peripheral draining LNs were stimulated with PMA and ionomycin, and treated with GolgiStop (all from BD Pharmingen) for 4 h at 37°C. Cells were then washed and stained for surface molecules and fixed with lysis buffer (BD Pharmingen). After treatment with Perm/Wash buffer (BD Pharmingen), cells were stained for IFN-γ. For intracellular Foxp3 staining, cells treated in a similar manner without in vitro stimulation. For the staining of Ag-bearing DC, mice were immunized with 500 μg of OVA-FITC (Molecular Probes) and CpG (500 μg) on the upper back. Draining LN were harvested 18 h after immunization, pooled cells from two mice were released with collagenase (Bayer), and CD11c$^+$ cells were positively selected using microbeads before staining with Abs to CD11c and CD40.

In vivo killing assay

Freshly isolated splenocytes from C56BL/6 mice were separately incubated in RPMI 1640 medium (Invitrogen Life Technologies) and 10% FCS with OVA peptide or control (2C) peptide at a concentration of 1 μg/ml for 1 h at 37°C. Control peptide-pulsed splenocytes were then labeled with 0.5 μM CFSE and OVA peptide-pulsed splenocytes with 5 μM CFSE in PBS to allow tracking of the different populations in vivo. Labeled cells were pooled in a 1:1 ratio and injected at 10^7 cells/mouse into the lateral tail vein. Disappearance of CFSE-labeled cells was tracked using FACS analysis of freshly isolated LN cells 24 h after the injection.

Epidermal sheet preparation

Ear or back skin from both UV-irradiated and nonirradiated groups was placed in 0.5 M ammonium thiocyanate (0.1 M phosphate buffer (pH 6.8); Sigma-Aldrich) at 37°C for 20 min. The epidermis was separated from dermis, washed with PBS, and dried at room temperature. After fixing with acetone, epidermal sheets were incubated with biotin-conjugated anti-mouse I-A^b (clone AF6-120.1; BD Pharmingen) for 1 h at room temperature followed by incubation with streptavidin-Alexa Fluor 488 conjugate (Molecular Probes) for 30 min. After washing with PBS, the sheets were mounted in mounting medium (Vector Laboratories) and images were captured with a Zeiss Axioplan epifluorescent microscope equipped with a COHO-CCD camera (Photometrics).

Protein contact hypersensitivity

Mice were immunized twice over 2 days with OVA protein (500 μg) and CpG (500 μg) beginning 24 h after 4 days UV irradiation or were irradiated for 4 days beginning 24 h following the second immunization as indicated. Seven days after the second immunization, each side of the right ear was challenged by applying OVA protein (100 μg) and CpG (100 μg) in 12.5 μl DMSO (Sigma-Aldrich) after tape-stripping twice. The left ear was challenged with DMSO only. The thickness of the ears was then measured with an engineer's micrometer (Mitutuyo) at 12, 24, 48, 76, and 96 h after challenge and changes in the left ear were subtracted from those on the right. In experiments involving the transfer of LN cells into naive mice, donor mice were immunized once with OVA protein (500 μg) and CpG (500 μg) beginning 24 h after 4 days UV irradiation. Two days later, CD4$^+$CD25$^+$ cells were isolated from LNs and spleens. A total of 2×10^6 of these cells was transferred by i.v. injection into naive mice. Recipients were then OVA-immunized with OVA protein (500 μg) and CpG (500 μg) on the back skin and this was repeated once 2 days later. Mice were challenged with OVA on the ears 7 days after the last immunization.

Statistical analysis

Groups were compared using two-tailed Student's *t* tests and results were displayed using Prism 3 (GraphPad software).

Results

UV irradiation impairs the transcutaneous priming of CTL to protein but not peptide Ag

UV irradiation impairs the sensitization to haptens subsequently applied to the irradiated skin area. We and others have demonstrated that the skin is an excellent site for the immunization to protein or peptide Ag (8, 15–17). TCI with protein or peptide Ag in the presence of cholera toxin adjuvant results in robust and durable Ag-specific CTL responses (16). We asked whether the induction of Ag-specific CTL is impaired when TCI proceeds through UV-irradiated skin. To this end, C57BL/6 mice were exposed to low dose UV radiation mainly in the UVB range (290–320 nm) on four consecutive days and then immunized with OVA

protein or the immunodominant K^b-restricted OVA-peptide Ag in the presence of cholera toxin adjuvant on the previously irradiated skin. We studied the priming of CTL to the K^b-restricted immunodominant peptide using adoptive transfer technology. Naive K^b-OVA-restricted CFSE-labeled OT-1 cells were adoptively transferred before irradiation and immunization and the level of proliferation and intracellular IFN-γ expression of these cells within the skin draining LNs was determined 3 days following immunization (Fig. 1*A*). As expected, application of either OVA peptide or protein resulted in the proliferation of OT-1 cells and

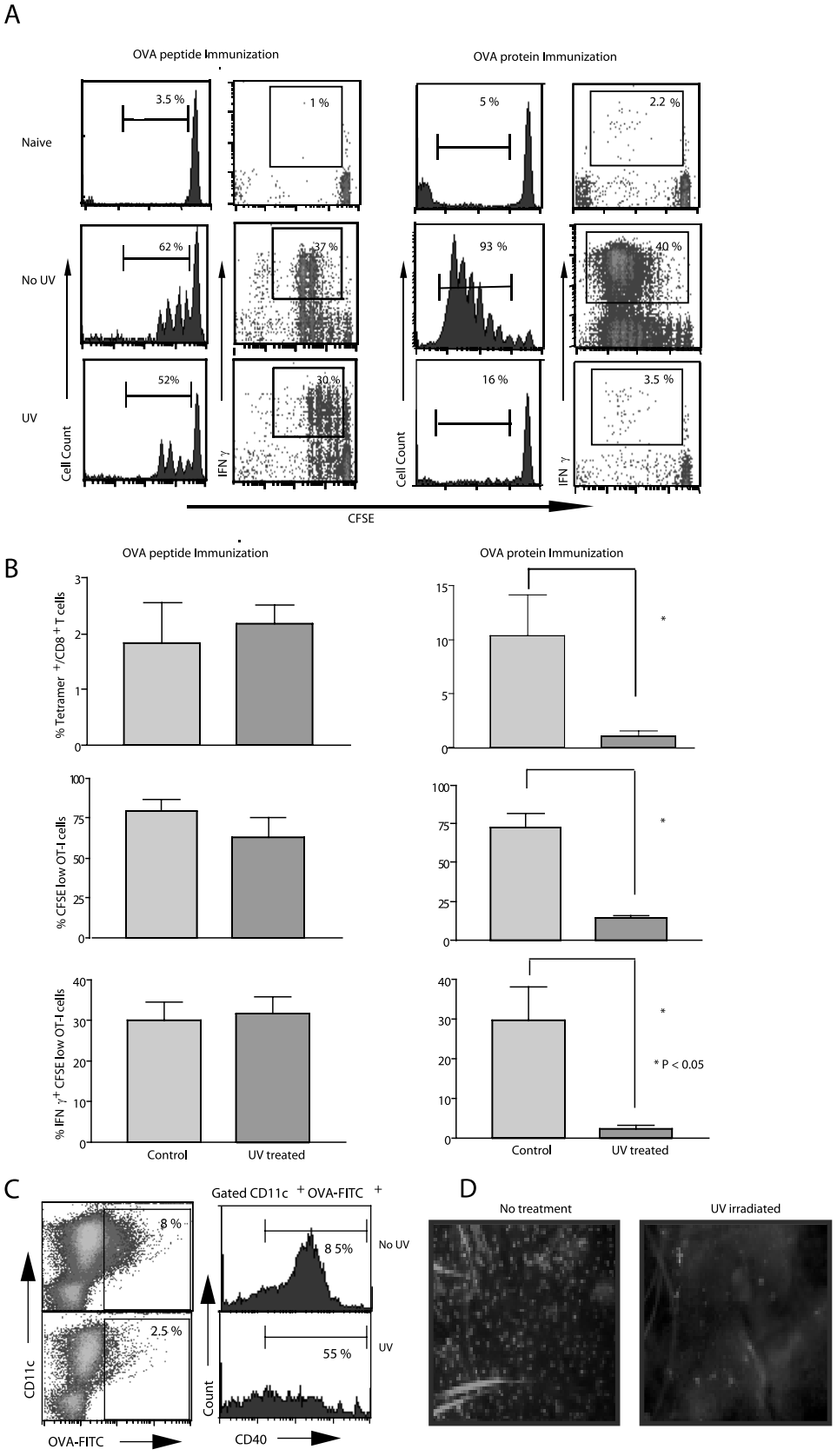


FIGURE 1. UV irradiation impairs the transcutaneous priming of CTL to protein but not peptide Ag. Mice (three per group) were irradiated with 1200 J/m^2 UVB daily for 4 days or sham-irradiated. Mice were then immunized on tape-stripped skin with cholera toxin and either OVA or the immunodominant K^b-restricted peptide, SIINFEKL (OVA peptide). CFSE-labeled OT-1 cells were adoptively transferred after the second dose of UV irradiation, and analysis was completed 3 days after immunization. UV light abrogated CTL responses to epicutaneous protein but not to peptide alone. *A*, Representative histograms (*left panels*) indicating serial CFSE dilution of proliferating OT-1 cells identified by gating on K^b-OVA-positive, CD8$^+$ T cells are shown. Numbers indicate the percentage of OT-1 cells that have divided at least once. Representative dot plots (*right panels*) indicating CFSE dilution and IFN-γ production by OT-1 cells in naive, sham-irradiated, and immunized or irradiated and immunized mice. Numbers represent the percentage of OT-1 cells having divided at least once that express IFN-γ. *B*, Aggregate data ($n = 3$) on the fraction of OT-1 cells among CD8$^+$ T cells recovered from the skin draining LN (*upper panel*), the percentage of OT-1 cells that have divided within the draining LN (*middle panel*), and the percentage of OT-1 cells expressing IFN-γ (*lower panel*). Error bars represent SEM. *C*, UV irradiation decreased the fraction of Ag-bearing CD11c$^+$ DCs within the skin draining LNs. Mice were immunized with OVA-FITC and CpG adjuvant following UV irradiation (UV) or sham irradiation (no UV). CD11c$^+$ DCs were isolated from the skin draining LNs 18 h later and analyzed for the presence of OVA-FITC and for CD40 expression (representative experiment of a series of three). *D*, Epidermal sheets were stained for class II MHC expression (indicative of LCs) following UV irradiation. Peptide-specific responses occurred despite an absence of LCs at the time of immunization. Original enlargement was ×20.

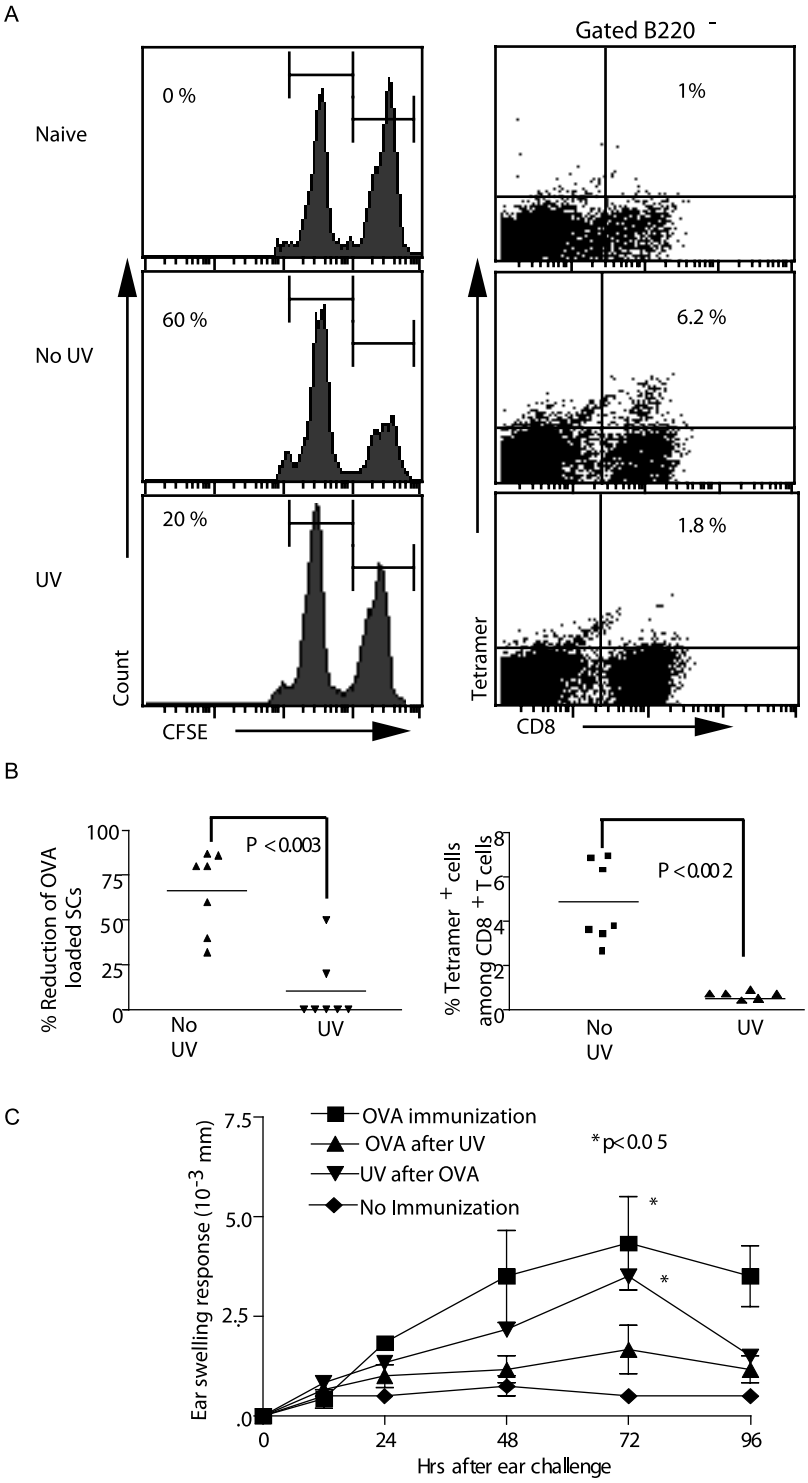


FIGURE 2. UV irradiation before TCI impairs the induction of protein contact dermatitis. Mice were irradiated with low-dose UV or sham-irradiated and then immunized with OVA protein and CpG adjuvant on tape-stripped skin. Mice received further irradiation or sham irradiation followed by a single boost. Draining LN were removed on day 14 and analyzed for the presence of OVA-specific CTL using tetramer staining as demonstrated by representative dot plots (*A*) on the *right*. Percentages indicate the proportion of K^b-OVA tetramer-positive $CD8^+$ T cells among all $CD8^+$ T cells. Aggregate data of three experiments ($n = 7$ mice/group) is shown. In vivo cytotoxicity of OVA-Ag-specific CTL was determined using CFSE-labeled and peptide-pulsed splenocytes. Splenocytes were pulsed with control peptide (low CFSE staining) or OVA peptide (high CFSE staining) and transferred to immunized mice. Survival was determined by FACS-based population analysis of skin draining LN cells 24 h later. Representative histograms with percent reduction of cognate peptide-pulsed splenocytes are depicted in *A* (*left figures*). Aggregate data ($n = 7$ mice/group) is presented in *B* (*left figure*). Effect of UV irradiation on the induction of protein contact hypersensitivity is depicted in *C*. Mice ($n = 3$/group) were immunized with OVA protein and CpG adjuvant. Mice were either irradiated with UV for four consecutive days before or for four consecutive days after immunization. For elicitation, OVA protein and CpG were applied to the ears 1 wk following immunization and ear swelling response was assessed daily. *, A significant difference between indicated data points and unimmunized control.

their full activation as determined by IFN-γ production. Proliferation was more marked, as determined by absolute numbers and CFSE dilution, following immunization with protein than peptide. Immunization through UV-irradiated skin profoundly inhibited the priming of OVA-specific CTL to protein immunogen but not to the class I MHC(K^b)-restricted peptide immunogen (Fig. 1, *A* and *B*). Immunization with FITC-labeled OVA allowed the detection of Ag-bearing DCs within the draining LN. Consistent with the diminished CTL responses in response to protein immunization, the fraction of OVA-bearing $CD11c^+$ DC was diminished in irradiated and immunized mice (Fig. 1*C*). Furthermore, Ag-bearing $CD11c^+$ DC within the draining LN expressed diminished levels of the costimulatory molecule, CD40. Surprisingly, CTL priming to transcutaneous class I MHC-restricted peptide administration was intact (Fig. 1, *A* and *B*) despite an absence of Langerhans cells (LC) following irradiation (Fig. 1*D*).

To determine whether CTL priming by TCI through UV-irradiated skin is impaired in the absence of the high CTL precursor frequency following adoptive transfer of OT-1 cells, mice were immunized with protein following UV or sham irradiation, boosted

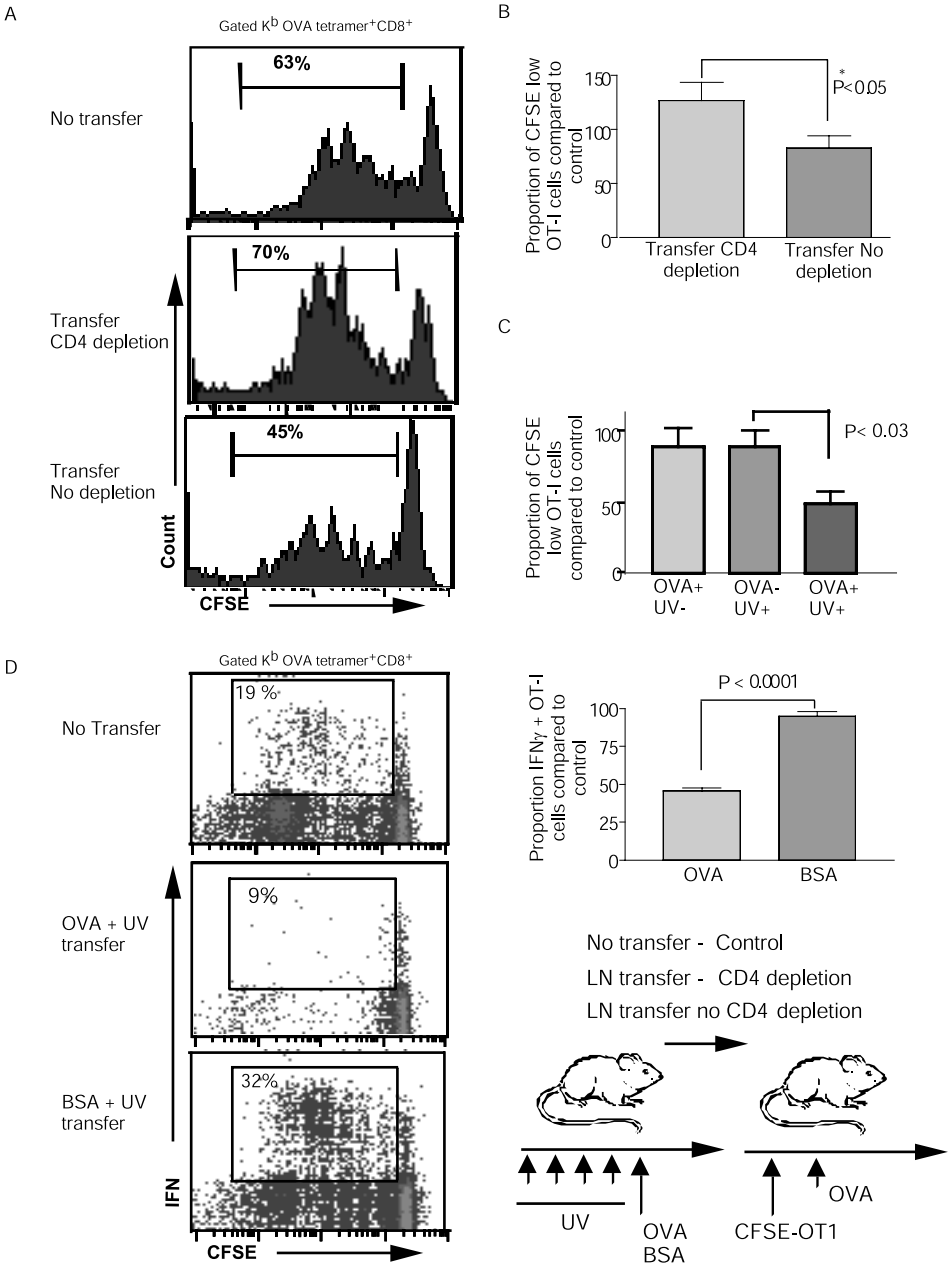


FIGURE 3. TCI with protein through UV-irradiated skin induces transferable Ag-specific tolerance. Donor mice were irradiated with UV before TCI with OVA. Two days after immunization, peripheral LN cells were obtained and $CD4^+$ T cells were depleted or not before adoptive transfer into naive mice. CFSE-labeled OT-1 $CD8^+$ T cells were cotransferred and the recipient mice were immunized transcutaneously with OVA. *A*, Representative histograms indicating OT-1 cell proliferation at 3 days following immunization. Numbers represent the percentage of OT-1 cells that divided at least once. *B*, Aggregate data from two experiments. The proportion of OT-1 cells that had proliferated at lease once was determined and normalized to mice immunized in the absence of adoptive transfer (no transfer, control) ($n = 3$ mice/group; bars indicate SEM). *C*, OT proliferation in immunized mice receiving donor cells from mice exposed to UV and immunization, UV irradiation only, or immunization only. *D*, OT-1 proliferative and IFN-γ responses in mice receiving $CD4^+$ cells from irradiated and OVA-immunized or control protein (BSA)-immunized donors. The results are normalized to values obtained in the absence of adoptive transfer (No transfer, control). A descriptive diagram of the experimental protocol used in this series of experiments accompanies this panel.

1 wk later (again following UV or sham irradiation) and CTL precursor frequencies were determined by K^b OVA-specific tetramer staining. In these experiments, CpG adjuvant was used as it is a potent inducer of Th1 immune responses and an effective adjuvant for the induction of CTL by the transcutaneous route (15). Immunization in the absence of UV irradiation resulted in 5 ± 0.7% OVA-specific CTL among the $CD8^+$ T cells in the peripheral LN, whereas the percentage of OVA-specific CTL detected following UV irradiation was significantly lower (0.7 ± 0.07%; $p < 0.002$) and similar to naive mice (Fig. 2, *A* and *B*, *right panels*). To determine whether the cytotoxic function of these cells was affected, splenocytes were differentially loaded with CFSE and pulsed with control or OVA peptide before transfer into immunized hosts (Fig. 2, *A* and *B*, *left panels*). A significant reduction in OVA peptide-pulsed spleen cells (labeled with a high concentration of CFSE) when compared with control peptide-pulsed spleen cells (labeled with a low concentration of CFSE) was detected in unirradiated and immunized but not irradiated and immunized hosts (66 ± 9% reduction vs 1% ± 7%; $p < 0.003$). To establish physiologic significance, we induced protein contact hypersensitivity in a fashion modeled upon standard hapten-mediated contact hypersensitivity experiments. Mice were immunized and then challenged with OVA protein on the ear 7 days following immunization and ear swelling was determined (Fig. 2*C*). Mice immunized with OVA demonstrated significant ear swelling with a maximum at 72 h following challenge. Mice treated with UV radiation immediately following immunization also demonstrated significant ear swelling, demonstrating that repeated low dose UV irradiation following immunization does not inhibit a protein contact elicitation response and is not systemically immunosuppressive. In contrast, mice immunized after UV irradiation developed minimal ear swelling. In allergic contact dermatitis and contact

hypersensitivity to haptens, both $CD8^+$ and $CD4^+$ T cells are activated in the draining LNs. Ear swelling responses are, however, predominantly due to $CD8^+$ T cells, and $CD4^+$ T cells contribute only when there is a deficient $CD8^+$ T cell pool (18). Ear swelling responses following OVA protein sensitization could be elicited by whole OVA protein, class I MHC-restricted $OVA_{254–267}$ peptide or class II MHC-restricted $OVA_{323–334}$ peptide (data not shown). Thus, in the absence of UV irradiation before protein immunization, either $CD8^+$ T cells or $CD4^+$ T cells could contribute to an ear swelling response in this model. The UV-mediated inhibition of ear swelling to protein elicitation observed may be due to inhibitory effects on the priming of Ag-specific $CD8^+$ T cells (as demonstrated here) and/or inhibitory effects on $CD4^+$ T cells.

TCI with protein through UV-irradiated skin induces transferable Ag-specific tolerance

In addition to unresponsiveness, sensitization to haptens through UV-irradiated skin induces hapten-specific tolerance. This tolerance is transferable as T cells from irradiated and immunized mice can be transferred into naive mice and these naive recipients are subsequently refractory to immunization with the same hapten (10). To determine whether protein immunization through UV-irradiated skin likewise induces tolerance, we performed adoptive transfer experiments using the skin draining LN cells of previously immunized mice (Fig. 3, *A* and *B*). Mice receiving LN cells from donors immunized following UV irradiation were immunized transcutaneously with OVA protein and CpG adjuvant. CTL generation was monitored by the response of adoptively cotransferred and CFSE labeled OT-1 cells. Mice receiving donor LN cells depleted of $CD4^+$ T cells demonstrated enhanced CTL generation (as determined by CFSE dilution and IFN-γ production) when compared with mice not receiving donor cells (Fig. 3*B*). In contrast, mice that received LN cells that were not depleted of $CD4^+$ T cells had diminished OVA-specific CTL proliferative responses compared with control mice not receiving donor cells. This inhibition of CTL priming was dependent upon UV irradiation and Ag exposure in the donors (Fig. 3*C*). The response was Ag-specific as only donors immunized through irradiated skin with OVA and not control protein were able to inhibit Ag-specific IFN-γ production by OT-1 cells in recipients (Fig. 3*D*). Thus, $CD4^+$ T cells from the LNs of UV-treated and protein-immunized mice prevented the subsequent priming of $CD8^+$ T cells to protein immunogen in an Ag-specific manner. Similar to immunization with full protein, T cells from donors immunized with the class II MHC-restricted $OVA_{323–334}$ peptide were able to inhibit the priming of OT-1 cells following TCI with OVA protein (data not shown). This suggested that the tolerance is mediated by $CD4^+$ T cells responding to the class II-restricted epitope of OVA.

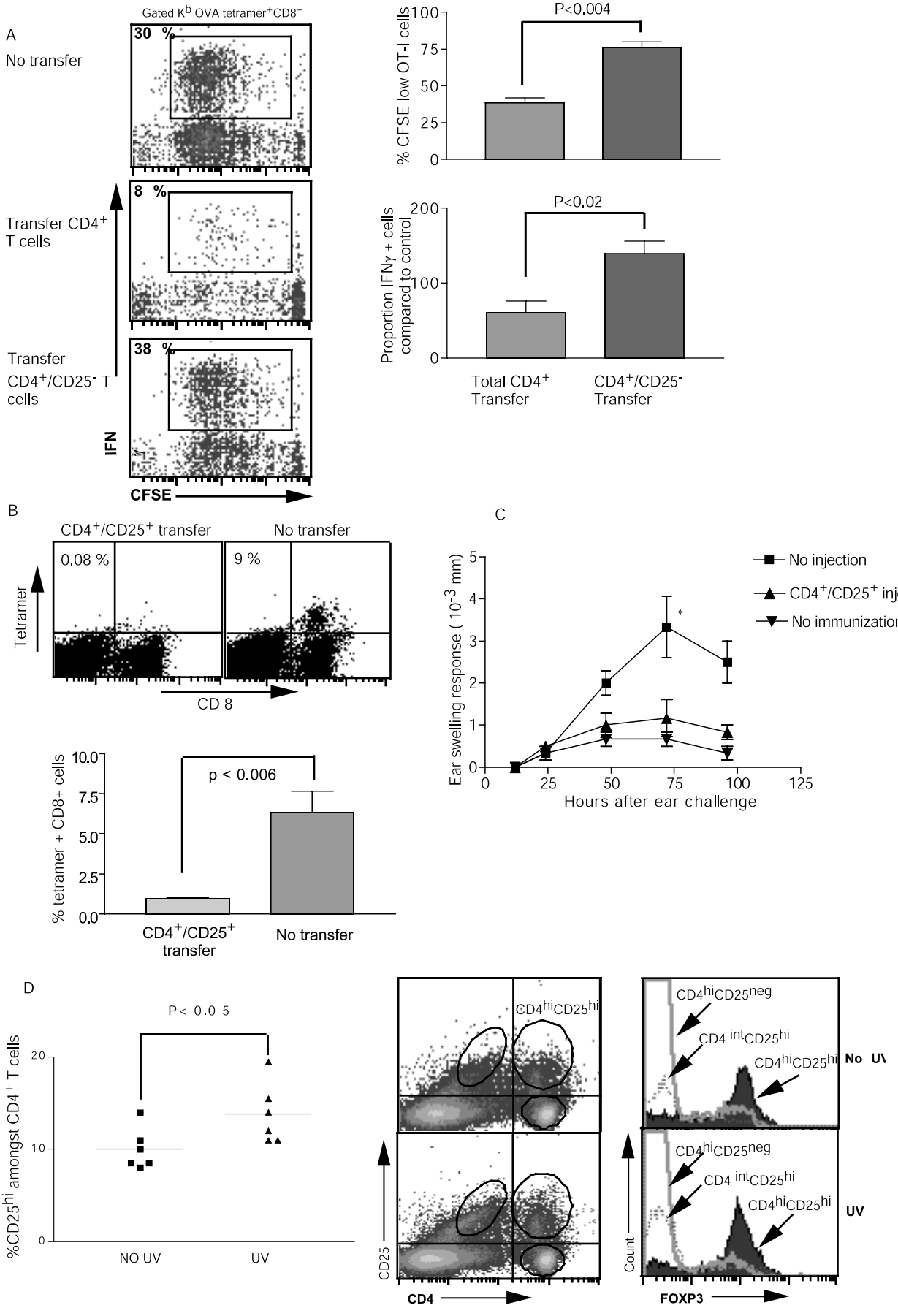
$CD4^+CD25^+$ T cells mediate Ag-specific tolerance following TCI through UV-irradiated skin

UV radiation-induced hapten-specific T_{reg} cells have recently been characterized as expressing CD4 and CD25 and thus belong to a increasingly well-characterized subset of T_{reg} cells (11). To determine whether the Ag-specific $CD4^+$ T cells that inhibit $CD8^+$ T cell priming belong to this subset, we used complementary approaches. In an initial approach, $CD4^+$ T cells were positively selected from the skin draining LN of UV-irradiated and immunized mice. In some experiments, these cells were further depleted of $CD25^+$ T cells before adoptive transfer. Recipient mice were next immunized with OVA and CpG adjuvant and CTL priming was assessed by OT-1 transfer (Fig. 4*A*). Mice receiving $CD4^+$ cells containing the $CD25^+$ subset demonstrated diminished CTL-priming responses, whereas mice receiving $CD4^+$ cells depleted of the $CD25^+$ subset did not. To confirm that the $CD4^+CD25^+$ T cells could potently inhibit CTL priming, $CD4^+CD25^+$ T cells from UV-immunized mice were next positively selected and transferred into naive animals (Fig. 4*B*). Recipient mice were then immunized and boosted once with OVA protein and CpG. OVA-specific CTL were enumerated using K^b-OVA tetramers. Mice receiving $CD4^+CD25^+$ T cells from OVA-immunized animals through UV-irradiated skin did not generate OVA-specific CTL. Furthermore, OVA protein contact hypersensitivity was completely abrogated in these animals (Fig. 4*C*). Comparison of the frequency of $CD4^+CD25^+$ T cells within the draining LN of UV-irradiated mice demonstrated an increase in the fraction of $CD4^+CD25^+$ T cells following UV irradiation (from $10 \pm 0.9\%$ of the $CD4^+$ T cell population to $14 \pm 1.3\%$ of the $CD4^+$ T cell population; $p < 0.05$). The expression of the Foxp3 transcription factor by T cells has been associated with the acquisition of regulatory function and studies support the use of this protein as a marker of T_{reg} cells (19, 20). To further characterize the $CD4^+CD25^+$ T cells induced by UV irradiation, they were stained for Foxp3 expression (Fig. 4*D*). High levels of Foxp3 were detected in the $CD4^{high}CD25^{high}$ cell subset in UV-exposed and control animals. Regardless of treatment status, the $CD4^+CD25^+$ gated cells were over 85% Foxp3 positive, consistent with a regulatory phenotype. Thus, $CD4^+CD25^+$ cells from UV-irradiated and OVA-immunized mice express Foxp3 and contain a subset of cells that prevent OVA-specific CTL priming and mediate Ag-specific tolerance to OVA protein.

Variable effect of IL-10 on protein tolerance induction through UV-irradiated skin

IL-10 significantly contributes to the UV suppression of contact hypersensitivity. UV irradiation of the skin results in release of IL-10 from different cells such as, keratinocytes, T cells, B cells,

FIGURE 4. $CD4^+CD25^+$ T cells mediate Ag-specific tolerance following TCI through UV-irradiated skin. *A*, Donor mice were irradiated or sham-irradiated before TCI with OVA and CpG adjuvant. Two days after immunization, skin draining LN were removed and $CD25^+$ cells were depleted or not. $CD4^+$ cells were next positively selected. Total $CD4^+$ T cells and $CD4^+/CD25^-$ T cells were cotransferred with OT-I $CD8^+$ T cells into unirradiated recipients that were then skin transcutaneously immunized with OVA protein. OT-I responses (proliferation and IFN-γ production) in the skin draining LNs were assessed 3 days later. The *left set* of figures show representative dot plots indicating OT-1 cell proliferation and IFN-γ production. Aggregate data (two experiments) is presented to the *right* ($n = 4$ mice/group). *B*, $CD4^+CD25^+$ T cells from donor-irradiated and immunized mice were purified and transferred into naive mice. These mice were next immunized s.c. with 100 μg of OVA protein and given 250 μg of CpG epicutaneously and boosted once 1 wk later. The frequency of Ag-specific CTL within the LN was determined at day 13 by tetramer staining. *Upper panels*, Representative dot plots and the numbers indicate the percent K^b-OVA-specific CTL among the $CD8^+$ T cells. Aggregate data is presented in the *lower panel* ($n = 3$). *C*, The induction of protein contact hypersensitivity is inhibited by the adoptive transfer of $CD4^+CD25^+$ T cells from OVA-immunized mice. *D*, UV light increases the proportion of $CD25^{+(high)}$ among the $CD4^+$ T cells in the skin draining LNs. Mice ($n = 6$) received either four daily UV treatments or sham irradiation. The percentage of $CD25^+$ T cells within the $CD4^{high}$ T cell population was determined (*left panel*). The $CD4^{high}CD25^{high}$ T cell subset but not the $CD4^{int}CD25^{high}$ or $CD4^{high}CD25^-$ subsets of T cells express high levels of intracellular Foxp3 with or without UV treatment (*right panel*).



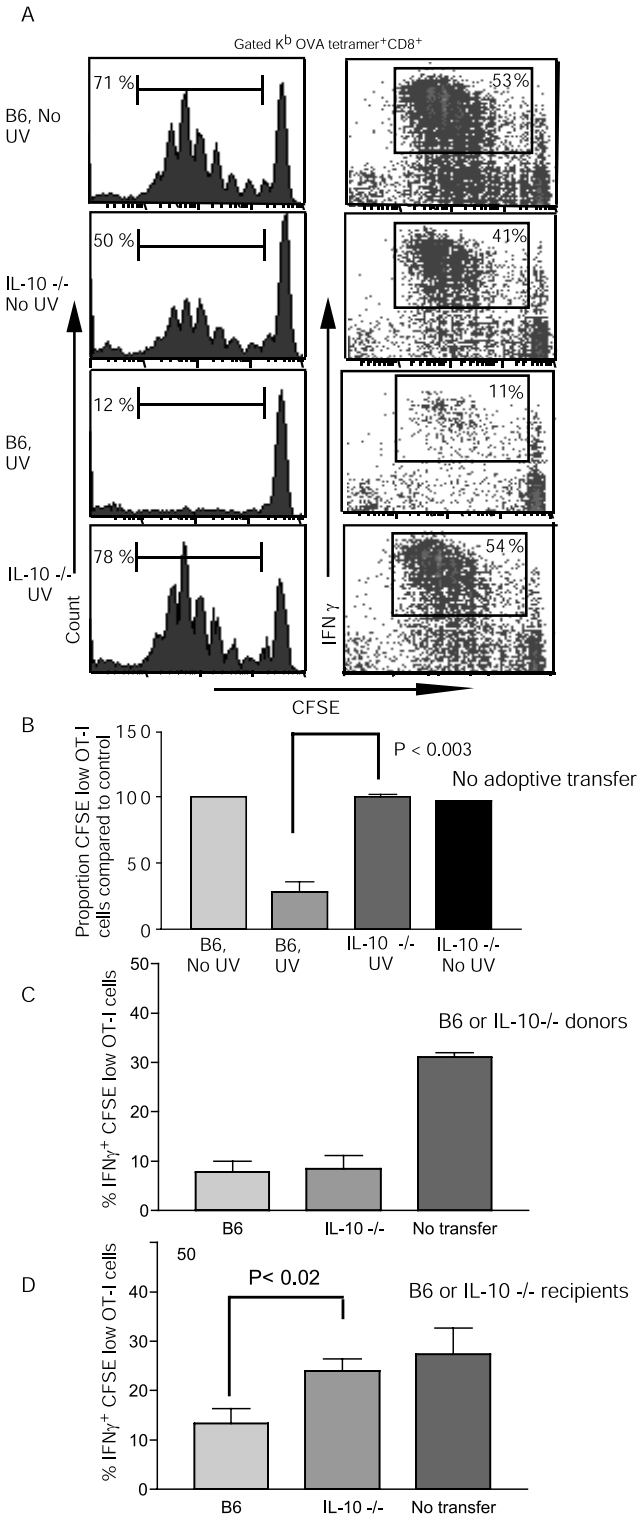


FIGURE 5. Variable effect of IL-10 on protein tolerance induction through UV-irradiated skin. *A* and *B*, IL-$10^{-/-}$ or IL-10-sufficient C57BL/6 animals were irradiated or sham-irradiated and immunized with OVA. OT-1 cells were adoptively transferred on day 2 of irradiation and before immunization. OT-1 cells from the skin draining LN were assayed for proliferation (*left histograms*) and IFN-γ production (*right dot plots*) 3 days postimmunization. Representative histograms and dot plots are presented in *A*, and aggregate data of two experiments ($n = 6$/group) are presented in *B*. *C*, IL-10-sufficient or -deficient animals were next used as donor following irradiation and immunization. $CD4^+$ T cells were transferred into recipient mice ($n = 6$/group), and these were next immunized with OVA and received OT-I $CD8^+$ T cells in cotransfer. IFN-γ production by OT-I T cells on day 3 was determined. IL-10-deficient $CD4^+$ T cells were able to transfer tolerance. Error bars indicate SEM. *D*, C57BL/6 donor mice were irradiated and immunized with OVA, and $CD4^+$ T cells were used for adoptive transfer into IL-10-deficient or -sufficient recipients. OT-I priming following transcutaneous OVA immunization with CpG adjuvant was determined. IL-10-sufficient (B6) but not deficient recipients were tolerized. Bars indicate SEM.

and infiltrating macrophages/monocytes to the skin. To investigate the role of IL-10 in the inhibition of OVA-specific CTL priming following protein immunization through UV-irradiated skin, we compared the effect of UV irradiation on CTL priming in IL-10-sufficient and IL-10-deficient mice (Fig. 5*A*). IL-$10^{-/-}$ mice did not demonstrate a decrease in OVA-specific CTL priming following UV irradiation in contrast to IL-10-sufficient animals (Fig. 5, *A* and *B*; $n = 6$/group; $p < 0.003$). To determine whether IL-10 is required for the generation of UV-induced T_{reg} cells, $CD4^+$ T cells from UV-irradiated and immunized IL-10-sufficient or -deficient donors were transferred into mice that were subsequently immunized with OVA. Surprisingly, donor cells from either IL-10-sufficient or -deficient animals were able to mediate suppression of $CD8^+$ T cell priming. Thus, UV-induced T_{reg} cells do not require IL-10 for generation and do not require T_{reg}-derived IL-10 for their tolerogenic activity. To determine whether UV-induced T_{reg} cells require IL-10 derived from other host cells for their tolerogenic activity, $CD4^+$ cells from UV-irradiated and immunized C57BL/6 hosts (IL-10 sufficient) were transferred into IL-10-sufficient or -deficient hosts. These mice were then immunized and CTL priming was assessed (Fig. 5*D*). Donor cells were unable to inhibit the priming of OT-1 cells in IL-10-deficient hosts indicating that IL-10 is required for the biologic activity of UV-T_{reg} cells but that this IL-10 is not from the UV-T_{reg} cells. Thus, host-derived IL-10 is necessary for maintaining the tolerance-inducing properties of T_{reg} cells generated through TCI of UV-irradiated skin.

Discussion

The skin is increasingly being studied as a site for effective and possibly painless immunization. In this study, we demonstrate that TCI through UV-irradiated skin prevents CTL priming to protein but not to class I MHC-restricted peptide Ag. In addition, protein or class II MHC-restricted peptide TCI through UV-irradiated skin can prevent the subsequent priming of CTL to protein Ag and protein contact hypersensitivity responses. This tolerance is mediated by Ag-specific UV-induced $CD4^+CD25^+$ T_{reg} cells.

Multiple methodologies are being pursued to induce durable Ag-specific tolerance. Targeting of Ag to immature DCs by means of DC-specific Ab or by infusion of Ag in the absence of inflammatory stimuli are simple means of inducing Ag-specific T_{reg}. A potential danger of these methods is the possibility of inadvertent inflammation during the tolerance induction, which has been shown not only to abrogate tolerance but also to have immunogenic sequelae (7). In this study, we demonstrate that application of protein or class II MHC peptide Ag to UV-irradiated skin, even in the presence of a bacterial toxin (cholera toxin) or strong Th1 adjuvant (CpG) prevents CTL priming and induces robust T_{reg} cells. The ensuing tolerance to CTL priming is resistant to subsequent priming and boosting with CpG adjuvant.

Although the findings presented in this study recapitulate in part what has been observed with hapten contact hypersensitivity models (21), the use of defined Ags has allowed a precise determination of the effects of UV light on Ag-specific CTL priming. In the absence of $CD4^+$ T cells (in MHC class II-deficient mice and $CD4^+$ T cell-depleted mice), UV irradiation has been shown to

have no effect on $CD8^+$ T cells responses to hapten-mediated contact hypersensitivity (22). We demonstrate clearly that UV irradiation does not affect the priming and expansion of CTL to class I MHC-restricted peptide Ag. The inability to inhibit CTL priming to peptide Ag following UV irradiation and the concomitant depletion of LC demonstrates that LC are dispensable for CTL induction. This is consistent with the recent observations that LC are not required for CTL priming following herpes simplex infection of the skin in mice (23) and that genetic ablation of langerin$^+$ DCs (LC) has no effect on $CD8^+$ T cell-mediated hapten contact hypersensitivity (24). Our observations contrasting the effects of protein and class I MHC-restricted peptide immunization indicate that the effect of UV on priming with protein Ag is markedly different than on priming with class I MHC-restricted peptide Ag. This suggests either that protein Ag is presented by a different APC (perhaps the LC) than class I MHC peptide Ag or that the APCs that present protein Ag are more susceptible to immune modulation by UV than the APCs that present only class I MHC-restricted Ag. Experiments using a contact hypersensitivity model and MHC class I- and class II-deficient animals showed that UV-induced suppression is secondary to a preferential activation of $CD4^+$ suppressor cells (22). Thus, the skin immune system may have a prominent role in systemic immune tolerance induction that is largely dependent upon $CD4^+$ T cells. This tolerance-inducing role of the skin may be missed in systems that are limited to the study of class I MHC Ags in the skin (25). Practical implications for the further development of TCI include the fact that for the potent induction of CTL, TCI should optimally be performed on UV-protected skin if protein Ags are used. If immunization is to proceed through non-UV-protected skin and potent CTL responses are desired, class-I MHC peptide epitopes may be preferred over protein immunogens.

Our observations suggest separate effects of UV irradiation on the inhibition of transcutaneous protein immunization and on the induction of tolerance: UV light irradiation diminished the proportion of OVA-bearing $CD11c^+$ DC within the draining LN and decreased the activation state of OVA-bearing DC as determined by decreased CD40 expression. This is consistent with previous observations detailing a deficient maturation of DC following UV irradiation (26) and deficient Th1 T cell priming by UV-irradiated DC or LC (27, 28). However, UV irradiation promoted the generation of $CD4^+CD25^+Foxp3^+$ T cells within the draining LN following immunization (Fig. 4*D*). Among these $CD4^+CD25^+Foxp3^+$ T cells were cells that had Ag-specific regulatory function in vivo. Our experiments clearly implicate these traditional $CD4^+CD25^+$ T cells as responsible for the transfer of tolerance observed. This can be deduced from the absence of tolerance following transfer of $CD4^+$ T cells depleted of $CD25^+$ T cells and from the induction of tolerance by transfer of $CD4^+CD25^+$ cells (Figs. 3 and 4). A further difference between the effect of UV on the inhibition of TCI and the effect of UV on tolerance induction is noted in the study of the IL-10 dependence of these effects. The inhibition of CTL priming to transcutaneous protein Ag following UV irradiation was IL-10-dependent (Fig. 5*B*), whereas the generation of UV-T_{reg} cells was IL-10-independent (Fig. 5*C*). Whether the initial inhibition of protein immunization seen here is a function of naturally present T_{reg} that are subsequently expanded remains to be determined. The induction of T_{reg} cells following UV irradiation has been associated with UV-induced DNA damage. UV-induced DNA damage induces LC to move from the skin into the draining LNs, and IL-12 can induce DNA repair limiting the number of UV-damaged LC in the draining LNs (29). It is thus possible that UV-induced DNA damage alters cutaneous APC and enhances their ability to activate the T_{reg} cells detected in our model.

Cutaneous suppressor T cells may be induced by repeated application of peptide Ag to the skin in the absence of adjuvant (30). These cells can mitigate autoimmune disease but primarily belong to a $CD25^-$ subset and require the strict absence of adjuvant activity for generation. In contrast, the skin-generated Ag-specific UV-T_{reg} cells described here are $CD4^+CD25^+$ and can be generated in the presence of adjuvant. The $CD4^+CD25^+$ T_{reg} cells induced are able to inhibit $CD8^+$ T cell proliferation and IFN-γ production in vivo, as noted by Piccirillo et al. (31) in vitro and in contrast to $CD4^+CD25^+$ T_{reg} cells induced in TCR transgenic models where $CD8^+$ T cell cytotoxicity alone is inhibited (32). Furthermore, although TLR activation of DCs has been shown to render APC resistant to $CD4^+CD25^+$ T_{reg} effects in vitro (33), repeated immunization using CpG, a TLR9 agonist, was unable to induce Ag-specific priming of $CD8^+$ T cells in the presence of the UV-T_{reg} described here. UV-T_{reg} were first characterized in terms of cytokine production and were shown to produce IL-10 (34). UV irradiation-induced hapten tolerance is IL-10-dependent (35). Our observations demonstrate that, while IL-10 is required for the ability of UV-T_{reg} cells to inhibit Ag-specific CTL priming, the source of IL-10 need not be the T_{reg} cells. $CD4^+CD25^+$ T_{reg} generated by other means have been shown to produce IL-10 in vivo (32, 36), and UV-T_{reg} may produce or induce IL-10 production in vivo (11). We find that IL-10 production by UV-T_{reg} is not required for their ability to prevent Ag-specific CTL priming in our system. To reconcile these observations, we propose that UV-T_{reg} cells induce IL-10 production by Ag-bearing APC or bystander cells before or coincident with the interaction of APC with cognate CTL and that this contributes to the inhibition of CTL priming.

The induction of Ag-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune homeostasis. One of the major mechanisms of peripheral tolerance is the active suppression of T cells by T_{reg} cells. The ability to use the skin to safely and robustly induce Ag-specific tolerance suggests that the immunization through UV-irradiated skin can be used to prevent untoward reactions to neo-Ags and possibly prevent autoimmune disease by limiting reactivity to self Ags. A further investigation of the mechanisms of this suppression may lead to safe and effective methodologies for tolerance induction using the skin.

Acknowledgments

We thank Drs. Rusung Tan and Megan Levings for a critical reading of the manuscript and Drs. YiQun Zhang and Hossain Najar for technical assistance and helpful comments.

Disclosures

The authors have no financial conflict of interest.

References

1. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
2. Belkaid, Y., and B. T. Rouse. 2005. Natural regulatory T cells in infectious disease. *Nat. Immunol.* 6: 353–360.
3. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory $CD25^+CD4$ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 167: 188–195.
4. Daniel, D., and D. R. Wegmann. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc. Natl. Acad. Sci. USA* 93: 956–960.
5. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199: 1401–1408.
6. Mahnke, K., Y. Qian, J. Knop, and A. H. Enk. 2003. Induction of $CD4^+/CD25^+$ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 101: 4862–4869.
7. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell

receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral $CD8^+$ T cell tolerance. *J. Exp. Med.* 196: 1627–1638.
8. Glenn, G. M., R. T. Kenney, S. A. Hammond, and L. R. Ellingsworth. 2003. Transcutaneous immunization and immunostimulant strategies. *Immunol. Allergy Clin. North Am.* 23: 787–813.
9. Toews, G. B., P. R. Bergstresser, and J. W. Streilein. 1980. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* 124: 445–453.
10. Elmets, C. A., P. R. Bergstresser, R. E. Tigelaar, P. J. Wood, and J. W. Streilein. 1983. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* 158: 781–794.
11. Schwarz, A., A. Maeda, M. K. Wild, K. Kernebeck, N. Gross, Y. Aragane, S. Beissert, D. Vestweber, and T. Schwarz. 2004. Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J. Immunol.* 172: 1036–1043.
12. Enioutina, E. Y., D. M. Visic, and R. A. Daynes. 2002. The induction of systemic and mucosal immunity to protein vaccines delivered through skin sites exposed to UVB. *Vaccine* 20: 2116–2130.
13. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
14. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76: 17–27.
15. Klimuk, S. K., H. M. Najar, S. C. Semple, S. Aslanian, and J. P. Dutz. 2004. Epicutaneous application of CpG oligodeoxynucleotides with peptide or protein antigen promotes the generation of CTL. *J. Invest. Dermatol.* 122: 1042–1049.
16. Kahlon, R., Y. Hu, C. H. Orteu, A. Kifayet, J. D. Trudeau, R. Tan, and J. P. Dutz. 2003. Optimization of epicutaneous immunization for the induction of CTL. *Vaccine* 21: 2890–2899.
17. Schutze-Redelmeier, M. P., S. Kong, M. B. Bally, and J. P. Dutz. 2004. Antennapedia transduction sequence promotes anti tumour immunity to epicutaneously administered CTL epitopes. *Vaccine* 22: 1985–1991.
18. Saint-Mezard, P., F. Berard, B. Dubois, D. Kaiserlian, and J. F. Nicolas. 2004. The role of $CD4^+$ and $CD8^+$ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur. J. Dermatol.* 14: 131–138.
19. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
20. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
21. Schwarz, T. 1999. Ultraviolet radiation-induced tolerance. *Allergy* 54: 1252–1261.
22. Krasteva, M., F. Aubin, S. Laventurier, J. Kehren, O. Assossou, J. Kanitakis, D. Kaiserlian, and J. F. Nicolas. 2002. MHC class II-KO mice are resistant to the immunosuppressive effects of UV light. *Eur. J. Dermatol.* 12: 10–19.
23. Allan, R. S., C. M. Smith, G. T. Belz, A. L. van Lint, L. M. Wakim, W. R. Heath, and F. R. Carbone. 2003. Epidermal viral immunity induced by $CD8\alpha^+$ dendritic cells but not by Langerhans cells. *Science* 301: 1925–1928.
24. Kissenpfennig, A., S. Henri, B. Dubois, C. Laplace-Builhe, P. Perrin, N. Romani, C. H. Tripp, P. Douillard, L. Leserman, D. Kaiserlian, et al. 2005. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22: 643–654.
25. Mayerova, D., E. A. Parke, L. S. Bursch, O. A. Odumade, and K. A. Hogquist. 2004. Langerhans cells activate naive self-antigen-specific CD8 T cells in the steady state. *Immunity* 21: 391–400.
26. Kitazawa, T., and J. W. Streilein. 2000. Studies on delayed systemic effects of ultraviolet B radiation on the induction of contact hypersensitivity. 3. Dendritic cells from secondary lymphoid organs are deficient in interleukin-12 production and capacity to promote activation and differentiation of T helper type 1 cells. *Immunology* 99: 296–304.
27. Simon, J. C., R. E. Tigelaar, P. R. Bergstresser, D. Edelbaum, and P. D. Cruz, Jr. 1991. Ultraviolet B radiation converts Langerhans cells from immunogenic to tolerogenic antigen-presenting cells. Induction of specific clonal anergy in $CD4^+$ T helper 1 cells. *J. Immunol.* 146: 485–491.
28. Simon, J. C., P. D. Cruz, Jr., P. R. Bergstresser, and R. E. Tigelaar. 1990. Low dose ultraviolet B-irradiated Langerhans cells preferentially activate $CD4^+$ cells of the T helper 2 subset. *J. Immunol.* 145: 2087–2091.
29. Schwarz, A., A. Maeda, K. Kernebeck, H. van Steeg, S. Beissert, and T. Schwarz. 2005. Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *J. Exp. Med.* 201: 173–179.
30. Bynoe, M. S., J. T. Evans, C. Viret, and C. A. Janeway, Jr. 2003. Epicutaneous immunization with autoantigenic peptides induces T suppressor cells that prevent experimental allergic encephalomyelitis. *Immunity* 19: 317–328.
31. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of $CD8^+$ T cell activation by $CD4^+CD25^+$ immunoregulatory cells. *J. Immunol.* 167: 1137–1140.
32. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc. Natl. Acad. Sci. USA* 100: 8886–8891.
33. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science* 299: 1033–1036.
34. Shreedhar, V. K., M. W. Pride, Y. Sun, M. L. Kripke, and F. M. Strickland. 1998. Origin and characteristics of ultraviolet-B radiation-induced suppressor T lymphocytes. *J. Immunol.* 161: 1327–1335.
35. Beissert, S., J. Hosoi, R. Kuhn, K. Rajewsky, W. Muller, and R. D. Granstein. 1996. Impaired immunosuppressive response to ultraviolet radiation in interleukin-10-deficient mice. *J. Invest. Dermatol.* 107: 553–557.
36. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. $CD25^+CD4^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.