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# Phenotypic and Functional Characterization of Ultraviolet Radiation-Induced Regulatory T Cells<sup>1</sup>

Akira Maeda,\* Stefan Beissert,<sup>†</sup> Thomas Schwarz,<sup>2\*</sup> and Agatha Schwarz\*

Sensitization through UV-exposed skin induces regulatory T cells (Treg). In contrast to the classical CD4<sup>+</sup>CD25<sup>+</sup> Treg that act contact dependent, UV-induced Treg (UV-Treg) suppress via IL-10, indicating a distinct subtype that requires further characterization. Depletion studies revealed that UV-Treg express the glucocorticoid-induced TNF family-related receptor (GITR) and the surface molecule neuropilin-1. The injection of T cells from UV-tolerized mice after depletion of UV-Treg into naive recipients enabled a contact hypersensitivity response, indicating that tolerization also induces T effector cells. Adoptive transfer experiments using IL-10-deficient mice indicated that the IL-10 required for suppression is derived from UV-Treg and not from host-derived cells. Activation of UV-Treg is Ag specific, however, once activated suppression is nonspecific (bystander suppression). Hence, speculations exist about the therapeutic potential of Treg generated in response to Ag that are not necessarily the precise Ag driving the pathogenic process. Thus, we studied the consequences of multiple injections of 2,4-dinitrofluorobenzene (DNFB)-specific Treg into ears of naive mice followed by multiple DNFB challenges. DNFB-specific Treg were injected once weekly into the left ears of naive mice and DNFB challenge was performed always 24 h later. After three injections, a challenging dose of DNFB was applied on the right ear. This resulted in pronounced ear swelling, indicating that the subsequent boosting of DNFB-specific Treg had caused sensitization of the naive mice against DNFB. These data demonstrate that UV-Treg express GITR and neuropilin-1 and act via bystander suppression. However, constant boosting of Treg with Ag doses in the challenging range results in final sensitization that might limit their therapeutic potential. *The Journal of Immunology*, 2008, 180: 3065–3071.

Ultraviolet radiation, in particular the mid-wave range (UVB; 290–320 nm), represents one of the most significant environmental factors affecting humans. Its hazardous effects on health include exacerbation of infectious diseases, skin cancer, and skin aging (1–3). These effects are partially mediated by the immunosuppressive properties of UV that are illustrated by the inhibition of cellular immune reactions such as contact hypersensitivity (CHS)<sup>3</sup> (4, 5). UV impairs sensitization to contact allergens applied directly to the UV-irradiated skin area (4, 5). In addition, hapten-specific tolerance develops that is due to the induction of suppressor/regulatory T cells (Treg) (6). Therefore, the suppression can be adoptively transferred into naive recipients in an Ag-specific fashion.

UV-induced Treg (UV-Treg), which suppress hapten-mediated CHS, express CD4, CD25 (7), and CTLA-4 (8), bind the lectin dectin-2 (9), may use the apoptosis-related Fas/Fas-ligand system (10) and secrete IL-10 upon hapten-specific stimulation (7, 8). The induction of UV-Treg is crucially dependent on the migration of

Langerhans cells, the major APC of the skin, into the lymph nodes and the presence of UV-induced DNA damage in the Langerhans cells (11). Accordingly, IL-12, which reduces UV-mediated DNA damage (12), prevents the induction of UV-Treg (11).

Because of their capacity to suppress immune reactions in a rather Ag-specific fashion, UV-Treg may exert a therapeutic potential that was also suggested for other types of Treg (13). When injected i.v., UV-Treg act suppressively only in naive but not in sensitized mice (14). This gave rise to the speculation that their therapeutic potential may be limited only to prevent but not to down-regulate already manifested immune-mediated diseases (14).

However, when UV-Treg were injected intracutaneously (i.c.) into the area of challenge of sensitized mice, the elicitation of CHS was suppressed in a hapten-specific fashion (7). When the ears of oxazolone (OXA)-sensitized mice were injected with 2,4-dinitrofluorobenzene (DNFB)-specific Treg and painted with DNFB before challenge with OXA, CHS against OXA was suppressed. Therefore, once activated in an Ag-specific manner, UV-Treg suppress in an Ag-independent fashion. This phenomenon has been initially described for regulatory type 1 (Tr1) cells and is named bystander suppression. Although they are able to inhibit T effector cells, UV-Treg do not suppress the elicitation of CHS upon i.v. injection because of their inability to migrate into the skin. This is due to the expression of the lymph node homing receptor L-selectin (CD62L) but not to that of the ligands for the skin homing receptors E- and P-selectin (7).

Recently, we identified a strategy to reprogram the migratory behavior of UV-Treg by exposing UV-Treg to different types of APC (15). DNFB-specific UV-Treg coincubated with epidermal Langerhans cells blocked the elicitation upon i.v. injection into DNFB-sensitized mice. In contrast, i.v. injection of UV-Treg coincubated with dendritic cells generated from bone marrow,

\*Department of Dermatology, University Kiel, Kiel, Germany; and <sup>†</sup>Department of Dermatology, University Münster, Münster, Germany

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<sup>2</sup> Address correspondence and reprint requests to Dr. Thomas Schwarz, Department of Dermatology, University Kiel, Schittenhelmstrasse 7, 24105 Kiel, Germany. E-mail address: tschwarz@dermatology.uni-kiel.de

<sup>3</sup> Abbreviations used in this paper: CHS, contact hypersensitivity; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; DNFB, 2,4-dinitrofluorobenzene; GITR, glucocorticoid-induced TNF family-related receptor; i.c., intracutaneous(ly); OXA, oxazolone; Treg, regulatory T cell; UV-Treg, UV-induced Treg.

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spleen, or lymph nodes did not inhibit the ear challenge. This demonstrated that migratory behavior can be reprogrammed by tissue-specific dendritic cells, which might have input for strategies trying to use Treg not only for the prevention but also for the treatment of immune-mediated diseases.

Therefore, we were interested in further characterizing UV-Treg phenotypically and functionally. In this article we show that UV tolerization does not only induce Treg but also T effector cells, because naive recipients that were injected with T cells from UV-tolerized mice after the depletion of Treg responded with a specific ear swelling response to challenge with DNFB. Depletion and selection experiments reveal that UV-Treg express the glucocorticoid-induced TNF family-related receptor (GITR) and the surface molecule neuropilin-1. Studies using IL-10-deficient mice indicate that IL-10, which mediates the bystander suppression, is derived from UV-Treg and not from host tissue cells. However, the constant boosting of Treg with Ag doses in the challenging range results in final sensitization of naive recipients, which may limit their therapeutic potential.

## Materials and Methods

### Animals

C57BL/6 mice and IL-10-deficient mice (C57BL/6 background) were purchased from Charles River Laboratories. Animals were housed under specific pathogen-free conditions. Animal care was provided by expert personnel in compliance with the relevant laws and institutional guidelines.

### Contact hypersensitivity

Mice were sensitized by painting 50  $\mu$ l of DNFB (Sigma-Aldrich) solution (0.5% in acetone/olive oil; 4:1) on the shaved back on day 0. On day 5, 20  $\mu$ l of 0.3% DNFB were applied to the left ear. OXA was applied at concentrations of 2% for sensitization and 0.5% for elicitation. Ear swelling was quantified with a spring-loaded micrometer 24 h after elicitation. CHS was determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear and expressed in centimeters  $\times 10^{-3}$  (mean  $\pm$  SD). Each group consisted of at least five mice. Each experiment was performed at least two times.

### UV irradiation

The shaved back was exposed to UV from TL12 fluorescent lamps (Philips) that emit most of their energy within the UVB range. Mice were exposed to UV (1.2 kJ/m<sup>2</sup>) daily for four consecutive days.

### Generation of UV-induced Treg

Donor mice were sensitized against DNFB through UV-exposed skin as described above. Five days after hapten application, spleens and regional lymph nodes were removed and single-cell suspensions were prepared. Cells ( $5 \times 10^7$ ) were injected i.v. into naive mice that were sensitized 24 h later. In some experiments, CD4<sup>+</sup>CD25<sup>+</sup> Treg were isolated in a two-step procedure according to the protocol of a magnetic separation kit (CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell isolation kit; Miltenyi Biotec). Purity of the CD4<sup>+</sup>CD25<sup>+</sup> cells was evaluated by flow cytometry using allophycocyanin-conjugated anti-mouse CD4 (BD Biosciences) and PE-labeled anti-CD25 Abs (Miltenyi Biotec). Treg were i.v. injected into naive mice. Recipients were sensitized against DNFB 24 h after injection and ear challenge was performed 5 days later. For i.c. injection,  $1 \times 10^6$  cells (100  $\mu$ l) were injected into the left ears of the recipient mice.

For labeling, cells were incubated with 5  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes) for 7 min in PBS, and then further incubated for 3 min in the presence of 10% FCS, followed by three washes with RPMI 1640 medium containing 10% FCS. The cell numbers were adjusted to  $2 \times 10^7$ /ml and 100  $\mu$ l of cells were injected i.c. into the left ears. Three hours later DNFB challenge was performed. After three injections once weekly, ears were obtained and cryosections cut. Sections were stained with a PE-conjugated Ab directed against CD25. Sections were analyzed by using an immunofluorescence microscope (Axiovert 40CFL, Carl Zeiss).

### Depletion and isolation of subpopulations

For depletion or selection subpopulations, lymphocytes obtained from regional lymph nodes and spleens were incubated with a PE-conjugated Ab

against mouse GITR (BD Biosciences). In the next step cells were incubated with microbeads coated with an Ab against PE (Miltenyi Biotec) followed by selection via magnetic bead separation using the autoMACS system (Miltenyi Biotec). For depletion or selection of neuropilin-1-positive cells, lymph nodes and spleen cells were incubated with a rabbit polyclonal Ab raised against neuropilin-1 (Santa Cruz Biotechnology). After washing, cells were incubated with goat anti-rabbit IgG microbeads (Miltenyi Biotec) followed by selection using the autoMACS system. The positive and negative fractions, respectively, were harvested, washed, and adjusted to the appropriate cell numbers and subsequently used for injection. The efficacy of depletion or isolation was determined by flow cytometric analysis (FC500; Beckman Coulter).

### Reverse transcription-PCR for IL-10

Excised ears were lysed with TRIzol reagent (Invitrogen Life Technologies) and total RNA was isolated according to manufacturer's protocol. One microgram of total RNA was reverse transcribed using a SuperScript cDNA synthesis kit (Invitrogen Life Technologies). After reverse transcription, cDNA was precipitated with 2.5 volume of cold ethanol and one-third volume of 3M sodium acetate buffer (pH 7.5) at  $-20^\circ\text{C}$  overnight. The pellet was rinsed with 70% ethanol and dissolved in RNase-free water. To perform PCR amplification on a semiquantitative basis, we determined the optimal concentration of each sample and a cycle number showing a logarithmic increase in amplification with primers specific for  $\beta$ -actin. Samples were then amplified using primers specific for murine IL-10. Primer sequences were as follows: 5'-TACCTGGTAGAAGTGATGCC-3' (IL-10 sense primer) and 5'-CATCATGTATGCTTCTATGC-3' (IL-10 antisense primer).

### Statistical analysis

Data were analyzed by Student's *t* test and differences were considered significant at  $p < 0.05$ .

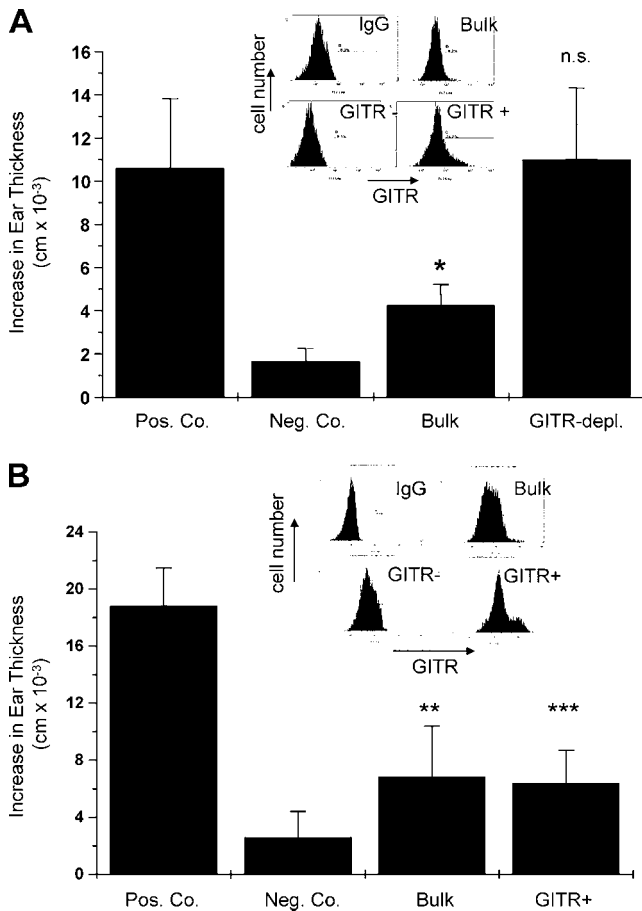
## Results

### UV-Treg express GITR and neuropilin

GITR, also known as TNFRSF18, is a member of the TNF/nerve growth factor (TNF-NGF) receptor gene superfamily and was found to be predominantly expressed on CD25<sup>+</sup>CD4<sup>+</sup> T cells and on CD25<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> thymocytes in normal naive mice (16). To determine whether UV-Treg express GITR, splenocytes and lymph node cells were obtained from C57BL/6 mice that were sensitized with DNFB through UV-exposed back skin. Cells were incubated with PE-conjugated Ab against mouse GITR followed by incubation with microbeads coated with Ab against PE. Negative selection was performed via magnetic bead separation. The negative fractions were subjected to FACS analysis that revealed successful depletion of GITR<sup>+</sup> cells (Fig. 1A, *inset*). Cells were injected into naive mice that were subsequently sensitized against DNFB. Although the mice that received bulk cells obtained from UV-tolerized mice were significantly suppressed in their CHS response, the mice that were injected with the GITR-depleted fraction mounted pronounced ear swelling (Fig. 1A). Because the transfer of suppression was lost upon depletion of GITR<sup>+</sup> cells, one can conclude that UV-Treg express GITR.

This was also confirmed by positive selection. Cells obtained from UV-tolerized mice were incubated with PE-conjugated Ab against mouse GITR, followed by incubation with microbeads coated with Ab against PE. After positive selection, cells were injected i.v. into naive mice that were subsequently sensitized against DNFB. The mice that received GITR-selected cells were equally suppressed in their sensitization as the mice that received bulk cells (Fig. 1B).

Neuropilin-1 is a receptor involved in axon guidance, angiogenesis, and the activation of T cells (17). It was recently found to be constitutively expressed on the surface of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells independently of their activation status (18). To determine whether UV-Treg also express neuropilin-1, depletion studies were performed. UV-Treg were depleted of neuropilin-1-expressing cells using an Ab directed against neuropilin-1 and injected into naive



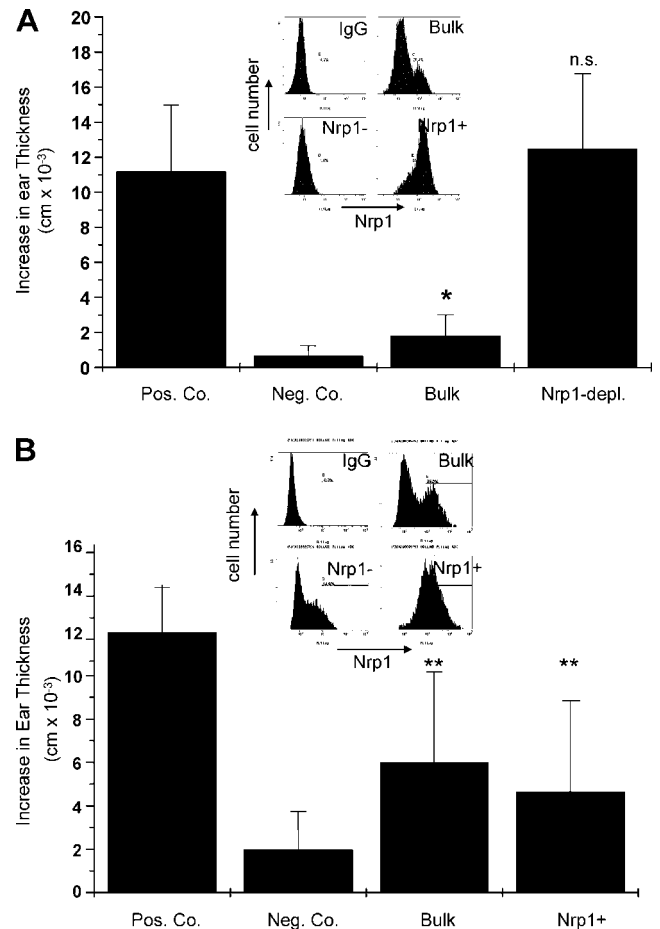
**FIGURE 1.** UV-Treg express GITR. *A*, Splenocytes and lymph node cells were obtained from mice that were UV-tolerized by the application of DNFB through UV-exposed skin. Cells were depleted of GITR<sup>+</sup> cells by magnetobead separation. Bulk cells or GITR-depleted (GITR-depl.) cells ( $5 \times 10^7$ ) were injected into naive syngeneic mice. Twenty-four hours later the recipients were sensitized against DNFB and ear challenge was performed 5 days thereafter. *B*, GITR expressing cells (GITR<sup>+</sup>) were isolated by magnetobead separation and injected into naive syngeneic mice. Twenty-four hours later the recipients were sensitized against DNFB and ear challenge was performed 5 days thereafter. Positive control (Pos. Co.) mice were sensitized and challenged with DNFB without injection, and negative control (Neg. Co.) mice were ear-challenged only. The ear-swelling response is expressed as the difference (centimeters  $\times 10^{-3}$ ; mean  $\pm$  SD) between the thickness of the challenged ear and the thickness of the vehicle-treated ear. The efficacy of depletion and selection was determined by flow cytometric analysis (FC500; Beckman Coulter). \*,  $p < 0.01$  vs positive control; \*\*,  $p < 0.0001$  vs positive control; \*\*\*,  $p < 0.00005$  vs positive control.

mice which were subsequently sensitized against DNFB. In contrast to bulk cells, neuropilin-1-depleted cells were not able to inhibit the sensitization of naive mice (Fig. 2A).

Positive selection confirmed these results. Cells obtained from UV-tolerized mice were incubated with a rabbit Ab against mouse neuropilin-1, followed by incubation with microbeads coated with goat anti-rabbit IgG. After positive selection, cells were injected i.v. into naive mice that were subsequently sensitized against DNFB. The mice that were injected with neuropilin-1-selected cells were suppressed in their sensitization response comparable with mice that had received bulk cells (Fig. 2B).

#### UV tolerization does not only induce Treg but also T effector cells

There is evidence that sensitization against haptens does not only induce T effector cells but universally also Treg. Glass et al. ob-



**FIGURE 2.** UV-Treg express neuropilin-1. *A*, Splenocytes and lymph node cells were obtained from mice that were UV-tolerized by the application of DNFB through UV-exposed skin. Cells were depleted of neuropilin-1-expressing cells by magnetobead separation. Bulk cells or neuropilin-1-depleted (Nrp1-depl.) cells ( $5 \times 10^7$ ) were injected into naive syngeneic mice. Twenty-four hours later recipients were sensitized against DNFB and ear challenge was performed 5 days thereafter. *B*, Neuropilin-1-positive cells (Nrp1<sup>+</sup>) were isolated by magnetobead separation and injected into naive syngeneic mice. Twenty-four hours later recipients were sensitized against DNFB and ear challenge was performed 5 days thereafter. Positive control (Pos. Co.) mice were sensitized and challenged with DNFB without injection, and negative control (Neg. Co.) mice were ear challenged only. The ear-swelling response is expressed as the difference (centimeters  $\times 10^{-3}$ ; mean  $\pm$  SD) between the thickness of the challenged ear and the thickness of the vehicle-treated ear. The efficacy of depletion and selection was determined by flow cytometric analysis (FC500; Beckman Coulter). \*,  $p < 0.0005$  vs positive control; \*\*,  $p < 0.05$  vs positive control.

served that conventional sensitization with haptens by epicutaneous painting is reduced when mice receive T cells from donors that were sensitized against DNFB (14). Although the reduction was moderate, from it the authors concluded that the T cells transferred should also contain a population with regulatory properties. Accordingly, Xu et al. could show that Ab-mediated depletion of CD4<sup>+</sup> T cells resulted in increased and prolonged CHS responses (19). This indicates that upon sensitization not only are T effector cells induced but concurrently also CD4<sup>+</sup> T cells as negative regulators of the response.

Thus, we asked whether in turn tolerization does not only induce Treg but also T effector cells. To address this issue, bulk T cells from mice, which were tolerized against DNFB by application of the hapten through UV-exposed skin, were depleted of



CD4<sup>+</sup>CD25<sup>+</sup> T cells. The remaining fraction was injected i.v. into naive mice that were challenged on the left ear 24 h after injection. In contrast to negative control mice that were only challenged, a significant ear swelling response was observed in mice that had received the T cell fraction depleted of UV-Treg (mean increase ear swelling  $0.75 \pm 0.5 \text{ cm} \times 10^{-3}$  vs  $4.0 \pm 1.82 \text{ cm} \times 10^{-3}$ ,  $p < 0.01$ ). This indicates that during UV tolerization not only Treg but also T effector cells are induced that, however, in the presence of Treg appear to be suppressed.

#### *IL-10 suppressing CHS is derived from UV-Treg and not from host cells*

Recently, we could show that the suppression of the elicitation of CHS upon i.c. injection of UV-Treg into the ears of sensitized mice is associated with an increased expression of IL-10 (7). In addition, injection of neutralizing IL-10 Ab prevented the suppression, indicating that the release of IL-10 is one of the major events during suppression (8). Although it was shown that activation of UV-Treg by APC coupled with the specific hapten induces the release of IL-10 in vitro, it was not clear whether the IL-10 required for suppression in vivo is released directly from UV-Treg or indirectly from host cells.

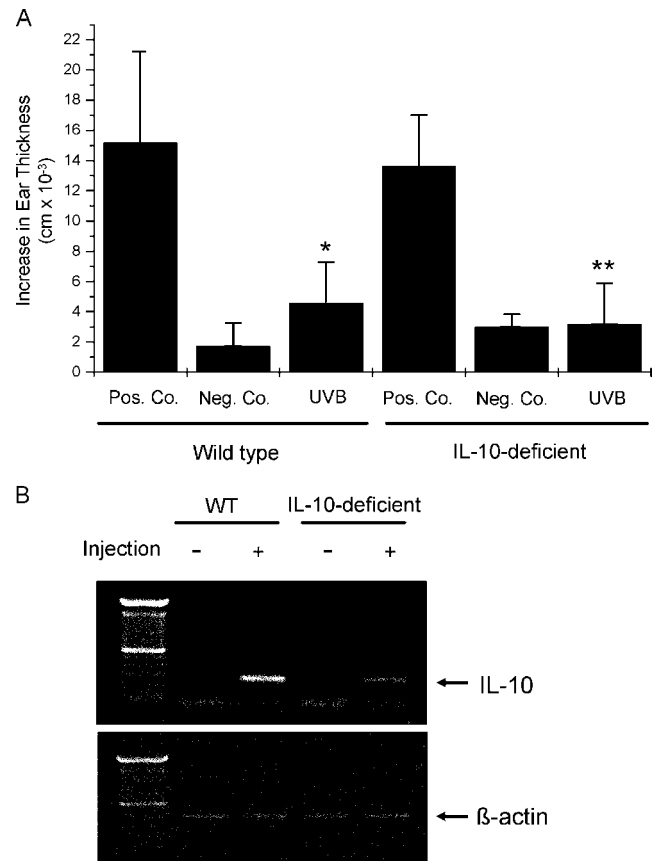
To address this issue, IL-10-deficient mice were used. DNFB-specific Treg were obtained from C57BL/6 wild-type mice that had been sensitized through UV-exposed skin. Cells were injected i.c. into the ears of IL-10-deficient mice that had been sensitized against DNFB 5 days earlier. Three hours after injection ear challenge was performed. Ear challenge was significantly suppressed in IL-10-deficient mice that had received UV-Treg from wild-type mice (Fig. 3A). A similar suppression was observed when UV-Treg were injected into sensitized wild-type mice, thus confirming previous results.

To corroborate these findings, semiquantitative RT-PCR for IL-10 was performed from the ears into which Treg were injected. No IL-10 transcripts could be detected in the uninjected ears of both wild-type and IL-10-deficient mice. Upon i.c. injection of UV-Treg into ears followed by DNFB challenge, IL-10 transcripts were found expressed in the ears of both wild-type and IL-10-deficient ears (Fig. 3B). In wild-type mice the transcripts appeared slightly stronger expressed, suggesting that the host cells also might produce IL-10. However, because the ear swelling response was equally suppressed in IL-10-deficient mice (Fig. 3A), this indicates that the amounts of IL-10 released from UV-Treg alone suffice to mediate the suppression. Based on these findings, one can conclude that it is the UV-Treg that release IL-10 and thereby suppress the CHS response.

#### *Subsequent activation of DNFB-specific UV-Treg by DNFB results in final sensitization*

We recently showed that UV-Treg exhibit the capacity to suppress the elicitation of CHS provided that they are present at the place of challenge. This can be achieved either by i.c. injection into the ears (7) or by reprogramming their migratory behavior via tissue-specific APC (15). Activation of DNFB-specific UV-Treg releasing IL-10 can only be achieved by DNFB but not by the unrelated hapten OXA. However, once activated Ag-specifically, they suppress other immune reactions as well (7). Because of the phenomenon of bystander suppression, speculations exist about the therapeutic potential of Treg generated in response to Ag that are not necessarily the precise Ag driving the pathogenic process (20).

Because of the therapeutic potential of this approach, we were interested in studying the consequences of multiple injections and activations of hapten-specific UV-Treg in an animal that is naive for the hapten against which the UV-Treg are directed. In partic-

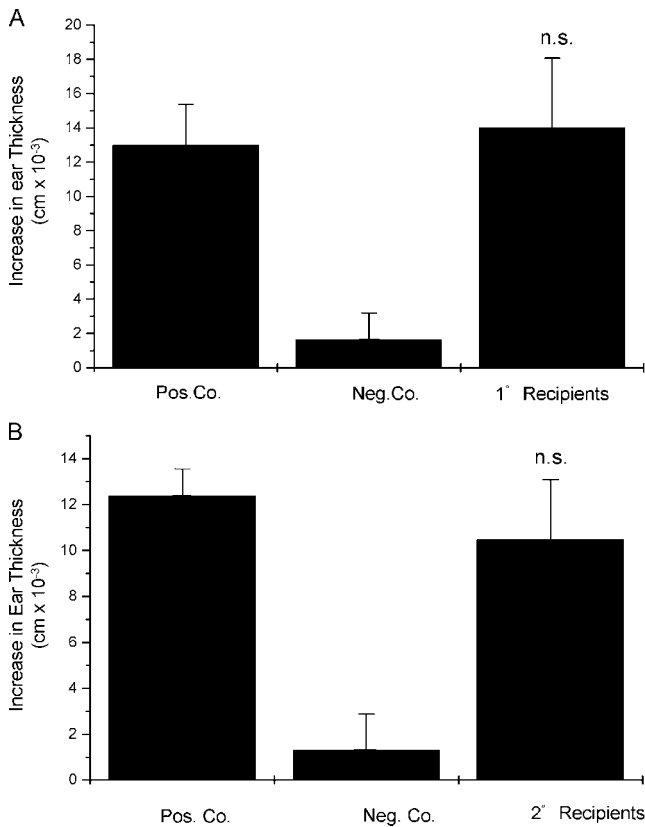


**FIGURE 3.** The IL-10 that suppresses CHS is derived from UV-Treg and not from host cells. **A**, Splenocytes and lymph node cells were obtained from wild-type mice that were UV tolerized by the application of DNFB through UV-exposed skin. Cells ( $1 \times 10^6$ ) were injected i.c. into the left ears of either wild-type or IL-10-deficient mice that had been sensitized against DNFB 5 days earlier. Three hours after injection, ear challenge was performed with 0.3% DNFB on the left ear. Positive control (Pos. Co.) mice were sensitized and challenged with DNFB without injection, and negative control (Neg. Co.) mice were ear challenged only. The ear-swelling response is expressed as the difference (centimeters  $\times 10^{-3}$ , mean  $\pm$  SD) between the thickness of the challenged ear and the thickness of the vehicle-treated ear. **B**, Ears that were injected with UV-Treg were obtained 24 h after challenge and RNA was extracted. RT-PCR was performed with primers for IL-10 and  $\beta$ -actin on a semiquantitative basis. Uninjected ears from wild-type (WT) and IL-10-deficient mice served as controls. \*,  $p < 0.005$  vs positive control; \*\*,  $p < 0.01$  vs positive control.

ular, we were interested in whether this finally results in sensitization or even tolerization against the hapten for which the UV-Treg are specific.

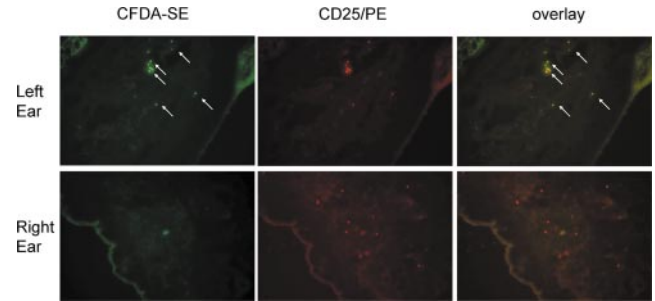
To address this issue, DNFB-specific UV-Treg were obtained from mice that were sensitized against DNFB through UV-exposed skin. Cells were injected i.c. into the left ears of naive mice. Three hours after injection ear challenge was performed with 0.3% DNFB on the left ear. Injections and challenges were performed on the left ear once weekly for 3 wk. Twenty-four hours after the third injection, 0.3% DNFB was applied on the right ear and ear swelling was measured 24 h after challenge. This resulted in a pronounced ear swelling response (Fig. 4A). This indicates that the subsequent injections and activations of DNFB-specific Treg did not result in tolerization against DNFB but, in contrast, may have caused sensitization of the initially naive recipient.

Because we have shown above that UV tolerization also induces T effector cells, one has to consider the possibility that not only Treg but also T effector cells were being injected i.c. into the left



**FIGURE 4.** Subsequent activation of DNFB-specific UV-Treg by DNFB results in final sensitization. *A*,  $CD4^+CD25^+$  Treg were obtained from mice that were UV tolerized by the application of DNFB through UV-exposed skin. Cells ( $1 \times 10^6$ ) were injected i.c. into the left ears of naive mice (primary ( $1^\circ$ ) recipients). Three hours after injection 0.3% DNFB was applied on the left ear. This procedure was repeated after 1 and 2 wk. Twenty-four hours after the third injection and the DNFB application, challenge with 0.3% DNFB was performed on the right ear. Twenty-four hours later ear swelling was measured. Positive control (Pos. Co.) mice were sensitized and challenged with DNFB without injection, and negative control (Neg. Co.) mice were ear-challenged only. The ear-swelling response is expressed as the difference (centimeters  $\times 10^{-3}$ ; mean  $\pm$  SD) between the thickness of the ear measured before and after challenge. *B*, Spleen and lymph nodes were obtained from the primary ( $1^\circ$ ) recipients and  $5 \times 10^7$  cells were injected i.v. into naive mice (secondary ( $2^\circ$ ) recipients). Twenty-four hours after transfer the mice were challenged on the left ear. Ear swelling was measured 24 h later. Positive control (Pos. Co.) mice were sensitized and challenged with DNFB without injection, and negative control (Neg. Co.) mice were ear challenged only. The ear-swelling response is expressed as the difference (centimeters  $\times 10^{-3}$ , mean  $\pm$  SD) between the thickness of the challenged ear and the thickness of the vehicle-treated ear.

ear that could finally migrate into the right ears and thus be responsible for the positive ear challenge. Although this appeared to be unlikely because  $CD4^+CD25^+$  cells were injected and thus the number of contaminating T effector cells should be minimal, cell tracking studies were performed. Cells were labeled with CFDA-SE before i.c. injection into the left ears of naive mice. After the third injection both left and right ears were cut and analyzed in a fluorescence microscope. CFDA-SE-positive cells could be detected in the left ears, which were injected with the labeled cells (Fig. 5). Staining with CD25 indicated that the vast majority of CFDA-SE-positive cells were also CD25-positive. In contrast, in the right ears only a few CD25-positive cells were found that, however, were negative for CFDA-SE and thus could



**FIGURE 5.** T cells injected i.c. do not migrate into the contralateral ear. Lymph node and spleen cells were obtained from mice that were UV tolerized by the application of DNFB through UV-exposed skin. Cells were labeled with CFDA-SE and injected i.c. ( $1 \times 10^6$ ) into the left ears of naive mice. Three hours after injection 0.3% DNFB was applied on the left ear. This procedure was repeated after 1 and 2 wk. Twenty-four hours after the third injection both ears were excised and cryosections were performed. Sections were stained with a PE-conjugated anti-CD25 Ab. Sections were analyzed under a fluorescence microscope. Arrows indicate double-positive cells.

not be classified as being derived from the pool of cells injected into the left ear. Hence, the positive ear challenge is not due to contaminating T effector cells but to sensitization by the subsequent activation of the UV-Treg via the application of DNFB.

To further confirm the sensitization against DNFB in this setting, T cells were obtained from the recipients described in Fig. 4*A* and injected into naive mice. Ear challenge was performed in these mice 24 h later. Animals that had received the T cells responded to the challenge with a significant ear swelling response (Fig. 4*B*). This indicates that the mice were sensitized by the T cell transfer, further implying that sensitized DNFB-specific T cells had to be present among the T cells injected. This proves that multiple injections of hapten-specific UV-Treg followed by boosting with the respective hapten do not result in tolerization but finally in sensitization.

## Discussion

Treg comprise a heterogeneous group of T cells that actively inhibit immune responses (21). They have been recognized as playing an important role in the prevention of autoimmunity, graft-vs-host disease, and transplant rejection (22–24). Clinically, there is great enthusiasm about the potential to develop strategies that can use Treg for therapeutic intervention. The complexity introduced by the varying types and activities of Treg has added an additional challenge. UV radiation has been recognized as representing an excellent tool for inducing Treg. However, UV tolerization does not only induce Treg but also T effector cells, because we demonstrate here that transfer of T cells from UV-tolerized mice upon depletion of  $CD4^+CD25^+$  Treg into naive mice enabled a specific ear swelling response upon challenge with DNFB. This is analogous to sensitization upon which not only T effector cells but also Treg are induced (14, 19). This implies that, irrespective of sensitization or tolerization, both T effector cells and Treg are induced. However, the predominance of either of these populations may determine the outcome of the response.

Several types of UV-Treg have been described; most of these belong to the CD4 type (13). Best characterized are the UV-Treg that suppress hapten-mediated CHS. These UV-Treg act in a strictly Ag-specific fashion. UV-Treg express CD4 and CD25 but exert their suppressive activity through the release of IL-10, which is more a characteristic feature of the Tr1 cells (25). Although there is some evidence at least in the murine system that

CD4<sup>+</sup>CD25<sup>+</sup> Treg can also release IL-10 (26), UV-Treg might represent a unique phenotype of Treg. Hence, it was the purpose of this study to further characterize these cells.

By performing depletion and positive selection studies we show that UV-Treg express GTR and neuropilin-1, two surface molecules that have been described as being expressed on Treg (16, 18). Whether the expression of these molecules by UV-Treg is of functional relevance for their suppressive activity as was observed for CTLA-4 (8) cannot be answered because Ab-blocking studies have not been performed.

Expression of *FoxP3*, a gene encoding a member of the Forkhead/winged-helix family of transcription regulators termed scurfin, has been described as being a unique feature of CD4<sup>+</sup>CD25<sup>+</sup> Treg (27). Overexpression of *FoxP3* in T cells induced a suppressor phenotype and an expansion of CD4<sup>+</sup>CD25<sup>+</sup> T cells (27–29). These findings indicated that *Foxp3* represents a key transcription factor in the development of natural CD4<sup>+</sup>CD25<sup>+</sup> Treg. Whether UV-Treg express *FoxP3* is currently under investigation.

In previous studies we could show that Ag-specific activation of UV-Treg by APC induces the release of IL-10 (7, 8). In addition, in vivo Ab-blocking experiments revealed that the inhibitory activity of UV-Treg is mediated by IL-10 (8). Although it is most likely true, it was not proven by these experiments that the IL-10-mediated suppression is derived from UV-Treg. It could not be excluded that Treg activate host cells to produce IL-10, which finally mediated the suppression. By using IL-10-deficient mice we now can demonstrate that the IL-10 required for suppression is derived from UV-Treg, because UV-Treg also exert their suppressive activity when injected into IL-10-deficient mice. This observation of course does not exclude the possibility that other subordinate mediators may be involved in the suppression as well.

We also have shown that once IL-10 is released upon Ag-specific activation by UV-Treg, it suppresses other immune reactions in a nonspecific fashion as has been described for bystander suppression (20). This suggests a therapeutic potential for the Treg generated in response to Ag that are not necessarily the precise Ag driving the pathogenic process (20). The proof of principle of this anticipation was demonstrated recently because it was possible to turn down a CHS response to OXA in OXA-sensitized DNFB-naive mice by injecting DNFB-specific Treg followed by DNFB activation (7).

Hence, we were interested in the long-term consequences of multiple injections of DNFB-specific UV-Treg followed by DNFB activation into DNFB-naive mice. In particular, we were interested in whether multiple applications of DNFB in challenge doses in the presence of Treg finally results in sensitization against DNFB or, in turn, even in tolerization. Therefore DNFB-specific UV-Treg were injected once weekly into the left ears of naive mice always followed by the application of DNFB in a challenging dose. After 3 wk a challenging dose was applied on the right ear that resulted in an ear swelling response in the same range as that for positive control mice which were conventionally sensitized and challenged. This indicated that the initially naive mice became sensitized after the three injections of UV-Treg with subsequent challenges.

Because we have shown in this study that T effector cells are also induced during tolerization, one could argue that the positive challenge upon multiple injections of UV-Treg might be caused by contaminating T effector cells. This, however, appears unlikely because CFDA-SE labeling showed no migration of i.c. injected T cells into the contralateral ears. The sensitization was further proven by the adoptive transfer of T cells from these mice into naive recipients that could be successfully challenged immediately

without the need of sensitization. We speculate that it is not the multiple injections of the DNFB-specific UV-Treg but the multiple challenges that finally sensitized the mice. This, however, implies that UV-Treg are not able to prevent the sensitization in the long term. Based on these findings, the enthusiasm for using bystander suppression to turn down specific immune responses through Treg generated in response to Ag that are not necessarily the precise Ag that are driving the pathogenic process might be toned down, because in the long term this could result in a further sensitization and a new pathologic process.

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## Disclosures

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