

***In vivo* reprogramming of UV radiation-induced regulatory T-cell migration to inhibit the elicitation of contact hypersensitivity**

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Background: Regulatory T (Treg) cells induced by UV radiation (UVR) inhibit only the induction and not the elicitation of contact hypersensitivity (CHS) because they migrate into the lymph nodes but not the skin. The tissue-homing receptor expression and migratory behavior of Treg cells can be altered by means of *in vitro* coinoculation with skin-derived antigen-presenting cells. On this *in vitro* treatment, Treg cells migrate into the skin and thus inhibit the elicitation of CHS.

Objective: We attempted to determine whether Treg cells can be induced by UVR in sensitized mice and manipulated entirely *in vivo* in such a way that they suppress the elicitation of immune responses.

Methods: Treg cells were induced by applying contact allergens onto UV-exposed skin in wild-type, langerin diphtheria toxin receptor knock-in, or depletion of Treg cell transgenic mice.

Results: UVR-induced Treg cells inhibit the elicitation of CHS in sensitized mice when stimulated by means of an antigen-specific boost through the skin. This requires cutaneous antigen-presenting cells that alter the migratory behavior of Treg cells and drive them out of the lymph nodes into the skin.

Conclusions: The indication is that antigen-specific Treg cells can be induced in sensitized hosts and manipulated in such a way that they suppress the elicitation of specific immune reactions. Because this is achieved entirely *in vivo* without invasive interventions, our findings might have important implications for strategies aiming to induce and use Treg cells in a therapeutic setting. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: Antigen-presenting cells, contact hypersensitivity, forkhead box protein 3, immunosuppression, *in vivo* reprogramming, Langerhans cells, regulatory T cells, tissue homing, skin, UV radiation

Abbreviations used

CD62L:	CD62 ligand
CFDA-SE:	Carboxyfluorescein diacetate succinimidyl ester
CHS:	Contact hypersensitivity
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
DEREG:	Depletion of regulatory T cells
DNFB:	2,4-Dinitrofluorobenzene
DT:	Diphtheria toxin
Foxp3:	Forkhead box protein 3
GFP:	Green fluorescence protein
Langerin-DTR:	Langerin diphtheria toxin receptor knock-in
LC:	Langerhans cell
LN:	Lymph node
Treg:	Regulatory T
UVR:	UV radiation
UVR-Treg:	UV radiation-induced regulatory T

Regulatory T (Treg) cells represent a subset of T cells that mediate peripheral tolerance.¹ Two types of Treg cells are distinguished. Natural Treg cells, which develop in the thymus, express CD4, CD25, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), CD62 ligand (CD62L), CD103, glucocorticoid-induced TNF receptor, neuropilin, CD152, CD103, and the transcription factor forkhead box protein 3 (Foxp3).² Induced Treg cells develop in the periphery from mature T cells, which are activated under suboptimal conditions, such as low antigen exposure, absence of costimulatory molecules, or the presence of inhibitory cytokines.³ The identification and characterization of Treg cells contributed tremendously to the understanding of the natural course of immune reactions and autoimmunity.⁴ They also opened new aspects of therapeutic intervention, although there is general consensus that Treg cells can suppress more effectively the activation of naive T cells than the effector function of already activated T cells.⁵

UV radiation (UVR), in particular the midwave range (UVB, 290–320 nm), has turned out to be a potent inducer of a subtype of Treg cells that express CD4, CD25, CTLA-4, CD62L, and Foxp3.⁶ As demonstrated in the model of contact hypersensitivity (CHS), application of contact allergens onto skin that has been exposed to UVR does not result in sensitization but rather in antigen-specific immunotolerance.⁷ This unresponsiveness is mediated through UVR-induced Treg (UVR-Treg) cells.⁸ Thus UVR-Treg cells should harbor therapeutic potential to shut down immune reactions in a specific fashion. Accordingly, intravenous injection of UVR-Treg cells into naive mice inhibits

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sensitization against the specific antigen, whereas all other immune reactions are not affected.⁸ However, the very same cells do not suppress the elicitation of CHS when injected intravenously into already sensitized recipients.⁹ This is due to the fact that UVR-Treg cells express the lymph node (LN)-homing receptor CD62L and thus migrate into the LNs but not into the skin.¹⁰ This led to the conclusion that UVR-Treg cells are ineffective once effector T cells are induced and thus are inferior to effector T cells. Along this line, Treg cells were assumed to be active only in naive and not in sensitized mice and therefore should act only in a preventive but not a therapeutic fashion. However, when UVR-Treg cells are injected into the area of hapten challenge, such as the ears of sensitized mice, the elicitation of CHS is suppressed,¹⁰ indicating that UVR-Treg cells have to get into the periphery to inhibit the effector phase of immune reactions.

The migratory behavior of naive T cells after priming is imprinted by the origin of the dendritic cell that presents the antigen.^{11,12} The same applies for UVR-Treg cells. Incubation of UVR-Treg cells with epidermal dendritic cells downregulates CD62L and induces the expression of ligands for the skin-homing receptors E- and P-selectin.¹³ Accordingly, UVR-Treg cells cocultured with epidermal dendritic cells migrate into the skin on intravenous injection and thus inhibit the elicitation of CHS in sensitized mice. This indicates that the migratory behavior of UVR-Treg cells can be altered by tissue-specific antigen-presenting cells. Because coinubation of UVR-Treg cells with dendritic cells is an *in vitro* approach, we sought to alter the migratory behavior of UVR-Treg cells through a similar approach entirely *in vivo*, with the ultimate aim of suppressing established immune reactions.

METHODS

Mice

Seven- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Langerin diphtheria toxin receptor knock-in (Langerin-DTR) mice generated by B. E. Clausen¹⁴ were bred in the central animal facilities. Mice were injected with diphtheria toxin (DT; Sigma-Aldrich, Taufkirchen, Germany) intraperitoneally and subcutaneously (400 ng each) to deplete langerin-positive cells. DERE (depletion of Treg cells) mice were generated by T. Sparwasser.¹⁵ Mice were injected intraperitoneally with DT ($3 \times 1 \mu\text{g}$) to deplete Treg cells. Animal care was provided by expert personnel in compliance with the laws and institutional guidelines. For all experiments, at least 6 mice per group were used.

CHS

Mice were sensitized by painting 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich; 50 μL in acetone/olive oil 4:1) on the shaved back or abdomen. Five days later, ear challenge was performed with 0.3% DNFB (20 μL) on the left ear. Ear swelling was measured with a spring-loaded micrometer 24 hours later. For sensitization against oxazolone (Sigma-Aldrich), 100 μL of a 2% solution was used, and for challenge, 20 μL of a 1% solution was used. The ear-swelling response was determined as the difference in ear thickness of the hapten-treated ear compared with that of the vehicle-treated ear and expressed in centimeters $\times 10^{-3}$ (means $\pm$ SDs).

Generation of UVR-Treg cells

Mice were exposed on their shaved backs to UVB (1.5 kJ/m^2) for 4 consecutive days by using TL12 lamps (Philips, Eindhoven, The Netherlands), which emit within the UVB range (290–320 nm), with a peak at 313 nm. Twenty-four hours after the last exposure, animals were sensitized with DNFB. Five days

later, LNs and spleens were collected, and single-cell suspensions were prepared. $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells were isolated with a magnet-activated cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) by using the $\text{CD4}^+\text{CD25}^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec). The purity of the $\text{CD4}^+\text{CD25}^+$ cells was determined by means of flow cytometric analysis (FC500; Beckman Coulter, Krefeld, Germany) with allophycocyanin-conjugated anti-CD4 (BD Biosciences, Mississauga, Ontario, Canada) and phycoerythrin-labeled anti-CD25 antibodies (Miltenyi Biotec). For adoptive transfer, 5×10^5 cells were injected intravenously. In some experiments bulk T cells obtained from LNs and spleens (5×10^7) were injected intravenously. For labeling, cells were incubated with 10 $\mu\text{mol}/\text{L}$ carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen, Karlsruhe, Germany) in PBS for 15 minutes at 37°C and then incubated for 30 minutes in the presence of 10% FCS, followed by 3 washes with RPMI 1640 containing 10% FCS.

Immunofluorescence

Ears were obtained from mice injected with CFDA-SE-labeled cells. Cryosections were subjected to fluorescence microscopy. In addition, sections were stained with a phycoerythrin-conjugated anti-CD25 antibody. Frozen sections obtained from DERE mice were stained with an anti-mouse/rat Foxp3 biotinylated antibody (eBioscience, Frankfurt, Germany) and as a second step with phycoerythrin-conjugated streptavidin (Beckman Coulter) or an Alexa Fluor 488-labeled antibody directed against green fluorescence protein (GFP; Invitrogen). Sections were analyzed with an immunofluorescence microscope (Axiovert 40 CFL; Carl Zeiss Corp, Hamburg, Germany).

Statistical analysis

Data were analyzed by using the Student *t* test. Differences were considered significant at a *P* value of less than .05.

RESULTS

Intravenously injected UVR-Treg cells on antigen-specific boost inhibit the elicitation of CHS in sensitized recipients

UVR-Treg cells specific for DNFB were obtained by exposing C57BL/6 mice on their shaved backs to UVR (1.5 kJ/m^2) on 4 consecutive days to address whether UVR-Treg cells can be manipulated *in vivo*. Twenty-four hours after the last exposure, DNFB was painted onto UVR-exposed skin. Five days thereafter, LNs and spleens were obtained, and $\text{CD4}^+\text{CD25}^+$ cells were isolated by means of magnetic bead separation. Cells (5×10^5) were injected intravenously into mice that had been sensitized against DNFB 5 days earlier. As demonstrated previously,^{9,10} elicitation of CHS with DNFB was unaffected in these recipients because the UVR-Treg cells locate to the LNs (Fig 1, A). Another group of recipient mice were boosted with DNFB on the abdomen 24 hours after adoptive transfer to activate the UVR-Treg cells in the LNs by skin-derived antigen-presenting cells. In this case the elicitation of CHS was significantly suppressed when compared with that seen in positive control mice or unboosted recipients (Fig 1, A).

The activation of UVR-Treg cells in the LNs has to be antigen specific because on a boost with the unrelated hapten oxazolone, intravenously injected DNFB-specific UVR-Treg cells did not suppress the ear-swelling response in DNFB-sensitized mice (Fig 1, B). UVR-Treg cells were labeled with CFDA-SE before injection into DNFB-sensitized mice to address whether the intravenously injected UVR-Treg cells on DNFB boost of the recipients migrate into the ears. CFDA-SE-labeled cells were found in the ears only in mice that received a DNFB boost before challenge (Fig 1, C). Double staining with CD25 indicated that the

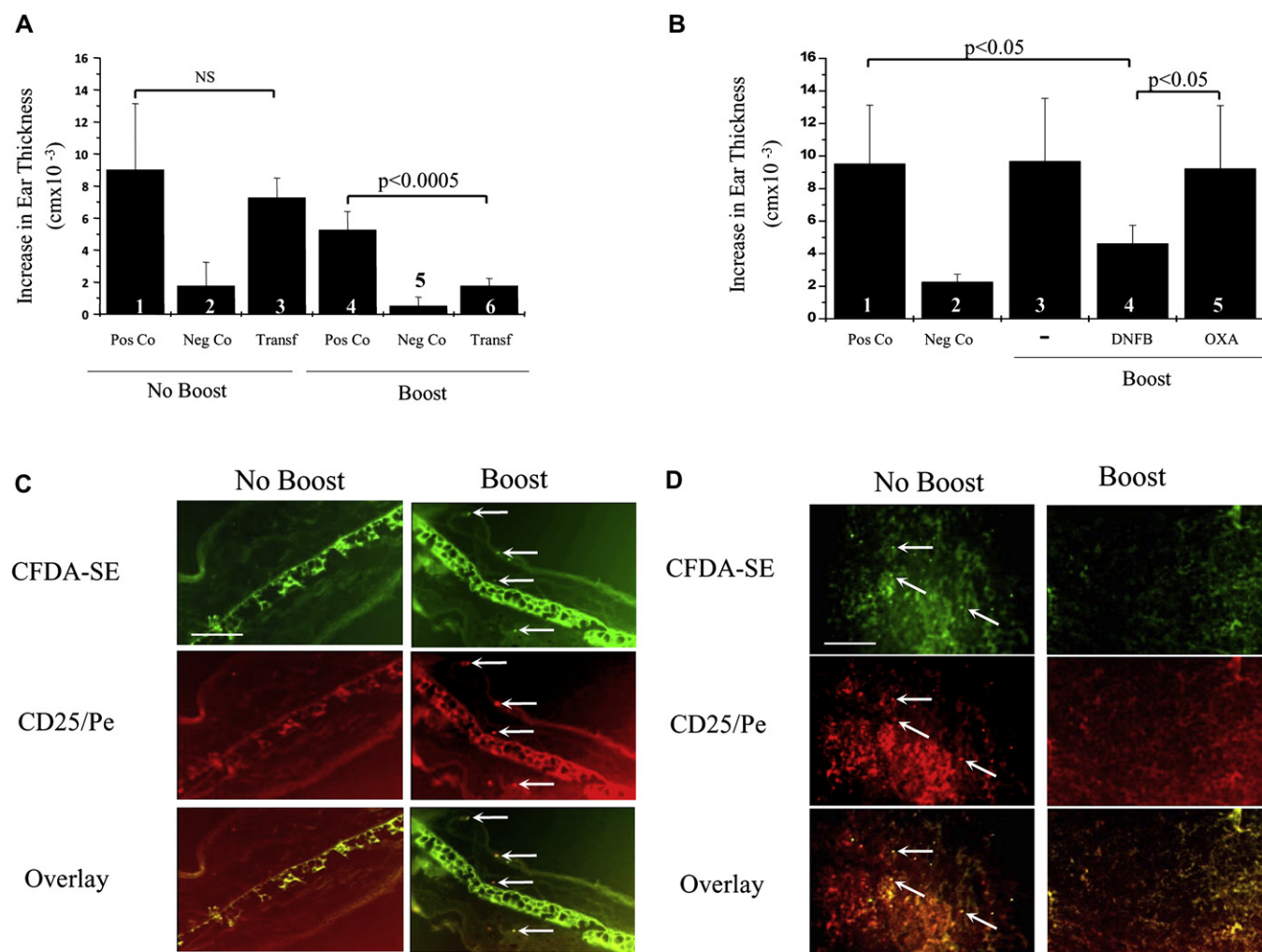


FIG 1. Intravenously injected UVR-Treg cells on boost inhibit the elicitation of CHS in sensitized recipients. **A**, CD4⁺CD25⁺ UVR-Treg cells were injected intravenously (5×10^5) into DNFB-sensitized mice (groups 3 and 6). Recipient mice were boosted with DNFB on the abdomen 24 hours after injection (group 6). Ears were challenged 24 hours later. Positive controls were sensitized and challenged (groups 1 and 4), and negative controls were challenged only (groups 2 and 5). Positive controls of the boost group received DNFB on the abdomen 24 hours before ear challenge (group 4). Ear swelling was measured 24 hours after challenge. NS, Not significant. **B**, DNFB-specific UVR-Treg cells were injected intravenously into DNFB-sensitized mice (groups 3-5). Recipients were boosted with DNFB (group 4) or oxazolone (OXA, group 5) 24 hours after transfer. Ears were challenged 24 hours thereafter. Group 3 received UVR-Treg cells without boost. **C** and **D**, CFDA-SE-labeled UVR-Treg cells were injected into sensitized mice. Mice were boosted or left untreated before ear challenge. Ears (Fig 1, C) and LNs (Fig 1, D) were cut and stained with an anti-CD25 antibody. Arrows indicate UVR-Treg cells. Bar = 100 μ m. Pe, Phycoerythrin.

CFDA-SE-labeled cells also express CD25. In turn, CFDA-SE-labeled cells were found in the LNs of unboosted but not boosted mice, supporting the hypothesis of emigration from the LNs on boost (Fig 1, D).

Cutaneous langerin-positive dendritic cells are required for modulating UVR-Treg cells

The skin harbors several types of antigen-presenting cells.¹⁶ For a long time, epidermal Langerhans cells (LCs) were regarded as the only antigen-presenting cell of the skin.¹⁷ There is accumulating evidence that dermal dendritic cells might be more potent in inducing sensitization responses through the skin than LCs.¹⁸ Langerin was initially described as a specific and exclusive

marker for LCs.^{19,20} Recently, it turned out that a small fraction of dermal dendritic cells can express langerin as well.²¹⁻²⁴ There is evidence that these cells do not represent LCs emigrating from the epidermis but rather a distinct subtype of dermal dendritic cells.¹⁶ Thus langerin is a useful marker for dendritic cells in the skin, although there is no doubt about the existence of dermal dendritic cells not expressing langerin.²³

To address whether the boost that appears to alter the migratory behavior of intravenously injected UVR-Treg cells is mediated through langerin-positive dendritic cells residing in the skin, we used Langerin-DTR mice. These mice harbor a targeted insertion of the human DT receptor into the langerin locus.¹⁴ Because the cytotoxicity of DT depends on receptor-mediated endocytosis²⁵ and murine cells are resistant to DT because of low affinity of

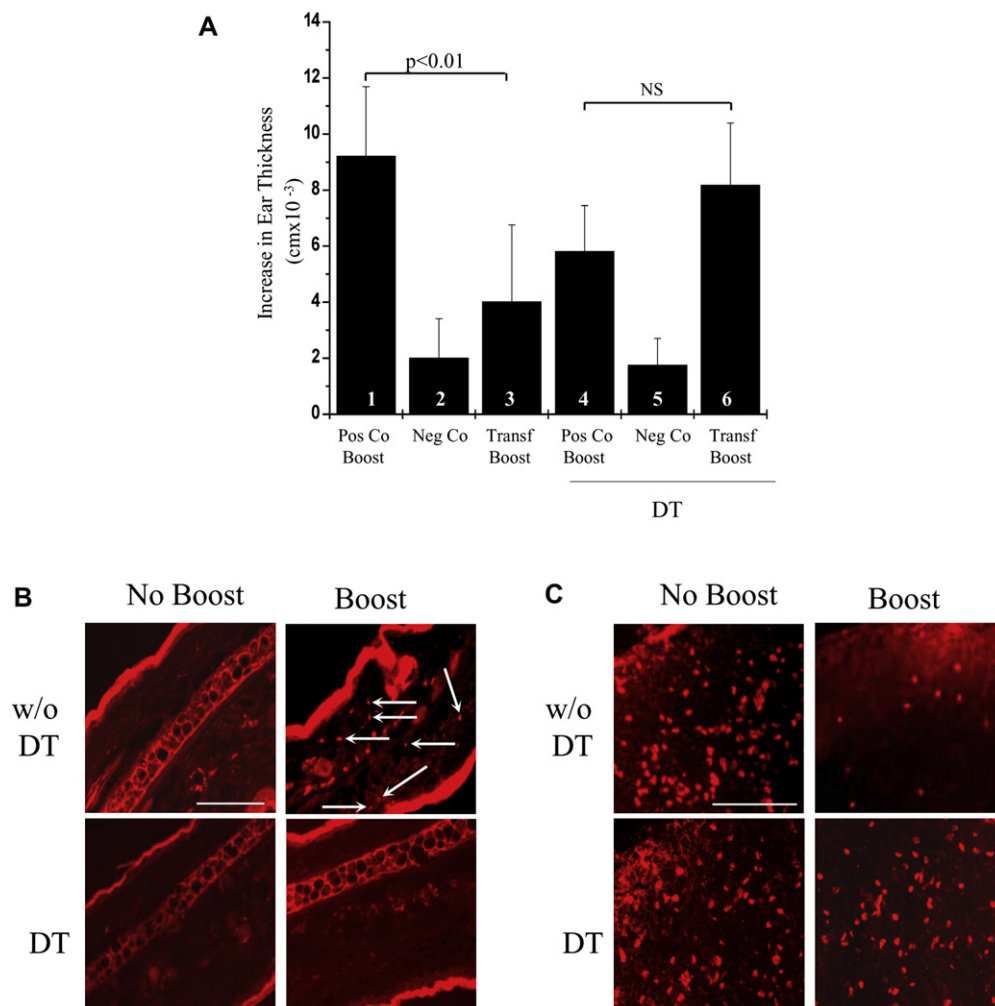


FIG 2. Cutaneous langerin-positive dendritic cells are required for modulating UVR-Treg cells. **A**, UVR-Treg cells were injected intravenously into DNFB-sensitized Langerin-DTR mice (groups 3 and 6). Group 6 was injected 48 hours after sensitization with DT. Recipients received a boost with DNFB on the abdomen 24 hours after transfer. Ear challenge was performed 24 hours thereafter. *NS*, Not significant. **B** and **C**, Ears (Fig 2, **B**) and LNs (Fig 2, **C**) were cut and stained with an anti-Foxp3 antibody. Arrows indicate UVR-Treg cells. Bar = 100 μ m.

the toxin for the rodent DT receptor, injection of DT into Langerin-DTR mice results in selective depletion of langerin-positive cells. The experiment described in Fig 1, A, was repeated with Langerin-DTR mice. On adoptive transfer of UVR-Treg cells, DNFB-sensitized recipients were boosted on the abdomen with DNFB. One group received an injection of DT 72 hours before adoptive transfer. Langerin-DTR mice that did not receive DT behaved like wild-type animals, as shown in Fig 1, A. As demonstrated in Fig 2, A, the boost resulted in a significant suppression of the ear-swelling response (group 3), confirming the results of Fig 1, A. Positive control mice receiving DT (group 4) had a moderately reduced ear-swelling response, confirming previous reports.¹⁴ On depletion of langerin-positive dendritic cells, the boost did not result in suppression in sensitized mice that had received UVR-Treg cells (group 6).

On immunohistochemistry with an anti-Foxp3 antibody, UVR-Treg cells could not be detected in the ears of boosted mice on depletion of langerin-positive dendritic cells (Fig 2, B). In turn, UVR-Treg cells were still detectable in the LNs under these conditions (Fig 2, C). This indicates that the boost that activates

UVR-Treg cells in the LNs to emigrate into the skin requires langerin-positive dendritic cells from the skin.

UVR-Treg cells can be induced and manipulated in sensitized mice

To avoid the disadvantages of isolation and *in vitro* cultivation of Treg cells, we asked whether UVR-Treg cells can be both induced and manipulated *in vivo* in sensitized mice. C57BL/6 mice were DNFB sensitized through the abdominal skin. Five days later, 2 groups of the DNFB-sensitized animals were UVR exposed for 4 days on the shaved back. Twenty-four hours after the last irradiation, DNFB was painted onto UVR-exposed skin to induce UVR-Treg cells in the sensitized mice. Ear challenge performed 5 days later resulted in a pronounced ear-swelling response, indicating that UVR-Treg cells were not induced or more likely trapped in the LNs and unable to move into the skin. When these mice received a DNFB boost on the flank 24 hours before challenge, the CHS reaction was significantly suppressed (Fig 3, A). This supports the hypothesis that (1) UVR-Treg cells

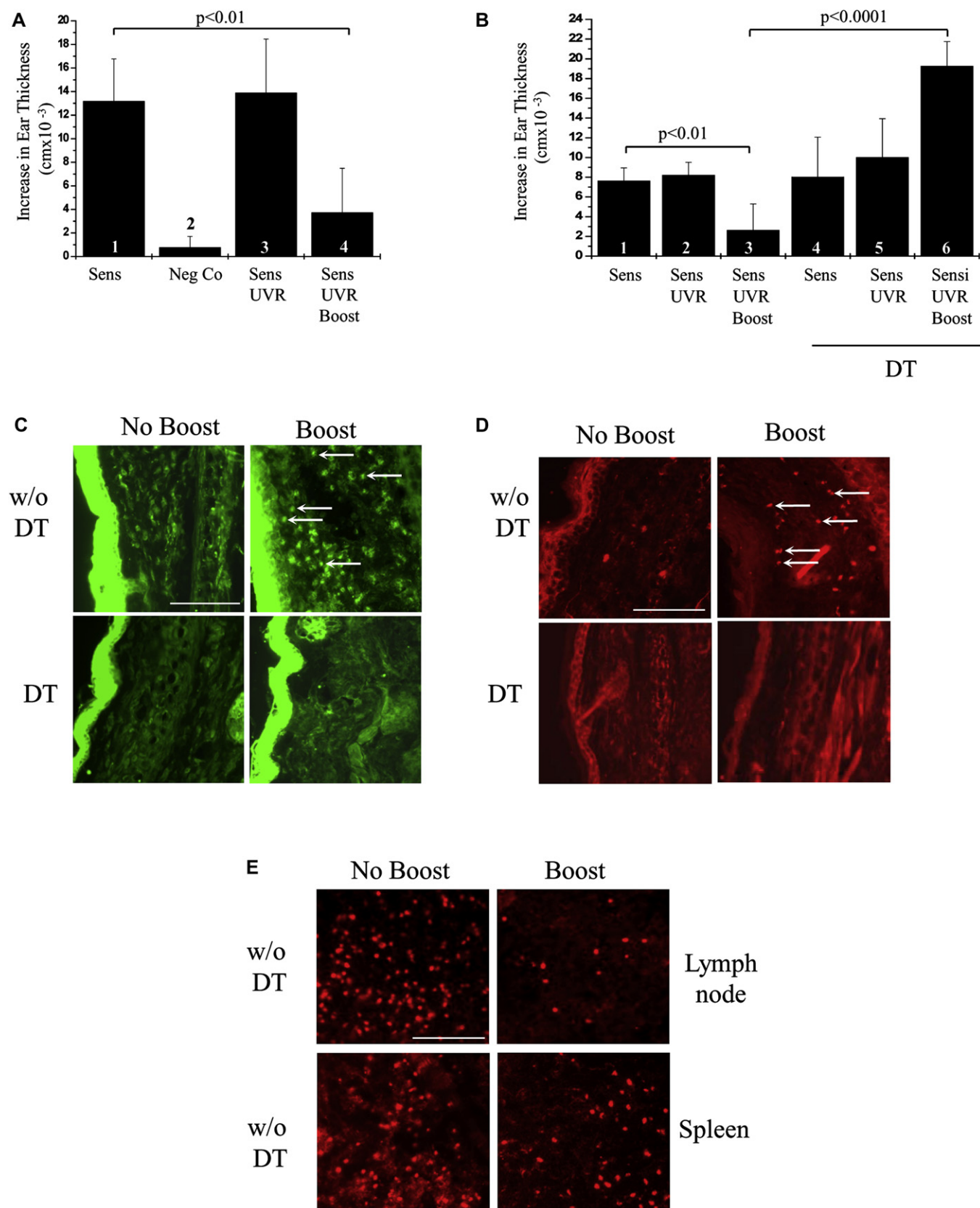


FIG 3. UVR-Treg cells expressing Foxp3 can be induced in sensitized mice. **A**, C57BL/6 mice were sensitized with DNFB on the abdomen (groups 1, 3, and 4). Five days later, mice were exposed to UVR. Twenty-four hours after the last exposure, DNFB was painted onto UVR-exposed skin (groups 3 and 4). Group 4 received a DNFB boost on the flank 5 days thereafter. Twenty-four hours later, ears were challenged. **B**, DEREG mice

can be induced in sensitized mice and (2) UVR-Treg cells on boost through the skin inhibit the effector phase of CHS.

DEREG mice were used to address whether the suppression of CHS in sensitized mice is mediated through UVR-Treg cells. They express a DTR-enhanced GFP fusion protein under control of the *Foxp3* gene locus, allowing depletion of Foxp3-positive Treg cells on DT injection.¹⁵ DEREG mice were treated identically as the mice shown in Fig 3, A. Only on a DNFB boost was the CHS reaction suppressed in the sensitized mice. However, when DEREG mice were treated with DT 48 hours after application of DNFB onto UVR-exposed skin, the CHS response was not suppressed on ear challenge (Fig 3, B). This demonstrates that the suppression caused by the boost is mediated through Foxp3⁺ UVR-Treg cells.

The emigration of the UVR-Treg cells induced by the boost into the ears was confirmed *in situ*. The ears of the DEREG mice were cut and subjected to immunofluorescence microscopy. Cryosections were stained with an anti-GFP antibody to enhance the fluorescence signal. Fluorescent cells were found only in the ears of mice subjected to a boost (Fig 3, C). On injection of DT, no GFP⁺ cells were found in unboosted or boosted mice. These findings were confirmed when ears were stained with an antibody directed against Foxp3 (Fig 3, D). LNs and spleens were obtained from DEREG mice that did not receive DT to study the effect of the boost on the distribution of the UVR-Treg cells in the LNs and spleens. On boost, much fewer cells were observed in the LNs than in unboosted mice (Fig 3, E), confirming the data shown in Fig 1, D. In contrast, the number of cells in the spleens was not remarkably altered by the boost (Fig 3, E). This confirms that Foxp3-expressing UVR-Treg cells generated in sensitized mice emigrate from the LNs into the skin on the antigen-specific *in vivo* boost and inhibit the elicitation of CHS.

DISCUSSION

UVR-induced immunosuppression is characterized by various features: it primarily affects T cell-mediated reactions, it is induced by low UVR doses in the physiologic range, and it does not act in a general but rather an antigen-specific fashion, thus differing from immunosuppression induced by immunosuppressive drugs.²⁶ The same UVR doses that suppress adaptive immune reactions induce innate immune responses, such as the production of antimicrobial peptides.²⁷ Because solar radiation disturbs the epidermal barrier, induction of antimicrobial peptides appears to be protective, yielding an antimicrobial defense. Along the same line, speculation exists that the suppression of T cell-mediated immune responses by physiologic UVR doses might be protective as well because most inflammatory reactions in the skin are T-cell driven,²⁸ implying that T cells in the skin are more harmful than beneficial.²⁶ As one of the most outer barrier organs, the skin is constantly exposed to potential allergens. In addition, the skin is an organ prone to autoimmunity, which is indicated by the fact that many autoimmune diseases involve the skin. Hence a

sort of constant physiologic immunosuppression by ambient UVR might be beneficial.²⁶

According to this concept, the primary task of UVR-Treg cells would be the inhibition of sensitization against specific antigens without disturbing other immune responses. UVR-Treg cells meet these criteria because they act in an antigen-specific fashion, as demonstrated by adoptive transfer experiments. UVR-Treg cells are quite well characterized: they express CD4, CD25,^{10,13} CTLA-4,²⁹ dectin-2,³⁰ glucocorticoid-induced TNF receptor family-related protein,³¹ neuropilin,³¹ and CD62L but not the ligands for the skin-homing receptors E- and P-selectin.^{10,13} Because of the expression of CD62L, they primarily locate to the LNs, where sensitization takes place. Hence they are in place and very effective in inhibiting sensitization. Because of their antigen specificity, UVR-Treg cells are attractive candidates for therapeutic intervention. However, several studies have demonstrated that UVR-Treg cells when injected intravenously only inhibit sensitization in naive subjects but not the elicitation of CHS in sensitized hosts.^{9,10} This might be due to the fact that UVR-Treg cells are trapped in the LNs and do not get out into the periphery, an essential prerequisite for suppressing elicitation of immune responses. In turn, the elicitation is suppressed on intracutaneous injection of UVR-Treg cells into sensitized mice.¹⁰

The expression pattern of tissue-homing receptors of UVR-Treg cells can be modified by tissue-specific antigen-presenting cells.¹³ On coinubation with dendritic cells derived from the skin, UVR-Treg cells downregulate CD62L and instead express the ligands for E- and P-selectin. Accordingly, cells treated in such a way on intravenous injection do not migrate into the LNs but into the skin, where they suppress the elicitation of CHS in sensitized mice. This indicates that UVR-Treg cells, in contrast to previous assumptions,⁹ exert the capacity to inhibit not only the induction but also the effector phase of immune responses provided they are at the site of T-cell reactivation. The inability of UVR-Treg cells to suppress the elicitation phase might also be explained by the expanded population of effector T cells, which might be less susceptible to UVR-Treg cell action or might be too many to be efficiently suppressed. This implies that inflammation-dependent homing of circulating UVR-Treg cells that might not be stuck in the LNs could be inefficient compared with homing of reprogrammed Treg cells expressing skin-homing receptors. Strategies to use Treg cells therapeutically include cell therapy with *in vitro*-activated and expanded Treg cells.⁵ This offers also the opportunity to reprogram their homing receptor expression and migration behavior. However, such cell therapy approaches are complex and expensive.

Hence we were interested whether the migratory behavior of intravenously injected UVR-Treg cells can be manipulated *in vivo*. Mice that were injected intravenously with DNFB-specific UVR-Treg cells received an antigenic boost with DNFB through the skin to imitate the *in vitro* situation and to stimulate UVR-Treg in the LNs. We expected that cutaneous antigen-presenting cells will carry the antigen into the LNs and restimulate the UVR-Treg cells, which in turn should alter their

were treated as in Fig 3, A. Forty-eight hours after DNFB painting, groups 4, 5, and 6 received DT. Twenty-four hours after the last injection, a DNFB boost was performed on the flank (groups 3 and 6). Twenty-four hours later, ears were challenged. C and D, Twenty-four hours after challenge, ears were cut and stained with antibodies against GFP (Alexa Fluor 488; Fig 3, C) and Foxp3 (phycoerythrin; Fig 3, D). Arrows indicate UVR-Treg cells. E, LNs and spleens from DEREG mice, which were treated as in Fig 3, A, but without injection of DT, were stained with an anti-Foxp3 antibody. Bar = 100 μ m.

tissue-homing receptor expression pattern and migrate into the skin. This appears to be the case because CFDA-SE-labeled UVR-Treg cells could be detected in the ears but not in the LNs on boosting. Accordingly, under these conditions, the elicitation phase of CHS was suppressed in sensitized mice, indicating that the UVR-Treg cells not only migrated into the skin but also exerted suppressive activities *in situ*.

The absolute number of UVR-Treg cells detected in the ears might appear rather low; however, this was also observed in previous studies.^{10,13} This might be due to the limitation of the method because *in situ* immunofluorescence is only a semiquantitative approach. We used this method primarily to answer the question of whether we find UVR-Treg cells in the ears after the boost. We occasionally found CFDA-SE⁺CD25⁺ cells in the ears. On first glance, this might appear to be in contrast to the observations by Vocanson et al,³² who found a much higher frequency of Treg cells in tissues. However, these findings cannot be strictly compared with ours because of differences in the design. First, they used BALB/c mice, whereas we used C57BL/6 mice. It is known that both strains differ quite remarkably in CHS. Second, they detected Foxp3⁺ Treg cells in draining and nondraining LNs by means of fluorescence-activated cell-sorting analysis, whereas we used immunofluorescence staining. In addition, the frequency of Treg cells in the skin was not analyzed.

The reprogramming of UVR-Treg cells by the boost was also confirmed by means of *in situ* staining against CD62L. We detected CD62L⁺ cells only in the LNs of unboosted but not boosted mice (data not shown). CD62L⁺ cells were not found in the ears of either boosted or unboosted mice. Because we can detect UVR-Treg cells only in the ears of the boosted mice but cannot identify CD62L⁺ cells there, one can conclude that the Treg cells have lost CD62L. The negative finding in the LNs is hard to interpret because this could imply that all cells are gone or that some cells are still there but have lost CD62L. Interestingly, the number of Treg cells in the spleens was not remarkably altered by the boost (Fig 3, E). This might be explained by the fact that skin-derived antigen-presenting cells primarily migrate into the LNs and not into the spleen on the cutaneous boost, suggesting that the majority of UVR-Treg cells are reprogrammed in the LNs and not in the spleen.

Cutaneous dendritic cells appear to be crucial for the boost because both the migration and suppression of the elicitation phase were lost when langerin-expressing dendritic cells, which represent the majority of cutaneous antigen-presenting cells, were depleted in Langerin-DTR mice. Accordingly, UVR-Treg cells could not be detected in the ears of boosted mice on depletion of langerin-positive dendritic cells. The appearance of UVR-Treg cells in the skin after an antigen-specific boost was also demonstrated in DERE mice.¹⁵ Depletion of Foxp3-expressing cells through injection of DT into DERE mice was associated with a loss of suppression of the elicitation of CHS in boosted mice. This implies that the suppression is mediated through UVR-Treg cells expressing Foxp3, thus confirming previous observations.²⁹

To avoid the disadvantages of isolation and *in vitro* cultivation of Treg cells, we finally asked whether UVR-Treg cells can be induced and manipulated *in vivo* in sensitized mice. Re-exposure of sensitized mice with the same antigen through UVR-exposed skin resulted in the induction of UVR-Treg cells. However, the cells were trapped in the LNs and were thus unable to suppress the elicitation of CHS. When the UVR-Treg cells induced *in vivo* were activated through an antigen-specific boost through the skin, they emigrated into the skin and suppressed the elicitation of CHS.

In conclusion, these data indicate that, at least in the murine model of CHS, antigen-specific Treg cells can be induced in sensitized mice *in vivo* by UVR. Treg cells generated in this way exert the capacity to inhibit the elicitation of CHS, provided they are stimulated to emigrate into the skin. This can be achieved by an antigen-specific boost through the skin. Here we demonstrate that antigen-specific Treg cells can be induced in sensitized hosts and manipulated in such a way that they suppress the elicitation of specific immune reactions. Because this is achieved entirely *in vivo* without invasive interventions, our findings might have important implications for strategies aiming to induce and use Treg cells in a therapeutic setting.

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Key messages

- Treg cells can be induced in sensitized hosts.
- Treg cells can be reprogrammed *in vivo*.
- This indicates a strategy to use Treg cells in a therapeutic setting.

REFERENCES

1. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. *Cell Mol Life Sci* 2009;66:2603-22.
2. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775-87.
3. Kabelitz D, Wesch D, Oberg HH. Regulation of regulatory T cells: role of dendritic cells and toll-like receptors. *Crit Rev Immunol* 2006;26:291-306.
4. Tang Q, Bluestone JA. Regulatory T-cell physiology and application to treat autoimmunity. *Immunol Rev* 2006;212:217-37.
5. Miyara M, Wing K, Sakaguchi S. Therapeutic approaches to allergy and autoimmunity based on FoxP3⁺ regulatory T-cell activation and expansion. *J Allergy Clin Immunol* 2009;123:749-55.
6. Schwarz T. 25 years of UV-induced immunosuppression mediated by T cells—from disregarded T suppressor cells to highly respected regulatory T cells. *Photochem Photobiol* 2008;84:10-8.
7. Toews GB, Bergstresser PR, Streilein JW. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J Immunol* 1980;124:445-53.
8. Elmetz CA, Bergstresser PR, Tigelaar RE, Wood PJ, Streilein JW. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J Exp Med* 1983;158:781-94.
9. Glass MJ, Bergstresser PR, Tigelaar RE, Streilein JW. UVB radiation and DNFB skin painting induce suppressor cells universally in mice. *J Invest Dermatol* 1990;94:273-8.
10. Schwarz A, Maeda A, Wild MK, Kernebeck K, Gross N, Aragane Y, et al. UV-induced regulatory T cells do not only inhibit the induction but can also suppress the effector phase of contact hypersensitivity. *J Immunol* 2004;172:1036-43.
11. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosenthal M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 2003;424:88-93.
12. Dudda JC, Simon JC, Martin S. Dendritic cell immunization route determines CD8 T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. *J Immunol* 2004;172:857-63.
13. Schwarz A, Maeda A, Schwarz T. Alteration of the migratory behavior of UV-induced regulatory T cells by tissue-specific dendritic cells. *J Immunol* 2007;178:877-86.
14. Bennett CL, van Rijn E, Jung S, Inaba K, Steinman RM, Kapsenberg ML, et al. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol* 2005;169:569-76.
15. Lahl K, Lodenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007;204:57-63.
16. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* 2008;8:935-47.

17. Stingl G, Tamaki K, Katz SI. Origin and function of epidermal Langerhans cells. *Immunol Rev* 1980;53:149-74.
18. Romani N, Clausen BE, Stoitzner P. Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. *Immunol Rev* 2010;234:120-41.
19. Valladeau J, Ravel O, Dezutter-Dambuyant C, Moore K, Kleijmeer M, Liu Y, et al. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 2000;12:71-81.
20. Takahara K, Omatsu Y, Yashima Y, Maeda Y, Tanaka S, Iyoda T, et al. Identification and expression of mouse Langerin (CD207) in dendritic cells. *Int Immunol* 2002;14:433-44.
21. Bursch LS, Wang L, Igyarto B, Kissenpfennig A, Malissen B, Kaplan DH, et al. Identification of a novel population of Langerin+ dendritic cells. *J Exp Med* 2007;204:3147-56.
22. Ginhoux F, Collin MP, Bogunovic M, Abel M, Leboeuf M, Helft J, et al. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med* 2007;204:3133-46.
23. Nagao K, Ginhoux F, Leitner WW, Motegi S, Bennett CL, Clausen BE, et al. Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc Natl Acad Sci U S A* 2009;106:3312-7.
24. Poulin LF, Henri S, de Bovis B, Devillard E, Kissenpfennig A, Malissen B. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J Exp Med* 2007;204:3119-31.
25. Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 1992;69:1051-61.
26. Schwarz T. The dark and the sunny sides of UVR-induced immunosuppression: photoimmunology revisited. *J Invest Dermatol* 2010;130:49-54.
27. Gläser R, Navid F, Schuller W, Jantschitsch C, Harder J, Schröder JM, et al. UV-B radiation induces the expression of antimicrobial peptides in human keratinocytes in vitro and in vivo. *J Allergy Clin Immunol* 2009;123:1117-23.
28. Robert C, Kupper TS. Inflammatory skin diseases, T cells, and immune surveillance. *N Engl J Med* 1999;341:1817-28.
29. Schwarz A, Beissert S, Grosse-Heitmeyer K, Gunzer M, Bluestone JA, Grabbe S, et al. Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J Immunol* 2000;165:1824-31.
30. Aragane Y, Maeda A, Schwarz A, Tezuka T, Ariizumi K, Schwarz T. Involvement of dectin-2 in ultraviolet radiation-induced tolerance. *J Immunol* 2003;171:3801-7.
31. Maeda A, Beissert S, Schwarz T, Schwarz A. Phenotypic and functional characterization of ultraviolet radiation-induced regulatory T cells. *J Immunol* 2008;180:3065-71.
32. Vocanson M, Rozieres A, Hennino A, Poyet G, Gaillard V, Renaudineau S, et al. Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of TH17/TH1 and regulatory T cells. *J Allergy Clin Immunol* 2010;126:280-9.