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Ultraviolet Radiation-Induced Regulatory T Cells Not Only Inhibit the Induction but Can Suppress the Effector Phase of Contact Hypersensitivity[1]

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Epicutaneous application of haptens to UV-exposed skin induces hapten-specific tolerance. This is mediated via regulatory T cells (Tr), as i.v. injection of T cells from UV-tolerized mice into naive animals renders the recipients unresponsive to the respective hapten. However, when UV-induced Tr are injected i.v. into sensitized mice, contact hypersensitivity (CHS) is not suppressed, suggesting that Tr inhibit the induction, but not the elicitation, of CHS and are inferior to T effector cells. As sensitization takes place in the lymph nodes, but elicitation occurs in the area of challenge, we postulated that Tr injected i.v. locate to the lymph nodes and not to the periphery and therefore only suppress the induction, not the elicitation, of CHS. Indeed, i.v. injection of Tr into sensitized mice did not inhibit CHS, although injection of Tr into the ears of sensitized mice suppressed the challenge. Inhibition was hapten specific, as injection of dinitrofluorobenzene (DNFB)-specific Tr into the ears of oxazolone (OXA)-sensitized mice did not affect challenge with OXA. However, when ears of OXA-sensitized mice were injected with DNFB-specific Tr and painted with DNFB before OXA challenge, CHS was suppressed. Inhibition correlated with the local expression of IL-10. Depletion studies and FACS analysis revealed that Tr express the lymph node-homing receptor L-selectin, but not the ligands for the skin-homing receptors E- and P-selectin, suggesting that UV-induced Tr, although able to inhibit T effector cells, do not suppress the elicitation of CHS upon i.v. injection, because they obviously do not migrate into the skin. *The Journal of Immunology,* 2004, 172: 1036–1043.

Ultraviolet radiation, in particular the midwave range (UVB, 290–320 nm), represents one of the most significant environmental factors affecting humans, not least because of its hazardous effects on health, which include exacerbation of infectious diseases, skin cancer, and premature skin aging (1–3). Some of these effects are mediated by the immunosuppressive properties of UV, which are best illustrated by the inhibition of cellular immune reactions, such as contact hypersensitivity (CHS)[3] (4, 5). Accordingly, UV exposure impairs sensitization to haptens, which are painted directly to the UV-irradiated skin area (4, 5). In addition to the failure to generate hapten sensitization, tolerance develops, as individuals treated in such a way cannot be resensitized against the same hapten at a later time point (4, 5). UV-induced tolerance is hapten specific, because the sensitization against other nonrelated haptens is not impaired (4).

Hapten-specific unresponsiveness can be adoptively transferred, because injection of lymph node cells and splenocytes obtained from UV-tolerized mice into syngeneic naive mice inhibits sensitization against the respective hapten in the recipients (6). Consequently, it has been suggested that UV-induced tolerance is mediated by hapten-specific T suppressor cells (reviewed in Ref. 7).

Although these experiments were performed almost 2 decades ago, the exact phenotype and, in particular, the mode of action of these cells remained unclear for quite a long time. Since the discovery that regulatory T cells (Tr) play an important role in the regulation and suppression of immune responses, respectively (8–10), several studies tried to further characterize UV-induced suppressor T cells, which are now also designated UV-induced regulatory T cells (11–14). Although in our view these names could be used interchangeably, we refer to the term Tr. Several types of UV-induced suppressor/regulatory T cells have been described; most of these belong to the CD4 type (7). There is recent evidence that they also express CD25 (13) and CTLA-4 (14). In addition, they secrete IL-10 upon hapten-specific stimulation (11, 14). The observation that UV-mediated tolerance and transfer of suppression can be inhibited by neutralizing anti-IL-10 Abs suggests that the release of IL-10 by UV-induced Tr plays an important role in photoimmunosuppression (14). Although the UV-induced suppression of CHS and DTH is clearly mediated via T cells, UV-induced suppression of tumor immunity appears to be mediated via NKT cells (15).

UV-induced Tr, however, exert suppressive activity only when injected i.v. into naive, but not sensitized, mice, implying that they inhibit only the afferent, not the efferent, limb of CHS. Thus, it was concluded that in the presence of T effector cells, Tr no longer exert any suppressive effect. This assumption was also supported by the finding that naive mice that were coinjected with T cells

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[3] Abbreviations used in this paper: CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; i.c., intracutaneous; OXA, oxazolone; Tr, regulatory T cell.

from sensitized and T cells from tolerized donors mount an ear-swelling response upon challenge (6, 16). It was concluded that 1) UV-induced Tr only inhibit the afferent, but not the efferent, phase of CHS; 2) UV-induced Tr are not active in the presence of effector T cells; and 3) UV-induced Tr are inferior to T effector cells (7, 16).

IL-10 has been demonstrated to be able to inhibit both the induction and the elicitation of CHS (17–19). Therefore, if the release of IL-10 is one of the major activities by which Tr mediate their suppressive effects, Tr, in principle, should be able to inhibit not only the afferent, but also the efferent, limb of CHS. During sensitization, the interaction between APC and T cells takes place in the lymph nodes, whereas elicitation occurs in the area of challenge. Therefore, we wondered whether the inability of Tr to inhibit the effector phase of CHS upon i.v. injection is due to the fact that they do not migrate into the skin.

In this study we show that intracutaneous (i.c.) injection of Tr into the ears of sensitized mice suppressed the ear challenge significantly in a hapten-specific fashion. Furthermore, we demonstrate that 2,4-dinitrofluorobenzene (DNFB)-specific Tr injected into the ears of mice sensitized against oxazolone (OXA) upon activation by epicutaneous application of DNFB also suppress the specific response against OXA. Activation of Tr was associated with an increased expression of IL-10. This indicates that activation of UV-induced Tr is hapten-specific, but once activated, their suppressive activity is nonspecific. UV-induced Tr belong to the $CD4^+CD25^+$ subtype and in addition express the lymph node-homing receptor L-selectin (CD62L), but not ligands for the skin-homing receptors, E- and P-selectin. Therefore, we propose that UV-induced Tr, by being able to inhibit T effector cells, do not suppress the elicitation of CHS upon i.v. injection because they obviously do not migrate into the skin.

Materials and Methods

Animals and reagents

C3H/HeN mice (8–10 wk old) were purchased from Harlan Winkelmann (Borchen, Germany). Animals were housed under specific pathogen-free conditions and treated according to institutional guidelines. The neutralizing anti-IL-10 Ab, SXC1 (20, 21), was provided in a collaboration with W. Müller (GBF, Braunschweig, Germany). As an isotype control, rat IgG (Sigma-Aldrich, St. Louis, MO) was used.

Contact hypersensitivity

Mice were sensitized by painting 50 μl of DNFB (Sigma-Aldrich) solution (0.5% in acetone/olive oil, 4/1) on the shaved back on day 0. On day 5, 20 μl of 0.3% DNFB was applied to the left ear, and the vehicle acetone/olive oil was applied to the right ear. OXA was applied at a concentration of 2% for sensitization and at 0.5% for elicitation. Ear swelling was measured in a blinded fashion with a spring-loaded micrometer (Mitutoyo, Kawasaki, Japan) 24 h after challenge. CHS was determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear in sensitized animals and was expressed in centimeters $\times 10^{-3}$ (mean $\pm$ SD). Mice that were ear-challenged without prior sensitization served as negative controls. Each group consisted of at least seven mice. Each experiment was performed at least twice.

UV irradiation

The shaved back was exposed to UV emitted from a bank of four TL12 fluorescent lamps (Philips, Eindhoven, The Netherlands) that emit most of their energy within the UVB range (290–320 nm), with an emission peak at 313 nm. Mice were exposed to UV daily for 4 consecutive days (1000 J/m²/exposure). Twenty-four hours after the last UV exposure, DNFB was applied carefully to the surface of the irradiated area as described above.

Adoptive transfer of immune response

Donor mice were tolerized against DNFB by painting DNFB to UV-exposed skin and are referred to as UV-tolerized mice. Five days later, spleens and regional lymph nodes were removed, and single-cell suspensions were prepared. The cell number was adjusted (see individual experiments), and cells were injected i.v. (200 μl) or i.c. (100 μl) into the ears

of the recipient mice. As demonstrated previously, i.v. injection of these cells into naive mice suppresses the induction of CHS in a hapten-specific fashion, indicating the presence of Tr within this population. For the sake of simplicity in the present manuscript, the term UV-induced Tr was used for the lymph node cells obtained from UV-tolerized mice, although we are fully aware of the fact that we did not inject pure Tr, but rather bulk cells containing Tr.

Depletion of subpopulations

For depletion of subpopulations, lymphocytes obtained from regional lymph nodes and spleens were incubated with microbeads coated with Abs against CD4, CD25, and CD62L (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by negative selection with the AutoMACS magnetic separation system (Miltenyi Biotec). The negative fractions were harvested, washed, and adjusted to the appropriate cell numbers for injection. In some experiments $CD4^+CD25^+$ T cells were obtained by magnetobead separation using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). The efficacy of depletion or isolation was determined by FACS analysis (BD Biosciences, Mountain View, CA).

Flow cytometry

For flow cytometric analysis, cells obtained from regional lymph nodes and spleens were incubated with microbeads coated with an Ab against CD4 (Miltenyi Biotec), followed by positive selection with the AutoMACS magnetic separation system (Miltenyi Biotec). $CD4^+$ subpopulations were first incubated with 25 μg/ml P- or E-selectin-IgG chimera (22) or 10 μg/ml affinity-purified rabbit Abs in HBSS with 3% FCS on ice for 30 min. Three-color staining was conducted with an allophycocyanin-conjugated Ab against CD4 (clone RM4-5; BD Biosciences, San Diego, CA), FITC-conjugated Ab against CD62L (clone MEL-14; BD Biosciences), and PE-labeled F(ab')$_2$ donkey anti-human IgG (Dianova, Hamburg, Germany). Analysis was performed with a FACSCalibur and CellQuest analysis software (BD Biosciences).

RT-PCR

Excised ears were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated. Five micrograms of total RNA was reverse transcribed. Reaction buffer (5 $\times$ 250 mM Tris-HCl (pH 8.3), 25 mM dNTP (each), and 250 mM KCl), 1 μl of RNase inhibitor (Promega, Madison, WI), and 0.8 μl of dT$_{15}$ were mixed and diluted to 38 μl with RNase-free water, heated to 65°C for 5 min in a water bath, and chilled on ice. After addition of 2 μl of avian myeloblastosis virus reverse transcriptase (Promega), samples were incubated at 42°C for 90 min, and the cDNA was precipitated with 2.5 vol of cold ethanol and one-third volume of 3 M sodium acetate buffer, pH 7.5, at -20°C overnight. The pellet was rinsed with 70% ethanol, evaporated in a vacuum exsiccator, and dissolved in RNase-free water.

To perform PCR amplification on a semiquantitative basis, we amplified all cDNA samples with primers specific for β-actin using various cycle numbers and dilution factors and thereby determined the optimal concentration of each sample and a cycle number showing a logarithmic increase in amplification. Samples were then amplified using primers specific for murine IL-10. For PCR amplification, we used a 50-μl reaction mixture containing 1 μg of cDNA, 200 mM dNTP (each), and 20 pmol of each primer pair. The PCR primers for murine IL-10 were as follows: sense primer, 5′-TACCTGGTAGAAGTGATGCC-3′; and antisense primer, 5′-CATCATGTATGCTTCTATGC-3′.

Statistical analysis

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

Results and Discussion

UV-Induced Tr inhibit sensitization only in naive, not sensitized, mice upon i.v. injection

It is well established that application of haptens to UV-exposed skin not only inhibits the induction of CHS, but also induces T cells that inhibit sensitization upon i.v. injection into naive mice (6). The adoptive transfer of suppression is hapten specific because the recipients can be sensitized against other unrelated haptens without any problems (6). As these T cells mediate suppression upon adoptive transfer, they were initially designated suppressor T cells. These types of cells have been recently renamed regulatory

T cells, a term that has become more accepted than the original name (23, 24). UV-induced Tr appear to be responsible for the mediation of hapten-specific tolerance (7). However, Tr act only in a suppressive fashion when they are injected i.v. into naive, but not sensitized, mice. Based on this finding it was concluded that UV-induced Tr can only inhibit the afferent, not the efferent, phase of CHS. Furthermore, UV-induced Tr were suggested to be active only in the absence of hapten-specific T effector cells and thus to be inferior to T effector cells (7, 16).

We could confirm these data because lymph node cells obtained from mice tolerized for DNFB by UV, subsequently referred to as DNFB-specific, UV-induced Tr, inhibited the CHS response against DNFB when injected into naive recipients (Fig. 1A). In contrast, when the same number of cells was injected i.v. into recipients that had been sensitized against DNFB, the ear-swelling response against DNFB was not affected (Fig. 1B). Both the phe-
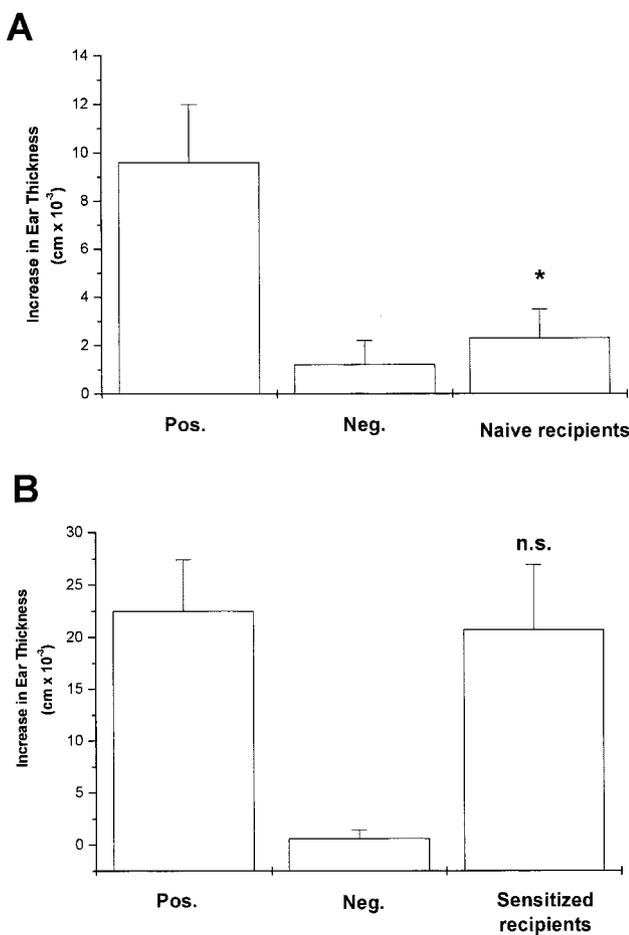

FIGURE 1. UV-induced Tr transfer suppression upon i.v. injection into naive, but not sensitized, recipients. A, Naive recipient mice were injected i.v. with 5×10^7 lymph node cells obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m² each time). Twenty-four hours after injection, mice were sensitized against DNFB, and 5 days later challenge with DNFB was performed on the left ear. Positive control mice were sensitized and challenged without transfer; negative control mice were ear-challenged only. Ear swelling was measured 24 h after challenge. The ear-swelling response is expressed as the difference (centimeters $\times 10^{-3}$, mean $\pm$ SD) between the thickness of the challenged ear compared with the thickness of the vehicle-treated ear. B, Cells obtained from UV-tolerized mice were injected i.v. into recipient mice that had been sensitized against DNFB 5 days before transfer. Twenty-four hours after transfer, ear challenge with DNFB was performed, and ear swelling was measured 24 h later. *, $p < 0.00001$.

notype and the mechanism of action of UV-induced Tr remain to be determined. However, there is evidence that Tr may act via the release of IL-10. This assumption is based on the observation that hapten-specific stimulation of Tr by APC results in the release of IL-10 (11, 14). In addition, adoptive transfer of CHS suppression upon i.v. injection of Tr can be prevented when the recipient mice are treated with a neutralizing Ab against IL-10 (14). Thus, IL-10 is able to inhibit the induction phase of CHS and to induce hapten-specific tolerance (17). IL-10 is also involved in the mediation of UV-induced immunosuppression because 1) UV stimulates the release of IL-10 (25); and 2) UV-induced immunosuppression can be reduced by neutralization of IL-10 (14, 26). In addition, several groups have shown that injection of IL-10 into sensitized mice inhibits the CHS response, indicating that IL-10 also has the ability to inhibit the effector phase of CHS (18, 19). Given that IL-10 is so potent in blocking both the afferent and the efferent phase of CHS and, secondly, that IL-10 is released by Tr upon Ag-specific stimulation, we were puzzled by the fact that UV-induced Tr inhibit only the induction, not the elicitation, of CHS upon injection into recipient mice. If IL-10 release is the or at least one of the crucial events by which UV-induced Tr exhibit their suppressive activity, we surmised that, in principle, they should be able to inhibit the effector phase of CHS as well and thus act in a suppressive way when injected into sensitized mice.

UV-induced Tr suppress the effector phase of CHS upon injection into the area of challenge

Primary sensitization takes place in the regional lymph nodes, whereas elicitation of CHS takes place in the area of challenge, i.e., in the ears in the CHS model. Therefore, we surmised that upon i.v. injection, Tr may primarily migrate into the regional lymph nodes, but not into the skin and ears, respectively. Due to the latter incapability, they should not be able to inhibit the effector phase in sensitized mice. Conversely, Tr should inhibit the effector phase when injected directly into the area of challenge. To address this issue, we obtained Tr from DNFB-tolerized mice and injected 1×10^6 cells (100 μl) into the ears of sensitized mice. Mere injection of the cells in this volume did not affect ear thickness when measured 3 h thereafter. However, the ear-swelling response in sensitized mice upon ear challenge was significantly reduced when Tr were injected i.c. into the ears 3 h before challenge (Fig. 2A). Inhibition of the effector phase of CHS was hapten specific, because injection of DNFB-specific Tr into the ears of mice that were sensitized against OXA did not affect the ear-swelling response evoked by the OXA challenge (Fig. 2B).

To address whether only the activation or also the suppressive activity of Tr is hapten specific, mice were sensitized against OXA, and 5 days later ear challenge with OXA was performed. As reported above (Fig. 2B), injection of DNFB-specific Tr did not affect ear swelling. However, if immediately after i.c. injection of DNFB-specific Tr, DNFB was applied to the ears, and 3 h later OXA was applied, the OXA-specific ear-swelling response was significantly reduced (Fig. 3A). In contrast, the application of OXA and DNFB on the same ear of OXA-sensitized mice without injecting UV-induced Tr did not impair the specific ear-swelling response to OXA (data not shown). This indicates that upon Ag-specific stimulation, DNFB-specific Tr can suppress an OXA-specific immune response, implying that the activation of UV-induced Tr, but not their suppressive activity, is Ag specific.

Because of the indications that UV-induced Tr may exert their suppressive activity via the release of IL-10, RNA was extracted from the ears, and semiquantitative PCR was performed. IL-10 transcripts were not detected in unchallenged ears (Fig. 3B). Likewise, no IL-10 was detected when DNFB-specific Tr were injected
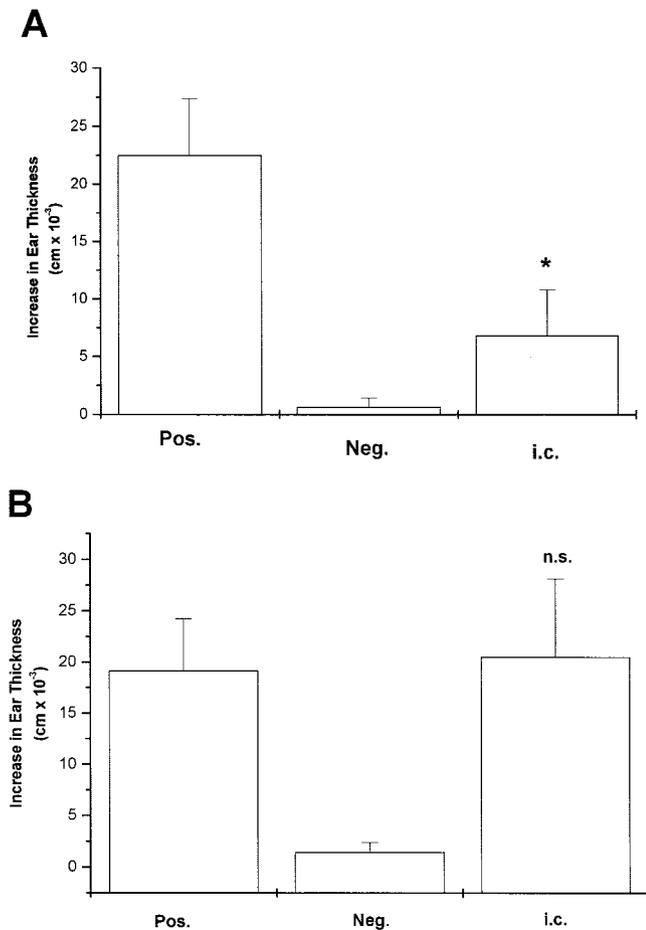
A

B

FIGURE 2. UV-induced Tr inhibit the elicitation of CHS upon i.c. injection into the area of challenge. *A*, Lymph node cells (1×10^6 in 100 μl) obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) were injected i.c. into the left ear of mice that had been sensitized against DNFB 5 days previously. Three hours after injection, challenge with DNFB was performed on the left ear. Positive control mice were sensitized and challenged with DNFB without injection; negative control mice were ear-challenged only. Ear swelling was measured 24 h after challenge. 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*, Lymph node cells (1×10^6 in 100 μl) obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) were injected i.c. into the left ear of mice that had been sensitized against OXA 5 days previously. Three hours after injection, challenge with OXA was performed on the left ear. Positive control mice were sensitized and challenged with OXA without injection; negative control mice were ear-challenged only. Ear swelling was measured 24 h after challenge. *, $p < 0.00001$.

A

B

FIGURE 3. Activation of Tr, but not their suppressive activity, is hapten specific. *A*, Lymph node cells (1×10^6 in 100 μl) obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) were injected i.c. into the left ear of mice that had been sensitized against OXA 5 days previously. Three hours after injection, challenge with DNFB (group 1) or with OXA (group 2) was performed on the left ear. In group 3, DNFB (0.3%) was applied on the left ear 3 h after injection. Three hours later, challenge with OXA was performed. Ear swelling was measured 24 h after challenge. 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. *, $p < 0.00005$. *B*, Mice that were sensitized against OXA were injected with DNFB-specific Tr i.c. into the ears and challenged as indicated. RNA was extracted from ears 18 h after challenge, and PCR was performed with primers for IL-10 and β-actin.

into ears that were subsequently challenged with OXA. In contrast, when DNFB was applied immediately after injection of DNFB-specific Tr into ears of OXA-sensitized mice, pronounced IL-10 expression was observed. An identical induction of IL-10 transcripts was observed when mice were, in addition, challenged with OXA (Fig. 3*B*). This implies that Tr may exert their suppressive activity via the release of IL-10 and thus is in accordance with previous observations (14). Furthermore, these findings indicate that the activation of Tr is Ag specific, but once activated the suppression that appears to be mediated via IL-10 is nonspecific. This phenomenon, called bystander suppression, has also been observed for other types of Tr (27).
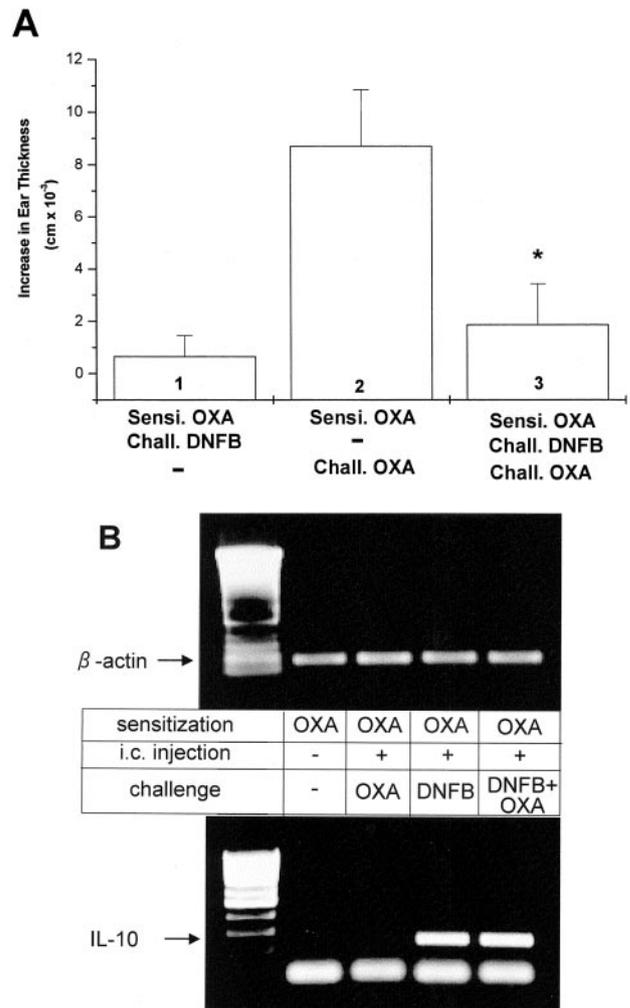
UV-induced Tr belong to the CD4$^+$CD25$^+$ subtype

Several subsets of Tr have been described in mouse and man (reviewed in Ref. 28). An important subtype of Tr expresses CD4 and CD25 and comprises 8–10% of the peripheral CD4$^+$ T cell subset in the mouse (29). In addition, these cells constitutively express the negative regulatory molecule CTLA-4 (30, 31). As there are recent indications that CD4$^+$CD25$^+$ T cells also may be involved in the mediation of UV-induced tolerance (13), we were interested to further characterize the phenotype of the UV-induced Tr that inhibit the effector phase of CHS. For that purpose, T cells were obtained from DNFB-tolerized mice and depleted of the CD25$^+$ subpopulation by magnetobead separation. Although injection of undepleted cells into the ears of DNFB-sensitized mice inhibited
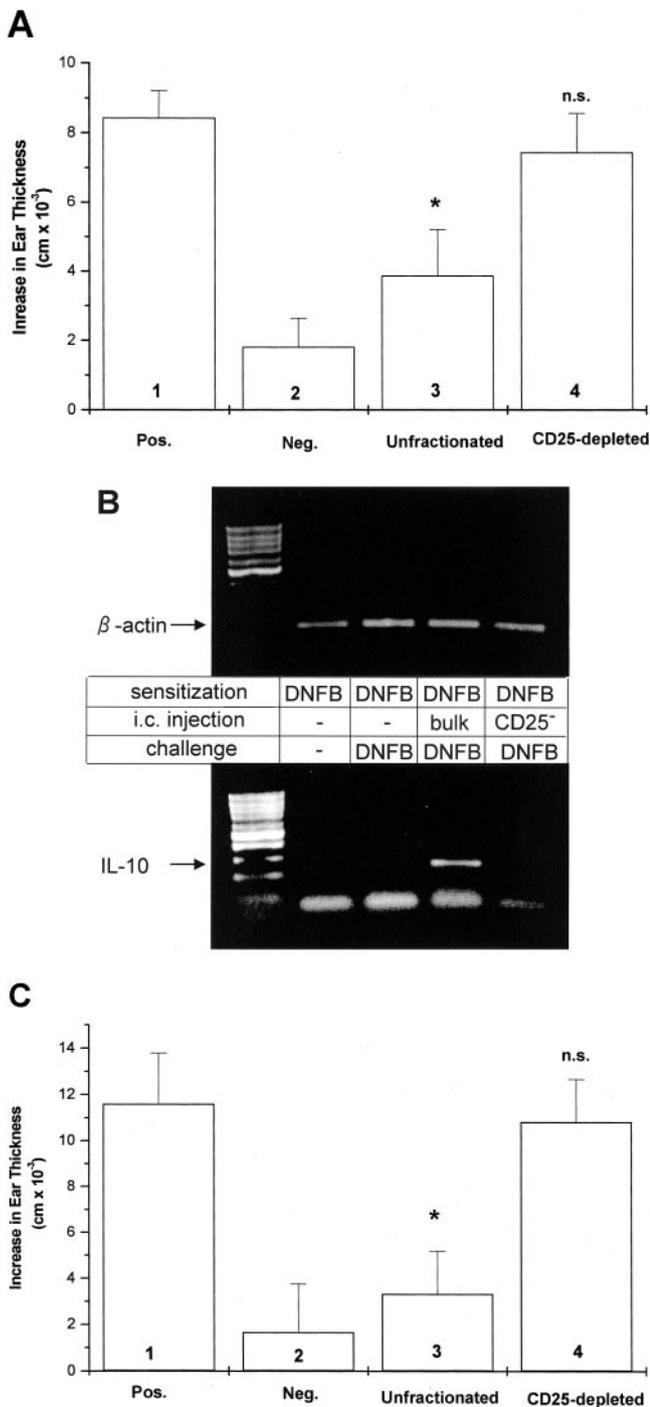
FIGURE 4. UV-induced Tr express CD25. *A*, Lymph node cells were obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) and depleted of CD25$^+$ cells by magnetobead separation. Unfractionated (group 3) or CD25-depleted (group 4) cells (1×10^6) were injected i.c. into the ears of mice that had been sensitized against DNFB 5 days previously (groups 1, 3, and 4). Three hours after injection, all groups were challenged on the left ear with DNFB. *B*, Mice were treated identically as in *A*. RNA was extracted from ears 18 h after challenge, and PCR was performed with primers for IL-10 and β-actin. *C*, Mice were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later (groups 1, 3, and 4). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 5×10^7 lymph node cells obtained from UV-tolerized donors. Group 3 received unfractionated cells, whereas group 4 received cells that had been depleted of CD25$^+$ cells by magnetobead separation. *, $p < 0.00005$.

the ear challenge, no suppression of the CHS response was observed upon injection of CD25-depleted T cells (Fig. 4*A*). Accordingly, induction of the expression of IL-10 was lost upon depletion of CD25$^+$ T cells (Fig. 4*B*). Depletion of the CD25$^+$ fraction from T cells obtained from DNFB-tolerized mice was also associated with a loss of the capacity to inhibit the induction of CHS when injected i.v. into naive mice (Fig. 4*C*), supporting the assumption that the same fraction of Tr is responsible for inhibition of the induction and elicitation of CHS. Likewise, inhibition of the elicitation of CHS was not observed when T cells obtained from DNFB-tolerized mice were depleted of CD4 (Fig. 5*A*). Accordingly, induction of the expression of IL-10 was lost upon depletion of CD4$^+$ T cells (Fig. 5*B*). This supports the idea that, as previously reported (13), UV-induced Tr belong to the CD4$^+$CD25$^+$ subtype, and the local release of IL-10 may be involved in suppression of the challenge upon i.c. injection of Tr.
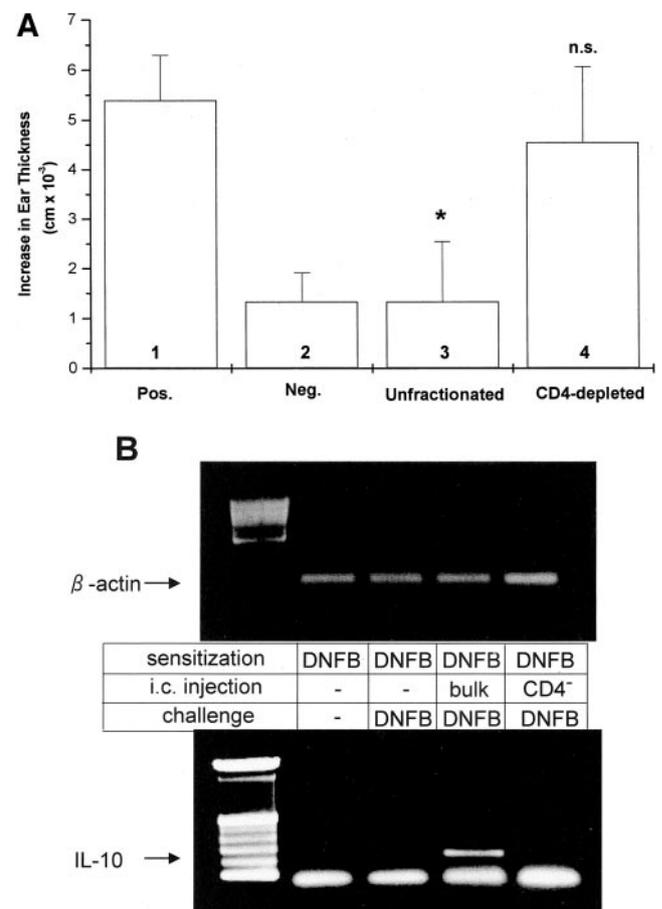

FIGURE 5. UV-induced Tr express CD4. *A*, Lymph node cells were obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) and depleted of CD4$^+$ cells by magnetobead separation. Unfractionated (group 3) or CD4-depleted (group 4) cells (1×10^6) were injected i.c. into the ears of mice that had been sensitized against DNFB 5 days previously (groups 1, 3, and 4). Three hours after injection, all groups were challenged on the left ear with DNFB. *B*, Mice were treated identically as in *A*. RNA was extracted from ears 18 h after challenge, and PCR performed with primers for IL-10 and β-actin. *C*, Mice were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later (groups 1, 3, and 4). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 5×10^7 lymph node cells obtained from UV-tolerized donors. Group 3 received unfractionated cells, whereas group 4 received cells that had been depleted of CD4$^+$ cells by magnetobead separation. *, $p < 0.0005$.

To prove that the induction of IL-10 expression demonstrated by PCR (Figs. 4B and 5B) is functionally relevant, Ab blocking experiments were performed. DNFB-specific Tr were obtained from UV-tolerized mice, and the CD4$^+$CD25$^+$ T cell fraction was isolated by magnetobead separation using a commercially available CD4$^+$CD25$^+$ regulatory T cell isolation kit. Intracutaneous injection of these cells into the ears of DNFB-sensitized mice significantly suppressed the ear-swelling response upon challenge (Fig. 6). In contrast, when mice were injected with a neutralizing anti-IL-10 Ab immediately after cell transfer, they revealed a pronounced ear-swelling response after challenge. In contrast, injection of rat IgG as an isotype control did not prevent suppression by Tr. This indicates that the suppressive activity of Tr is mediated via IL-10.

UV-induced Tr express the lymph node-homing receptor CD62L

The fact that UV-induced Tr only inhibit the induction, but not the elicitation, of CHS upon i.v. adoptive transfer, but suppress the elicitation of CHS when injected i.c. into the area of challenge, implies that these differences in the activities of Tr may be related to migration of the cells into different areas. Intravenous injection of T cells from hapten-sensitized donors confers the capacity to express an ear-swelling response upon challenge in the recipients (6). In addition, it has been demonstrated that the T cells responsible for transferring sensitization migrate into the skin due to the expression of the skin-homing receptor cutaneous lymphocyte-associated Ag, which is an inducible carbohydrate modification of the P-selectin glycoprotein ligand-1 (32, 33). Furthermore, T cells responding to an Ag down-regulate the lymph node-homing receptor L-selectin (CD62L) as they develop into activated effector cells. This is accompanied by an up-regulation of the proinflammatory adhesion molecules LFA-1, CD44, and VLA-4, which implies that after their release into the circulation, they traffic to sites of Ag deposition and inflammation (34, 35). As i.v. injection of UV-induced Tr inhibits the sensitization that takes place in the lymph nodes, but not the elicitation phase of CHS in the ears, we surmised that Tr upon i.v. injection may migrate primarily into the lymph nodes.
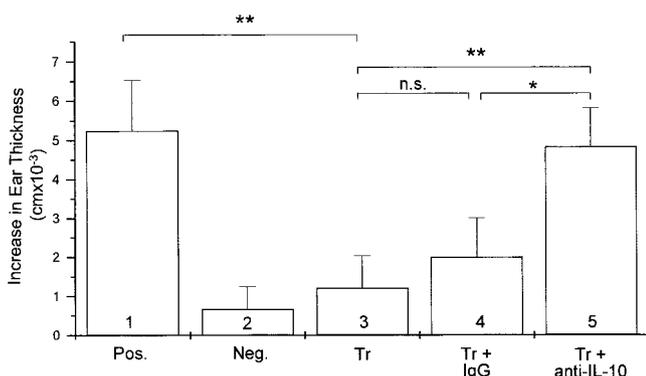
Therefore, we studied whether UV-induced Tr express the lymph node-homing receptor L-selectin (CD62L). When T cells obtained from DNFB-tolerized mice via UV were depleted of the CD62L$^+$ fraction, i.v. injection into naive mice did not inhibit the induction of CHS (Fig. 7A). This indicates that UV-induced Tr express CD62L. In addition, we have evidence that the expression of CD62L on UV-induced Tr is of functional relevance by blocking studies using MEL14, an Ab that inhibits CD62L. When UV-induced Tr were injected into naive mice, sensitization was significantly suppressed (7.2 ± 1.2 cm × 10^{-3} ear swelling) compared with positive control mice, which showed a pronounced ear-swelling response after sensitization (15.2 ± 2.1). Incubation of Tr with MEL14 Ab before injection was associated with a loss of transfer of suppression, because recipients of MEL14-treated Tr revealed a normal CHS response (14.3 ± 2.1). This implies that UV-induced Tr indeed require the expression of the lymph node-homing receptor CD62L to exert their suppressive activity.
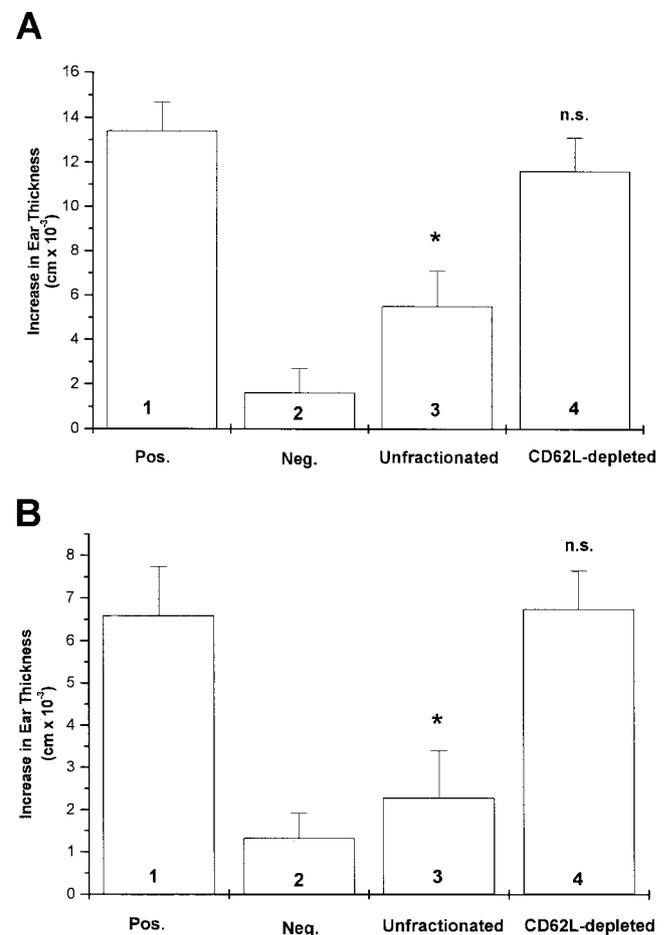

FIGURE 7. UV-induced Tr express CD62L. A, Lymph node cells were obtained from donors that were tolerized against DNFB by application of DNFB to UV-exposed skin (four times, 1000 J/m^2 each time) and depleted of CD62L$^+$ cells by magnetobead separation. Mice were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later (groups 1, 3, and 4). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 5 × 10^7 lymph node cells obtained from UV-tolerized donors. Group 3 received unfractionated cells, whereas group 4 received cells that had been depleted of CD62L$^+$ cells by magnetobead separation. B, Unfractionated (group 3) or CD62L-depleted (group 4) cells (1 × 10^6) were injected i.c. into the ears of mice that had been sensitized against DNFB 5 days previously (groups 1, 3, and 4). Three hours after injection, all groups were challenged on the left ear with DNFB. *, p < 0.0001.

FIGURE 6. Neutralization of IL-10 reverses the suppressive effect of i.c. injected Tr. T cells were obtained from mice that were tolerized against DNFB by application of DNFB to UV-exposed skin. CD4$^+$CD25$^+$ T cells were obtained by magnetobead separation using a CD4$^+$CD25$^+$ Regulatory T Cell Isolation kit (Miltenyi Biotec). Cells (8 × 10^5) were injected i.c. into the ears of mice that had been sensitized against DNFB 5 days previously (groups 3–5). Immediately after cell transfer, ears were injected with a neutralizing anti-IL-10 Ab (100 μg; group 5) or rat IgG (100 μg) as an isotype control (group 4). Three hours after injection, all groups were challenged with DNFB. Positive control mice were sensitized and challenged (group 1); negative mice were challenged only (group 2). Ear swelling was measured 24 h later. *, p < 0.05; **, p < 0.0005.

Likewise, i.c. injection of CD62L-depleted Tr into the ears of DNFB-sensitized mice did not inhibit the challenge with DNFB, although the ear-swelling response was significantly reduced when undepleted Tr were injected i.c. (Fig. 7B). This confirms the above-mentioned assumption (see Fig. 4) that the same fraction of Tr is responsible for inhibition of the induction and elicitation of CHS. Furthermore, the data indicate that UV-induced Tr express CD62L and thus may preferentially migrate into the lymph nodes upon i.v. injection.

It has recently been shown that $CD4^+$ T cells migrate into inflamed skin only if they express ligands for E- and P-selectin (36, 37). In addition, it was demonstrated that L-selectin does not contribute to the effector phase of CHS responses (37), and that T cells responding to an Ag down-regulate L-selectin (35). Therefore, we anticipated that UV-induced Tr do not express the ligands for E- and P-selectin. Triple FACS analysis (Fig. 8) indicated that $CD4^+CD62L^+$ T cells obtained from UV-tolerized mice do not bind E- and P-selectin chimeras, indicating that, in fact, these cells do not express the ligands for the skin-homing receptors E- and P-selectin.
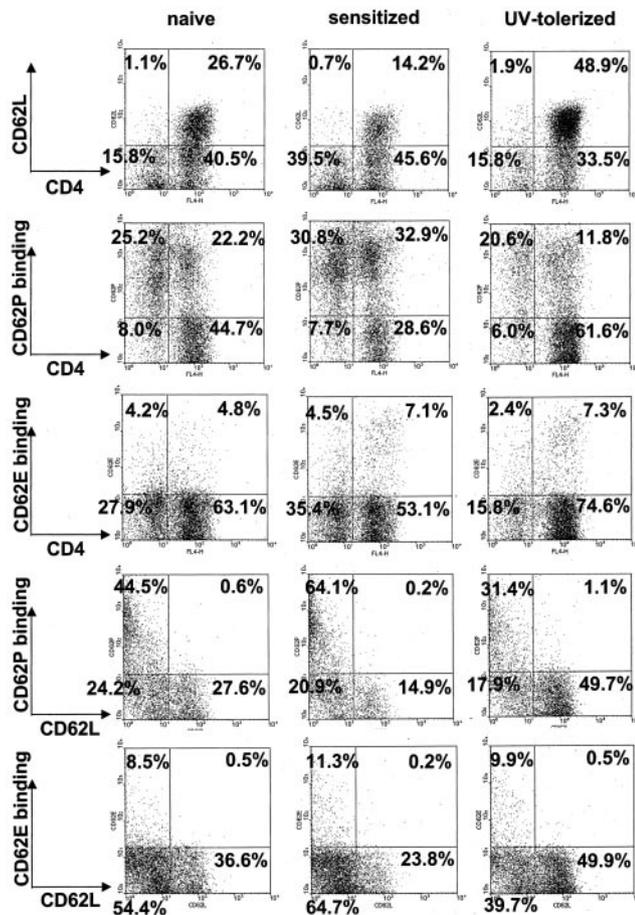
Conclusion

Since the discovery of UV-induced suppressor/regulatory T cells more than two decades ago it was accepted that these cells only inhibit the induction but not the elicitation phase of CHS and thus are inferior to T effector cells (7). The present study demonstrates that the failure to inhibit the efferent limb of CHS is due to the fact that UV-induced Tr may not migrate into skin but probably into the lymph nodes because they express CD62L but not the ligands for the skin homing receptors E- and P-selectin. Accordingly, i.c. injection of Tr into the ears of sensitized mice inhibits the challenge. This inhibition is Ag-specific, however, the specificity applies only for the activation of the Tr. If once activated in a hapten-specific way they inhibit CHS responses in a non-specific fashion. Tr appear to exert this activity via the release of IL-10 and thus via a mechanism of bystander suppression. Because of the capacity of bystander suppression, speculations exist about the therapeutic potential of Tr which could be generated in response to Ag known to be present in the target organ that are not necessarily the precise Ag that drives the pathogenic response (27). However, the present study shows that this strategy will only be successful if the Tr home to the target organ which does not apply at least for UV-induced Tr.

Acknowledgments

FIGURE 8. UV-induced Tr do not express ligands for E- and P-selectin. Cells obtained from regional lymph nodes and spleens of naive, sensitized, or UV-tolerized mice were incubated with microbeads coated with an Ab against CD4, followed by positive selection with the AutoMACS magnetic separation system. $CD4^+$ subpopulations were incubated with a P- or E-selectin-IgG chimera. Three-color staining was conducted with an allophycocyanin-conjugated Ab against CD4, FITC-conjugated Ab against CD62L, and PE-labeled F(ab')$_2$ donkey anti-human IgG. Analysis was performed with a FACSCalibur.

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