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Evidence for Functional Relevance of CTLA-4 in Ultraviolet-Radiation-Induced Tolerance[1]

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Hapten sensitization through UV-exposed skin induces hapten-specific tolerance that can be adoptively transferred by injecting T lymphocytes into naive recipients. The exact phenotype of T cells responsible for inhibiting the immune response and their mode of action remain unclear. Evidence exists that CTLA-4 negatively regulates T cell activation. We addressed whether CTLA-4 is involved in the transfer of UV-induced tolerance. Injection of lymph node cells from mice that were sensitized with dinitrofluorobenzene (DNFB) through UV-irradiated skin inhibited induction of contact hypersensitivity against DNFB in the recipient animals. When CTLA-4$^+$ cells were depleted, transfer of suppression was lost. Likewise, significantly fewer lymphocytes enriched for CTLA-4$^+$ cells were necessary to transfer suppression than unfractionated cells. Expression of CTLA-4 appears to be functionally relevant, since in vivo injection of a blocking anti-CTLA-4 Ab was able to break UV-induced tolerance and inhibited transfer of suppression. Upon stimulation with dendritic cells in the presence of the water-soluble DNFB analogue, DNBS, CTLA-4$^+$ T cells from DNFB-tolerized mice secreted high levels of IL-10, TGF-β, and IFN-γ; low levels of IL-2; and no IL-4, resembling the cytokine pattern of T regulatory 1 cells. Ab blocking of CTLA-4 resulted in inhibition of IL-10 release. Accordingly, transfer of tolerance was not observed when recipients were treated with an anti-IL-10 Ab. Hence we propose that T cells, possibly of the T regulatory 1 type, transfer UV-mediated suppression through the release of IL-10. Activation of CTLA-4 appears to be important in this process. *The Journal of Immunology,* **2000, 165: 1824–1831.**

Ultraviolet radiation can be regarded as one of the most significant environmental factors affecting humans. Its health hazardous effects include exacerbation of infectious diseases, skin cancer, and premature skin aging (1–3). Several of these are mediated by the immunosuppressive properties of UV, which are best demonstrated by the inhibition of cellular immune reactions, such as contact hypersensitivity (CHS)[3] (4, 5). Accordingly, cutaneous exposure to UV impairs sensitization to haptens applied directly to the UV-irradiated skin area (4, 5). In addition to the failure to generate hapten sensitization, tolerance develops, since individuals treated in this way cannot be resensitized against the same hapten at a later time point (4, 5). UV-induced tolerance is hapten specific, as the sensitization against another nonrelated hapten is not affected (4). Hapten-specific unresponsiveness can be adoptively transferred; injection of lymph node cells and splenocytes from UV-tolerized mice into syngeneic naive mice inhibits sensitization against the relevant hapten in the recipients (6) (see scheme in Fig. 1). Consequently, it has been suggested that UV-induced tolerance is mediated by hapten-specific T suppressor cells (reviewed in Ref. 7). Despite the clear transfer experiments, this concept is controversial, mainly because the cells transferring hapten-specific unresponsiveness are still poorly characterized, and their mode of action is unclear in major parts (7).

CTLA-4 (CD152) is an important T cell regulatory molecule that is expressed on the surface of activated T cells (reviewed in Ref. 8). Like CD28, CTLA-4 exhibits the ability to ligate the B7 family molecules B7-1 (CD80) and B7-2 (CD86). However, unlike CD28, cross-linking of CTLA-4 down-regulates IL-2 production and cell cycle progression, whereas blockade of CTLA-4 signaling prolongs T cell activation. Hence, CTLA-4 is regarded as a negative regulatory T cell-associated molecule (9, 10). Furthermore, administration of anti-CTLA-4 Ab to mice during the initial response to tolerogenic Ag protected Ag-specific T cells from becoming anergic and promoted their differentiation into effector cells (11, 12). Taken together, CTLA-4 appears to be an important player in the regulation of immune responses and might also play a role in tolerance induction.

In the present study we addressed whether CTLA-4 is involved in UV-induced tolerance. Here, we show that T cells transferring hapten-specific UV-mediated tolerance express CTLA-4 on their surface. However, CTLA-4 not only appears to be a marker for T cells mediating this effect, but also seems to be functionally relevant, since in vivo injection of an Ab that blocks CTLA-4 resulted in the loss of tolerance and transfer of suppression. Upon in vitro expansion, CTLA-4$^+$ T cells transferring suppression secrete high levels of IL-10, TGF-β, and IFN-γ and low levels of IL-2 but no IL-4, resembling a T regulatory 1 (Tr1)-like cytokine pattern. Transfer of tolerance was inhibited when recipients were treated with an anti-IL-10 Ab. Thus, we propose that T cells, probably of the Tr1 type, transfer UV-mediated suppression through the release of IL-10. Activation of CTLA-4 appears to play an important role in this process.

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[3] Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; DNBS, 2,4-dinitrobenzenesulfonic sodium salt; Tr, regulatory T cells.

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Materials and Methods

Animals

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.

Abs and reagents

Hamster anti-murine Ab to CTLA-4 (clone UC10-4F10) was produced as described previously (13). In vivo neutralization of IL-10 was performed with a rat anti-IL-10 mAb (clone JES5-2A5; BioSource, Ratingen, Germany). IL-10, IFN-γ, and TGF-β in the supernatant were determined by ELISA (R&D Systems, Wiesbaden, Germany). The IL-2-dependent cell line CTLL-2 was used for the detection of IL-2, while the IL-4-dependent cell line CT.4S was used for detection of IL-4.

Contact hypersensitivity

Mice were sensitized by painting 25 μl of 2,4,-dinitrofluorobenzene (DNFB; Sigma, St. Louis, MO) solution (0.5% in acetone/olive oil, 4/1) on the shaved back on day 0 as previously described (14). On day 5, 20 μl of 0.3% DNFB was applied to the left ear; the vehicle acetone/olive oil was applied to the right ear. Ear swelling was quantitated with a spring-loaded micrometer (Mitutoyo, Kawasaki, Kanagawa, 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 as centimeters $\times 10^{-3}$ (mean $\pm$ SD). The ear swelling response was measured in a blinded fashion. Mice that were ear-challenged without prior sensitization served as negative controls. Resensitization was performed as described above through shaved abdominal skin 14 days after the first challenge. The second challenge was performed on the right ear 5 days after the second sensitization. Each group consisted of at least seven mice. Each experiment was performed at least twice.

UV irradiation

The shaved back was exposed to UV radiation from a bank of four TL12 fluorescent lamps (Philips, Eindhoven, The Netherlands), which 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/m^2/exposure). 24 h after the last UV exposure DNFB was applied carefully to the surface of the irradiated area as described above. As reported previously, the UV regimen applied does not cause generalized systemic immunosuppression, but induces hapten-specific tolerance (14, 15).

Adoptive transfer of immune response

Donor mice were treated as indicated, spleens and regional lymph nodes were removed thereafter, and single-cell suspensions were prepared. The cell number was adjusted (see individual experiments), and 200 μl was injected i.v. into each recipient mouse. Recipients were sensitized 24 h later by epicutaneous application of DNFB on the shaved abdomen. After 5 days, mice were challenged on the left ear, and ear swelling was evaluated 24 h later.

Depletion of subpopulations

For in vitro depletion of CTLA-4$^+$ subpopulations, lymphocytes obtained from regional lymph nodes and spleens were incubated with magnetobeads (Dynabeads M-450 tosylactivated, Dynal, Oslo, Norway) that were coated with a purified hamster mAb directed against murine CTLA-4 (UC10-4F10-11) according to general protocols (Cell Separation and Protein Purification, Technical Handbook, Dynal). After incubation for 2 h at 4°C, magneto-separation was performed by placing the tubes into a magnet field (Dynal) for 4 min. The supernatant containing the CTLA-4$^-$ cells was removed. Cells were washed and adjusted to 5×10^8 cells/ml for i.v. injection. CTLA-4$^+$ cells bound to the magnetobeads were detached by incubating cells overnight in cell culture medium at 37°C in a humidified atmosphere containing 5% CO_2. Magnetobeads that had spontaneously detached from the cells after overnight incubation were removed with a magnet. Remaining (CTLA-4$^+$) cells were harvested, washed, and adjusted for i.v. injection. The efficacy of separation was determined by flow cytometry (EPICS XL, Coulter, Miami, FL).

Cytokine induction in vitro

T lymphocytes were prepared from mice that were tolerized by applying DNFB onto UV-irradiated skin and separated into CTLA-4$^-$ and CTLA-4$^+$ fractions by magnetobead separation. T cells (5×10^6/ml) were incubated with dendritic cells (DC; 1×10^6/ml) that were isolated from bone marrow of syngeneic naive mice as described previously (14). Co-incubations were performed in the absence or the presence of the water-soluble DNFB analogue 2,4-dinitrobenzenesulfonic sodium salt (0.1 mM DNBS). Supernatants were tested for IL-2, IL-4, IL-10, IFN-γ, and TGF-β as described above.

Statistical analysis

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

Results

Transfer of UV-induced suppression is lost upon depletion of CTLA-4$^+$ T cells

To determine whether T lymphocytes transferring UV-mediated suppression of CHS express CTLA-4, adoptive transfer experiments were performed. C3H/HeN mice were sensitized by topical application of DNFB onto UV-exposed skin. Since this procedure results in hapten-specific tolerance (14), animals treated in this way are subsequently referred to as UV tolerized. Ten days after hapten application, spleen and regional lymph node cells were obtained for i.v. injection into naive syngeneic mice (Fig. 1). Before transfer, cells were depleted from CTLA-4$^+$ cells by magnetobead separation. Unfractionated cells served as a control. Depletion was efficient, as confirmed by FACS analysis (Fig. 2). Unfractionated cells contained a CTLA-4$^+$ and a CTLA-4$^-$ subpopulation (Fig. 2*A*); the CTLA-4$^+$ population was lost after CTLA-4 depletion (Fig. 2*B*). For transfer of UV-induced immunosuppression, 1×10^8 cells of the unseparated or the CTLA-4-depleted fraction were injected i.v. into naive mice. Recipients were sensitized with DNFB 24 h after transfer, their ears were challenged 5 days later, and ear swelling was measured 24 h thereafter. As previously reported (16), the CHS response in mice receiving unfractionated cells from UV-tolerized mice was remarkably suppressed (Fig. 3*A*, group 3) compared with that in mice that were just sensitized and challenged (Fig. 3*A*, group 1). In contrast, transfer of CTLA-4 depleted cells did not inhibit subsequent sensitization (Fig. 3*A*, group 4). Sham depletion control, i.e., incubation of lymphocytes with magnetobeads not coated with the anti-CTLA-4 Ab, did not

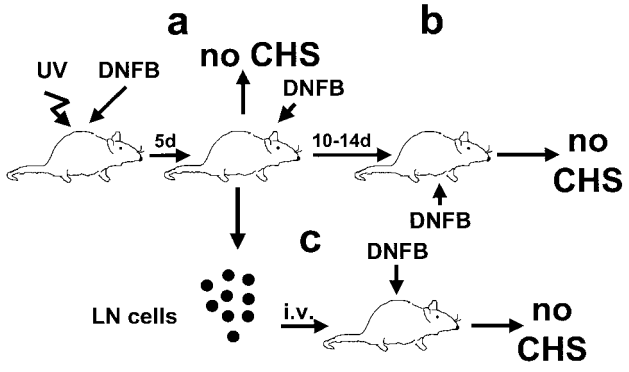

FIGURE 1. Induction of hapten-specific, transferable tolerance by UV radiation. Mice are exposed to UV radiation (four doses of 1000 J/m^2) on the shaved back skin. Twenty-four hours after the last exposure hapten (0.5% DNFB) is applied to the irradiated skin. Ear challenge performed 5 days later with 0.3% DNFB does not cause a swelling response, indicating that UV radiation inhibits induction of contact hypersensitivity (CHS; *a*). Resensitization of the same animals by application of DNFB onto abdominal skin also fails to induce a CHS response against DNFB, indicating that hapten-specific tolerance has developed (*b*). The i.v. injection of lymph node cells obtained from UV-irradiated and DNFB-treated mice 5–14 days after treatment into naive syngeneic mice inhibits sensitization of the recipients against DNFB, indicating that hapten-specific suppression is transferable (*c*).

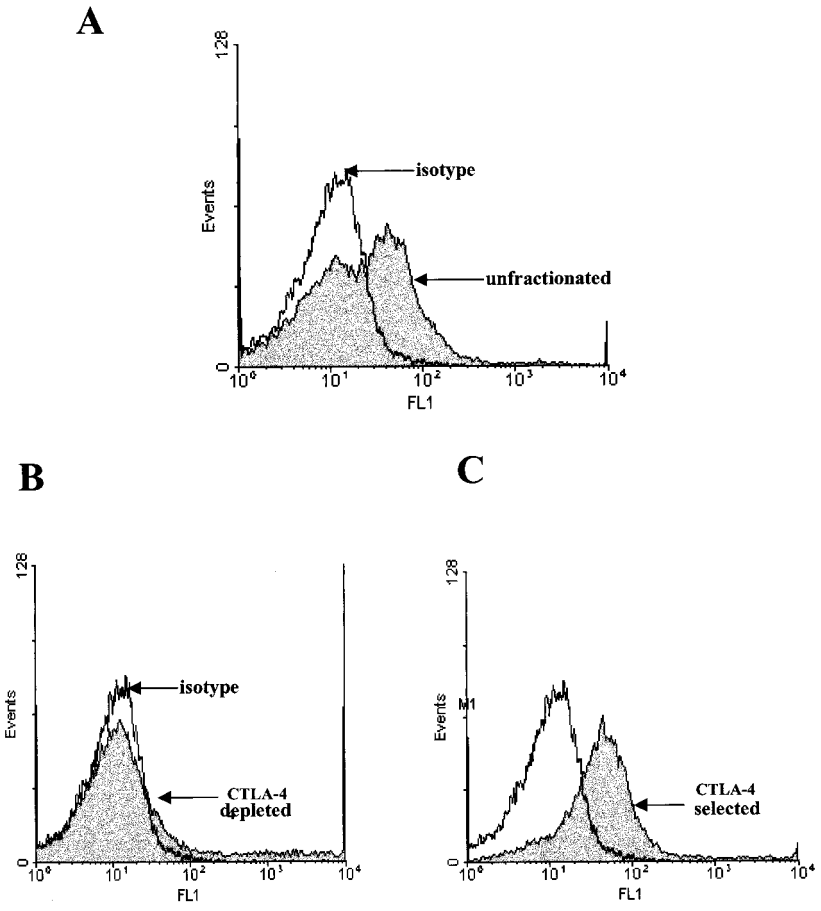

FIGURE 2. Efficacy of depletion and enrichment of CTLA-4$^+$ T cells by magnetobead separation. Lymph node cells and splenocytes from UV-tolerized mice were either depleted of (*B*) or enriched for (*C*) CTLA-4$^+$ cells. The efficacy of depletion or enrichment was evaluated by FACS analysis using a hamster anti-CTLA-4 mAb. Histograms show fluorescence intensity (*x*-axis) vs cell number (*y*-axis).

affect the transfer of suppression (data not shown). Thus, T cells mediating tolerance in UV-induced immunosuppression appear to express CTLA-4 on their surface.

CTLA-4$^+$ cells obtained from UV-tolerized mice transfer suppression

To further confirm that T cells transferring suppression reside in the CTLA-4$^+$ fraction, spleen and regional lymph node cells from UV-tolerized mice were obtained and subjected to CTLA-4 magnetobead separation as described above. This time, however, CTLA-4$^+$ cells were collected from the beads and used for transfer. Successful positive selection was determined by FACS analysis (Fig. 2*C*). Cells (5×10^5) injected i.v. significantly inhibited sensitization of the recipients against DNFB (Fig. 3*B*, group 3). Note that when using unfractionated cells, 1×10^8 cells were necessary to transfer suppression at comparable levels (Fig. 3*A*). The fact that a much lower number of CTLA-4$^+$ cells suffices to mediate complete suppression indicates that the relevant cells reside in the CTLA-4$^+$ population. With decreasing the number of CTLA-4$^+$ cells, the inhibitory effect was gradually reduced (data not shown).

Cytokine secretion pattern

The data obtained to date suggest that CTLA-4 can be used as a marker for T cells transferring unresponsiveness in the model of UV-induced tolerance. Thus, we tried to expand these T cells in vitro. Lymph node cells and splenocytes were obtained from UV-tolerized mice and separated into CTLA-4$^+$ and CTLA-4$^-$ cells. Cells were put into culture with bone marrow-derived DC. Cells were cultured for 72 h in the absence or the presence of the water-soluble DNFB analogue DNBS. Supernatants were harvested and tested for the amounts of IL-2, IL-4, IL-10, IFN-γ, and TGF-β (Table I). IL-4 was detectable in none of the samples. Incubation of CTLA-4$^+$ cells with DC and DNBS did not alter the low constitutive IL-2 secretion, but remarkably enhanced the secretion of IL-10, IFN-γ, and TGF-β. Stimulation of the CTLA-4$^-$ cell fraction with DC and DNBS caused a similar increase in IFN-γ release, while IL-10 and TGF-β were only moderately induced. The cytokine secretion pattern observed in CTLA-4$^+$ cells (high release of IL-10, IFN-γ, and TGF-β; no release of IL-4; and moderate levels of IL-2) is similar to that of the recently described Tr1 cells (17, 18). This cytokine secretion pattern was even more pronounced, especially excessive IL-10 release, when cells were stimulated and cultured for 11 days (Table II).

UV-induced tolerance is inhibited by blocking CTLA-4

Since CTLA-4 can serve as a marker for T cells that mediate UV-induced tolerance, we next asked whether CTLA-4 is of functional relevance for the development of tolerance to haptens applied through UV-irradiated skin. To address this issue, mice were sensitized through UV-exposed back skin and challenged on the left ear 5 days later. After 14 days, mice were resensitized through the nonirradiated shaved abdominal skin and challenged on the right ear 5 days later. One group of animals (Fig. 4, group 4) was injected i.p. 3 h before resensitization with an Ab that blocks CTLA-4. Mice that were initially sensitized through UV-exposed skin did not show an ear swelling response after resensitization with the same hapten through non-UV-exposed skin, indicating that hapten-specific tolerance was induced (Fig. 4, group 3). In contrast, mice that received a single dose of the blocking anti-

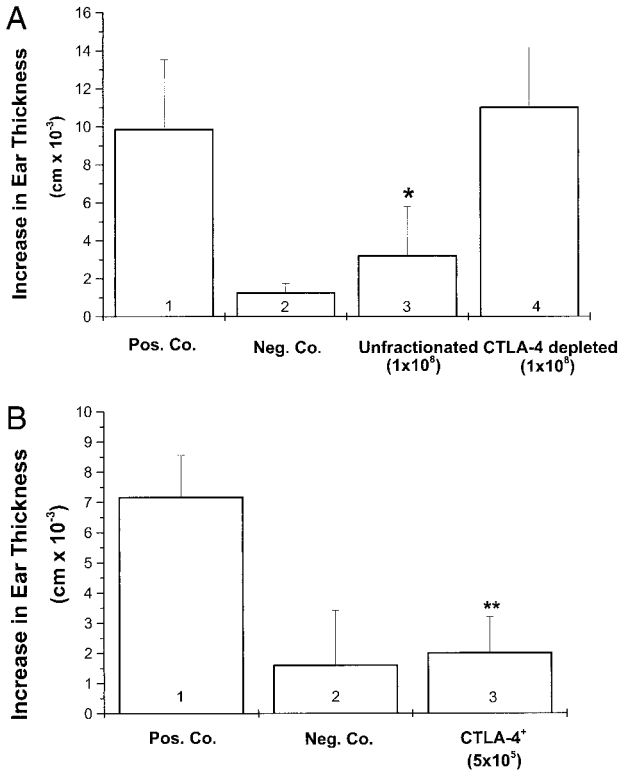

FIGURE 3. CTLA-4$^+$ cells obtained from UV-tolerized mice transfer suppression. *A*, Mice were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later (groups 1, 3, and 4). Negative control mice were ear challenged only (group 2). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 1×10^8 spleen and lymph node cells obtained from syngeneic animals that had been sensitized against DNFB through UV-exposed skin 10 days earlier. Group 3 received unfractionated cells, while group 4 received cells that had been depleted of CTLA-4$^+$ cells by magnetobead separation. *B*, Mice were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later (groups 1 and 3). Negative control mice were ear challenged only (group 2). After enrichment for CTLA-4$^+$ cells by magnetobead separation, 5×10^5 CTLA-4$^+$ cells were injected i.v. into naive recipients 24 h before sensitization (group 3). 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. *, $p < 0.01$; **, $p < 0.0005$ (vs positive control).

CTLA-4 Ab before resensitization (group 4) revealed a pronounced ear swelling response comparable to that of positive control mice that were sensitized and challenged but not UV irradiated (group 1).

Blockade of CTLA-4 in the recipients inhibits transfer of suppression

Next we studied whether transfer of suppression is inhibited when CTLA-4 is functionally blocked in the recipient animals. Lymph node cells from UV-tolerized mice were injected into recipient animals that were immediately sensitized against DNFB. Three hours before adoptive cell transfer and 24 h after sensitization, one group of animals (Fig. 5, group 4) was injected i.p. with an anti-CTLA-4 Ab (100 μg). The CHS response was measured 5 days later. Compared with the positive control (group 1), injection of cells from UV-irradiated and DNFB-treated mice (group 3) suppressed the generation of CHS in the recipient animals (Fig. 5). The suppressive activity on the CHS response by the adoptive cell transfer was completely inhibited when the recipient mice were injected with anti-CTLA-4 Ab (group 4). Application of an isotype control had no effect (data not shown). Together, these findings and the data presented in Fig. 4 indicate that CTLA-4 is of functional relevance in mediating UV-induced tolerance and in transferring hapten-specific suppression.

Blockade of CTLA-4 suppresses IL-10 release in vitro

IL-10 has been implicated to be involved in mediating UV-induced tolerance (reviewed in Ref. 19). Since 1) transfer of tolerance is critically dependent on CTLA-4$^+$ T cells, which, as demonstrated in Table I, upon in vitro stimulation secrete high levels of IL-10; and 2) CTLA-4 blockade inhibits UV-induced tolerance in vivo (Fig. 4), we studied whether blockade of CTLA-4 suppresses IL-10 secretion. Therefore, the neutralizing anti-CTLA-4 Ab was added to CTLA-4$^+$ lymphocytes from UV-tolerized mice that were propagated in the presence of DC and DNBS for 11 days. The CTLA-4 Ab only marginally affected the secretion of IL-2 and IFN-γ, but significantly reduced enhanced IL-10 release (Table II).

Neutralization of IL-10 inhibits transfer of tolerance

The data obtained to date suggest that 1) CTLA-4 signaling is crucial for mediation and transfer of tolerance; and 2) blockade of CTLA-4 impairs regulatory T cells in their ability to release IL-10. These observations imply that transfer of tolerance is critically dependent on IL-10. Hence, we determined whether neutralization of IL-10 in the recipients is associated with a loss of hapten-specific unresponsiveness. Lymph node cells from UV-tolerized mice were injected into recipient animals that were immediately sensitized against DNFB. Three hours before adoptive cell transfer and 24 h after sensitization, one group of animals was injected i.p. with an anti-IL-10 Ab (250 μg for each injection). CHS response was measured 5 days later. Compared with the positive control (Fig. 6, group 1), injection of cells from UV-irradiated and DNFB-treated mice suppressed generation of CHS in the recipient animals (Fig. 6, group 3). The suppressive activity on the CHS response by the

Table I. *Cytokine release by CTLA4$^+$ and CTLA4$^-$ lymph node cells obtained from UV-tolerized mice*[a]

	IL-2 (U/ml)	IFN-γ (pg/ml)	IL-4 (U/ml)	IL-10 (pg/ml)	TGFβ (pg/ml)
CTLA-4$^+$					
DC	0.3 $\pm$ 0.02	90.8 $\pm$ 1.5	ND	90.0 $\pm$ 3.6	78.3 $\pm$ 5.4
DC + DNBS	0.5 $\pm$ 0.01	1450.9 $\pm$ 5.5	ND	319.0 $\pm$ 1.1	739.3 $\pm$ 29
CTLA-4$^-$					
DC	2.4 $\pm$ 0.5	120.1 $\pm$ 3.5	ND	39.0 $\pm$ 1.5	98.5 $\pm$ 17.7
DC + DNBS	5.4 $\pm$ 0.8	1300.5 $\pm$ 6.5	ND	73.9 $\pm$ 2.0	168.8 $\pm$ 18.2

[a] Lymph node cells from UV-tolerized mice were separated into CTLA4$^+$ and CTLA4$^-$ fractions that (5×10^6/ml) subsequently were coincubated with DC (1×10^6/ml) in the absence of presence of DNBS (0.1 mM). Seventy-two hours later, supernatants were tested for the amounts of IL-2, IFN-γ, IL-4, IL-10, and TGFβ.

Table II. *Blockade of CTLA-4 inhibits IL-10 release by CTLA-4$^+$ lymph node cells obtained from UV-tolerized mice*[a]

	IL-2 (U/ml)	IFN-γ (pg/ml)	IL-4 (U/ml)	IL-10 (pg/ml)
DC	0.78 ± 0.01	390 ± 8.9	ND	30 ± 4.5
DC + DNBS	2.7 ± 0.04	900 ± 5.5	ND	845 ± 17
DC + DNBS + anti-CTLA-4 Ab	1.7 ± 0.02	855 ± 17.5	ND	320 ± 7.5

[a] CTLA-4$^+$ lymph node cells (5×10^6/ml) obtained from UV-tolerized mice were coincubated with DC (1×10^6/ml) in the absence or presence of DNBS (0.1 mM) for 11 days. CTLA-4 was blocked by adding a hamster anti-CTLA-4 Ab. Supernatants were tested for the amounts of IL-2, IFN-γ, IL-4, and IL-10.

adoptive cell transfer was reversed when the recipient mice were injected with anti-IL-10 Ab (group 4), while injection of an isotype control had no effect (data not shown). These findings indicate that the transfer of UV-induced tolerance is critically dependent on IL-10.

Discussion

The consequences of hapten application onto UV-irradiated skin are 2-fold: 1) CHS is not induced; and 2) hapten-specific tolerance develops (Fig. 1). Since both impairment of CHS and induction of hapten-specific tolerance are caused by the identical procedure, it was postulated for quite a long time that the same mechanisms are involved and that induction of tolerance is the direct consequence of the inhibition of CHS induction. However, there is accumulating evidence that the molecular basis of UV-induced tolerance is different from the mechanism responsible for UV-impaired induction of CHS (20). Whereas a key event in the suppression of CHS by UV irradiation appears to be the depletion of Langerhans cells from the epidermis (4, 21), the mechanisms that cause tolerance still remain unclear in major parts (reviewed in Refs. 7 and 22). Nevertheless, unresponsiveness can be adoptively transferred by injecting T cells from UV-tolerized mice into naive animals (6). These recipients cannot be sensitized against the specific hapten, whereas their sensitizing response to another unrelated hapten is not affected. The cells transferring suppression have been poorly characterized, but certainly belong to the T cell group (6, 16, 23). Here we show that they express CTLA-4 on their surface, since depletion of CTLA-4$^+$ cells resulted in complete loss of transfer of suppression. Likewise, when lymph node cells and splenocytes were enriched for CTLA-4$^+$ cells, a significantly lower number of cells sufficed to transfer suppression. Glass et al. proposed that T suppressor cells are universally induced during sensitization (24). Vice versa, one could hypothesize that primed effector cells are induced during tolerization. Provided these putative cells would be CTLA-4$^-$, depletion of these cells during CTLA-4 enrichment could also contribute to enhanced suppression following transfer. Whether this is the case is currently under investigation.

CTLA-4, however, cannot be regarded as a specific marker for the cells transferring suppression, since it is likely that only a minority of cells transferred is responsible for mediating hapten-specific unresponsiveness in the recipient. Nevertheless, CTLA-4 can serve as a marker to enrich these T cells for in vitro propagation. When lymph node cells from UV-tolerized mice were coincubated with DC, differences in the cytokine secretion pattern between CTLA-4$^+$ and CTLA-4$^-$ became obvious. CTLA-4$^+$ cells upon stimulation with DC and the specific hapten released high amounts of IL-10, TGF-β, and IFN-γ and low levels of IL-2, but no IL-4. In contrast, identically treated CTLA-4$^-$ cells behaved similarly in terms of IFN-γ, IL-2, and IL-4, while the pronounced induction of IL-10 and TGF-β secretion was not observed. Hence the cytokine pattern of our CTLA-4$^+$ cells resembles that of Tr1 cells described recently by Groux et al. (17, 18). Tr1 cells are induced by chronic activation of lymphocytes in the presence of IL-10; are CD4$^+$ and

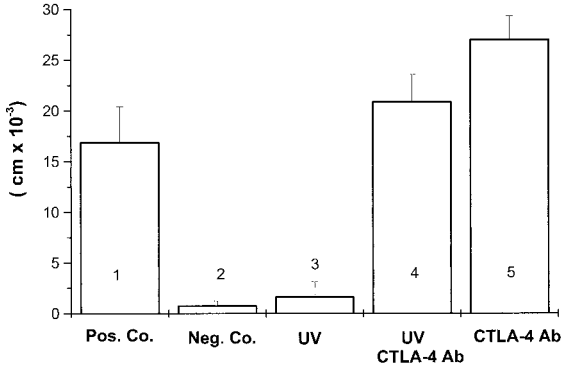

FIGURE 4. UV-induced tolerance is inhibited by blocking CTLA-4. Mice (groups 3 and 4) were treated daily with UV (1000 J/m^2) on 4 consecutive days on the shaved back, sensitized through UV-exposed back skin 24 h after the last UV exposure, and challenged on the left ear 5 days later. After 14 days, mice were resensitized through the nonirradiated shaved abdominal skin and challenged on the right ear 5 days later. Group 4 was injected i.p. with an Ab-blocking CTLA-4 (100 μg) 3 h before resensitization. Positive control mice were double-sensitized and challenged (group 1); negative control animals were ear challenged only (group 2). Group 5 was treated identically as group 1, but in addition was injected with the anti-CTLA-4 Ab before resensitization. Ear swelling was measured 24 h after challenge. The ear swelling response is expressed as the difference (centimeters $\times 10^{-3}$, mean ± SD) between the thickness of the challenged left ear and that of the ear measured immediately before challenge.

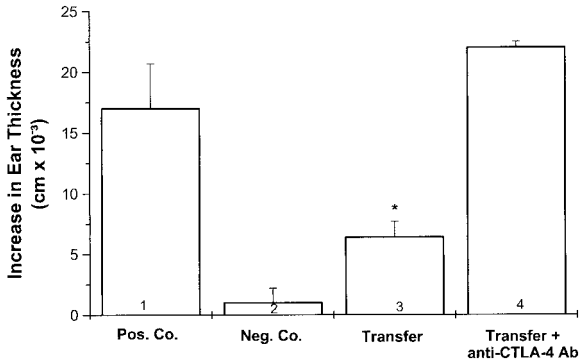

FIGURE 5. Blockade of CTLA-4 in the recipients inhibits transfer of tolerance. Mice (groups 1, 3, and 4) were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later. Negative control mice were ear challenged only (group 2). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 1×10^8 spleen and lymph node cells obtained from syngeneic animals that had been sensitized against DNFB through UV-exposed skin 10 days earlier. Group 4 was injected i.p. 3 h before cell transfer with an anti-CTLA-4 Ab (100 μg). Ear swelling was measured 24 h after challenge. The ear swelling response is expressed as the difference (centimeters $\times 10^{-3}$, mean ± SD) between the thickness of the challenged ear and the thickness of the vehicle-treated ear. *, $p < 0.001$ vs positive control.

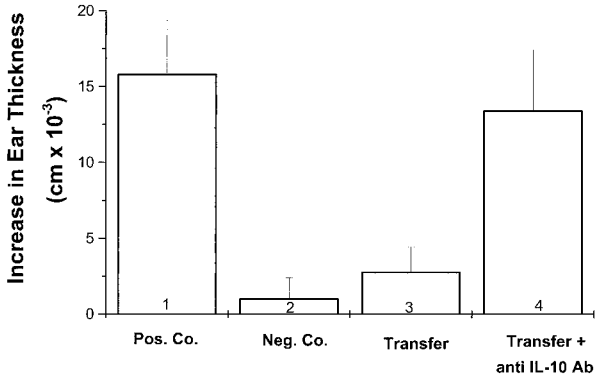

FIGURE 6. Neutralization of IL-10 inhibits transfer of tolerance. Mice (groups 1, 3, and 4) were sensitized on the back with DNFB, and ear challenge with DNFB was performed 5 days later. Negative control mice were ear challenged only (group 2). Twenty-four hours before sensitization, groups 3 and 4 were injected i.v. with 1×10^8 spleen and lymph node cells obtained from syngeneic animals that had been sensitized against DNFB through UV-exposed skin 10 days earlier. Group 4 was injected i.p. 3 h before and 24 h after sensitization with an anti-IL-10 Ab (250 μg each). 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.

$\alpha\beta^+$; and produce IL-10, IFN-γ, and TGF-β with little or no IL-2 and IL-4. Induction of IL-10 secretion in our CTLA-4$^+$ cells was only observed when DC and the specific hapten were added. Coincubation with DC in the presence of TNBS, the water-soluble analogue of the unrelated hapten TNCB, did not induce IL-10 production (data not shown), implying that IL-10 secretion by CTLA-4$^+$ cells is hapten specific. In addition, we can exclude that the preparation of CTLA-4$^+$ cell by itself could promote Ag-nonspecific triggering causing IL-10 release, since transfer of CTLA-4$^+$ T cells obtained from DNFB-tolerized donors into naive mice did not suppress sensitization of the recipients against the unrelated hapten oxazolone (data not shown).

There is convincing evidence that IL-10 plays an important role in mediating the immunosuppressive effects of UV radiation (25–27). Several studies suggested that Th2 cells mediate the suppressive effects of UV radiation by down-regulating Th1 functions through their production of IL-10 (reviewed in Ref. 19 and 28). Our cells, however, do not appear to belong to the Th2 type due to the absence of IL-4 production and their significant IFN-γ production. Shreedar et al. were the first to clone T cells with suppressive activity from UV-exposed mice that were sensitized with FITC (23). Cells cloned were CD4$^+$, CD8$^-$, TCR-$\alpha\beta^+$, MHC restricted, and specific for FITC. They produced IL-10, but not IL-4 or IFN-γ, whereas cells from unirradiated animals produced high amounts of IFN-γ and little IL-4 and IL-10. T cells from UV-exposed mice blocked APC functions and IL-12 production. Even more importantly, injection of cloned T cells into untreated recipients suppressed the induction of CHS against FITC. The phenotype of these cells resembled that of Tr1 cells to some extent, but, in contrast to Tr1 cells, failed to secrete IFN-γ. Hence, Shreedar et al. tentatively designated these cells Tr2 cells (23). The differences between our observations and those of Shreedar may be due to differences in the experimental approaches. Shreedar analyzed cloned cells, whereas we cultured our cells for 2 wk at the longest. While Shreedar used FITC as a hapten, we performed our studies with DNFB. There are indications that the outcome and the type of immune reaction may depend on the type of hapten (29).

CTLA-4 not only appears to be a marker for the T cells transferring UV-mediated suppression but also seems to be functionally relevant. Injection of an Ab that blocks CTLA-4 (13) into UV-tolerized mice enabled sensitization against the respective hapten. These data indicate for the first time that blockade of CTLA-4 can break UV-induced tolerance. In vivo blockade of CTLA-4 slightly enhanced sensitization (Fig. 4). However, this potentiation was never so pronounced that this effect could be responsible for the restoration of the CHS response in tolerized animals. Tang et al. recently showed that treatment of mice with a soluble form of CTLA-4 (CTLA-4Ig) during primary sensitization induced long lasting unresponsiveness against DNFB (29). CTLA-4Ig binds to B7 (CD80/86) with 20-fold higher avidity than CD28 and thus inhibits the interaction of CD28 with its B7 counterparts (30). Blockade of costimulation through the B7-CD28 pathway by CTLA-4Ig has been shown to inhibit a variety of in vivo immune responses, in some instances leading to Ag-specific tolerance as demonstrated by Tang et al. (29). In contrast to CTLA-4Ig, the anti-CTLA-4 Ab that we used for the in vivo studies does not interfere with B7-CD28 interaction, but only inhibits CTLA-4 signaling. Since in vivo blockade of CTLA-4 results in the loss of tolerance (Fig. 4) and the loss of suppressive transfer (Fig. 5), functional CTLA-4 signaling in T cells appears to be crucial in mediating UV-induced tolerance. Taking into account that transfer of suppression is critically dependent on T cells expressing CTLA-4, the in vivo blocking data indicate that activation of CTLA-4 on these cells is critically involved in the mediation and transfer of tolerance.

The underlying mechanism by which CTLA-4 signaling mediates tolerance remains to be determined. Since upon in vitro stimulation with hapten-coupled DC, CTLA-4$^+$ cells released high amounts of IL-10, one mechanism by which the anti-CTLA-4 Ab could break tolerance is by inhibiting the release of IL-10 that has been suggested to be crucial in tolerance induction (31, 32). Accordingly, the blocking Ab reduced in vitro IL-10 release significantly, when added to CTLA-4$^+$ cells stimulated with DC in the presence of the specific hapten. Thus, we postulate that ligation of CTLA-4 results in enhanced release of IL-10, which ultimately mediates UV-induced immunosuppression. In this context it is important to mention that induction of release of immunosuppressive cytokines upon ligation of CTLA-4 has been previously observed; engagement of CTLA-4 by cross-linking Ab induces TGF-β (33), which is a potent inhibitor of T cell-mediated immune responses both in vitro and in vivo (34–38). Accordingly, stimulation of CTLA-4$^+$ cells obtained from UV-tolerized mice with hapten-coupled DC induced the release of TGF-β. Whether this is due to CTLA-4 ligation in our system cannot be answered, since we have not yet performed Ab blocking studies in vitro.

Since blockade of CTLA-4 in our experiments was able to break tolerance in vivo, on the one hand, and to inhibit IL-10 secretion in vitro, on the other hand, we surmised that the T cells obtained from UV-tolerized mice exert their regulatory activity in the recipient animals by release of IL-10. In this case, the inhibitory activity of the adoptive transfer should be suppressed when IL-10 is neutralized in the recipients. Indeed, animals receiving T cells from DNFB-tolerized donors could be normally sensitized against DNFB when they were injected with an anti-IL-10 Ab. Injection of the same amounts of anti-IL-10 Ab into naive mice before sensitization caused a slight, but not significant, enhancement of the subsequent ear swelling response (data not shown). Hence, it is unlikely that the restoration of the ear swelling response by the anti-IL-10 Ab in mice receiving T suppressor cells is just due to a potentiation of CHS reactivity. Consequently, these findings indicate that transfer of UV-mediated tolerance is critically dependent

on IL-10. Although we cannot exclude other possibilities with absolute certainty, e.g., that the anti-CTLA-4 Ab could operate through cell killing or redirecting cell traffic or homing, we suspect that triggering of IL-10 release is the most likely mechanism. Hence, induction of immunosuppressive cytokines, especially of IL-10, following ligation of CTLA-4 may represent a mechanism by which the CTLA-4 molecule functions as a negative regulator of T cell responses.

On the first glance, inhibition of transfer of suppression by in vivo blockade of IL-10 may appear to be in contrast to previous observations made by Rivas and Ullrich (25). In this study inhibition of CHS in the recipient animals after adoptive transfer was not affected by injecting anti-IL-10 Ab. The most likely explanation for these differences is that Rivas and Ullrich studied systemic UV-induced immunosuppression, while we used the local model. There is ample evidence that although both systemic and local immunosuppressions inhibit the induction of CHS and induce hapten-specific tolerance, their effects are mediated by different mechanisms (39, 40).

Since TGF-β release was also induced by CTLA-4$^+$ T cells upon stimulation with DC, the question arises of whether TGF-β is also involved in mediating transfer of UV-induced suppression. In contrast to the clear-cut data when blocking IL-10 in vivo, injection of a neutralizing anti-TGF-β Ab did not inhibit transfer of suppression (data not shown). However, since neither detailed dose-response studies nor injections with different kinetics were performed, it cannot be excluded with absolute certainty that TGF-β may be involved as well.

Based on the present data we propose the following scenario for UV-induced tolerance. A subtype of CTLA-4$^+$ T cells is induced after tolerization by application of haptens through UV-exposed skin. Upon resensitization, these CTLA-4$^+$ T cells may meet the APC carrying the specific hapten in the regional lymph nodes. Triggering of CTLA-4 by B7 molecules expressed on the APC induces the release of IL-10, which subsequently inhibits resensitization, thereby rendering the respective animal tolerant to this hapten. The same process may occur when CTLA-4$^+$ T cells from UV-tolerized animals are adoptively transferred into naive recipients. Since the CTLA-4$^+$ T cells become only activated when the specific hapten is presented, this may explain the hapten specificity of UV-mediated tolerance. However, once IL-10 is released in an Ag-specific manner, its immunosuppressive effects should be general (bystander suppression) (18). Whether this is the case in vivo is currently under investigation. How IL-10 might inhibit resensitization also remains to be determined. It recently was shown that induction of apoptosis via the Fas/FasL system is crucial for the induction of UV-induced tolerance (41, 42). Hence, we are currently investigating whether IL-10 affects apoptotic pathways in DC or T cells.

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