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of October 25, 2021.

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J Immunol 2007; 178:877-886; ;
doi: 10.4049/jimmunol.178.2.877
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Alteration of the Migratory Behavior of UV-Induced Regulatory T Cells by Tissue-Specific Dendritic Cells¹

Agatha Schwarz,² Akira Maeda,² and Thomas Schwarz³

UV radiation-induced regulatory T cells (UV-Treg) inhibit the sensitization but not the elicitation of contact hypersensitivity when injected i.v. Because UV-Treg express the lymph node homing receptor CD62 ligand, upon i.v. injection they migrate into the lymph nodes but not into the periphery and therefore inhibit sensitization but not elicitation. We tried to modify the migratory behavior of UV-Treg with the aim to get them into the periphery and thereby to suppress the effector phase of immune reactions. Because the tissue selective homing of T effector cells is determined by tissue-specific dendritic cells (DC), we attempted to reprogram the migratory behavior of UV-Treg by DC. 2,4-Dinitrofluorobenzene (DNFB)-specific UV-Treg coincubated with epidermal Langerhans cells (LC) blocked the elicitation upon i.v. injection into DNFB-sensitized mice. In contrast, i.v. injection of UV-Treg not incubated with LC did not inhibit the ear challenge. The same negative effect was observed for UV-Treg coincubated with DC from bone marrow, spleen, or lymph nodes. This effect was not due to different maturation stages as checked by MHC class II expression of the different DC types. Incubation with LC but not with bone marrow-derived DC down-regulated the expression of CD62 ligand on UV-Treg. Accordingly, CFDA-SE labeled UV-Treg coincubated with LC were found in the ears but not in the lymph nodes upon i.v. injection. This finding shows that the migratory behavior can be reprogrammed by tissue-specific DC and may have input on strategies trying to use Treg not only for the prevention but also for the treatment of immune-mediated diseases. *The Journal of Immunology*, 2007, 178: 877–886.

Ultraviolet radiation, in particular the mid-wave range (UVB, 290–320 nm), represents one of the most significant environmental factors affecting humans. Its hazardous effects on health include skin cancer, skin aging, and exacerbation of infectious diseases (1). A variety of these effects are partially mediated by the immunosuppressive properties of UV radiation, which are best illustrated by the inhibition of cellular immune reactions, such as contact hypersensitivity (CHS)⁴ (2, 3). UV radiation inhibits the sensitization to contact allergens if they are applied directly to the UV-exposed skin area (2, 3). In addition, hapten-specific tolerance develops because the very same animals cannot be sensitized against the same hapten at a later time point, although other immune reactions are not suppressed (2). This unresponsiveness can be adoptively transferred because injection of splenocytes and lymph node cells obtained from UV-tolerized mice into naive mice inhibits the sensitization against the respective hapten in the recipients (4). Accordingly, it was suggested that UV-induced tolerance is mediated via the induction of hapten-specific T suppressor cells, which are nowadays called regulatory T cells (Treg) (5).

Several types of UV-induced Treg (UV-Treg) have been described; most of these belong to the CD4 type (5). Best characterized are the UV-Treg that suppress hapten-mediated CHS. These cells express besides CD4 also CD25 (6), the negative regulatory molecule CTLA-4 (7) and bind the lectin dectin-2 (8). In addition, they secrete IL-10 upon hapten-specific stimulation and may use the apoptosis-related Fas/Fas ligand system (7, 9). The induction of Treg by UV radiation is critically dependent on the migration of Langerhans cells (LC), the primary APC of the skin, into the lymph nodes and the presence of UV-induced DNA damage in the LC (10) because IL-12, which reduces UV-mediated DNA damage (11), prevents the generation of UV-Treg.

As UV-Treg inhibit immune reactions in a rather Ag-specific fashion, these cells may harbor therapeutic potential as suggested also for other types of Treg (12). Treg have been shown to exert the capacity to prevent the manifestation of autoimmune diseases (13). In turn, depletion of Treg results in autoimmune phenomena (14). The therapeutic potential of Treg may even further increase, should they be able to act not only in a preventive but also in a curative fashion. UV-Treg, however, exert suppressive activity only when i.v. injected into naive but not into sensitized mice (15). This implied that they inhibit only the afferent but not the efferent limb of CHS. This led to the final conclusion that UV-Treg are not active in the presence of T effector cells and thus are inferior to T effector cells (15). This observation gave rise to the speculation that UV-Treg only act in naive and not in sensitized hosts and thus that their therapeutic potential may be limited only to prevent but not to cure already manifested immune-mediated diseases (16).

Recently, we could demonstrate the UV-Treg specific for the contact allergen 2,4-dinitrofluorobenzene (DNFB) did not inhibit the effector phase of CHS when injected i.v. into DNFB-sensitized mice, but suppressed the ear swelling response when intracutaneously injected into the ears of DNFB-sensitized mice (6). Furthermore, it was shown that DNFB-specific UV-Treg injected into the

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Received for publication June 20, 2006. Accepted for publication November 6, 2006.

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¹ This work was supported by grants from the German Research Foundation (SFB 415, A16; SCHW1177/1-1). T.S. is a recipient of the CERES 2004 Research Award.

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⁴ Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cell; BMDC, bone marrow-derived DC; DNFB, 2,4-dinitrofluorobenzene; CD62L, CD62 ligand; DNBS, dinitrobenzenesulfonic acid-sodium salt; LC, Langerhans cell; Treg, regulatory T cell; PSGL-1, P-selectin glycoprotein ligand-1.

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ears of mice sensitized against oxazolone upon activation by epicutaneous application of DNFB also suppressed the specific response against oxazolone. Activation of UV-Treg was always associated with an increased expression of IL-10. This indicated that activation of UV-Treg is hapten-specific but once activated their suppressive activity is nonspecific, a phenomenon called by-stander suppression. Together, this demonstrated for the first time that UV-Treg in fact can inhibit the effector phase of CHS, provided they are present in the area where the challenge takes place. Thus, we surmised that the inability of Treg to inhibit the effector phase of CHS upon i.v. injection is due to the fact that they do not migrate into the skin. Accordingly, FACS analysis of tissue homing receptors on UV-Treg revealed that they express the lymph node homing receptor L-selectin (CD62 ligand (CD62L)) but not the ligands for the skin homing receptors E- and P-selectin (6). This implied that UV-Treg upon i.v. injection locate primarily to the lymph nodes but do not get into the periphery and thus inhibit the sensitization that takes place in the lymph nodes. However, because they do not get into the periphery upon i.v. injection, they are not able to suppress the effector phase of CHS, which takes place in the periphery, in the CHS model in the skin of the ears.

Because of the capacity of by-stander suppression, speculations exist about the therapeutic potential of Treg, 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 (17). However, according to the described findings, this strategy will only be successful if the Treg home into the target organ, which does not apply at least for i.v. injected UV-Treg (6). Hence, we were interested to identify strategies by which the migration behavior of UV-Treg can be reprogrammed with the final aim to get them into the periphery and thereby to suppress effector phases of immune reactions.

The mechanisms by which the migration behavior of T effector cells is determined are quite well studied. Activated effector/memory T cells migrate preferentially to tissues that are connected to the secondary lymphoid organs where the Ag is first encountered (18). The route by which the Ag is administered plays an important role in this process (19). Accordingly, it was shown that the intracutaneous but not the i.v. injection of bone marrow-derived dendritic cells (BMDC) induced skin homing T cells with up-regulated expression of the ligand for E-selectin. In contrast, i.p. injection induced T cells expressing the gut homing integrin $\alpha_4\beta_7$. However, not only the route but also the APC involved appear to play an important role in imprinting tissue-specific address codes onto T effector cells, which allows proper tissue homing (20). It was shown that the imprinting of gut tropism is mediated by DC from Peyer's patches. DC obtained from Peyer's patches, peripheral lymph nodes, or spleen induced equal activation of CD8⁺ T cells but only DC from Peyer's patches induced high levels of $\alpha_4\beta_7$ that were associated with the ability to home to the small intestine. In addition, it was recently reported that DC also exert the capacity to reprogram polarized tissue-selective homing receptor patterns of T cells (21). Skin homing memory T cells stimulated *ex vivo* were reprogrammed by intestinal DC cells to a gut homing phenotype.

Whether similar regulatory mechanisms controlling the homing behavior apply also for Treg is completely unclear. In seeking for strategies to get UV-Treg into the periphery with the final aim to suppress effector phases of immune responses, we asked whether the migration behavior of UV-Treg can be reprogrammed by tissue-specific DC. In this study, we show that UV-Treg after cocubation with epidermal LC inhibit the effector phase of CHS when injected i.v. into sensitized mice. In contrast, UV-Treg cocubated with DC from lymphoid organs fail to inhibit the effector

phase of CHS upon i.v. injection. Cocubation of UV-Treg with LC but not with APC derived from lymphoid organs results in a down-regulation of the expression of the lymph node homing receptor CD62L and in turn in an up-regulation of the ligand for the skin homing receptor P-selectin on UV-Treg. These data show that the migratory behavior of UV-Treg can be altered by tissue-specific DC. This observation may have important implications on strategies trying to use UV-Treg not only for the prevention but also for the treatment of immune-mediated diseases.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles River Breeding Laboratories. Animal care was provided by expert personnel in compliance with the relevant laws and institutional guidelines.

CHS measurements

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

UV irradiation

The shaved back was exposed to UV radiation from TL12 fluorescent lamps (Philips), which emit most of their energy within the UVB range. Mice were exposed to UV radiation (1.5 kJ/m^2) daily for 4 consecutive days.

Generation of UV-induced Treg

Donor mice were sensitized against DNFB through UV-exposed skin as described above. Five days after hapten application, spleens, and regional lymph nodes were removed and single-cell suspensions prepared. Briefly, CD4⁺CD25⁺ Treg were isolated in a two-step procedure, according to the protocol of a magnetic separation kit (CD4⁺CD25⁺ Regulatory T Cell Isolation kit; Miltenyi Biotec). A total of 5×10^5 of Treg were i.v. injected into the sensitized or naive recipient mice. Naive recipients were sensitized against DNFB 24 h after injection and ear challenge was performed 5 days later. Recipients that had already been sensitized before injection were challenged on the left ear 24 h after adoptive transfer.

Isolation of APC

Epidermal LC were obtained from ears of C57BL/6 mice that were divided into two pieces and floated (upper side epidermis, lower side dermis) on Dispase II solution (2.4 U/ml; Roche Diagnostics). After 2 h incubation at 37°C, the epidermis was separated from the dermis and passed through a nylon mesh to obtain a single-cell suspension. To separate MHC class II-positive LC, epidermal cells were incubated with a PE-conjugated rat anti-mouse I-A/I-E Ab (BD Biosciences). MHC class II-labeled cells were separated using microbeads conjugated with a monoclonal anti-PE Ab (Anti-PE MicroBeads; Miltenyi Biotec).

BMDC were generated by culture of bone marrow cells in the presence of GM-CSF as described (22). Briefly, bone marrow was collected from tibias of female C57BL/6 mice. Erythrocytes were lysed and the remaining cells were passed through a nylon mesh to remove small pieces of bone debris. The cells were washed, resuspended, and cultured in petri dishes (BD Biosciences) at a density of $1 \times 10^6/\text{ml}$ for 2 h. Nonadherent cells were collected and 1×10^6 cells/ml were placed in 24-well plates in RPMI 1640, supplemented with 5% FCS, 50 μ M 2-ME, 1% nonessential amino acids, 20 μ g/ml gentamicin (all from PAA Laboratories), and 100–200 U/ml GM-CSF (BD Biosciences). Two-thirds of medium was replaced every 2 days and for the last 48 h LPS (50 ng/ml; Sigma-Aldrich) was added. Nonadherent cells were harvested on days 1, 4, or 8. The expression of surface molecules characteristic for adult DC (MHC class II, CD86/B7-2) was determined by flow cytometry using an anti-MHC class II PE-conjugated Ab (BD Biosciences) or a rat anti-mouse anti-CD86 Ab (Beckman Coulter).

Lymph node-derived DC were isolated from regional lymph nodes obtained from naive mice. After mincing, cell suspension was filtered and

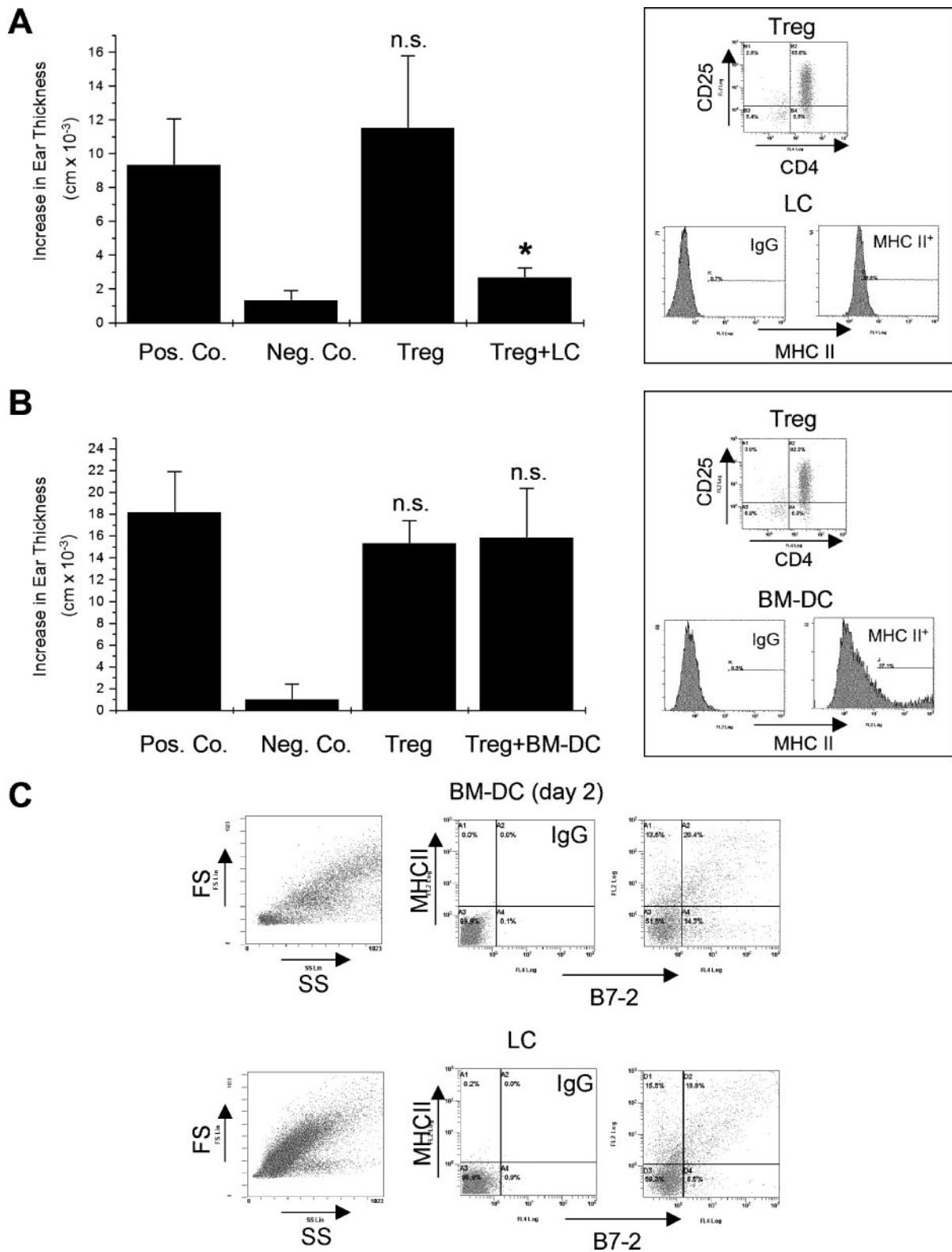


FIGURE 1. UV-Treg cocultured with LC but not with BMDC inhibit the effector phase of CHS. CD4⁺CD25⁺ Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. *A*, CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence (Treg+LC) of LC obtained from the epidermis of naive mice. *B*, CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence (Treg+BM-DC) of DC obtained from the bone marrow of naive mice. MHC class II expression of DC was measured by FACS analysis (*inset*). After coculture, cells were harvested and injected i.v. into recipients which had been sensitized against DNFB 5 days earlier. At 24 h after injection recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm \times 10⁻³) of the challenged ear and that of the vehicle-treated ear. *C*, LC and BMDC used were analyzed for MHC class II and B7-2 expression by FACS analysis. n.s., Not significant. *, $p < 0.0001$ vs positive control.

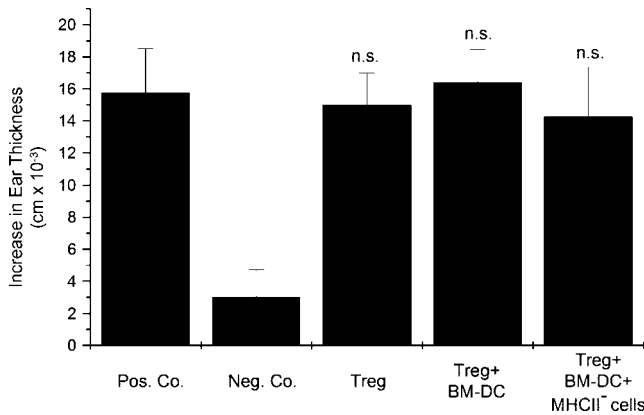


FIGURE 2. MHC class II-negative cells obtained from the epidermis do not affect UV-Treg. Epidermal LC were isolated as described in *Materials and Methods*. To obtain the immature contaminating cells, MHC class II-positive cells were depleted by magnetic bead separation. The MHC class II-negative fraction (MHCII⁻) was added to UV-induced Treg that were cocultured in the absence (Treg) or presence of BMDC (Treg+BM-DC). After coculture, the nonadherent cells were harvested and 5×10^5 cells were injected i.v. into sensitized mice. At 24 h after transfer, recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm $\times 10^{-3}$) of the challenged and that of the vehicle-treated ear. n.s., Not significant.

washed. CD11c⁺ lymph nodes cells were fractionated with CD11c Microbeads and MACS columns (CD11c, N418 Microbeads; Miltenyi Biotec).

To isolate spleen-derived DC, spleens were obtained from naive mice and pooled, and cell suspension prepared by mechanical disruption. Spleen capsules were cut into fine pieces and passed through a cell strainer (100 μ m). Cell suspension was filtered and washed. CD11c⁺ cells were isolated by using CD11c Microbeads and MACS columns, as previously described for lymph node-derived DC.

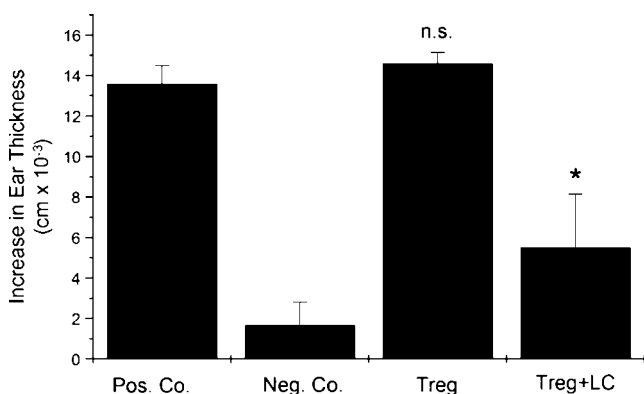


FIGURE 3. UV-Treg cocultured with LC inhibit the effector phase of CHS. UV-Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence of LC (Treg+LC). After coculture for 48 h, nonadherent cells were harvested and depleted for MHC class II-positive cells via magnetic bead separation using FITC conjugated anti-mouse MHC class II (clone 2G9) and anti-FITC microbeads. 5×10^5 (MHC II neg. cells) were injected i.v. into recipient mice that had been sensitized against DNFB 5 days earlier. 24 h after transfer, recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm $\times 10^{-3}$) of the challenged and that of the vehicle-treated ear. n.s., Not significant. *, $p < 0.0005$ vs positive control.

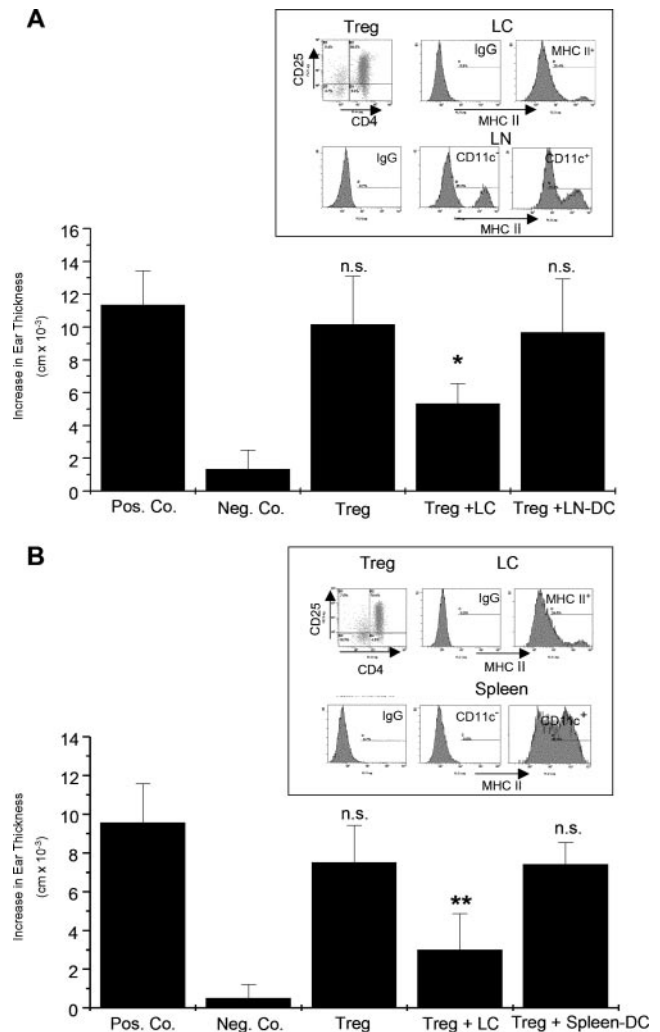


FIGURE 4. UV-Treg cocultured with lymph node (LN)- or spleen-derived DC do not inhibit the effector phase of CHS. CD4⁺CD25⁺ Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. **A**, CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence (Treg+LC) of LC obtained from the epidermis of naive mice or from CD11c⁺ DC (Treg+LN-DC) obtained from lymph nodes of naive mice. **B**, CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence (Treg+LC) of LC obtained from the epidermis of naive mice or from CD11c⁺ DC (Treg+Spleen-DC) obtained from spleens of naive mice. MHC class II expression of DC was measured by FACS analysis (*insets*). After coculture, cells were harvested and injected i.v. into recipients that had been sensitized against DNFB 5 days earlier. At 24 h after injection recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm $\times 10^{-3}$) of the challenged and that of the vehicle-treated ear. n.s., Not significant. *, $p < 0.0005$ vs positive control; **, $p < 0.0001$ vs positive control.

Coculture experiments

APC (LC, BMDC, spleen-derived DC, or lymph node-derived DC; 1×10^6 /ml each) were pulsed for 30 min with 1.0 mM DNBS (dinitrobenzenesulfonic acid-sodium salt), the water soluble derivative of DNFB, washed and suspended in RPMI 1640 supplemented with 10% FCS and 1% MEM (Non Essential Amino Acids; PAA Laboratories). UV-Treg were added at a ratio of 1:3. After incubation for 48 h, the nonadherent cells were obtained, washed and either injected into mice or subjected to FACS analysis. In some experiments, the nonadherent cells were depleted of MHC class II-positive cells via magnetic bead separation using FITC-conjugated anti-mouse MHC class II (clone 2G9) and anti-FITC microbeads.

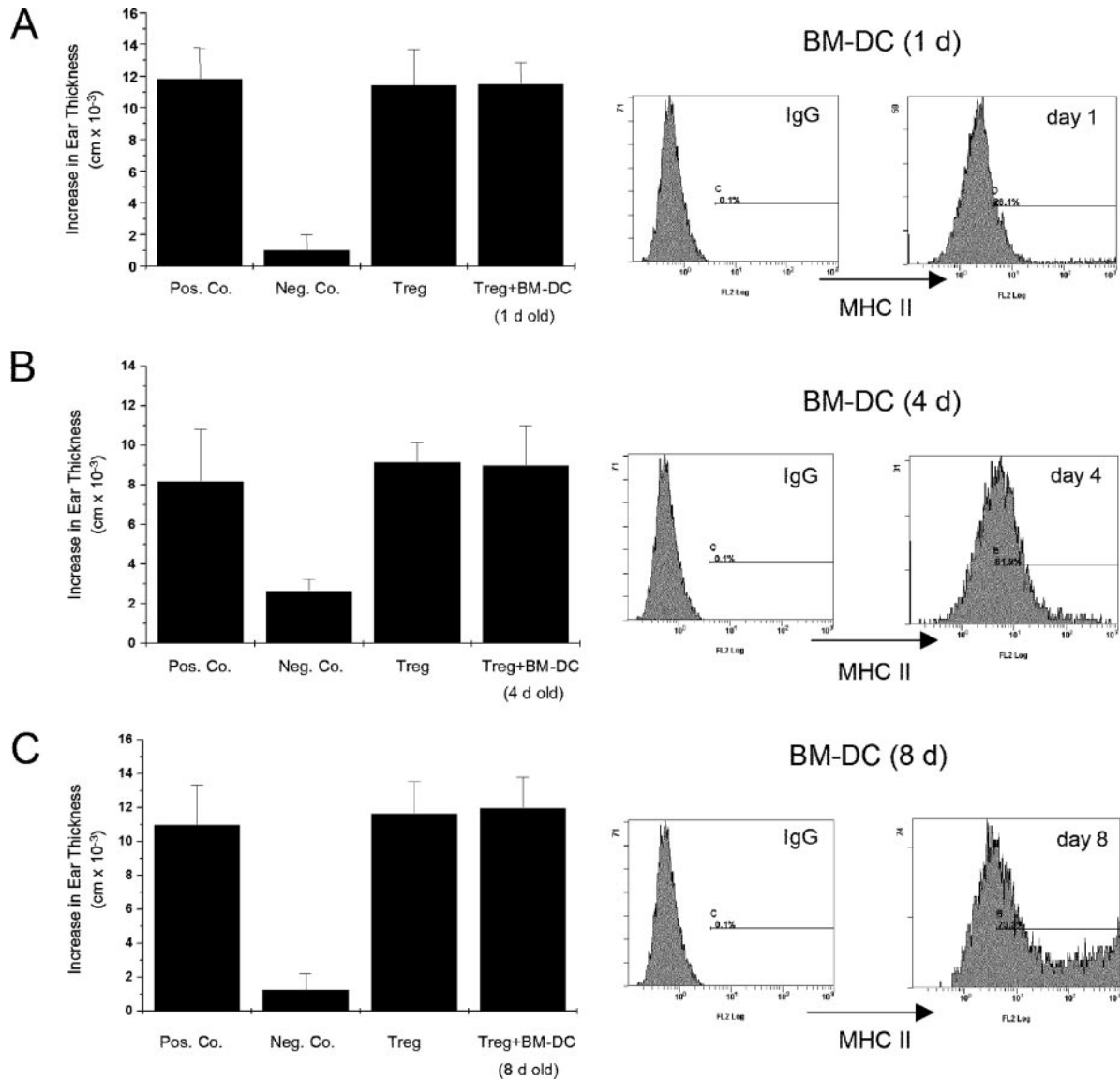


FIGURE 5. UV-Treg coincubated with BMDC of different maturation stages do not inhibit the effector phase of CHS. DC were obtained from the bone marrow of naive mice and cultured for 1 (A), 4 (B), or 8 (C) days. Maturation was evaluated by MHC class II staining (right inset). CD4⁺CD25⁺ Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. CD4⁺CD25⁺ Treg were cultured in the absence (Treg) or presence (Treg+BM-DC) of BMDC. After coculture, cells were harvested and injected i.v. into recipients that had been sensitized against DNFB 5 days earlier. At 24 h after injection, recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm \times 10⁻³) of the challenged and that of the vehicle-treated ear.

FACS analysis

Purity of the CD4⁺CD25⁺ cells was evaluated by flow cytometry using allophycocyanin-conjugated anti-mouse CD4 (BD Biosciences) and PE-labeled anti-CD25 Abs (Miltenyi Biotec). MHC class II and B7-2 expression on APC was measured by using PE-conjugated anti-mouse I-A/I-E Abs (BD Biosciences) and anti-mouse CD86 Abs (Beckman Coulter). For staining of L-selectin (CD62L) and the ligand for P-selectin (CD162) we used purified rat anti-mouse mAbs directed against CD62L and CD162, respectively (both BD Biosciences). Analysis was performed with a FC 500 CXP FACS analyzer (Beckman Coulter).

CFDA-SE labeling

Cells were incubated with 5 μ M CFDA-SE (Molecular Probes) for 7 min in PBS, and then further incubated for 3 min in the presence of 10% FCS, followed by three washes with RPMI 1640 medium containing 10% FCS. The cell numbers were adjusted to 2.5 \times 10⁶/ml and 200 μ l of cells were injected i.v. into recipients, which had been sensitized against DNFB 5 days earlier. At 24 h after injection ears and lymph nodes were obtained

and cryosections cut. Sections were stained with a PE-conjugated Ab directed against CD25. Sections were analyzed by using an immunofluorescence microscope (Axiovert 40CFL; Carl Zeiss).

Statistical analysis

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

Results

UV-induced Treg coincubated with LC inhibit the elicitation of CHS upon i.v. injection

To determine whether LC can affect the migration behavior of UV-Treg, UV-Treg were obtained from C57BL/6 mice that were sensitized with DNFB through UV-exposed back skin. Intravenous injection of these cells into mice that were sensitized with 0.5% DNFB 5 days earlier did not inhibit the elicitation of CHS in the

recipients because the ear swelling response upon application of 0.3% DNFB was not suppressed (Fig. 1A). This observation is in accordance with previous findings (6, 15). However, when DNFB-specific UV-Treg were coincubated with epidermal LC and DNBS, the water-soluble analog of DNFB, for 48 h, the ear swelling response was significantly suppressed upon i.v. injection of these cells into DNFB-sensitized recipients (Fig. 1A). When DNFB-specific Treg were coincubated with LC but instead with DNBS with 2,4,6-trinitrobenzenesulfonic acid sodium salt, the water soluble analog of the unrelated hapten trinitrochlorobenzene, the CHS response against DNFB was not suppressed upon i.v. injection of these cells into DNFB-sensitized mice (data not shown). Thus LC are able to alter the function of UV-Treg only in the presence of the specific Ag, indicating that this process is Ag-specific.

In contrast, when DNFB-specific UV-Treg were coincubated with BMDC and DNBS, the elicitation of CHS was not suppressed upon i.v. injection of these cells into DNFB-sensitized mice (Fig. 1B). It is important to notice that LC and BMDC did not significantly differ in the expression of MHC class II molecules as checked by FACS analysis (Fig. 1, insets) as well as by B7-2 expression (Fig. 1C), indicating that the stage of maturation was similar in both APC populations. Thus, we speculated that the alteration of UV-Treg by APC may not be because of differences in the maturation stage but because of the differences in the tissue origin of DC.

The FACS profile shown in Fig. 1C also indicated that UV-Treg were not coincubated with pure LC but also with contaminating cells being negative for MHC class II and B7-2. Thus, one could argue that it is not the LC but maybe this population, which alters the activity of UV-Treg. To exclude this possibility, the LC cell preparation was depleted of MHC class II-positive cells. The remaining MHC class II-negative cell fraction was added to UV-Treg that were coincubated with BMDC. After incubation for 48 h, UV-Treg were injected i.v. into sensitized mice, which were ear challenged 24 h thereafter. Both UV-Treg alone and UV-Treg preincubated with BMDC did not suppress the ear swelling response, confirming the data obtained in Fig. 1B. Addition of the MHC class II-negative cell fraction of the LC preparation had no impact on the effect of UV-Treg on the ear swelling response (Fig. 2). This finding indicates that the alteration of UV-Treg demonstrated in Fig. 1A is due to the presence of LC and not due to the contaminating MHC class II-negative epidermal cells.

In the experiments discussed, the nonadherent fractions of the coincubations were injected into sensitized mice. Although the majority of this fraction consists of UV-Treg, nonadherent DC may be present as well. To prove that the inhibition of the ear challenge is caused by the injected UV-Treg and not by the contaminating DC, UV-Treg were coincubated with LC for 48 h. Finally, the nonadherent fraction was depleted of MHC class II-positive cells, thereby eliminating the contaminating DC. Injection of the MHC class II depleted fraction into sensitized mice inhibited the ear challenge in the same fashion as observed in Fig. 1A (Fig. 3). The same results as in Fig. 1B were obtained when MHC class II-positive cells were depleted from the coincubations of UV-Treg with BMDC (data not shown). Hence, UV-Treg and not remaining nonadherent DC are mediating the effects on the ear challenge. Thus, in the following experiments, the nonadherent fractions obtained from coincubations were injected.

DC obtained from lymphoid tissue do not alter the behavior of UV-Treg

Because the alteration in the suppressive behavior of UV-Treg was only induced by LC but not by BMDC, we speculated that this alteration may be induced by APC obtained from peripheral but

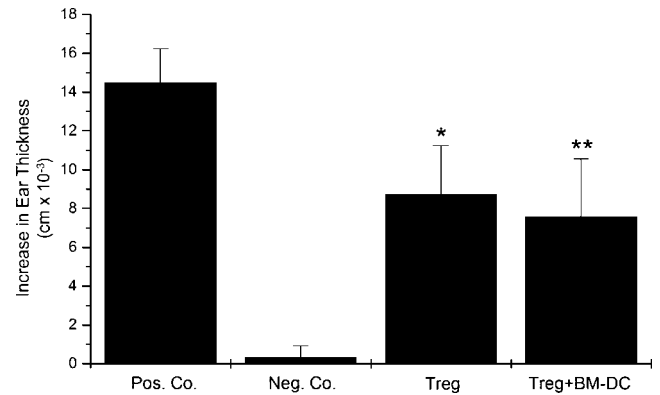


FIGURE 6. BMDC do not inhibit the suppressive activity of UV-Treg. UV-Treg were cocultured in the absence (Treg) or presence of BMDC (Treg+BM-DC). After 48 h nonadherent cells were harvested and 5×10^5 cells were injected intracutaneously into the left ears of recipients that had been sensitized against DNFB 5 days earlier. Three hours after injection, recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness ($\text{cm} \times 10^{-3}$) of the challenged and that of the vehicle-treated ear. n.s., Not significant. *, $p < 0.01$ vs positive control; **, $p < 0.005$ vs positive control.

not from lymphoid organs. Therefore, DNFB-specific UV-Treg were coincubated with CD11c⁺ DC obtained either from lymph nodes or spleens from naive mice. Again, only Treg costimulated with epidermal LC, but neither with lymph node-derived (Fig. 4A) nor with spleen-derived APC (Fig. 4B) were able to inhibit the elicitation of CHS when injected i.v. into DNFB-sensitized recipients. This response suggests that only APC from peripheral but not from lymphoid organs exhibit the capacity to reprogram the suppressive activity of UV-Treg.

In contrast to BMDC, MHC class II expression was higher in DC obtained from the spleen or lymph nodes when compared with LC. We were not able to develop techniques to isolate DC from these organs with lower MHC class II expression. This difference might nourish the argument that the alteration of UV-Treg is rather due to maturation differences of the DC than to the tissue origin of the DC. To prove whether the maturation stage of DC has a significant impact on the alteration of UV-Treg, BMDC were obtained and cultured for several days. With increasing length of cultivation the MHC class II expression increased, which is a suitable surrogate marker for maturation (Fig. 5). DNFB-specific UV-Treg were incubated with BMDC that were cultured for 1, 4, or 8 days as described. At 48 h after coincubation, UV-Treg were injected i.v. into DNFB-sensitized mice, and ear challenge was performed 24 h after injection. Mice that had received Treg coincubated with BMDC were not suppressed in their CHS response, irrespective of whether UV-Treg were coincubated with more immature or mature DC (Fig. 5). Together, these data support the hypothesis that the alteration of the functional behavior of UV-Treg by DC is rather related to the tissue origin of the DC than the maturation stage.

BMDC do not impair the suppressive activity of UV-Treg

The fact that UV-Treg coincubated with BMDC do not inhibit the effector phase when injected i.v. could be due to an impairment of the suppressive activity of UV-Treg by BMDC. To address this issue, UV-Treg were coincubated with BMDC for 48 h. After incubation UV-Treg were obtained and injected intracutaneously into the ears of DNFB-sensitized mice. As a control, UV-Treg,

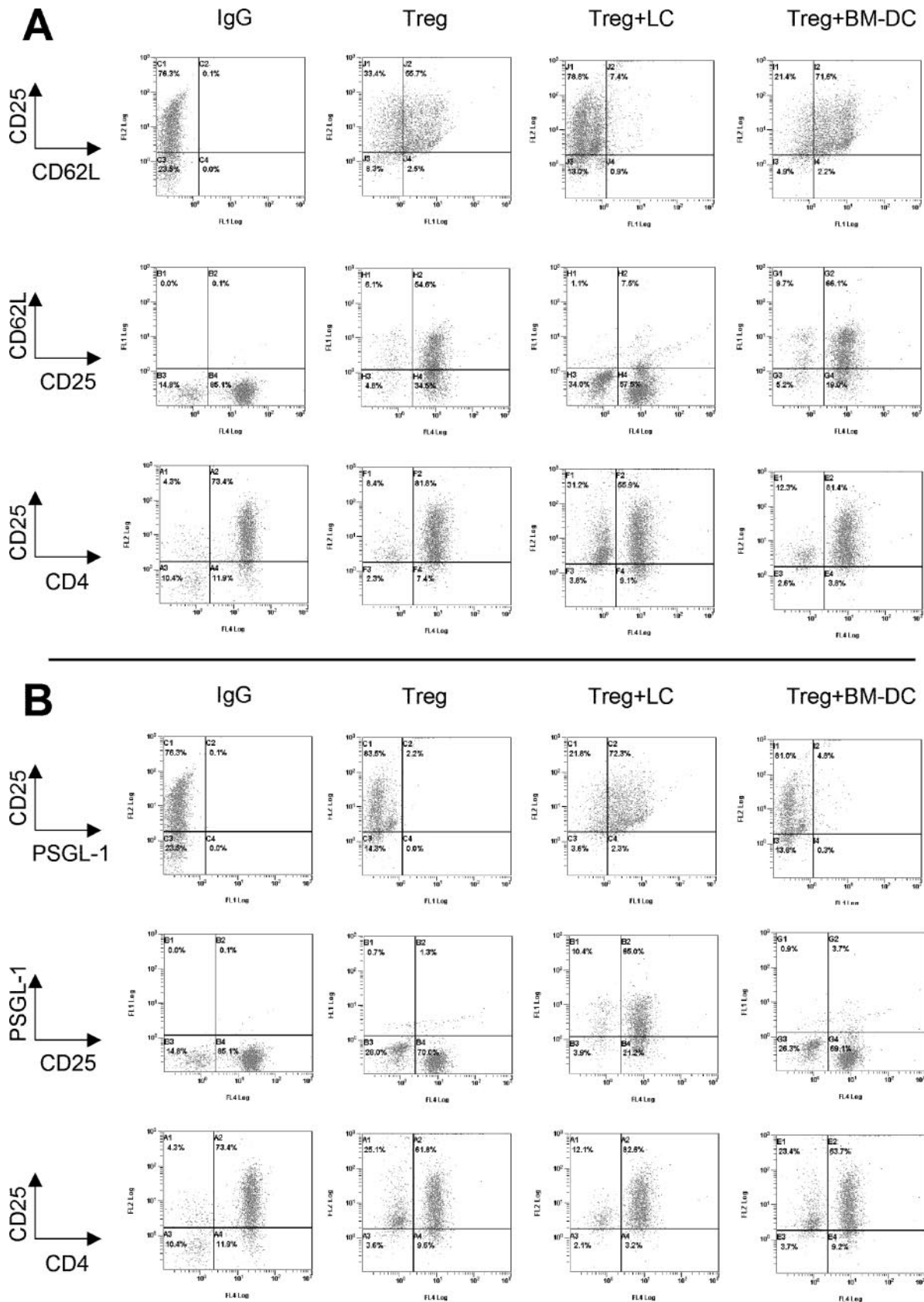
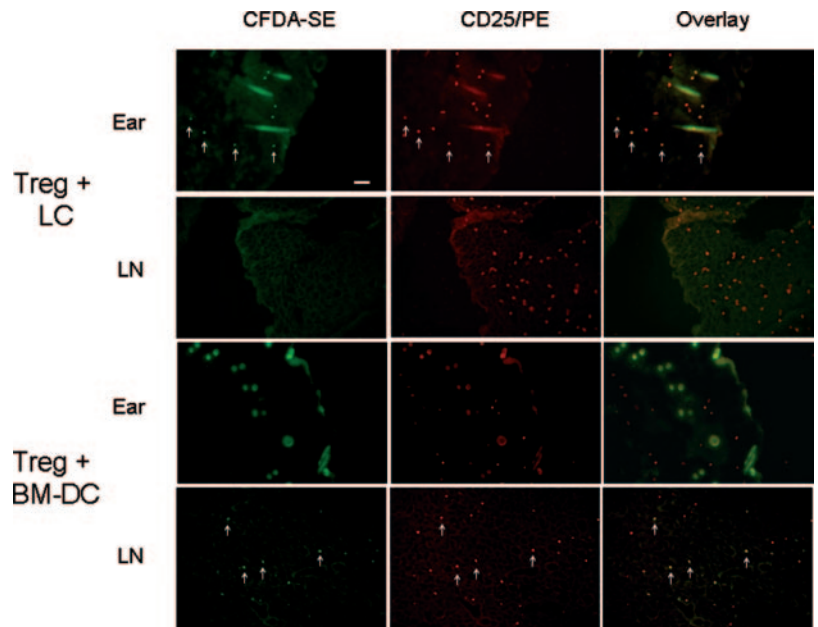


FIGURE 7. Differential alteration of the expression of tissue homing receptors on UV-Treg by LC and BMDC. $CD4^+CD25^+$ Treg were obtained from donors that were tolerized against DNFB by application of DNFB onto UV-exposed back skin. After coincubation with LC (Treg+LC) or BMDC (Treg+BM-DC) for 48 h, cells were stained with purified rat anti-mouse mAb against CD62L (A) or PSGL-1/CD162 (B). Three-color staining was conducted with an allophycocyanin-conjugated Ab against CD4, FITC-conjugated anti-rat IgG, and PE-labeled anti-mouse CD25 Ab. IgG controls for CD62L and PSGL-1 staining are shown in the *left column*. Analysis was performed with an FC 500 CXP FACS Flow Cytometer.

FIGURE 8. In situ detection of UV-Treg upon i.v. injection. UV-Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. UV-Treg were cultured in the presence of LC obtained from the epidermis of naive mice (Treg+LC) or of DC obtained from the bone marrow of naive mice (Treg+BM-DC). After coculture for 48 h, cells were harvested and labeled with 5 μ M CFDA-SE at 37°C for 7 min. A total of 5×10^5 cells were injected i.v. into recipients that had been sensitized against DNFB 5 days earlier. At 24 h after injection, recipient mice were challenged on the left ear. Left ears and lymph nodes of the recipients were obtained and cryosections were cut. These sections were stained with a PE-conjugated Ab directed against CD25. Sections were analyzed under a fluorescence microscope. Arrowheads indicate representative double-positive UV-Treg (Scale bar, 100 μ m).



which were not incubated with BMDC, were included in this experiment. Intracutaneous injection of UV-Treg into the ears of sensitized mice down-regulated the ear swelling response upon challenge, thus confirming previous observations (6). The same degree of suppression was observed, when UV-Treg that were coincubated with BMDC were injected intracutaneously (Fig. 6). This indicates that UV-Treg have retained their suppressive activity despite the coincubation with BMDC. Hence, BMDC do not impair the suppressive activity of UV-Treg. The fact that intracutaneous but not i.v. injection of these cells resulted in suppression suggested that this could be due to differences in the tissue migration of UV-Treg.

LC but not BMDC alter the expression of tissue homing receptors on UV-Treg

The migration behavior of T cells is critically determined by the expression of tissue homing receptors (23). 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 (PSGL-1) (24). 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 in the circulation, they traffic to sites of Ag deposition and inflammation (25, 26). Accordingly, it was recently observed that UV-Treg express CD62L but not the ligands for the skin homing receptors E- and P-selectin (6). This explains that upon i.v. injection UV-Treg migrate into the lymph nodes where they can inhibit the sensitization of naive mice. Due to the lack of the expression of skin homing receptors they do not migrate into the periphery and thus are not able to inhibit the challenge, which in the CHS model takes place in the skin of the ears.

Because UV-Treg incubated with LC inhibit the ear challenge in sensitized mice upon i.v. injection, they should be able to migrate into the periphery. Therefore, we studied whether LC may alter the expression of the tissue homing receptors of UV-Treg. To address this issue, UV-Treg were obtained from UV-exposed and DNFB-

treated mice and isolated for CD4 and CD25 by magnetobead separation. Cells were incubated either with LC or with BMDC. After an incubation period of 48 h the expression of CD62L and PSGL-1 was determined by FACS analysis. Untreated UV-Treg strongly expressed CD62L (Fig. 7A), confirming previous findings (6). Coincubation with LC significantly down-regulated CD62L expression, whereas CD62L expression was not altered upon coincubation with BMDC (Fig. 7A). In turn, untreated UV-Treg did not express PSGL-1 (Fig. 7B). However, upon coincubation with LC but not with BMDC, UV-Treg expressed PSGL-1. Together, this demonstrates that LC shift the tissue homing receptor expression of UV-Treg into a "skin-prone" phenotype. The down-regulation of CD62L and the up-regulation of PSGL-1 might explain why in contrast to untreated UV-Treg, which migrate into the lymph nodes upon i.v. injection, UV-Treg coincubated with LC migrate into the periphery and thus are able to inhibit the effector phase of CHS in sensitized mice, when i.v. injected.

To further confirm the alteration of the migration behavior of UV-Treg, UV-Treg were labeled with CFDA-SE before incubation with either LC or BMDC. Cells were finally injected i.v. into sensitized mice. 24 h after injection ears and lymph nodes cells were obtained. Cryosections were analyzed by immunofluorescence microscopy. In mice injected with UV-Treg coincubated with BMDC, CFDA-SE-labeled cells could be detected in the lymph nodes but not in the ears (Fig. 8). In turn, CFDA-SE-labeled cells were found in the ears but not in the lymph nodes of mice that were injected with UV-Treg coincubated with LC. To consolidate these findings, cryosections were stained with a PE-conjugated Ab directed against CD25. CD25-positive cells could be detected in the ears and lymph nodes of all mice. However, overlay analysis detected double-positive cells only in the ears of mice that received UV-Treg coincubated with LC, and only in the lymph nodes of mice that received UV-Treg coincubated with BMDC. Hence, these data confirm our conclusion that LC can reprogram the migratory behavior of UV-Treg to enter the skin.

Upon coincubation with LC UV-Treg do not inhibit sensitization of CHS

These experiments suggest that epidermal APC in contrast to APC from lymphoid organs alter the migration behavior of UV-Treg,

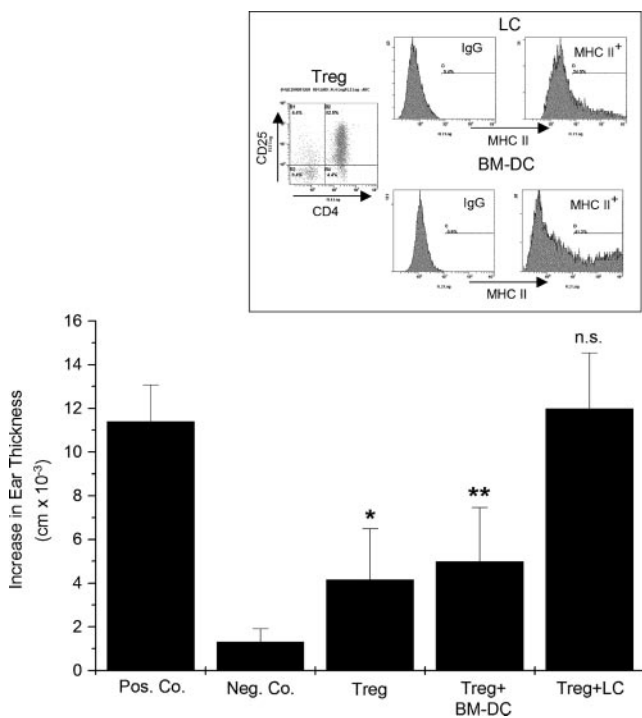


FIGURE 9. UV-Treg coincubated with BMDC but not with LC inhibit the sensitization phase of CHS. $CD4^+CD25^+$ Treg were obtained from UV-tolerized donors by application of DNFB through UV-exposed back skin. $CD4^+CD25^+$ Treg were cultured in the absence (Treg) or presence (Treg+LC) of LC obtained from the epidermis of naive mice or of DC obtained from the bone marrow of naive mice (Treg+BM-DC). After coculture, nonadherent cells were harvested and injected i.v. into naive mice. Recipients were sensitized against DNFB 24 h later. 5 days later recipient mice were challenged on the left ear. Positive control mice were sensitized and challenged (Pos. Co.), whereas negative control animals were only challenged (Neg. Co.). Ear swelling is expressed as the mean \pm SD difference between the thickness (cm \times 10⁻³) of the challenged and that of the vehicle-treated ear. n.s., Not significant. *, $p < 0.0005$ vs positive control; **, $p < 0.001$ vs positive control.

yielding entry into the periphery upon i.v. injection. Therefore, UV-Treg treated in such a way inhibit the effector phase of CHS, which takes place in the periphery. In turn, according to their homing receptor expression pattern, these cells do not enter into the lymph nodes (Fig. 8) because they do not express CD62L. Consequently, this subtype of UV-Treg should not be able to inhibit the sensitization when injected i.v. into naive mice, as sensitization takes place in the lymph nodes. To prove this hypothesis, UV-Treg were coincubated with LC or with BMDC as described. After incubation for 48 h the nonadherent cells of the respective cocultures were injected into naive mice, which were sensitized 24 h after injection. Five days later ear challenge was performed. The CHS response was significantly inhibited in mice that had received UV-Treg coincubated with BMDC, indicating that sensitization was prevented (Fig. 9). In contrast, a profound ear swelling response could be induced in mice that had received UV-Treg which were incubated with LC, suggesting that UV-Treg treated in such a way did not inhibit sensitization. The inability of Treg coincubated with LC to inhibit sensitization can be explained by the fact that due to altered tissue receptor expression they migrate into the periphery but not into the lymph nodes where sensitization takes place.

Discussion

UV radiation has been recognized as a suitable tool to induce Treg. At least in the model of CHS, UV-Treg act in an Ag-specific fash-

ion and inhibit the sensitization when injected i.v. into naive mice. UV-Treg, however, exert suppressive activity only when injected i.v. into naive but not into sensitized mice. Thus, it was concluded that 1) UV-Treg only inhibit the afferent but not the efferent phase of CHS, 2) UV-Treg are not active in the presence of effector T cells, and 3) UV-Treg are inferior to T effector cells (6, 15).

IL-10 has been demonstrated to be able to inhibit both the induction and the elicitation of CHS (27–29). Therefore, if the release of IL-10 is one of the major activities by which UV-Treg mediate their suppressive effects, UV-Treg in principal should be able to inhibit not only the afferent but also the efferent limb of CHS. During sensitization interaction between APC and T cells takes place in the lymph nodes, whereas during elicitation in the area of challenge. Thus, it was postulated that the inability of UV-Treg to inhibit the effector phase of CHS upon i.v. injection is due to the fact that they do not migrate into the skin (6). This hypothesis was confirmed by the finding that UV-Treg injected intracutaneously into the ears of sensitized mice suppressed the effector phase of CHS. This finding indicated that UV-Treg can suppress T effector cells, provided they are at the place of the effector phase of the immune reaction. The absence of UV-Treg in the periphery upon i.v. injection is due to the expression of tissue homing receptors. UV-Treg express CD62L, but not the ligands for the skin homing receptors E- and P-selectin (6).

Thinking in terms of a therapeutic use of UV-Treg, the identification of strategies by which UV-Treg can be reprogrammed to get into the periphery appears to be crucial (30). In this study, we show that coincubation of epidermal LC in the presence of the specific Ag alters the migration behavior of UV-Treg. Upon coincubation with epidermal LC but not with BMDC, UV-Treg suppress the effector phase of CHS when injected i.v. into sensitized recipients. This alteration is associated with a change of the tissue homing receptors expressed on UV-Treg. Upon coincubation with LC, the lymph node homing receptor CD62L is down-regulated and the ligand for P-selectin up-regulated. This pattern of homing receptors might allow the entry into the skin. Accordingly, CFDA-SE-labeled UV-Treg were detected in situ in the skin of the ears upon i.v. injection only when they were coincubated with LC before injection. In turn, CFDA-SE-labeled UV-Treg coincubated with BMDC were found in the lymph nodes upon i.v. injection. The fact that the CFDA-SE-labeled cells detected in situ were indeed UV-Treg was supported by double staining against CD25. This result indicates that the alteration of the expression of the tissue homing receptors of UV-Treg by APC is of functional relevance. We do not yet know the molecular mechanisms by which LC alter the tissue homing receptor expression. Promoter methylation does not appear to be involved in the down-regulation of CD62L because preliminary experiments using the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine did not prevent the down-regulation (data not shown). Although Boyden chamber experiments have not been performed, it is highly likely that cell contact between LC and UV-Treg is required for the alterations because the migration behavior of UV-Treg was not altered upon incubation with supernatants obtained from LC cultures (data not shown).

We can also exclude that the BMDC inhibit UV-Treg in their suppressive activity for two reasons. First, UV-Treg coincubated with BMDC still retained their capacity to prevent the sensitization when injected i.v. into naive mice (Fig. 9). Second, upon coincubation with BMDC UV-Treg still were able to suppress the ear challenge, when injected intracutaneously into the ears of sensitized mice (Fig. 6). The latter experiment further confirms that the tissue distribution might be crucial for UV-Treg to inhibit the effector phase of CHS.

It was striking that only epidermal LC but not APC obtained from lymphoid organs altered the migration behavior of UV-Treg. Neither upon cocubation with BMDC nor with DC obtained from lymph node or spleens, were UV-Treg able to suppress the effector phase of CHS upon i.v. injection. The MHC class II expression was much higher in DC obtained from lymph node or spleens. Thus, one could argue that the changes observed are due to different maturation stages of DC. However, this appears to be unlikely because the MHC class II and B7-2 expression of BMDC was similar to that of the LC used. In addition, BMDC cultured for several days yielding different maturation stages did not convey the capacity to alter the activity of UV-Treg. Thus, we speculate that not the stage of maturation but rather the tissue origin of the DC is primarily responsible for this effect. A similar phenomenon was already observed for T effector cells because skin homing memory T cells stimulated ex vivo could be reprogrammed by intestinal DC to a gut homing phenotype (21). However, it is not yet clear whether the reprogramming of UV-Treg by LC is specific for the skin or whether UV-Treg treated in such a way also enter other peripheral organs like the gut. Studies using DC obtained from the gut are currently under way to address the issue of whether the alteration of the migration behavior of UV-Treg by DC is strictly organ-specific or just dissects the migration to lymphoid organs vs periphery. It recently has been shown that Treg compartmentalization determines the suppressive activity in vivo and might have significant implications for anti-inflammatory therapies targeting recruitment mechanisms (31). Although it remains to be determined whether our observations apply not only for UV-Treg but also for naturally occurring Treg, the present study describes a strategy that can interfere in the compartmentalization process, which may be relevant when using Treg not in a prophylactic but in a therapeutic setting.

Acknowledgments

We are grateful to Martina Wedler, Susanne Dentel, Nadine Tüxen, and Tina Steffen for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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