FoxP3$^+$ regulatory T cells essentially contribute to peripheral CD8$^+$ T-cell tolerance induced by steady-state dendritic cells

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Peripheral T-cell tolerance is thought to significantly contribute to the prevention of autoimmunity, and it has been shown that antigen-presenting steady-state dendritic cells efficiently induce peripheral tolerance. We previously showed that dendritic-cell–induced tolerance is a T-cell–intrinsic process that depends on coinhibitory molecules such as programmed death-1. Here we specifically analyze the involvement of FoxP3$^+$ regulatory T cells, which are known to be important for maintenance of self-tolerance. We show that antigen presentation by steady-state dendritic cells failed to induce peripheral tolerance in the absence of FoxP3$^+$ regulatory T cells but induced protective CD8$^+$ T-cell–mediated immunity instead. Regulatory T-cell–depleted mice had massively increased numbers of dendritic cells in lymph nodes. Dendritic cells isolated from mice without regulatory T cells had up-regulated costimulatory molecules and showed stronger T-cell stimulatory capacity ex vivo, suggesting that regulatory T cells contribute to peripheral tolerance by keeping the dendritic cells in an immature state. Using blocking antibodies, we demonstrate that CTLA-4 but not IL-10 is necessary for control of dendritic cells by regulatory T cells.

Treg | tolerance | DC | CD8$^+$ T cells

Most autoreactive T cells are deleted in the thymus by so-called "negative selection." Although this process is efficient, the presence of autoreactive T cells in every healthy individual demonstrates that it is not complete (1). Peripheral, mature autoreactive T cells are kept in check by peripheral tolerance, which acts through a variety of mechanisms that are not necessarily mutually exclusive and that include unresponsiveness/anergy, regulation/suppression, and deletion.

We and others have recently demonstrated that dendritic cells (DCs) play a central role in the induction of peripheral tolerance (2–4). Using transgenic mice that allow the inducible expression of viral cytotoxic T lymphocyte (CTL) epitopes selectively by DCs (DIETER mice), we showed that presentation of CTL epitopes by steady-state DCs induces robust tolerance in antigen-specific CD8$^+$ T cells. We found that this tolerance depends on signaling via the inhibitory receptors programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) and follows a recessive mechanism such as induction of anergy in, or deletion of, CD8$^+$ T cells specific for the antigens presented by the steady-state DCs. Using adoptive transfer of naive T cells into tolerant mice, we did not find any evidence for involvement of a dominant suppressive mechanism such as the induction of antigen-specific regulatory T cells (Treg cells) or the production of immunosuppressive cytokines. We did not, however, formally address the contribution of Treg cells to peripheral tolerance induced by steady-state DCs in DIETER mice (3, 4).

CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, first characterized by their immunosuppressive properties (5, 6), comprise ≈10–15% of all peripheral CD4$^+$ T cells in mice. The forkhead box transcription factor FoxP3 is the best marker for Treg cells at present (7–11). That Treg cells play a critical role in the control of autoimmunity is illustrated by the fact that loss-of-function mutations in the FoxP3 gene, as well as depletion of Treg cells (12, 13), result in fatal autoimmune disease in humans and mice (14–17). Furthermore, naturally occurring FoxP3$^+$ Treg cells were shown to regulate inflammatory disorders such as colitis and immune responses to transplants, tumors, vaccines, and infectious agents (6, 18, 19).

To investigate the role of FoxP3$^+$ Treg cells in peripheral tolerance of CD8$^+$ T cells, we crossed DIETER mice to DEREG mice, thus generating a system in which FoxP3$^+$ Treg cells can be depleted by injection of diphtheria toxin (DT) (13) and in which peripheral tolerance results from induced antigen presentation by steady-state DC (3). We found that the induction of peripheral tolerance by steady-state DC was severely impaired in the absence of regulatory T cells. Rather than inducing tolerance, antigen presentation by steady-state DCs resulted in priming of a functional CTL response.

Results

Transient Antigen Presentation by Steady-State DCs Primes Protective Immunity in the Absence of FoxP3$^+$ Regulatory T Cells. DIETER and DEREG/DIETER chimeras were injected with tamoxifen (TAM) to induce presentation of lymphocytic choriomeningitis virus (LCMV) $GP_{33–41}$ and β-$gal_{497–505}$ by ≈5% of CD11c^{high} cells (3). Half of the mice received multiple injections with DT that resulted in absence of GFP$^+$ FoxP3$^+$ cells in DEREG/DIETER mice for at least 8 days. We measured the frequency of LCMV $GP_{33–41}$- and β-$gal_{497–505}$-specific T cells 8 days after TAM injection. As expected, we found that antigen presentation by steady-state DCs did not induce measurable expansion of endogenous CD8$^+$ T cells specific for LCMV $GP_{33–41}$ or β-$gal_{497–505}$, and we had shown previously that this treatment induced robust and antigen-specific peripheral tolerance (3). In contrast, TAM injection of DT-treated DEREG/DIETER mice induced LCMV $GP_{33–41}$- and β-$gal_{497–505}$-specific responses (Fig. 1*A*), suggesting that antigen presentation by steady-state DCs results in priming if FoxP3$^+$ Treg cells are absent. To test whether the expanded CD8$^+$ T cells can execute effector function, we chal-

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lenged the four groups of mice with 200 pfu of the LCMV strain WE at day 8 and measured splenic viral titers 5 days later. We found no evidence for protective immunity in any of the experimental groups except for DEREG/DIETER mice treated with TAM and DT, which had a significantly lower virus load in their spleens (Fig. 1*B*).

Transient Depletion of FoxP3$^+$ Regulatory T Cells Changes the Phenotype and Number of DCs in Lymph Nodes. We have shown previously that antigen-presenting steady-state DCs induce peripheral tolerance of CD8$^+$ T cells. Therefore, mechanisms that impede DC maturation may contribute to self-tolerance, and there is experimental evidence that Treg cells interfere with DC maturation (11, 20). To test whether the short-term specific depletion of FoxP3$^+$ cells resulted in phenotypic changes of endogenous DCs in our model system, we depleted Treg cells from DEREG mice during 5 days and analyzed the surface expression of costimulatory molecules associated with DC maturation such as CD40, CD70, CD80, and CD86 and of the coinhibitory molecules PD-L1 and PD-L2 on ex vivo isolated DCs from peripheral and mesenteric lymph nodes and spleens. We found a significant up-regulation of the costimulatory molecules CD40, CD80, and CD86 but surprisingly also of the coinhibitory molecules PD-L1 and PD-L2 on DCs upon depletion of FoxP3$^+$ Treg cells (Fig. 2*A*). In addition to displaying a more activated phenotype, the number of CD11c$^+$ cells in peripheral and mesenteric lymph nodes, but not in the spleen, also increased significantly in the absence of FoxP3$^+$ Treg cells (Fig. 2*B*).

To ascertain that the observed phenotypic up-regulation of activation markers reflected a biologically relevant functional change in DC activation state, we sought to directly assess the ex vivo T-cell stimulatory capacity of the DCs isolated from Treg-cell–depleted DEREG mice. DCs were isolated from pooled peripheral lymph node cells from DT-treated DEREG mice and nontransgenic littermates by magnetic cell sorting of CD11c-expressing cells. Purified DCs were used to stimulate allogenic BALB/c responder T cells purified by magnetic sorting against CD90.2, and proliferation was monitored by ^{3}H-thymidine incorporation. DCs isolated from Treg-cell–depleted animals were significantly better at stimulating an allogenic response (Fig. 2*C*), indicating that depletion of regulatory T cells in vivo results in a functionally relevant activation of DCs that can explain their relative inability to induce peripheral tolerance.

Control of DC Activation and Peripheral CD8$^+$ T Cell Tolerance by Regulatory T Cells Depends on CTLA-4 but Not on IL-10. To gain insight into how Treg cells impact on DC-induced peripheral CD8 T cell tolerance, we blocked the action of two molecules that are critically involved in suppression by Treg cells, namely IL-10 and CTLA-4 (21, 22). Treatment of DIETER mice with TAM plus an antibody that blocks the IL-10 receptor (IL-10R, clone 1B1.3a) did not result in priming of CD8$^+$ T cells (Fig. 3*A*), which makes it unlikely that Treg cells control DC-induced peripheral tolerance through IL-10. As expected, DIETER mice treated with TAM plus anti-IL-10R were not protected against a subsequent challenge infection with LCMV (Fig. 3*B*), which we used as a readout for CTL priming. In contrast, blocking CTLA-4 signaling upon induction of antigen presentation by steady-state DC (TAM plus anti-CTLA-4) mirrored the effect of Treg cell depletion: A considerable number of LCMV GP$_{33–41}$-specific CD8$^+$ T cells were primed (Fig. 3*A*), which protected mice against a subsequent challenge infection with LCMV (Fig. 3*B*), suggesting that Treg cells control DC-induced peripheral tolerance at least partially through CTLA-4 interactions. Because depletion of Treg resulted in increased DC numbers and in phenotypic and functional DC maturation (Fig. 2), we tested whether blocking of CTLA-4 or the IL-10R had the same effect. Blocking of the IL-10R did not result in changes of the DC maturation status, but we observed a trend of increased numbers, which was not significant (Fig. 3*C*). Because the IL-10R blockade did not affect DC-induced peripheral tolerance (Fig. 3, *A* and *B*), this result was expected. Blocking of CTLA-4, however, induced a significantly higher expression of CD80 on DC and a trend of increased numbers (Fig. 3*C*). The expression of other surface molecules including CD86, CD40, and CD70 as well as PD-L1 and PD-L2 was higher after anti-CTLA-4 treatment in some, but not in all experiments (Fig. 3*C*).

Discussion

We have previously shown that antigen presentation by steady-state DCs did not induce measurable expansion of endogenous, antigen-specific CD8$^+$ T cells, but instead resulted in robust peripheral tolerance (3). Interestingly, when this happened in the absence of Treg cells, endogenous LCMV GP$_{33–41}$-specific CD8$^+$ T cells expanded to 1–2% of total CD8$^+$ T cells and mediated an ≈10-fold reduction of virus titers after LCMV challenge. This response, however, is considerably weaker than that primed by mature DCs, in which case 10–20% LCMV GP$_{33–41}$-specific CD8$^+$ T cells and full protection to subsequent LCMV challenge were observed (3). Furthermore, when PD-1 interactions were blocked, 8–12% LCMV GP$_{33–41}$-specific CD8$^+$ T cells and full protection to subsequent LCMV challenge were observed (4). However, the response in the absence of Treg cells is comparable in magnitude to the response observed when only CTLA-4 interactions were blocked. This is of particular interest in the light of experiments demonstrating a crucial role of CTLA-4 in the suppressive function of Treg cells (22–25).

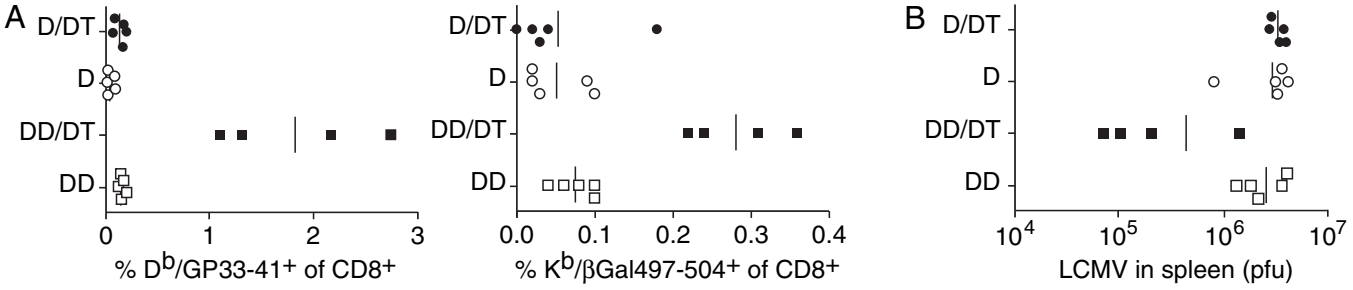

Fig. 1. Antigen presentation by steady-state DCs induces protective immunity instead of tolerance in the absence of FoxP3$^+$ Treg cells (*A*) DIETER (D) and DEREG/DIETER (DD) mice were injected i.p. with 1 μg DT or PBS on days −1, 1, 3, and 5 and with 2 mg TAM on day 0. LCMV GP$_{33–41}$/D^b- and β-Gal$_{497–505}$/K^b-specific CD8$^+$ T cells were quantified in the blood on day 8 by staining with MHC class I tetramers. Each point in the graph represents an individual mouse and a representative experiment of three is shown. Student's *t* test: tetramer GP$_{33–41}$/D^b: DD vs. DD/DT, $P = 0.0017$; β-Gal$_{497–505}$/K^b: DD vs. DD/DT, $P = 0.0003$; all other comparisons: not significant (NS). (*B*) Mice depicted in *A* were challenged on day 8 with 200 pfu LCMV–WE and the splenic virus titers were determined on day 13 using a focus-forming assay. Each symbol represents an individual mouse. One representative experiment of two is shown. Student's *t* test: DD vs. DD/DT, $P = 0.0148$; all other comparisons: NS.

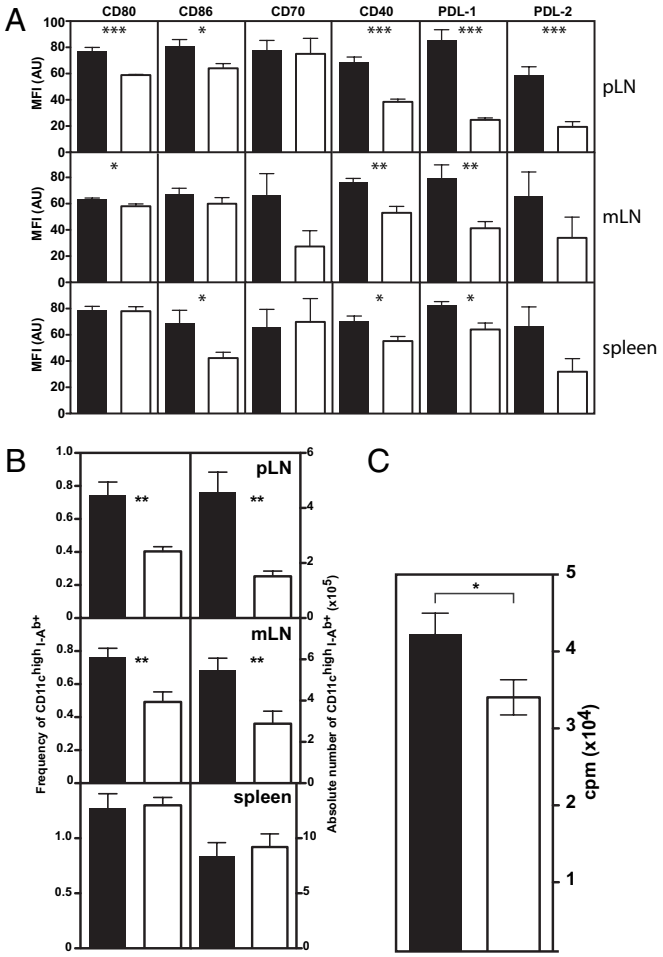

Fig. 2. Depletion of FoxP3$^+$ Treg cells changes the phenotype, numbers, and T-cell stimulatory capacity of DCs in vivo. Age- and sex-matched DEREG mice and wild-type littermates (six mice per group) were injected i.p. with 1 μg DT or with PBS on days 0 and 2 and spleens, peripheral lymph nodes (pooled inguinal, brachial, axillary, and submandibular), and mesenteric lymph nodes were removed on day 5, digested with collagenase and DNaseI, stained with appropriate antibodies, analyzed by FACS or purified by magnetic sorting, and used as stimulator cells in an allogenic T-cell proliferation assay. (*A*) Median fluorescence intensities (MFI) for CD80, CD86, CD70, CD40, PD-L1, and PD-L2 of CD11c^{high}I-A^{b+} DCs from peripheral lymph nodes (*Upper*), mesenteric lymph nodes (*Middle*), and spleens (*Lower*) of DT-treated DEREG (solid bars) and wild-type littermates (open bars). (*B*) The frequency (*Left*) and total number (*Right*) of I-A^{b+} CD11c^{high} in peripheral lymph nodes (*Upper*), mesenteric lymph nodes (*Middle*), and spleen (*Lower*) were determined. (*C*) Purified T cells from BALB/c mice were stimulated with titrated numbers of DCs purified from DT-treated DEREG mice (solid bars) or wild-type littermates (open bars) at a T:DC cell ratio of 4. *P* values (Student's *t* test): $***P < 0.0005$, $**P < 0.005$, $*P < 0.05$. One of three independent experiments is shown.

Several mechanisms by which Treg cells suppress T-cell responses have been proposed (reviewed in ref. 26): Tregs may outcompete effector T cells for access to antigen-presenting DCs; they may directly interact with conventional T cells, thus inactivating or eliminating them; or they may modulate the activation state and function of DCs. It was shown that, upon short contact with Treg cells, DCs down-regulate costimulatory molecules (20, 27), up-regulate IL-10 (28) and the coinhibitory molecule B7-H3, and are compromised in the stimulation of naive T cells (27). The suppressive impact of Treg cells on steady-state DCs can largely be overruled by inflammatory stimuli such as lipopolysaccharides (11, 28, 29), which is consistent with the fact that pathogens usually induce strong immune responses despite the presence of regulatory mechanisms. Most of these studies used in vitro cocultures of DCs and Treg cells, in which the ratio Treg:DC as well as the duration of contact presumably exceeded those found under physiological circumstances. A recent in vivo study, however, confirmed the Treg cell–mediated impact on the phenotype and the function of DCs (27). In addition, a recent study using DT-mediated depletion of FoxP3$^+$ cells in a transgenic model similar to the DEREG mice used here, described up-regulation of CD40 and CD80 on DC and an increase in numbers of DCs in secondary lymphoid organs in the context of autoimmunity following Treg cell depletion (12). We show here that depletion of Treg cells resulted in a significant up-regulation of costimulatory molecules such as CD40, CD80, and CD86 on DCs as well as a twofold increase of their number in secondary lymphoid organs. Increased migration as well as increased proliferation of DCs or their precursors may account for the increase of DCs in lymph nodes upon Treg cell depletion, and there is evidence for both. It has been suggested that Treg cells inhibit the production of CCR5 ligands, thus limiting the CCR5-dependent recruitment of DC to the lymph nodes (30). A recent study has clearly demonstrated that Treg cell depletion results in increased division of DC and precursor DC in lymphoid organs, although the molecular mechanism through which Treg cells keep the numbers of DCs in check remains to be identified (31). The observed increase in DC numbers following Treg cell depletion will presumably result in an increased number of antigen-presenting DCs upon TAM injection of DEREG/DIETER mice. But it is unlikely that this mere increase in the number of antigen-presenting DCs is the reason for priming instead of tolerance induction in the absence of Treg cells, because injection of higher doses of TAM (up to 6 mg), which also leads to an increase in the number of antigen-presenting DCs, does not induce priming.

We had previously shown that peripheral CD8$^+$ T-cell tolerance induction following the presentation of CD8$^+$ T-cell epitopes on steady-state DCs is a T-cell–intrinsic phenomenon involving deletion or anergy of the T cells recognizing their antigen on steady-state DCs (4). Antigen presentation in the absence of DC activation did not lead to a dominant suppressive tolerance that affected T cells of other specificities, which made the induction of regulatory T cells by the steady-state DC unlikely. This is difficult to reconcile with the results presented here at first glance but may be explained by multiple levels or mechanisms of peripheral tolerance induction, which probably operate in concert. DCs constitutively express ligands for coinhibitory molecules such as CTLA-4 and PD-1, which are expressed at a low level on naive T cells, and the result of the interaction between naive T cells and DCs under steady-state conditions usually is tolerance. Inflammatory stimuli induce DC maturation, and the costimulatory interactions will overrule the inhibitory signals, resulting in T-cell activation. If naive T cells scan DCs in the steady state, they will contact many resting DCs that transmit inhibitory signals, but also a few activated or mature DCs that are presumably present in every individual. But, because the inhibitory signals outnumber the activating ones, tolerance ensues, except in situations in which the inhibitory interactions are prevented, as we have shown before (4). Our data suggest that Treg cells act on a different level, keeping the DCs in an immature state or increases the threshold for DC activation and that Treg cells employ CTLA-4 but not IL-10 to execute this effect. At present it is unclear whether this results from a direct interaction between DCs and Treg or whether the absence of Treg allows activation of other cell types, which then modulate the DC phenotype.

In addition to CTLA-4 and IL-10, TGF-β was shown to be involved in peripheral T-cell homeostasis and tolerance (reviewed in ref. 32). For example, in vitro and in vivo experiments showed that Treg-cell–mediated immunosuppression depends on TGF-β-Receptor-II expression by CD4$^+$ and CD8$^+$ T cells (33–35). Furthermore, activation of the biologically inactive TGF-β-precursor by $\alpha_v\beta_8$ integrin-expressing DCs was found to be required for TGF-β-mediated immunosuppression (36). This suggests that the cooperation between DCs and Treg cells in peripheral tolerance induction may involve the activation of

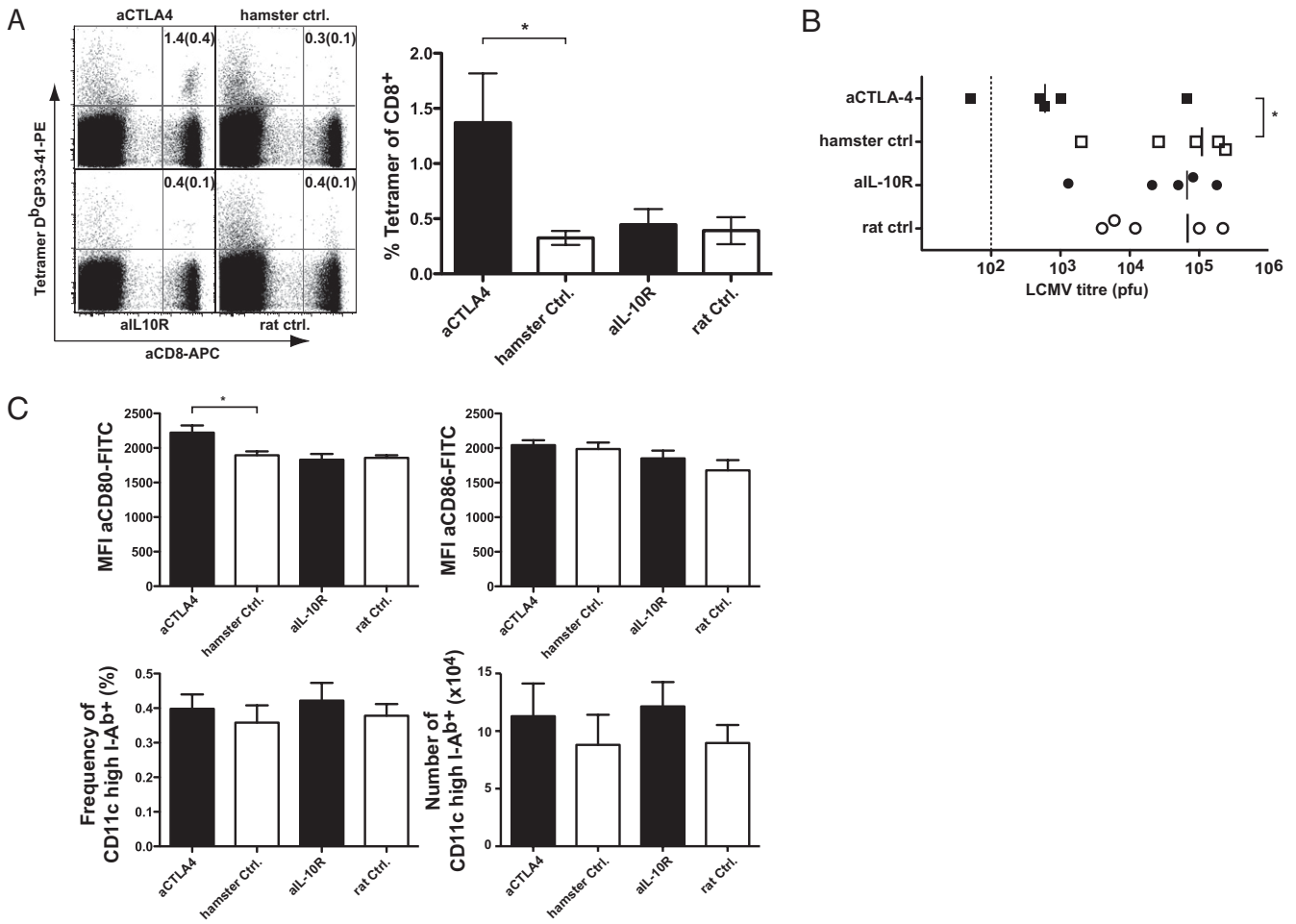

Fig. 3. CTLA-4 but not IL-10 is involved in Treg cell-mediated suppression of DC activation and CTL priming in the immunological steady state. DIETER mice were injected with 2 mg TAM plus blocking antibodies against CTLA-4 or IL-10R or respective control antibodies. (*A*) LCMV $GP_{33–41}/D^b$-specific $CD8^+$ T cells were quantified in the blood on day 8 by staining with MHC class I tetramers. (*B*) Mice depicted in *A* were challenged on day 8 with 200 pfu LCMV–WE and the splenic virus titers were determined on day 13 using a focus-forming assay. (*C*) To determine whether the blockade of CTLA-4 or the IL-10R resulted in DC activation, C57BL/6 mice were injected i.v. with anti-CTLA-4 or anti-IL-10R or the respective control antibodies. Three days later, activation state and numbers of DC were assessed by flow cytometry. Student's *t* test (*A* and *C*) or Mann–Whitney test (*B*): $*P < 0.05$. One representative of three independent experiments is shown.

Treg-cell–derived TGF-β-precursor molecules by $\alpha_v\beta_8$-expressing DCs. In addition, in vitro data with CTLA-4-deficient Treg cells suggest that TGF-β expression by Treg can compensate for the lack of CTLA-4 (37).

Our data indicating that the CTLA-4 blockade impairs the induction of $CD8^+$ T-cell tolerance by steady-state DCs to a similar extent as Treg cell depletion do not unambiguously prove the crucial role of CTLA-4 for Treg cell function, because we cannot exclude the involvement of coinhibitory signals through CTLA-4 expressed by $CD8^+$ T cells. However, the suggestion that the role of CTLA-4 in $CD8^+$ T-cell tolerance is linked mainly to Treg cells is supported by a recent study showing that proliferation and activation of CTLA-4-deficient $CD8^+$ T cells, upon transfer in lymphopenic animals, are effectively suppressed by CTLA-4 competent Treg cells (25).

Taken together, we have shown that $FoxP3^+$ regulatory T cells are required for efficient induction of $CD8^+$ T-cell tolerance upon presentation of $CD8^+$ T-cell epitopes on resting DCs. In the absence of Treg cells, DCs had an activated phenotype and were present in increased numbers in secondary lymphoid organs. Thus, Treg cells seem to be required to maintain the nonactivated phenotype of DCs that is essential for peripheral T-cell tolerance.

Materials and Methods

Mice. DIETER double transgenic mice allow TAM-inducible presentation of three LCMV-derived CTL epitopes ($GP_{33–41}/D^b$, $GP_{34–41}/K^b$ and $NP_{396–404}/D^b$) and one β-galactosidase-derived CTL epitope ($\beta\text{-gal}_{497–504}/K^b$) by $CD11c^{high}$ cells (DCs)(3). DIETER mice were bred with DEREG (13) mice that allow DT-mediated depletion of $FoxP3^+$ Treg cells. All mice were generated on a C57BL/6 background. DEREG, DIETER, and C57BL/6 mice were obtained from the Institute of Laboratory Animal Science (University of Zurich) or from the central animal facility of the Johannes Gutenberg University of Mainz and were bred and maintained under specific pathogen-free conditions. Experiments were performed with age- and sex-matched mice and were carried out in accordance with the Swiss federal and cantonal laws on animal protection or with permission of the Landesuntersuchungsamt Rheinland-Pfalz. To increase the number of available DIETER and DEREG/DIETER mice, bone marrow chimeras were generated as described (3).

LCMV. LCMV strain WE (LCMV–WE) was propagated on L929 fibroblast cells at a low multiplicity of infection. LCMV titers were determined in spleen 5 days after infection as described (38).

Treatment of Mice. Cre recombinase activity resulting in antigen presentation by steady-state DCs was induced in vivo by injecting DIETER or DEREG/DIETER mice i.p. with 2 mg TAM as described (3). $FoxP3^+$ cells were depleted from DEREG/DIETER mice by i.p. injection of 1 μg DT (Sigma Chemical) in 100 μL PBS at day −1, +1, +3, and +5 relative to TAM injection or with PBS as control. This

regimen depleted GFP$^+$FoxP3$^+$ cells from DEREG/DIETER mice to undetectable levels at least until day 8.

In some experiments, DIETER mice were injected with 2 mg TAM i.p. together with antibodies blocking CTLA-4 or the IL-10 receptor, as follows. Mice were injected i.v. with 500 μg hamster–anti-mouse CTLA-4 (clone 4F10) or with 500 μg hamster–anti-DNP (clone UC8-1B9) as a control, or alternatively, with 250 μg rat–anti-mouse IL-10R (clone 1B1.3a) or with 250 μg rat–anti-LCMV nucleoprotein (clone VL4) as control in PBS. All antibodies were purified from hybridoma culture supernatants using a HiTrap Protein G Sepharose HP column (GE Healthcare).

Priming of endogenous, $GP_{33–41}/D^b$- and $\beta\text{-gal}_{497–504}/K^b$-specific CD8$^+$ T cells was measured 8 days after TAM injection. Functionality of $GP_{33–41}/D^b$-specific CD8$^+$ T cells was measured by their capacity to protect against a subsequent i.v. infection with 200 pfu LCMV–WE.

Peptides. LCMV-derived peptides $GP_{33–41}$ (KAVYNFATC, H-2D^b) and β-galactosidase-derived peptide $\beta\text{-gal}_{497–504}$ (ICPMYARV) were purchased from NeoMPS in immunograde quality.

Staining with Tetrameric MHC Class I–Peptide Complexes. Tetrameric peptide–MHC complexes were generated and staining was performed as described (3, 39). Samples were measured with a FACS Calibur (Becton Dickinson) and analyzed using FlowJo Analysis Software (Tree Star).

DC Isolation and Phenotyping. DEREG or C57BL/6 mice were injected i.p. with 1 μg DT or with PBS at day 0 and 2 or with blocking antibodies at day 2. Spleens, peripheral lymph nodes (pooled inguinal, brachial, axillary, and submandibular lymph nodes), and mesenteric lymph nodes were removed at day 5 and were digested with collagenase II (Worthington)/DNaseI (Sigma-Aldrich). Single-cell suspensions were counted, incubated with anti-CD16/CD32 (0.5 μg/mL) to block FcγR, and stained for CD80-FITC (clone 16-10A1), CD86-APC (clone GL1), CD70-PE (clone FR70), PDL-1-PE, PDL-2-FITC, CD40-APC, or the respective isotype controls, plus CD11c-PE-Cy7 (clone HL3) plus H-2I-A^b-biotin (clone AF6-120.1), followed by streptavidin-APC-Cy7 in 25 μL FACS buffer for 20 min at 4°C. All antibodies were obtained from Becton Dickinson or eBioscience. Cells were washed twice and analyzed by flow cytometry (FACS Canto, Becton Dickinson) using FlowJo Analysis Software (Tree Star). Median Fluorescence Intensities (MFI) were calculated and the MFI of the isotype controls was subtracted.

Allogenic T-Cell Stimulation Assay. DCs were isolated as described above, incubated with anti-CD16/CD32 (0.5 μg/mL) to block FcγR followed by magnetic enrichment using CD11c-microbeads and MS columns (Miltenyi). Responder T cells were isolated from BALB/c spleens using CD90.2 microbeads and LS columns (Miltenyi).

DC and 10^4 allogenic responder T cells were incubated in T:DC cell ratios from 1 to 32 in round-bottom 96-well plates in RPMI 1640 culture medium (Gibco) supplemented with 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 10 IU penicillin, 100 μg/mL streptomycin, and 10% FCS. After 96 h, [^{3}H]-thymidine was added to the cultures (0.5 μCi/well). [^{3}H]-Thymidine uptake was assessed by scintillation counting after an additional 18 h of culture.

Statistical Analysis. Statistical analysis was performed with the Student's *t* test or Mann–Whitney test using Prism 4 software (GraphPad Software).

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