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# Lentiviral-Mediated Transcriptional Targeting of Dendritic Cells for Induction of T Cell Tolerance In Vivo<sup>1</sup>

Christiane Dresch,<sup>2,3\*</sup> Stephanie L. Edelmann,<sup>2\*</sup> Peggy Marconi,<sup>†</sup> and Thomas Brocker<sup>4\*</sup>

**Dendritic cells (DCs) are important APCs able to induce both tolerance and immunity. Therefore, DCs are attractive targets for immune intervention. However, the ex vivo generation and manipulation of DCs at sufficient numbers and without changing their original phenotypic and functional characteristics are major obstacles. To manipulate DCs in vivo, we developed a novel DC-specific self-inactivating lentiviral vector system using the 5' untranslated region from the DC-STAMP gene as a putative promoter region. We show that a gene therapy approach with these DC-STAMP-lentiviral vectors yields long-term and cell-selective transgene expression in vivo. Furthermore, transcriptionally targeted DCs induced functional, Ag-specific CD4 and CD8 T cell tolerance in vivo, which could not be broken by viral immunization. Tolerized CTL were unable to induce autoimmune diabetes in a murine autoimmune model system. Therefore, delivering transgenes specifically to DCs by using viral vectors might be a promising tool in gene therapy. *The Journal of Immunology*, 2008, 181: 4495–4506.**

**D**endritic cells (DCs)<sup>5</sup> are specialized APCs located in peripheral and lymphoid tissues, where they sample environmental Ags and sense inflammatory signals via pattern recognition receptors (1, 2). In response to microbial stimuli and as part of their normal turnover, DCs leave tissues and migrate to draining lymphoid organs, where they present peptides from peripheral Ags in the context of MHC molecules to T lymphocytes.

Inflammatory signals trigger DCs to “mature” and up-regulate MHC and costimulatory molecules, thereby increasing their Ag presentation capacities for efficient T cell priming (1). As a consequence, DCs are able to induce protective Ag-specific T cell responses (1). In the absence of inflammation and foreign Ags, DCs present peptides derived from self-Ags. Under such noninflammatory conditions DCs are not fully “mature” and serve to induce tolerance of self-reactive T cells. This contributes to protection from autoimmunity by functional inactivation of potentially harmful T cells (3, 4).

Inactivation of Ag-specific T cells would be beneficial to treat autoimmune disease, transplant rejection, and allergies. Accordingly, the injection of Ag-loaded immature DCs into humans was

shown to functionally inhibit Ag-specific T cells by inducing regulatory T cells (5, 6). However, functional T cell tolerance was rather short-lived and fully functional Ag-specific T cells reappeared 6 mo after the DC treatment (5). In different approaches, DCs were genetically modified by transfection with viral or nonviral vectors to introduce genes encoding for Ags or molecules capable of modulating DC functions (7, 8). However, ex vivo transfection or transduction may induce DC maturation and therefore neutralize their capacities to induce T cell tolerance (9). In addition, transfer of ex vivo-manipulated DCs will not allow long-term treatments due to the restricted DC life span after transfer (10).

Induction of T cell tolerance was also achieved by targeting Ag to DCs in vivo with Ags coupled to DC-specific Abs (11, 12). However, different DC subsets have different capacities to process and present Ags to CD4 or CD8 T cells. Accordingly, the outcome of Ag-targeting approaches depends on the DC subtype targeted (13). In addition, to achieve tolerance of cytotoxic CD8 T cells, Ags need to be presented for longer time periods and single-dose applications are inefficient in this context (14).

DCs also contribute substantially to central tolerance induction in the thymus. There they present self-Ags to developing thymocytes, deleting those with high-avidity TCRs specific for self-Ags (15). Thymic DCs acquire self-Ags from thymic epithelial cells (16) and are involved in the thymic generation of regulatory T cells (17).

Taken together, manipulation of DCs for tolerance induction seems to be an attractive approach, especially if long-term T cell tolerance can be achieved. We therefore set out to develop a self-inactivating (SIN) lentiviral system that allows transcriptional targeting of DCs in vivo to achieve stable and long-lasting expression of Ag at levels sufficient for tolerization of CD4 and CD8 T cells. We demonstrate that DC-specific expression of Ags leads to both elimination and functional inactivation of CD4 and CD8 T cells in peripheral organs and thymus. In bone marrow (BM) chimeras generated with such a DC-specific lentiviral vector, expression of Ag is long-lasting, tolerance is induced and cannot be broken by immunization.

## Materials and Methods

### Mice

C57BL/6, OT-I, OT-II, and RIP-OVA<sup>low</sup> mice were maintained and bred in the animal facility of the Institute for Immunology (Munich, Germany). OT-I and OT-II mice have transgenic V $\alpha$ 2/V $\beta$ 5 TCRs specific for

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<sup>5</sup> Abbreviations used in this paper: DC, dendritic cell; SIN, self inactivating; eGFP, enhanced GFP; MOI, multiplicity of infection; HSC, hematopoietic stem cell; BM, bone marrow; RIP, rat insulin promoter; gB, glycoprotein B.

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OVA<sub>257–264</sub>/H2-K<sup>b</sup> and OVA<sub>323–329</sub>/I-A<sup>b</sup>, respectively (18, 19). RIP-OVA<sup>low</sup> mice express OVA under control of the rat insulin promoter (20). All animal experiments have been approved by the ethical committee of the state of Bavaria.

### Lentiviral constructs

To generate DC-STAMP-eGFP, the DC-STAMP promoter was amplified by PCR from total genomic DNA of C57BL/6 mice using specific oligonucleotide primers (5'-GCTGAGAGGCCTGAAAACAC-3' and 5'-CAGAGACTTTTAAACCTGTCTTCT-3') to amplify a 2552-bp fragment. The latter was digested with *Bbs*I, resulting in a product of 1704 bp covering the region between -1565 bp and +131, considering +1 as the first bp of transcription initiation of DC-STAMP. This fragment was digested with *Pst*I, blunt-ended with Klenow enzyme, and digested with *Age*I for cloning into FUGW (21). As control for lentiviral treatment (control virus), eGFP was removed from the original FUGW. The vector was digested with *Xba*I and *Age*I, blunt-ended with Klenow enzyme, and religated. To obtain the lentiviral vector encoding the membrane-bound form of OVA, the eGFP cDNA in DC-STAMP-eGFP was replaced with a chimeric transferrin receptor OVA cDNA (22) creating DC-STAMP-OVA.

### Generation and titration of lentiviral vector stocks

To generate lentiviral vector stocks, 293T cells were transfected by standard calcium phosphate transfection. Briefly,  $6 \times 10^6$  cells were plated 18 h before transfection with 20  $\mu$ g of vector-DNA, 15  $\mu$ g of pCMV $\Delta$ R8.2, and 10  $\mu$ g of pMD2G (VSV-G). Supernatants were routinely generated 24–48 h after transfection by overnight incubation in 293T growth medium at 37°C. Vector stocks were filtered (0.45- $\mu$ m filter; Nalgene) before use. The virus titer was determined by spin infection ( $300 \times g$ , 2 h, 32°C) of NIH3T3 cells with serial dilutions of virus-containing supernatant in the presence of 8  $\mu$ g/ml polybrene followed by genomic DNA purification (DNeasy Tissue Kit; Qiagen) and real-time quantitative PCR. In brief, the virus backbone was amplified using specific primers (5'-TGAAAGCGAAAGGGAAACCA-3' and 5'-CCGTGC GCGCTTCAG-3') and the single-copy housekeeping gene *Bdnf* was also amplified (5'-ACGACATCACTGGCTGACAC-3' and 5'-CATAGACAT GTTGGCGCATC-3'). Each sample was measured in duplicates using SYBR Green I (Roche). Standard curves were generated using serial dilutions of DNA from a plasmid containing the region amplified with the primers described above.

### BM chimeras

BM cells of at least 6-wk-old C57BL/6, OT-I, or OT-II mice were harvested 4 days after i.v. injection of 5-fluorouracil (150 mg/kg body weight; Amersham Pharmacia). The cells were prestimulated for 2 days in serum-free Stemline hematopoietic stem cell expansion medium (Sigma-Aldrich) supplemented with penicillin-streptomycin (Life Technologies and Invitrogen) and a growth factor mixture containing human IL-6 (25 ng/ml), murine IL-3 (10 ng/ml), and murine stem cell factor (50 ng/ml). Recombinant growth factors were purchased from Strathmann Biotech. Cells were transduced by spin infection ( $300 \times g$ , 2 h, 32°C) with cell-free stocks of lentiviral vectors in the presence of protamine sulfate (4  $\mu$ g/ml). If desired, the transduction procedure was repeated 20–26 h after the first round. After the final transduction,  $1\text{--}3 \times 10^6$  cells/mouse were injected i.v. in lethally irradiated (550 rad, days -2 and 0) C57BL/6 recipients. When OT-I mice were the BM donors, CD8<sup>+</sup> cells were depleted by magnetic sorting before injection.

### Analysis of transgene expression

Expression of GFP was measured by flow cytometry combined with mAbs specific for mouse as well as streptavidin reagents, all purchased from BD Biosciences/BD Pharmingen, Caltag Laboratories, or eBioscience). H-2K<sup>b</sup>/OVA<sub>257–264</sub> and H-2K<sup>b</sup>/HSVgB<sub>498–505</sub> tetramers were purchased from Pro-Immune. Flow cytometry was performed on a FACSCalibur (BD Biosciences) instrument and analyzed with CellQuest (BD Biosciences) or FlowJo software (Tree Star). For flow cytometry, organs were prepared as single-cell suspensions according to standard protocols. For OVA expression analysis, total RNA was extracted using a micro- to midi-RNA extraction kit (Invitrogen) from CD11c<sup>+</sup> or CD11c<sup>-</sup> DCs isolated from the spleen or thymus of DC-STAMP-OVA or control virus chimeras by positive selection using CD11c microbeads (Miltenyi Biotec), and one-step RT-PCR was performed (SuperScript One-Step; Invitrogen) with the primers OVA forward 5'-CGT GGA TTC TCA AAC TGC AA-3' and reverse 5'-GAC TTC ATC AGG CAA CAG CA-3' amplifying a product of 317 bp. For  $\beta$ -actin, the mouse/rat  $\beta$ -actin PCR primer pair (R&D Systems)

amplified a product of 302 bp from RNA and a product of 766 bp from genomic DNA, serving as a control for DNA contamination.

### In vivo cytotoxic T cell assay

C57BL/6 erythrocyte-depleted splenocytes were incubated in the presence or absence of 10  $\mu$ M OVA<sub>257–264</sub> peptide or HSVgB<sub>498–505</sub> peptide for 2 h at 37°C and 5% CO<sub>2</sub>. Peptide-loaded cells were labeled with a high (1.7  $\mu$ M) concentration of CFSE (Molecular Probes), whereas unloaded cells were labeled with a low concentration (0.2  $\mu$ M). Equal numbers of CFSE<sup>high</sup> and CFSE<sup>low</sup> cells were mixed and  $20 \times 10^6$  cells/mouse were administered i.v.; 15–18 h later, mice were sacrificed and spleen cell suspensions were analyzed by flow cytometry.

### In vivo and in vitro T cell proliferation assay

For the in vivo assay, mice received the indicated number of OT-I T cells isolated from spleen and lymph nodes of OT-I Thy1.1 mice by negative selection using the MACS CD8-T cell isolation kit (Miltenyi Biotec). T cells were labeled with 5  $\mu$ M CFSE (Molecular Probes). Positive B6 controls received rIgG $\alpha$ OVA-OVA immunocomplexes. The complexes were formed with 25  $\mu$ g of rIgG $\alpha$ OVA (Valeant Pharmaceuticals) and 1  $\mu$ g of OVA (Sigma-Aldrich) during 30 min at 37°C. After 3 days, mice were sacrificed and spleen cell suspensions were analyzed by flow cytometry. For the in vitro proliferation assay, DCs were isolated from spleen by positive selection using CD11c microbeads (Miltenyi Biotec). DCs were cultured with OT-I T cells labeled with 2.5  $\mu$ M CFSE at 37°C and 5% CO<sub>2</sub>. Cells were analyzed by flow cytometry after 3 days.

### Diabetes induction in RIP-OVA<sup>low</sup> mice

RIP-OVA<sup>low</sup> mice received  $1 \times 10^6$  OT-I T cells isolated from spleen and lymph nodes of OT-I mice by negative selection using a MACS CD8-T cell isolation kit (Miltenyi Biotec) and were immunized the next day with rIgG $\alpha$ OVA-OVA (or rIgG in the mock controls) immunocomplexes and 20  $\mu$ g/mouse of CpG nucleotides (InvivoGen). The complexes were formed with 25  $\mu$ g of rIgG $\alpha$ OVA (Valeant Pharmaceuticals) and 1  $\mu$ g of OVA (Sigma-Aldrich) during 30 min at 37°C. The level of glucose in urine was measured with test sticks (Diabur; Roche Diagnostics) before and after immunization. Mice with glucose concentrations  $>5.6$  mmol/L were considered diabetic.

### Intracellular cytokine staining

Splenocytes ( $10 \times 10^6$ ) were restimulated in 1 ml with 10  $\mu$ g of SIINFEKL in the presence of 2  $\mu$ l of GolgiPlug (BD Biosciences) for 4 h. Intracellular staining for IFN- $\gamma$  and TNF- $\alpha$  was performed using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol.

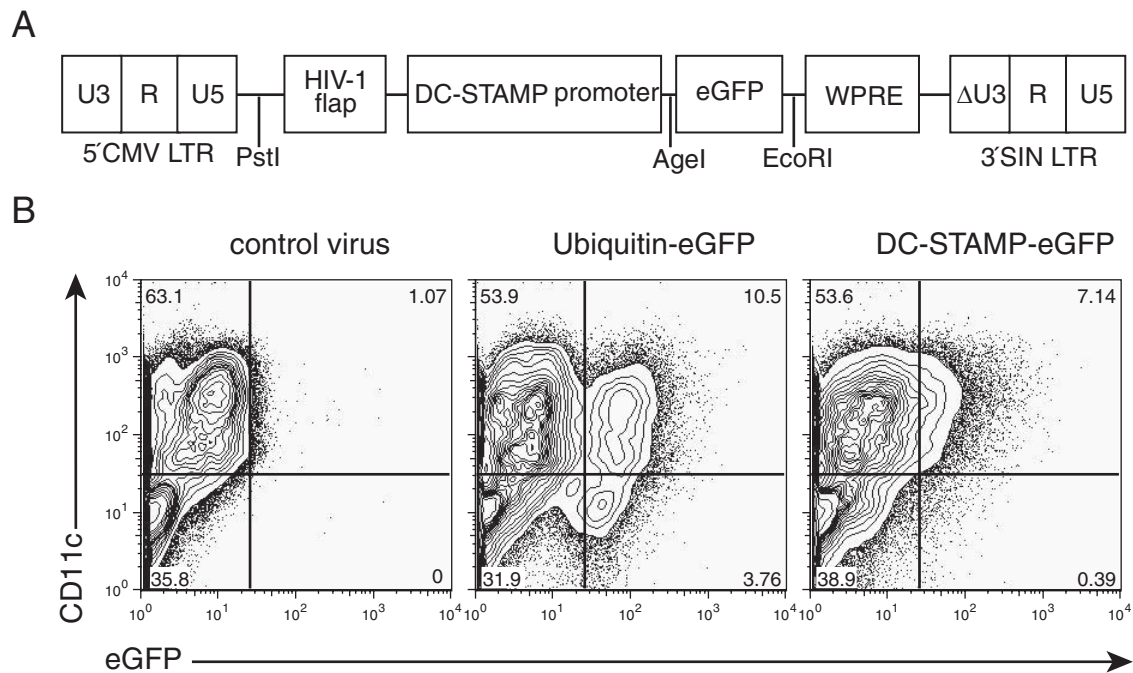
### Statistical analysis

Data were analyzed using the Student *t* test (GraphPad Prism version 4.03 software). A value of  $p < 0.05$  was considered significant. All experiments were composed by a number of at least three mice per group, unless otherwise stated.

## Results

### The murine DC-STAMP promoter mediates transcription of transgenes in DC in vitro

To develop a viral vector that confers transgene expression selectively to DCs, we cloned the 5' untranslated region of the gene encoding the mouse DC-specific transmembrane protein (DC-STAMP) into a SIN lentiviral vector (Fig. 1A). The promoter from DC-STAMP (pDC-STAMP) was chosen since DC-STAMP is specifically expressed by both immature and mature DCs and is highly conserved between different species (23, 24). The usage of a SIN vector allows elimination of virus-derived control elements after virus integration. It therefore leaves DC-STAMP as the only functional lentivirus-transmitted promoter/enhancer region, increasing safety and eliminating undesired interactions between viral and internal promoters (25, 26). To test the function of this DC-STAMP-GFP-SIN lentivirus vector, we transduced BM-derived DCs in vitro. In this study, CD11c<sup>+</sup> DCs from DC-STAMP-GFP vector-transduced cultures showed expression of GFP, as detected by flow cytometry (Fig. 1B). This was in contrast to the original FUGW lentiviral vector, where GFP expression was controlled by



**FIGURE 1.** DC-STAMP-eGFP SIN lentiviral vector drives transgene expression in DCs in vitro. *A*, Schematic representation of lentiviral-based SIN vector, employing the murine DC-STAMP promoter to control expression of eGFP cDNA. LTR, long terminal repeat; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element;  $\Delta$ U3, deletion in the U3 region. *B*, BM cells from C57BL/6 mice were cultured in the presence of GM-CSF and transduced 1 day later (MOI 0.5) with ubiquitin eGFP (FUGW), DC-STAMP-eGFP or control virus (ubiquitin promoter without cDNA). Cells were analyzed for GFP expression by flow cytometry at day 9 of culture. The data shown are representative of two independently performed experiments.

the ubiquitin promoter, generating also GFP<sup>+</sup>CD11c<sup>-</sup> cells (Fig. 1*B*). These data indicated that the 5' untranslated region of DC-STAMP was sufficient to control transgene expression in the DC-STAMP-GFP SIN lentiviral vector. Taken together, these data suggest that the pDC-STAMP used in the SIN lentivirus supports expression of transgenes in DC.

#### *pDC-STAMP targets transgene expression to DC in vivo*

To evaluate specificity of expression regulated by the DC-STAMP promoter in vivo, we transplanted hematopoietic stem cells (HSCs) transduced with the DC-STAMP-GFP-SIN vector into lethally irradiated mice (Fig. 2). Eight weeks post reconstitution, GFP expression was analyzed in leukocyte populations isolated from spleen (Fig. 2*A*). Besides the main DC subpopulations such as CD11b<sup>-</sup>CD8<sup>+</sup> DCs, CD11b<sup>+</sup>CD8<sup>-</sup> DCs, and plasmacytoid DCs, only a small percentage of CD11c<sup>-</sup>CD11b<sup>+</sup> monocytes expressed eGFP. Because monocytes are potential precursors of various DC subpopulations (27), DC-STAMP may be expressed in this transitional developmental state between monocytes and DCs. Although we have no proof for such a scenario, similar results were obtained in transgenic mice with DC-selective transgene expression controlled by the mouse CD11c promoter (28). After transduction of HSCs with different low virus concentrations (multiplicity of infection (MOI) ranging between 0.4 and 1.4), the high DC selectivity of transgene expression was maintained. In contrast, no or only little expression could be detected in CD4<sup>+</sup> or CD8<sup>+</sup> T cells, B cells, or NK cells (Fig. 2, *A* and *B*).

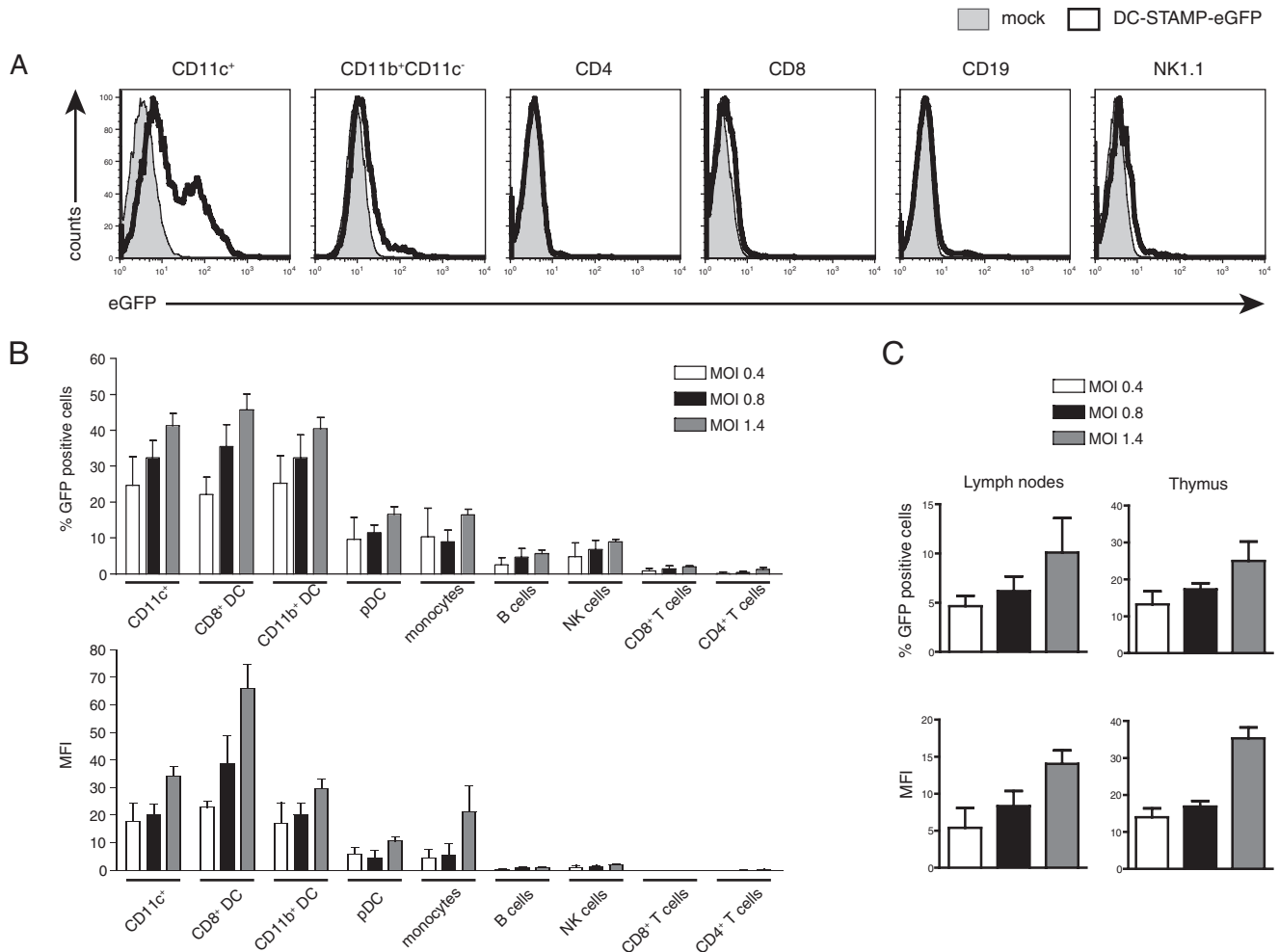
The specific GFP expression in DC populations could be observed in spleen, lymph nodes, and thymus (Fig. 2, *B* and *C*, and data not shown), although it was more pronounced in DCs present in spleen. We suggest that the lower percentage of DCs expressing the transgene in thymus and lymph nodes (Fig. 2*C*)

could be the result of different origins of DCs in such organs. It is known that 50% of DCs present in lymph nodes are from migratory origin, such as Langerhans cells, whereas a significant part of the DCs present in thymus under normal conditions are derived from lymphoid precursors (29). Taken together, lentiviral SIN vectors employing pDC-STAMP are suitable tools to target transcription of genes to a large proportion of DCs in vivo.

Next, we determined whether expression of Ag from DC-STAMP lentiviral vectors would lead to functional presentation of transgenic antigenic peptides by DCs. To this end, we replaced GFP in the DC-STAMP-GFP vector (Fig. 1*A*) by cDNA encoding for a chimeric transferrin receptor chicken OVA as a membrane-bound, nonsecreted model Ag (22). Next, we generated BM chimeras using this DC-STAMP-OVA vector. To analyze expression of the OVA transgene, we isolated mRNA from purified CD11c<sup>+</sup> DCs and CD11c<sup>-</sup> non-DCs from different organs and performed RT-PCR (Fig. 3*A*). These data confirm our findings from the FACS analysis of the GFP vector-transduced BM chimeras (Fig. 2), as in the thymus, OVA could only be detected in CD11c<sup>+</sup> cells (Fig. 3*A*, *top panel*). Also in spleen, the main signal can be detected in CD11c<sup>+</sup> DCs, while a weaker signal in the CD11c<sup>-</sup> fraction probably corresponds to CD11b<sup>+</sup> monocytes (Fig. 3*A*, *bottom panel*) that were found previously to express GFP to a certain extent (Fig. 2).

To determine whether transgenic Ag was properly processed and presented by DCs, purified DCs from these chimeras were coincubated as APC with CD8<sup>+</sup> OT-I T cells expressing a TCR specific for OVA<sub>257-264</sub> peptide in the context of H-2K<sup>b</sup>. Ag-specific T cell expansion was monitored using CFSE-labeled OT-I cells (Fig. 3*B*). T cells cocultured with DCs from control chimeras reconstituted with empty vector-transduced BM did not proliferate at all (Fig. 3*B*). In contrast, DCs from DC-STAMP-OVA lentivirus



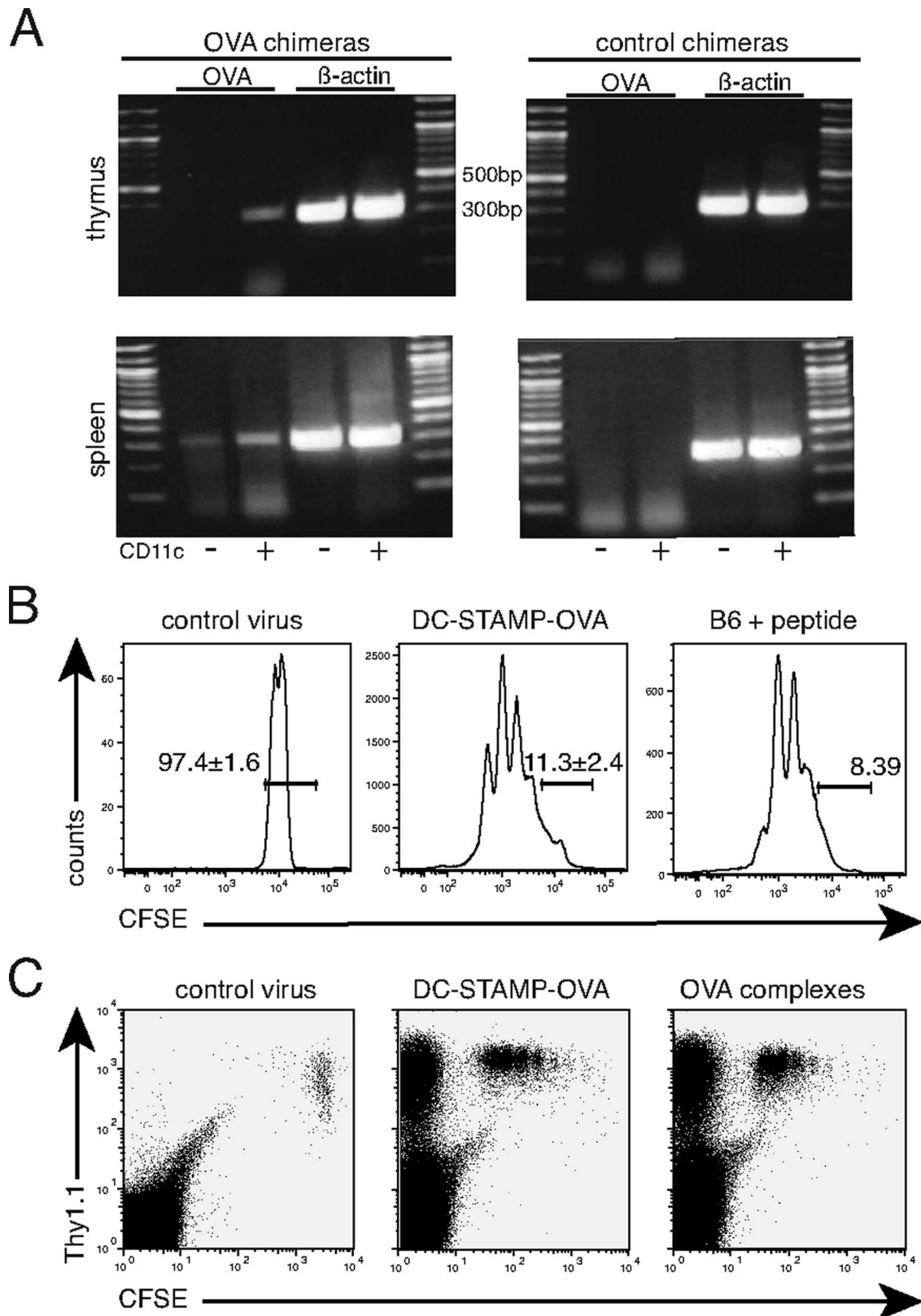


**FIGURE 2.** The murine DC-STAMP promoter targets GFP expression to DCs in vivo. BM HSCs from 5-Fluorouracil-treated C57BL/6 donor mice were transduced with the indicated MOI of DC-STAMP-eGFP and  $1-3 \times 10^6$  cells were injected into lethally irradiated recipient mice. After at least 8 wk after transplantation, chimeras were sacrificed and cells were analyzed by flow cytometry. *A*, FACS analysis of the indicated leukocyte populations from spleen. One representative example with MOI of 1.4 is shown. *B*, Percentage (*upper panel*) and mean fluorescence intensity (MFI; *lower panel*) of eGFP<sup>+</sup> cells within the indicated lymphocyte populations from chimeras generated with HSCs transduced with the indicated MOI. *C*, Percentage (*upper panel*) and mean fluorescence intensity (*lower panel*) of eGFP<sup>+</sup>CD11c<sup>+</sup> cells in thymus and lymph nodes from the same chimeras as in *B*. The mean values represent a group of three (MOI 0.8) or four animals (MOI 0.4 and 1.4). One of two independent experiments with similar results is shown.

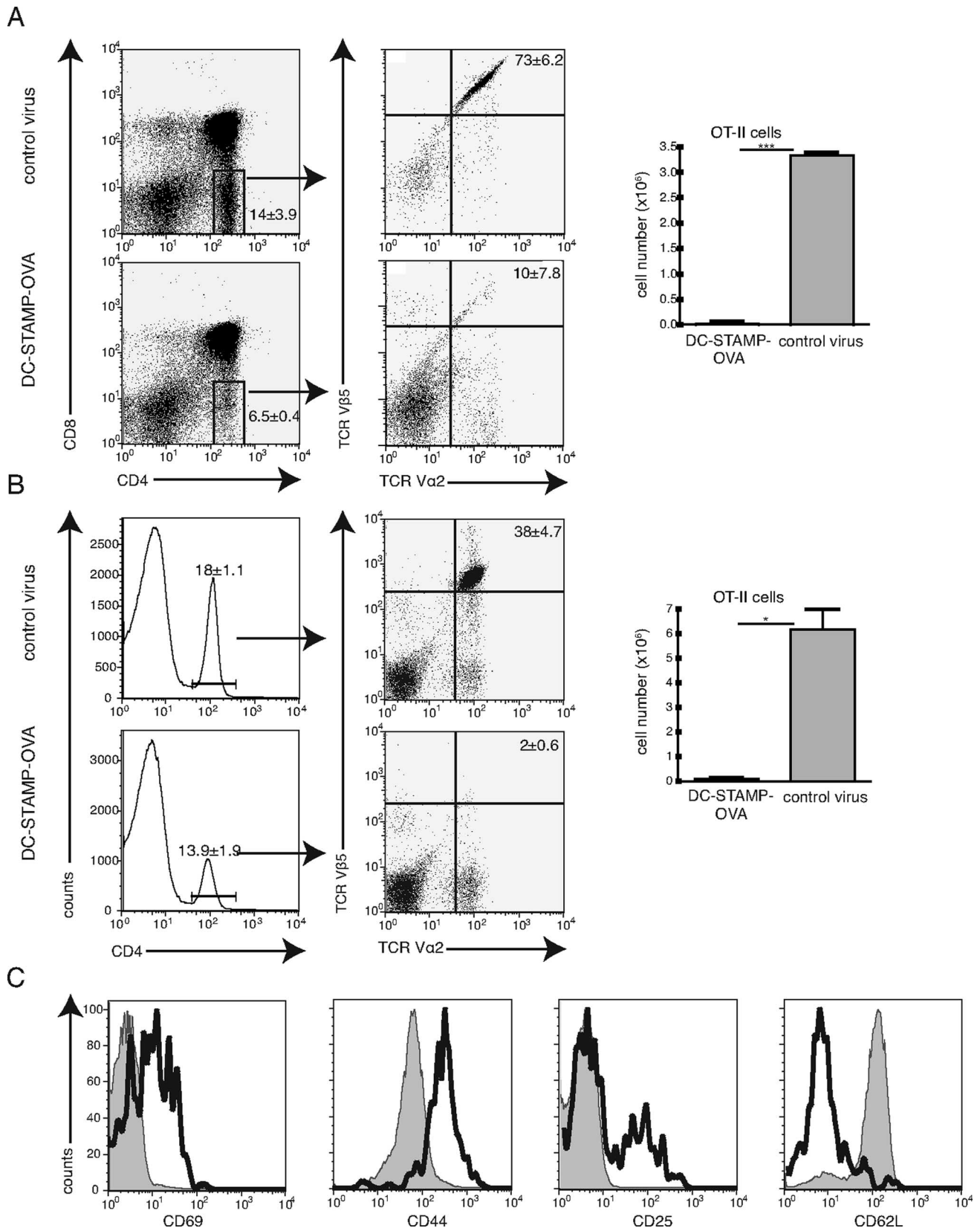
chimeras induced vigorous proliferation of CD8 T cells (Fig. 3*B*). The observed proliferation was comparable to OT-I cell division induced by DCs loaded with the preprocessed OVA<sub>257-264</sub> peptide (Fig. 3*B*). To test whether a similar functional Ag presentation would appear in vivo, we transferred CFSE-labeled OT-I T cells into the different chimeras and monitored CFSE dilution (Fig. 3*C*). Also in vivo, OT-I T cells readily proliferated in DC-STAMP chimeras to comparable levels as in OVA-immunized wild-type mice (Fig. 3*C*). These data indicate that OVA is presented functionally by DCs in vivo and because these chimeras were used 10 mo after reconstitution, these data also indicated that DC-STAMP lentiviral vectors achieved long-term expression of transgenes without detectable silencing. Furthermore, by analyzing maturation surface markers of DCs (MHC class II, CD80, CD86; data not shown) in chimeras, we could not detect alterations of DC phenotypes, indicating that virus-encoded transgene expression did not influence DC biology. Taken together, these data indicated that expression of OVA cDNA from the DC-STAMP-OVA vector led to long-term expression of transgenes resulting in immunologically detectable peptide presentation by DCs.

#### *DC-STAMP lentivirus vector-mediated transgene expression is sufficient to delete Ag-specific CD4 T cells*

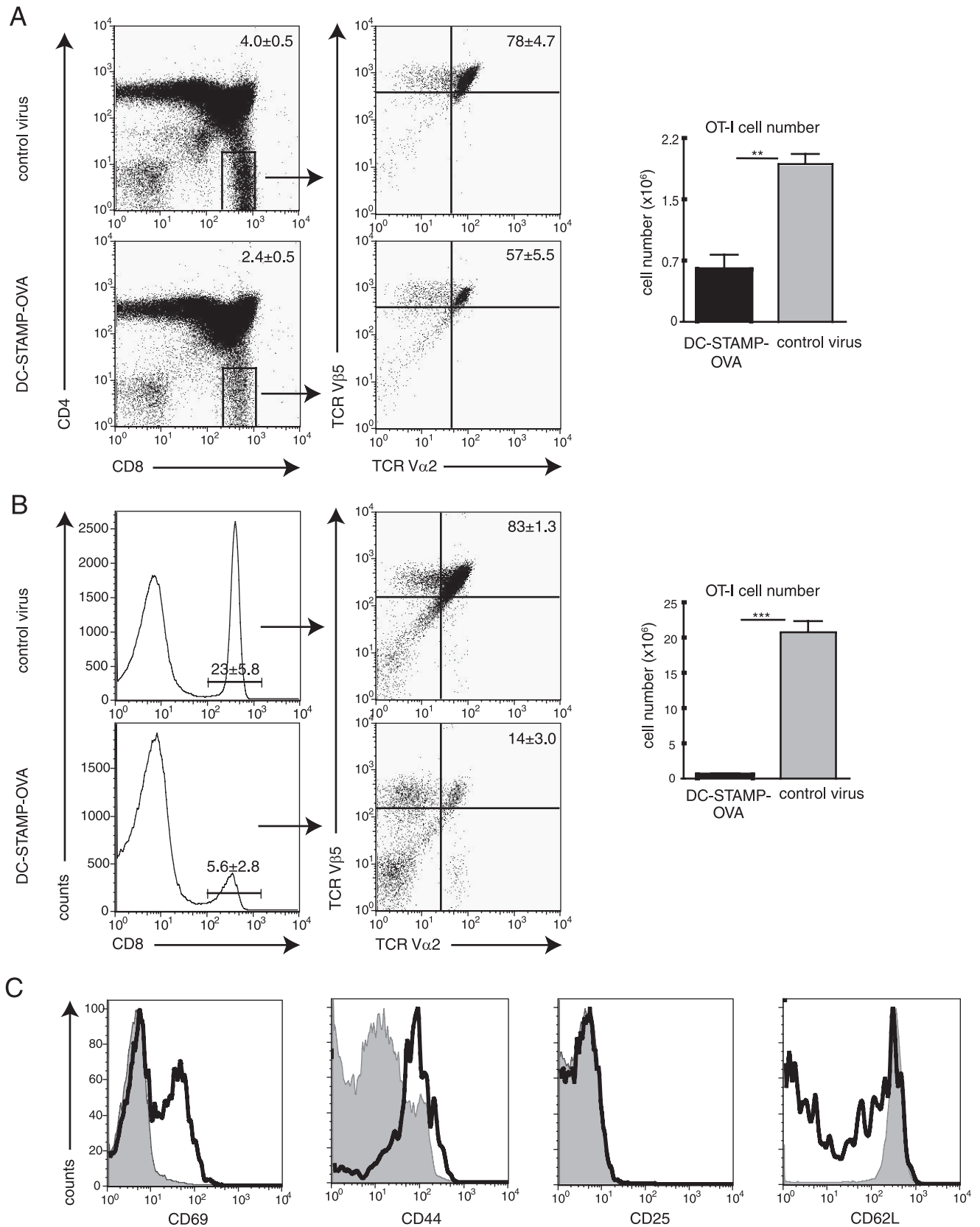
We evaluated whether Ag presentation by vector-driven transgene expression was sufficient to influence development and function of OVA-specific CD4 T cells in vivo. To this end, we transduced BM from Ly5.2<sup>+</sup> mice transgenic for the OVA-specific, MHC class II-restricted OT-II TCR (18) with DC-STAMP-OVA or control vector and generated BM chimeras in lethally irradiated congenic Ly5.1-positive B6 recipients. Development of OT-II T cells was severely disrupted in the thymus of chimeras generated with the DC-STAMP-OVA vector, but not in control chimeras (Fig. 4*A*). The frequencies of mature CD8<sup>-</sup>CD4<sup>+</sup> thymocytes were reduced by >2-fold in DC-STAMP-OVA chimeras as compared with control chimeras (Fig. 4*A*). Further analysis with Abs specific for TCRV $\alpha$ 2 and TCRV $\beta$ 5, the  $\alpha\beta$ TCR combination of OT-II T cells, revealed >7-fold reduced frequencies of OT-II thymocytes in DC-STAMP-OVA chimeras (Fig. 4*A*). The reduction of OT-II cells in thymus of DC-STAMP-OVA chimeras was not due to a



**FIGURE 3.** DC-STAMP promoter-driven expression of OVA results in long-term functional expression of antigenic complexes in DCs. *A*, CD11c-positive and -negative thymic or splenic cells from DC-STAMP-OVA chimeras and control virus chimeras were purified by magnetic bead sorting and RNA was prepared. RT-PCR analysis from these samples shows the presence of OVA mRNA predominantly in the CD11c-positive fraction, as identified by amplification of a 317-bp fragment for OVA and a control 302-bp fragment for  $\beta$ -actin by agarose gel electrophoresis. *B*, DCs ( $0.5 \times 10^5$ ) isolated from spleens of DC-STAMP-OVA chimeras and control virus chimeras were cultured *in vitro* with  $1 \times 10^5$  CFSE-labeled Thy1.1<sup>+</sup> OT-I cells. At day 3 of culture, OT-I cells were analyzed after gating on Thy1.1<sup>+</sup> T cells. Mean ( $n = 3$ ) values are displayed. As the positive control, DCs were isolated from a normal B6 mouse and loaded with 0.1  $\mu$ g of SIINFEKL peptide. *C*, Ten months after BM transplantation, CFSE-labeled  $5 \times 10^6$  OT-I Thy1.1 cells were adoptively transferred into DC-STAMP-OVA chimeras and control virus chimeras or normal C57BL/6 mice that received OVA-IgG Ab immunocomplexes (OVA complexes, positive control), and all mice were immunized *i.v.* with 30  $\mu$ g of LPS. After 3 days, OT-I cells from spleens were analyzed by flow cytometry after gating on CD8<sup>+</sup> T cells.

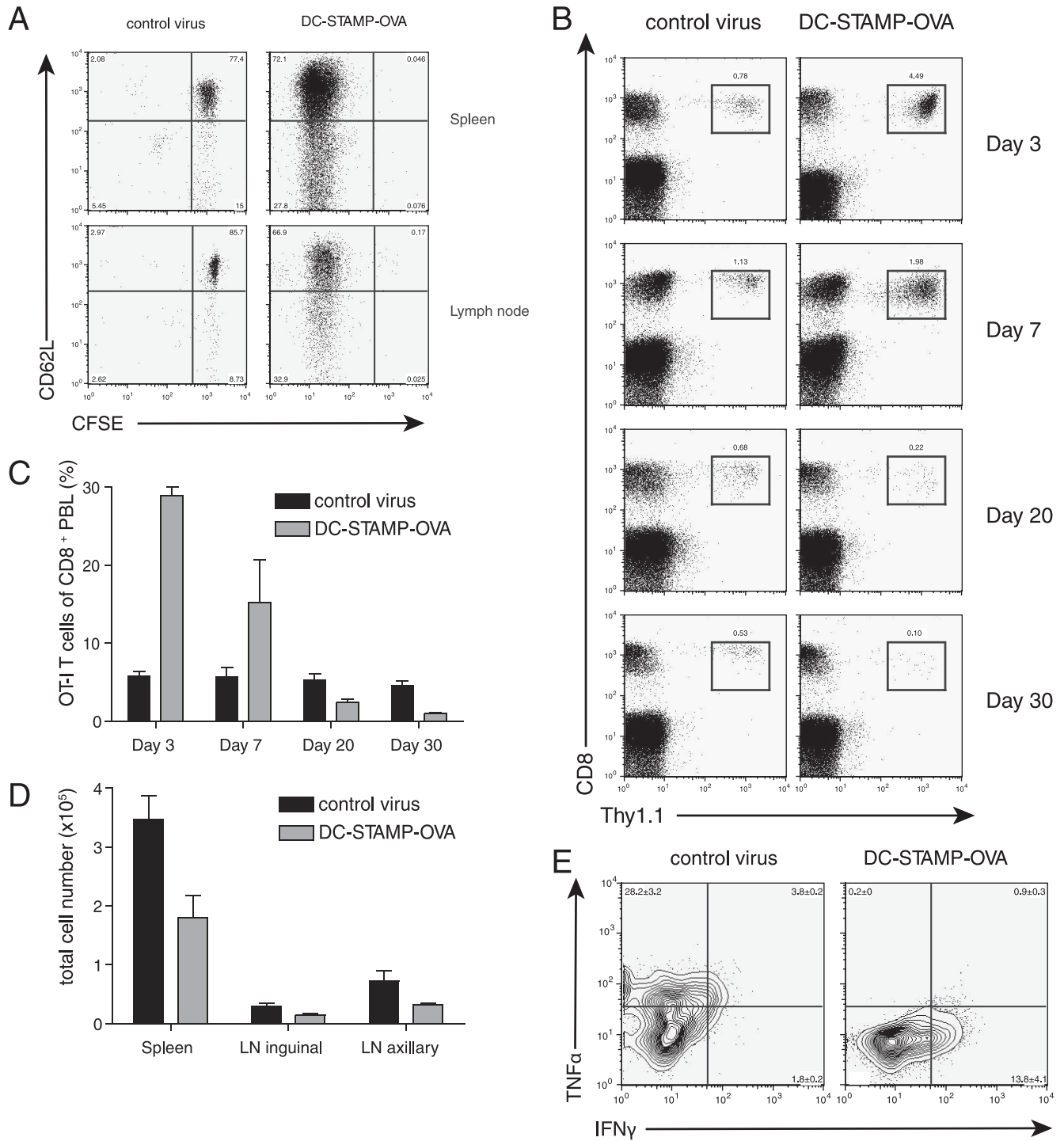


**FIGURE 4.** DC-STAMP promoter-regulated expression of OVA in DCs leads to Ag-specific central tolerance induction in TCR-transgenic CD4 T cells. BM HSCs from OT-II mice were transduced with DC-STAMP-OVA or control lentivirus vector and BM chimeras were generated. After at least 5 wk posttransplantation, chimeras were sacrificed and cells were analyzed by flow cytometry. **A**, Thymocytes were gated on single-positive CD4<sup>+</sup> cells and OT-II cells were identified according to their expression of TCRV $\alpha$ 2 and TCRV $\beta$ 5.1/5.2; total numbers of OT-II cells in both types of chimeras were compared (\*\*\*,  $p = 0.0003$ ; Student's  $t$  test). **B**, Expression of V $\alpha$ 2 and V $\beta$ 5.1/5.2 on CD4<sup>+</sup> OT-II cells from spleen; total numbers of OT-II cells in both types of chimeras were compared (\*,  $p = 0.0169$ ; Student's  $t$  test). **C**, OT-II cell from spleens were identified as described in **B** and expression of the indicated surface molecules was analyzed in DC-STAMP-OVA (open histogram) and control virus (gray histogram) chimeras. Data are representative of three independent experiments with at least three mice per group.



**FIGURE 5.** OVA-expressing DCs induce Ag-specific deletion of TCR-transgenic CD8 T cells. BM HSCs from OT-I mice were transduced with DC-STAMP-OVA or control lentivirus vector and BM chimeras were generated. After at least 5 wk posttransplantation, mice were sacrificed and cells were analyzed by flow cytometry. **A**, Identification of OT-I thymocytes according to expression of CD8, TCRV $\alpha$ 2, and TCRV $\beta$ 5 as indicated by gates and quadrants. Total numbers of OT-I T cells were determined (\*\*,  $p = 0.004$ ; Student's  $t$  test). **B**, CD8<sup>+</sup>TCRV $\alpha$ 2<sup>+</sup>TCRV $\beta$ 5<sup>+</sup> OT-I cells from spleens were identified by flow cytometry. Total numbers of OT-I cells from spleens of both types of chimeras were compared (\*\*\*,  $p = 0.0002$ ; Student's  $t$  test). **C**, OT-I T cells from spleens were identified as shown in **B** and expression of the indicated surface molecules was analyzed in DC-STAMP-OVA (open histogram) and control virus (gray histogram) chimeras. The data are representative of two independent experiments with at least three mice per group.



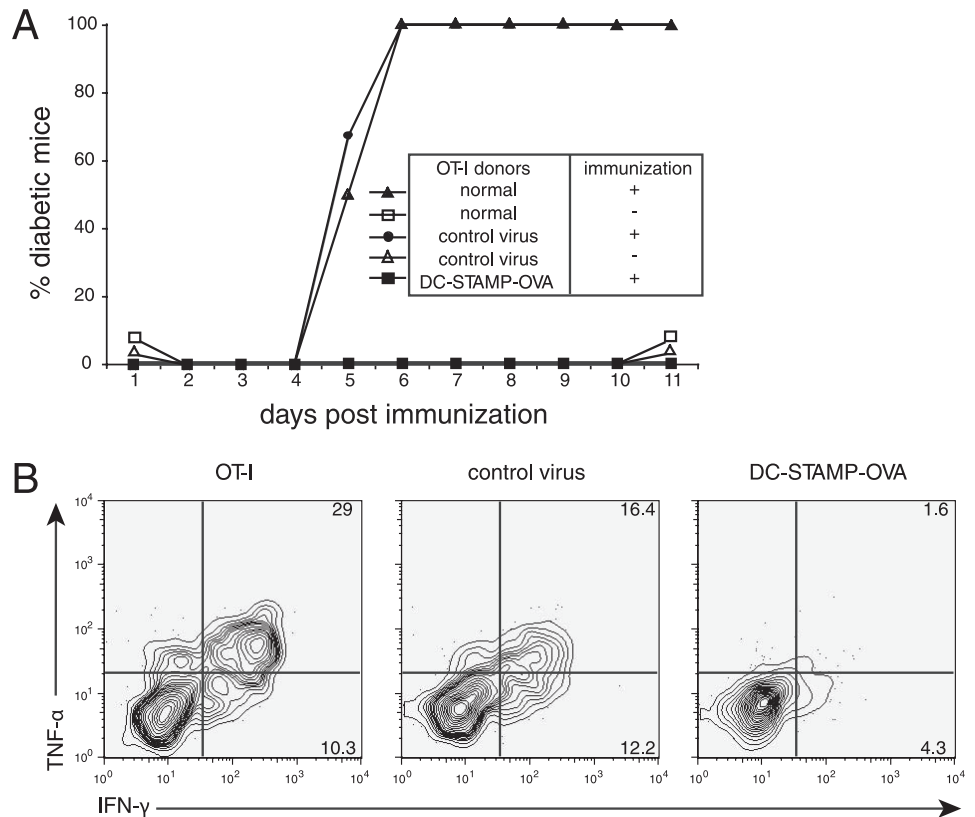


**FIGURE 6.** Peripheral tolerance of CD8 T cells by clonal deletion and anergy. *A*, CFSE-labeled Thy1.1-positive OT-I T cells ( $1.5 \times 10^6$ ) were adoptively transferred into DC-STAMP-OVA or control virus chimeras. After 3 days OT-I T cells from spleens and lymph nodes were analyzed by flow cytometry by gating on Thy1.1<sup>+</sup> cells (data not shown). One of three experiments with similar outcome is shown. *B–E*, Thy1.1<sup>+</sup> OT-I T cells ( $5 \times 10^6$ ) were adoptively transferred into DC-STAMP-OVA or control virus chimeras. *B*, PBL from all mice were analyzed by flow cytometry 3, 7, 20, and 30 days later for the presence of CD8<sup>+</sup>Thy1.1<sup>+</sup> OT-I T cells. Numbers indicate the mean ( $n = 3$ ) percentage of OT-I T cells of PBL (cells in gate). *C*, The respective frequencies of OT-I T cells of CD8<sup>+</sup> PBL are shown. *D*, After 34 days, spleen and lymph nodes were isolated and the total cell number of OT-I Thy1.1<sup>+</sup> T cells was determined by flow cytometry. *E*, Intracellular cytokine staining for TNF- $\alpha$  and IFN- $\gamma$  is shown after gating on Thy1.1<sup>+</sup> cells that were restimulated with OVA<sub>257–264</sub>-pulsed spleen cells for 4 h in vitro.

reduced chimerism, since >99% of thymocytes and lymphocytes from all chimeras were of donor phenotype (Ly5.1<sup>-</sup>Ly5.2<sup>+</sup>; data not shown). As a result, presentation of OVA by thymic DCs led to a >80-fold reduction in total numbers of OVA-specific CD8<sup>-</sup>CD4<sup>+</sup> OT-II thymocytes in DC-STAMP-OVA chimeras (Fig. 4A).

The spleens (Fig. 4B) and lymph nodes (data not shown) of these chimeras were analyzed for the presence of OT-II T cells. In spleen, lower frequencies of total CD4<sup>+</sup> lymphocytes (Fig. 4B, left panel) were due to a nearly 20-fold reduction of TCRV $\alpha$ 2<sup>+</sup>V $\beta$ 5<sup>+</sup> OT-II cell frequencies, resulting in an approximate 50-fold

**FIGURE 7.** Lentiviral targeting of Ag expression to DCs generates functionally inactivated CD8 T cells. RIP-OVA<sup>low</sup> mice received  $1 \times 10^6$  OT-I T cells from DC-STAMP-OVA chimeras, control virus chimeras, or normal OT-I mice. One day later, mice were immunized with either OVA-IgG Ab immunocomplexes and CpG nucleotides (filled symbols) or CpG nucleotides alone (open symbols). *A*, Diabetes induction was monitored for 20 days and mice with  $>5.6$  nmol/L glucose in urine were considered diabetic. The percentage of diabetic mice over time is shown. One representative experiment of two with  $n = 3$ –5 mice/group is shown. *B*, In a parallel experiment, mice ( $n = 2$ –3) were sacrificed at day 15 after OT-I transfer and splenocytes were restimulated with SIINFEKL peptide for 4 h. Intracellular cytokine staining for TNF- $\alpha$  and IFN- $\gamma$  is shown after gating on Ly5.1-positive cells.



reduction in the total numbers of OT-II T cells in DC-STAMP-OVA chimeras (Fig. 4*B*). The few remaining OT-II cells in DC-STAMP-OVA chimeras were analyzed for surface markers of T cell activation (Fig. 4*C*). The elevated levels of CD25, CD44, and CD69 expression and down-regulation of CD62L on these OT-II cells probably reflected Ag experience of OT-II T cells (Fig. 4*C*).

Taken together, the above findings show that efficient central CD4 T cell tolerance can be induced by DCs lentivirally targeted to express Ag.

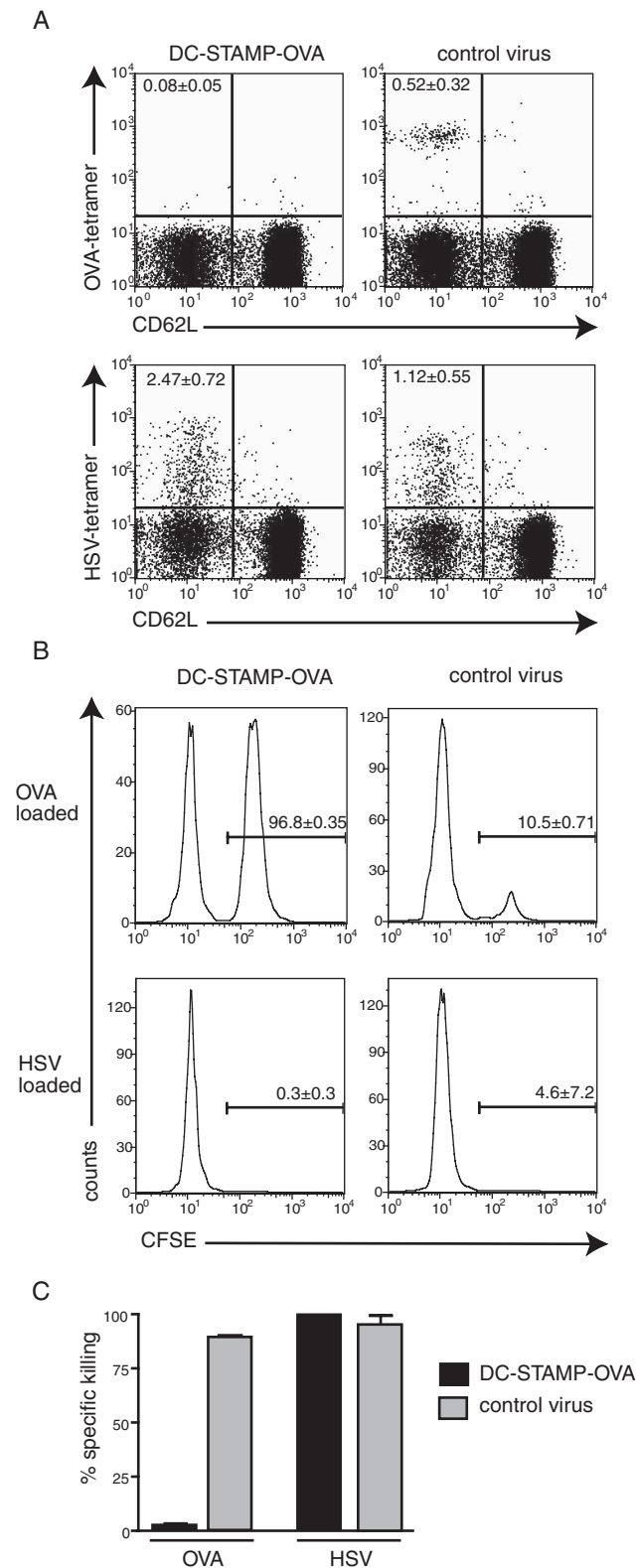
#### Transgene expression driven by pDC-STAMP induces tolerance of Ag-specific CD8 T cells

To ask whether DC-specific expression of Ag would also induce tolerance of CD8<sup>+</sup> T cells, we generated BM chimeras using BM cells from the OVA-specific CD8<sup>+</sup> TCR-transgenic OT-I mouse strain. Analysis of the DC-STAMP-OVA chimeras revealed a significant, nearly 2-fold reduction of the frequency of mature CD8<sup>+</sup> thymocytes (Fig. 5*A*; Student's *t* test,  $p = 0.021$ ). Further analysis revealed that significantly fewer CD8 thymocytes were of the OT-I phenotype TCRV $\alpha 2^+V\beta 5^+$  in DC-STAMP-OVA chimeras as compared with control chimeras (Fig. 5*A*; Student's *t* test,  $p = 0.007$ ). This resulted in a 4-fold reduction of total OT-I thymocyte numbers (Fig. 5*A*). Compared with deletion in OT-II chimeras (Fig. 4), central deletion of CD8 T cells was less efficient, probably due to lower efficacies of thymic DCs to mediate central deletion of CD8 vs CD4 thymocytes (16).

However, when OT-I T cells from peripheral lymphoid organs of these chimeras were analyzed, we consistently detected a  $>4$ -fold reduction of CD8<sup>+</sup> T cell frequencies from spleen (Fig. 5*B*; Student's *t* test,  $p = 0.009$ ) and lymph nodes (data not shown). Of those, only 14% as compared with  $>80\%$  in control chimeras were of the OT-I phenotype (TCRV $\alpha 2^+V\beta 5^+$ , Fig. 5*B*; Student's *t* test,  $p = 0.0001$ ). Together, this resulted in a 50-fold reduction of absolute OT-I T cell numbers as compared with control chimeras

(Fig. 5*B*). The remaining peripheral OT-I T cells displayed elevated levels of CD69 and CD44 and reduced CD62L expression as evidence of T cell activation or Ag experience (Fig. 5*C*). In contrast to OT-II T cells (Fig. 4*D*), CD25 expression was not modulated (Fig. 5*C*). These findings are in accordance with previous reports, where tolerance induction of CD8 T cells by model tissue Ag in transgenic mice was accompanied by up-regulation of CD69 and CD44, reduction of CD62L, and no modulation of CD25 (30).

We next wondered whether transgene expression in peripheral DCs could mediate peripheral deletional tolerance and contribute to low peripheral OT-I T cell numbers found in DC-STAMP-OVA chimeras. To test this possibility, we transferred CFSE-labeled OT-I T cells into control or DC-STAMP-OVA chimeras and detected after 3 days a strong proliferation selectively in DC-STAMP-OVA chimeras (Fig. 6*A*). Further monitoring of chimeras revealed that after an initial expansion phase the OT-I frequencies (Fig. 6, *B* and *C*) and total numbers (Fig. 6*D*) decreased over the next 5 wk beyond those found in control chimeras. To test the OT-I T cells from the different hosts for production of effector cytokines, we stimulated them with antigenic peptide *in vitro*. In agreement with a previous report (31) naive OT-I T cells from the OVA-negative environment in control chimeras produced primarily TNF- $\alpha$ , but only low amounts of IFN- $\gamma$  (Fig. 6*E*). In contrast, OT-I T cells from DC-STAMP-OVA chimeras were defective in TNF- $\alpha$  production, but produced IFN- $\gamma$  (Fig. 6*E*). To determine whether OT-I T cells from OVA-expressing chimeras could differentiate into effector T cells and exert autoimmune aggression *in vivo*, the RIP-OVA<sup>low</sup> mouse model was used. In this strain, transgenic OVA expression in the pancreas is controlled under the rat insulin promoter (RIP) and serves as a model self-Ag (20). When OT-I T cells are transferred into RIP-OVA<sup>low</sup> mice, they are ignorant due to low expression levels of OVA. However, upon Ag-specific immunization, transferred OT-I T cells may become activated, destroy the OVA<sup>+</sup> pancreatic  $\beta$  islet cells, and the mice develop



**FIGURE 8.** Ag expression in DCs induces functional tolerance of a polyclonal CD8 T cell repertoire. BM HSCs from C57BL/6 mice were transduced with DC-STAMP-OVA or control lentivirus vectors and BM chimeras were generated. After at least 10 wk after transplantation, chimeras were immunized by i.v. injection of  $4 \times 10^6$  PFU of recombinant HSV-expressing OVA. **A**, Seven days after immunization, leukocytes were isolated from peripheral blood of DC-STAMP-OVA or control virus chimeras and stained with Abs specific for CD8 (data not shown), CD62L as well as H2K<sup>b</sup>-OVA or H2K<sup>b</sup>-HSVgB tetramers and the frequencies of

diabetes (32). Upon transfer into these recipients, RIP-OVA<sup>low</sup> mice were immunized with OVA and all mice that received naive OT-I T cells from control chimeras or wild-type OT-I donors developed diabetes with similar kinetics (Fig. 7). In marked contrast, none of the mice receiving OT-I cells from DC-STAMP-OVA-chimeras developed disease, indicating their functional tolerance (Fig. 7A). Next, we isolated the OT-I T cells from different origins 15 days after transfer into the RIP-OVA<sup>low</sup> recipients to determine their capacities to produce effector cytokines (Fig. 7B). As expected, OT-I T cells from wild-type mice and those from control chimeras developed after their transfer into RIP-OVA<sup>low</sup> mice effector functions. This resulted in diabetes (Fig. 7A) and production of both TNF- $\alpha$  and IFN- $\gamma$  (Fig. 7B). In contrast, OT-I cells from DC-STAMP-OVA mice could neither induce diabetes (Fig. 7A) nor produce TNF- $\alpha$  (Fig. 7B). In accordance with a previous report on tolerized CD8 T cells (33), OT-I cells were not completely defective in cytokine production, but rather showed impaired IFN- $\gamma$  production (Figs. 6E and 7B).

#### Lentiviral targeting of DCs generates a tolerant polyclonal CD8 T cell repertoire

To further evaluate whether polyclonal CD8 T cell populations also could be tolerized efficiently, we next generated chimeras with lentivirally transduced C57BL/6-HSCs. To elicit potent CTL responses, we immunized these chimeras with a recombinant herpes simplex type 1 vector, HSV-OVA-encoding OVA (34). Monitoring of expanding OVA-specific and HSV glycoprotein B (gB)-specific CD8 T cells with MHC tetramers revealed the complete absence of OVA-specific CD8 T cells in DC-STAMP-OVA chimeras, but not in controls (Fig. 8A). In contrast, both groups of chimeras were able to mount HSVgB-specific CTL responses (Fig. 8A). We next monitored the specific cytotoxic activity induced by this immunization (Fig. 8B). Using an in vivo killer assay, we revealed that the DC-STAMP-OVA chimeras were not able to specifically lyse OVA-positive target cells, while their ability to kill HSVgB<sup>+</sup> targets was normal (Fig. 8, B and C). This data showed that lentiviral targeting of DCs induced Ag-specific tolerance also in T cells with normal precursor frequencies and that tolerance cannot be broken by Ag-specific immunization.

#### Discussion

DCs are the main APCs of the immune system able to induce both tolerance and immunity. In this study, we describe a new approach to specifically and permanently modify DCs. By using a lentiviral vector with transcriptional control of a transgene by the DC-specific DC-STAMP promoter, we were able to transduce HSCs and obtain transgene transcription predominantly in DCs and in some monocytes. In the present study, we identified monocytes as CD11b<sup>+</sup>CD11c<sup>-</sup> cells. As it was shown that monocytes may be progenitors of DCs (35), it is difficult to differentiate between "real" monocytes and DC precursors. In contrast, plasmacytoid DCs expressed DC-STAMP-controlled transgenes only at low levels (Fig. 2). Interestingly, transgene expression was lower in lymph nodes and thymus when compared with spleen (Fig. 2), what could be consequence of the different origins of resident and

H-2K<sup>b</sup>/OVA- or H-2K<sup>b</sup>/HSV-specific cells among all CD8 T cells were analyzed by flow cytometry. **B**, On day 7 after immunization, a CFSE-based in vivo cytotoxic T cell assay was performed and the specific lysis of OVA-SIINFEKL or HSVgB-SSIEFARL peptide-loaded, CFSE-labeled target cells was determined by flow cytometry. The data are representative of two independent experiments with two to four mice per group. **C**, Percentage of specific killing in graphic presentation.



migratory DCs in the respective organs (29). Therefore, the DC-STAMP lentivirus vector system may be a valuable tool to study DC origin and development.

Gene therapy is an efficient method to induce tolerance when the identity of target Ags in autoimmune diseases, transplant rejection, or other T cell-mediated indications are known (36–40). However, in previous studies tolerance induction was achieved with conventional retroviral vectors leading to transgene expression in multiple cell types. In this case, transgene expression can be harmful, as for example vector integration in the proximity of a protooncogene promoter induced uncontrolled exponential clonal proliferation of T cells in some of the patients treated by gene therapy (41, 42). Our approach to focus gene expression selectively to DCs, a cell type with a low propensity for proliferative disorders, could minimize the potential danger of viral enhancers introduced by gene therapy.

Numerous studies report the *in vitro* modification of human DCs for boosting immune responses against cancer (43) or induction of tolerance (5, 6). A major drawback of these studies was the requirement to obtain sufficient amounts of viable DCs for application. Moreover, *ex vivo* manipulation may induce functional changes in DCs, and the route of DC application can also influence the experimental outcome. Although the mechanisms of tolerance induction by DCs are still not completely understood, it is accepted that in normal noninflammatory conditions DCs present constantly self-Ags to maintain tolerance. As by lentiviral transduction of HSCs, DCs have no direct contact with viral vectors, transgene expression should result in tolerance as DCs remain in the steady state. Monitoring of DC surface markers indicated that transgene expression indeed did not induce DC maturation (data not shown). Moreover, induction of CD8 T cell tolerance depends on long-term exposure of T cells to Ag-presenting DCs *in vivo* (14), and *in vivo* imaging has shown that multiple brief DC-CD8 T cell contacts were required over prolonged periods of time for efficient tolerance induction (44). Therefore, the lentiviral vector system presented here is advantageous because it allows the modification of autologous BM cells for permanent and continuous output of genetically modified tolerogenic steady-state DCs to lymphoid organs. In contrast to ubiquitously expressed retroviral systems described previously (45), lentivirus-driven DC-specific transgene expression was not silenced.

Although we demonstrated that transgene expression by DCs was sufficient to induce central tolerance of CD4 and CD8 T cells, central deletion of Ag-specific CD8 T cells was less complete as compared with deletion of CD4 thymocytes. These results are in accordance with previous findings suggesting that thymic DCs are more specialized in CD4 than in CD8 T cell deletion (16). However, it should be stressed that the comparison of negative selection efficacies might be biased in favor of CD4 T cells, as OT-II thymocytes recognize an additional Ag. They can interact via their TCRV $\beta$ 5 segment with the endogenous superantigen Mtv-9 in context of MHC class II I-A<sup>b</sup> (46). Therefore, OT-II cells can be deleted by two thymic Ags (OVA, Mtv-9), while OT-I cells recognize only one Ag (OVA). In addition, it is possible that cross-presented self-Ags, normally expressed by thymic epithelial cells and acquired by DCs for presentation via MHC class I, are leading to more efficient deletion as compared with Ags expressed and directly presented by DCs. Since lentivirally encoded Ag was expressed by DCs, we could not distinguish direct and cross-presentation of Ag, although both forms of presentation should be possible. Since the membrane-bound OVA fusion protein used in our studies was shown to generate both effective MHC class II- and MHC class I-restricted T cell responses (22), it is not likely that defective access of Ag to the MHC class I compartment was re-

sponsible for less efficient central deletion of CD8 T cells. Although negative selection in the thymus is crucial for tolerance induction, peripheral tolerance is important to control autoreactive T cells that have escaped central deletion (47). In our chimeras, peripheral DCs deleted a significant portion of peripheral OT-I cells or rendered them anergic (Figs. 6 and 7). These results indicate that lentiviral targeting of DCs recruits both complementary mechanisms of tolerance, clonal deletion and anergy. However, because OT-I (and OT-II) chimeras represent a rather artificial situation, with too high numbers of thymocytes expressing the same TCRs, it is unlikely that in a normal setting with polyclonal T cells a similar pressure will be set on the negatively selecting thymic DC population. Therefore, we assume that Ag-expressing thymic DCs will be able to delete Ag-specific thymocytes occurring at naturally low frequencies with even higher efficiencies. Lentiviral targeting of DCs imposed robust tolerance induction even in the face of artificially high precursor frequencies of Ag-specific TCR-transgenic T cells. However, normal polyclonal CD8 T cells were also functionally tolerized, demonstrating that lentiviral DC targeting can lead to functional tolerance in a more physiological setting (Fig. 8).

Taken together, our results indicate that the lentiviral vector-mediated, DC-specific expression of Ags is a potent method to induce and maintain Ag-specific central and peripheral T cell tolerance and may be of clinical relevance for therapeutic application in transplantation or autoimmune disease.

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## Disclosures

The authors have no financial conflict of interest.

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