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Peripheral T Cells Re-Enter the Thymus and Interfere with Central Tolerance Induction

Stephanie L. Edelmann,* Peggy Marconi,† and Thomas Brocker*

The thymus mainly contains developing thymocytes that undergo thymic selection. In addition, some mature activated peripheral T cells can re-enter the thymus. We demonstrated in this study that adoptively transferred syngeneic Ag-specific T cells can enter the thymus of lymphopenic mice, where they delete thymic dendritic cells and medullary thymic epithelial cells in an Ag-specific fashion, without altering general thymic functions. This induced sustained thymic release of autoreactive self-Ag–specific T cells suggested that adoptively transferred activated T cells can specifically alter the endogenous T cell repertoire by erasing negative selection of their own specificities. Especially in clinical settings in which adoptively transferred T cells cause graft-versus-host disease or graft-versus-leukemia, as well as in adoptive tumor therapies, these findings might be of importance, because the endogenous T cell repertoire might be skewed to contribute to both manifestations. ***The Journal of Immunology*****, 2011, 186: 5612–5619.**

The thymus is a primary lymphoid organ in which progenitor cells enter and develop into mature CD4 and CD8 single-positive (SP) T cells. The intrathymic developmental process is determined by positive- and negative-selection events shaping the preimmune T cell repertoire. Positive selection promotes survival of thymocyte clones expressing TCRs with low-affinity binding, whereas negative selection removes those with no or high-affinity binding to self-peptide MHC, because the latter could potentially cause autoimmune diseases (1). Finally, properly selected mature T cells leave the thymus for peripheral organs and rarely come back to their organ of origin (2).

More recently, several reports showed that back-migration can occur, and mature peripheral T cells may re-enter the thymus (2–5). Apparently, $\sim 10^5$ mature T cells from the periphery can be accommodated in a specific thymic niche (3) and mainly localize to the medulla (6). Multiple possible functions have been assigned to these cells, such as maintenance of medullary thymic epithelial cells (mTECs) or direct mediation of thymocyte selection in certain experimental settings (7–10). None of these observed effects are classical functions for T cells and, therefore, were discussed as epiphenomena (2). Peripheral T cells, which re-enter the thymus of normal mice, are not naive but rather are activated and cycling (11). Upon activation, T cells change their homing receptors to mediate effector functions in all tissues of the body, including the thymus (6). In lymph nodes (12) and tissues (13), cytotoxic effector T cells can eliminate dendritic cells (DCs) in an Ag-specific manner. In this article, we present evidence that they can also remove APCs upon return to the thymus. We report that adoptively transferred mature, peripheral T cells re-enter the thymus and target thymic DCs and mTECs presenting cognate Ag. Because both cell types are key mediators of thymic central tolerance induction by negative selection, this process is efficiently prevented. As a consequence, nascent endogenous T cells with self-Ag–specific TCR and autoimmune potential can develop. In conventional mouse models and clinical settings of graft-versus-host disease (GVHD), donor T cells recognize allo-MHC and, therefore, can damage many cells and tissues of the host (14–18). Specifically in the thymus, donor T cells cause thymic dysfunction, insufficient endogenous T cell output, and reduced negative selection, summarized as thymic GVHD (19, 20). In contrast, we demonstrated interference only with negative selection by transferred Ag-specific T cells in the absence of generalized thymic damage. Only thymic cells that express and present Ag are deleted, whereas the majority of host cells and the general thymic functions remain intact. Possible beneficial implications of our findings for cancer therapies, such as graft-versus-leukemia, or adoptive T cell transfers in immune-depleted cancer patients are discussed.

Materials and Methods

Mice

C57BL/6, OT-I, and RIP-mOVA mice were maintained and bred in the animal facility of the Institute for Immunology. CD8 T cells from OT-I mice express transgenic TCRs (vα2 and vβ5.1/5.2) specific for $OVA_{257-264}$/H2-K^b (21, 22). RIP-mOVA mice express a membrane-bound form of OVA under control of the rat insulin promoter (23). All animal experiments were approved by the local ethical committee.

Lentiviral vectors

The DC–STAMP–OVA virus contains a membrane-bound form of OVA (fused to the transferrin receptor) under the control of a 1704-bp promoter fragment of DC-STAMP. As control virus, a modified form of FUGW (24) was used, which contains the ubiquitin promoter without any transgene. Both lentiviral constructs were described previously (25).

Generation and titration of lentiviral stocks

For virus production, 293FT cells (Invitrogen) were transfected using the standard calcium phosphate method with 20 μg vector-DNA, 15 μg pCMVΔR8.2, and 10 μg pMD2G (VSV-G). Supernatants were collected for three consecutive days starting 1 d after transfection, filtered (0.45 μm filter; Nalgene), and concentrated using Centricon filter devices (Plus-70; Millipore). Aliquots were snap-frozen and stored at −80°C. To determine

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Abbreviations used in this article: BM, bone marrow; DC, dendritic cell, GVHD, graft-versus-host disease; mTEC, medullary thymic epithelial cell; qPCR, quantitative PCR; SP, single positive; Treg, regulatory T cell.

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viral titers, NIH3T3 cells were transduced with various virus dilutions using spin infection (300 × *g*, 2 h at 32°C) in the presence of Polybrene (8 μg/ml; Sigma-Aldrich). After 4 h at 37°C, virus was removed, and cells were incubated for two more days. DNA was isolated (DNeasy Blood & Tissue Kit, Qiagen) and analyzed by quantitative PCR (qPCR) (LightCycler FastStart DNA Master^{PLUS} SYBR Green; Roche) on a LightCycler Carousel-based system (Roche). Viral integration (forward: 5′-TGAAAGCGAAAGGGAAACCA-3′, reverse: 5′-CCGTGCGCGCTT-3′) was analyzed per cell (BDNF forward: 5′-ACGACATCACTGGCTGACAC-3′, BDNF reverse: 5′-CATAGACATGTTTGCGGCATC-3′). Standard curves were generated with serial dilutions of plasmids containing the relevant template DNA, and absolute quantification was used to calculate the viral titers.

Generation of bone marrow chimeras

Recipient mice were lethally irradiated with two separate doses (2× 550 rad) using a Cesium source (Gammacell 40; AECL) and supplied with drinking water containing neomycin (1.2 g/l; Sigma-Aldrich) for 5 wk. Chimeras were analyzed 8–10 wk after bone marrow (BM) transfer. BM was harvested from tibiae and femurs of donor mice and depleted of erythrocytes (Mouse Erythrocyte Lysing Kit; R&D Systems), and 5–10 × 10^6 cells were injected i.v. per mouse. If required, CD8 depletion was performed by magnetic bead separation using CD8 microbeads (Miltenyi Biotec). To generate chimeras with genetically modified marrow, donor mice were injected i.v. with 5-Fluorouracil (150 mg/kg body weight; InvivoGen), and BM was isolated after 4 d. This stem cell-enriched BM was depleted of erythrocytes and cultured in serum-free medium (stemline II hematopoietic stem cell expansion medium, Sigma-Aldrich) with 1% penicillin/streptomycin (Invitrogen). If required, CD8 depletion was performed by magnetic bead separation using CD8 microbeads (Miltenyi Biotec) before culturing. The cells were stimulated with a cytokine mixture (Miltenyi Biotec) containing murine IL-3 (10 ng/ml), murine stem cell factor (50 ng/ml), and human IL-6 (50 ng/ml). At day 3 of culture, cells were spin-infected (300 × *g*, 2 h at 32°C) with cell-free stocks of lentivirus (multiplicity of infection between 1 and 15) in the presence of protamine sulfate (4 μg/ml; Sigma-Aldrich). After four additional hours of incubation at 37°C, the virus was removed, and 1–3 × 10^6 cells per recipient mouse were injected i.v. the next day.

Isolation of OT-I T cells

Naive OT-I T cells (Ly5.1 or Thy1.1) were isolated from lymph nodes, unless stated otherwise, and purified with magnetic bead separation using negative selection ($CD8^+$ T cell isolation kit, Miltenyi Biotec). The indicated number of cells was mixed directly with the BM and injected into irradiated recipient mice. For one set of experiments, OT-I T cells were labeled with 5 μM CFSE (Molecular Probes), according to the manufacturer's protocol.

Flow cytometry

Organs were prepared as single-cell suspensions, according to standard protocols, and the following mAbs (from BD Bioscience or eBioscience) were used for staining: CD4 (L3T4), CD8α (53-6.7), Thy1.1 (OX-7), Ly5.1 (A20), vα2 (B20.1), vβ5.1/5.2 (MR9-4), CD44 (Pgp-1, Ly-24), Ly6C (AL-21), CD62L (MEL-14), PD-1 (J43), CD69 (H1.2F3), CD24 (M1/69), Qa-2 (1-1-2), and IFN-γ (XMG1.2). H-$2K^b$/$OVA_{257–264}$ and H-$2K^b$/$HSVgB_{498–505}$ tetramers were purchased from ProImmune. Flow cytometry was performed on a FACSCalibur or FACSCanto II instrument (Becton Dickinson) and analyzed with FlowJo software (TreeStar).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Micro Kit (Qiagen), and cDNA synthesis was performed with the SSIII First-Strand Synthesis SuperMix for quantitative RT-PCR (Invitrogen). The TaqMan Assay was performed with the LightCycler TaqMan Master Kit (Roche) and the Universal ProbeLibrary Set mouse (Roche) on a CFX96 Real Time System (BIO-RAD) using the following primers (MWG Biotech) and probes: CD11c: forward, 5′-CCAGTTGGAGCTTCCAGTAAA-3′ and reverse, 5′-CCTTTTCTGAGGTTGAGAAGTTAAG-3′; probe #46; OVA: forward, 5′-GCTATGGGCATTACTGACGTG-3′ and reverse, 5′-TGCTGAGGAGATGCCAGAC-3′; and probe #41.

Immunization

The adoptively transferred population of OT-I T cells (Thy1.1) was depleted with a single i.p. injection of 200 μg Thy1.1-Ab (clone HIS51; eBioscience). The control groups without adoptively transferred OT-I T cells were treated with IgG2a κ isotype control (eBioscience). After 2 d, the absence of adoptively transferred OT-I T cells was confirmed in the blood, and all mice were immunized i.v. with 4 × 10^6 PFU of a replication-deficient, rHSV-1 vector encoding OVA (26).

Intracellular cytokine staining

Splenocytes (10 × 10^6) were restimulated in 1 ml culture medium (RPMI, 10% FCS) with 2 μg SIINFEKL or SSIEFARL (both from Polypeptide Group) in the presence of 2 μl GolgiStop (BD Biosciences) for 4 h. Intracellular staining for IFN-γ was performed using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's protocol.

Monitoring of diabetes

Glucose levels were determined with test sticks (Diabur, Roche), and hyperglycemia was defined as levels >5.6 mmol/l. After the onset of diabetes, mice were sacrificed or received i.p. injections of insulin (0.14 U/g body weight; Actraphane 50, NovoNordisk) twice daily for the remainder of the study.

Histology

Organs were embedded in OCT compound (Sakura Finetek), snap-frozen, and cut in 5-μm sections on a cryostat instrument (Jung Frigocut 2800 E; Leica). Sections were air-dried for ≥1 h, fixed with acetone (−20°C for 10 min), and stained by standard protocol with CD8-PE (clone 53-6.7; eBioscience). Analysis was performed on a BX41 microscope equipped with a F-view II camera and cellˆF software (all from Olympus).

Statistical analysis

The *p* values were calculated with the Student *t* test using PRISM software (Graph Pad). Error bars represent SD.

Results

Homeostatically expanding activated CD8 T cells re-enter thymus and pancreas

To analyze the potential functions of thymus-homing mature T cells, we used one of the best-studied autoimmune mouse models. RIP-mOVA mice, which express the membrane-bound form of chicken OVA in pancreatic β cells, as well as thymic mTECs (27), were lethally irradiated and reconstituted with BM from syngeneic OT-I mice expressing an OVA-specific TCR (Fig. 1*A*). When $CD8^+$ OT-I T cells were depleted from OT-I BM before reconstitution (Fig. 1*A*), the recipient chimeras showed no signs of diabetes at early (Fig. 1*B*) or late time points after reconstitution (data not shown). In contrast, chimeras receiving nondepleted BM developed lethal diabetes (Fig. 1*B*), which was caused by highly activated $CD44^{high}Ly6C^{high}$ OT-I T cells (Fig. 1*E*) infiltrating the pancreas (Fig. 1*C*, 1*D*). In the same mice, we also found substantial numbers of OT-I T cells in spleen (Fig. 1*D*, 1*E*) and thymus (Fig. 1*F*, 1*H*). Thymic OT-I T cells were $CD24^{low}$Qa-2^{high}, markers of mature T cells (Fig. 1*G*). Also, in the absence of cognate Ag or MHC mismatches, substantial numbers of homeostatically expanding CD8 OT-I T cells were seen to enter the thymus of normal C57BL/6 mice upon adoptive transfer (Fig. 2*A*). However, in contrast to T cells in spleen, lymph nodes, and pancreas, thymic immigrants had undergone more rounds of division ($CFSE^-$; Fig. 2*B*) and were highly activated ($CD62L^{low}$; Fig. 2*B*). In keeping with previous reports from allogeneic settings (28), our data showed that homeostatic proliferation generates T cells that are able to enter the thymus independently of cognate Ag.

Thymus re-entry of peripheral T cells leads to Ag-specific removal of thymic APCs and deficient negative selection

We next wondered whether these highly activated T cells display effector functions within the thymic microenvironment. To investigate this, we used a lentiviral-transduction system that was described previously (25). Lentiviral transduction of BM stem cells with virus carrying the DC-STAMP promoter allows specific transcriptional targeting of transgenes to DCs (25). We generated

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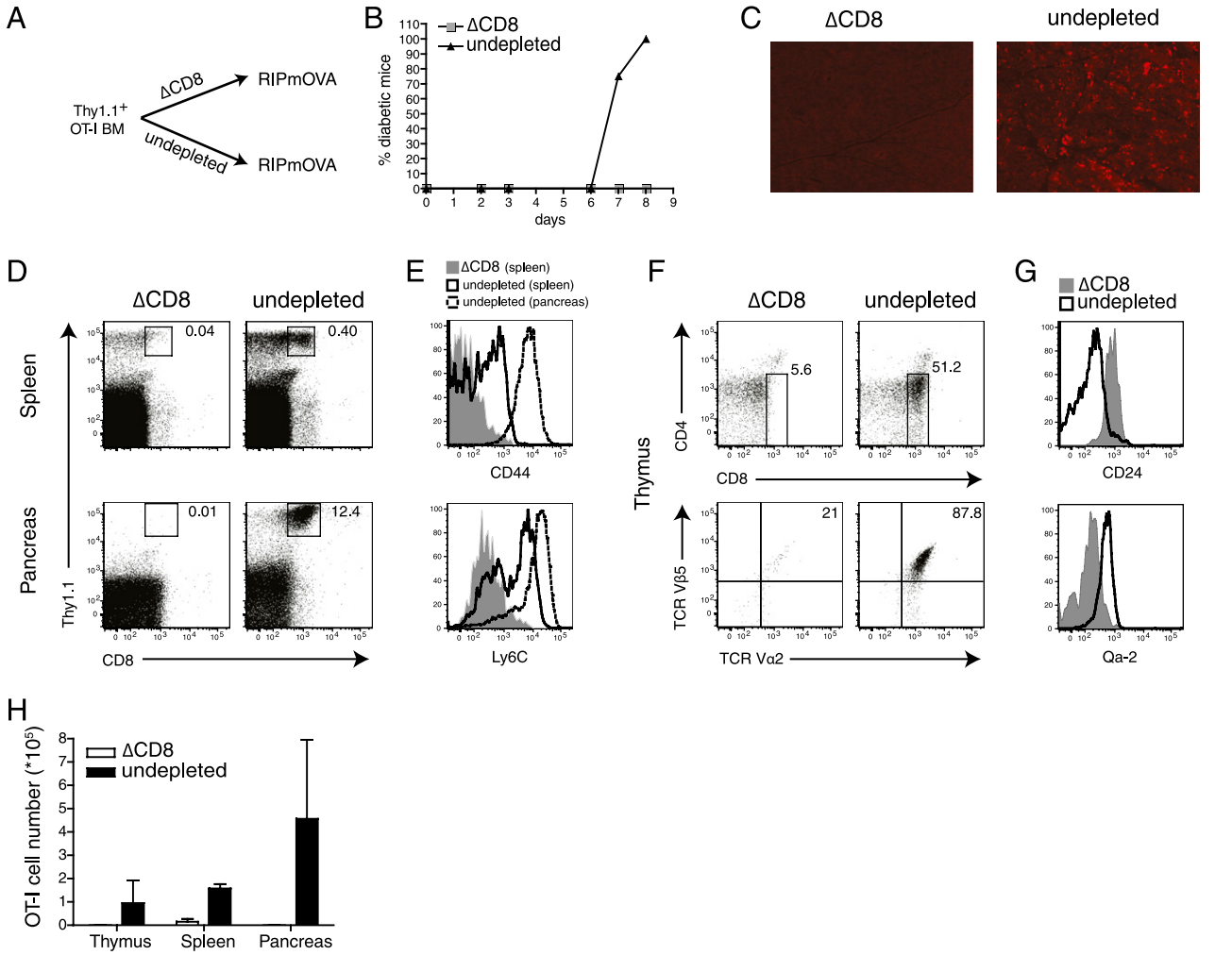

FIGURE 1. OT-I T cells induce diabetes in lymphopenic RIP-mOVA mice. *A*, Thy1.1$^+$ OT-I BM was transferred undepleted or after depletion of CD8 T cells (ΔCD8) into lethally irradiated RIP-mOVA mice. *B*, Mice were monitored for onset of diabetes and sacrificed 8 d after BM transfer. *C*, Immunofluorescence staining of pancreatic sections for CD8 (original magnification ×10). Frequency (*D*) and surface phenotype (*E*) of donor-derived Thy1.1$^+$ CD8 T cells in spleen and pancreas. *F*, Flow cytometric analysis of Thy1.1$^+$ donor-derived cells in the thymus. The frequency of CD8$^+$ SP T cells and the percentage of TCR Vα2$^+$ and Vβ5.1/5.2$^+$ cells among them are indicated. *G*, Developmental status of OT-I T cells in the thymus. *H*, Total cell numbers of Thy1.1$^+$ OT-I T cells in the indicated organs. Data are representative of three independent experiments with similar results (n = 3–4 mice per group).

BM chimeras using T cell-depleted BM from Thy1.1$^+$ OT-I donors, which was transduced with DC–STAMP–OVA or control lentivirus and injected into lethally irradiated hosts. The DC–STAMP–OVA–treated BM was used alone or was mixed with low numbers of Ly5.1$^+$ OT-I T cells (Fig. 3*A*). The use of different congenic markers allowed tracking of the origin of OT-I T cells. We then monitored the development of Thy1.1$^+$ BM-derived endogenous OT-I T cells in thymi without OVA (control virus, Fig. 3*A*) or in the presence of their cognate Ag OVA on thymic DCs (DC–STAMP–OVA lentivirus, Fig. 3*A*) (25). In the third group

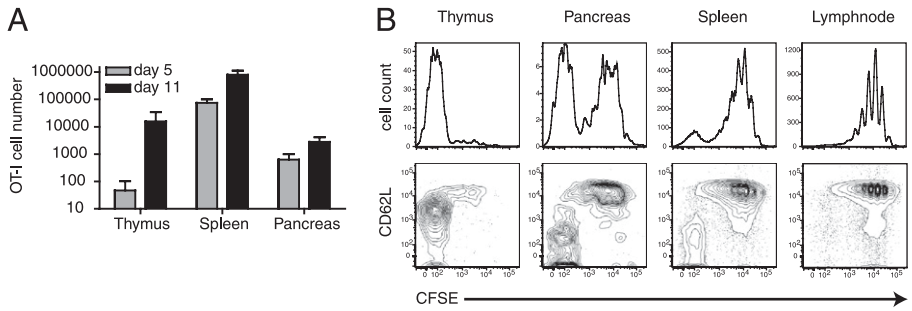

FIGURE 2. Most activated OT-I T cells can enter the thymus. Lethally irradiated B6 mice were reconstituted with syngeneic BM (3×10^6) together with CFSE-labeled Ly5.1$^+$ OT-I T cells (6.5×10^6) and analyzed at days 5 and 11 posttransfer. *A*, Total cell number (log scale) of adoptively transferred OT-I T cells in thymus, spleen, and pancreas at days 5 and 11. *B*, CFSE profile and CD62L expression of OT-I T cells in the indicated organs at day 11. OT-I T cells were identified based on the expression of CD8 and the congenic marker (Ly5.1). Data are representative of two independent experiments with similar outcomes (n = 3 per group).

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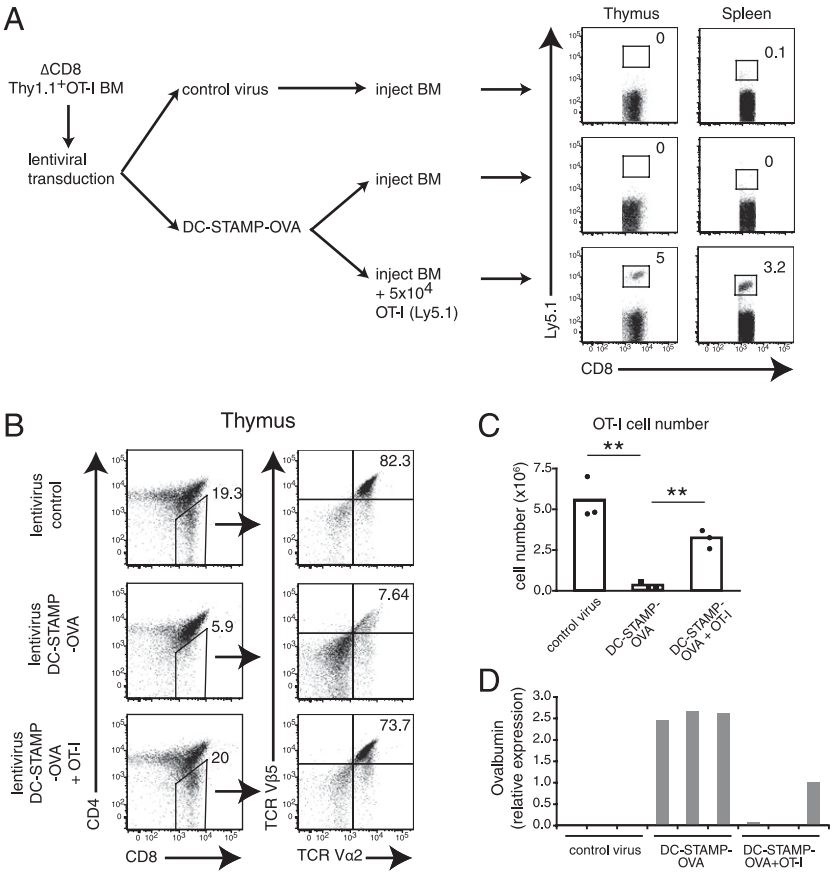

FIGURE 3. Adoptively transferred OT-I T cells disturb DC-mediated negative selection of BM-derived T cells. *A*, Thy1.1$^+$ OT-I BM was depleted of CD8 T cells (ΔCD8) and transduced with control lentivirus or lentivirus encoding OVA under control of the DC-STAMP promoter (DC–STAMP–OVA). The DC–STAMP–OVA lentivirus-treated BM was injected into irradiated B6 mice alone or with 5×10^4 Ly5.1$^+$ OT-I T cells. The presence of these cells is demonstrated in representative flow cytometric analyses of thymus and spleen (*right panels*) after gating on CD8 T cells. *B*, Flow cytometric analysis of thymocytes after gating on donor-specific (Thy1.1) cells (data not shown). Expression of OT-I TCR Vα2 and Vβ5.1/5.2 on CD8 SP cells identifies them as BM-derived OT-I T cells. *C*, Total cell number of BM-derived OT-I T cells in the thymus. $^{**}p$ = 0.001–0.01. *D*, RNA from thymocytes was analyzed by qPCR. Expression of OVA is shown relative to CD11c. Results are representative of three independent experiments, with three or four mice per group.

(DC–STAMP–OVA + Ly5.1$^+$OT-I, Fig. 3*A*), thymic DCs expressing OVA could be potential targets for activated re-entering Ly5.1$^+$ OT-I T cells. If back-migrated T cells indeed function to kill those DCs, negative selection should be obstructed, thereby allowing endogenous Thy1.1$^+$ OT-I T cells to develop. As expected, adoptively transferred Ly5.1$^+$ OT-I T cells homeostatically expanded and could be found in thymus, spleen and other organs (Fig. 3A, data not shown). Although the endogenous BM-derived Thy1.1$^+$ OT-I T cells developed normally in chimeras generated with control lentivirus-treated BM (Fig. 3*B*, 3*C*), their frequencies (Fig. 3*B*) and total numbers (Fig. 3*C*) were diminished to background levels in DC–STAMP–OVA chimeras as a result of negative selection, as previously reported (Fig. 3*B*, 3*C*) (25). When OT-I T cells were transferred into control chimeras, no interference with the development of endogenous OT-I thymocytes was observed (data not shown). In contrast, the presence of back-migrated OT-I T cells in DC–STAMP–OVA chimeras strongly interfered with negative selection, as observed by the development of the endogenous Thy1.1$^+$ donor-derived OT-I T cell population at near-normal frequencies (Fig. 3*B*, 3*C*). To demonstrate that OT-I T cell development was due to lack of OVA expression, we performed qPCR on thymic cells from the different groups (Fig. 3*D*). This analysis revealed that back-migrating Ly5.1$^+$ OT-I T cells caused the disappearance of DC-specific OVA mRNA expression, whereas OVA remained detectable in thymi of DC–STAMP–OVA–treated chimeras (Fig. 3*D*). Because we showed previously in DC–STAMP–OVA BM chimeras that thymic expression of OVA is confined to DCs (25), our data suggested that mature T cells expand homeostatically in lethally irradiated hosts and enter the thymus, where they eliminate thymic DCs. This allows endogenous thymocytes to develop in the absence of negative selection.

In addition to thymic DCs, mTECs are a key mediator of central tolerance induction. These cells efficiently express and present self-Ags and might serve as an Ag source for thymic DCs (29). We used RIP-mOVA mice to study whether mTECs could also be targets for mature re-entered thymic T cells (Fig. 4). Following lethal irradiation of RIP-mOVA hosts, we adoptively transferred Thy1.1$^+$ ΔCD8–OT-I BM alone or with Ly5.1$^+$OT-I T cells and monitored their expansion and accumulation in various tissues (Fig. 4*A*, data not shown). As a consequence of transgenic OVA expression by pancreatic β cells, the pancreas is damaged (30), followed by the onset of diabetes in all animals of this group (data not shown). As previously shown, mTECs of RIP-mOVA mice express transgenic OVA (27) and, therefore, induce efficient negative selection of Thy1.1$^+$OT-I T cells by thymic deletion in mice reconstituted with ΔCD8-BM (Fig. 4*B*). As a consequence, relatively low frequencies of BM-derived Thy1.1$^+$CD8$^+$ T cells expressing the transgenic OT-I TCR Vα2Vβ5 combination could be found in the thymus (Fig. 4*B*, 4*C*, ΔCD8). In contrast, development of endogenous Thy1.1$^+$OT-I T cells was much more efficient in RIP-mOVA hosts that had also received peripheral Ly5.1$^+$OT-I T cells (Fig. 4*B*, 4*C*, ΔCD8 + OT-I). Although OT-I T cells undergoing negative selection were CD69^{high}Thy1.1^{low} and showed a phenotype of recent activation, probably by Ag recognition (31, 32) (Fig. 4*D*, *upper panel*), BM-derived OT-I T cells that could develop unopposed by negative selection had a naive surface phenotype (CD69^{low}Thy1.1^{high}) (Fig. 4*D*, *lower panel*).

Re-entered mature T cells induce development of self-reactive endogenous T cells

We next wanted to monitor whether the observed inhibited thymic negative selection would also occur in normal mice with polyclonal T cell repertoires and if this would lead to the presence of functional

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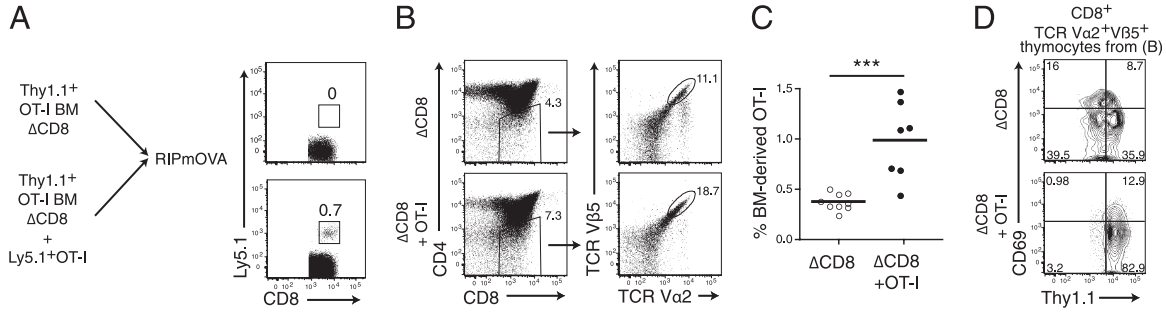

FIGURE 4. Adoptively transferred OT-I T cells disturb mTEC-mediated negative selection of BM-derived T cells. *A*, Thy1.1$^+$ OT-I BM was depleted of CD8 T cells (ΔCD8) and injected into lethally irradiated RIP-mOVA mice alone or mixed with 5×10^4 Ly5.1$^+$ OT-I T cells (ΔCD8+OT-I). Chimeras were analyzed at day 22 after transfer. Onset of diabetes was observed at day 7, and the diabetic animals were treated with insulin twice daily until the end of the experiment. Representative flow cytometric analysis of thymocytes after gating on CD8 SP thymocytes (*right panels*). *B*, Thymocytes were analyzed for CD4 and CD8 expression by flow cytometry after gating on the Thy1.1 marker (data not shown) to identify cells derived from donor BM. The frequency of endogenous OT-I TCR Vα2 and Vβ5.1/5.2$^+$ cells was determined (*right panels*). *C*, Percentage of BM-derived OT-I T cells in the thymus of ΔCD8→RIP-mOVA ($n = 9$) and ΔCD8+OT-I→RIP-mOVA chimeras ($n = 7$) was compared. ***$p = 0.0003$. *D*, Frequency of CD69^{hi} and Thy1.1^{lo} cells in the BM-derived OT-I population (from gate on CD8$^+$TCRVα2$^+$Vβ5$^+$ in *B*). Data are representative of two independent experiments with four to nine mice per group.

peripheral T cells. To this end, we generated C57BL/6 chimeras using C57BL/6-BM transduced with control or DC–STAMP–OVA lentivirus. The DC–STAMP–OVA–treated BM was injected alone or with low numbers of Thy1.1$^+$ OT-I T cells (Fig. 5*A*). Before immunization, we removed the adoptively transferred Thy1.1$^+$ OT-I T cells by injection of a Thy1.1-specific depleting Ab (Fig. 5*B*). We then immunized mice with replication-deficient HSV-expressing rOVA and monitored the induced CD8 T cell responses (Fig. 5*C*). As expected, all chimeras mounted efficient polyclonal CD8 T cell responses against the unrelated MHC class I K^b-restricted HSV-gB epitope SSIEFARL (Fig. 5*C*). In contrast, although control chimeras also showed T cell responses to the K^b-restricted OVA epitope SIINFEKL, DC–STAMP–OVA chimeras did not, because their T cell repertoire was devoid of OVA-specific cells due to lentivirus-mediated OVA expression in thymic DCs, leading to central tolerance (Fig. 5*C*) (25). However, when adoptively transferred OT-I T cells were present and acted to remove OVA-expressing thymic DCs in DC–STAMP–OVA chimeras, OVA-specific peripheral CD8 T cells were detectable in peripheral organs by MHC tetramers (Fig. 5*C*) and intracellular cytokine staining (Fig. 5*D*). These data demonstrated that thymus re-entry of mature CD8 T cells can lead to Ag-specific removal of mTECs and DCs with concomitant Ag-specific loss of central tolerance and the resulting appearance of self-reactive peripheral T cells.

Discussion

Remigration of peripheral T cells to the thymus is a rare event in normal adult immunocompetent animals (reviewed in Ref. 2). In contrast, when T cells are lacking congenically, such as in SCID-deficient (33) or RAG-deficient (7) animals, the numbers of adoptively transferred back-migrated thymic T cells can increase substantially. Depletion of T cells by irradiation or chemical ablation (chemotherapy) does not seem to enhance the migration of adoptively transferred naive T cells to the thymus (11, 34). However, back-migration is favored when T cells are activated by lymphopenic proliferation and allo-activation (28, 35). Yet, when CFSE-labeled C57BL/6 T cells were transferred into irradiated syngeneic C57BL/6-recipients, only low numbers of undivided, naive donor T cells were found in the thymi of the recipients (35). Also, we found similarly low C57BL/6 T cell numbers in syngeneic thymi, when the analyses were performed early after transfer (day 5, Fig. 2*A*). However, at day 11, the numbers of syngeneic T cells in recipient thymi had increased substantially (Fig. 2*A*) and the back-migrated syngeneic T cells had undergone several rounds of proliferation (Fig. 2*B*). These data indicated that substantial back-migration is also possible in syngeneic transfer systems, although it might take longer until T cells reach the "permissive" activation status by lymphopenic proliferation in the absence of antigenic TCR triggering. T cells expanding in lymphopenic conditions gain a memory T cell phenotype and respond rapidly with cytotoxic effector functions, when stimulated with cognate Ag (36).

In the absence of lymphopenia, adoptively transferred T cells are prone to tolerance induction, when cognate Ag is recognized in the absence of appropriate costimuli. It is unclear whether adoptively transferred and properly activated T cells in nonlymphopenic hosts would kill thymic mTECs and DCs expressing cognate Ag. Although peripheral DCs might also be proper CTL targets in normal mice (37), it is unknown whether T cells can also eliminate thymic APCs. If so, this would certainly contribute to perpetuation of certain autoimmune diseases, in which autoreactive T cells are activated in peripheral organs by self-Ag and can alter negative selection upon back-migration to the thymus. In addition, the transfer of purified CD8 T cells devoid of Foxp3$^+$ regulatory T cells (Tregs) might favor lymphopenic expansion and back-migration, because Tregs also substantially suppress expansion of cotransferred T cells in allogeneic settings (35) and negatively regulate GVHD (38, 39). Because Tregs are present in normal nonlymphopenic hosts, they might control adoptively transferred T cells effectively.

In some of our experiments (Figs. 3, 5), negative selection was almost completely inhibited, because normal frequencies of OT-I T cells were reappearing. It was shown previously that 1% of Ag-positive thymic DCs are sufficient to reduce 80% of CD4 T cells (40) or 60% of CD8 T cells (41) by negative selection. Therefore, the level of inhibition of negative selection observed in our experiments argues for a decrease in OVA$^+$ DCs below this threshold. The elimination of APCs presenting self-Ag leads to an increase in the generation of self-reactive T cells. As a consequence, endogenous self-reactive T cells are generated.

Yet another mechanism could involve removal of DC precursors instead of mature thymic DCs. If the DC-STAMP promoter was active in precursor cells, then cotransferred OT-I T cells would interfere with thymic repopulation by OVA$^+$ thymic DCs. Such a mechanism could certainly be of importance for gene products that are expressed by DC precursors. However, there is evidence from precursor microarray data that DC-STAMP is not expressed

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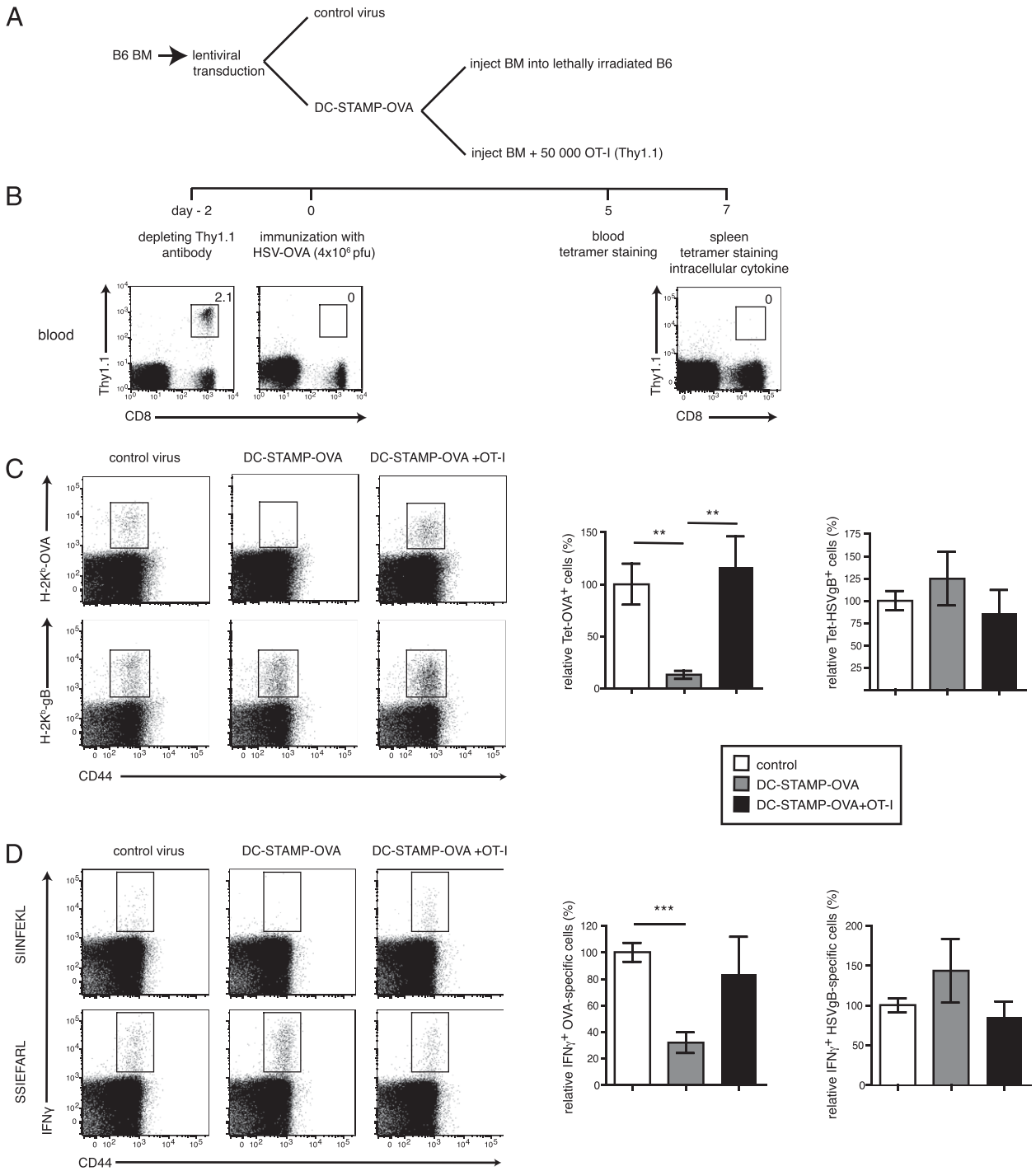

FIGURE 5. Adoptively transferred OT-I T cells disturb negative selection of a polyclonal CD8 T cell repertoire. *A*, B6 BM was transduced with control lentivirus or DC–STAMP–OVA lentivirus. The DC–STAMP–OVA lentivirus-treated BM was injected into lethally irradiated B6 recipients alone or together with 5×10^4 Thy1.1$^+$ OT-I T cells. *B*, After ≥8 wk, the adoptively transferred OT-I T cells were removed by treatment with a depleting Thy1.1 Ab 2 d before immunization and remained undetectable. Representative stainings of blood samples at the indicated time points are shown. *C*, Percentage of tetramer$^+$ cells (H-2K^b-OVA and H-2K^b-gB) in the splenic CD8 T cell population 7 d after immunization. Representative dot blots are shown, and data from two independently performed experiments (n = 2–3 per group) were pooled as data relative to control and statistically analyzed. *D*, Spleen suspensions were stimulated in vitro with the OVA peptide SIINFEKL or the gB peptide SSIEFARL and analyzed after 5 h for surface expression of CD44 and production of IFN-γ by intracellular staining. Representative flow cytometric data are shown after gating on the CD8$^+$ population. Representative dot blots are shown, and data from two independently performed experiments (n = 2–3 per group) were pooled as data relative to control and statistically analyzed. $^{**}p$ = 0.001–0.01, $^{***}p < 0.001$.

in common DC precursors (42). Also, our own data (S. Edelmann and T. Brocker, unpublished observations) indicated that DC-STAMP is only active in mature CD11c^{hi} DCs in vitro. In addition, ≥50% of thymic DCs originate from extrathymic DCs that home to the thymus in a more mature stage (43). Because we showed effective suppression of negative selection, all thymic DC subsets must be targets, including thymic DCs of mature extrathymic origin.

An alternative mechanism for elimination of negative selection could be competition for peptide/MHC complexes on the surface of DCs. To address this possibility, we performed in vivo experiments in which OT-I T cells were transferred into chimeras reconstituted

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with DC–STAMP–OVA–transduced BM from OT-II mice (data not shown). Because OT-II T cells recognize OVA peptide in the context of MHC class II, whereas OT-I cells are MHC class I restricted, no competition should occur. Because in these experiments negative selection of OT-II cells was also inhibited by OT-I cells (data not shown), we concluded that competition for MHC complexes on the surface of thymic DCs is not the mechanism responsible for our findings.

Our model of selective thymic DC or mTEC removal is clearly different from GVHD, which is complex (17, 18, 44) and has been elaborated using various animal models (45–51). In allogeneic BM transplantation, donor T cells can broadly damage the host thymus (thymic GVHD) by secretion of IFN-γ (20) or by mechanisms involving FasL and TRAIL (35). This overall destruction of the thymic epithelium impairs endogenous T cell development, which is essential for long-term immunity and survival. In addition to failure in reconstitution of the T cell compartment, the broad damage can lead to the loss of negative selection and appearance of T cells with antihost reactivity (19). It was shown more recently that severe damage of thymic architecture occurs and that thymic DCs are deleted during thymic GVHD (52). The protection of DCs from T cell attack by treatment of mice with keratinocyte growth factor could protect mice from GVHD (52). These data showed that DCs are also targets in models of classical GVHD, and their removal certainly contributes to disease. In contrast, our data showed that DC removal can also occur in disease-free settings (Figs. 3, 5), leading only to alteration of the T cell repertoire in the absence of peripheral expression of cognate Ag. Our findings suggested that, in addition to overall thymic destruction in allogeneic settings, Ag-specific adoptively transferred T cells can selectively eliminate APCs (DCs or mTECs) in an Ag-specific manner. Therefore, thymus–re-entering T cells could also play beneficial roles in therapies, such as the highly successful adoptive transfer of tumor-specific T cells in immune-depleted cancer patients (53); tumor-specific T cells are adoptively transferred into lymphopenic cancer patients, where they expand, gain effector functions, and are able to remove tumor cells with high efficiency (53). Because thymic DCs and mTECs present tumor (self-)Ags (54, 55), their possible removal might positively enhance the endogenous antitumor repertoire of the patients, similar to the appearance of OVA-specific T cells in our mouse model system (Fig. 5). However, because single mTECs express many self-Ags in addition to those recognized by the back-migrating T cells, mTEC eradication could also lead to a collateral loss of self-tolerance. It will be interesting to investigate the potential relevance of our findings to adoptive-therapy treatments in clinical settings.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu. Rev. Immunol.* 21: 139–176.
2. Sprent, J., and C. D. Surh. 2009. Re-entry of mature T cells to the thymus: an epiphenomenon? *Immunol. Cell Biol.* 87: 46–49.
3. Bosco, N., J. Kirberg, R. Ceredig, and F. Agenès. 2009. Peripheral T cells in the thymus: have they just lost their way or do they do something? *Immunol. Cell Biol.* 87: 50–57.
4. Hale, J. S., and P. J. Fink. 2009. Back to the thymus: peripheral T cells come home. *Immunol. Cell Biol.* 87: 58–64.
5. Ceredig, R. 2009. The impact of cell re-entry into the primary lymphoid organs on lymphocyte repertoire and functionality. *Immunol. Cell Biol.* 87: 13–15.
6. Hirokawa, K., M. Utsuyama, and T. Sado. 1989. Immunohistological analysis of immigration of thymocyte-precursors into the thymus: evidence for immigration of peripheral T cells into the thymic medulla. *Cell. Immunol.* 119: 160–170.
7. Kirberg, J., N. Bosco, J. C. Deloulme, R. Ceredig, and F. Agenès. 2008. Peripheral T lymphocytes recirculating back into the thymus can mediate thymocyte positive selection. *J. Immunol.* 181: 1207–1214.
8. Surh, C. D., J. Sprent, and S. R. Webb. 1993. Exclusion of circulating T cells from the thymus does not apply in the neonatal period. *J. Exp. Med.* 177: 379–385.
9. Tian, C., J. Bagley, D. Forman, and J. Iacomini. 2004. Induction of central tolerance by mature T cells. *J. Immunol.* 173: 7217–7222.
10. Webb, S. R., and J. Sprent. 1990. Induction of neonatal tolerance to Mlsa antigens by CD8+ T cells. *Science* 248: 1643–1646.
11. Agus, D. B., C. D. Surh, and J. Sprent. 1991. Reentry of T cells to the adult thymus is restricted to activated T cells. *J. Exp. Med.* 173: 1039–1046.
12. Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. Martín-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia, and F. Sallusto. 2007. L-selectin-negative CCR7− effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. *Nat. Immunol.* 8: 743–752.
13. Yang, J., S. P. Huck, R. S. McHugh, I. F. Hermans, and F. Ronchese. 2006. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 103: 147–152.
14. Ferrara, J. L., K. R. Cooke, and T. Teshima. 2003. The pathophysiology of acute graft-versus-host disease. *Int. J. Hematol.* 78: 181–187.
15. Ferrara, J. L., and G. Yanik. 2005. Acute graft versus host disease: pathophysiology, risk factors, and prevention strategies. *Clin. Adv. Hematol. Oncol.* 3: 415–419, 428.
16. Goker, H., I. C. Haznedaroglu, and N. J. Chao. 2001. Acute graft-vs-host disease: pathobiology and management. *Exp. Hematol.* 29: 259–277.
17. Higman, M. A., and G. B. Vogelsang. 2004. Chronic graft versus host disease. *Br. J. Haematol.* 125: 435–454.
18. Ho, V. T., and R. J. Soiffer. 2001. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* 98: 3192–3204.
19. van den Brink, M. R., E. Moore, J. L. Ferrara, and S. J. Burakoff. 2000. Graft-versus-host-disease-associated thymic damage results in the appearance of T cell clones with anti-host reactivity. *Transplantation* 69: 446–449.
20. Hauri-Hohl, M. M., M. P. Keller, J. Gill, K. Hafen, E. Pachlatko, T. Boulay, A. Peter, G. A. Holländer, and W. Krenger. 2007. Donor T-cell alloreactivity against host thymic epithelium limits T-cell development after bone marrow transplantation. *Blood* 109: 4080–4088.
21. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
22. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76: 17–27.
23. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184: 923–930.
24. Lois, C., E. J. Hong, S. Pease, E. J. Brown, and D. Baltimore. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295: 868–872.
25. Dresch, C., S. L. Edelmann, P. Marconi, and T. Brocker. 2008. Lentiviral-mediated transcriptional targeting of dendritic cells for induction of T cell tolerance in vivo. *J. Immunol.* 181: 4495–4506.
26. Lauterbach, H., K. M. Kerksiek, D. H. Busch, E. Berto, A. Bozac, P. Mavromara, R. Manservigi, A. L. Epstein, P. Marconi, and T. Brocker. 2004. Protection from bacterial infection by a single vaccination with replication-deficient mutant herpes simplex virus type 1. *J. Virol.* 78: 4020–4028.
27. Gallegos, A. M., and M. J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200: 1039–1049.
28. Tian, C., J. Bagley, and J. Iacomini. 2007. Homeostatic expansion permits T cells to re-enter the thymus and deliver antigen in a tolerogenic fashion. *Am. J. Transplant.* 7: 1934–1941.
29. Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annu. Rev. Immunol.* 24: 571–606.
30. Kurts, C., F. R. Carbone, M. Barnden, E. Blanas, J. Allison, W. R. Heath, and J. F. Miller. 1997. CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J. Exp. Med.* 186: 2057–2062.
31. Guehler, S. R., J. A. Bluestone, and T. A. Barrett. 1996. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. *J. Exp. Med.* 184: 493–503.
32. Zheng, B., S. Han, and G. Kelsoe. 1996. T helper cells in murine germinal centers are antigen-specific emigrants that downregulate Thy-1. *J. Exp. Med.* 184: 1083–1091.
33. Surh, C. D., B. Ernst, and J. Sprent. 1992. Growth of epithelial cells in the thymic medulla is under the control of mature T cells. *J. Exp. Med.* 176: 611–616.
34. Kadish, J. L., and R. S. Basch. 1977. Hematopoietic thymocyte precursors. III. A population of thymocytes with the capacity to return ("home") to the thymus. *Cell. Immunol.* 30: 12–24.
35. Na, I. K., S. X. Lu, N. L. Yim, G. L. Goldberg, J. Tsai, U. Rao, O. M. Smith, C. G. King, D. Suh, D. Hirschhorn-Cymerman, et al. 2010. The cytolytic

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molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J. Clin. Invest.* 120: 343–356.
36. Goldrath, A. W., L. Y. Bogatzki, and M. J. Bevan. 2000. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J. Exp. Med.* 192: 557–564.
37. Ronchese, F., and I. F. Hermans. 2001. Killing of dendritic cells: a life cut short or a purposeful death? *J. Exp. Med.* 194: F23–F26.
38. Hoffmann, P., J. Ermann, M. Edinger, C. G. Fathman, and S. Strober. 2002. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 196: 389–399.
39. Taylor, P. A., C. J. Lees, and B. R. Blazar. 2002. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99: 3493–3499.
40. Anderson, G., K. M. Partington, and E. J. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J. Immunol.* 161: 6599–6603.
41. Merkenschlager, M., M. O. Power, H. Pircher, and A. G. Fisher. 1999. Intrathymic deletion of MHC class I-restricted cytotoxic T cell precursors by constitutive cross-presentation of exogenous antigen. *Eur. J. Immunol.* 29: 1477–1486.
42. Felker, P., K. Seré, Q. Lin, C. Becker, M. Hristov, T. Hieronymus, and M. Zenke. 2010. TGF-beta1 accelerates dendritic cell differentiation from common dendritic cell progenitors and directs subset specification toward conventional dendritic cells. *J. Immunol.* 185: 5326–5335.
43. Li, J., J. Park, D. Foss, and I. Goldschneider. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J. Exp. Med.* 206: 607–622.
44. Pavletic, S. Z., S. L. Carter, N. A. Kernan, J. Henslee-Downey, A. M. Mendizabal, E. Papadopoulos, R. Gingrich, J. Casper, S. Yanovich, D. Weisdorf; National Heart, Lung, and Blood Institute Unrelated Donor Marrow Transplantation Trial. 2005. Influence of T-cell depletion on chronic graft-versus-host disease: results of a multicenter randomized trial in unrelated marrow donor transplantation. *Blood* 106: 3308–3313.
45. Hamilton, B. L., M. J. Bevan, and R. Parkman. 1981. Anti-recipient cytotoxic T lymphocyte precursors are present in the spleens of mice with acute graft versus host disease due to minor histocompatibility antigens. *J. Immunol.* 126: 621–625.
46. Parkman, R. 1986. Clonal analysis of murine graft-vs-host disease. I. Phenotypic and functional analysis of T lymphocyte clones. *J. Immunol.* 136: 3543–3548.
47. Hamilton, B. L., and R. Parkman. 1983. Acute and chronic graft-versus-host disease induced by minor histocompatibility antigens in mice. *Transplantation* 36: 150–155.
48. Anderson, B. E., J. M. McNiff, D. Jain, B. R. Blazar, W. D. Shlomchik, and M. J. Shlomchik. 2005. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. *Blood* 105: 2227–2234.
49. Zhang, C., I. Todorov, Z. Zhang, Y. Liu, F. Kandeel, S. Forman, S. Strober, and D. Zeng. 2006. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood* 107: 2993–3001.
50. Jaffee, B. D., and H. N. Claman. 1983. Chronic graft-versus-host disease (GVHD) as a model for scleroderma. I. Description of model systems. *Cell. Immunol.* 77: 1–12.
51. Via, C. S., and G. M. Shearer. 1988. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol. Today* 9: 207–213.
52. Zhang, Y., E. Hexner, D. Frank, and S. G. Emerson. 2007. CD4+ T cells generated de novo from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease. *J. Immunol.* 179: 3305–3314.
53. Rosenberg, S. A., N. P. Restifo, J. C. Yang, R. A. Morgan, and M. E. Dudley. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat. Rev. Cancer* 8: 299–308.
54. Cloosen, S., J. Arnold, M. Thio, G. M. Bos, B. Kyewski, and W. T. Germeraad. 2007. Expression of tumor-associated differentiation antigens, MUC1 glycoforms and CEA, in human thymic epithelial cells: implications for self-tolerance and tumor therapy. *Cancer Res.* 67: 3919–3926.
55. Gotter, J., B. Brors, M. Hergenhahn, and B. Kyewski. 2004. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J. Exp. Med.* 199: 155–166.

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