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Peripheral T Lymphocytes Recirculating Back into the Thymus Can Mediate Thymocyte Positive Selection

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The thymus continuously produces T lymphocytes that contribute to the maintenance of the peripheral T cell pool. Since peripheral recirculating T cells represent a very minor population among total thymocytes in normal animals, the relationship between the thymus and secondary lymphoid organs is generally considered unidirectional. Recently, several reports have described the presence of recirculating T cells in the thymus, raising issues regarding their possible function. In this article, we show that the niche for recirculating T cells in the thymus, i.e., their absolute number, is the same in lymphopenic and normal mice. Using a novel combination of TCR-transgenic mice in which the ligand necessary for positive selection of host T cells is only expressed by transferred donor T cells, we show that mature T cells recirculating back to the thymus can mediate positive selection. ***The Journal of Immunology,*** **2008, 181: 1207–1214.**

For continuous T cell production, the thymus must receive multipotent progenitors derived from the bone marrow. These progenitors have recently been called thymus-settling cells (TSP)[3] and enter the thymus at the corticomedullary junction (1). TSP, which initially express neither CD4 nor CD8 (double negative), migrate to the cortex and become CD4 and CD8 double positive (DP). Following TCR-positive selection, mature CD4 or CD8 single-positive (SP) cells migrate back into the medulla. On their way, they traverse the corticomedullary junction where it is thought that negative selection takes place (2). Once in the medulla, it is generally believed that all cells migrate out. However, the possibility that the medullary thymocytes constitute an equilibrium between cells produced in the cortex and recirculating peripheral T cells is rarely considered.

Previous reports had indicated that either transfer of labeled mature T cells into neonatal mice or activated T cells into adult animals resulted in some T cells entering the thymus (3–5). More recently, using new tools, such as transgenic, gene knockout and gene reporter mice, we and others have readdressed this question (6–8). These reports have shown that some SP cells in the thymus are in fact recirculating T cells that may play a role in negative selection of the TCR repertoire (9, 10). Herein, using parabiosis, T cell transfers into normal or lymphopenic hosts and a combination of TCR-transgenic mice where the ligand necessary for positive selection of host T cells is only expressed by transferred T cells, we quantify and further characterize the thymic recirculating T cell pool. In addition, we show that mature T cells recirculating back to the thymus are involved in positive selection.

Materials and Methods

Mice

All mice were on the C57BL/6 background (B6). B6 mice (Ly5.2) were obtained from Iffa-Credo. B6 Ly5.1 congenic (B6.Ly5^1) and B6 mice expressing GFP (B6.GFP) (11) were obtained from the Centre de Distribution Typage et Archivage animal. B6 mice deficient for the pre-Tα gene (B6 pT$\alpha^{-/-}$, referred to as B6.pTαko) were a gift from Dr. H. J. Fehling (University of Ulm, Ulm, Germany) (12). B6 mice deficient for the *RAG-2* gene (B6 RAG-2$^{-/-}$, referred to as B6.Ragko) were obtained from Dr. A. Rolink (University of Basel, Basel, Switzerland). OT1 TCR-transgenic mice, OVA-specific, H-2K^b-restricted, Vα2Vβ5, were provided by Drs. S. Degermann and E. Palmer; P14 TCR-transgenic mice, lymphocytic choriomeningitis virus gp33 specific, H-2D^b-restricted, Vα_2Vβ8, were provided by the CDTA. Hemagglutinin (HA) TCR-transgenic mice, influenza virus HA-specific, I-E^d restricted, were described before (13). All of these TCR-transgenic mice were bred with B6.Ragko or B6.Ragko.Ly5^1 to generate the following strains: B6 RAG-2$^{-/-}$ P14 TCR transgenic, referred to as B6.Ragko.P14tg, B6 RAG-2$^{-/-}$ OT1 TCR-transgenic, referred to as B6.Ragko.OT1tg, and B6 RAG-2$^{-/-}$ Ly5.1 HA TCR transgenic, referred to as B6.Ragko.Ly5^1.HAtg. Mice harboring the H-2^{bm1} haplotype in a RAG$^{-/-}$ background, referred to as B6.Ragko.K^{bm1}, have been described previously (14). To obtain B6 RAG-2$^{-/-}$ OT1 TCR-transgenic K$^{b-/-}$ mice, referred to as B6.Ragko.OT1tg.K^{b}ko, B6.Ragko.OT1tg were subsequently bred with K^b-deficient mice (15). All mice were bred at the animal facilities of the Commissariat á l'Energie Atomique (Grenoble, France) or at the Max Planck Institute of Immunobiology (Freiburg, Germany). Parabiosis was conducted as previously described (16). For fetal liver reconstitution, livers were dissociated by pipetting and cells were transferred into adult recipient animals that were irradiated from a ^{137}Cs source. For intrasplenic CFSE injection, 75 μl of a 400 μM CFSE solution freshly diluted in PBS was injected into the two extremities of the spleen (150 μl total) while animals were under anesthesia with ketamine and xylazine. All animals were kept under specific pathogen-free conditions and 6- to 10-wk-old mice were used for experiments that were conducted according to institutional guidelines.

Cell preparation and flow cytometry

Single-cell suspensions were prepared from thymus, pooled inguinal, axillary, brachial, and cervical lymph nodes (LN) or spleen. For flow cytometry, cells were washed and resuspended in PBS containing 2% FCS and

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[3] Abbreviations used in this paper: TSP, thymus-settling cell; DP, double positive; LN, lymph node; SP, single positive; tg, transgenic; HA, hemagglutinin; PI, propidium iodide; CK, cytokeratin; WT, wild type; HSA, human stable Ag.

0.01% sodium azide. Viable cells were counted in a hemocytometer following staining with trypan blue. The total number of T cells was calculated from the frequency determined by FACS analysis and the total number of living cells recovered per organ.

The following mAbs were used: anti-CD4 (RM4-5 or GK1.5), anti-CD5 (53-7.3), anti-CD8 (53-6.7), anti-CD8b.2 (53-5.8), anti-CD19 (1D3), anti-CD24 (human stable Ag (HSA), M1/69) anti-CD44 (IM7), anti-Ly5.1 (A20), anti-Ly5.2 (104), anti-TCRβ (H57-597) (BD Pharmingen) and anti-Vβ Abs: anti-Vβ5 (MR9-4.5), anti-Vβ8 (F23.1), the other anti-Vβ have been described previously (17). Cell surface staining was performed as previously described (16) and acquisitions were performed on FACSCalibur, FACSLSRII, or CANTO instruments (BD Biosciences). Analyses were performed with CellQuest, DIVA (BD Biosciences), or FlowJo (Tree Star) softwares. Dead cells were excluded from the analysis by a combination of light scatter and/or absence of propidium iodide (PI) staining. Cell sorting was performed either on a MoFlo (DakoCytomation) or FACSAria (BD Biosciences) and the purity of sorted cells was >98%.

Confocal microscopy

GFP$^+$CD4$^+$ LN T cells from GFP-transgenic mice were purified by sorting and injected into B6.pTαko hosts. After 7 days, animal were perfused with PBS/4% paraformaldehyde, spleen was collected, postfixed overnight at 4°C in 4% paraformaldehyde, incubated in 30% sucrose for 24 h, and embedded in frozen medium (Cryomatrix; Therm Shandon). Immunofluorescence staining has been performed as detailed previously (18). Cryostat sections were immunolabeled with anti-CD4, anti-CD8, anti-cytokeratin (CK), and/or anti-CK18. CK5 and CK18 Abs were provided by Dr. J. Gill (University of Basel). Fluorescent images were obtained with a Leica (TCS SP2) confocal microscope. Due to perfusion with paraformaldehyde, no peripheral lymphocytes are found in vessels.

Results

Re-entry of peripheral T cells in different thymi following parabiosis

Using parabiosis, we have recently shown that in lymphopenic B6 pT$\alpha^{-/-}$ mice, referred to as B6.pTαko, a considerable proportion of SP thymocytes represent recirculating peripheral T cells (6). To investigate T cell recirculation back to the thymus in more detail, we performed parabiosis experiments combining immunocompetent mice (B6) with immunodeficient animals in which SP cells, and eventually DP cells also, are absent: 1) B6 RAG-2$^{-/-}$ Ly5.1 mice, referred to as B6.Ragko.Ly5^1, in which DP and SP cells are absent and 2) B6 RAG-2$^{-/-}$ Ly5.1 HA TCR transgenic, referred to as B6.Ragko.Ly5^1.HAtg mice, which on this MHC background (H-2^b) do not positively select the HA-TCR, leading to an accumulation of DP and an absence of SP cells. Thus, we parabiosed (B6 with B6.Ragko.Ly5^1) and (B6 with B6.Ragko.Ly5^1.HAtg) and the thymi of the immunodeficient mice were analyzed after 14 days for the presence of mature T cells derived from the immunocompetent partner. Results obtained (Fig. 1) show that independently of the DP/SP composition, mature T cells from the partner mouse could be readily detected in the thymus (6). Analysis of the thymus of the B6.Ragko.Ly5^1 mice parabiosed with B6 mice showed that only partner-derived SP cells were present. The absence of DP cells in the thymus and of progenitor B cells in the bone marrow (data not shown) indicates that in these short-term parabiosis experiments, there was no exchange of lymphoid progenitors, including TSP.

Recirculation in non parabiosed mice

In the above parabiosis experiments, we could show that mature T cells recirculate back to the thymus. To quantify the number of mature T cells capable of entering the thymus of lymphopenic and normal mice, an alternative experimental approach was undertaken. Genotypically distinct (GFP$^+$) mature T cells were adoptively transferred into either B6.pTαko or B6 host mice and their thymi were analyzed after 14 days. In lymphopenic B6.pTαko hosts, after injection of 25×10^6 GFP$^+$ lymph node cells, the level of T cell chimerism in the spleen was 47 ± 5% (proportion of GFP$^+$ cells among TCRβ^+ cells). The proportion of GFP$^+$TCRβ^+ cells in the thymus was 1.9% (range, 0.5–6.7, n = 5). Assuming that injected GFP$^+$ and host-derived (GFP$^-$) mature T cells recirculate equally well to the thymus, we calculated that the thymus of B6.pTαko mice contains ~10^5 recirculating, peripheral T cells (Fig. 2*A*). As expected, in nonlymphopenic B6 mice, the degree of T cell chimerism in the spleen was only 1.4 ± 0.5% and the proportion of GFP$^+$TCRβ^+ cells in the thymus was 0.00097 ± 0.00049% (i.e., frequency of 0.97 ± 0.49 × 10^{-5}). Using these values, we calculated that the total number of recirculating T cells in the thymus of normal B6 mice was approximately the same as in lymphopenic B6.pTαko mice, namely, 10^5 cells (Fig. 2*A*). That this number represents an increased proportion in B6.pTαko mice reflects the reduced thymus cellularity of these mice. Further phenotypic analysis indicated that more CD4$^+$ than CD8$^+$ T cells recirculated to the thymus.

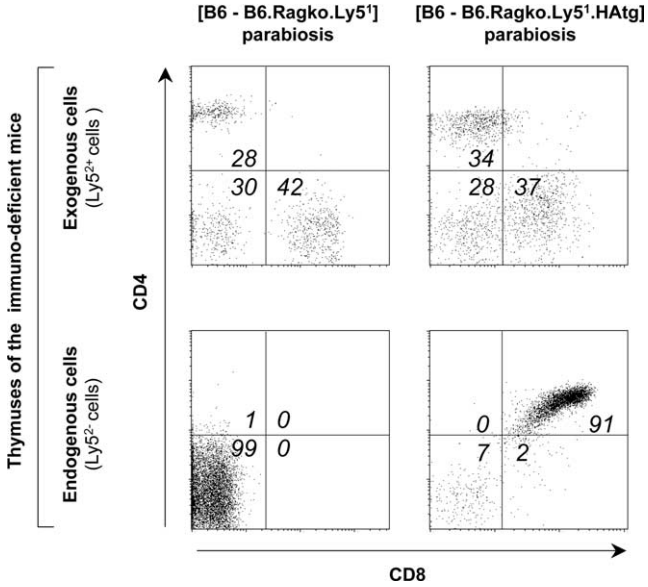

FIGURE 1. Migration of peripheral T cells from immunocompetent mice into the thymus of immunodeficient hosts following parabiosis. Immunocompetent B6 mice were parabiosed with immunodeficient mice devoid of SP and DP cells in the thymus (B6.Ragko.Ly5^1, *left panels*) or lacking SP thymocytes only (B6.Ragko.Ly5^1.HAtg, *right panels*). Two weeks after surgery, animals were sacrificed. Peripheral lymphoid organs (data not shown) and thymi of the immunodeficient animals were collected and analyzed for the presence of lymphocytes originating from the immunocompetent parabiosis partner. Endogenous cells were distinguished from recirculating cells originating from the contralateral parabiont by the expression of the relevant Ly5 allelic marker. *Upper graphs,* CD4 and CD8 expression among cells that originate from the immunocompetent animal; *lower graphs*, the same profile for endogenous sessile thymocytes of the immunodeficient animals. Results are representative of three to five independent parabiosis pairs for each combination. Results obtained for (B6 and B6.pTαko) parabiosis have been published previously.

To complement the above results, an independent experimental approach was devised to increase the proportion of peripheral cells that would be labeled. To this end, CFSE was directly injected into the spleen of mice and, 7 days later, the proportion of CFSE$^+$ cells in lymphoid organs was analyzed (Fig. 2*B*). Assuming that mature T cells recirculate independently of CFSE labeling, we estimated that the total number of mature recirculating T cells in the thymus of B6 mice was 8.6 ± 2.0 × 10^4. Similar experiments were conducted with B6.pTαko mice and the calculated number of recirculating cells was similar. Importantly, these results indicate that

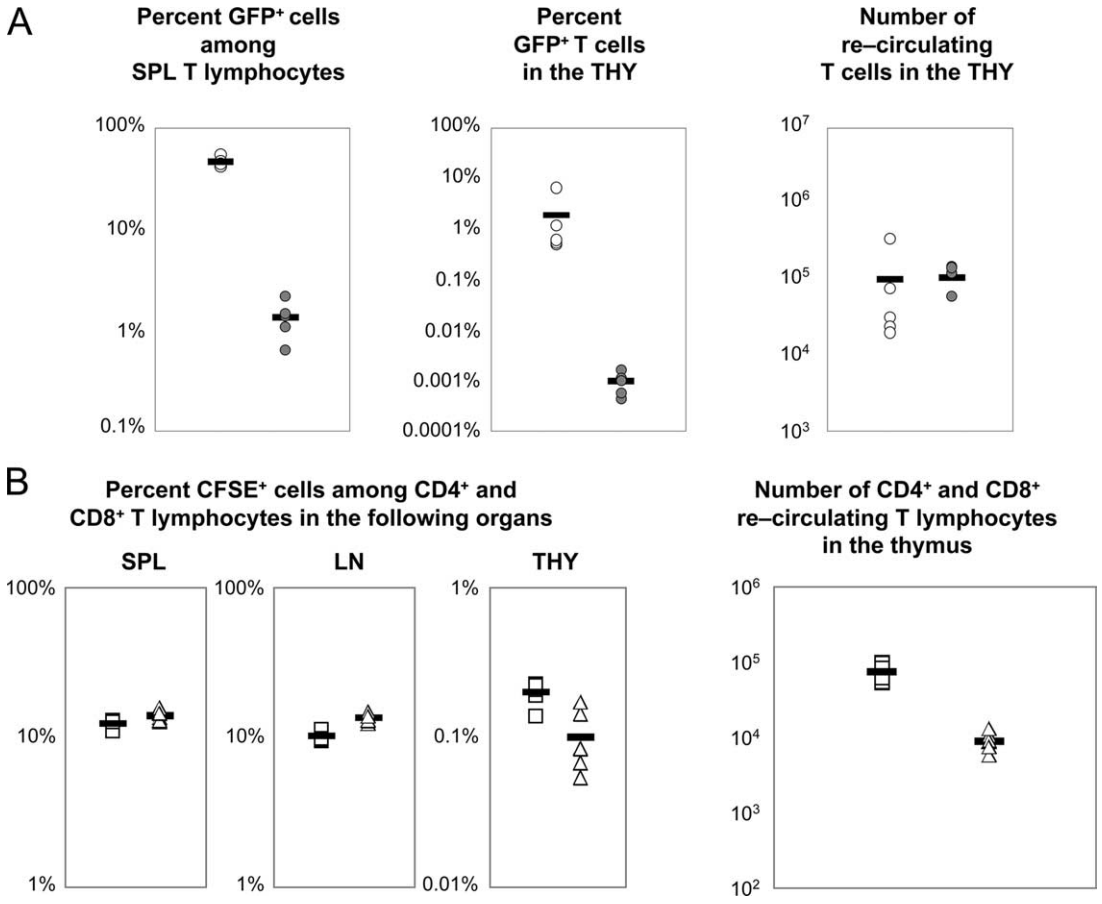

FIGURE 2. Quantification of "peripheral" mature T cells recirculating into the thymus. *A*, Numbers of recirculating T cells in B6.pTαko and B6 mice thymi following adoptive transfer of GFP$^+$ cells. *Left panel*, Percentage of GFP$^+$ cells among TCRβ^+ cells in the spleen (SPL) of B6.pTαko (○) or B6 (●) mice injected with 25×10^6 GFP$^+$ LN cells 2 wk earlier. *Middle panel*, Percentage of GFP$^+$TCRβ^+ cells in the thymus (THY) of the same animals. *Right panel*, Based on these frequencies and thymus cellularity, the total number of recirculating cells present in the thymus of B6.pTαko and B6 mice was calculated, assuming that injected GFP$^+$ and host-derived GFP$^-$ mature T cells recirculate equally well to the thymus. Five animals per group were analyzed and the experiment was performed twice varying the quantity of injected donor GFP$^+$ cells (2×10^6, 6×10^6, or 2×10^7; data not shown); the calculated overall number of recirculating T cells was independent of the number of peripheral LN GFP$^+$ cells transferred. *B*, Numbers of recirculating T cells in B6 mice following intrasplenic CFSE labeling. Shown is the percentage of labeled cells 7 days after intrasplenic injection of CFSE among CD4$^+$ (□) and CD8$^+$ (△) T cells in the peripheral lymphoid organs (spleen and LN) and in the thymus (*left panel*). Based on the determined frequencies of recirculating T cells and the thymus cellularity, we calculated the number of peripheral CD4$^+$ and CD8$^+$ T cells present in the thymus of normal mice, assuming that mature T cells recirculate to the thymus irrespective of CFSE labeling (*right panel*). Six mice were used in this experiment which was reproduced twice.

recirculation of T cells back into the thymus is not a unique property of lymphopenic mice and, irrespective of the thymus size or genotype, the thymus can accommodate $\sim 10^5$ recirculating peripheral T cells (19).

Two groups using RAG-2 promoter-driven GFP mice (RAG2p-GFP) have also recently shown that mature phenotype thymocytes contain a population of recirculating cells (7, 8). In RAG2p-GFP mice, thymocytes are initially generated as GFP$^+$ cells, which upon migration to the periphery rapidly lose GFP expression; thymus recirculating cells are identified being GFP$^-$. Based on two different methods, namely, GFP$^+$ cell transfer and intrasplenic CFSE injection, we found that in wild-type (WT) animals on average $\sim$1% of thymic SP cells are recirculating lymphocytes, whereas Hale et al. (7) suggest that it is $\sim$5% in 5- to 9-wk-old mice. However, our approaches, in which peripheral cells are positively stained, might slightly underestimate the phenomenon if a proportion of peripheral T cells in the thymus have a slow turnover. In contrast, use of RAG2p-GFP mice might lead to an overestimation due to premature loss of GFP expression in newly generated SP thymocytes before emigration from the thymus and nondiscrimination of $\alpha\beta$ and $\gamma\delta$ T cells within the SP populations.

In certain mice displaying a partial block in T cell production and concomitant peripheral lymphopenia, the absolute number of SP thymocytes is relatively high compared with the number of DP cells; i.e., the ratio SP:DP is higher than in WT controls. This is the case for mice deficient for genes involved in T cell development (12, 20), in mice in which genes encoding molecules expressed by epithelial cell supporting T cell maturation have been deleted (21), as well as for mouse models of T cell lymphopenia (6). Interestingly, the SP:DP ratio is also elevated in cervical thymi (22). Instead of being caused by an increased rate of survival, positive selection, or cell division of SP cells, in these situations a considerable fraction of SP cells may represent recirculating mature T cells, as in B6.pTαko mice.

Phenotype, diversity, and localization of recirculating T cells

To determine whether T cells recirculating to the thymus represented a particular subpopulation of peripheral T cells, we used the CFSE labeling approach described above to determine the proportion of naive vs memory phenotype T cells among recirculating cells. As shown in Fig. 3*A*, the CD44 profile of splenic and thymic CFSE$^+$CD4$^+$ cells differed with 29% splenic and 52% thymic

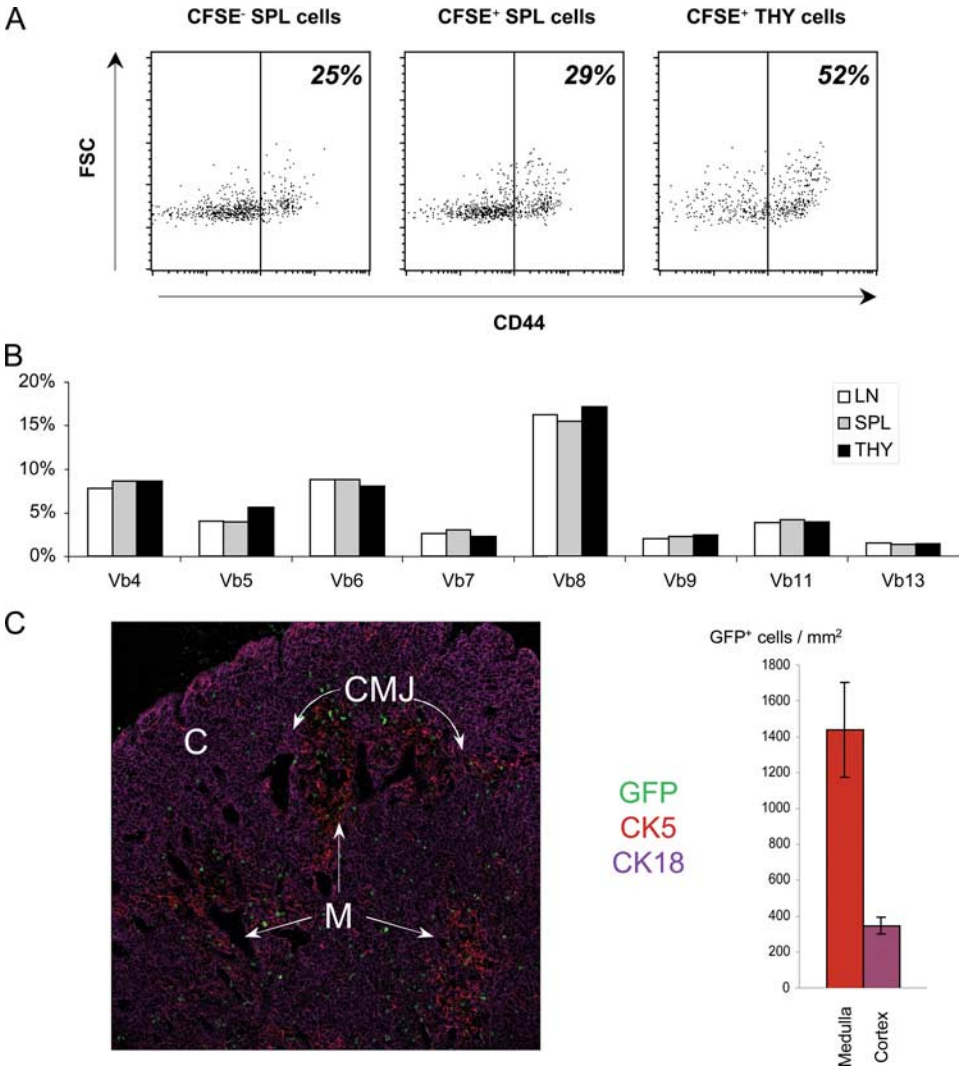

FIGURE 3. Phenotype, diversity, and localization of recirculating T cells. *A*, CD44 expression on CFSE$^+$ T cells in the spleen and the thymus of B6 animals injected with CFSE intrasplenically. Phenotypic analysis of splenic CD4$^+$CFSE$^-$ cells (*left panel*), splenic CD4$^+$CFSE$^+$ cells (*middle panel*), and thymic CD4$^+$ CFSE$^+$ cells (*right panel*) for forward scatter (FSC) vs CD44 from a mouse presented in Fig. 2*C*. Data presented were obtained from one animal representative for the group. *B*, TCR Vβ representation among B6.Ly5^1 donor T cells in the lymphoid organs of B6.pTαko recipients. The graph depicts the percentage of T cells expressing the given Vβ element within the B6.Ly5^1 CD4 SP T cell population (CD4$^+$CD8$^-$PI$^-$Ly5^{1+}) in the LN (□), spleen (▨), and thymus (■) of B6.pTαko recipient mice (Ly5^2). *C*, Localization of transferred GFP$^+$ LN T cells recirculating into the thymus of B6.pTαko recipients. Thymus sections were stained for GFP (green), CK5 to stain medullary epithelial cells (red), and CK18 to stain cortical epithelial cells (cyan). The histogram bars show distribution of GFP$^+$ cells in the cortex or the medulla based on analysis of thymic sections from two recipient mice.

CD4$^+$ cells being CD44^{high}. In a series of five such experiments, the proportion of CD44^{high}CD4$^+$ splenic cells was 24 ± 4% while in the thymus it was 50 ± 2%. Thus, there was an enrichment for CD44^{high} cells among T cells recirculating to the thymus.

To determine whether there was a bias in TCRVβ usage by recirculating cells, LN cells from B6.Ly5 ^{1} mice were transferred into B6.pTαko hosts. After 11 days, cells from spleen, LN, and thymus were stained with anti-Ly5 ^{1} and a panel of Vβ-specific mAbs. These experiments were performed using B6.pTαko hosts because the proportion of donor-derived cells in the thymus required for a complete Vβ repertoire analysis was insufficient using B6 mice. Results obtained from a pool of animals (Fig. 3*B*) showed that, first, the Vβ repertoire of recirculating T cells in the thymus was diverse and, second, that there was no bias in the representation of different Vβ specificities. This result was confirmed by parabiosis experiments between B6 RAG-2$^{-/-}$ P14 TCR-transgenic mice (Vβ8$^+$), referred to as B6.Ragko.P14tg, with B6 RAG-2$^{-/-}$ OT1 TCR-transgenic mice (Vβ5$^+$), referred to as B6.Ragko.OT1tg: T cells from the partner mouse were clearly present in each contralateral thymus 7 days after surgery (data not shown).

Function of recirculating T cells

To localize the recirculating T cells in the thymus, LN T cells from GFP-transgenic mice were purified by cell sorting and injected into B6.pTαko hosts. After 7 days, thymic sections were prepared and immunolabeled for cytokeratin-5 and cytokeratin-18 to stain medullary epithelial cells and cortical epithelial cells, respectively. Three-color confocal microscopy (Fig. 3*C*) showed that donor GFP$^+$ cells were found predominantly in the medulla. However, GFP$^+$ cells could also be found throughout the cortex. Because positive selection of DP thymocytes takes place in the cortex, we designed experiments to determine whether recirculating mature T cells could support positive selection of DP cells, a function commonly attributed to cortical epithelial cells only.

In line with this, our analysis of positive selection of OT1 TCR-expressing cells in recipient mice lacking the restricting class I MHC molecule H-2K^b had led to the surprising finding that the presence of H-2K^b on cells of the hematopoietic lineage was already sufficient for positive selection. Thus, when B6.Ragko.OT1tg fetal liver cells were used to reconstitute irradiated mice of H-2^{bm1} haplotype (B6.Ragko.K^{bm1}) or mice deficient for K^b (B6.Ragko.K^{b}ko) (14, 15), the recipient thymi contained a reduced, but significant, compartment of mature SP thymocytes (CD4$^-$CD8$^+$Vα2$^+$HSA$^-$), in comparison to thymi of control mice that express H-2K^b also on thymic epithelial cells (Fig. 4*A*). By analogy to published data, the hematopoietic cell type supporting positive selection in this situation may include thymocytes (23, 24) and/or recirculating T cells. Indeed, this type of positive selection on bone marrow-derived cells is efficient in *trans*, as shown

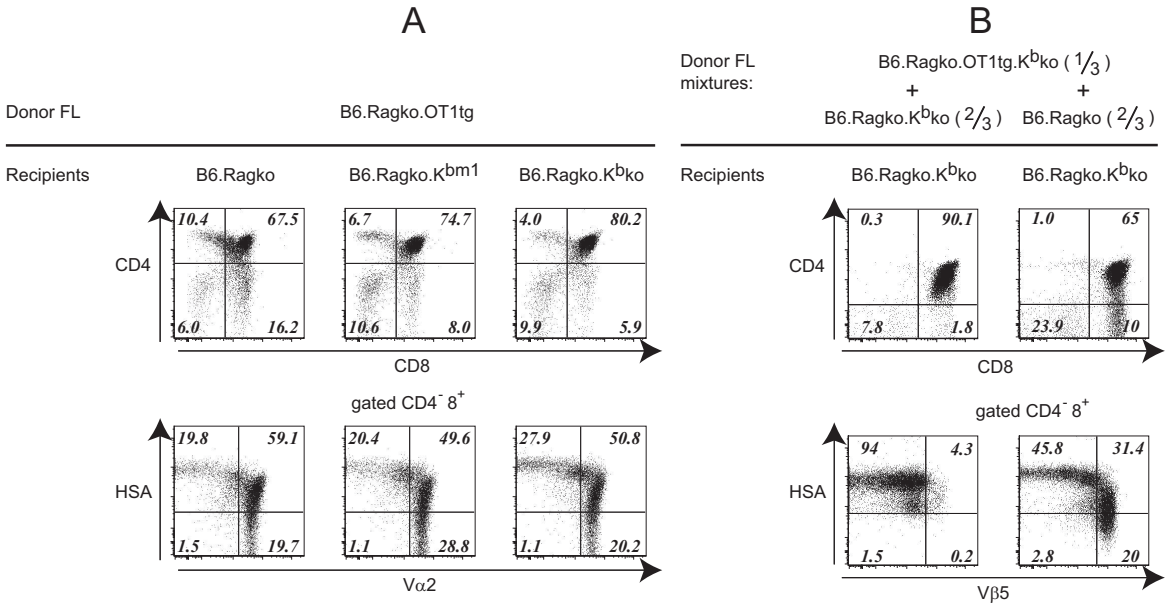

FIGURE 4. Positive selection of OT1 thymocytes on cells of hematopoietic origin. *A*, The equivalent in cells from one-third of a B6.Ragko.OT1tg fetal liver, gestational age day 14.5, was transferred i.v. into the indicated recipient mice that had received 650 rad of total body irradiation the day before. Six to 8 wk after reconstitution, recipient thymocytes were analyzed as shown. Thymocyte numbers were $13.0 \pm 1.8 \times 10^6$, $11.7 \pm 0.2 \times 10^6$, and $7.8 \pm 0.3 \times 10^6$ for B6.Ragko, B6.Ragko.K^{bm1}, and B6.Ragko.K^bko recipients, respectively. Absence of positive selection of OT1 T cells in the absence of K^b is shown in Fig. 5*B* (*upper left panel*) for B6.Ragko.OT1tg.K^bko mice, as well as in Fig. 4*B*. *B*, Irradiated B6.Ragko.K^bko recipient mice were prepared as in Fig. 4*A*, but reconstituted with day 15.5 fetal liver cells that were a mixture of cells from B6.Ragko.OT1tg.K^bko (one-third of final inoculum) and either B6.Ragko.K^bko or B6.Ragko (two-thirds of final inoculum) embryos. Thymocyte numbers were $9.7 \pm 1.6 \times 10^6$ and $2.5 \pm 1.3 \times 10^6$ for recipients receiving B6.Ragko.OT1tg.K^bko fetal liver cells admixed with B6.Ragko.K^bko or B6.Ragko fetal liver cells, respectively.

by reconstituting irradiated B6.Ragko.K^bko mice with a mixture of B6.Ragko and B6.Ragko.OT1tg.K^bko fetal liver cells (Fig. 4*B*). In contrast, when these mice were reconstituted with a mixture of B6.Ragko.K^bko and B6.Ragko.OT1tg.K^bko fetal liver cells, there was no evidence of positive selection, as expected (Fig. 4*B*).

To directly investigate whether recirculating peripheral T cells expressing H-2K^b class I molecules could mediate positive selection of OT1 thymocytes, we transferred peripheral P14 TCR-transgenic H-2K^b-expressing cells into B6 RAG-$2^{-/-}$ OT1 TCR-transgenic $K^{b-/-}$ mice (referred to as B6.Ragko.OT1tg.K^bko). Since P14 T cells are restricted to H-2D^b, a MHC class I molecule expressed normally in H-2K^b-deficient recipient mice, their survival would be ensured. Three weeks after injection of P14 cells, lymphoid organs of recipient mice were collected, stained for CD4, CD8, HSA, PI, and Vβ (Vβ5 or Vβ8) and the number of V$\beta5^+$ OT1 (host) and V$\beta8^+$ P14 (donor) $CD8^+$ cells was determined. As expected, V$\beta8^+$ P14 T cells were detected in the spleen ($8.0 \pm 0.4 \times 10^5$), pooled LN ($1.6 \pm 0.3 \times 10^6$), and thymus ($8.2 \pm 0.3 \times 10^4$) of recipient mice (Fig. 5*A*). In the thymus, we also detected $3.1 \pm 0.9 \times 10^4$ $CD4^-CD8^+HSA^-V\beta5^+$ cells suggesting that P14 T cells that recirculate into the thymus provided the H-2K^b-restricting element necessary for the positive selection of OT1 T cells (Fig. 5, *B* and *C*). In the peripheral lymphoid organs of B6.Ragko.OT1tg.K^bko mice, we observed few $CD4^-CD8^+HSA^-V\beta5^+$ cells ($1.5 \pm 0.6 \times 10^6$) and their number was not elevated after transfer of P14 LN cells ($1.0 \pm 0.5 \times 10^6$). This suggested that OT1 SP cells arising in the thymus of B6.Ragko.OT1tg.K^bko mice after engraftment with P14 LN cells are newly generated SP thymocytes and not rare preexisting cells that underwent homeostatic expansion. In the above experiment, the vast majority of the injected cells were $CD8^+$ P14tg T cells and positive selection of OT1 cells is likely to be mediated by recirculating peripheral T lymphocytes. However, to exclude that positive selection was mediated by other cells types, a similar experiment was conducted with purified T cells. Results obtained showed that purified P14.K^{b+} T cells mediate the positive selection of OT1 thymocytes; as expected, expression of K^b on the transferred P14 T cells is essential (Fig. 6, *A* and *B*). No positive selection was observed when peripheral mature K^b-expressing non-B and non-T cells (from B6.Ragko donors) were transferred into B6.RAGko.OT1tg.K^bko recipients, suggesting that under these experimental conditions, T cells are those cells that migrate back to the thymus most efficiently to mediate positive selection. Positive selection of OT1 cells was detected in B6.Ragko.OT1tg.K^bko mice injected with 2×10^7 $CD3^+TCR\beta^+$ purified T cells from B6.Ly5^1 donors, showing that polyclonal T cells recirculating back into the thymus can also mediate thymocyte-positive selection (data not shown).

Discussion

Whether mature, peripheral T cells can recirculate back to the thymus has been the subject of debate (8). Early observations indicated that this phenomenon might be restricted to the neonatal period, a time when the periphery is lymphopenic, or to the recirculation of some activated T cells back to the adult thymus (5). Clearly, during an ongoing antiviral immune response, some degree of priming is seen among mature thymocytes (25, 26). Using either parabiosis (6) or transfer of CFSE-labeled cells (27), we have previously shown that in a situation of peripheral lymphopenia, the contribution of recirculating peripheral T cells to the pool of mature "thymocytes" was significant. Using complementary approaches, including adoptive transfer of genetically marked T cells and the intrasplenic injection of CFSE, in this study we quantify this recirculation in detail and analyze the phenotype and TCR Vβ repertoire of recirculating cells. As expected, T cells recirculating to the thymus had a diverse TCR Vβ repertoire and were enriched

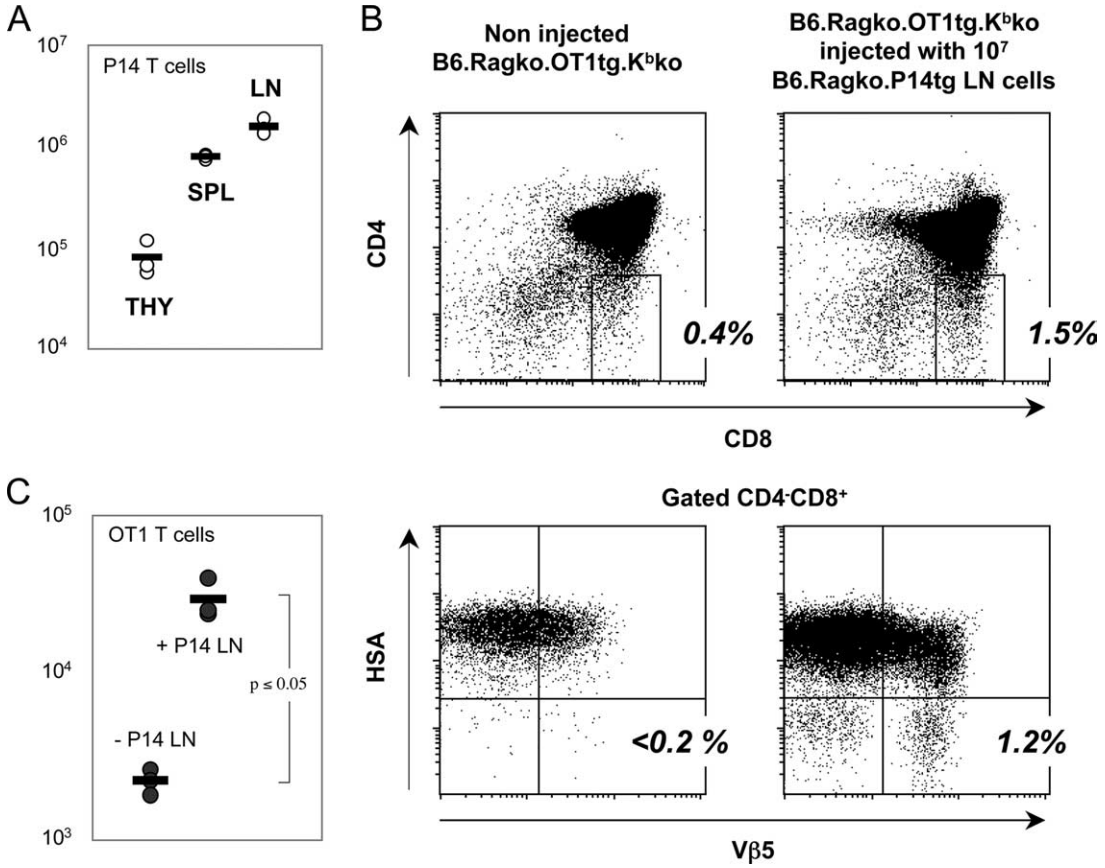

FIGURE 5. Positive selection of OT1 thymocytes on recirculating K^b-expressing cells. *A*, Monoclonal P14 T cells migrate and survive efficiently in the spleen, LN, and thymus following transfer into B6.Ragko.OT1tg.Kbko mice. B6.Ragko.P14tg LN cells (10^7) were transferred into B6.Ragko.OT1tg.K^bko recipients i.v. Three weeks later the percentage of $CD8^+CD4^-HSA^-PI^-V\beta8^+$ cells in the various lymphoid organs of the recipients were determined by FACS and the absolute number of $CD8^+CD4^-V\beta8^+$ cells was calculated ($n = 3$). *B*, Positive selection of OT1 thymocytes on recirculating P14 LN cells expressing K^b class I MHC molecules. Thymocytes of the animals described in *A* were stained for CD8, CD4, HSA, PI, and Vβ5. The *upper dot plots* show CD4 vs CD8 expression of viable thymocytes in the noninjected control B6.Ragko.OT1tg.K^bko mice (*left dot plot*) and B6.Ragko.OT1tg.K^bko mice that had received B6.Ragko.P14tg cells (*right dot plot*). The percentage of $CD8^+CD4^-$ cells is indicated for the depicted region. Among these cells, HSA vs Vβ5 expression was evaluated (*lower panels*): injected P14tg T cells are expected in the *lower left quadrant* ($CD8^+CD4^-HSA^-PI^-V\beta5$) and OT1tg SP T cells, if generated, in the *lower right quadrant* ($CD8^+CD4^-HSA^-PI^-V\beta5^+$). *C*, Number of OT1 SP thymocytes generated in the recipient mice. The number of OT1 SP thymocytes was calculated based on the FACS stainings shown in *B* and thymus cellularity for noninjected B6.Ragko.OT1tg.K^bko mice (*left*, −P14 LN; $n = 3$) and B6.Ragko.OT1tg.K^bko injected with 10^7 B6.Ragko.P14tg LN cells (*right*, +P14 LN; $n = 3$).

in $CD44^{high}$, so-called "memory-like" cells (28). Because it had already been shown that recirculating T cells can mediate negative selection of developing thymocytes (9, 10), their localization to the medulla might have been expected (3, 4). In this study, we show that medullary recirculating cells were clearly detectable outside of blood vessels and intermingled with host thymocytes. Moreover, we also observed recirculating T cells in the cortex, the site where positive selection is presumed to occur (1, 2). This observation led us to investigate whether recirculating T cells might be involved in positive selection by using novel TCR-transgenic strains. In B6.RAGko.OT1tg.K^bko mice, because of the absence of expression by thymic epithelial cells of the H-$2K^b$ molecule necessary for the positive selection of OT1 TCR, thymocyte development is arrested at the DP stage (14). When these mice are injected i.v. with B6.Ragko.P14tg $CD8^+$ T cells, some recirculate to and enter the recipient thymus, thereby providing H-$2K^b$ molecules necessary for the positive selection of host OT1Tg thymocytes.

Despite being able to readily detect $CD8^+HSA^-V\beta5^+$ (OT1) cells in the thymus, we did not detect increased number of these cells in the periphery, presumably because the H-$2K^b$ ligand required for the survival of OT1 T cells was not expressed at sufficient frequency or not on the relevant cells to allow for their survival. The failure of donor T cells to survive in the absence of their selecting ligand in the periphery may also explain why the transfer of B6.Ly5[1] T cells into β_2-microglobulin-deficient mice did not result in an efficient positive selection of host $CD8^+$ thymocytes: in the absence of peripheral MHC class I molecules, injected cells will not survive and hence not migrate in sufficient numbers to the thymus to support positive selection (data not shown). The above results indicate that class I molecules and, by implication, other self -Ags expressed by recirculating T cells may be involved in positive selection.

Indeed, there has been an increasing realization that thymocyte-positive selection can be mediated by cells other than thymic epithelial cells. Thus, and as recently reviewed by Berg (29), such "unconventional" positive selection may apply to $CD4^+$ NKT cells (30), $FoxP3^+$ regulatory T cells, and so-called "innate" $CD8^+$ T cells (31). Because in our case, the number of positively selected cells was quite small, this precluded detailed analysis of their innate vs conventional phenotype. The relative "efficiency" of epithelial vs T cell positive selection is difficult to directly estimate. However, by intrathymic injection of 10^7 MHC class I-bearing fibroblasts into β_2-microglobulin-deficient mice, Pawlowski

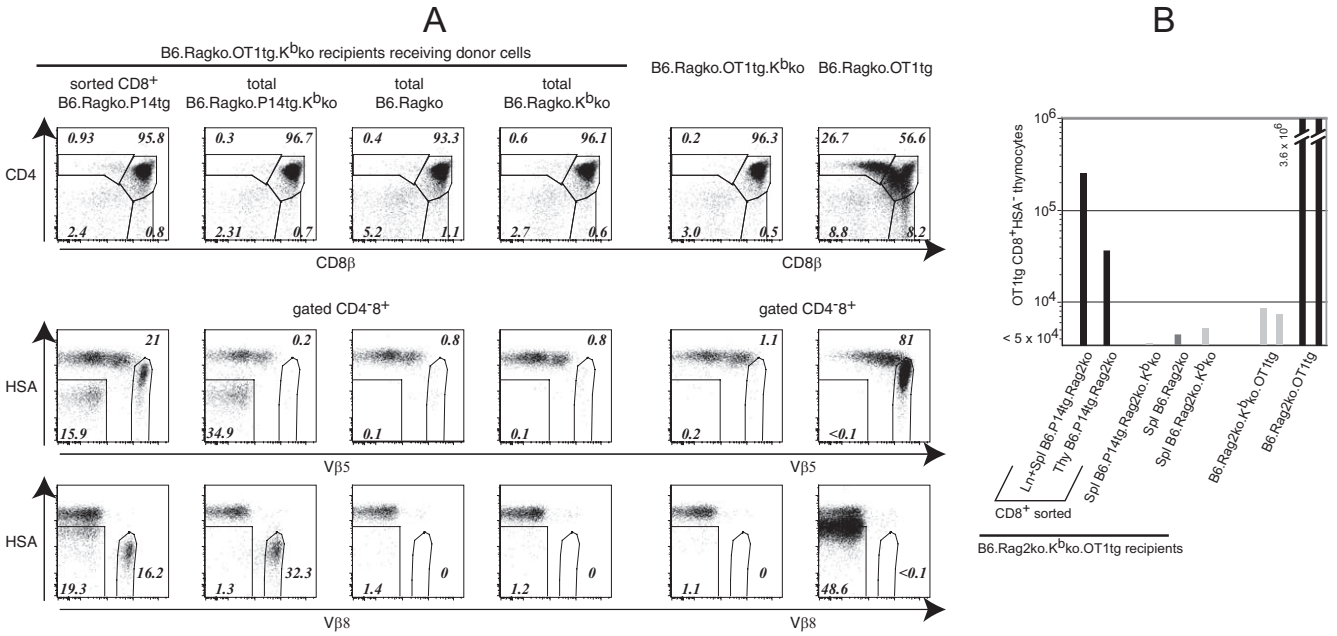

FIGURE 6. Positive selection of OT1 thymocytes on recirculating K^b-expressing T cells. *A*, Positive selection of OT1 thymocytes on recirculating P14 T cells expressing K^b class I MHC molecules. Four $\times$ 10^6 cell sorter-purified peripheral $CD8^+$ P14 T lymphocytes from B6.Ragko.P14tg mice, or 5 $\times$ 10^6 peripheral cells from B6.Ragko.P14tg.K^bko mice, or 4 $\times$ 10^6 peripheral cells from B6.Ragko, or 4 $\times$ 10^6 peripheral cells from B6.Ragko.K^bko mice, respectively, were adoptively transferred into B6.Ragko.OT1tg.K^bko recipients i.v. Ten weeks later, thymocytes were stained for CD8β, CD4, HSA, PI, and Vβ5 or Vβ8. The *upper dot plots* show CD4 vs CD8β expression of viable thymocytes from the experimental mice as well as noninjected control B6.Ragko.OT1tg.K^bko and B6.Ragko.OT1tg mice. Among $CD8^+CD4^-$ cells, HSA vs Vβ5 or HSA vs Vβ8 expression was evaluated to determine whether there was positive selection for OT1 T cells (Vβ5) and whether injected P14 T cells (Vβ8) had recirculated into the thymus. *B*, Number of OT1 SP thymocytes generated in the various recipient mice. From the above FACS stainings and total thymus cellularity, the number of OT1 SP thymocytes was calculated for B6.Ragko.OT1tg.K^bko mice injected with sorted B6.Ragko.P14tg $CD8^+$ peripheral T cells (n = 1), sorted B6.Ragko.P14tg thymocytes (n = 1), total B6.Ragko.P14tg.K^bko peripheral cells (n = 1), total B6.Ragko peripheral cells (n = 1), or total B6.Ragko.K^bko peripheral cells (n = 1) and control mice: B6.Ragko.OT1tg.K^bko (n = 2) and B6.Ragko.OT1tg (n = 2).

et al. (32) demonstrated a complete restoration of $CD8^+$ SP-positive selection with normal numbers, TCR repertoire, and effector function of the selected cells.

More recently, two groups (23, 24), developed mouse models in which thymocytes were the only MHC class II-expressing cells. In these mice, $CD4^+$ SP T cells were selected as efficiently as in control mice, although the functionality of these cells was later questioned (33). In a normal mouse adult thymus, ~50,000 thymic epithelial cells are present and presumably able to mediate most thymocyte-positive selection by contacting several cells simultaneously and because of their high MHC expression density. Numerically, this leads to the selection of ~10^7 $CD4^+$ and 2 $\times$ 10^6 $CD8^+$ mature SP T cells per WT thymus (and ~5–7 $\times$ 10^6 V$\beta5^+$ $CD8^+$ mature SP T cells per B6.Ragko.OT1tg thymus). In our experimental system, the presence of ~10^5 transferred T cells intrathymically generated only 2 $\times$ 10^4–2 $\times$ 10^5 mature V$\beta5^+CD8^+$ SP T cells. Thus, the efficiency of positive selection by recirculating T cells is relatively low in our experimental system and might be very modest under physiological conditions, but this does not preclude functional consequences for this process.

In order for central tolerance to be maximally efficient, the thymus must ensure that developing cells are exposed to the maximum repertoire of self-Ags. This is probably ensured, to a large extent, by AIRE-mediated regulation of peripheral tissue-specific self-Ag expression by epithelial cells (34). However, we would propose that this may be complemented by the direct import of self-Ags expressed by recirculating lymphocytes including B cells (3, 9), T cells (6, 9) or, as recently shown, dendritic cells (35). In practical terms, recirculation of lymphocytes to the thymus could be envisioned as a group of Trojan horses in that they could be used to introduce Ags necessary for both positive and negative selection of the T cell repertoire.

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Disclosures

The authors have no financial conflict of interest.

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