The Role of the Thymus and Recent Thymic Migrants in the Maintenance of the Adult Peripheral Lymphocyte Pool

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Summary

The thymus is essential for the initial seeding of T cells to the periphery, but its role in maintaining the adult T cell pool remains poorly defined. We investigated whether changes to the rate of T cell export could form part of the mechanism(s) controlling the homeostatic regulation of the size and composition of the peripheral T cell pool. Using neonatal thymi grafted under the kidney capsule, we found that irrespective of whether the pool was oversupplied (by thymic grafts) or undersupplied (due to neonatal thymectomy), the thymic export rate was constant from both the host and graft thymus, and the periphery remained constant in size. Recent thymic emigrants (RTE) were also tracked to determine the extent of their acceptance into the T cell pool of a normal mouse. As a population, RTE are phenotypically mature, but were distinct from resident T cells in the periphery, being released in a CD4/CD8 ratio approximately twice that of established peripheral T cells. This export ratio is similar to that of T cells in the mature thymic compartment, but soon after entry into the periphery, the ratio falls, indicating separate thymic and peripheral regulation of the CD4/CD8 ratio. RTE may also be preferentially incorporated into the periphery, causing displacement of resident T cells, thus maintaining the size of the peripheral pool. Although not vital for the maintenance of a functional T cell pool, the acceptance of RTE in a "full" peripheral pool would ensure that the T cell receptor repertoire is kept diverse and that the T cell population encompasses a broad range of naive as well as memory T cells.

Key words: thymus • thymic emigration • peripheral T cell pool • T cell homeostasis • thymus grafting

One of the more controversial areas of T lymphocyte physiology involves the maintenance of remarkably stable peripheral T cell levels throughout adult life (1). A central question surrounds the precise role of the thymus in this homeostasis. Specifically, does replenishment occur through self-renewal, continual export from the thymus, or both? Given that the thymus can respond to external stimuli such as changing steroid levels (2, 3), can the rate of thymic export also be modulated to regulate the size of the T cell pool?

Based on a reported daily export rate of ~1% of thymocytes (4), a normal young mouse thymus would seed up to 5×10^7 T cells each month, yet the peripheral T cell pool remains essentially constant in size. This stability must be achieved either through systematic downregulation of thymocyte emigration, loss of peripheral T cells, or both. In the young adult mouse, there is no evidence to support the former; however, coupled with age-related thymic atrophy, a slowing if not cessation of T cell export from the adult thymus would provide an effective means of preventing oversupply to the intact T cell pool.

Thymocyte emigration rates as low as 10^4 T cells per day have been described for 1-yr-old mice (4), but the role of the thymus should not be regarded as insignificant in older mice. We have found recently that the atrophied thymus of aged animals clearly supports thymopoiesis, and that age-related falls in export rates may be less than reported previously, with up to 10^5 T cells exported daily from mice >2 yr old (Sutherland, J.S., M.A. Price, S.P. Berzins, and R.L. Boyd, manuscript in preparation). Although thymocyte export in adult mice is the dominant source of all naive T cells (5), population maintenance must be at least in part peripherally based, because the thymus alone is incapable of replacing the number of T cells lost daily through natural attrition (6). Therefore, in terms of maintaining the pool size, the progressively atrophying thymus of normal healthy adult mice would appear to be redundant after initial seeding. Bromo deoxyuridine (BrdU)[1]

[1]*Abbreviations used in this paper:* BrdU, bromo deoxyuridine; RAG-2, recombination activating gene-2; RTE, recent thymic emigrant(s).

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labeling experiments have demonstrated that the peripheral T cell pool in adult thymectomized mice can be maintained through the proliferation of memory T cells and the longevity of naive cells (7, 8). However, such a reliance on expansion must cause a progressive loss of repertoire diversity, potentially leading to disease susceptibility (9). Therefore, the thymus has intrinsic value as a support of population diversity.

Whether the rate of T cell export is modulated as part of the regulation of pool size has yet to be addressed directly. Wallis and colleagues demonstrated that T cells continued to be exported in mice with multiple thymic grafts, but changes to the rate of T cell export from the grafted and host thymus were not investigated (10, 11). If recent thymic emigrants (RTE) were more susceptible to deletion than resident peripheral T cells in grafted and indeed in normal mice, the need to regulate thymic export would be negated. In this regard, excess naive B cells enter the circulating pool on a transient basis, only to be deleted in the parenchyma of lymphoid organs after 1–3 d (12). Although RTE clearly accumulate and possibly expand in lymphopenic conditions, their longevity in normal or hyperthymic environments has yet to be clearly addressed (8, 10). Deletion of RTE in excess of peripheral requirements would remove the need to control RTE export in order to keep the peripheral T cell pool constant in size.

Obviously, a key problem is the simultaneous maintenance of T cell diversity and population size. Although still controversial, the homeostatic regulation of naive and memory cell populations may provide a solution. In normal animals, the memory T cell compartment is maintained in a thymic-independent manner by cytokine- or antigen-stimulated proliferation (13, 14). Naive T cells seldom divide, and their inherent longevity, coupled with the contribution of thymic export, can maintain a steady population level (8, 15). However, what is not clear is whether resident naive cells in a saturated pool are favored for survival over RTE (8, 10), or whether RTE specifically displace long-lived naive cells that may carry a redundant specificity (16).

In this study using mice with additional thymi grafted under the kidney capsule, we have addressed the role of the thymus in an already saturated peripheral pool. Specifically, does a negative feedback on thymocyte emigration assist in the regulation of the peripheral T cell pool; are RTE more susceptible to deletion than resident T cells when the peripheral pool is "full"; and is the RTE population functionally or phenotypically distinct from other T cells of the peripheral T cell pool?

Materials and Methods

Mice. C57BL/6J mice were obtained from Monash University and the Walter and Eliza Hall Institute Central Animal Houses and were housed under conventional conditions.

Thymic Grafting. 5–6-wk-old female mice were anesthetized by intraperitoneal injection of 0.3 ml of 0.3 mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5 mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline. Fur was removed from the left abdomen, and an incision was made to expose the kidney. Thymic lobes removed previously from newborn pups were then grafted under the left kidney capsule, the wound was closed with surgical clips, and the mouse was placed in a warm environment until it recovered. Unless otherwise specified, mice were grafted with two lobes. Upon harvest, mice with unsuccessful grafts (i.e., one or zero successful lobes present) were excluded from analysis.

Cell Suspensions. Mice were killed by CO_2 asphyxiation at various time points after thymic grafting. In order of removal, separate cell suspensions were obtained from blood, lymph nodes, spleen, and thymus (both in situ and grafted). Blood lymphocytes were obtained by heart puncture, with red blood cells lysed by incubation in red cell lysis buffer (8.9 g/liter ammonium chloride) for 10 min at 4°C. Organs were pushed gently through a 200-μm sieve in cold PBS–FCS (1%)–Azide (0.02%). Cell concentration and viability were determined in duplicate using a hemocytometer and ethidium bromide/acridine orange staining viewed under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, Germany). The cell suspensions were then washed by centrifugation (650 g_{max}, 5 min, 4°C), and the cell pellet was resuspended in PBS-FCS-Azide.

FITC Labeling of Thymocytes. Details of this technique are similar to those described elsewhere (4). In brief, animals were anesthetized, and the chest was opened (or the kidney was exposed, in the case of grafted mice) to expose the thymic lobes. Each lobe was injected with ~10 μl of 350 μg/ml FITC (in PBS) which typically resulted in random labeling of 30–60% of the thymocyte population (70–80% for grafted thymi). The wound was closed with a surgical staple, and the mouse was warmed until fully recovered from anesthesia. Mice were typically killed by CO_2 asphyxiation ~24 h after grafting, and lymphoid organs were removed for analysis. Instruments were washed after removal of each organ, and the FITC-injected thymus was always removed last to avoid cross-contamination of samples.

Quantification of Migrant Populations in Host Mice. Migrant cells were detected in two ways. For detection of RTE <24 h after export, the thymus (graft or host) was injected with FITC, and lymphoid organs were removed ~24 h later. After cell counts, samples were stained with anti–CD4-PE and anti–CD8-biotin (detected with streptavidin–Tri-color [Caltag Laboratories, Inc., Burlingame, CA]), then analyzed by flow cytometry. Migrant cells were identified as live-gated $FITC^+$ cells expressing either CD4 or CD8 (to omit autofluorescing cells and doublets). The percentages of $FITC^+$ CD4 and CD8 cells among live-gated cells were added to provide the total migrant percentage for lymph nodes and spleen, respectively.

Calculation of Daily Export Rate. The daily export rate was calculated by taking into account (*a*) the percentage of thymocytes stained $FITC^+$, (*b*) total cell counts for thymus, spleen, and lymph nodes (pooled from mesenteric, inguinal, and axillary nodes), (*c*) the proportion of T cells within these organs that were $FITC^+$, thereby indicating export from the FITC-injected thymus, and (*d*) the time between FITC injection and harvest. These data allowed the total number of cells exported from the injected thymus (or thymus graft) in the previous 24 h to be calculated. The peripheral pool was considered equal to the total number of spleen cells plus twice the total number of lymph node cells (pooled from mesenteric, inguinal, and axillary nodes) (1).

The number of emigrants up to 8 wk after export was calculated in a similar fashion, except that migrant cells were identified by the expression of the Ly5.2 antigen (indicating export from the $Ly5.2^+$ graft) rather than through FITC staining of thymocytes.

Flow Cytometry. Cell suspensions were stained in V-bottomed tubes (3×10^6 cells/test) by gently resuspending in 40 μl of appropriate mAb or secondary conjugate and incubating for 20 min at 4°C. Between incubations, the cells were washed by the addition of 1 ml PBS-FCS-Az and centrifuged (650 g_{max}, 5 min, 4°C). Data was collected on 7×10^4 viable cells of the total population of each sample, and where necessary, on an additional $3–5 \times 10^3$ viable cells from gated populations (e.g., $FITC^+$ RTE). Selective gating on forward light scatter versus side light scatter was used to eliminate red blood cells and dead cells from analysis. Flow cytometry data were analyzed using Lysis II and CellQuest software (Becton Dickinson, Mountain View, CA).

Thymectomy. In some experiments, mice were thymectomized at either day 3 (neonatal thymectomy) or at 4 wk (adult thymectomy) of age. In brief, mice were anesthetized and the chest was opened to expose the thymic lobes which were removed by suction. For adult mice, the wound was closed using surgical staples, and the mouse warmed until fully recovered from anesthesia. For newborn thymectomized mice, the wound was cleaned but not closed, and the pup was returned to the mother. At the completion of the experiment, the chest cavity was always checked, and mice with thymic remnants were excluded from further analysis.

Antibodies. For flow cytometric analysis, PE-labeled anti-CD25, PE-labeled anti-CD69, PE-labeled anti-CD4, biotinylated anti-CD8a, biotinylated anti-CD44, and FITC-labeled CD45.2 (Ly5.2) were purchased from PharMingen (San Diego, CA); biotinylated anti-CD4 and streptavidin–Tri-color conjugate were purchased from Caltag Laboratories, Inc. FITC Isomer 1 (for intrathymic injection) was purchased from Becton Dickinson.

Results

Graft Reconstitution

After transplantation of neonatal Ly5.2 thymic lobes beneath the kidney capsule of Ly5.1 congenic mice, recipient mice were examined for evidence of emigration from the grafted thymus and reconstitution of the grafted thymus with host stem cells. At each time point examined, including the time of engraftment, the grafted neonatal thymus had all major CD4 and CD8 T cell subpopulations present in normal proportions. Within 7 d, the grafts had clearly exported donor RTE (detected by the Ly5.2 congenic phenotype), and circulating stem cells from the host had entered the graft. Alongside these cells were host cells of mature nonactivated phenotype (data not shown). These cells were low in number, and were absent by week 2. By 3 wk after grafting, reconstituting cells from the host were either $CD4^+CD8^+$ or $CD4^-CD8^-$, with the remaining $LY5.2^+$ cells largely of a more mature phenotype. Beyond 3 wk, host-derived cells had matured sufficiently to be exported alongside the graft-derived $Ly5.2^+$ cells. From this point, the thymic pool of $Ly5.2^+$ (donor) cells diminished rapidly until, by 4 wk after grafting, no $Ly5.2^+$ thymocytes remained in the graft. Thus, all thymocytes transplanted with the graft had been exported from the graft thymus or been deleted through normal selection processes, illustrating the absence of long-term resident thymocytes. Both the size and rate of export from the grafted thymi were relatively stable from 4 wk after transplantation, but fluctuated considerably in the 2–3-wk period, possibly due to the variable contribution of host-derived reconstituting cells (Fig. 1) and slight variations in the time to vascularize the transplanted thymus.

Peripheral Pool Size of Thymus-grafted Mice

This experiment involved 30 age-matched female C57Bl6/J mice, 15 of which were grafted with 2 neonatal thymic lobes, and 15 were sham-grafted. Mice were killed 8 wk af-

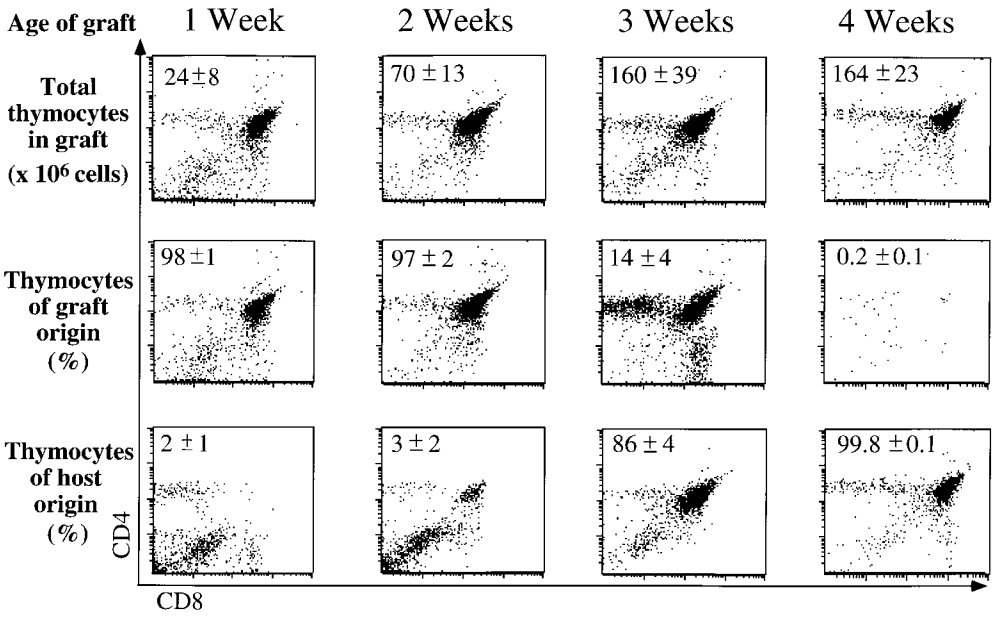

Figure 1. $Ly5.2^+$ thymic lobes grafted beneath the kidney capsule of Ly5.1 congenic mice are progressively reconstituted by host-derived cells. Fig. 1 shows graft- and host-derived thymocytes at markedly different stages of maturity. Despite the different profiles of thymocytes of graft and host origin, the profile of the total thymocytes in the graft remains normal. The progression of $Ly5.2^+$ cells through the various thymus compartments is shown by the increasingly mature phenotype over the first 3 wk, coupled with the decreasing proportion of total thymocytes they represent. By week 4, the grafted thymus has been reconstituted completely by host-derived stem cells, indicative of the absence of long-term resident thymocytes. Profiles are typical examples of results. 5–10 mice were used at each time point.

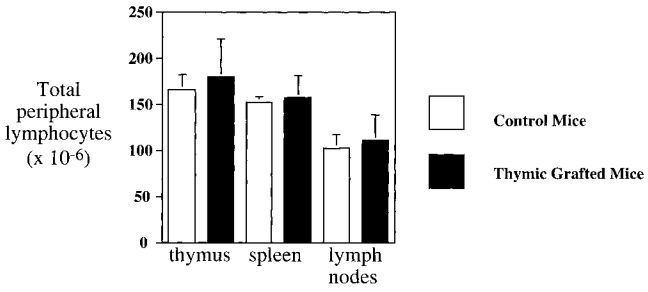

Figure 2. The size of the peripheral T cell pool does not increase when an additional thymus is grafted beneath the kidney capsule. Examination of lymphoid organs 8 wk after engraftment of two neonatal thymic lobes revealed no significant difference between the size of the thymus, spleen, or lymph nodes of grafted animals and those of nongrafted littermates. Results are from 16 grafted and 9 nongrafted animals. Error bars represent 1 SD from the mean.

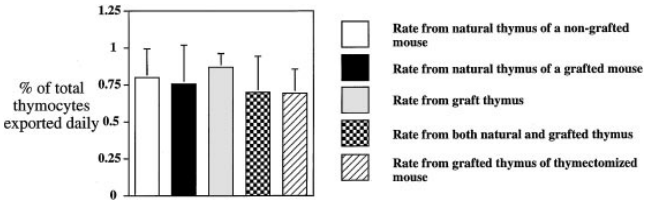

Figure 3. Thymic export rates, as measured by intrathymic FITC injection, are not reduced in thymic-grafted mice. T cell emigration rates from the mature (8-wk-old) thymus of normal mice was compared with that of thymic-grafted mice. Despite no significant difference in the size of the peripheral T cell pools of grafted and nongrafted mice, the rate of T cell export remained constant. Both in situ and grafted thymi, irrespective of the presence or absence of the other, exported T cells at the rate of ~0.7% of total thymocytes per day. This illustrated that grafted thymi were exporting cells in a proportion similar to that of an in situ thymus, and that no change to export rates from either thymus was induced by an increase in T cell supply to the peripheral pool. Each group contained 4 mice (total of 20). Error bars represent 1 SD from the mean.

ter grafting, and total lymphocyte number of thymus (host), spleen, and lymph nodes was measured for each mouse. Surprisingly, despite the increased number of potential RTE, no significant differences were found between the T cell populations of grafted and nongrafted mice (Fig. 2). Grafted thymic lobes were phenotypically identical to the host thymus.

T Cell Export as a Function of Thymocyte Number

Separate groups of identically treated grafted mice were tested for (*a*) migration from the natural thymus, (*b*) migration from the natural thymus of a grafted mouse, (*c*) migration from the grafted thymus, and (*d*) migration from both the natural and the grafted thymus. In addition, migration was also checked from the grafted thymus of neonatally thymectomized mice. In each case, the thymus tissue was injected intrathymically with FITC, and peripheral lymphoid organs were harvested 24 h later to determine export rates.

The rate of export from the host thymus in grafted and control mice was compared. Expressed as a percentage of total thymocytes, the host thymus exported ~0.7% of total thymocytes daily (representing ~10^6 cells), irrespective of the presence or absence of additional thymic grafts. This rate corresponds to previously reported studies (4, 7). We also examined migration rates from thymic grafts and found that once established, they also exported ~0.7% of total thymocytes, irrespective of whether the host thymus (or other grafted thymi) was present.

In grafted mice, the total number of RTE exported daily was ~0.7% of the pooled total of thymocytes from the natural and grafted thymi (Fig. 3). Hence, the rate of RTE export was under autonomous control, and was unrelated to the number or size of thymi present. These results support and extend previous work demonstrating that thymic lymphopoiesis is not affected by the presence of multiple thymus grafts (17).

Hyperthymic Mice

Having established that mice would maintain a peripheral lymphocyte pool of constant size despite being grafted with two thymic lobes, we examined the effects of up to nine thymic grafts on the peripheral pool. In these mice, the T cell pool 6 wk after grafting was increased 30% over control mice, demonstrating that the homeostatic control of the T cell population size could be overcome. The increase was not compensated for by the loss of B lymphocytes or other leukocytes, confirming independent homeostatic regulation of the T and B cell pools (Fig. 4).

The CD4/CD8 Ratio of RTE Decreases in the Periphery

The CD4/CD8 ratio of peripheral T cells exported from the thymus <24 h earlier was measured and found to be significantly different from that of the resident T cells of the peripheral lymphocyte pool. In this experiment, migrant cells exported <24 h earlier were identified by intrathymic FITC labeling of thymocytes. Migrant cells present in the periphery for up to 1 or 3 wk were identified by expression

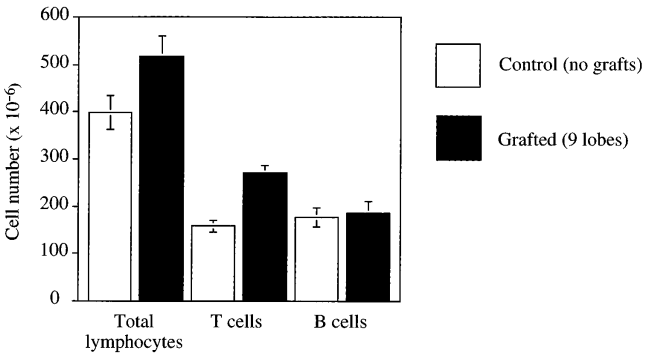

Figure 4. Homeostatic control of total lymphocyte numbers can be overcome. Nine neonatal thymic lobes were grafted beneath the kidney capsule of 6-wk-old mice. Mice were killed 8 wk after grafting, and lymphocyte numbers and phenotypes were evaluated. The total lymphocyte pool increased by 15% over age-matched controls, due to a 30% increase in the T cell pool. B cell numbers were not affected by grafting. Four mice were used in each group (total of eight). Error bars represent 1 SD from the mean.

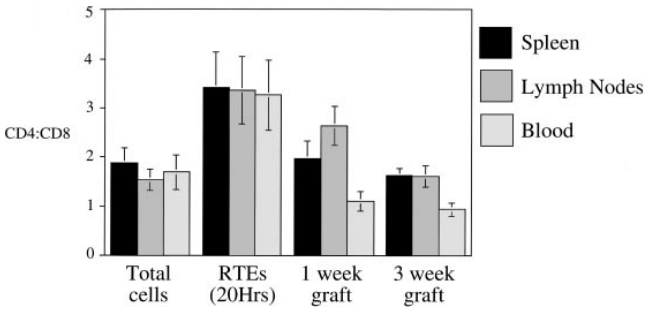

Figure 5. The CD4/CD8 ratio of RTE is distinct from that of peripheral T cells. For RTE detected in the periphery after intrathymic FITC injection, the CD4/CD8 ratio was 3.5, unlike the typical 1.5:1 ratio observed in the periphery. No difference was seen between the CD4/CD8 ratio of RTE from in situ thymi and those of grafted thymi. Using grafted Ly5.2 congenic thymi, migrant cells were examined 1 and 3 wk after export, and the CD4/CD8 ratio was found to vary between lymphoid organs. At both week 1 and week 3 after grafting, the spleen and lymph nodes carried recently exported cells that were still of a ratio higher than that of the resident cells but lower than that of the RTE. Significantly, cells in the blood exported within 3 wk of assay had a ratio lower than that of surrounding residents. For the overall pool and the RTE, 45 mice were used for spleen and lymph node results, 18 for blood. Five mice were used at the 1-wk time point, and 10 at the 3-wk time point. Error bars represent 1 SD from the mean.

of the Ly5.2 congenic marker restricted to cells of graft origin.

Similar to the mature intrathymic T cells, RTE contained almost four times as many CD4 as CD8 cells, compared with a ratio of <2:1 seen among resident peripheral T cells. This difference was consistent for RTE from in situ or grafted thymi, indicating a regulation intrinsic to the thymus. To examine more closely the kinetics of this change in the CD4/CD8 ratio, Ly5.2 thymi were grafted into Ly5.1 recipients, and the spleen, lymph nodes, and blood were examined over a 3-wk time course. Although at any one time point, it is impossible to determine exactly when cells had been exported, the trend in both spleen and lymph nodes was clearly a shift in the CD4/CD8 ratio from that seen among RTE (3.5:1) to that of resident cells (1.5:1). Barring changing phenotypes, selective proliferation and deletion of RTE must have occurred after thymic export to selectively decrease the CD4/CD8 ratio without greatly altering the size of the exported T cell population. Strikingly, however, the ratio seen in the blood was threefold below that seen among RTE and even below that of resident cells, implicating the blood as a possible effector site of homeostatic regulation (Fig. 5).

Longevity of RTE in the Periphery

To examine the degree to which RTE are accepted and retained in a full peripheral lymphocyte pool, the total number of cells exported from a grafted thymus over an 8-wk period was compared with the number of cells of graft origin in the periphery at the end of the 8 wk. The daily rate of T cell export from grafted thymic lobes of varying age was calculated using FITC injection, enabling the number of cells exported over the 8 wk after grafting to be estimated (see below). The actual number of graft origin cells present in the periphery of mice was then determined through the expression of the Ly5.2 antigen.

Migration. Six time points were examined for migration rates from the graft thymus and the presence of Ly5.2 cells in the periphery. Each time point comprised 3–10 grafted mice (plus controls) for each experiment. Migration was lowest in the first week after grafting ($>10^5$/d) but quickly reached a level of $\sim 0.8 \times 10^5$ cells/d that was essentially maintained to 8 wk after grafting (Fig. 6). A peak of emigration was observed at day 14 after grafting, coinciding with the considerable expansion of host stem cell–derived thymocytes and the accompanying rapid thymic growth. This minor increase in the migration rate, though consistent, was transient, and fell to ~0.7% of total thymocytes exported per day by day 28.

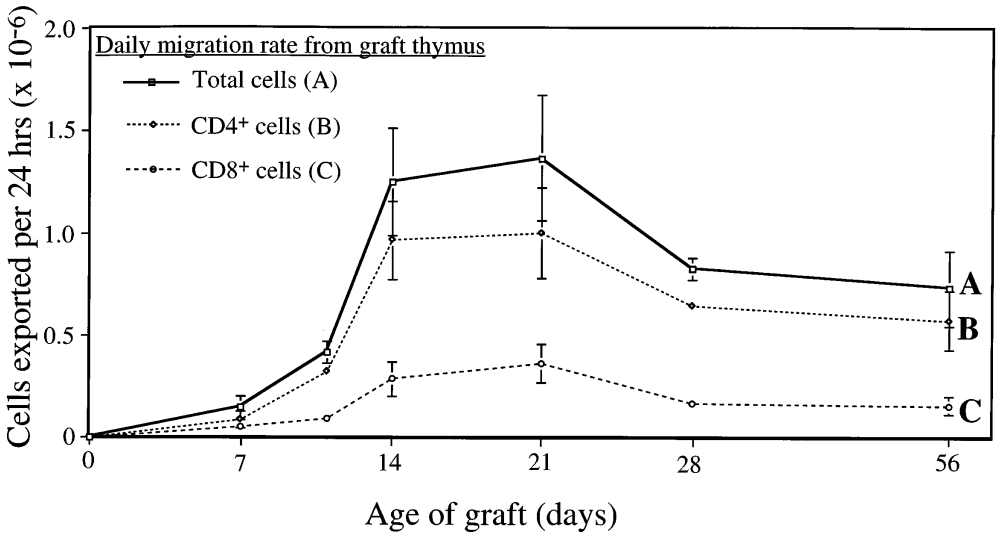

Figure 6. The T cell export rate was correlated with the age of the graft by FITC injection at five time points up to 4 wk after grafting and again at 8 wk. The export rate rose steadily in the first 2 wk after grafting, and remained at $0.6–0.8 \times 10^6$ cells/d until the final time point. A transient but consistent peak began at day 14 of 1.25×10^6 cells/d, which may be associated with the extremely rapid growth of the graft at this time (see also Fig. 1). Each of the six time points represents the mean export rate from groups of three to seven mice. Error bars represent 1 SD from the mean.

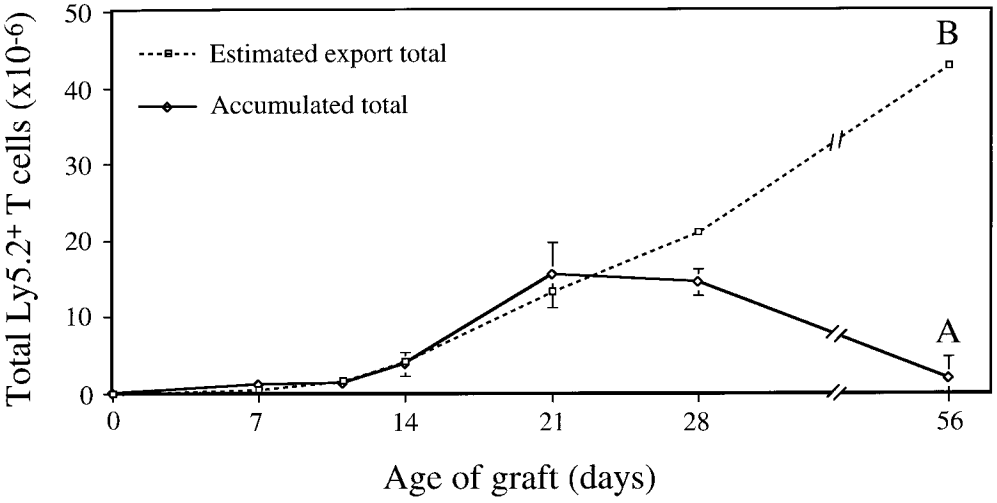

Figure 7. T cells exported to a full periphery are accepted into the T cell pool, presumably at the expense of longer-term resident cells. The number of cells of graft origin (*Ly5.2*$^+$) present in the peripheral pool at various time points (*A*) was compared with the number estimated to have been exported from the time of grafting (*B*), calculated using previously determined migration rates. In the 21 d after grafting, the number of graft origin cells found in the periphery matched closely the number estimated to have been exported over the same period at each of five time points. This suggests that RTE accumulated in the full periphery at the expense of resident T cells. Beyond 3 wk, the export of Ly5.2$^+$ cells fell progressively due to reconstitution by host precursor cells. From this time, the peripheral Ly5.2$^+$ population decayed, due in part to no new Ly5.2$^+$ cells being exported. Each of the seven time points represents the mean of 3–10 mice. Error bars represent 1 SD from the mean.

Accumulation. The predictability of the export rate of the grafted lobes enabled the total number of cells exported to be estimated. The actual number of thymic graft–derived cells present was compared with the total number of cells calculated to be exported by the graft at five time points within the initial 4-wk period (7, 11, 14, 21, and 28 d after grafting) and again after 8 wk (Fig. 7). Using 3–10 grafted mice per time point, the number of accumulated Ly5.2$^+$ T cells (migrants from graft lobes) at each time point from day 0 to day 28 matched consistently the number of cells predicted to have left the graft since the graft was transplanted, indicative of a steadily accumulating population that was neither rapidly expanding nor being deleted.

Decay. From 4 to 8 wk after grafting, the number of Ly5.2$^+$ T cells in the periphery fell dramatically. This coincided with the full reconstitution of the graft thymus by host stem cells, resulting in no further Ly5.2$^+$ T cells being exported. After day 21, reconstituting host stem cell–derived cells began to be exported, meaning that RTE from the graft were no longer exclusively Ly5.2$^+$. After day 28, no Ly5.2$^+$ cells remained in the graft, and RTE became exclusively Ly5.2$^-$.

Therefore, examination of the peripheral compartment from day 28 allows the longevity of previously exported Ly5.2$^+$ T cells to be examined. More importantly, the question of whether the displacement of peripheral T cells by RTE is random or selective can also be addressed.

If the daily loss of peripheral T cells (equivalent to the number of RTE) occurred through random displacement alone, the rate of decay can be calculated with the formula $X(t) = X_0(1 - a)^t$, where $X(t)$ is the number of remaining Ly5.2$^+$ cells as a function of time, X_0 is the starting number of Ly5.2$^+$ cells present 28 d after grafting, a is the ratio of the number of total thymic migrants to the total number of T cells in the peripheral pool susceptible to replacement by RTE, and t is the number of days over which the decay is to be estimated.

Export rates of $\sim 10^6$cells/d each for the grafted and natural thymus (see Figs. 2 and 3) indicate that the export of RTE in grafted mice is unlikely to exceed 2×10^6/d beyond 4 wk after grafting. As RTE have been shown recently to displace naive but not memory T cells (16), only the naive cells of the total T cell pool are subject to displacement. Consistent with previous reports (8, 18, 19), we found that naive T cells, identified by low expression of CD44, represented >50% of the total peripheral T cell pool in mice grafted for 4 wk (9-wk-old mice; data not shown). The total peripheral T cell pool of these mice was $\sim 1.8 \times 10^8$ cells (Fig. 2). Therefore, we conservatively estimated the naive pool to be 50% of the total T cell pool (50% of 1.8×10^8 cells).

At 4 wk after grafting, there was an Ly5.2$^+$ starting population of $\sim 14.5 \times 10^6$ graft-derived cells (see Fig. 7) in an overall population of 9.0×10^7 naive T cells (50% of 1.8×10^8 cells). Assuming total RTE to be 2×10^6/d, random displacement kinetics would predict that 53% of the Ly5.2$^+$ cells present at 4 wk would remain at 8 wk, i.e., $14.5 \times 10^6 \times [1 - (2 \times 10^6/9.0 \times 10^7)]$[28]. Instead, Ly5.2$^+$ cells were barely detectable. Of five mice grafted for 8 wk, an average of 1.9×10^6 Ly5.2$^+$ cells remained, rather than the expected 7.7×10^6. Although we stress that the kinetics of this loss have yet to be determined, such a dramatic loss clearly suggests nonrandom displacement of RTE after 4 wk.

Using the same rationale, displacement estimates can also be calculated for the CD4 and CD8 pools. As shown previously, the CD4/CD8 ratio among RTE from both normal and graft thymi was 3.5:1. Therefore, in a total daily RTE population of 2×10^6, $\sim 1.55 \times 10^6$ will be CD4$^+$, and the remaining will be 0.45×10^6 CD8$^+$. RTE were entering an overall naive pool of 9.0×10^7 cells that can itself be

subdivided into CD4 and CD8 components of 5.4×10^7 and 3.6×10^7 cells, respectively (peripheral CD4/CD8 ratio of ~1.5:1). At 4 wk after grafting, 14.5×10^6 Ly5.2$^+$ T cells of graft origin remained in the peripheral naive pool. Of these, 8.7×10^6 were CD4$^+$, and 5.8×10^6 were CD8$^+$. Assuming random displacement kinetics, cell loss for the CD4 and CD8 pools can now be estimated.

Due to a higher CD4/CD8 ratio among RTE than peripheral T cells, random displacement of resident T cells by RTE would cause a more rapid turnover of peripheral CD4 than CD8 T cells. Applying the same formula for random displacement used previously, 3.9×10^6 CD4$^+$ and 4.1×10^6 CD8$^+$ cells of graft origin are expected to remain after 8 wk. However, in this study, the mean totals remaining from five mice grafted for 8 wk were 0.8×10^6 CD4$^+$ cells and 1.1×10^6 CD8$^+$ cells. Again, although the precise kinetics of these losses have yet to be determined, the loss of resident CD4$^+$ and CD8$^+$ cells of graft origin from the periphery clearly suggests nonrandom displacement of resident peripheral T cells by RTE from 4 wk after export.

Activation Status of RTE. The possibility remained that the apparent linear accumulation of migrating T cells was due to compensating levels of proliferation and deletion. Although some evidence does exist for division among naive cells (5, 7, 8), most researchers report that RTE are nondividing cells of naive phenotype (8, 20). Migrant cells were nevertheless examined for activation markers and for evidence of significant proliferation.

Ly5.2$^+$ cells from Ly5.1$^+$ mice grafted 1, 2, or 3 wk previously were examined by flow cytometry for the activation markers CD25 and CD69. As the samples contained peripheral Ly5.2$^+$ cells exported any time between grafting and harvest, examination of cells over the 3-wk period after migration was possible. Without exception, Ly5.2$^+$ cells from each time point expressed levels of CD25 and CD69 similar to or lower than surrounding resident cells, indicating a nonactivated phenotype. Similarly, FACS®-sorted Ly5.2$^+$ cells incubated in overnight culture with tritiated thymidine showed no evidence of proliferation (data not shown).

Discussion

In this study using adult mice with a normal T cell repertoire, we examined the possible influence of increased peripheral T cell numbers on the regulation of thymocyte export, the comparative phenotype of RTE to mature thymic and resident peripheral T cells, and the influence of thymic migrants on the status of the peripheral T cell pool. Grafting two neonatal thymic lobes beneath the kidney capsule produced the equivalent of a second fully functional thymus, yet no increase in the size of the peripheral T cell pool occurred. However, grafting nine lobes produced a much larger additional mass of thymus tissue that caused a 30% increase in the peripheral T cell pool 8 wk after grafting. Taken together, these experiments indicated that the pool size is regulated homeostatically, but with a degree of elasticity, facilitating the incorporation of additional T cells in some circumstances.

More importantly, while the T cell pool of mice with two additional thymic lobes remained constant, demonstrating effective homeostatic regulation, the host and grafted thymus each continued to export cells at the normal rate of ~1% of total thymocytes per day. Furthermore, grafts in neonatally thymectomized mice also exported cells at the same rate, despite having a reduced peripheral T cell pool and presumably a need for additional T cells. These results are in general agreement with earlier work demonstrating the lack of an external feedback mechanism regulating thymus lymphopoiesis (17, 20, 21).

Collectively, these data demonstrate that the rate of thymic migration is not influenced either by the size of the peripheral pool or by increased levels of circulating RTE. Furthermore, although the size of the T cell pool is clearly subject to homeostatic regulation, it does not occur at the level of RTE export.

It could be argued that T cells continued to be exported from the thymus but were specifically excluded from becoming long-term residents in the spleen and lymph nodes. Surplus B lymphocytes, for example, enter the lymphoid organs but not the B cell areas, and are deleted within 2–3 d (12). A similar scenario among T cells would not be seen using traditional labeling of RTE with FITC, because the poor persistence of the FITC label limits tracking to 24–48 h after staining. However, grafting thymic lobes from congenic Ly5.2$^+$ mice into Ly5.2$^-$ mice allowed long-term analysis of migrant cells, and hence the issue of whether RTE became true residents could be clearly addressed in two ways. Immunohistology of lymph nodes and spleen of grafted mice showed that migrant cells entered and persisted in T cell areas of both spleen and lymph nodes (data not shown), and flow cytometry showed that most RTE persisted for at least 3 wk in the periphery despite the overall pool remaining constant in size.

By measuring the rate of emigration at various times after grafting, a correlation between the age of the graft and the number of T cells exported daily could be made. A period of 3 wk was chosen because for the first 3 wk after grafting, only Ly5.2$^+$ cells are exported from the graft. Using the Ly5 congenic system, the predicted number of RTE leaving the graft over the 3-wk period was then compared with the actual number of Ly5.2$^+$ cells remaining in the periphery at the end of that time.

The exported cells were entering a periphery of normal size, already seeded by ~10^6 T cells each day from the host thymus. As the overall number of peripheral T cells did not increase significantly in grafted mice despite the clear increase in RTE exported daily, homeostasis must be achieved through increased deletion of peripheral T cells. However, the fate of RTE in this circumstance remained unclear. If RTE had been selectively targeted for deletion as a means of controlling pool size, the accumulated total of Ly5.2$^+$ cells of graft origin after 3 wk would be substantially less than the total number released. Conversely, a

number of peripheral Ly5.2$^+$ cells higher than that actually released would indicate that RTE proliferated after export, perhaps as a means of establishing residency.

Instead, at each of the time points examined over 3 wk, the number of accumulated Ly5.2$^+$ T cells in the lymphoid organs matched closely the predicted number of cells exported. Although the vigorous growth of the grafted thymus at this time means that migration rates may vary slightly from predicted values, most RTE appear to have successfully entered the peripheral pool, presumably at the expense of resident T cells. Therefore, the host thymus in a normal healthy individual may exert considerable influence over the peripheral TCR repertoire beyond the initial seeding of the pool.

RTE of both graft and host thymi were essentially CD25$^-$ and CD69$^-$, indicating that peripheral establishment was not due to proliferation, but rather to accumulation (8). Furthermore, graft origin cells stained 4 wk after grafting expressed generally low levels of CD44 compared with surrounding resident cells, and RTE isolated by cell sorting showed little evidence of spontaneous tritiated thymidine uptake in short-term culture, all of which indicated minimal proliferation (data not shown). This correlates well with studies by Tough and Sprent (7) and Tanchot and Rocha (8, 16) demonstrating that RTE in both normal and lymphopenic mice are small nondividing cells of naive phenotype.

This study also provides information on the important question of the physiology of peripheral T cells—what is the survival capacity of thymic migrants relative to resident T cells? The longevity of naive phenotype T cells is generally regarded as dependent on the level of cellular competition within the peripheral pool (5, 7, 22), although the possible selective deletion of RTE has also been described (23). Leuchars et al. (11) also reported that thymus-grafted mice showed a large reduction in the peripheral levels of T cells of graft origin after 3 wk, although they were unable to measure directly the rate of supply or the longevity of these cells.

Given the homeostatic regulation of the size of the peripheral pool, any increase in number of peripheral T cells due to thymic emigration must be balanced by a corresponding loss of resident cells. This could either be by random displacement or by selective loss among RTE, naive T cells, or memory T cells. Very recently, using the H-Y–specific TCR transgenic RAG-2–deficient mouse model, Tanchot and Rocha (16) suggested in a series of experiments that the loss was due to random displacement. However, our model provides results consistent with thymic migrants having a selective survival advantage over resident T cells for up to 3–4 wk after export, at which time they themselves become selectively disadvantaged by new RTE.

The major difference between our grafted thymus model and the H-Y–specific TCR transgenic RAG-2–deficient mouse model used by Tanchot and Rocha, apart from the diversity of the TCR repertoire, is that random displacement kinetics were seen in RAG-2–deficient mice with only very low peripheral levels ($\sim 4 \times 10^6$ cells) of TCR transgenic CD8$^+$ T cells before bone marrow reconstitution. Because the mechanisms of peripheral lymphocyte homeostasis are largely unknown, the effect of removing or modifying any elements of a normal peripheral pool is difficult to predict. Hence, the displacement of TCR transgenic CD8$^+$ T cells from RAG-2–deficient mice in the week after bone marrow reconstitution may differ significantly from displacement of resident CD4$^+$ and CD8$^+$ T cells by RTE in normal mice.

In our study of thymic grafted mice, an average of 14.5×10^6 Ly5.2$^+$ cells was present in the periphery 4 wk after grafting. At week 8, random displacement kinetics predict almost 8×10^6 would remain; however, only $\sim 2 \times 10^6$ cells persisted. Separate analysis of both CD4 and CD8 cells demonstrated that although the extent of decay was greater for CD4$^+$ than CD8$^+$ cells, the cell loss from within both pools was far in excess of that predicted by random displacement kinetics alone.

Some reduction with time is expected, because by 3–4 wk, the grafted thymus is almost fully reconstituted by host Ly5.2$^-$ cells that accordingly make up an increasing proportion of the RTE. Beyond 4 wk, virtually no Ly5.2$^+$ cells are released, removing the only means by which the naive Ly5.2$^+$ T cell pool could be maintained homeostatically. A progressive loss of Ly5.2$^+$ cells would then be expected, as Ly5.2$^-$ RTE from both the host and grafted thymus displace cells in the periphery, including some Ly5.2$^+$ cells. Although random displacement of resident T cells by RTE would increase in thymus-grafted mice, this does not explain fully the marked loss of peripheral Ly5.2$^+$ cells observed in our study.

A more precise analysis of the displacement of peripheral cell loss by RTE in normal mice is currently being undertaken to determine the mechanisms and location of cell deletion and to establish whether the cell loss is linear or exponential. At present, there are three possible explanations for the differences observed between the Tanchot and Rocha study, advocating random displacement (16), and our own, suggesting nonrandom displacement. First, the discrepancies may be a direct consequence of the different in vivo models used (i.e., TCR transgenic, bone marrow–reconstituted RAG-2$^{-/-}$ mice versus thymus-grafted normal mice); second, CD4$^+$ and CD8$^+$ T cells or subsets therein may exhibit different decay kinetics; and third, TCR specificities may affect the survival potential of T cells. These possibilities are currently under investigation.

The initial survival of most RTE in our study suggests that a window of at least 3 wk may exist for the T cell to respond to antigen and become established in the memory compartment. After 3 wk without activation, the naive T cell may become more susceptible to displacement, allowing new TCR specificities to be introduced to the pool from the thymus. Indeed, in the Tanchot and Rocha study, nonreactive "tolerant" T cells were preferentially lost in the presence of thymic migrants.

Although the dominance of one T cell subset over the other can occur in transgenic models (24), the ratio of CD4/CD8 T cells in normal mice is controlled homeostatically (1). However, analysis of the CD4/CD8 ratio of RTE in the spleen, lymph nodes, and blood revealed it to be twice that of the resident T cell population (3.5:1 compared with 1.5:1). This trait has been reported recently in chickens (25), and provides further evidence that the RTE population is under thymic rather than peripheral regulation. 3 wk after grafting, donor Ly5.2$^+$ T cells in the lymph node and spleen still had slightly raised CD4/CD8 ratios compared with the resident population, but the blood ratio was significantly lower. This suggests that the "correction" of the CD4/CD8 ratio does not occur simultaneously throughout the peripheral pool, but is achieved initially at the level of recirculating T cells and is then reflected in the secondary lymphoid organs as T cells reenter these regions.

Perhaps most significant is that correction occurs at all. Given the small proportion of the total peripheral T cell pool represented by RTE, the overall CD4/CD8 ratio is barely affected by that of the migrant cells. Yet within a week of export, the CD4/CD8 ratio among migrant cells is largely corrected to that of resident T cells. Therefore, migrant cells are clearly a separate population, and are regulated accordingly. Whether the change in CD4/CD8 ratio among RTE is due to peripheral regulation or to prior thymic signaling is currently being investigated.

The reduction in CD4/CD8 ratio after thymic export without any overall loss of migrants (up to 3 wk after export) implies that some CD4$^+$ cells are deleted, with a similar proportion of CD8$^+$ RTE proliferating. Interestingly, previous reports on the proliferation of RTE using BrdU uptake confirm that although RTE are largely nondividing, a minor subpopulation of emigrants shows evidence of recent division (7, 26). The majority of these cells were CD8$^+$, which may account for the shift in the CD4/CD8 ratio of exported cells towards that of resident cells.

In summary, this study demonstrates that T cells within the thymus and peripheral lymphocyte pools are regulated independently of each other. The thymus supports thymopoiesis and exports T cells at a predictable rate, unaffected by the level of peripheral T cells or the presence of abnormally high levels of RTE caused by thymic grafting. However, the thymus does rely on the periphery for a continual supply of blood-borne precursors, as no thymocytes persist as long-term residents to enable true autonomy. After export from the thymus, the RTE population is clearly distinct from the resident peripheral T cells. The transition from thymic to peripheral regulation is reflected in the change of the CD4/CD8 ratio of RTE cells induced upon entry to the peripheral pool. This appears to occur primarily among circulating cells, and could result from the partial loss of CD4$^+$ RTE and corresponding proliferation of CD8$^+$ cells. RTE are largely accepted into the peripheral pool, providing a constant refurbishing of the TCR repertoire, but a failure to contact antigen within 3 wk of export may result in susceptibility to displacement by new RTE. The precise mechanisms underlying the homeostatic regulation of the peripheral T pool and the fate of displaced cells are currently under investigation.

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References

1. Rocha, B., N. Dautigny, and P. Pereira. 1989. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur. J. Immunol.* 19:905–911.
2. Grossman, C.J. 1985. Interactions between the gonadal steroids and the immune system. *Science*. 227:257–261.
3. Dardenne, M., and W. Savino. 1994. Control of thymus physiology by peptidic hormones and neuropeptides. *Immunol. Today*. 15:518–523.
4. Scollay, R., E.C. Butcher, and I.L. Weissman. 1980. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210–218.
5. Tough, D.F., and J. Sprent. 1994. Lymphocyte life-span and memory. *Science*. 265:1395–1400.
6. Pereira, P., and B. Rocha. 1991. Post-thymic in vivo expansion of mature αβ T cells. *Int. Immunol*. 3:1077–1080.
7. Tough, D.F., and J. Sprent. 1994. Turnover of naive and memory-phenotype T cells. *J. Exp. Med.* 179:1127–1135.
8. Tanchot, C., and B. Rocha. 1995. The peripheral T cell repertoire: independent homeostatic regulation of virgin and activated CD8$^+$ T cell pools. *Eur. J. Immunol.* 25:2127–2136.
9. Mackall, C.L., F.T. Hakim, and R.E. Gress. 1997. T-cell regeneration: all repertoires are not created equal. *Immunol. Today*. 18:245–251.
10. Wallis, V.J., E. Leuchars, M. Chaudhuri, and A.J.S. Davies. 1979. Studies on hyperlymphoid mice. *Immunology*. 38:163–171.
11. Leuchars, E., V.J. Wallis, M.J. Doenhoff, A.J.S. Davies, and J. Kruger. 1978. Studies of hyperthymic mice. *Immunology*. 35: 801–809.
12. Cyster, J.G., and C.C. Goodnow. 1995. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity*. 3:691–701.
13. Tough, D., F.P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type 1 interferon in vivo. *Science*. 272:1947–1951.
14. Ahmed, R. 1996. Tickling memory T cells. *Science*. 272: 1904.

15. Takeda, S., H. Rodewald, H. Arakawa, H. Bluethmann, and T. Shimuzu. 1996. MHC class 2 molecules are not required for survival of newly generated $CD4^+$ T cells, but affect their long term life span. *Immunity*. 5:217–228.
16. Tanchot, C., and B. Rocha. 1997. Peripheral selection of T cell repertoires: the role of continuous thymic output. *J. Exp. Med*. 186:1099–1106.
17. Metcalf, D. 1963. The autonomous behaviour of normal thymus grafts. *Aust. J. Exp. Biol. Med. Sci*. 41:437–440.
18. Bradley, L.M., M. Croft, and S.L. Swain. 1993. T-cell memory: new perspectives. *Immunol. Today*. 14:197–199.
19. Barrat, F., B.M. Lesourd, A. Louise, H.-J. Boulouis, S. Vincent-Naulleau, D. Thibault, M. Sanaa, T. Neway, and C. Pilet. 1997. Surface antigen expression in spleen cells of C57Bl/6 mice during ageing: influence of sex and parity. *Clin. Exp. Immunol*. 107:593–600.
20. Gabor, M.J., R. Scollay, and D.I. Godfrey. 1997. Thymic T cell export is not influenced by the peripheral T cell pool. *Eur. J. Immunol*. 27:2986–2993.
21. Miller, J.F.A.P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev*. 47: 437–520.
22. Tanchot, C., M.M. Rosado, F. Agenes, A.A. Freitas, and B. Rocha. 1997. Lymphocyte homeostasis. *Semin. Immunol*. 9: 331–337.
23. Sprent, J. 1993. Lifespans of naive, memory and effector lymphocytes. *Opin. Immunol*. 5:433–438.
24. Hanahan, D. 1990. Transgenic mouse models of self-tolerance and autoreactivity by the immune system. *Annu. Rev. Cell Biol*. 6:493–537.
25. Vainio, A., and K. Katevuo. 1996. Thymocyte emigration in the chicken: an over-representation of $CD4^+$ cells over $CD8^+$ in the periphery. *Immunology*. 89:419–423.
26. Penit, C., and F. Vasseur. 1997. Expansion of mature thymocyte subsets before emigration to the periphery. *J. Immunol*. 159:4848–4856.