Thymus-autonomous T cell development in the absence of progenitor import

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Thymus function is thought to depend on a steady supply of T cell progenitors from the bone marrow. The notion that the thymus lacks progenitors with self-renewal capacity is based on thymus transplantation experiments in which host-derived thymocytes replaced thymus-resident cells within 4 wk. Thymus grafting into T cell–deficient mice resulted in a wave of T cell export from the thymus, followed by colonization of the thymus by host-derived progenitors, and cessation of T cell development. Compound *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mutants lack competitive hematopoietic stem cells (HSCs) and are devoid of T cell progenitors. In this study, using this strain as recipients for wild-type thymus grafts, we noticed thymus-autonomous T cell development lasting several months. However, we found no evidence for export of donor HSCs from thymus to bone marrow. A diverse T cell antigen receptor repertoire in progenitor-deprived thymus grafts implied that many thymocytes were capable of self-renewal. Although the process was most efficient in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ hosts, γ_c-mediated signals alone played a key role in the competition between thymus-resident and bone marrow–derived progenitors. Hence, the turnover of each generation of thymocytes is not only based on short life span but is also driven via expulsion of resident thymocytes by fresh progenitors entering the thymus.

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Abbreviations used: DN, double negative; ETP, early thymic progenitors; HSC, hematopoietic stem cell; LAM, linear amplification-mediated; SCID, severe combined immunodeficient; SP, single positive; W, white spotted kit alleles.

It is generally accepted that thymocytes are short-lived cells that are continuously replaced by new progenitors of adult bone marrow origin. Under normal steady-state conditions, the total pool of thymocytes in a thymus graft is replaced within 4 wk by a new generation of cells originating from the bone marrow (Berzins et al., 1998). After transplantation of wild-type thymus grafts into severe combined immunodeficient (SCID), or *Rag2*$^{-/-}$ recipient mice, the thymus exports a single wave of thymus-derived T cells that seed the peripheral lymphoid organs. The thymus is then colonized by developmentally arrested T cell progenitors from the host SCID or *Rag2*$^{-/-}$ bone marrow, and additional T cell production stops (Frey et al., 1992; Takeda et al., 1996). It has therefore been assumed that the thymus lacks self-renewing progenitors, or long-term resident thymocytes, and that thymus function is absolutely dependent on uninterrupted import of progenitors from the bone marrow.

The earliest stages of intrathymic T cell development depend on growth factor receptor signals, mediated by the receptor tyrosine kinase Kit and IL-7R (Rodewald et al., 1997), which drive proliferation of pro–T cells to yield a population of cells numerous enough to generate a broad repertoire of TCR β chains. In contrast to SCID or *Rag2*$^{-/-}$ mice which have a block at the CD4$^-$CD8$^-$ (double negative [DN]) 3 (CD4$^-$CD8$^-$CD25$^+$CD44$^-$) stage, *Kit* and γ_c (component of the IL-7 receptor) double-deficient mice are completely devoid

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of T cell progenitors, including the earliest stages (Rodewald et al., 1999). Mice triple-deficient for *Kit*, γ_c, and *Rag2* (termed *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$) lack T, B, and NK cells. Because of their Kit defect, niches in the bone marrow of *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice can readily be colonized by Kit$^+$ (wild-type) hematopoietic stem cells (HSCs), allowing HSC engraftment across histocompatibility barriers without irradiation (Waskow et al., 2009). These mice have no endogenous T cell development because of the lack of Kit and IL-7R function in their bone marrow progenitors.

To address the fate of a thymus in the complete absence of developmentally competent bone marrow progenitors, we have transplanted normal wild-type thymus into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice. The thymus grafts did not export HSC that could engraft in the bone marrow, even in the most HSC-deprived *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients. However, we noticed thymus-autonomous T cell development in the absence of progenitor replenishment from the bone marrow. Here, we characterize this previously unrecognized capacity of the thymus to sustain productive T cell development under conditions of bone marrow progenitor deprivation, and show that normal thymocyte turnover is not only regulated by cell-intrinsic life span but also by competitive progenitor replacement.

RESULTS AND DISCUSSION

Thymus autonomy under conditions of T cell progenitor deprivation

To search for signs of T cell progenitor (Lambolez et al., 2006) or HSC export from the thymus, and to analyze the long-term fate of thymus grafts in the absence of progenitor competition from the bone marrow, we transplanted thymus lobes from newborn wild-type mice into adult *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients (see Materials and methods for donor and host MHC and congenic markers). At 10 wk after transplantation, thymus grafts were analyzed by flow cytometry for cells of donor (thymus) versus host origin, and for expression of thymocyte differentiation markers (Fig. 1 A). In thymus grafts implanted into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients, the donor cell CD4 and CD8 expression profile resembled that of a normal thymus, with CD4 and CD8 double-positive (DP) thymocytes representing the largest cellular fraction (Fig. 1, A and B). In *Rag1*$^{-/-}$ host controls, DP thymocytes were absent in the grafts by 6 wk (Fig. 1, A and B), as expected (Frey et al., 1992; Takeda et al., 1996). Thus, in *Rag1*$^{-/-}$ hosts, thymocyte development of thymus-resident cells was fully exhausted by this time, and ~95% cells in the grafts were host-derived DN thymocytes, blocked at the DN 3 stage. In contrast, even at later times in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice, a large proportion of cells were of donor thymus origin, and overall donor cell numbers, although heterogeneous between grafts, were on average several orders of magnitude higher in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ compared with *Rag1*$^{-/-}$ hosts (Fig. 1 B).

The fact that T cell development in the thymus grafts appeared to be ongoing after >2 mo, as further demonstrated below, raised the possibility that T cell progenitors had been exported from the wild-type thymus (Lambolez et al., 2006) and resided in an extrathymic host tissue from which they might continuously seed the thymus. The endogenous thymus in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice is reconstituted after wild-type HSC transplantation (Waskow et al., 2009). If thymus-derived HSCs or T cell progenitors were available systemically, one should expect to find active T cell development not only in the graft but also in the endogenous recipient thymus. In 75% (43/57) of the graft recipients, T cell development was restricted to the grafts, whereas the endogenous thymi harbored only recirculating graft-derived mature T cells and no developing thymocytes (Fig. 1 A). In line with the notion that the thymus can export T cell progenitors (Lambolez et al., 2006), we found T cell development of the donor thymus type in the graft and in the endogenous thymus in the remaining 25% (14/57) of the hosts. However, neither in the cases of T cell development restricted to the thymus graft nor of T cell development in the graft and in the endogenous thymus, was there evidence for a contribution of donor HSC. Thymus-derived HSCs were undetectable in the bone marrow of thymus graft recipients (Fig. 1 C), and recipient spleens harbored T cells (Fig. 1 D), NKT cells (Fig. 1 E), and only very few myeloid cells of donor origin (Fig. 1 F). Reconstitution of splenic B cells of donor origin was variable, in that 33% (19/57) of the recipients showed no B cells (Fig. 1 D), 42% (24/57) had low percentages (<2% of splenocytes), and 21% (12/57) had robust (>2% of splenocytes) B cell numbers (the remaining 2/57 mice were not analyzed for B cells). In the absence of HSC engraftment, and in view of the frequent B cell potential in the newborn thymus (Ceredig et al., 2007), it is likely that B cells arose from intrathymic progenitors. In addition to 57 thymus recipients without evidence for HSC engraftment, we observed one recipient mouse with overt donor thymus-derived HSC engraftment (not depicted), demonstrating that HSC export from the thymus is a rare event, which is consistent with the notion that the thymus does not harbor HSCs (Matsuzaki et al., 1993).

Collectively, thymus-autonomous T cell development can proceed in the grafts in the absence of continuous T cell progenitor import. The data imply that thymocytes do not vanish solely due to their cell-intrinsic life span, which is on the order of days (Penit et al., 1988; Egerton et al., 1990; Huesmann et al., 1991), but that thymocyte turnover is also regulated by competition between thymus-resident cells and new progenitors entering from the bone marrow. The fact that *Rag1*$^{-/-}$ bone marrow–derived thymocytes suppressed ongoing T cell development in the grafts indicates that the competition occurs at the DN stages.

Normal intrathymic differentiation during thymus-autonomous T cell development

The presence of DP thymocytes in thymus grafts suggests, but does not prove, ongoing and productive intrathymic T cell development, i.e., the progression along well-known stages of TCR αβ T cell development. To address this question, we transplanted thymus grafts from newborn

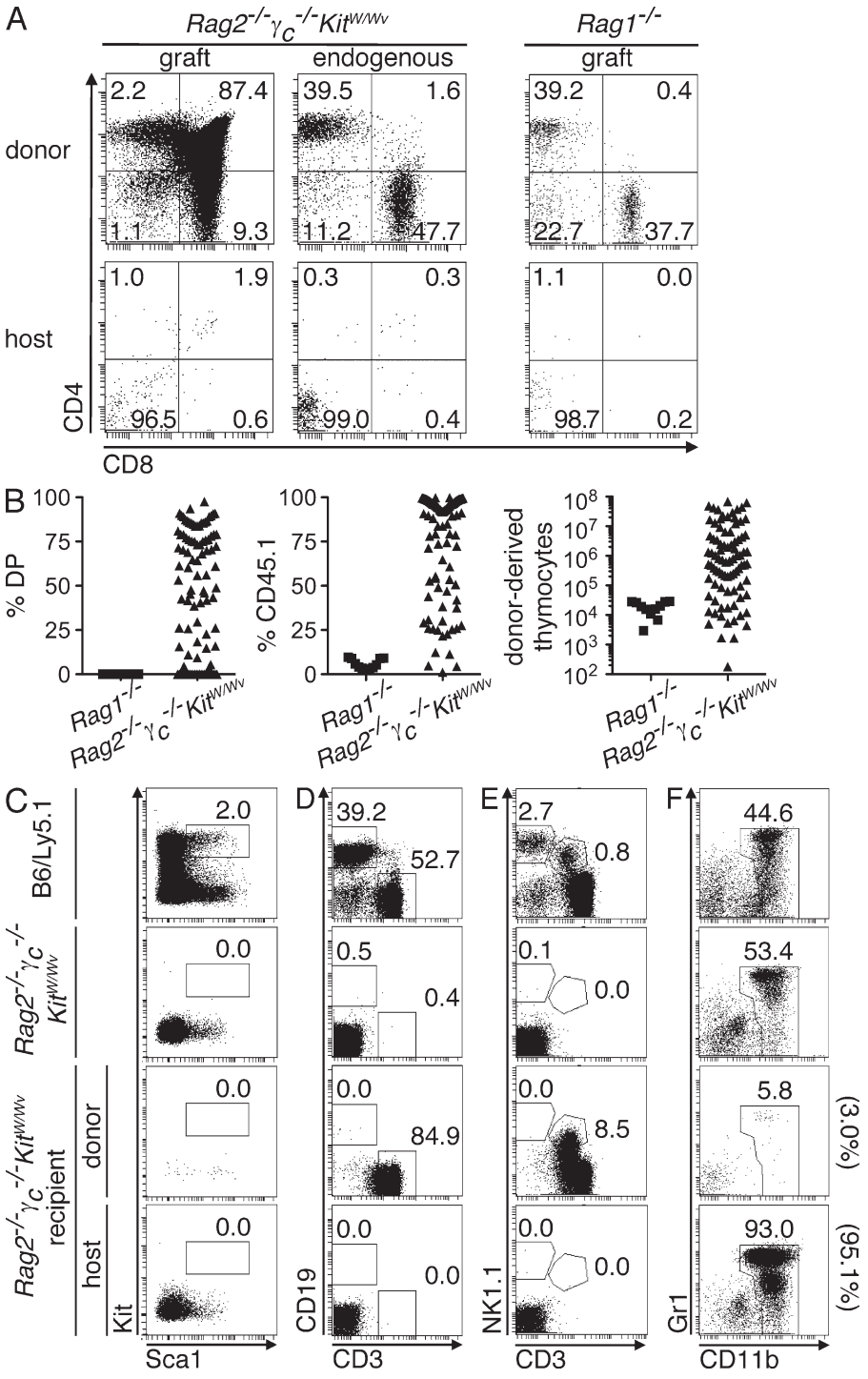

Figure 1. T cell development in thymus grafts in the absence of donor HSC engraftment. (A) Newborn wild-type thymi were transplanted into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients (n = 57), and analyzed 9–11 wk later. One thymus graft representative of sustained T cell development (left column), and the endogenous thymus of the same recipient (middle column) are shown. *Rag1*$^{-/-}$ recipients (n = 3) were transplanted as controls and analyzed at 6 wk (right column). Dot plots display cells pregated for thymus donor (CD45.1$^+$) or host origin (CD45.1$^-$), and stained for CD4 and CD8 expression. Numbers indicate percentages of cells in each quadrant. (B) CD45.1$^+$ thymus grafts implanted into CD45.1$^-$ *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ (n = 57) or *Rag1*$^{-/-}$ (n = 7) recipients were analyzed 9–11 wk later by flow cytometry for the percentage of DP thymocytes (left), for the percentage of CD45.1$^+$ donor cells per total cells (middle), and for absolute numbers (right) of CD45.1$^+$ donor-derived thymocytes within each graft. Each symbol represents an individual lobe. (C–F) CD45.1$^+$ wild-type mice (B6/Ly5.1), nontransplanted *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice, and a representative *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipient (n = 57) at 9–11 wk after thymus transplantation were analyzed for the presence of HSCs (Lin$^-$Sca1$^+$Kit$^+$; C; gated on Lin$^-$), T cells (CD3$^+$CD19$^-$), B cells (CD3$^-$CD19$^+$; D; total splenocytes), NK cells (CD3$^-$NK1.1$^+$), NKT cells (CD3$^+$NK1.1$^+$; E; gated on CD19$^-$ splenocytes), and myeloid cells (CD11b$^+$ cells expressing varying levels of Gr1; F; gated on CD4$^-$CD8$^-$CD3$^-$CD19$^-$ Ter119$^-$ splenocytes). Percentages shown in F next to the corresponding FACS plots refer to percentage of donor (CD45.1$^+$) or host (CD45.2$^+$) cells. Numbers indicate percentages of cells in each gate.

transgenic RAG2p-GFP donor mice (Yu et al., 1999). GFP expression indicates *Rag2* expression (Yu et al., 1999) in developing thymocytes. Consistent with *Rag2* expression in thymocytes but not in mature T cells, and with the longer half-lives of GFP compared with Rag proteins (Nagaoka et al., 2000; McCaughtry et al., 2007), GFP$^+$ thymocytes in RAG2p-GFP mice included mostly DP and single-positive (SP) cells, whereas GFP$^-$ cells were predominantly mature recirculating T cells (Fig. 2 A). Similarly, in RAG2p-GFP grafts placed for 9–10 wk in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ hosts, GFP$^+$ cells were mostly DP and, to a variable extent, also SP thymocytes, whereas GFP$^-$ cells were mature T cells. Consistent with thymus-autonomous T cell development, the endogenous thymus lacked de novo generated GFP$^+$ thymocytes and contained only mature GFP$^-$ T cells. Hence, in the RAG2p-GFP reporter system, GFP-expressing cells in both the normal thymus and in progenitor-deprived thymus grafts were immature thymocytes, whereas post-GFP expressers were mature T cells.

Productive T cell development should result in T cell export from the grafts. GFP expression from the RAG2p-GFP

transgene is a hallmark of recent thymic emigrants in the periphery (Boursalian et al., 2004). *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice bearing RAG2p-GFP thymus grafts were bled over time to search for graft-derived recent thymic emigrants (Fig. 2 B). Staining for either CD4 or CD8 versus GFP showed that both CD4$^+$ and CD8$^+$ T cells were exported early after transplantation. At later time points, mostly CD8 T cells (and NKT cells; not depicted) continued to be released from the grafts in 16/19 analyzed recipients, whereas in 3/19 mice both CD4 and CD8 T cells continued to be produced (not depicted). Together, analyses of RAG2p-GFP thymi grafted into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice, and peripheral blood of the hosts support the view of continuous and productive T cell development in the grafts.

Kinetics and proliferation during thymus-autonomous T cell development

To track the kinetics of T cell development in the grafts, *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients received 1 mg BrdU 9 wk after transplantation. 9-wk-old wild-type mice were injected in parallel. Mice were analyzed after 2 h, or after a 5-d chase. After 2 h, gated BrdU$^+$ thymocytes were mostly immature

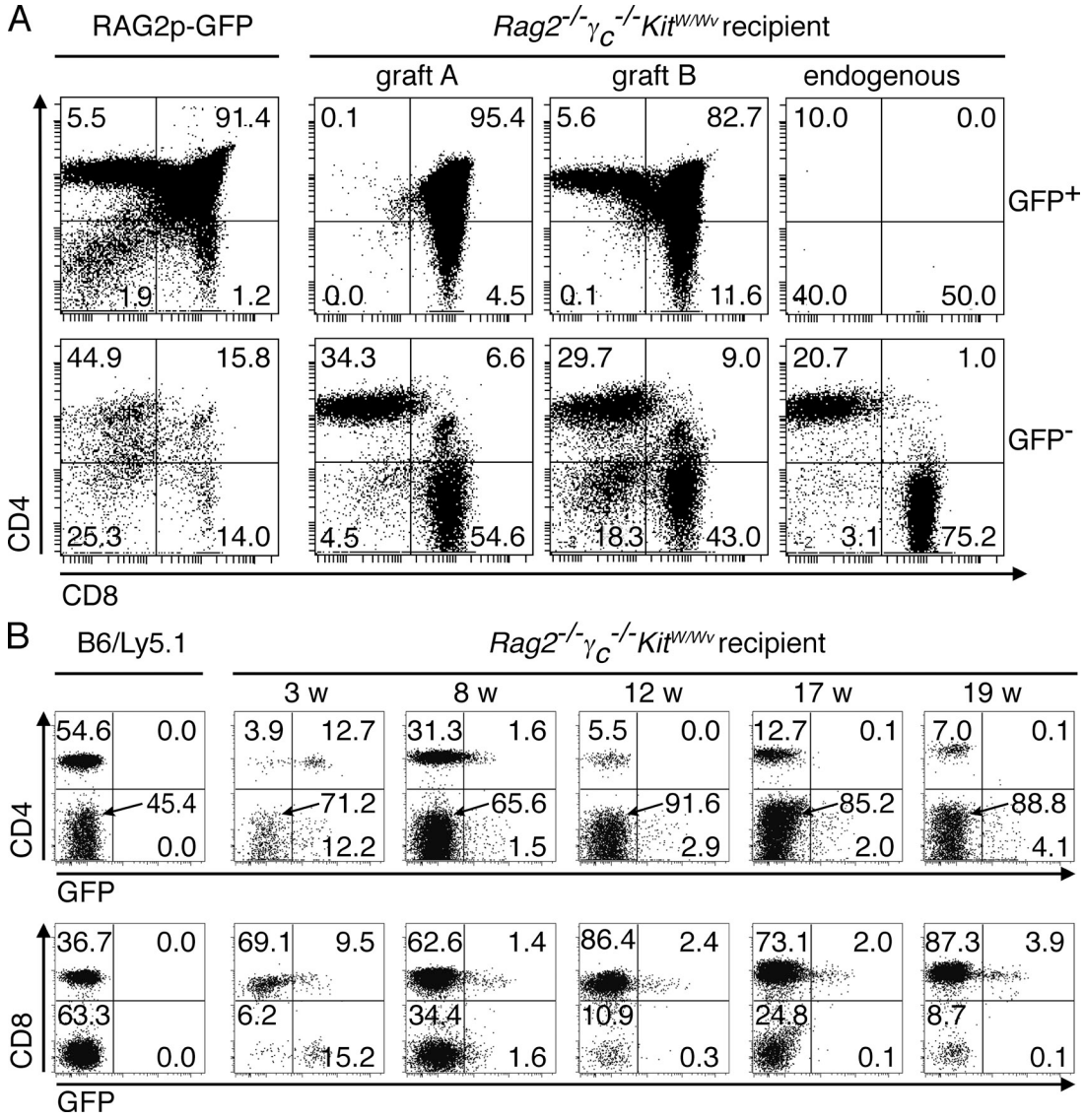

Figure 2. Sustained T cell development and T cell export visualized by RAG2p-GFP reporter. (A) Newborn thymi from RAG2p-GFP reporter mice were transplanted into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients. RAG2p-GFP control thymus, 2 RAG2p-GFP grafts 10 wk after transplantation (graft A and graft B are two lobes from the same thymus that were separated at the time of transplantation), and the endogenous thymus of the *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ host are shown. Expression of CD4 and CD8 was analyzed in CD45.1$^+$ donor cells, gated for GFP$^+$ and GFP$^-$. Plots are representative of one out of four experiments, each with four to seven recipients. (B) CD45.1$^+$CD3$^+$ cells from peripheral blood of a B6/Ly5.1 control and from a representative *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ host bearing RAG2p-GFP grafts are shown. A total of 19 recipient mice were analyzed at the indicated time points in weeks (w) for the presence of GFP$^+$CD4$^+$ (top row) and GFP$^+$CD8$^+$ (bottom row) recent thymic emigrants. Numbers indicate percentages of cells in each quadrant.

CD8 SP and DP cells, which was comparable between the normal thymus and thymus grafts (Fig. 3 A). After the chase, the proportion of DP thymocytes declined, whereas the proportion of SP thymocytes increased among BrdU-labeled cells (Fig. 3 A), indicating that the kinetics of TCR αβ T cell development was comparable in the normal thymus and in progenitor-deprived thymus grafts.

To analyze rates of intrathymic proliferation and decay (label retention), we quantified thymocyte subsets from normal thymi and from thymus grafts for their frequencies of $BrdU^+$ cells (Fig. 3 B). 2 h and 5 d after injection of BrdU (as described above), we compared DN, DP, CD4 SP, and CD8 SP subsets. Overall, frequencies were very similar, comparing normal thymi and thymus grafts, except that the numbers appeared more heterogeneous in the grafts, and label retention seemed to be reduced in DP and SP cells in the grafts. The latter finding may point at a higher population turnover in the progenitor-deprived thymus grafts. However, the overall comparable frequency of $BrdU^+$ cells at 2 h indicates similar proliferation rates.

The most immature T cell progenitors in the normal thymus are early thymic progenitors (ETP; $CD44^+CD25^-Kit^+$ within the $CD3^-$ DN1 compartment), followed by DN2 ($CD44^+CD25^+$), to DN3 ($CD44^-CD25^+$) to DN4 ($CD44^-CD25^-$) stages.

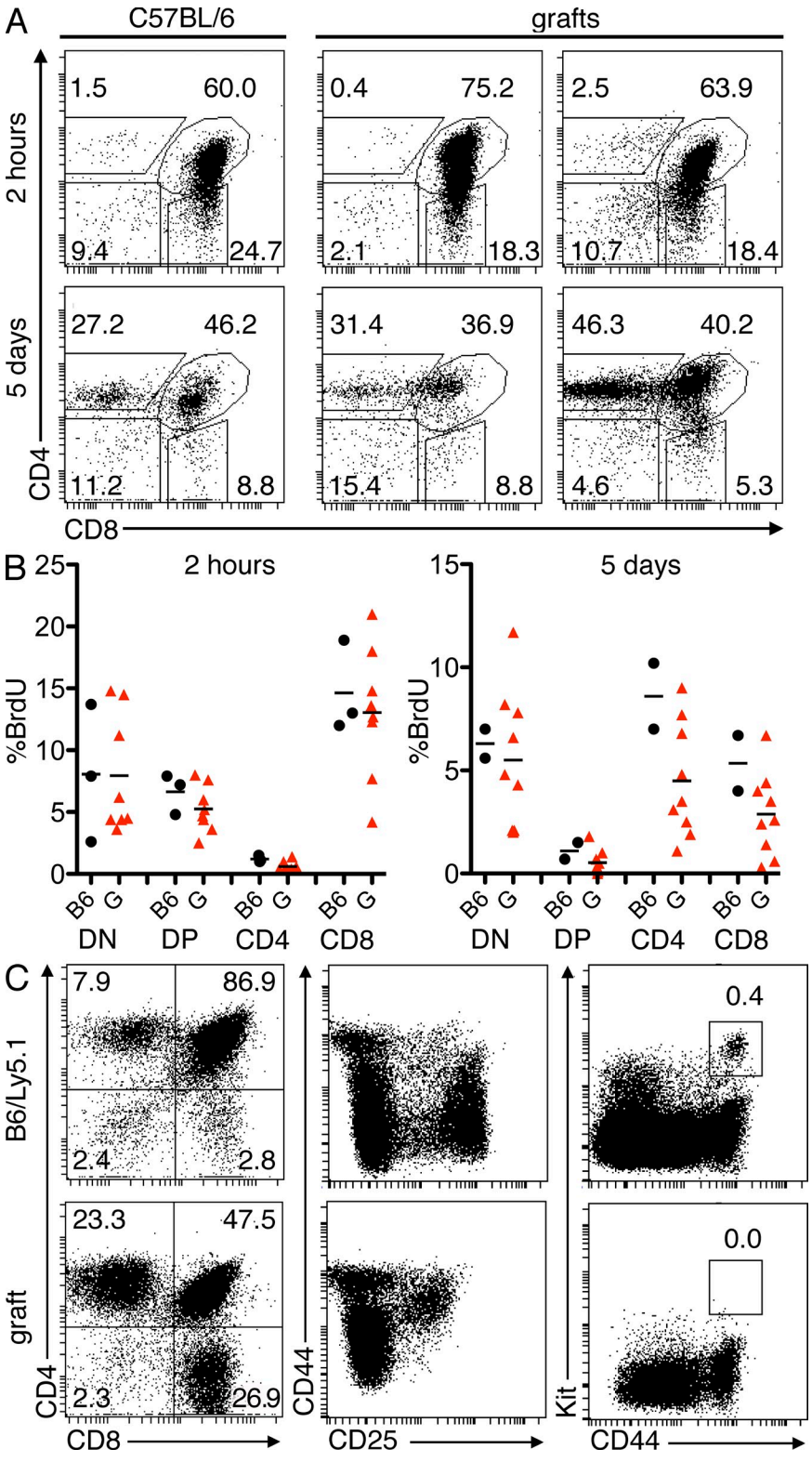

Figure 3. Intrathymic differentiation in the absence of continuous progenitor import. (A and B) 9 wk after thymus transplantation, *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients and C57BL/6 control mice received 1 mg BrdU and thymocytes were analyzed 2 h or 5 d later. Dot plots in A show expression of CD4 and CD8 by $BrdU^+$ cells and represent examples of individual thymus grafts. Numbers indicate percentages of cells in each region. (B) Percentages of $BrdU^+$ cells within each indicated population (DN, DP, CD4, and CD8) were analyzed 2 h and 5 d after BrdU injection. Results from one experiment are shown, where each dot is an individual C57BL/6 thymus (B6) or an individual thymus graft (G). The 2-h pulse labeling was done in 3 independent experiments, in which 3, 4, or 8 grafts with DP were analyzed. The 5-d chase experiment was performed once, and 9 grafts with DP were analyzed. (C) B6/Ly5.1 control thymus, and a representative graft after 10 wk in a *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ host, were analyzed for the indicated markers. Cells stained for CD44 and CD25 were pregated as $Lin^-CD45.1^+$ donor cells, and cells stained for CD44 and Kit were pregated as $Lin^-CD25^-CD45.1^+$ donor cells. Numbers indicate percentages of cells in each gate. A total of 3 independent experiments were performed, in each of which 3–10 grafts with cellularities >10^6 cells were analyzed.

Although ETPs were readily identified in the normal thymus, we could not detect an ETP phenotype in thymus grafts (Fig. 3 C), and normal DN2 and DN3 stages were also absent (Fig. 3 C). An unusual $CD44^{low}CD25^{low}$ population (Lambolez et al., 2006), which lacked expression of Kit, was evident in the grafts. Hence, T cell development proceeded in the absence of normal canonical stages.

TCR diversity in the absence of progenitor colonization of the thymus

Thymocytes at and beyond the pre–TCR (pTα-TCRβ) stage bear clonal rearrangements of their TCR β loci, and DP thymocytes also carry TCR α rearrangements (von Boehmer, 2004). TCR repertoire analyses should provide estimates on numbers of unique thymocytes that can be generated in a thymus deprived of de novo colonizing progenitors. The diversity of TCR α and β rearrangements was analyzed by sequencing of cDNA from total thymocytes. We used an adapted version of the standard and nonrestrictive (nr) linear amplification-mediated (LAM) PCR (nr/LAM-PCR; Schmidt et al., 2007; Paruzynski et al. 2010), followed by 454 pyrosequencing and bioinformatic data mining for rearranged TCR α and β DNA sequences (see Materials and methods; and not depicted). The data are displayed as Vβ-Jβ (Fig. 4 A), and Vα-Jα (Fig. 4 B) pairings. Although the grafts still harbored a large diversity, we found greater holes in the repertoire compared with the two normal thymus controls (Fig. 4 A, B). In addition, we observed clonal dominance based on TCR β CDR3 sequence analysis, i.e., individual sequences were overrepresented in the grafts. This was true for TCR β rearrangements in three out of four grafts (grafts #2, #3, and #4; Fig. 4 A). Display of the 10 most prevalent CDR3 sequences (in relation to all TCR β CDR3 sequences) indicated that, in three out of four grafts, few sequences predominated in the repertoire (Fig. 4 C). For the TCR α locus, we found fewer VJ pairings in the grafts compared with the normal thymus (Fig. 4 B), and greater clonal dominance was observed in two out of four grafts (grafts #1 and #2 for TCR α Fig. 4 D).

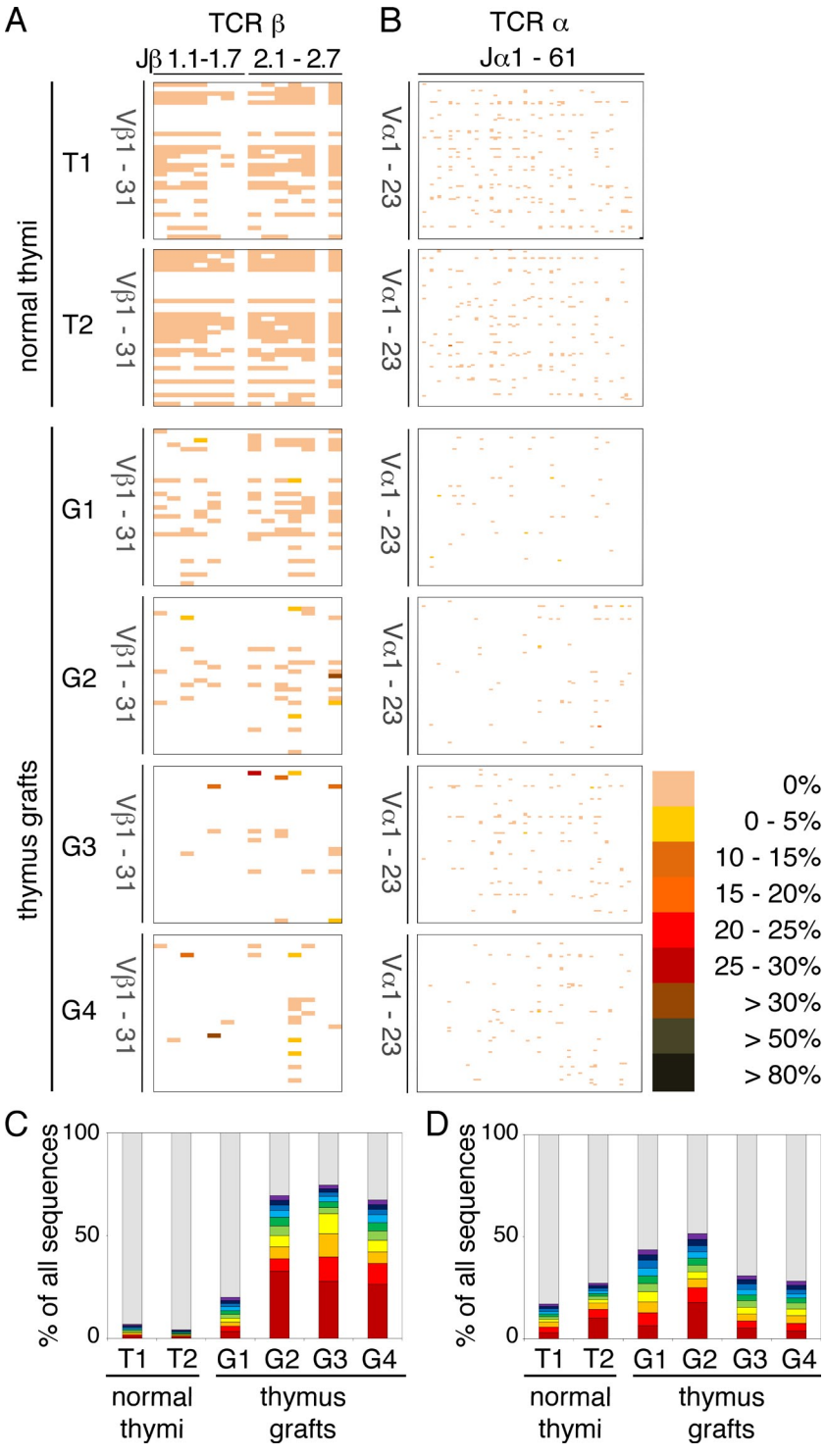

Figure 4. TCR diversity under conditions of thymus autonomy. TCR α and β chain analysis was performed on cDNA of total thymocytes derived from control mice (T; $n = 2$) or from grafts (G; $n = 4$). (A and B) Frequencies of V-J pairings are represented as heat maps for TCR β (A) and TCR α (B) chains. Each graph displays the number of times (in percentage; color in the bottom of panel B) defined V and J segments were paired in the TCR rearrangements identified. (C and D) The quantitative contribution of individual CDR3 clonotypes is represented as retrieval frequency (percentage of all TCR sequences) for β (C) and for α (D) chains, and was determined by counting identical CDR3 nucleotide sequences. The 10 most frequent CDR3 clonotypes are shown as colored bars, gray bars represent the remaining TCR sequences. Grafts were analyzed 10 wk after transplantation.

Collectively, diverse TCR rearrangements were generated or maintained under conditions of autonomous T cell development. However, the fact that repertoire holes became larger, and that clonal predominance was more evident than in a normal thymus, reflects constraints on repertoire generation. This may point to limitations in the number of cells perpetuating T cell development, or to exhaustion of the recombination machinery. Nevertheless, the highly polyclonal picture indicates that many different thymocytes contribute to autonomous T cell development. Alternatively, few early progenitors with TCR loci in germline configuration may persist, and these could continuously generate diversely rearranged thymocytes.

Key role for γ_c in permissiveness for autonomous T cell development

Sustained T cell development was permissive in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$, but not in *Rag1*$^{-/-}$ hosts (Fig. 1 A). To determine which of the signaling pathways disrupted in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice regulates this process, we grafted newborn thymus lobes into single mutant *Kit*$^{W/Wv}$ or $\gamma_c^{-/-}$, or double mutant *Rag2*$^{-/-}\gamma_c^{-/-}$ mice (Fig. 5 A; data from all recipients are summarized in Fig. 5 B). *Kit*-deficient host progenitors were developmentally competent and suppressed graft-autonomous T cell development. In *Rag2*$^{-/-}\gamma_c^{-/-}$ recipients, T cell development was exclusively of graft origin, but in $\gamma_c^{-/-}$ mutants, T cell development of host bone marrow and donor thymus origin were concurrent. Mutations in Kit (*Kit*$^{W/Wv}$) were not essential, but an effect of Kit was revealed by increased frequencies of grafts with active T cell development in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ (84%) as opposed to *Rag2*$^{-/-}\gamma_c^{-/-}$ (71%) hosts. Comparison of $\gamma_c^{-/-}$ and *Rag2*$^{-/-}\gamma_c^{-/-}$ recipients showed no obvious effect of the additional *Rag*-deficiency (Fig. 5 B).

The findings reported here, as well as data from Peaudecerf et al. (in this issue), have implications for the flow of T cell progenitors from the bone marrow to the thymus, the forces driving thymocyte turnover, and the plasticity of thymocyte life span. Intrathymic T cell development has been viewed as a continuum of differentiating cells, despite of evidence for noncontinuous, gated progenitor import into the thymus (Foss et al., 2001). It is currently impossible to measure the flow of cells from bone marrow to thymus, and we can only speculate whether this route is continuously used or not. The constant distribution of thymocyte subsets in adult mice could argue against discontinuous colonization. However, data from Peaudecerf et al. (2012) and from our study show that the thymus can switch to autonomous function in the absence of de novo progenitor colonization, raising the possibility that this mechanism provides the thymus with a buffering function to overcome a temporary shortage of progenitor supply from the bone marrow without concomitant loss of T cell production. Such mechanism may mask interrupted progenitor colonization.

Under conditions of progenitor deprivation, thymocytes did not only persist but remained productive, i.e., they progressed through stages of T cell development. This is a violation of the long-held notion that thymocytes are cell-intrinsically short lived, and that thymus function is absolutely dependent on ongoing

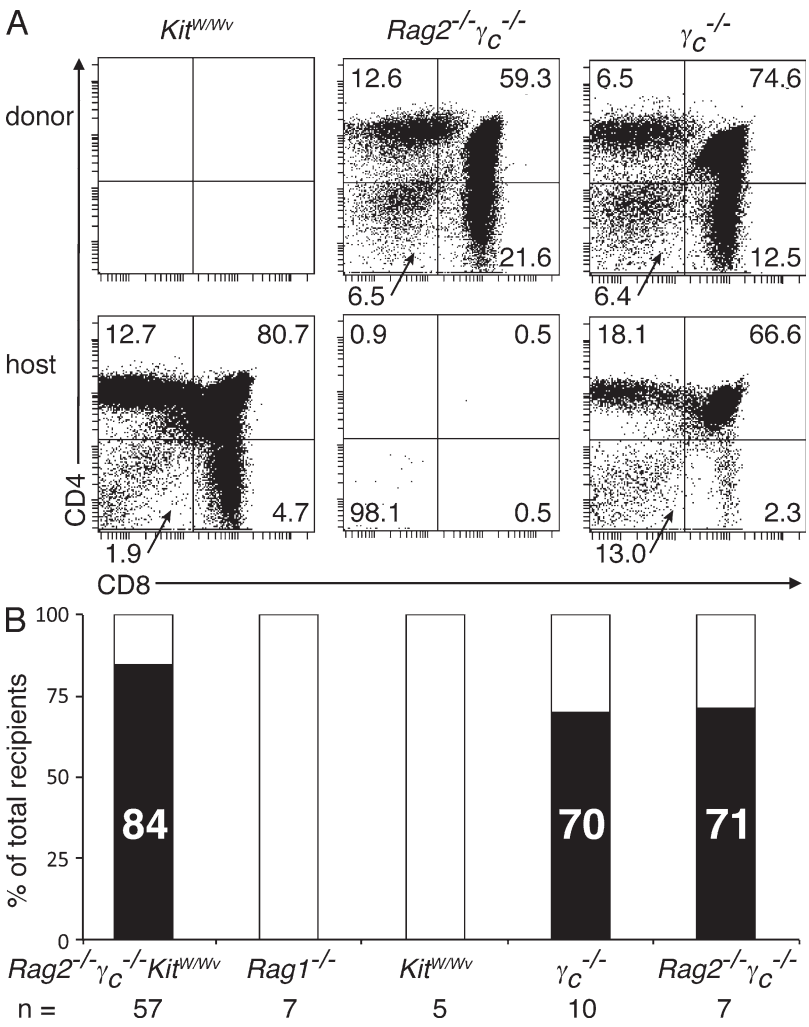

Figure 5. Competition between thymus-intrinsic and -extrinsic T cell progenitors is regulated by cytokine receptors. (A) Newborn thymus lobes were grafted into the indicated recipients. After 9 wk, cells of donor thymus and host origin were analyzed for CD4 and CD8 expression. Numbers are percentages of cells in each quadrant. Numbers of analyzed recipients are displayed in B. (B) Summary of the proportions of persistent (filled bars) versus exhausted (open bars) T cell development in thymus grafts. Exhausted refers to recipients in which both grafted lobes contained only circulating SP T cells but no DP thymocytes, whereas persistent indicates recipients with ongoing T cell development (presence of DP and SP cells) in at least one lobe. Number (n) indicates the total number of recipients. Data in B were obtained 9 to 11 wk after transplantation.

replacement by new progenitors of bone marrow origin. Our data and that of Peaudecerf et al. (2012) now demonstrate that thymocyte turnover is not only a result of short-lived thymocytes but that the time thymocytes spend in the thymus is also dependent on the competition between old and new cells. We have experimentally abrogated this competition, and found sustained T cell development for up to 10 wk. The fact that *Rag*$^{-/-}$ progenitors suppressed thymus-autonomous T cell development suggests that the competition between former and newer generations of thymocytes takes place at DN stages. In our hands, thymus-autonomous T cell development occurred in the absence of the canonical TN stages of T cell development; i.e., we find DP thymocytes in the absence of DN2 and DN3 stages. In contrast, Peaudecerf et al. (2012) report very long lasting persistence of CD25$^+$ DN stages, and TCR repertoire analysis found no evidence for gaps in the TCR diversity, whereas we noticed restrictions in the diversity. It remains to be determined whether the thymus can achieve autonomous functions from several different yet to be characterized progenitors.

The competition between thymus-resident and newly imported progenitors is regulated, at least in part, by growth factors. Early T cell development depends on signaling via Kit and IL-7R/γ_c, and thymus stromal cells provide the ligands, Kit ligand and IL-7. We have abrogated these pathways separately and found that γ_c plays a pivotal role. Kit deficiency on its own does not seem to play a role, akin to Rag deficiency, but combining mutations in *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice was most proficient in abrogation of competition from the host. These data imply that thymocyte survival in stromal cell niches is regulated in part by IL-7 and that newly arriving and formerly resident thymocytes compete for such, possibly trophic, signals. It remains an intriguing challenge to identify the mechanisms underlying this crucial step of competition. Although old and new thymocytes in one and the same mouse nominally have the same age, the data suggest that new thymocytes are more efficient in utilization of limited growth factor signals in the thymus. This newly uncovered process may not only contribute to the regulation of thymocyte turnover but also serve as a quality control device that keeps thymocytes young by ensuring that always the most recent generation of thymocytes has the greatest chance to conduct active T cell development. We have noticed T cell acute lymphoblastic leukemia (T-ALL) arising in 56% of thymus recipients beyond 16 wk (unpublished data). Thymus-autonomous T cell development in the absence of progenitor import may thus not only have bearings on the physiology of thymocyte dynamics but also on the origin of T cell leukemia in the thymus.

MATERIALS AND METHODS

Mice. *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ mice (Waskow et al., 2009) have H-2^{jxb} haplotypes and are CD45.2$^+$. To obtain congenic histocompatible donor thymi, B6.SJL-*Ptprc*^{a} Pep3^b/BoyJ (H-2^b; CD45.1$^+$) mice, termed B6/Ly5.1 (The Jackson Laboratory) were crossed to WB *Kit*$^{+/+}$ (H-2^j) mice (Japan-SLC). The resulting WBB6.SJL F1 *Kit*$^{+/+}$ (H-2^{jxb}; CD45.1$^+$ CD45.2$^+$) animals were used as donors for newborn thymus. To visualize *Rag2*-expressing thymocytes and recent thymic emigrants, RAG2p-GFP transgenic B6/Ly5.1 (Yu et al., 1999; Hale et al., 2006) x WB *Kit*$^{+/+}$ F1 were used in some experiments as newborn thymus donors. WBB6F1 *Kit*$^{W/Wv}$ mice (H-2^{jxb}) were generated from WB *Kit*$^{W/+}$ and C57BL/6 *Kit*$^{Wv/+}$ parents (Japan-SLC). *Rag2*$^{-/-}\gamma_c^{-/-}$ and $\gamma_c^{-/-}$ mice (Shinkai et al., 1992; Cao et al., 1995) were obtained from Taconic, and *Rag1*$^{-/-}$ mice (Mombaerts et al., 1992) were provided by J. Reimann (Internal Medicine I, University of Ulm, Ulm, Germany). All mice were bred and kept in individually ventilated cages in the mouse facility of the University of Ulm. All animal experiments were approved by the Regierungspräsidium Tübingen.

Thymus transplantation. The thymus was isolated from newborn mice of the genotypes described above, and the two lobes of each thymus were physically separated. Each host received one thymus, i.e., two lobes, and each lobe was placed in one extremity of the kidney, as described (Rodewald et al., 1995). Some recipients were periodically bled from the tail vein, and the peripheral blood was analyzed for the presence of T cells.

Flow cytometry. Organs were harvested and single-cell suspensions were prepared in PBS/5% FCS. Cells were blocked with 100 µg/ml mouse IgG (Jackson ImmunoResearch Laboratories) for 15 min and stained for 30 min in an appropriate diluted antibody staining solution. Antibodies were purchased from BD, eBioscience, or Invitrogen and were as follows: CD3 bio (500A2), CD3 PE (145-2C11), CD3 APC-Cy7 (17A2), CD4 bio (GK1.5), CD4 PE (H129.19), CD4 PE-Cy7 (GK1.5), CD8 bio (53–6.7), CD8 PE (53–6.7), CD8 APC (53–6.7), CD11b bio (M1/70.15), CD11b PE (M1/70.15), CD11c PE (HL3), CD19 bio (1D3), CD19 PE (1D3), CD19 PE-Cy5.5 (1D3), CD25 PE (PC61), CD44 PE-Cy5.5 (IM7), CD45.1 bio (A20), CD45.1 PE Cy7 (A20), CD45.2 PerCP-Cy5.5 (104), CD117 APC (2B8), NK1.1 bio (PK136), NK1.1 PE (PK136), Gr1 bio (RB6-8C5), Gr1 PE (RB6-8C5), Gr1 APC (RB6-8C5), Ter119 bio (Ter119), Ter119 PE (Ter119), and Sca1 PE-Cy5.5 (Ly-6A/E). Streptavidin QDot605 was purchased from Invitrogen, and Streptavidin APC-Cy7 was obtained from BD. For definition of HSC, the lineage cocktail (Lin) was composed of CD3, CD4, CD8, CD11b, CD11c, Gr1, CD19, NK1.1, and Ter119. In thymocytes defined by expression of CD44 and CD25, the Lin cocktail included CD3, CD8, CD11b, CD11c, CD19, NK1.1, Gr1, Ter119, and γδ TCR.

For blood analysis, the blood was collected from the tail vein directly into tubes containing potassium-EDTA (SARSTEDT). 500 µl of PBS/5% FCS was added to dilute the blood and underlaid with FICOLL-Paque PLUS (GE Healthcare). Samples were centrifuged at 250 *g* in a bench centrifuge for 20 min at room temperature, the interface was collected, and cells were washed with PBS/5% FCS. Staining was performed as indicated above.

BrdU analysis. 1 mg BrdU (Sigma-Aldrich) per mouse was injected intraperitoneally into *Rag2*$^{-/-}\gamma_c^{-/-}$*Kit*$^{W/Wv}$ recipients 9 wk after transplantation and into 9-wk-old wild-type control mice. A group of mice was sacrificed and analyzed 2 h later, and the second group was sacrificed and analyzed 5 d later. For BrdU flow cytometry analysis, cells were stained with extracellular antibodies followed by intracellular staining using the BrdU flow kit (BD) according to the manufacturer's recommendations.

Analysis of TCR V gene diversity. Individual TCR rearrangements were sequenced independently of complex multiplex PCR. RNA was isolated from 2 × 10^7 total thymocytes using RNAzol B (IsoTex Diagnostics) according to the manufacturer's recommendations. A DNase I (Invitrogen) digest step was performed, and cDNA was synthesized with the Transcriptor High Fidelity kit (Roche) using oligo dT primers. 1 µg of cDNA, an amount corresponding to 0.5–10^6 thymocytes, was used for the analysis of TCR diversity by an adapted nr/LAM PCR (Schmidt et al. 2007; Paruzynski et al. 2010). The resulting PCR products were purified by Agencourt Ampure beads, and an exponential PCR was performed to add the 454 specific amplification and sequencing adaptors to both ends of the amplicons. To sequence different samples in parallel, adaptors containing 6–10 bp barcode were used. 40 ng of DNA

were amplified using the following PCR program: initial denaturation for 120 s at 95°C, 12 cycles at 95°C for 45 s, 58°C for 45 s, 72°C for 60 s, and final elongation for 300 s at 72°C. Raw amplicon sequences were separated according to the introduced barcode, further trimmed, and aligned to a reference set of TCR genes using BLAT. CDR3 clonotypes were defined as the sequences occurring between the last conserved cysteine of the V region and the conserved phenylalanine in the FGXG motif of the J region.

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Author contributions: V. Martins designed research, performed most experiments, analyzed data, and wrote the manuscript. S. Schlenner and V. Madan contributed to thymus grafting and analyses. E. Ruggiero, M. Schmidt, and C. von Kalle made TCR diversity analyses. P. J. Fink provided congenic mice harboring the RAG reporter and analyzed data, and H. R. Rodewald designed research, analyzed data and wrote the manuscript.

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