Clonable progenitors committed to the T lymphocyte lineage in the mouse bone marrow; use of an extrathymic pathway

Sussan Dejbakhsh-Jones*, Marcos E. García-Ojeda*, Devavani Chatterjea-Matthes, Defu Zeng, and Samuel Strober†

Department of Medicine, Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA 94305

Edited by Irving L. Weissman, Stanford University School of Medicine, Stanford, CA, and approved April 16, 2001 (received for review November 27, 2000)

We searched for clonable committed T cell progenitors in the adult mouse bone marrow and isolated rare ($\approx 0.05\%$) cells with the Thy-1^{hi}CD2⁻CD16⁺CD44^{hi}CD25⁻Lin⁻ phenotype. *In vivo* experiments showed that these cells were progenitors committed only to reconstituting the T cell lineage of irradiated Ly5 congenic hosts. Reconstitution of the thymus was minimal compared with that of the bone marrow, spleen, and lymph nodes. At limiting dilutions, donor T cell reconstitution of the spleen frequently occurred without detectable donor cells in the thymus. Progenitors were capable of rapidly reconstituting athymic hosts. In conclusion, the clonable bone marrow progenitors were capable of T cell reconstitution predominantly by means of an extrathymic pathway.

T cell progenitor | extrathymic maturation | T cell reconstitution | thymus

Committed T cell progenitors (CTP) that are restricted only to the T cell lineage have been reported to develop in the mouse fetal blood, liver, or spleen before they emigrate to the thymus for further maturation (1–5). However, the presence of multipotent or bipotent early progenitors in the adult and fetal thymus capable of generating non-T lineage cells suggested that commitment to the T lineage occurs only within the thymus (6–12). A recent study indicated that the CD90⁺/CD117^{lo}/CD3⁻ progenitor cells in the fetal blood and spleen that have been reported to be CTP (1) expressed the NK1.1 marker, and were actually bipotent progenitors capable of generating both T and natural killer (NK) cells despite the expression of such T lineage-associated genes as GATA-3, TCF-1, TCR- β , and pT α (13). The latter study suggested that commitment to the T cell lineage occurred after fetal progenitors emigrate to the thymus and during subsequent rearrangement of the TCR β chain genes (12, 13).

The object of the current study was to search for CTP in the adult bone marrow that are capable of generating only mature T cells *in vivo* and that can be cloned by *in vivo* limiting dilution as reported for hematopoietic stem cells and common lymphoid progenitors cells (14–16). In particular, we studied a rare population ($\approx 0.05\%$) of T cell progenitors in the marrow (Thy-1.2^{hi}CD2⁻CD16⁺CD44^{hi}Lin⁻) that are able to generate CD4⁺ TCR $\alpha\beta$ ⁺ and CD8⁺ TCR $\alpha\beta$ ⁺ T cells *in vitro* (17). The phenotype of these progenitors is similar to that reported for early T cell progenitors in the fetal thymus (18).

Materials and Methods

Mice. Congenic strains of wild-type C57BL/6 Ly5.2 and C57BL/6 Ly5.1 mice were bred and maintained in the Department of Comparative Medicine animal facility (Stanford University School of Medicine, Stanford, CA). Male and female mice were used at 8 to 12 weeks of age. In addition, C57BL/6-RAG-2^{-/-} mice (B6.SJL; Rag2) and C57BL/6 nu/nu mice expressing the same Ly5.1 allele as the Ly5.1 wild-type mice were purchased from Taconic Farms.

Immunofluorescent Staining and Sorting of Cells. Bone marrow cells were harvested from the femur and tibia as described (19). Staining and sorting of cell suspensions with fluorochrome or

biotin-conjugated mAbs, including FcR blocking and propidium iodide gating, have been described (20). For sorting candidate progenitor cells in the bone marrow, cells were first enriched by incubation with biotin-conjugated anti-Thy-1.2 mAb (5a-8, Caltag, South San Francisco, CA), incubated further with streptavidin-conjugated immunomagnetic beads, and positively selected on MACS-MS magnetic separation columns (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. The enriched cells were stained and sorted thereafter. The following mAbs were conjugated with fluorochromes as described (21): FITC-conjugated anti-Ly5.2 (A20.1.7), biotin-conjugated anti-Ly5.1 (ALI-4A2), and allophycocyanin (APC) conjugated anti-CD44 (IM-781). Conjugated mAbs and reagents were purchased from PharMingen, including FITC and phycoerythrin (PE) anti-CD4 (CT-CD4), PE anti-CD8 (CT-CD8a), PE and FITC anti-TCR $\alpha\beta$ (H57-597), PE anti-CD25 (PC61-5.3), FITC and PE anti-Mac-1 (M1/70.15), and Texas red streptavidin. PE and FITC anti-B220 (RA3-6B2), PE anti-Gr-1 (RB6-8C5), PE anti-NK1.1 (PK136), FITC anti-CD16/32 (2.4G2), PE and FITC anti-CD2 (RM2-5), APC anti-CD3 (2C11), PE anti-V β 3 (KJ25), FITC anti-V β 6 (RR4-7), FITC anti-V β 8 (F23.1), and biotin anti-CD11c (HL3) were purchased from PharMingen. Cells detected by the 2.4G2 mAb are referred to as CD16⁺ in the text. In some experiments, spleen cells were enriched for Thy-1⁺ and CD11c⁺ cells by incubating cells with biotin mAbs directed against these markers, incubated with streptavidin beads, and positively selected on magnetic separation columns (Miltenyi Biotech). In studies of splenic-dendritic cells, spleen cell fragments were treated with collagenase type II (Worthington) and DNase I (Roche Molecular Biochemicals) before making single-cell suspensions according to a protocol designed to optimize the dendritic cell yield as described (22).

Adoptive Transfer of Progenitor Cells and Monitoring. C57BL/6 Ly5.1 host mice were given a single dose of lethal whole-body irradiation (either 800 cGy or 950 cGy) from a 200 kV (20 mA) source (Philips Medical Systems, Milford, CT) at a rate of 72.5 cGy/min. Candidate progenitor cells from Ly5.2 congenic donors mixed with bone marrow cells from RAG-2^{-/-} Ly5.1 donors were injected i.v. into the lateral tail vein within 24 h of irradiation. The lymphoid tissues of recipient mice were harvested at serial time points, and monitored for their content of donor-type (Ly5.2) cells by immunofluorescent staining.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CTP, committed T cell progenitors; TCR, T cell antigen receptor; PE, phycoerythrin; NK, natural killer.

*S.D.-J. and M.E.G.-O. contributed equally to this work.

†To whom reprint requests should be addressed at: Division of Immunology and Rheumatology, Department of Medicine, Clinical Sciences Research Building, Stanford Medical Center, 300 Pasteur Drive, Stanford, CA 94305-5166. E-mail: sstrober@stanford.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. Absolute number of Ly5.2⁺ cells in adoptive hosts

Host*	Number of cells per tissue × 10 ⁶		
	Bone marrow	Spleen	Thymus
Irradiated host			
2 weeks	11 ± 5	2.1 ± 0.4	0.10 ± 0.01
3 weeks	12 ± 5	5 ± 2	2 ± 1
4 weeks	4.7 ± 0.6	8 ± 4	0.9 ± 0.4
8 weeks	10 ± 4	4 ± 1	3 ± 1
WT C57BL/6	1.0 ± 0.3 [†]	17 ± 2 [‡]	119 ± 27 [‡]

Values shown are means ± SE.

*There were three mice for each time point, and three mice in the WT (wild-type) C57BL/6 control group.

[†]Absolute number of T cells.

[‡]Absolute number of thymocytes.

Sorted Thy-1^{hi}CD2⁻Lin⁻ cells (1×10^4) were injected i.v. into whole-body irradiated (800 cGy) C57BL/6 Ly5.1 wild-type hosts along with 1×10^6 unfractionated marrow cells from C57BL/6 Ly5.1 RAG-2^{-/-} donor mice. Fig. 1D shows two-color flow cytometric analyses of the marrow, spleen, peripheral lymph nodes, and thymus from the hosts at 2, 4, or 8 weeks after the cell injections. Boxes enclose the TCRαβ⁺ T cells that are derived from the sorted Thy-1^{hi}CD2⁻ Ly5.2⁺ candidate progenitor cells.

At 2 weeks after the cell injection, about half of the marrow and spleen cells were TCRαβ⁺ T cells derived from the Ly5.2⁺ progenitors. However, only 5.6% of the lymph node cells were TCRαβ⁺ Ly5.2⁺ T cells, and about 1% of thymus cells were TCRαβ⁺ Ly5.2⁺. After 4 and 8 weeks, the percentage of TCRαβ⁺ Ly5.2⁺ cells in the marrow and spleen remained in the range of about 30–50%, and the percentage of TCRαβ⁺ Ly5.2⁺ cells in the lymph nodes increased to 31.5% at 8 weeks. Similarly, the bright TCRαβ⁺ Ly5.2⁺ cells in the thymus increased from 1.1% at 2 weeks to 45.2% at 8 weeks. At 8 weeks, more than 90% of thymocytes were donor-type (Ly5.2⁺) and about 35% were CD4⁺CD8⁺. The pattern in all tissues at 8 weeks remained stable at 12 weeks (data not shown).

Table 1 shows the mean ± SE absolute number of Ly5.2⁺ donor cells in the bone marrow (both femurs), thymus, and spleen at serial time points after the cell injection. The number of lymph nodes obtained from host mice was too variable for accurate analysis. The number of donor cells in the marrow was stable in the range of 5×10^6 to 11×10^6 from 2 to 8 weeks. The mean number of donor cells in the spleen was 2.1×10^6 at 2 weeks, and increased to the range of 4×10^6 to 8×10^6 thereafter. The number of donor cells in the thymus increased about 20-fold from 2 to 3 weeks (0.1 to 2×10^6 cells), and was 0.9 and 3×10^6 thereafter. Comparison of the absolute numbers of donor T cells in the bone marrow of irradiated hosts to that of untreated wild-type mice showed that the reconstituted marrow T cells were 10-fold higher than in untreated mice, but reconstituted spleen cells were about 4-fold lower at 8 weeks (Table 1). Reconstituted thymocytes were about 40-fold lower. Thus, robust reconstitution in the bone marrow was associated with minimal reconstitution of the thymus.

Because it was possible that the outgrowth of Ly5.2 T cells in the hosts given purified Ly5.2 progenitor cells and RAG-2^{-/-} Ly5.1 marrow cells may have been derived from a small number of mature bone marrow T cells contaminating the progenitor cells, a control group was set up in which hosts were given RAG-2^{-/-} Ly5.1 marrow cells and 1×10^4 sorted CD4⁺ and CD8⁺TCRαβ⁺ T cells from the bone marrow of Ly5.2 donors instead of 1×10^4 purified progenitor cells. Fig. 1D shows that the expansion of the mature T cells in the lymphoid tissues of the hosts was minimal after 4 weeks and that the bone marrow, spleen, lymph nodes, and thymus contained 1% or less of

Fig. 2. Sorted Thy-1.2^{hi}CD2⁻Lin⁻ bone marrow cells generate CD4⁺ and CD8⁺ T cells in Ly5 congenic hosts. (A) Two-color analyses of the gated Ly5.2⁺ donor cells in the lymphoid tissues harvested from lethally irradiated C57BL/6 Ly5.1 hosts 2 and 8 weeks after injection of 1×10^4 sorted Thy-1^{hi}CD2⁻Lin⁻ C57BL/6 Ly5.2 marrow cells and 1×10^6 marrow cells from C57BL/6 Ly5.1 RAG-2^{-/-} mice, as shown in Fig. 1D. Staining for CD4 vs. CD8 is shown. (B) Two-color flow cytometric analyses of the spleen from hosts at 4 weeks with T cell (TCRαβ or combined CD4 and CD8), B cell (B220), granulocyte (Gr-1), macrophage/myeloid cell (Mac-1), and NK cell (NK1.1) vs. Ly5.2 markers. Boxes enclose donor-type (Ly5.2) cells that stain for the different cell lineages. (C) Staining of the spleen after enrichment of the cells with anti-CD11c mAb and immunomagnetic beads. (Upper) CD11c vs. Ly 5.2 markers. CD11c⁺Ly5.2⁻ and CD11c⁺Ly5.2⁺ cells are enclosed in boxes. (Lower) CD11c vs. Ly5.2 after depletion of Mac-1⁺ and B220⁺ cells by gating. (D) One-color analysis of gated Ly5.2⁺TCRαβ⁺ donor cells in the host spleen stained for CD44 and CD45RB markers at 8 weeks (open profiles). Shaded profiles show the same analysis of gated TCRαβ⁺ T cells from the spleen of untreated wild-type donors.

TCRαβ⁺Ly5.2 T cells. Thus, contaminating mature T cells from the bone marrow had little capacity to expand in the host lymphoid tissues. This result stands in contrast to previous reports that show dramatic expansions of wild-type or transgenic donor CD4⁺ or CD8⁺ T cells after transfer to T cell-deficient hosts (29–32). However, all of these studies used T cells obtained from the lymph nodes and spleen (29–32) that did not have the unusual phenotypic and functional characteristics of T cells in the marrow (19).

To study the expression of CD4 vs. CD8 markers on Ly5.2⁺ donor cells in the tissues of the hosts coinjected with Ly5.2 purified progenitors and Ly5.1 RAG-2^{-/-} marrow cells, gated

Ly5.2⁺ cells from each tissue were examined at 2 and 8 weeks. Fig. 2A shows that at least 90% of gated cells in the marrow and lymph nodes at 2 weeks were single-positive T cells with CD8⁺ cells predominating over CD4⁺ cells by a ratio of about 4:1 or 5:1. Almost all of the CD8⁺ T cells in the spleen and lymph nodes expressed the CD8 β phenotype (data not shown). About 75% of donor spleen cells were single-positive T cells with a predominance of CD8⁺ cells. At 2 weeks, CD4⁺CD8⁺ double-positive T cells accounted for 12% and 34% of donor cells in the spleen and marrow, respectively. The Ly5.2⁺ cells in the thymus at 2 weeks were almost all CD4⁺CD8⁺ double-positive T cells. At 8 weeks, the intensity of staining of Ly5.2⁺ CD4⁺CD8⁺ cells in the marrow, spleen, lymph node, and thymus was similar and bright, and double-positive T cells disappeared from the spleen.

The splenic single-positive TCR $\alpha\beta$ ⁺ T cells at 8 weeks expressed very high levels of both CD44 and CD45RB in comparison with the levels expressed by most splenic T cells in untreated wild-type mice (Fig. 2D). The phenotype of these progenitor-derived T cells differed also from the CD44^{hi} CD45RB^{lo} activated-memory phenotype of expanded mature spleen and lymph node CD4⁺ T cells transferred to T cell-deficient hosts (30–32). The unique CD44^{hi}CD45RB^{hi} phenotype of T cells derived from progenitors in reconstituted hosts was found amongst both CD4⁺ and CD8⁺ T cells (data not shown). CD4⁺CD8⁺ cells persisted in the lymph nodes, spleen, and marrow (Fig. 2A); the latter cells were either TCR $\alpha\beta$ ⁺ or expressed the Thy-1^{hi}CD44^{hi}CD16^{hi}CD2[–]Lin[–] phenotype of the progenitors (data not shown).

The high percentage of TCR $\alpha\beta$ ⁺ cells amongst Ly5.2⁺ cells in hosts given RAG-2^{–/–} marrow cells as shown in Fig. 1 indicated that the Ly5.2⁺ progenitors were biased toward the T cell lineage. A more detailed analysis of a variety of lineage markers vs. the Ly5.2 marker in the spleen at 4 weeks is shown in Fig. 2B. Whereas TCR $\alpha\beta$ ⁺Ly5.2⁺ and CD4⁺ and CD8⁺ Ly5.2⁺ cells accounted for 66.5% and 65.3%, respectively, of all spleen cells, the percentage of B220⁺, Gr-1⁺, Mac-1⁺, or NK1.1⁺ Ly5.2⁺ cells accounted for less than 0.01% of spleen cells. The non-T cell lineage donor cells accounted for less than 0.01% of cells in the bone marrow, lymph nodes, and thymus also (data not shown). Thus, few if any B cells, granulocytes, macrophages, or NK cells, respectively, developed from the injected Ly5.2⁺ progenitor cells that seem to be CTP. The inability of the latter progenitors to generate myeloid/erythroid cells was also shown *in vitro*, because no outgrowth of methyl-cellulose colonies at day 7 was detected after plating 200 CTP in cultures containing stem cell factor, IL-11, Flt3, IL-3, granulocyte/macrophage colony-stimulating factor, erythropoietin, and thrombopoietin. In contrast, 59 myeloid, 3 erythroid, 2 megakaryocytic, and 11 mixed colonies were detected at the same time point after plating 100 sorted Thy-1^{lo}Sca-1^{hi}c-Kit^{lo} hematopoietic stem cells.

Because NK cells represent only a few percent of cells in the normal spleen or marrow, it was possible that a small percentage of the progeny of the purified progenitors were members of the latter lineages and could be detected only after appropriate enrichment. Accordingly, bone marrow cells from adoptive hosts were enriched for Thy-1⁺ cells by using immunomagnetic beads, because NK cells express this marker (33). The enriched cells were analyzed for NK1.1⁺ cells; these accounted for 3.2% of all harvested cells, but progenitor-derived Ly5.2⁺NK1.1⁺ cells accounted for only 0.01% of enriched cells (data not shown).

To determine whether Ly5.2 dendritic cells were reconstituted by using 1×10^4 purified progenitors, spleen fragments from adoptive hosts were harvested at 4 weeks. Single-cell suspensions were enriched for dendritic cells with anti-CD11c immunomagnetic beads, because the latter cells express this marker (34). Fig. 2C Upper shows the staining pattern of the enriched cells for CD11c vs. Ly5.2. Whereas 78% of CD11c⁺Ly5.2[–] cells were present, 0.003% of enriched cells were CD11c⁺Ly5.2⁺ (Ly5.2

Fig. 3. Limiting dilution analysis of donor T cell reconstitution of lethally irradiated (950 cGy) C57BL/6 Ly5.1 hosts 4 weeks after i.v. injection of graded numbers of sorted C57BL/6 Ly5.2 Thy-1^{hi}CD2[–]Lin[–] (donor) bone marrow cells coincubed with 1×10^6 C57BL/6 Ly5.1 RAG-2^{–/–} whole bone marrow cells. (A and B) Poisson analyses of reconstitution of spleen and thymus, respectively, with groups of 10 hosts for each cell dose. (C and D) Two-color analysis of TCR $\alpha\beta$ vs. Ly5.2 markers of the spleen and thymus, respectively, of a single 4-week host given 100 sorted Ly5.2 marrow progenitor cells i.v. This pattern was observed in three of seven reconstituted mice. (E and F) Similar analyses of a single 4-week host given 250 sorted cells. This pattern was observed in 3 of 10 mice. Boxes in C–F enclose donor-type (Ly5.2) cells.

progenitor-derived) cells. Because CD11c⁺ cells contain B cells, macrophages, and dendritic cells, the immunomagnetic bead-enriched spleen cells were gated to remove B220⁺ and Mac-1⁺ cells (B cells and macrophages), and CD11c vs. Ly5.2 marker were analyzed thereafter (Fig. 2C Lower). The residual dendritic cells accounted for about 5.1% of the gated spleen cells, and were almost all Ly5.2[–] cells. CD11c⁺B220[–]Mac-1[–]Ly5.2⁺ cells were 0.002% of enriched spleen cells. Thus B, myeloid, and dendritic cells derived from the Ly5.2 progenitors were not clearly detected (as judged by flow cytometry). These experiments with Ly5.2 progenitors were repeated to improve the yield of dendritic cells by treating host spleen fragments with collagenase before preparing the single-cell suspensions (22). After immunomagnetic bead enrichment, the percentage of Ly5.2⁺CD11c⁺ cells in the host spleen was still less than 0.1% (data not shown).

Limiting Dilution Assays. Limiting dilution assays were used to determine the frequency of progenitors in the sorted Thy-1⁺CD2[–]Lin[–] bone marrow cells that were capable of reconstituting mature T cells in the lethally irradiated congenic hosts. Instead of injecting 1×10^4 sorted Ly5.2 cells along with 1×10^6 RAG-2^{–/–} Ly5.1 marrow cells, graded numbers of sorted cells (500, 250, 100, and 50) were injected i.v. with the constant number of RAG-2^{–/–} marrow cells into groups of 10 irradiated mice for each sorted-cell dose. After 4 weeks, the spleen and thymus cells from each host were harvested and stained for TCR $\alpha\beta$ vs. Ly5.2 markers. The percentage of hosts without detectable (<0.05%) TCR $\alpha\beta$ ⁺Ly5.2⁺ T cells in the spleen was plotted against the number of sorted cells injected (Fig. 3A). Poisson statistics predict that two-thirds of the hosts in a group

will be reconstituted by an average of one progenitor cell per mouse. Thus, for reconstitution of spleen T cells, there was approximately 1 progenitor cell in 75 sorted cells that were injected. A similar analysis for reconstitution of the thymus was approximately 1 progenitor in 180 sorted cells (Fig. 3B).

Near unit dilution (100 sorted cells injected), three of seven mice with detectable donor T cells showed reconstitution of the spleen without detectable donor Ly5.2⁺ cells in thymus (Fig. 3C and D, respectively). At that dilution, four of seven mice showed donor cell reconstitution of both the spleen and the thymus. When 250 sorted cells were injected, 3 of 10 mice showed reconstitution of the spleen (Fig. 3E) without detectable donor cells in the thymus (Fig. 3F), and 7 of 10 mice showed reconstitution of the spleen and thymus.

These experiments indicated that the sorted progenitors can use an extrathymic pathway for reconstitution of the spleen. Extrathymic reconstitution was tested also by injecting 250 sorted cells into a group of irradiated athymic C57BL/6 Ly5.1 nu/nu hosts instead of euthymic hosts. Reconstitution of TCR $\alpha\beta$ ⁺ T cells was detected in the latter hosts, and the mean $\pm$ SE of the absolute number of progenitor-derived TCR $\alpha\beta$ ⁺ Ly5.2⁺ T cells in the spleen and lymph nodes was 21 ± 8 and $3.8 \pm 0.2 \times 10^5$, respectively, at 8 weeks ($n = 4$). The absolute number of donor T cells in the same tissues of irradiated euthymic hosts given 250 sorted progenitors was 12 ± 4 and $5.2 \pm 0.8 \times 10^5$, respectively, at 8 weeks ($n = 3$). Ratios of CD4⁺:CD8⁺ T cells were similar in the two types of hosts (data not shown).

TCR β Chain Gene Rearrangements and V β Repertoire. Purified Thy-1⁺CD2⁻Lin⁻ marrow cells from untreated wild-type mice were obtained by using anti-CD3 mAb instead of anti-TCR $\alpha\beta$ to gate on Lin⁻ cells (see *Materials and Methods*). The sorted Thy-1⁺CD2⁻Lin⁻ marrow cells failed to show rearrangement of the V β 8 gene segment as determined by a PCR analysis of amplified genomic DNA (Fig. 4A Upper). Primers were chosen to hybridize with the V β 8 gene segment and with an intron located between the J β 2 gene cluster and the C β 2 exon. Whereas amplified, genomic DNA from untreated wild-type spleen cells showed a clear band of amplified DNA of appropriate size, no amplified DNA was detected by using the same amount of genomic DNA from sorted marrow cell progenitors. However, genomic DNA from sorted donor-type (Ly5.2⁺) spleen cells obtained 4 weeks after the injection of 1×10^4 sorted Ly5.2 marrow progenitor cells into irradiated Ly5.1 hosts showed an amplified band of DNA with primers for V β 8 (Fig. 4A Upper), which is indicative of gene rearrangement. The sensitivity of the PCR analysis for cells with rearrangements of the V β 8 gene segment was studied by making mixtures of a cloned BALB/c B cell lymphoma line (BCL₁) without V β 8 gene rearrangements and untreated wild-type C57BL/6 spleen cells. All mixtures contained a constant number (1×10^6) of B cells and graded numbers of spleen cells with 10-fold decrements from 1×10^6 to 1×10^0 , such that the ratio of spleen cells:BCL₁ cells varied from 1:1 to $1:1 \times 10^6$. Fig. 4B shows that an amplified band of V β 8 DNA was still detected at the cell ratio of $1:1 \times 10^4$, but was not detected thereafter. Thus, the PCR analysis can detect about one V β 8 rearranged cell amongst 0.5×10^5 cells, because V β 8⁺ T cells account for about 20% of normal spleen T cells (20). The same analysis was performed to study the rearrangement of the V β 7 gene with primers that hybridized specifically with the V β 7 gene segment. Again, the sorted progenitor cells failed to show an amplified V β 7 band, but the appropriate size band was observed in the host spleen 4 weeks after the progenitor cell injection (data not shown).

Because rearrangements of the D β -J β gene segments can precede rearrangements of the V β gene segment during early T cell maturation, PCR analysis for D β gene segment rearrangement was performed with a primer that hybridized with the D β 1

Fig. 4. V β gene rearrangements, T cell gene expression, and V β surface expression in sorted donor (Ly5.2) bone marrow cells and/or their progeny. (A Upper) PCR amplification of genomic DNA isolated from $\approx 1 \times 10^4$ sorted or unfractionated cells by using primers that hybridize with the V β 8 exon and J β 2-C β 2 intron gene segments. Lane 1, size markers from ϕ X174 digested with *Hinf*I; lane 2, DNA from sorted Thy-1^{hi}CD2⁻Lin⁻ marrow cells; lane 3, DNA from sorted Ly5.2⁺ cells from the spleen of C57BL/6 Ly5.1 lethally irradiated hosts injected with 1×10^4 sorted Ly5.2 marrow cells and 1×10^6 whole marrow cells from C57BL/6 RAG-2^{-/-} Ly5.1 mice 4 weeks earlier; lane 4, DNA from C57BL/6 Ly5.2 wild-type unfractionated spleen cells; lane 5, DNA from control tk⁻ L cell line; lane 6, no DNA added to PCR. Arrows show amplified bands of appropriate size. (A Lower) PCR amplification of the same DNA samples as in A Upper with primers that hybridize with a C α exon gene segment. (B) PCR amplification of the same gene segments in A with genomic DNA from cell mixtures with a constant number (1×10^6) of BALB/c B cell lymphoma (BCL₁) cells and graded numbers of C57BL/6 spleen cells. Lane 1, size markers; lanes 2–8, 10-fold decrements of added spleen cells starting from 1×10^6 and ending at 1×10^0 , respectively; lane 9, BCL₁ cells without added spleen cells. (C) PCR amplification of the D β 1 and J β 2-C β 2 intron gene segments with genomic DNA from sorted or unfractionated cells. Lanes are labeled as in A. (D) Reverse transcription-PCR analysis of mRNA from sorted or unfractionated cells for RAG-1, RAG-2, pT α , and β -actin genes. Lane 1, sorted Thy-1^{hi}CD2⁻Lin⁻ marrow cells; lane 2, sorted CD4⁺ and CD8⁺ TCR $\alpha\beta$ ⁺ T cells from the spleen; lane 3, sorted CD4⁺CD8⁺ TCR $\alpha\beta$ ⁺ thymocytes. (E) Two-color flow cytometric analyses of gated Ly5.2⁺ cells from the spleen of lethally irradiated Ly5.1 hosts given 250 sorted Thy-1^{hi}CD2⁻Lin⁻ Ly5.2 marrow cells and 1×10^6 C57BL/6 RAG-2^{-/-} Ly5.1 marrow cells 4 weeks earlier. Staining of Thy-1.2 vs. V β 3, V β 6, and V β 8 is shown. Boxes enclose V β ⁺Thy-1.2⁺ cells.

segment, and the J β 2-C β 2 intron primer was the same as described above. Fig. 4C shows that an amplified DNA band was not detected with genomic DNA from sorted progenitor cells, but a band of the appropriate size was detected by using either normal spleen cells or spleen cells from irradiated hosts 4 weeks after the progenitor cell injection. Fig. 4A Lower shows positive control PCR amplifications of the C α exon 1 gene segment for all genomic DNA samples used in Fig. 4A and C.

The bipotent T and NK progenitors in the fetal blood and spleen reported elsewhere (13) expressed the RAG-1, RAG-2, and pT α genes that precede V β gene rearrangement. To compare similar gene expression in sorted progenitors in the adult bone marrow to those reported in the fetus, RNA was extracted from sorted progenitors from the bone marrow of wild-type C57BL/6 mice, and reverse transcription-PCR analysis was

performed. Fig. 4D shows that the sorted progenitors expressed the RAG-1, RAG-2, and pT α genes but that sorted CD4⁺ and CD8⁺ TCR $\alpha\beta$ ⁺ spleen cells from normal C57BL/6 mice did not. On the other hand, sorted CD4⁺CD8⁺ thymocytes from the same mice expressed all three genes as expected (Fig. 4D). β -Actin controls were obtained to assure adequacy of the RNA yields.

In further experiments, the spleen cells from 10 hosts injected with 250 sorted cells (receiving an average of about three progenitor cells per mouse) were stained to determine the percentage of donor (Ly5.2⁺) T cells expressing three different V β receptors (V β 3, V β 6, and V β 8). The two-color flow cytometric analyses of each V β vs. Thy-1.2 with gated Ly5.2⁺ cells are shown in Fig. 4E (boxes enclose V β ⁺Thy-1.2⁺ cells). The percentages of the three V β T cell subsets (8.7% to 24.4%) were similar to the percentages amongst T cells in the normal C57BL/6 spleen (20). Thus, limited numbers of progenitor cells can reconstitute the V β subsets without obvious skewing toward a predominant type.

Discussion

Our recent study showed that the sorted Thy-1^{hi}CD2⁺Lin⁺ marrow cells generated CD4⁺ and CD8⁺ single-positive T cells *in vitro* by means of double-positive intermediary cells (17). Injection of 1×10^4 of these sorted marrow cells from Ly5.2 donor mice into lethally irradiated Ly5.1 congenic hosts along with 1×10^6 marrow cells from C57BL/6 RAG-2^{-/-} Ly5.1 donors was able to reconstitute the bone marrow, spleen, lymph nodes, and thymus with Ly5.2⁺ donor-type TCR $\alpha\beta$ ⁺ T cells as early as 2 weeks later. The earliest and largest expansion of donor-type single-positive T cells occurred in the bone marrow, and the latest and least occurred in the thymus.

Whereas the absolute number of donor T cells in the host marrow exceeded the absolute number of T cells in the bone marrow of untreated wild-type mice, the number in the thymus was about 40-fold lower. In contrast, reconstitution of irradiated hosts with 1×10^3 Thy-1^{lo}Sca-1^{hi}c-Kit^{lo}Lin⁺ hematopoietic stem cells (15, 16) resulted in an absolute number of thymocytes and bone marrow T cells that was similar to that of untreated mice (data not shown). This result suggested that progenitors used in the present study may have reconstituted T cells in the marrow and peripheral lymphoid tissues predominantly through an

extrathymic pathway, and that thymus reconstitution was severely restricted. The ability of progenitors to reconstitute by means of an extrathymic pathway was confirmed by using athymic nu/nu hosts. The latter hosts rapidly developed similar numbers of donor T cells in the spleen and lymph nodes as compared with euthymic hosts, given equal limiting numbers (250 per host) of progenitor cells. It is unlikely that mature marrow T cells that contaminate the sorted progenitor cells were the source of donor T cells in the host lymphoid tissues, because injection of 1×10^4 mature marrow TCR $\alpha\beta$ ⁺ T cells failed to reconstitute hosts. Reconstituted CD4⁺ and CD8⁺ T cells had an unusual CD44^{hi}CD45RB^{hi} phenotype.

The Thy-1^{hi}CD2⁺Lin⁺ marrow progenitors were committed to the T cell lineage and failed to reconstitute detectable B (B220⁺) cells, granulocytes (Gr-1⁺), macrophages (Mac-1⁺), NK (NK1.1⁺) cells, or dendritic (CD11c⁺B220⁺Mac-1⁺) cells in the bone marrow or spleen of the hosts. The inability to differentiate into myeloid and erythroid cells was confirmed by *in vitro* culture experiments. The T cell commitment of the progenitors occurred in the marrow before the rearrangement of the TCR β chain genes, because PCR analysis of genomic DNA obtained from the purified progenitors failed to detect rearrangements of the D β 1, V β 7, or V β 8 gene segments. The CTP expressed the RAG-1, RAG-2, and pT α genes as did the bipotent NK and T cell progenitors reported elsewhere (13), but the CTP have a more restricted developmental potential.

In conclusion, the study shows that commitment of progenitors to the T cell lineage occurs in the bone marrow before the rearrangement of the TCR genes. Furthermore, these unusual progenitors are capable of only limited reconstitution at the thymus of the adoptive hosts, and the bone marrow and peripheral lymphoid tissues can be reconstituted by means of an extrathymic pathway.

We thank Drs. T. Miyamoto and I. L. Weissman, Department of Pathology, Stanford University for assays of myeloid/erythroid colonies and for help in the design and analysis of experiments. We are grateful to Dr. J. W. Tung for providing C α primers, A. Mukhopadhyay for technical assistance, and V. Cleaver and M. Hansen for preparation of the manuscript. The studies were supported by National Institutes of Health Grants AI-43013, HL-58250, and HL-57443, and by Grant AI-37683S1 (to M.E.G.-O.).

- Rodewald, H. R., Kretzschmar, K., Takeda, S., Hohl, C. & Dessing, M. (1994) *EMBO J.* **13**, 4229–4240.
- Rodewald, H. R. (1995) *Curr. Opin. Immunol.* **7**, 176–187.
- Kawamoto, H., Ohmura, K. & Katsura, Y. (1997) *Int. Immunol.* **9**, 1011–1019.
- Kawamoto, H., Ohmura, K., Fujimoto, S. & Katsura, Y. (1999) *J. Immunol.* **162**, 2725–2731.
- Kawamoto, H., Ohmura, K. & Katsura, Y. (1998) *J. Immunol.* **161**, 3799–3802.
- Wu, L., Antica, M., Johnson, G. R., Scollay, R. & Shortman, K. (1991) *J. Exp. Med.* **174**, 1617–1627.
- Rodewald, H. R., Moingeon, P., Lucich, J. L., Dosiou, C., Lopez, P. & Reinherz, E. L. (1992) *Cell* **69**, 139–150.
- Carlyle, J. R., Michie, A. M., Furlonger, C., Nakano, T., Lenardo, M. J., Paige, C. J. & Zuniga-Pflucker, J. C. (1997) *J. Exp. Med.* **186**, 173–182.
- Shortman, K. & Wu, L. (1996) *Annu. Rev. Immunol.* **14**, 29–47.
- Matsuzaki, Y., Gyotoku, J., Ogawa, M., Nishikawa, S., Katsura, Y., Gachelin, G. & Nakauchi, H. (1993) *J. Exp. Med.* **178**, 1283–1292.
- Ardavin, C., Wu, L., Li, C. L. & Shortman, K. (1993) *Nature (London)* **362**, 761–763.
- Carlyle, J. R. & Zuniga-Pflucker, J. C. (1998) *Immunol. Rev.* **165**, 63–74.
- Carlyle, J. R. & Zuniga-Pflucker, J. C. (1998) *Immunity* **9**, 187–197.
- Kondo, M., Weissman, I. L. & Akashi, K. (1997) *Cell* **91**, 661–672.
- Spangrude, G. J., Heimfeld, S. & Weissman, I. L. (1988) *Science* **241**, 58–62.
- Uchida, N. & Weissman, I. L. (1992) *J. Exp. Med.* **175**, 175–184.
- Dejbakhsh-Jones, S. & Strober, S. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 14493–14498.
- Rodewald, H. R., Awad, K., Moingeon, P., D'Adamio, L., Rabinowitz, D., Shinkai, Y. & Reinherz, E. L. (1993) *J. Exp. Med.* **177**, 1079–1092.
- Zeng, D., Lewis, D., Dejbakhsh-Jones, S., Lan, F., Ojeda-Garcia, M., Sibley, R. & Strober, S. (1999) *J. Exp. Med.* **189**, 1073–1081.
- Garcia-Ojeda, M. E., Dejbakhsh-Jones, S., Weissman, I. L. & Strober, S. (1998) *J. Exp. Med.* **187**, 1813–1823.
- Akashi, K. & Weissman, I. L. (1996) *Immunity* **5**, 147–161.
- Vremec, D. & Shortman, K. (1997) *J. Immunol.* **159**, 565–573.
- Taubenberger, J. K., Reid, A. H., Izon, D. & Boehme, S. A. (1996) *Cell. Immunol.* **171**, 41–47.
- Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J. & Ouellette, B. F. (1998) *Nucleic Acids Res.* **26**, 1–7.
- Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J. & von Boehmer, H. (1994) *Science* **266**, 1208–1212.
- Schmidt-Wolf, I. G., Liang, O., Dejbakhsh-Jones, S., Wang, H., Cheng, L., Holm, B., Bell, R. & Strober, S. (1993) *J. Immunol.* **151**, 5348–5353.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, E. W. (1990) *J. Mol. Biol.* **215**, 403–410.
- Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, V., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., et al. (1992) *Cell* **68**, 855–867.
- Goldrath, A. W. & Bevan, M. J. (1999) *Immunity* **11**, 183–190.
- Beutner, U. & MacDonald, H. R. (1998) *Int. Immunol.* **10**, 305–310.
- Kieper, W. C. & Jameson, S. C. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 13306–13311.
- Muranski, P., Chmielowski, B. & Ignatowicz, L. (2000) *J. Immunol.* **164**, 3087–3094.
- Herberman, R. B., Nunn, M. E. & Holden, H. T. (1978) *J. Immunol.* **121**, 304–309.
- Metlay, J. P., Witmer-Pack, M. D., Agger, R., Crowley, M. T., Lawless, D. & Steinman, R. M. (1990) *J. Exp. Med.* **171**, 1753–1771.