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Stepwise Development of Committed Progenitors in the Bone Marrow That Generate Functional T Cells in the Absence of the Thymus

Marcos E. García-Ojeda,²* Sussan Dejbakhsh-Jones,²* Devavani Chatterjea-Matthes,* Aditi Mukhopadhyay,* Andrew BitMansour,* Irving L. Weissman,† Janice M. Y. Brown,* and Samuel Strober³*

We identified committed T cell progenitors (CTPs) in the mouse bone marrow that have not rearranged the TCRβ gene; express a variety of genes associated with commitment to the T cell lineage, including GATA-3, T cell-specific factor-1, Cβp, and Id2; and show a surface marker pattern (CD44⁺CD25⁻CD24⁺CD5⁻) that is similar to the earliest T cell progenitors in the thymus. More mature committed intermediate progenitors in the marrow have rearranged the TCR gene loci, express Vα and Vβ genes as well as CD3ε, but do not express surface TCR or CD3 receptors. CTPs, but not progenitors from the thymus, reconstituted the αβ T cells in the lymphoid tissues of athymic nu/nu mice. These reconstituted T cells vigorously secreted IFN-γ after stimulation in vitro, and protected the mice against lethal infection with murine CMV. In conclusion, CTPs in wild-type bone marrow can generate functional T cells via an extrathymic pathway in athymic nu/nu mice. The Journal of Immunology, 2005, 175: 4363–4373.
 Farms), and RAG-1−/− C57BL/6 CD45.2 mice (Taconic Farms) were used at 8–12 wk old. All experiments were performed in compliance with the National Institutes of Health and institutional guidelines, and were approved by the Stanford Administrative Panel on Laboratory Animal Care.

Antibodies

Fluorochrome-conjugated anti-CD2 (RM-25), CD3ε (145-2C11), CD4 (RM-45), CD11b (Mac-1, M70/15), CD16/32 (2.4G2), CD25 (IL-2Rα, 7D4), CD45R (B220, RA3-6B2), CD45.1 (Ly-5.2, A20.1.7), CD45.2 (Ly-5.1, A1-4A2), CD122 (IL-2Rβ, TM-β1), CD127 (IL-7Rα, B12-1), GR-1 (RB6-8C5), NK1.1 (PK136), TCRβ (H5-5^7^), and TCRγδ (GL3) mAbs were obtained from BD Pharmingen. Fluorochrome-conjugated CD4 (CT-CD4), CD8α (CT-CD8), CD25 (PC61-5.3), CD90.2 (Thy-1.2, 5a-8), rat IgG2b, and hamster IgG1 isotype controls were obtained from Caltag Laboratories. The following Abs were conjugated as previously described and directed to (3): CD3ε (KT31.1), CD4 (GR1.5), CD8α (55-6.7), CD11b (Mac-1, M70/12), CD45 (heat stable Ag, M1/69), and CD16 (M81/14), CD25 (B220, RA3-6B2), CD45.1 (Ly-5.2, A20.1.7), CD45.2 (Ly-5.1, A1-4A2), CD90.2 (Thy-1.2, 5a-8), CD117 (c-kit, 2B8), GR-1 (RB6-8C5), NK1.1 (136TC, and Sca-1 (E13-161).

Cell preparation and immunofluorescent staining

Single cell suspensions of bone marrow, spleen, and thymus cells were obtained, as previously described (1, 2). All single cell suspension samples were pretreated with purified anti-CD16/32 at saturation to block FcγRIIA/B receptors. Following CD16/32 block, the samples were incubated with various combinations of fluorochrome- or biotin-conjugated Abs at saturation for 15–20 min on ice. In the case of biotin-conjugated reagents, counterstaining for 5 min with fluorochrome-conjugated streptavidin was performed. After staining, cells were washed and resuspended in fresh staining medium containing PBS without calcium or magnesium (BioWhittaker) with 1% FBS (HyClone) before sorting or analysis. Stained samples were resuspended in staining medium with propidium iodide at 0.5–1 μg/ml. Eight-color FACS analysis and sorting were done using a highly modified dual laser (488-nm argon and 543-nm He/Ne) (Becton Dickinson) with four-decade logarithmic amplifiers (5) and a Moflo cytometer (4) equipped with dual laser (488-nm argon and 599-nm dye lasers) FACS III (BD Biosciences) with four-decade logarithmic amplifiers (5) and a Moflo cytometer (4).

Isolation of CTPs and CIPs

The isolation of CTPs has been previously described (1). In brief, bone marrow of adult mice was enriched by incubation with biotin-conjugated anti-Thy-1.2 mAb (5a-8; Caltag Laboratories), incubated further with streptavidin-conjugated immunomagnetic beads, and positively selected on MACS-LS magnetic separation columns (Miltenyi Biotec), according to the manufacturer’s specifications. For molecular genetics and extended survival studies, BM was depleted of Thy-1.2highLin−/− cells were depleted of Thy-1.2−/−Lin−/− whole lymph node cells from C57BL/6 mice, nu/nu, or CTP-reconstituted nu/nu mice were used for cytokine stimulation in triplicate in a final volume of 0.2 ml/well. These cells were incubated for 48 h in the absence or presence of plate-bound anti-CD3 mAb and rhIL-2 and treated with a combination of 20 ng/ml PMA plus 10 μg/ml combination of anti-CD3 mAb and CD28 mAb (clones 2C11 and 5A12, respectively). Following incubation, the covered wells were washed three times in complete RPMI 1640 medium.

Cytokine production assay

Protection against lethal murine CMV (MCMV) infection

Nuclei were reconstituted with 500 CTP, prepared as described above. As controls, nu/nu mice were reconstituted with 2 × 10^4 RAG-1−/− (CD45.2) bone marrow cells alone or together with 1 × 10^7 whole lymph node cells from C57BL1/6 mice. Stocks of reconstituent-type MCMV strain RM427 (8) were grown and titrated on NIH 3T3 cells (American Type Culture Collection, Rockville, MD). Following culture, the supernatants were harvested and frozen at −80°C. ELISA tests were performed following the manufacturer’s specifications using CytoScreen II, IL-2, IL-4, and IFN-γ mouse ELISA kits (BioSource International).

Isolation of total RNA and RT-PCR technique

Total RNA was extracted from sorted cells using the RNeasy Mini kit (Qiagen). RNA was reverse transcribed using random hexamer primers, followed by PCR amplification. Optimization for PCR conditions for β-actin message, to be used as an internal standard, were established by titration of the number of amplification cycles using primers specific for β-actin, followed by densitometry analysis to measure ethidium bromide fluorescence of agarose gels. PCR products were visualized by ethidium bromide staining, RAG-1, RAG-2, and IL-7Rα primers were used for RT-PCR, as previously described (6). Additional primers used for RT-PCR were designed using oligo4.0 (National Biosciences) with the sequences found in GenBank (7) under the listed accession numbers: CD3ε (M23376), 5′-CACCTCGTCTACTCTGTGTC-3′ and 5′-TTA CAGAACACTCACGGAG-3′; GATA-3 (X55123), 5′-GGGGCCTC TTGCTGTTACC-3′ and 5′-CTCTTGTGTTGCTGTTACC-3′; T cell-specific factor-1 (TCF-1) (X61385), 5′-CTACCTCGCTCCCTCACTGC-3′ and 5′-TGTAGACTGCTCGTCTAAGC-3′; βF (A000664), 5′-TACAAAGGCTCCAGAAGAC-3′; pre-Tα (U16958), 5′-GGCTC CACCCATCACACTGTC-3′ and 5′-CCATTATCACAGGGCAGATAC-3′; pre-Tα2, 5′-TGTTGCTTGTGCTGTTACC-3′ and 5′-GGGA CAGTATGTCAGAGCGATC-3′; TCR β (X03672), 5′-TGTTGCTAAGGACTCTCTG-3′ and 5′-AC CAGACAGCTTCTGTTGCC-3′; CCR-9 (A13236), 5′-TCTGCA CCTGGTCTC-3′ and 5′-CCAGTTGGTTGGTGGTGC-3′; CXCR-4 (U59760), 5′-CTAGGATGAGGACTCTC-3′ and 5′-ACCTCTACAGACGGCTTCTC-3′; Aiolos (A001293), 5′-ATCGAGA GCTGGGCGGCTCACC-3′ and 5′-GTTGGTGGCTGTTACC-3′; TM-1 (A13236), 5′-CTGCTA CCCTTACCAC-3′ and 5′-GTTGGTTGGTGGTGC-3′. PCR was performed in a single set of primers, except for β-F analysis, which used two nested primer pairs (pre-Tα1 and pre-Tα2).

RT-PCR analysis

Total RNA was extracted from 3–6 × 10^5 sorted cells using an RNeasy Mini kit (Qiagen). RNA was reverse transcribed using random hexamer primers, and amplified by PCR using the following forward and reverse primers: 5′-TGTTGCTAAGGACTCTCTG-3′ and 5′-ACACAGACAGCATGCTTCTG-3′; GATA-3 (M38252), 5′-AGGCTACGTCAGAGCGATC-3′ and 5′-GTTGGTGGCTGTTACC-3′; TM-1 (A13236), 5′-CTGCTACCCCTTACCAC-3′ and 5′-GTTGGTGGCTGTTACC-3′; and 5′-CAAC CTTCTCCACAAATGTG-3′ for the Vo8-2 rearrangement; and 5′-GTTGACCTGTTCCGTGTAAGC-3′; E2a (BO18286), 5′-CAGTGACGCAAGTCTGATG-3′ and 5′-TGAATGTTGGTGGTGC-3′; and 5′-TTGATA AACCTGGAGGAGG-3′. PCR was performed following the manufacturer’s specifications using CytoScreen II, IL-2, IL-4, and IFN-γ mouse ELISA kits (BioSource International).

Protection against lethal murine CMV (MCMV) infection

Briefly, male BALB/c host mice were given a single dose of lethal whole body irradiation (950 cGy) from a 200 kV (20 mA) source (Phillips Medical Systems) at a rate of 72.5 cGy/min. Within 24 h after irradiation, the host mice were transplanted i.v. with 1.5 × 10^7 T cell-depleted bone marrow and graded numbers of whole lymph node cells from C57BL6/6 mice, as previously described (10). Treatment with neomycin and penicillin was started concomitantly with the transplantation and terminated between 3 and 4 wk after transplantation. The mice were weighed weekly and monitored daily for survival and signs of GVHD, including pyrexia (bunchy back), alopecia (hair loss), skin lesions, diarrhea, and fluid wasting. Male body weight and survival were determined 100 days after transplantation. Chimerism, as evidenced by the presence of H-2Kb+ cells in the spleen, was confirmed in all hosts by flow cytometry.

Infection of graft-vs-host disease (GVHD)

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Results
Extended surface marker phenotype of CTPs and CIPs

Multicolor analysis of surface markers on CTPs and CIPs was performed to look for patterns reported to be present on other early progenitor cells, such as common lymphoid progenitors (CLPs), and hemopoietic stem cells (HSCs) in the marrow, and thymic and early fetal blood progenitors (11–15). One-color analyses of key markers were compared after bone marrow cells enriched with anti-Thy-1.2 immunomagnetic beads were gated for CD3^+ B220^+ Mac-1^-NK1.1^- (Lin^-) cells, as shown in Fig. 1A. These cells were gated further for Thy-1.2^high cells, and the gated cells were analyzed for expression of CD2. Two discrete populations of Thy-1.2^highCD2^- and Thy-1.2^highCD2^+ cells (CTPs and CIPs, respectively) were observed, as shown in Fig. 1B. As previously shown (1), the gated CTPs expressed higher levels of CD16 than the CIPs (Fig. 1D). In contrast, the CIPs expressed increased levels of CD44, CD5, and CD122 (IL-2Rβ) as compared with the CTPs (Fig. 1, C, G, and K, respectively). Fig. 1E shows that the large majority of both CTPs and CIPs express little or no CD25 (IL-2Rα). However, there is a small subset of CTPs that is CD25^+. The CTPs and CIPs expressed intermediate levels of CD24 (HSA) (Fig. 1F). Taken together, the data indicate that the CTPs have a CD44^-CD25^-CD16^medium/lowCD24^-CD5^+ phenotype similar to the most undifferentiated T cell progenitor described previously in the adult thymus, and in the fetal thymus and blood (11, 13, 14). In contrast to the CTPs, the earliest progenitors in the thymus do not express high levels of Thy-1 (16). With the exception of CD25, the CIPs have the CD44^highCD16^medium/CD24^-CD5^+ phenotype, characteristic of T cell progenitors further along the T cell developmental pathway (11, 13, 14).

We evaluated the expression of Sca-1, c-Kit, and IL-7Rα on the CTPs and CIPs. The CTPs are predominantly Sca-1^- (Fig. 1H), c-Kit^- (Fig. 1I), and IL-7Rα^- (Fig. 1J). However, a small subset of CTPs is Sca-1^low and IL-7Rα^low. In contrast, the CIPs are Sca-1^low (Fig. 1H), c-Kit^- (Fig. 1I), and IL-7Rα^low (Fig. 1J). Background staining with isotype-matched irrelevant mAbs showed a peak channel of fluorescence that approximated channel 1 (data not shown), and overlapped with negative staining for CD25, Sca-1, CD5, CD127, and CD122 on CTPs.

In additional experiments, the expression of CD3, CD4, and CD8 on CTPs and CIPs was compared with that of mature splenic T cells. Fig. 1, L, M, and N, shows one-color analyses for the intensity of staining of each marker on the gated cell populations. For mature T cells, spleen cells were stained for CD3, CD4, or CD8 vs light scatter, and the intensity of staining for each positive population was determined. Whereas the mature T cells showed bright staining for CD3, CD4, and CD8, the intensity staining of the latter markers on CIPs and CTPs was dull and similar to background (Fig. 1, L, M, and N).

Expression of T cell lineage genes

We analyzed CTPs and CIPs for expression of T cell lineage genes by RT-PCR analysis using bone marrow cells enriched with anti-Thy-1 beads and then sorted for Thy-1.2^highCD3^- B220^- Mac-1^-NK1.1^- (Thy-1.2^highLim^-) cells that were either CD2^- or CD2^+. Sorted CD4^-CD8^- double-negative (DN) thymocytes, used as a control population, showed intense RT-PCR product bands for Rag-1, Rag-2, pre-Tα, and PU.1 genes (Fig. 1O). The control cells confirmed previous studies that showed Rag-1, Rag-2, pre-Tα, and PU.1 gene expression in the DN population (17–20). In contrast, the sorted CIPs and control sorted splenic TCRαβ T cells showed either undetectable or faint bands for these genes. The sorted CIPs had intense bands for both the TCR Vβ8 and CD3ε genes, as did the sorted splenic control T cells and DN thymocytes. These genes are expected to be expressed in the latter control cells because the pre-TCR complex that contains both a rearranged TCRβ chain and CD3ε is present in the CD44^-CD25^-DN thymus subpopulation (11). However, only the CIPs and sorted T cells had intense bands for Vα8 indicative of TCRα chain gene rearrangement and expression. The CTPs expressed little or no RT-PCR product for either the rearranged TCRβ, TCRα, or CD3ε genes. This is consistent with the absence of a rearranged TCR Vβ8 gene segment. Furthermore, both CTP and CIP cells expressed very low levels of RNA for Notch-1 reported to be involved in T lineage commitment (21), whereas we could readily find PCR products for this receptor in sorted T cells and DN thymocyte populations.

Both CTPs and CIPs expressed RNA for the IL-7Rα gene and for the T cell-specific transcription factor genes GATA-3 and TCF-1 (22). Moreover, the CTPs expressed RNA for TCR β, indicative of sterile transcripts made during the earliest stages of T cell development in extrathymic microenvironments (23). All the populations tested had RNA for Aiolos and E2a genes, expressed only in lymphoid cells, as well as low, but detectable levels of Id-2 RNA, which is restricted to NK and T cell development (24–27).

Finally, both populations were tested for expression of RNA of the chemokine receptors CCR9 and CXCR4. CCR9 is expressed on both thymocytes and mature T cells (28), and is the receptor for the thymus-expressed chemokine (29). CXCR4 is the receptor for stromal cell-derived factor-1, a chemokine produced in multiple tissues, including the thymus and bone marrow (30). Both the CTP and CIP populations showed intense bands for these chemokine receptors (Fig. 1O).

Reconstitution of T cells in nu/nu hosts using wild-type CTPs

Our previous studies showed that CTPs generated mature T cells in the lymphoid tissues of irradiated euthymic adoptive hosts (2, 3). In the current study, CTPs were examined for their capacity to generate T cells in athymic hosts. CTPs obtained from the marrow of wild-type CD45.1 C57BL/6 mice were sorted (Fig. 2), and 250 were injected i.v. into groups of five CD45.1 C57BL/6 athymic nu/nu hosts that were co-injected with 1 × 10^5 CD45.2 Rag-1^-/- marrow cells. The host lymph node, spleen, and bone marrow cells were harvested and analyzed 1, 2, 4, and 8 wk later. Two-color analysis of cells stained for TCRαβ vs CD45.1 was performed at each time point. At 1 wk, CD45.1^- cells accounted for <1% of cells in all three tissues (data not shown). However, at 2 wk, TCRαβ^- T cells derived from the CTPs accounted for 31.2% of lymph node cells (Fig. 2). The percentage increased to 49.5% at 8 wk. In control animals injected with Rag-1^-/- marrow cells alone, only 0.2% of lymph node cells were TCRαβ^- T cells at 8 wk. Background control staining was up to 0.1%. Control nu/nu mice had ~0.1–0.8% of TCRαβ^- T cells in the lymphoid tissues before irradiation and cell transfer (bottom panels, Fig. 2).

Spleen cells of the nu/nu hosts injected with CTPs contained 11.5% of TCRαβ^- CD45.1^- cells at 2 wk, and the percentage rose to 39.8% at 8 wk. In the absence of CTPs, the host spleen cells contained 0.01% of the CD45.1^- T cells. A similar pattern was observed in the bone marrow, although the percentage of T cells was much lower than in the lymph nodes and spleen, as expected. It was possible that CD45.1^- T cell outgrowth from injected CTPs were generated from mature TCRαβ^- T cells that contaminated the purified CTPs. Accordingly, control irradiated athymic hosts were injected with Rag-1^-/- marrow and 250 sorted bone marrow CD45.1 TCRαβ^- T cells (BMTC) instead of CD45.1 CTPs from the Thy-1.2-enriched donor bone marrow. Hosts that received
FIGURE 1. Comprehensive analysis of CTPs and CIPs for expression of surface markers and genes found in pro-T cells, CLP, HSC, and DN progenitors in the thymus. Bone marrow cells were analyzed for lineage markers (A), and gated Lin− cells were gated for Thy-1.2+ cells and then analyzed for Thy-1.2 vs CD2 in B. Cells in boxes in B were gated for CTPs and CIPs. CTPs (open histograms) and CIPs (shaded histograms) were studied for expression of CD44 (C), CD16 (D), CD25 (E), CD24 (F), Sca-1 (H), c-Kit (I), CD127 (J), and CD122 (K) using a fluorochrome not used for gating. Gated CIPs and CTPs were also studied for expression of CD3, CD4, and CD8. Gated CD3+ CD4− and CD8− cells from the normal spleen are shown for comparison in L. CD3, M, CD4, and N, CD8. Thick lines show spleen cells, thin lines show CIPs, and dashed lines show CTPs. Data shown display one of three representative experiments for surface staining. Total RNA from sorted CTPs and CIPs was analyzed for expression of genes associated with T cell differentiation by RT-PCR in O. cDNA was prepared and then amplified by PCR using gene-specific primer pairs, as indicated. Sorted splenic TCRαβ cells, and CD4+ CD8− DN thymocytes from C57BL/6 mice were used as controls.
the sorted T cells had <1% TCRαβ+ T cells in the lymph nodes, spleen, and bone marrow at 8 wk (Fig. 2). In additional experiments, we tested the ability of 250 sorted CD4−CD8−CD44hiCD25− cells from the CD45.1 wild-type thymus (stage I DN thymocytes) to reconstitute the irradiated nu/nu hosts. Hosts that received the CD44hiCD25− thymic progenitors had <1% TCRαβ+ T cells in the lymphoid tissues at 4 wk (Fig. 2).

The kinetics of the increase in the absolute number of CTP-derived T cells in the spleen, lymph nodes, and bone marrow are shown in Table I. At 2 wk after injection of 250 CTPs, a mean of 1.3 × 10^6 CD45.1+ T cells was harvested from the spleen, 0.54 × 10^6 from cervical and axillary lymph nodes, and 0.21 × 10^6 from the marrow cells of both femurs and tibias. The absolute numbers in the spleen and bone marrow increased ~2-fold from 2 to 8 wk, and those in lymph nodes declined slightly (Table I). The total number of CD45.1+ T cells in all three tissues (~3 × 10^6) indicates an expansion of ~10,000-fold as compared with that of the injected CTPs (250 cells). As compared with untreated wild-type
injection of 250 sorted TCR
CTP-derived T cells at 8 wk were
irradiated congenic were used instead of wild-type CTPs to reconstitute the T cells of
(data not shown). When 250 sorted CD4
CTPs in the
expected, because wild-type stem cells fail to develop into functional
row of wild-type C57BL/6 donors into irradiated congenic
nu/nu hosts resulted in minimal T cell reconstitution as compared with that with 250 wild-type CTPs (Table I). The latter result was ex-
bected, because wild-type stem cells fail to develop into functional
in the
bone marrow after adoptive transfer (3). The injection of 250 sorted TCRβ+ T cells (BMTC) from the wild-
type bone marrow resulted in <0.001 × 10^6 T cells in the lymphoid tissues of the
nu/nu hosts at 8 wk (Table I). When the dose of sorted T cells was increased to 1 × 10^6 cells, then ~0.1 × 10^6 donor T cells were found in the spleen at 8 wk as compared with the 2.1 × 10^6 splenic T cells generated by 40-fold fewer CTPs (data not shown). When 250 sorted CD4^CD8^-CD44^{high}CD25^- DN stage I (DNSI) thymocytes were used instead of CTPs, then the number of T cells that developed from the thymic progenitors was <0.001 × 10^6 in each tissue (Table I). Injection of 1 × 10^4 sorted CD4^CD8^thymus cells (DN thymus cells) resulted in a similar lack of T cell outgrowth (Table I).

To determine whether CD45.1^+ cells derived from the injected CTP were restricted to the T cell lineage, host spleen cells harvested at 4 wk were stained for a variety of markers that identify B cells (B220), macrophages (Gr-1), and NK cells (NK1.1), in addition to T cells (TCRβ) and T cell-associated markers (CD4 and CD8). Fig. 3A shows that ~17% of host spleen cells contained CD45.1^+ TCRβ^+ cells, and <1% of the spleen cells were CD45.1^+ cells expressing the non-T cell lineage markers (B220, Gr-1, Mac-1, NK1.1). A small population of CD45.1 TCRβ^{low/-} and CD45.1 CD4^-CD8^- cells was observed in the spleen (Fig. 3B, top panels). To further characterize these cells, CD45.1-gated cells were analyzed for Thy-1.2 vs CD2 and TCRβ^+ vs CD2 markers. Fig. 3B shows that 99.6% of the gated CD45.1 cells expressed both Thy-1.2 and CD2 markers, and ~12% of these fail to express high levels of TCRβ or any of the other markers. Thus, the latter cells derived from the injected CTP
express the Thy-1^+CD2^+Lin^- phenotype present on intermediate
T cell progenitors that are rapidly generated by CTPs in vitro (1), and are present also in the wild-type bone marrow (Fig. 1B).

The expression of CD4 vs CD8 markers on gated CD45.1 TCRβ^+ cells in the host spleen at 4 and 8 wk is also shown in Fig. 3B. Approximately 65–68% of the gated cells were single-
positive CD4^+ cells, and ~26–28% were single-positive CD8^+ cells. The latter cells were almost all CD8αβ^+ cells, as determined by staining for CD8α vs CD8β (data not shown). A small popu-
lation (2.8%) of gated cells was CD4^-CD8^- double-positive cells at 4 wk, and a discrete population of the latter cells was not observed at 8 wk. Some CD4^-CD8^- T cells were observed at both time points (Fig. 3B). Thus, almost all cells derived from the injected CTPs were members of the T cell lineage, and among the latter cells almost all were single-positive CD4^+ or CD8^+ T cells.

To provide an estimate of the precursor frequency of functional progenitors, we injected 100, 150, or 250 CTPs into groups of 10 irradiated
nu/nu hosts, and observed the fraction without T cell reconstitution. After injection, 0 of 10, 6 of 10, and 10 of 10 hosts, respectively, showed reconstitution of the lymph nodes 4 wk later. This suggests that the precursor frequency is in the range of 1 precursor in ~100–250 purified CTPs. Wild-type CD45.1 CTPs generated a normal repertoire of Vβ receptors near limit dilution (250 CTPs injected), as judged by the percentage of Vβ3^+, Vβ6^+, and Vβ8^- (2.8, 7.0, and 19.4%, respectively) among lymph node T (Thy-1.2^+) cells in reconstituted hosts at 4 wk (Fig. 4A). These percentages were similar to those reported among TCRβ^+ T cells in the normal C57BL/6 spleen (31).

### Rearrangements of Vβ genes and Vβ repertoire in reconstituted nu/nu hosts

We performed RT-PCR analysis on mRNA from the sorted CTPs from wild-type C57BL/6 bone marrow to look for expression of Vβ8 and Vα8 genes, and failed to detect an appropriate size cDNA
fragment (Fig. 4B). However, the sorted CD45.1^+ TCRβ^+ T cells derived from CTPs injected into the congenic nu/nu hosts showed expression of both genes (Fig. 4B). Sorted lymph node T cells from wild-type mice showed expression of both genes also, but an equivalent number of whole nu/nu lymph node cells failed to provide a detectable signal (Fig. 4B). Samples from all cell sources showed a dense band for β-actin after amplification (Fig. 4B).

The results using adoptive nu/nu C57BL/6 congenic hosts indi-
cated that the transferred CTPs generate mature CD4^+ and CD8^-cells derived from the injected CTPs.

### Table I. Absolute number of donor-derived T cells in lymphoid tissues of adoptive hosts (mean ± SE × 10^6)

<table>
<thead>
<tr>
<th>Time after Cell Injectiona (weeks)</th>
<th>Host Tissues Analyzedb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bone Marrowc</td>
</tr>
<tr>
<td>WT-CTP 2</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>WT-CTP 4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>WT-CTP 8</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td>WT-BMTC 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>nu/nu-CTP 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WT-HSC 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WT-DN thymus cells 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WT-DNSI 4</td>
<td>&lt;0.001</td>
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*a A total of 250 CTPs, BMTC, CD44^{high}CD25^- thymocytes (DNSI) or hematopoietic stem cells (HSC), or 1 × 10^4 CD4^-CD8^- thymocytes (DNTC) from wild-type (WT) or nu/nu C57BL/6 donors injected along with 1 × 10^6 bone marrow cells from RAG-1^-/- C57BL/6 mice sharing CD45 marker of host.

*b Lethally irradiated C57BL/6 nude host tissues stained for donor-type CD45 markers (n = 4).

*c Bone marrow cells harvested from two femurs and tibias from each host.

dCells harvested from cervical and axillary lymph nodes.
T cells with a full Vβ receptor repertoire. To study negative selection, we injected 500 CTPs from the bone marrow of Thy-1.1 BALB/c mice donors along with 10^6 Thy-1.2 BALB/c RAG-2−/− bone marrow cells into lethally irradiated Thy-1.2 nu/nu BALB/c hosts. We compared the Vβ receptor repertoires of sorted CTP-derived T cells (Thy-1.1) from the spleens of BALB/c nu/nu hosts 4 wk after the adoptive transfer with that of wild-type BALB/c and C57BL/6 T cells.

The percentages of Vβ receptor-expressing cells in the spleen among gated Thy-1.2− bone marrow cells into lethally irradiated Thy-1.2 nu/nu BALB/c hosts. We compared the Vβ repertoires of sorted CTP-derived T cells (Thy-1.1−) from the spleens of BALB/c nu/nu hosts 4 wk after the adoptive transfer with that of wild-type BALB/c and C57BL/6 T cells.

The percentages of Vβ receptor-expressing cells in the spleen among gated Thy-1.2− cells were determined, and the ratios of each Vβ to that of the predominant nondeleted Vβ8− cells were compared. As shown in Table II, statistically significant reductions of these Vβm:Vβ8 ratios (p < 0.05; Student’s t test of independent means) were observed for Vβ3, Vβ5, Vβ11, and Vβ12 receptors that are expected to be deleted in BALB/c as compared with wild-type C57BL/6 mice (Table II). The Vβ receptor deletion pattern observed in the CTP-reconstituted nu/nu BALB/c-adoptive hosts was similar to that observed in the wild-type BALB/c mice (Table II). Thus, negative selection of the CTP-derived T cells occurred extrathymically in vivo.

**Function of T cells in reconstituted nu/nu hosts**

To determine whether the T cells in the lymph nodes of the CTP-reconstituted nu/nu mice were functional, in vitro assays of cytokine secretion were performed in which 10^7 lymph node cells from the latter mice were harvested 8 wk after reconstitution with 250 CTPs and compared with an equal number of lymph node cells obtained from untreated C57BL/6 wild-type mice and untreated C57BL/6 nu/nu mice. Cells were stimulated with a combination of anti-CD3 and anti-CD28 mAbs bound to the surface of 96-well plates, and control cultures contained an irrelevant (anti-TNP) mAb or no mAb. Supernatants were collected at 48 h and cytokines were measured by ELISA.

**FIGURE 3.** Sorted wild-type Thy-1.2highCD2−Lin− bone marrow cells reconstitute CD4+ and CD8+ T cells in nu/nu hosts. A, Staining of irradiated nu/nu host spleen cells 4 wk after injection of 10^6 RAG-2−/− CD45.2 marrow cells and 250 wild-type CD45.1 CTPs for T cell (TCRβ or combined CD4 and CD8), B cell (B220), granulocyte (Gr-1), macrophage (Mac-1), and NK cell (NK1.1) vs CD45.1 markers. B, Gated CD45.1 cells from the spleen at 4 wk were stained for TCRβ vs CD2 and CD4 vs CD8 in two top panels. The gated CD45.1 cells were stained for Thy-1.2 vs CD2 in the lower left panel. Staining for CD4 vs CD8 on CD45.1-gated spleen cells at 8 wk is shown in lower right panel.
and sorted TCR-challenged with 1 week after the injection of the cells, the three groups of mice were, and amplification of lane 5). PCR with no cDNA template added is shown in lane 4, lane 2 (Fig. 5, D and E). Whereas the injection of 0.5 and 2 × 10^5 wild-type lymph node cells caused diarrhea, weight loss, and the death of 20 and 100% of the allogeneic hosts, respectively, during a 100-day observation period, all mice in the groups given either no lymph node cells, or 2 × 10^5 CTP-reconstituted nu/nu mice, or untreated wild-type mice (Fig. 5, D and E). Thus, the CTP-reconstituted lymph node cells had little capacity to induce lethal GVHD even when the absolute number of lymph node CD4^+ T cells injected was at least as high as that of untreated wild-type CD4^+ T cells (2 × 10^5 nu/nu reconstituted vs 0.5 × 10^5 wild-type lymph node cells injected, respectively).

Discussion

Our data show that the CTPs in the normal marrow have the CD16^+CD44^lowCD25^−CD25^+CD5^− phenotype and the unarranged TCR Vβ gene pattern of immature T cell precursors that are similar to stage I DN progenitors in the thymus (11, 14). CTPs rapidly generate CIPs with rearranged Vβ genes in vitro (1). In the thymus, rearrangement of the TCRβ locus is mirrored by the down-regulation of CD16 with the concomitant up-regulation of CD2 (14) and CD5 (13). The current phenotypic analysis shows that decreased expression of CD16 and increased expression of CD2 and CD5 are associated with rearrangement of Vβ genes in the CIPs. In the thymus, up-regulation of CD25 and down-regulation of CD44 expression indicate progression through the T cell developmental pathway (11). Unlike DN thymocytes, the CIPs do all survived without weight loss until day 56 when they were sacrificed for harvesting of tissues (Fig. 5C). At that time, ~50% of cells in the lymph nodes were TCRαβ^+ T cells derived from CTPs (CD45.1). The group given marrow and wild-type lymph node cells remained healthy also until sacrifice at day 56.

Histopathological analysis of tissue sections of the liver stained with H&E was obtained either at the time of death in the group given marrow cells alone or at the time of sacrifice for the two other groups. Multiple patchy areas of liver parenchymal necrosis associated with inflammatory cell infiltrates were observed in the group given marrow cells alone, and no evidence of necrosis or inflammation was observed in the group given marrow and wild-type lymph node cells (data not shown). Although some areas of necrosis and inflammation were observed in the livers from mice given marrow cells and CTPs, the extent was reduced as compared with that of mice given marrow cells alone.

Protection against MCMV is predominantly mediated by CD8^+ T cells (32). We also tested the functional capacity of lymph node cells from CTP-reconstituted C57BL/6 nu/nu mice to induce lethal GVHD in BALB/c mice, because tissue injury in this disease is mediated predominantly by CD4^+ T cells (10). Groups of lethally irradiated (950 cGy) BALB/c mice were given injections of T cell-depleted wild-type C57BL/6 bone marrow cells alone or with 0.5 × 10^5 or 2 × 10^5 lymph node cells from CTP-reconstituted nu/nu mice, untreated nu/nu mice, or untreated wild-type mice (Fig. 5, D and E). The irradiated CD45.2 nu/nu hosts were reconstituted with 2 × 10^6 bone marrow cells alone or RAG-1^−/− lymph node cells from CD45.1 wild-type mice. Control mice received either RAG-1^−/− bone marrow cells alone or RAG-1^−/− bone marrow cells and 1 × 10^7 lymph node cells from CD45.1 wild-type mice. Three weeks after the injection of the cells, the three groups of mice were challenged with 1 × 10^6 PFU of MCMV, and mice were monitored for weight loss, clinical signs, and survival thereafter. As shown in Fig. 5C, mice reconstituted with RAG-1^−/− bone marrow cells alone all died by 24 days after the MCMV challenge. These mice developed diffuse abdominal swelling (ascites) ~1 wk before they died. The mice reconstituted with marrow cells and wild-type CTPs remained healthy after the MCMV injection, and the presence of IL-4 and IL-10, and all culture supernatants, including those with wild-type lymph node cells, contained <100 pg/ml cytokine after stimulation with anti-CD3/CD28 mAb (data not shown).

In additional experiments, CTP-reconstituted nu/nu mice were challenged with MCMV to determine whether the extrathymically derived T cells afforded protection against a lethal viral infection. The irradiated CD45.2 nu/nu hosts were reconstituted with 2 × 10^6 bone marrow cells from CD45.2 RAG-1^−/− mice and 500 CTPs from CD45.1 wild-type mice. Control mice received either RAG-1^−/− bone marrow cells alone or RAG-1^−/− bone marrow cells and 1 × 10^7 lymph node cells from CD45.1 wild-type mice. Three weeks after the injection of the cells, the three groups of mice were challenged with 1 × 10^6 PFU of MCMV, and mice were monitored for weight loss, clinical signs, and survival thereafter. As shown in Fig. 5C, mice reconstituted with RAG-1^−/− bone marrow cells alone all died by 24 days after the MCMV challenge. These mice developed diffuse abdominal swelling (ascites) ~1 wk before they died. The mice reconstituted with marrow cells and wild-type CTPs remained healthy after the MCMV injection, and

![FIGURE 4.](image-url)

**FIGURE 4.** Vβ receptor expression of donor T cell reconstitution of lethally irradiated nu/nu hosts with wild-type CTPs. A, Representative staining of lymph node cells for Thy-1.2 vs Vβ3, Vβ6, or Vβ8 receptors is shown for a host given 250 CTPs. B, RT-PCR analysis of mRNA for Vβ8 and Vα8 gene expression using specific primer pairs that hybridized to reverse-transcribed cDNA obtained from control untreated nu/nu lymph node cells (lane 1), sorted wild-type donor Thy-1.2^highLin^-CD2^- CTPs (lane 2), sorted donor-type CD45.1^+ TCRαβ^+ T cells from lymph nodes of irradiated nu/nu hosts reconstituted with 250 wild-type CTPs (lane 3), and sorted TCRαβ^+ T cells from the lymph nodes of unirradiated wild-type C57BL/6 mice (lane 4). PCR with no cDNA template added is shown in lane 5, and amplification of β-actin T cell cDNA is shown for all samples to ensure adequacy of RNA yields and amplifications.
not show either up-regulation of CD25 nor down-regulation of CD44. Thus, they are not similar to stages II, III, or IV of thymic T cell development, as judged by surface receptor expression. Both CTPs and CIPs expressed transcripts for GATA-3 and TCF-1, transcription factors exclusively expressed on cells of the T cell lineage (22, 33).

FIGURE 5. Extrathymically derived T cells secrete IFN-γ, protect against MCMV infection, but do not induce GVHD. A and B, Cytokine analysis after stimulation with plate-bound anti-CD3/CD28 mAb. A total of 1 × 10⁶ unfractionated lymph node cells from C57BL/6 wild-type, C57BL/6 nu/nu, and CTP-transplanted C57BL/6 nu/nu mice (4 wk posttransplant) was cultured for 48 h in medium (left bars) with or without stimulation by plate-bound anti-TNP mAb (middle bars) and plate-bound anti-CD3/CD28 mAb (right bars). ELISA was performed to determine the levels of IL-2 (A) or IFN-γ (B). Mean and SD from six independent experiments are shown. Asterisks show no cytokine was detected. C, Lethally irradiated (950 cGy) CD45.2 C57BL/6 nu/nu mice were injected with 2 × 10⁶ CD45.2 RAG-1⁻/⁻ bone marrow cells, alone or together with 500 CD45.1 CTP, or 10⁵ CD45.1 C57BL/6 wild-type lymph node cells. Three weeks posttransplant, the mice were challenged with 10⁶ PFU of MCMV (clone RM427). Host mice were monitored for signs of MCMV infection and survival over a period of 56 days; RAG-1⁻/⁻ bone marrow alone (—) (n = 5), RAG-1⁻/⁻ bone marrow and 500 CD45.1 CTPs (— —) (n = 10), and RAG-1⁻/⁻ bone marrow and 10⁵ wild-type CD45.1 lymph node cells (——) (n = 5). One experiment of two with similar outcomes is shown. D and E, Unfractionated lymph node cells from C57BL/6 nu/nu, or CTP-reconstituted C57BL/6 nu/nu mice (4 wk posttransplant), or from C57BL/6 wild-type mice, together with a constant number (1.5 × 10⁶) of T cell-depleted bone marrow cells from C57BL/6 wild-type mice were injected i.v. into lethally irradiated (950 cGy) BALB/c hosts. Control hosts received marrow without lymph node cells. D, Host survival over a 100-day period is shown for groups of 10 mice; no lymph node cells (——), 2 × 10⁵ nu/nu lymph node cells (— —), 2 × 10⁵ CTP-reconstituted nu/nu lymph node cells (— — —), 0.5 × 10⁵ wild-type lymph node cells (——), and 2 × 10⁵ wild-type lymph node cells (—— —). Each point represents the mean and SD.

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Although we could not detect RNA for CD3ε or TCR Vβ8, in the CTPs, the CIPs expressed RNA for both. However, the CTP gene loci was transcriptionally active, as evidenced by the expression of sterile TCR Cβ RNA, but showed no V-J rearrangements. Both CTPs and CIPs expressed RNA for IL-7Ra, Aiolos, an Ikaros-related transcription factor only expressed on lymphoid cells (24, 25), and for Id2 (26), a transcription factor involved in fate decision between T vs NK cells. The lack of Notch1 expression in CTPs and CIPs and presence in thymocytes may reflect differences in the kinetics of expression during thymic vs extrathymic T cell development and commitment. Together, the data indicate that CTPs are early committed progenitors, as judged by both surface markers and the expression of a variety of T cell-specific genes.

After adoptive transfer, wild-type CTPs reconstituted mature TCRαβ T cells in the lymph nodes, spleen, and bone marrow of adoptive $nu/nu$ hosts at ~2 wk after cell transfer, as judged by staining for the TCRαβ and the congeneric CD45 markers. We could not determine the kinetics of expansion of progenitors vs mature T cells at 1 wk, because there were too few CTP-derived cells to analyze. HSCs from the bone marrow of wild-type donors and CTPs from $nu/nu$ donors were unable to reconstitute the lymphoid tissues. This was expected, because HSCs generate only functionally defective CTPs in the $nu/nu$ bone marrow (3). Reconstitution with wild-type CTPs indicates that the $nu/nu$ lymphoid tissues can support the extrathymic maturation of T cells after the CTP stage of development has been achieved. Control infusions of mature TCRαβ T cells from the wild-type bone marrow showed that expansion of TCRαβ T cells that contaminate the sorted CTPs cannot account for the reconstitution of mature T cells in the irradiated $nu/nu$ lymphoid tissues. Lack of homeostatic expansion of bone marrow TCRαβ T cells was expected, because these T cells produce markedly reduced levels of IL-2 as compared with peripheral T cells (10). In contrast to the CTPs, CD4 CD8 CD44/CD25 stage I progenitors in the thymus failed to reconstitute $nu/nu$ hosts despite their similarities in surface phenotype and T cell lineage gene expression.

We found that the injection of 250 CTPs containing one to two precursors generated a normal distribution of Vβ receptors among reconstituted splenic T cells when compared with the distribution among splenic T cells in untreated wild-type BALB/c mice. In addition, the marked reduction in the percentage of T cells expressing Vβ3, Vβ5, Vβ11, and Vβ12 as compared with that of C57BL/6 wild-type mice showed that the reconstituted T cells had been appropriately negatively selected. Our previous in vitro studies suggested that the bone marrow stroma can perform the role of positive and negative selection during extrathymic T cell maturation, in the same way that the thymic stroma performs this function during intrathymic T cell maturation (31).

Functional tests of lymph node T cells from CTP-reconstituted $nu/nu$ hosts showed that the cells vigorously secreted IFN-γ and but little IL-2 after in vitro stimulation with anti-CD3 and anti-CD28 mAbs. The reconstituted hosts were afforded protection against lethal MCMV infection. Protection against MCMV infection has been reported to be mediated predominantly by CD8+ T cells (32). The results suggest that the CTP extrahymically derived CD8+ T cells are immunocompetent. These results are consistent with a recent report that CLP (12) can generate CD8+ T cells in irradiated adoptive hosts that contribute to the protection against lethal MCMV infection (34). Interestingly, the protective CD8+ T cells appear to be generated extrahymically, because the percentage of CLP-derived CD8+ T cells in the spleen and protection against MCMV infection were similar in the thymectomized as compared with euthymic allogeneic hosts (34). CLP-protected hosts had reduced viral loads. The meager secretion of IL-2 and inability of the CTP-derived lymph node cells to mediate GVHD in irradiated BALB/c hosts in the current study indicated that the CTP-derived CD4+ T cells were defective as compared with wild-type CD4+ T cells.

In conclusion, we show that CTPs and CIPs are committed T cell lineage progenitors that express T cell-specific genes and gene rearrangements previously reported in the thymus. The data provide evidence that commitment to the T cell lineage can occur in the bone marrow as well as in the thymus, and that these marrow progenitors can generate mature T cells via an extrathymic pathway in athymic $nu/nu$ mice.

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Disclosures

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References


