Similar Rates of Production of T and B Lymphocytes in the Bone Marrow

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Summary

The rate of renewal of T lymphocytes in the bone marrow of euthymic C57BL/Ka and athymic nu/nu BALB/c mice was estimated by in vivo labeling with bromodeoxyuridine. T lymphocytes accounted for 16–18% of marrow cells in euthymic mice as judged by immunofluorescent staining with monoclonal antibodies for Thy-1, CD3, and α/β T cell antigen receptor markers. About 70% of marrow cells expressed receptors (Mac-1, Gr-1, B220) for myeloid, macrophage, and B lineage cells. Approximately 13% of cells in the athymic bone marrow expressed α/β T cell receptors. Sorted marrow T cells proliferated in response to stimulation with anti-α/β antibodies in vitro and showed functional rearrangements of Vβ and Jβ genes. Sorted non-T cells did not respond to stimulation in vitro, and all Vβ and Jβ gene rearrangements identified were nonfunctional. In vivo labeling studies indicated that \(17 \times 10^6\) bone marrow T cells are renewed daily in euthymic mice and \(14 \times 10^6\) are renewed in athymic mice. Approximately \(11 \times 10^6\) mature B cells (immunoglobulin M \(^+\)) are renewed daily in the bone marrow of the latter mice. To determine whether marrow precursors can give rise to T cells directly, marrow cells from euthymic and athymic mice were depleted of T cells by cell sorting and incubated in vitro for 48 h in the absence of exogenous growth factors or thymic stromal cells. Examination of the cells after culture showed that 10–12% stained brightly for α/β T cell receptors. Although functional rearrangements of Vα and Jα genes were not detected before culture, the majority of rearrangements were functional after culture. The emergence of the bright α/β T cells in culture was dependent on depletion T cells from the marrow cells before culture. The results suggest that most marrow T cells are generated in the marrow itself.

T cell precursors present in the bone marrow and fetal liver of normal mice migrate to the thymus and differentiate into α/β and γ/δ T cells (1–5). Extrathymic maturation of both α/β and γ/δ T cells in nonlymphoid and lymphoid tissues has been reported also (6–9). There is a gradual development of T cells in the peripheral lymphoid tissues and a more rapid development of bone marrow T cells in congenitally athymic nude mice (10–12). The pathways of extrathymic maturation of T cells are still unclear. However, several laboratories have provided evidence that T cell precursors in the bone marrow and spleen can differentiate into T cells in vitro in the absence of the thymus. Differentiation required 1–3 wk of culture in media enriched with growth factors such as IL-3 and IL-2 (13–17). The precursor populations used for in vitro studies were obtained from T cell–depleted bone marrow of athymic or euthymic mice (13, 14, 17), neonatal spleen (16), and from multilineage hematopoietic colonies from the spleen (15).

The majority of T lymphocytes in the euthymic and athymic mouse bone marrow express the α/β TCR without the CD4 or CD8 receptors (11, 12, 18–20). These T cells appear to be extrathymically derived, since their numbers are not reduced in athymic mice. The object of the current study was to determine the source and daily rate of production of marrow T cells in euthymic and athymic mice. Previous estimates of the production of mature B cells in the euthymic bone marrow range from 5 to \(16 \times 10^6\) cells/d using a variety of methods, including incorporation of [3H]thymidine (21), cell replenishment after depletion with hydroxyurea (22), and labeling of cells with bromodeoxyuridine (23). Using the latter technique, the production of marrow T cells expressing the α/β receptors was estimated to be 17 and \(14 \times 10^6\) cells/d in euthymic and athymic mice, respectively, in the present study. The production of mature IgM \(^+\) B cells in the athymic mice was estimated to be \(11 \times 10^6\)/d. The source of the bone marrow α/β T cells appears to be marrow precursors, which...
developed functional rearrangements of the T cell antigen receptor β chain genes and surface α/β receptors in vitro within 48 h.

Materials and Methods

Animals. Normal C57BL/Ka, BALB/c, or nu/nu Balb/c male mice, 6–12 wk old, were obtained from the Department of Laboratory Animal Medicine, Stanford University.

Immunofluorescent Staining and Flow Cytometry Analysis of T Cell Receptors. Single cell suspensions of spleen and bone marrow cells were stained with fluorochrome-conjugated mAbs and analyzed on a FACS®-Flow Cytometry Systems, Mountain View, CA) using FACSCAN® software developed in the L. Herzenberg Laboratory (Stanford, University), as described in detail previously (12, 19). PE-conjugated anti-CD4 (L3T4) and anti-CD8 (Ly-2) antibodies were obtained from Caltag Laboratories (South San Francisco, CA). Fluorescein-conjugated anti-α/β (H57-597) and anti-CD3 (145-2C11) hamster mAbs, and anti-Thy-1.2 (53-2.1), anti-Gr-1 (RB6-8C5), and anti-B220 (RA3-6B2) rat mAbs, were obtained from Pharmingen (San Diego, CA). PE-conjugated rat monoclonal anti–MAC-1 (M1/70.15) antibodies were obtained from Caltag Laboratories. PE-conjugated rat anti-IgM antibodies (331) were obtained from A. Kantor and L. Herzenberg (Department of Genetics, Stanford University). Biotinylated anti-NK1.1 (PK136) mouse mAbs were obtained from Pharmingen, and biotinylated anti-Vα2 (B20.6) rat mAbs were a gift from Professor N. Minato (Kyoto University, Kyoto, Japan). Control PE-conjugated rat IgG2b and fluorescein-conjugated hamster IgG antibodies were obtained from Caltag Laboratories. Control biotinylated rat IgG2b and mouse IgG2a antibodies as well as fluorescein-conjugated streptavidin were obtained from Pharmingen. All experimental staining reagents were used at saturation, and control reagents were used at concentrations identical to the latter with similar fluorochrome conjugation ratios. All stainings were performed in the presence of saturating concentrations of rat monoclonal anti-Fc receptor antibodies to reduce nonspecific binding (24). Cell sorting was performed with a FACSTAR® as described in detail previously (12, 19). A 10-channel gap was placed between sorted positive and negative cells. Light scatter gating excluded only erythroid cells and dead cells.

Immunofluorescent Staining for Bromodeoxyuridine. Bromodeoxyuridine was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in distilled water for each experiment. Spleen cells were harvested from mice given the label, and immunofluorescent staining for α/β receptors was performed as above. Thereafter, cells were fixed in methanol and stained with fluorescein-conjugated antithromodeoxyuridine mouse mAbs (Becton Dickinson Immunocytometry Systems) according to the procedure of Forster et al. (23). Background control staining was performed with PE-conjugated hamster IgG antibody and fluorescein-conjugated mouse IgG antibody. Cells were analyzed using a FACSTAR®. Gateing for α/β* cells used thresholds set such that <2% of cells stained with control reagent were above the threshold.

Density Gradient Separation of Marrow Cells. Single cell suspensions of bone marrow were prepared as described previously (12). Marrow cells were separated on a discontinuous Percoll (Pharmacia LKB, Uppsala, Sweden) gradient using 10% steps between 40 and 70% Percoll. A low to middle density fraction corresponding to a density of 1.060–1.068 g/ml with ~one-third of the original marrow cells was harvested from this fraction (12, 24).

Amplification of DNA by PCR. Lysates were isolated from sorted or cultured bone marrow cells by adding 1.0 ml distilled water to 1 × 105 cells. DNA was amplified by the PCR with 5′ primers, which hybridized to Vα7 gene segments, and 3′ primers, which hybridized to the intron between Jα2.6 and Ca2 genes. Limiting dilutions of lysates were made such that at least one-third of 30–60 replicate reactions was negative, and the remainder gave a single band on 1.5% agarose gels. Amplification was carried out in two stages. In the first, synthetic oligonucleotide 5′ and 3′ primers were TACCTGTACAAAAGATGAGGAAGAT and TCCCTAGCTT-GCGAGAGAGC, respectively. The reaction buffer consisted of 50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM MgCl2, 100 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 2–5 U of Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT). Reaction volumes were 100 μl under 100 μl of light mineral oil. 30–35 cycles of denaturation (1 min at 95°C), primer annealing (2 min at 55°C), and extension (3 min at 72°C) were followed by 7 min additional extension. Temperature cycling was carried out in an automated heating/cooling block (DNA Thermo Cycler; Perkin-Elmer Cetus Instruments).

In the second stage, a second primer pair that hybridized to the base sequences within the first amplified DNA fragment was used, and the amplification conditions were as described above except that the annealing temperature was maintained at 50°C. The second primer pair was GAGGATCTTCCCTGATTGGATTCT (Vα7) and ACAACGTTGGCAC (intren). In some experiments, the Vα15 genes were amplified instead of the Vα7 genes. The first and second stage primers for Vα15 were AGAACCATCTGTGAAG-TGGAACCT and TATGCTTCTTATCATGAGCATTTC; and the intron primers were the same as before.

Nucleotide Sequencing. After amplifications by PCR, a portion of the product was purified from a 1.5% agarose gel by cutting the appropriate DNA band from the gel and binding the DNA to glass powder (GeneClean; Bio 101, Inc., Vista, CA). For direct sequencing, the purified products were denatured by incubation at 100°C for 3 min and immediately snap frozen on dry ice to minimize renaturation. Sequencing by dideoxy chain termination (25) was performed with the specific primers of the PCR using Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH). α-32P-dATP was incorporated into the sequencing reactions. Since genomic DNA was used as a template for amplification by PCR, the method described enabled us to determine the putative Jα elements by the size of the amplified product. Confirmation of the appropriate Vα7, Vα15, and Jα genes was obtained by sequence analysis and comparison with the previously reported sequences.

Culture of Bone Marrow Cells. In some experiments, 4–8 × 105 sorted bone marrow cells were cultured in 25-cm2 plastic flasks (T-25; Corning Glass, Inc., Corning, NY) at 1 × 104 cells/ml in tissue culture medium, RPMI 1640, with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT). Cultures were maintained at 37°C for 48 h in a humidified atmosphere with 5% CO2. At the end of the culture period, cells were harvested with a Pasteur pipette and washed three times in tissue culture medium before immunofluorescent staining or extraction of genomic DNA.

Stimulation of Bone Marrow with Anti-α/β TCR Antibodies. Unfractionated, sorted Thy-1.2+ or Thy-1.2− bone marrow cells were incubated at 37°C in 5% CO2 in 200 μl of culture medium in 96-well, flat-bottom plastic plates coated with either hamster monoclonal anti-α/β antibodies (5 μg/ml) or hamster serum IgG (5 μg/ml) as described elsewhere (20). [3H]thymidine was added on day 5, and cells from triplicate wells were harvested 18 h later and counted in a Beta Plate Counter (Wallac, Gaithersburg, MD).
**Results**

*T Lymphocytes in the Euthymic Bone Marrow.* Fig. 1 shows the results of immunofluorescent staining used to carefully quantitate the T cells in the bone marrow and spleen of normal 8–12-wk-old male C57BL/Ka mice. Cells were stained with a mixture of PE-conjugated anti-CD4 and anti-CD8 antibodies and counterstained with fluorescein-conjugated antibodies to the α/β TCRs or CD3 or Thy-1.2 receptors after saturation concentrations were determined. About 54% of spleen cells stained brightly for α/β receptors, which are coexpressed with either CD4 or CD8 receptors (A). B and C show that 21–23% of marrow cells stain above background (E) with anti-α/β receptor and anti-Thy-1.2 antibodies. The large majority of the Thy-1.2+ and α/β+ marrow cells did not stain above background levels with anti-CD4 and anti-CD8 antibodies (compare B, C, and E). The mean (± SE) percentage of Thy-1.2+ and α/β+ cells in the marrow of six normal C57BL/Ka mice was 18 ± 3 and 17 ± 2%, respectively.

To determine whether antibodies directed to the α/β receptors stain specifically only cells that express Thy-1.2 receptors, the cells in C were sorted into Thy-1.2+ and Thy-1.2- cells. The latter cells were stained again with monoclonal anti-α/β as well as anti-CD4 and anti-CD8 antibodies (D). Fewer than 1% of cells stained positively for α/β receptors (D). In F and G, C57BL/Ka bone marrow cells were stained with monoclonal anti-α/β and anti-CD3 antibodies, respectively, and counterstained with anti-CD4 and anti-CD8 antibodies. Again, a similar percentage of cells was stained above background (I) for CD3 and α/β receptors (21%). Restaining of a purified population of CD3- cells obtained by sorting cells from G showed <1% of cells stained positively for α/β.
Table 1. \[^{[H]}\text{Thymidine Incorporation of Bone Marrow Cells after Stimulation In Vitro with Monoclonal Anti-α/β Antibodies or Hamster IgG}\]

| Bone marrow cells                          | [H]Thymidine incorporation |
|--------------------------------------------|---------------------------|
|                                            | Anti-α/β antibodies       | Hamster IgG               |
|                                            | Mean cpm ± SE             |                           |
| Unfractionated (5 \times 10^6 cells)       | 101,753 ± 4,197           | 9,183 ± 2,373             |
| Unfractionated (1 \times 10^6 cells)       | 13,437 ± 2,775            | 9,880 ± 2,557             |
| Sorted Thy-1.2+ (1 \times 10^5 cells)      | 202,435 ± 28,509          | 6,462 ± 359               |
| Sorted Thy-1.2- (1 \times 10^5 cells)      | 384 ± 10                  | 378 ± 18                  |

receptors (H). The mean percentage of CD3+ cells in the marrow of six normal C57BL/Ka mice was 16 ± 2%. These results indicate that the Thy-1.2, CD3, and α/β receptors are coexpressed in marrow cells.

J–M show that the α/β+ cells in the marrow are distinct from myeloid cells, macrophages, pre-B, and B lymphocytes. C57BL/Ka marrow cells were stained with a combination of PE-conjugated monoclonal anti-Gr-1, anti–Mac-1, and anti-B220 antibodies (J). The combination of PE-conjugated antibodies stained 72% of cells included in the upper box. The lower box included cells within the background thresholds (J). A sorted population of the latter was stained with anti-α/β antibodies (open areas) and hamster serum IgG (shaded area) (L). About 75% of the sorted cells stained with the anti-α/β antibodies above the background control. Before sorting, only 16% of cells stained above background (K). M compares the staining intensities of these sorted cells and spleen cells. The majority of the α/β+ cells in the marrow are dull compared with those in the spleen, and <2% of α/β+ cells in the lymph node or blood have these characteristics (data not shown).

Stimulation of Marrow T Cells with Anti-α/β TCR Antibodies. To determine whether staining and sorting of Thy-1.2+ and Thy-1.2− cells in the bone marrow separate functional T cells from residual cells, these sorted cells were stimulated in vitro with anti-α/β TCR antibodies coated onto plastic plates. [H]Thymidine incorporation of the two populations was measured thereafter. Table 1 shows that the mean response of 1 \times 10^6 Thy-1.2+ cells (202,435 cpm) was markedly increased as compared with either 1 \times 10^6 unfractionated marrow cells (13,437 cpm) or 1 \times 10^6 Thy-1.2− marrow cells (384 cpm). Table 1 also shows [H]Thymidine incorporation of controls in which hamster IgG was substituted for anti-α/β antibodies. In four of four replicate ex-

![Figure 2](image-url)
periments, sorted Thy-1.2− cell responses were <1,000 cpm for both experimental and control stimulations.

T and B Lymphocytes in the Athymic Bone Marrow: Fig. 2 compares the staining patterns of T and B lymphocytes in the bone marrow and spleen from 8–12-wk-old male euthymic BALB/c, and athymic, nu/nu BALB/c, mice. A and F show that ~34 and 5% of euthymic and athymic spleen cells, respectively, stained positively for the α/β TCR as well as CD4 and/or CD8 receptors. Approximately 35 and 75% of euthymic and athymic spleen cells, respectively, stained positively for surface IgM (mature B cells) (B and G). Staining of the euthymic and athymic bone marrow was analyzed using cells in the “lymphocyte gate” (C and H) (containing 40 and 32% of marrow cells, respectively) to provide data on cells with light scatter characteristics of small lymphocytes. D shows that a discrete population of 5% of euthymic cells stained brightly for surface IgM (mature B cells), and 13% of cells stained above background for α/β receptors. IgM+ and α/β+ cells in athymic marrow were 8 and 12%, respectively, of cells in the “lymphocyte gate” (J). Background staining with irrelevant control fluorochrome-conjugated antibodies was <1% (E and I). Similar percentages of IgM+ and α/β+ cells were observed in the athymic marrow using the lymphocyte gate or a gate to include all live nucleated cells. The mean percentages of IgM+ and α/β+ cells using the latter gate were 7 ± 1 and 13 ± 2%, respectively.
Renewal Rate of T and B Lymphocytes in the Bone Marrow. To determine the rate of renewal of $T$ cells in the bone marrow of normal C57BL/Ka mice, bromodeoxyuridine was added to the drinking water of groups of experimental mice for 12, 24, 48, and 72 h. Control mice were given drinking water without bromodeoxyuridine. Experimental and control mice were killed at each time interval, and the bone marrow cells from both groups were stained before fixation with monoclonal anti-$\alpha/\beta$ antibodies, and then counterstained after fixation with monoclonal anti-bromodeoxyuridine antibodies to detect incorporation of the latter label into DNA.

Fig. 3 A shows an example of the profiles of gated $\alpha/\beta^+$ fixed cells (open) from a control mouse given no label as compared with background staining with hamster IgG (shaded). The $\alpha/\beta^+$-gated cells from control and experimental mice were analyzed for the staining of bromodeoxyuridine shown in B, C, and D at 24, 48, and 72 h, respectively. Shaded and open profiles show the staining of control and experimental mice, respectively. The percentage of cells staining above background controls was determined by subtracting the shaded area from the open profile obtained using the experimental mice. A progressive shift of the fluorescein staining intensity with time was observed from ~31% at 24 h to 83% at 72 h. The mean percentages of $\alpha/\beta^+$ cells that stained above control levels for bromodeoxyuridine incorporation were 21, 32, 54, and 85% at 12, 24, 48, and 72 h, respectively, for groups of four mice. SEs were <10% of the mean values.

Fig. 3 E shows an example of the profile of gated IgM$^+$ fixed cells as compared with background staining in marrow from control BALB/c nu/nu mice given no label. Experimental mice were given bromodeoxyuridine in the drinking water for 24, 48, and 72 h. F, G, and H show the shift in staining for the label (33, 52, and 84%, respectively) in the gated $\alpha/\beta^+$ cells as compared with control mice not given the label. I shows an example of the profile of gated IgM$^+$ cells as compared with background staining. J, K, and L show the shift in incorporated bromodeoxyuridine above control levels at 24, 48, and 72 h. Approximately 48, 76, and 88% of cells incorporated the label at these time points, respectively. In a repeat series of experiments, 32, 56, and 82% of gated $\alpha/\beta^+$ cells incorporated the label at 24, 48, and 72 h, and 50, 72, and 86% of gated IgM$^+$ cells incorporated the label at the same time points.

To determine a minimum estimate of the daily production of $\alpha/\beta^+$ and IgM$^+$ cells in the euthymic C57BL/Ka and BALB/c nu/nu marrow, the total number of cells in the marrow of a single mouse is multiplied by the percentage of $\alpha/\beta^+$ or IgM$^+$ marrow cells, and multiplied again by the percentage of the $\alpha/\beta^+$ or IgM$^+$ marrow cells incorporating bromodeoxyuridine in 24 h. The total number of marrow cells has been estimated at $3.2 \times 10^8$ per mouse (26). The mean percentages of IgM$^+$ and $\alpha/\beta^+$ cells in the bone marrow of four nu/nu mice gated to include all live nucleated cells were 7 ± 1 and 13 ± 2%, respectively. Therefore, the mean numbers of $\alpha/\beta^+$ and IgM$^+$ cells in the nu/nu mice are $\sim 4.2 \times 10^7$ ($3.2 \times 10^8 \times 13\%$) and $2.2 \times 10^7$ ($3.2 \times 10^8 \times 7\%$), respectively. Since $\sim 33\%$ of the $\alpha/\beta^+$ and 48% of the IgM$^+$ cells incorporated bromodeoxyuridine in 24 h (Fig. 3), the estimated daily production of $\alpha/\beta^+$ and IgM$^+$ cells in nu/nu BALB/c mice is $\sim 14 \times 10^6$ and $11 \times 10^6$, respectively. The mean number of $\alpha/\beta^+$ cells in the marrow of C57BL/Ka mice is $\sim 5.4 \times 10^7$ ($3 \times 10^8 \times 17\%$). The estimated daily production of $\alpha/\beta^+$ cells is $\sim 17 \times 10^6$ ($5.4 \times 10^7 \times 32\%$).

In Vitro Generation of T Cells by Bone Marrow Precursors. The rapid renewal of bone marrow T cells in athymic as well as in euthymic mice suggested that the bone marrow is the source of these T cells. Previous studies have indicated that precursor cells in the bone marrow and spleen can give rise to cells during in vitro culture in the absence of thymic stroma (13-17). To determine whether C57BL/Ka marrow precursors can generate $\alpha/\beta^+$ T cells in vitro, marrow cells were fractionated on a discontinuous Percoll density gradient, and a low density fraction was obtained to enrich for hematopoietic progenitors (24). This fraction was stained with PE-conjugated anti-CD4 and anti-CD8 antibodies and counterstained with anti-$\alpha/\beta$ antibodies. The stained cells were sorted into CD4$^+$CD8$^-$ $\alpha/\beta^+$ and CD4$^+$CD8$^+$ $\alpha/\beta^+$ cells. Reanalysis of the sorted CD4$^+$CD8$^-$ $\alpha/\beta^+$ cells is shown in Fig. 4 A. Analysis of unsorted marrow cells is shown in B.

Sorted CD4$^+$CD8$^-$ $\alpha/\beta^+$ were cultured in vitro for 48 h in medium containing fetal bovine serum without exogenous growth factors. Fig. 4 E shows that ~12% of cells harvested at the end of the culture period stained brightly for $\alpha/\beta$ receptors. Less than 1 in $1 \times 10^4$ cells stained with a similar intensity before culture (A). In four replicate experiments, the percentage of bright $\alpha/\beta^+$ cells varied from 12 to 18%, and the yield of nucleated cells harvested from the cultures was always >75%. When unsorted bone marrow cells were cultured under the same conditions, a discrete bright population of $\alpha/\beta^+$ cells could not be detected after 48 h in four replicate experiments (compare B and F).

Additional cultures of CD4$^+$CD8$^-$ $\alpha/\beta^+$ cells obtained from the bone marrow of euthymic and athymic BALB/c mice were also established. C and D show the analyses of the cells from euthymic and athymic mice, respectively, before culture. G and H show that ~22 and 11% of bright $\alpha/\beta^+$ cells developed after a 48-h incubation period of the euthymic and athymic cells, respectively. The appearance of the bright $\alpha/\beta^+$ cells again required depletion of the dull $\alpha/\beta^+$ cells before cultures (data not shown). Background stainings of C57BL/Ka CD4$^+$CD8$^-$ $\alpha/\beta^+$, C57BL/Ka unsorted marrow cells, and sorted euthymic and athymic BALB/c CD4$^+$CD8$^-$ $\alpha/\beta^+$ cells after culture are shown in I, J, K, and L, respectively.

CD4$^+$CD8$^-$ $\alpha/\beta^+$ T cells that express the NK1.1 receptors have been identified in the thymus and bone marrow of normal C57BL mice (18, 27). The NK1.1$^+$ CD4$^+$CD8$^-$ $\alpha/\beta^+$ thymocytes are skewed toward the expression of V$_{a7}$ and V$_{a8.2}$ receptors (28). CD4$^+$CD8$^-$ $\alpha/\beta^+$ bone marrow T cells are skewed toward the expression of V$_{b2}$ receptors (17). Immunofluorescent staining of NK1.1, V$_{a7}$, and $\alpha/\beta$ receptors after culture of sorted $\alpha/\beta^+$ bone marrow cells from C57BL/Ka mice was studied in the present series of
Two-color flow cytometric analysis of cultured bone marrow cells. Low density C57BL/Ka bone marrow cells were harvested from a discontinuous Percoll density gradient and stained for CD4, CD8, and \( \alpha/\beta \) receptors. Cells were sorted into two populations: CD4\(^{-}\)CD8\(^{-}\)\(\alpha/\beta\)\(^{-}\) and CD4\(^{-}\)CD8\(^{-}\)\(\alpha/\beta\)\(^{+}\). Analysis of the negative population is shown in A, and that of the unsorted low density marrow cells is shown in B. After culture of the negative population for 48 h, cells were harvested, stained again, and analyzed (E). Box 1 encloses a discrete population of bright cells staining for \( \alpha/\beta \) receptors, and the percentage of cells within the box is shown. Similar analysis of unsorted cells cultured for 48 h is shown in F. The percentage of cells in Box 1 is shown for comparison. I and J show background staining of 48-h cultures of sorted and unsorted cells with fluorescence-conjugated hamster IgG and rat IgG antibodies. C and D show sorted CD4\(^{-}\)CD8\(^{-}\)\(\alpha/\beta\)\(^{-}\) low density marrow cells from BALB/c and nu/nu BALB/c mice, respectively, before culture. G and H show the sorted BALB/c and nu/nu BALB/c cells after culture, respectively. Boxes 2 and 3 enclose populations of brightly staining cells. K and L show the background staining of cultured BALB/c and nu/nu BALB/c cells, respectively.

Experiments to determine whether newly formed \( \alpha/\beta^{+} \) cells coexpress the NK1.1 or V\(\beta\)\(^{2}\) receptors. Fig. 5, A and B, shows immunofluorescent staining with fluorescent-conjugated anti-\(\alpha/\beta\) antibodies versus forward light scatter before and after culture, respectively. Approximately 17% of cells stained brightly after culture, and <1% stained above background before culture. C and D show the staining patterns of the cultured cells with biotinylated anti-V\(\beta\)\(^{2}\) and anti-NK1.1 antibodies followed by fluorescent-conjugated streptavidin, respectively. Less than 1% of cells stained above the background, and boxes are shown for comparison to B. Positive control staining for the anti-V\(\beta\)\(^{2}\) and anti-NK1.1 antibodies was performed on C57BL/Ka splenic T cells enriched on nylon wool columns (open profile, E) and on C57BL/Ka thymocytes gated to exclude CD4\(^{+}\) and CD8\(^{+}\) cells by flow cytometry (open profile, F). Approximately 5 and 12% of cells stained above background (shaded profiles) in E and F, respectively.

Rearrangement of TCR \( \beta \) Chain Genes before and after Culture. Sorted CD4\(^{-}\)CD8\(^{-}\)\(\alpha/\beta\)\(^{-}\) and \(\alpha/\beta^{+}\) cells were obtained from the low density fraction of C57BL/Ka marrow cells. Rearrangements of the \( \beta \) chain genes in the two populations were studied to determine the proportions that were functional and nonfunctional. Genomic DNA was obtained from the sorted cells, and the junctional regions involving rearrangements of the V\(\beta\)7 and V\(\beta\)15 genes with the J\(\beta\)2 gene cluster were amplified using the PCR as described previously for T cell clones with V\(\beta\)7 and V\(\beta\)15 genes joined to J\(\beta\)2 segments (29). The nucleotide sequences of the amplified DNA fragments were determined.

Fig. 6 shows that none of the 18 junctions involving V\(\beta\)7 and J\(\beta\)2 gene rearrangements were functional from the

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sorted CD4−CD8−α/β− marrow cells, but that 16 out of 17 junctions were functional in the α/β+ marrow cells. The proportion of functional rearrangements involving the Vβ15 and Jβ2 genes found in the CD4−CD8+α/β− and α/β+ cells were 0 out of 22 and 14 out of 16, respectively (sequence data not shown). The results show that functional rearrangements of Vβ genes in the marrow are confined to T cells that express surface α/β receptors.

Genomic DNA was obtained also from cultures of CD4−CD8−α/β− cells after the 48-h culture period, and the proportion of functional and nonfunctional rearrangements of the Vβ genes was determined. Fig. 6 shows that 22 out of 27 junctions involving rearrangements of the Vβ7 and Jβ2 genes were functional. In the case of Vβ15 genes, 14 out of 18 were functional. Thus, there was a marked increase in cells expressing surface α/β receptors as well as in cells with functional rearrangements of the β chain genes after the brief culture period.

Discussion
To measure the renewal rate of T cells in the bone marrow, enumeration of cells staining with three anti-T cell antibodies was performed. C57BL/Ka bone marrow cells gated to exclude erythrocytes and dead cells showed dull staining of ~16−18% of cells with monoclonal anti-Thy-1.2, anti-CD3, and anti-α/β TCR antibodies. Depletion of the Thy-1.2+ or CD3+ cells by cell sorting before staining with anti-α/β antibodies resulted in the depletion of cells staining above background with the latter antibodies. The results indicate that the same subset of cells are stained by the three anti-T
Sorted αβ Cells
Before Culture | Sorted αβ Cells
CD4+ | Sorted αβ Cells
CD8+ | Sorted αβ Cells
Before
Culture | Sorted αβ Cells
CD4+ | Sorted αβ Cells
CD8+ | Sorted αβ Cells
After
Culture

|Va | Va | Va | Va |
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Figure 6. Predicted amino acid sequences encoded by junctions of Vg7 and Jg2 genes of sorted C57BL/Ka bone marrow cells. Sorted bone marrow cells obtained before or after culture were lysed in distilled water, and limiting dilutions were made such that replicate amplifications of DNA using the PCR with Vg7 and Jg2 C2b intron primers produced a single band on 1.5% agarose gels. The nucleotide sequences of the DNA in each band were determined. The predicted amino acid sequences shown start with amino acids encoded by the last two codons of the Vg7 genes and within a sequence represent nonfunctional junctions that resulted in an end with the first two codons of the Jg2 genes from the junctions. Dashes were 5 and 10 times more concentrated than those from the cultured bone marrow was obtained from molecular genetic studies and assays of in vitro function. Genomic DNA from sorted CD4+CD8α/β- and α/β+ cells was used as a template for amplification of the junctions between the Vg7 and Vg15 genes and the Jg2 gene cluster using the PCR. The nucleotide sequences showed that all junctions from the CD4+CD8α/β- cells were nonfunctional because of out-of-frame readings of the Jg2 genes or the insertion of a stop codon, and that almost all junctions from the α/β+ cells were functional. The results indicate that almost all cells with functionally rearranged β chain genes in the bone marrow expressed α/β surface receptors. The significance of the nonfunctional rearrangements in CD4+CD8α/β- cells is unclear. These cells may represent failed attempts of cells in the T cell lineage to rearrange TCR genes.

In vitro functional studies showed that the sorted Thy-1.2+ marrow cells proliferated after stimulation by anti-α/β antibodies coated onto plastic plates. The Thy-1.2- cells did not proliferate above background levels. The data provide further evidence that Thy-1.2+ marrow cells coexpress the α/β receptors and confirm a previous report that sorted CD4+CD8α/β+ marrow T cells proliferate in response to anti-TCR antibodies.

Subsequent studies of the quantitation of T and B cells in the bone marrow of euthymic BALB/c mice showed that IgM+ cells and α/β+ cells accounted for ~5 and 13% of cells, respectively, in the lymphocyte gate. This gate contains cells with light scatter characteristics of small lymphocytes. In similar experiments with athymic BALB/c mice, IgM+ and α/β+ cells accounted for ~8 and 12% of cells, respectively. The percentage of these cells was similar when marrow cells were gated to exclude only erythrocytes and dead cells (mean IgM+ cells, 7%; mean α/β+ cells, 13%). This finding indicates that both T and B cells in the bone marrow have a wide range of light scatter characteristics and are not restricted to the lymphocyte gate. The majority of non-T cells in the latter gate were B220+ (B cell lineage), and few were Mac-1+ or Gr-1+ (macrophages and granulocytes). In contrast, the majority of non-T cells outside of the lymphocyte gate were Mac-1+ or Gr-1+, and the minority were B220- (data not shown).

The turnover rate of bone marrow α/β T cells was determined by labeling newly formed cells in vivo by adding bromodeoxyuridine to the drinking water of mice for variable periods of time. Cells that have recently divided incorporate the label and can be stained with antibromodeoxyuridine antibodies. Accordingly, cells from the bone marrow of euthymic C57BL/Ka and athymic BALB/c mice were obtained after in vivo labeling for 12, 24, 48, or 72 h. Cells were stained with anti-α/β antibodies and gated to include only the α/β+ cells. The latter cells were counterstained to detect bromodeoxyuridine incorporation. Approximately 31-33% of α/β+ cells from the euthymic and athymic mice were labeled with bromodeoxyuridine within 24 h. These percentages of renewed cells are similar to those of marrow lymphocytes expressing low levels of Thy-1 receptors using the method of hydroxyurea depletion (30). Approximately 48-50% of IgM+ gated cells from athymic BALB/c mice were labeled within 24 h. Based on previous estimates of the
cells obtained from either euthymic or athymic BALB/c mice were obtained when sorted CD4-CD8-δ~3- bone marrow appeared. The bright T cells accounted for fetal bovine serum without growth factors or thymic stromal preciably reduced in the athymic mice. A likely source for cells.

mine whether marrow precursors can generate T cells in short depleted bone marrow cell cultures were performed to deter-

eral lymphoid tissues (17). Accordingly, sorted CD4-CD8-δ/β- cells from C57BL/Ka bone marrow were cultured for 48 h in medium containing fetal bovine serum without growth factors or thymic stromal cells.

After 48 h, a discretely staining population of α/β T cells that was ~40 channels brighter than the dull T cells in fresh bone marrow appeared. The bright T cells accounted for 12-18% of the cells harvested from the cultures. Similar results were obtained when sorted CD4-CD8-α/β- bone marrow cells obtained from either euthymic or athymic BALB/c mice were cultured for 48 h. At least 10% of the harvested cells stained brightly for α/β receptors.

Previous studies have shown that NK1.1 receptors are ex-

receptors. Although bright c~/β + T cells were detected before culture, since the presence of the latter cells exerts an inhibitory influence on bright T cell development. A possible explanation of these results is that newly formed bright α/β T cells mature in the marrow and down-regulate the expression of the α/β receptors or emigrate from the marrow during the maturation process. The rate of production of bright α/β T cells from precursors may be regulated in the normal marrow. Depletion of mature dull cells may accelerate the production of the immature bright cells as part of the regulatory process. Similarly, depletion of mature dull cells may accelerate production of bright cells in vitro.

Although none of the rearrangements of Vδ7 or Vδ15 genes with Jα2 genes were functional before culture of CD4-CD8-α/β- marrow cells from C57BL/Ka mice, at least 75% were functional after culture. The results indicate that the development of the bright α/β cells during culture is due to the generation of new functional rearrangements of the TCR genes in marrow T cell precursors rather than to the expression of preexisting functional rearrangements. Additional recent evidence indicates that the bone marrow is the most likely site for the generation of the marrow α/β T cells, since circular DNA deleted during α chain gene recom-

conjugation is present in athymic BALB/c marrow cells, and mRNA for the RAG-1 and RAG-2 recombinase enzymes is found in B220- marrow cells (33).

The fate of the marrow T cells with regard to efflux to the periphery and in situ cell death is unknown at present. Previous reports indicate that marrow T cells inhibit immune responses (12, 19) and may maintain a "neutral" marrow environment to prevent dysregulation of hematopoiesis by conventional CD4 and CD8 T cell activation.
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