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B Lymphopoiesis in the Thymus

Koichi Akashi,2,3 Lauren I. Richie,3 Toshihiro Miyamoto, William H. Carr, and Irving L. Weissman

The thymus has been regarded as the major site of T cell differentiation. We find that in addition to αβ and γδ T cells, a significant number (~3 × 10^4 per day) of B220^+ IgM^+ mature B cells are exported from the thymus of C57BL/6 mice. Of these emigrating B cells, we estimate that at least ~2 × 10^4 per day are cells which developed intrathymically, whereas a maximum of ~0.8 × 10^4 per day are cells which circulated through the thymus from the periphery. The thymus possesses a significant number of pro-B and pre-B cells that express CD19, VpreB, A5, and pax-5. These B cell progenitors were found in the thymic cortex, whereas increasingly mature B cells were found in the corticomedullar and medullary regions. Other lymphoid cells, including NK cells and lymphoid dendritic cells, are not exported from the thymus at detectable levels. Thus, the thymus contributes to the formation of peripheral pools of B cells as well as of αβ and γδ T cells. The Journal of Immunology, 2000, 164: 5221–5226.

*Abbreviations used in this paper: CLP, common lymphoid progenitor; RTE, recent thymic emigrants.*

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Materials and Methods

**Mouse strains**

The congenic strains of mice, C57BL6-Ly5.2 or C57BL6-Ly5.1 mice, were used. The C57BL6-Ly5.1/Ly5.2 mice were made by crossing C57BL6-Ly5.2 with C57BL6-Ly5.1 (F1). The strains differed only at the Ly5 allele, and this difference made it possible to detect donor-derived cells. C57BL6-TCRβ-deficient mice (13) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained in the animal care facility at Stanford University School of Medicine and were used at 4–8 wk of age.

**Labeling of thymocytes and flow cytometric analysis**

The technique for in vivo intrathymic labeling of thymocytes with FITC has been previously described (14). Briefly, 10 μl of FITC (1 ng/ml; Sigma, St. Louis, MO) was injected into both thymic lobes of 5- to 8-wk-old C57BL6 mice. Single-cell suspensions were made from the spleen and the lymph nodes, including cervical, axillary, submandibular, inguinal, brachial, and mesenteric lymph nodes. Cells were stained with PE or allophycocyanin-conjugated anti-IgM (clone 331; obtained from Dr. L. A. Herzenberg, Stanford University, Stanford, CA), Mac-1 (M1/70), Gr-1 (8C5), cocyanin-conjugated anti-IgM (clone 331; obtained from Dr. L. A. Herzenberg); PE; or anti-CD3 (KT31.1), anti-CD4 (GK1.5), anti-CD8 (53-7.8), and/or anti-CD11b (ITM11.6), Abs. Anti-IA^β, anti-CD11c, anti-αβTCR, and anti-γδTCR Abs were purchased from Pharmingen (San Diego, CA). In analyzing CD4^+CD8^− RTEs, cells were additionally stained with Texas Red-conjugated anti-CD4 and anti-CD8 Abs. For thymic B cell progenitor analysis, thymocytes were stained with FITC-conjugated anti-CD3 (S7) and anti-mouse IgM, PE-conjugated anti-CD19, allophycocyanin-conjugated B220, and Texas Red-conjugated anti-CD4 and anti-CD8 Abs (PharMingen). Cells were analyzed by a highly modified five-color FACS Vantage (Becton Dickinson, Mountain View, CA) (9). Dead...
FIGURE 1. Changes in the FITC level of thymocytes after intrathymic injection of FITC. A, Decrease in the number of FITC + thymocytes after intrathymic injection of FITC. The vertical bars represent SD in each time point of analysis (each point represents five mice). B, Levels of FITC in thymocytes 6 h after FITC injection. Limit of FITC intensity detectable on FACS. Each vertical broken line represents a scale for a two-fold difference in FITC intensity. Therefore, the number on top of each vertical line indicates the time of cell division that allows the cells on the lines to remain detectable by FACS.

Estimation of mature T and B cell immigrants into the thymus

Single spleen cell suspension was obtained from C57BL6-Ly5.1/Ly5.2 mice. Myeloid spleen cells were removed by incubation with Gr-1, Mac-1, and Ter119 Abs before negative selection using anti-rat IgG-conjugated immunomagnetic beads (Dynal, Oslo, Norway). The purified 1 × 10^6 splenocytes from adult Ly5.1 × Ly5.2F1 mice were injected into 1.5- to 3-week-old Ly5.1 mice. The percentages of donor-derived mature αβ T cells and B cells in the secondary lymphoid organs were analyzed 7, 18, and 24 days postinjection, when the injected Ly5.1 mice reached the age of 4–5 wks.

Immunohistochemical staining

Sections of the thymus were cut from frozen samples at 4 microns and were fixed with acetone for 10 min (15). Samples were treated with a Vector Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) and counterstained with Gill’s hematoxylin (Medical Chemical, Fairfield, NJ). Sections of the thymus were fixed with acetone for 10 min (15). Sections were incubated with Streptavidin HRP (Caltag, South San Francisco, CA), visualized with 3-amino-9-ethylcarbazole as the chromagen for 20 min, and counterstained with Gill’s hematoxylin (Medical Chemical, Fairfield, NJ). Isotype-matched rat IgG was used as a negative control.

RT-PCR analysis

Total RNA was isolated from 1000 purified thymic pro-B and pre-B cells. cDNA was analyzed for the presence of pas-3, VpreB, or A5 by amplification of 36, 32, or 32 cycles, respectively. The following primers were used: pas-5-forward, CTA CAG GCT CCG TGA CGC AG; pas-5-reverse, TCT CGG CCT GTG ACA ATA GG (Tanneal, 65°C; expected length, 439 bp; Ref. 17); VpreB-forward, GTC TGA ATT CCT CCA GAG CCT AAG ATC CC; VpreB-reverse, CAG GTC TAG AGC CAT GGC CTG GAC GTC TG (Tanneal, 60°C; expected length, 400 bp; Ref. 18); A5-forward, GGG TCT AGT GGA TGG TGT CC; and A5-reverse, CAA AAC TGG GGC TTA GAT GG (Tanneal, 60°C; expected length, 205 bp; Ref. 19).

Results

Recent thymic emigrants include B cells as well as αβ and γδ T cells

FITC was injected intrathymically to label cells in the thymus, and the spleen and lymph nodes were later analyzed for the presence of RTE. After intrathymic FITC injection, the percentage of labeled cells in the thymus was followed over time (Fig. 1A). Six hours postinjection, 95% of the thymocytes were FITC + (Fig. 1A) in comparison with noninjected controls. From 6 to 48 h postinjection, the percentage of FITC-labeled cells decreased in a nearly linear fashion. One of the main causes of the loss of FITC might be cell division, resulting in a 50% decrease in surface labeling per cell division (20), although it is possible that the loss of FITC label could be due to normal turnover of membrane proteins in the absence of division (21). As shown in Fig. 1B, the intensity of FITC signals at 6 h postinjection was strong enough to detect more than 85% of FITC-labeled cells after one or two cell divisions and approximately 50% of cells after four cell divisions. Because the estimated cycling time for thymocytes is ~8–12 h (22), the majority of progeny from cycling FITC-labeled thymocytes might maintain detectable levels of FITC at least 24 h after injection. At 24 h postinjection, ~84% of thymocytes retained detectable levels of FITC, indicating that the bulk of thymocytes can be estimated to turn over in ~6 days. This estimate is in accordance with turnover rates of thymocytes in previous reports (23). Because only ~1% of thymocytes emigrate per day (14), the major loss of labeled cells is likely due to apoptosis of thymocytes that either failed positive selection or were deleted by negative selection (22, 24).

The phenotype of FITC-labeled thymic emigrants in the spleen is shown in Fig. 2. Approximately 95% of FITC + recent thymic emigrants were CD4 + CD8 — or CD4 + CD8 + single-positive αβ T cells (Fig. 2A). The remaining ~5% of FITC + RTEs were CD4 + CD8 — double-negative cells that were composed of CD3 + TCR γδ + T cells, B220 + IgM + mature B cells, and some CD3 + TCR γδ — T cells (Fig. 2C). The majority of B220 + IgM + mature B cells expressed IgD, but only a minority were CD5 + (Fig. 2C). The rate of appearance of FITC-labeled RTEs is almost linear in the periphery up to 24 h postinjection (Fig. 3). The profiles of RTE in the lymph nodes were similar to those in the spleen (data not shown). Because most lymphocytes collect in the spleen...
and lymph nodes, it can be estimated that the thymus exports at least 1.2 × 10⁶ αβ T cells, ~3 × 10⁴ γδ T cells, and ~3 × 10⁵ B cells per day to the periphery (Fig. 3). The estimated numbers of αβ and γδ T cell emigrants are compatible with previous reports (14, 25). On the other hand, Mac-1⁺Gr-1⁺ granulocytes/macrophages, CD3⁻NK1.1⁺ NK cells, and CD8α⁻⁻CD11c⁻⁺ MHC-class II⁺ dendritic cells were not detectable in the RTE (FITC⁺) fractions throughout these experiments (Fig. 2C). The lack of FITC⁺ granulocytes/macrophages indicates that the FITC label did not leak into the circulation, and therefore, unincorporated FITC does not persist in a form that would be available for labeling newly immigrating or circulating cells.

A significant fraction of B cells that are exported from the thymus developed in the thymus

It is important to know whether the export of B cells from thymus reflects B cell production in the thymus or results from FITC labeling of B cells that have migrated into the thymus from the periphery. We tested whether or not peripheral B cells could re-enter the thymus and contribute to the thymic B cell pool. We found that B cell progenitors in the thymus did not leak into the circulation, and therefore, unincorporated FITC does not persist in a form that would be available for labeling newly immigrating or circulating cells.

Table I. Chimerism of mature T and B cells 18 days after injection of mature lymphocytes

<table>
<thead>
<tr>
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<th>Spleen</th>
<th>LN</th>
<th>Blood</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature B cells</td>
<td>5.3 ± 1.2</td>
<td>6.7 ± 1.6</td>
<td>5.1 ± 1.8</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Mature T cells</td>
<td>4.1 ± 1.3</td>
<td>3.7 ± 1.6</td>
<td>3.9 ± 0.9</td>
<td>0.01 ± 0.002</td>
</tr>
</tbody>
</table>

* A total of 1 × 10⁶ myeloid cell-depleted (Mac-1⁻Gr-1⁻ Ter119⁻) splenocytes from C57BL/6 (Ly5.1/Ly5.2) mice were injected i.v. into C57BL/6 (Ly5.2) mice. B220⁺ IgM⁺ B cells and TCRαβ⁺ αβ⁺ T cells were analyzed. Data are shown as the mean ± SD in four chimeric mice.

FIGURE 3. Numbers of FITC⁺ αβ T (A), γδ T (B), and B cell (C) emigrants in the spleen and lymph nodes. The vertical bars represent SD in each time point of analysis (each point represents five mice).

FIGURE 4. Donor-derived mature B cells (Ly5.1⁺Ly5.2⁺) in lymphoid organs 18 days after injection of mature splenic lymphocytes into 2-wk-old mice (Ly5.1⁺Ly5.2⁺). Percentages of donor-type B220⁺IgM⁺ B cells (Ly5.1⁺Ly5.2⁺) are ~5–7% in lymph nodes, blood, and spleen but only 0.6% in the thymus. Data from a representative mouse are shown (see Table I).

It has been reported that B cell progenitors are present in the thymus and that isolated thymic B cell progenitors can intrathymically differentiate into mature B cells after reinsertion into the thymus (28). The phenotype of thymic B cell progenitors is similar to that of bone marrow B cell progenitors (29). In the C57BL/6 strain, the thymus contains significant numbers of immature B220⁺CD43⁻IgM⁻ pre-B cells (~1.8 × 10⁴/thymus) and B220⁺CD43⁻IgM⁺ pro-B cells (~1.2 × 10⁴/thymus) (Fig. 5A and Table II). These thymic B220⁺ B cell progenitors coexpressed CD19 but not NK1.1 (Fig. 5B). The thymic IgM⁺ B cells were composed of IgD⁻ and IgD⁺ B cells as in bone marrow B cells. Although thymic B cells are reported to express a broad range of CD5 in the C3H mouse strain (28, 30, 31), B cell progenitors in the thymus develop in the thymus and that isolated thymic B cell progenitors can intrathymically differentiate into mature B cells after reinsertion into the thymus (28).
CD5 expression was almost limited to the pro-B cell fraction in the C57BL/6 strain thymi (Fig. 5B).

The thymic pro-B and pre-B cells were purified and analyzed for expression of early B cell-related molecules such as VpreB and A5 as well as a transcription factor, pax-5. All of these are detectable in the thymic pro-B cells and were increasingly expressed in pre-B cells (Fig. 6). The expression pattern of these molecules was consistent with that in bone marrow pro-B and pre-B cells (data not shown).

Thymic B lymphopoiesis was significantly enhanced in C57BL/6-TCRβ-deficient mice in which thymic T cell development is impaired. The TCRβ-deficient thymi had more than a 10-fold increase in the frequencies (Fig. 5A) and more than a 10-fold increase in absolute numbers of both mature B cells and B cell progenitors in comparison to wild-type thymi (Table II).

In immunohistochemical stainings of normal and TCRβ-deficient thymi, IgM+ B cells reside mainly in the corticomedullar junction and in the medulla, whereas B220+ cells were found throughout the thymus, indicating that immature B220+ IgM+ B cells mainly reside in the cortex through the corticomedullar junction (Fig. 7). Accordingly, thymic B lymphopoiesis might occur concomitantly with B cell progenitor migration from the cortex to the medulla of the thymus, mirroring T lymphopoiesis (32).

Discussion

We demonstrate that the adult C57BL mouse thymus physiologically generates and exports a significant number of B cells into the peripheral B cell pool. The findings of B cell development in the thymus and the recently described αβ T cell development in the bone marrow (33, 34) challenge the paradigm of lymphocyte classification into bone marrow-derived B cells and thymus-derived T cells. Our study also suggests that the thymus may not actively export NK cells and dendritic cells into the periphery or that the level of export may be below the level of detection in this study.

The thymus possesses B cell progenitors of each stage that are similar to those in bone marrow. This suggests that the thymic microenvironment can fully support B as well as T cell maturation. Adult thymi have been shown to be capable of generating B cells after intrathymic injection of hematopoietic stem cells or CLPs (9, 35). Although it has not been shown that the CLPs themselves migrate from the bone marrow to the thymus, it is possible that CLPs or one of their immediate offspring could migrate to and seed the thymus. We have previously reported that IL-7, which is presumably secreted from thymic epithelial cells, promotes survival of thymocytes undergoing positive selection through the up-regulation of Bcl-2 (1, 4). IL-7 is essential also for Ig gene rearrangement in developing B cells (5). Because IL-7 is known to exist in the thymic milieu, IL-7 would be available for the intrathymic development of B cells as well as T cells (36). Therefore, the thymic microenvironment should be able to physiologically support B lymphopoiesis as well as T lymphopoiesis.

Recent studies have shown that, in CD3-ε transgenic mice (37) and Notch1-deficient mice (38), the number of thymic B cells significantly increases in association with severe impairment of thymic T cell development. It is possible that alterations of these genes could skew the commitment of immature thymic progenitors toward the B cell lineage (37, 38). However, we demonstrate in

![Figure 5](http://www.jimmunol.org/Downloaded/from/by_guest_on_April_15_2017)

**Figure 5.** Phenotypic analysis of thymic B cell compartments in either C57BL/6- or C57BL/6-TCRβ-deficient mice. A, B220/IgM profiles of CD4+ CD8+ thymocytes (upper panels) and B220/CD43 profiles of CD4+ CD8+ thymocytes (lower panels). B, Additional surface phenotypic analysis of thymic B cell progenitors: the CD19/CD43 (a) and CD5/CD43 (b) profiles of B220+ IgM+ cells, the B220/NK1.1 profile of CD4+ CD8+ cells, and the IgM/IgD profile of B220+ cells.

![Figure 6](http://www.jimmunol.org/Downloaded/from/by_guest_on_April_15_2017)

**Figure 6.** RT-PCR analysis of sorted thymic B cell progenitors. The sorted CD4+ CD8+ IgM+B220+CD43+ pro-B cells and CD4+ CD8+ IgM+B220+CD43+ pre-B cells increasingly expressed pax-5, VpreB, and A5.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Pro-B</th>
<th>Pre-B</th>
<th>Mature B</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>1.20 ± 0.10 (0.006)</td>
<td>1.81 ± 0.14 (0.010)</td>
<td>4.96 ± 0.14 (0.028)</td>
</tr>
<tr>
<td>TCRβ-deficient C57BL/6</td>
<td>10.60 ± 2.77 (0.530)</td>
<td>25.20 ± 3.93 (1.260)</td>
<td>84.12 ± 6.15 (4.206)</td>
</tr>
</tbody>
</table>

* Data are shown as the mean ± SD in four mice in each strain.

a Percentage of total cells.
this study that the disruption of T cell development simply by a TCRβ gene knockout also results in a relative and absolute increase in thymic B cell compartments. Accordingly, these data collectively suggest that in normal thymi, rapid expansion of T cells might take up most of the microenvironmental niches, preventing efficient thymic B cell maturation.

The presence of B cell development in and export from the thymus has potential implications for the immune system. For example, B cells that develop in the thymus may have a different repertoire of Ig receptors than do bone marrow-derived B cells, resulting from local Ags and stimuli mediating their positive and negative selection. This may allow a more diverse set of Ig receptors in the periphery. It is also possible that the thymic B cells may contribute to the cellular interactions that mediate positive and negative selection of maturing T cells (39), including Ig isotype and idotype as selecting elements (40). Thus, it is important to clarify the role of these ectopically but physiologically developed thymic B cells in the immune system.

Acknowledgement

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References


FIGURE 7. Immunohistochemical staining of a TCRβ-deficient thymus. The thymus from a TCRβ-deficient mouse was stained with MD-2 Abs that react with medullar epithelial cells (A), B220 Abs (B), and anti-IgM Abs (C). Cx, Cortex; M, medulla.