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A SURFACE MEMBRANE DETERMINANT SHARED BY SUBPOPULATIONS OF THYMOCYTES AND B LYMPHOCYTES[1]

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Utilizing a quantitative fluorescence assay with the fluorescence-activated cell sorter (FACS), we have demonstrated that a rabbit antiserum obtained by immunization with cells of a mouse IgM-producing plasma cell tumor (MOPC104E) is reactive with at least two surface determinants, designated Th-B and ML2, on subpopulations of normal murine lymphocytes. The ML2 determinant is restricted to B lymphocytes. The Th-B determinant is shared by splenic B lymphocytes and a large subpopulation of thymocytes, the latter of which express a 3-fold higher density of Th-B on their surface than do the B lymphocytes. Neither Th-B nor ML2 were found on peripheral T cells or on brain, liver, or kidney cells. The available evidence suggesting that Th-B may be a stem cell determinant that is lost upon maturation is discussed.

Lymphocytes in the mouse have been separated into thymus-dependent (T)[3] and bone marrow-derived (B) classes, each class expressing distinctive cell surface antigens and functions (1). Recent studies have suggested that a high degree of functional heterogeneity exists within the T and B lymphocyte classes. In addition to the maturational heterogeneity of the thymus cell population as a whole (2, 3), "mature" T lymphocytes that participate in graft-*vs*-host and cytotoxic responses display functional heterogeneity with respect to homing properties and sensitivity to the cytotoxic effects of anti-lymphocyte and anti-Thy 1.2 antibodies (4). B lymphocytes display heterogeneity with respect to T dependence and specificity of stimulation, burst size, and longevity of clones derived from stimulated cells, and the immunoglobulin class of antibody expressed on their surface membranes (5–7).

A large number of antisera reactive with T and B lymphocyte membrane determinants have been prepared in an attempt to delineate these subpopulations and their maturational sequences (2, 3, 6). Yutoku *et al.* (8) have reported that antibodies raised against intact MOPC104E plasmacytoma cells were cytotoxic for plasma cells and a subpopulation of thymocytes. Utilizing a quantitative fluorescence assay employing a fluorescence-activated cell sorter (FACS), we have extended these findings to show that the antiserum is reactive with at least two determinants, one of which is shared by B lymphocytes and an immature subpopulation of thymocytes.

MATERIALS AND METHODS

Mice. Male and female mice from inbred strains of BALB/cN, CSW/H_z, NZB/H_z, and SJL/J used in these experiments were obtained from our own colonies at Stanford.

Antisera. A rabbit antiserum prepared against a saline EDTA extract of mouse thymus (RAMT) and absorbed with mouse bone marrow was shown to be specific for T cells (9). Rabbit anti-mouse Ig (RAMIG) was prepared by repeated injections of rabbits with ammonium sulfate prepared mouse Ig in complete Freund's adjuvant. Goat anti-mouse IgM was prepared by repeated injections of goats with MOPC104E myeloma protein in complete Freund's adjuvant. Goat anti-rabbit Ig (GARIG) was prepared by repeated injections of goats with DEAE-cellulose-purified rabbit immunoglobulin in complete Freund's adjuvant. Anti-Thy 1.2 (anti-θ) was prepared by Dr. G. Michael Iverson from ascites fluid elicited by i.p. injection of Sarcoma 180 cells into AKR mice after immunization with CBA thymocytes according to the method of Reif and Allen (10). Rabbit anti-MOPC104E (RAM104E) was prepared by Yutoku *et al.* (8) by injection of viable MOPC104E cells in complete Freund's adjuvant into New Zealand rabbits. The rabbit antiserum was absorbed *in vivo* by i.p. injection of 1 ml of antiserum into 10 to 12 week old BALB/cN mice. The mice were bled 8 and 18 hr later. The pooled *in vivo* absorbed RAM104E (RAML, rabbit anti-mouse lymphocyte) absorbed *in vitro* by incubating 200 to 500 μl of RAML with 4×10^8 BALB/cN spleen or thymus cells at room temperature for 30 min. The cells were eliminated by centrifugation at $300 \times G$ and the serum was recovered. The absorption was repeated two to three times as necessary until no activity against the absorbing cell populations remained. BALB/cN anti-keyhole limpet hemocyanin (KLH, Pacific Biomarine, Venice, Calif.) was prepared by i.p. injection of 100 μg alum-precipitated KLH into 6- to 8-week-old BALB/cN mice. Four weeks later the mice were boosted with 100 μg aqueous KLH administered i.v. The mice were bled 7 and 10 days after the boost. The sera were pooled, ammonium sulfate precipitated, and the 7S fraction was purified by chromatography on Sephadex G-200.

All serum preparations were deaggregated by centrifugation at $50,000 \times G$ for 1 hr sterilized by passage through a 0.22 μ Millipore filter, and stored at 4°C. Fluorescein conjugates of the ammonium sulfate-purified Ig of GARIG and AKR anti-

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[3] Abbreviations used in this paper: T, thymus dependent; B, bone marrow derived; FACS, fluorescence activated cell sorter; RAMT, rabbit anti-mouse thymocyte; RAMIG, rabbit anti-mouse immunoglobulin; GARIG, goat anti-rabbit immunoglobulin; RAM104E, rabbit anti-MOPC104E plasmacytoma cells; RAML, rabbit anti-mouse lymphocyte (*in vivo* absorbed RAM104E); KLH, keyhole limpet hemocyanin; D-PBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; MBLA, mouse bone marrow-derived lymphocyte antigen.

Thy 1.2 were prepared according to the method of Cebra and Goldstein (11).

Cell suspensions. Mice were sacrificed by cervical dislocation and the spleens, thymus, and peripheral lymph nodes were excised. Cell suspensions were prepared by cutting the organs into small fragments and gently pressing them between two glass slides in Dulbecco's phosphate-buffered saline (D-PBS) (12) supplemented to 5% v/v with fetal calf serum (FCS). Bone marrow cells were obtained by gently passing D-PBS through the femur and tibia with a 25-gauge needle. All cell suspensions were dispersed by aspiration with a Pasteur pipette. Connective tissue and tissue clumps were eliminated by passage through nylon screen. Erythrocytes were eliminated by treatment with NH_4Cl buffered in Gey's solution.

Fluorescence staining of cells. Pelleted cells (1×10^7) were resuspended in 0.1 ml of the labeling reagent (0.5 mg/ml RAMT, 1.2 mg/ml RAMIG, 0.5 mg/ml fluoresceinated anti-Thy 1.2, or the relevant dilution of RAM104E) and left at room temperature for 20 min. The cell suspension was pelleted through FCS and subsequently washed in 2 ml of medium. Except in the case of directly fluoresceinated anti-Thy 1.2, the cell pellets were resuspended in 0.1 ml of fluoresceinated GARIG at 0.4 mg/ml and left at room temperature for 20 min. The cells were washed as above, resuspended to 2 to 5×10^6 cells/ml in D-PBS and held on ice for analysis.

Labeling of Fc receptor. Fluoresceinated antigen-antibody complexes were prepared by adding a 10- to 20-fold excess of 7S anti-KLH to fluoresceinated KLH, incubating 2 hr at 37°C, and then overnight at 4°C. The complexes were pelleted by centrifugation at 50,000 × G and resuspended in D-PBS to 1 mg protein/ml. Pelleted cells (1×10^7) were resuspended in 0.1 ml of the fluoresceinated complexes and incubated at 37°C for 30 min. The cells were pelleted through 1 ml of FCS and subsequently washed in 2 ml of D-PBS.

Fluorescence microscopy. A Zeiss microscope with an HB200 mercury arc (OSRAM) light source was used. Cells were examined with dark field illumination with a Zeiss 1.2/1.4 NA oil immersion planochromat objective with an iris diaphragm. Combination of a Zeiss PIL 546 NM excitation filter and two layers of Kodak Wratten No. 23A gelatin barrier filter were used to detect fluorescence.

Cell separation. Immunoglobulin-bearing cells were removed from spleen cell suspensions by passage through nylon wool according to the method of Julius *et al.* (13). Briefly, 0.6 g nylon wool (LP-1) Leuko-Pak Leukocyte Filters, Fenwal Laboratories, Morton Grove, Ill.) was packed into the barrel of a 12-ml syringe up to the 6-ml mark and thoroughly wetted with D-PBS. The column was incubated at 37°C for at least 1 hr and washed with 50 to 80 ml of warm (37°C) D-PBS. A total of 10^8 cells in 2 ml of D-PBS were loaded onto the column and subsequently washed into the nylon wool with 1 ml of warm D-PBS. The columns were sealed with parafilm and incubated at 37°C for 45 min. The column was then washed slowly with 24 ml of warm D-PBS and the effluent, which contained a highly pure population of T cells, was collected. The nylon wool was then removed from the column, eluted with 50 to 80 ml of cold (4°C) D-PBS, and the eluate, which was enriched for Ig-bearing cells, was collected. Viability, as determined by trypan blue dye exclusion, was usually greater than 90%.

Cells labeled with fluoresceinated anti-Thy 1.2 were separated from unlabeled cells with a FACS as previously described (14–16). In the latest version, FACS-1, (made by Becton Dickinson Electronics Laboratory, 506 Clyde Avenue, Mountain View, Calif. 94043) of this instrument, cells are observed individually in suspension in the central stream of a 70-μ diameter coaxial liquid jet as it passes through a laser beam. One photo detector system provides a measure of cell size by detecting light scattering characteristics and a second photo detector system provides a quantitative measure of fluorescence intensity by using appropriate optical filters. The liquid jet is later broken into uniform-sized droplets, and those droplets containing the desired cells are charged electrically and deflected in an electric field. The cell populations separated in these experiments were processed at a rate of 5000 viable cells per second, which permitted a substantial enrichment of positive fractions and virtually 100% pure negative fractions (see Table I).

Analysis of cell populations on the FACS. Analysis of cell populations on the FACS involved the same principles as involved in cell separation described above. Cells were processed at 500 to 1000 cells per second and the intensity of fluorescence (pulse height) was recorded for each individual cell on the pulse height analyzer. The level of background fluorescence was determined by analyzing appropriate negative controls (e.g., cells labeled with normal rabbit serum). Thus the percentage of labeled cells was obtained by counting the number of cells giving fluorescence signals above background and dividing by the total number of viable cells examined. Light scattering signals distinguish live from dead lymphocytes (16). This calculation was performed by the FACS analyzer and was based on analysis of 5 to 10×10^4 individual viable cells.

RESULTS

Exposure of single cell suspensions of thymus or spleen from 12-week-old BALB/cN to a 1:5 dilution of RAML resulted in labeling approximately 50% of the cells in both populations (Figs. 1 and 2). This level of labeling represents saturation of available binding sites insofar as further increases in concentration of RAML did not increase the number of cells labeled (Fig. 1). At saturation (1:3 dilution of RAML), there was a substantial difference between the fluorescence distributions (i.e., the number of available binding sites per cell) of thymus and spleen cell populations (Fig. 2). The labeled spleen cell population displayed a lower fluorescence intensity (median

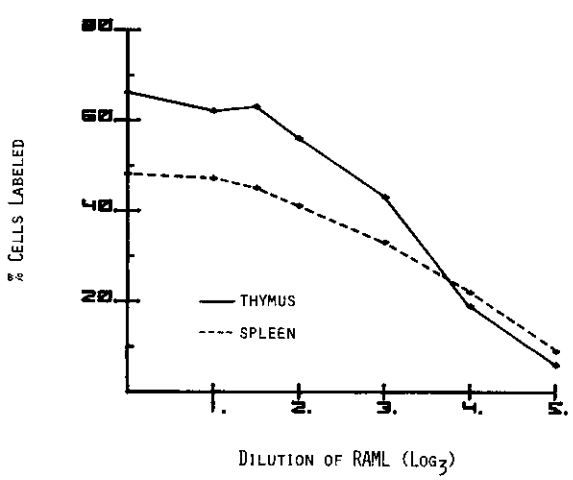

Figure 1. Labeling of spleen and thymus with RAML. 10^7 BALB/cN spleen or thymus cells were incubated with diluted RAML 20 min at room temperature, washed, and stained with fluoresceinated GARIG. The percentage of labeled cells was determined by analysis on the FACS.

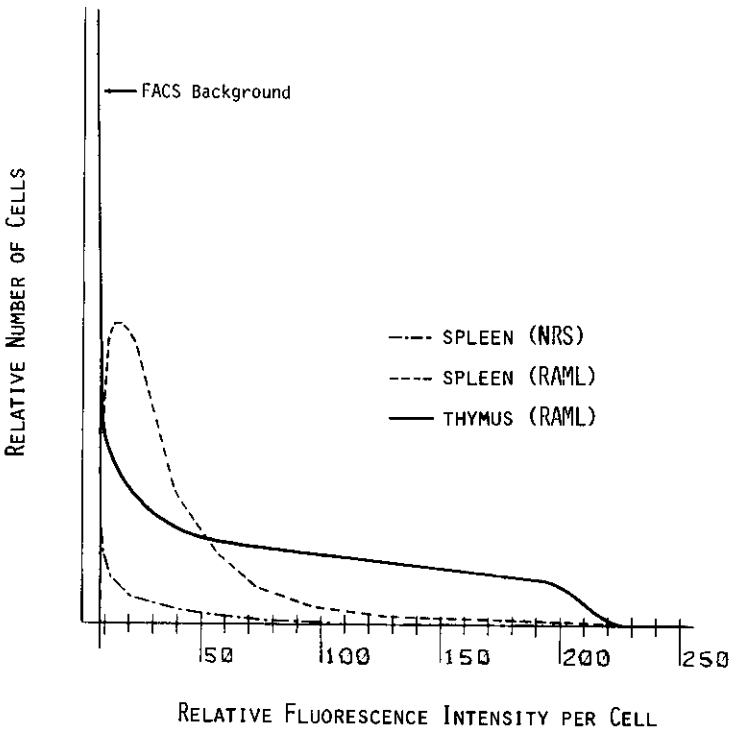

Figure 2. Fluorescence distribution of RAML-labeled spleen and thymus lymphocytes. 10^7 BALB/cN spleen or thymus cells were incubated 20 min at room temperature with a 1:3 dilution of RAML and stained with fluoresceinated GARIG. The fluorescence distribution was obtained by accumulative analysis of 2×10^4 individual viable cells on the FACS. The percentage of cells labeled by RAML was calculated by determining the difference between the percentage of cells with a fluorescence intensity greater than 7 units (FACS background) in specifically (RAML) and nonspecifically (NRS) labeled populations.

fluorescence intensity of 30 to 40 units) than the labeled thymocyte population (median fluorescence intensity of 80 to 90 units).

To determine whether the RAML was labeling T or B lymphocytes in the spleen, spleen cells from 12-week-old BALB/cN mice were labeled with fluoresceinated anti-Thy 1.2 and separated on the FACS into a Thy 1.2-enriched population (88% Thy 1.2 positive, Fraction I) and a Thy 1.2 negative population (< 1% Thy 1.2 positive, Fraction II) (Table I). The two cell fractions were then labeled with the RAML. Eighty to ninety percent of the Thy 1.2 negative cells were labeled with RAML, whereas only 8% of the Thy 1.2 positive fraction labeled with RAML (Table I). To obtain further evidence that the cells labeling with RAML in spleen were indeed B cells, spleen cells were passed through nylon wool and the effluent and eluted fractions were labeled with RAML and/or fluorescent antigen-antibody complexes. Less than 2% of the effluent (splenic T) population labeled with RAML (Table II). Of the eluted fraction, 73% labeled with RAML, almost all of which also bound antigen-antibody complexes (Table II).

To exclude the possibility that RAML was reacting with Ig on the surface of the splenic B lymphocytes, spleen cells were stripped of their membrane Ig by treating them with RAMIG and incubating them at 37°C for 90 min. After such treatment, less than 10% of the cells had demonstrable Ig on their surface and yet no diminution in labeling with RAML was observed (Table III). Similar treatment with a goat anti-IgM reagent also had no effect (Table III).

To determine if labeling of thymocytes with RAML was a property of any of the known subpopulations of thymocytes, thymus cells were labeled with anti-Thy 1.2 and separated on the FACS into three major fractions as described by Weissman *et al.* (17): large cells with a high Thy 1.2 density, which are the rapidly dividing subcapsular thymocytes; medium size cells with a low Thy 1.2 density, which are the cortisone-resistant medullary thymocytes; and small cells with an intermediate Thy 1.2 density. The separated fractions were then labeled with RAML. The fluorescence due to the previous anti-Thy 1.2 label contributed only a minor increase in background since RAML-treated cells counter stained with fluoresceinated GARIG have a fluorescence intensity 50 to 100 times higher than anti-Thy 1.2-labeled cells. The RAML did not label the medium-sized cortisone-resistant thymocytes but did label almost all (94%) of the large, rapidly dividing thymocytes (Table IV). RAML labeled half of the small cells with moderate Thy 1.2 density (Table IV). The determinants detected by RAML were not strain-specific since RAML labeled 54%, 38%, and 38% of CSW, NZB, and SJL spleen cells, respectively, and 53%, 49%, and 41% of CSW, NZB, and SJL thymocytes, respectively (Table V).

To determine whether the RAML was reacting with the same or different determinants on thymocytes and splenic B lymphocytes, RAML was absorbed extensively *in vitro* with either spleen or thymus cells from 12-week-old BALB/c mice. Absorption of RAML with thymocytes eliminated its reactivity with thymus cells but did not significantly alter its reactivity with spleen cells (Table VI), indicating that a determinant exists on splenic B cells that is not present on thymocytes. However, absorption of RAML with spleen cells completely eliminated its reactivity with both spleen and thymus (Table VI) indicating that the determinant present on thymocytes is also present

TABLE I

Reactivity of Thy 1⁺ vs Thy 1⁻ spleen cells with RAML

Spleen Fraction[a]	% Cells labeled[b] with:	
	Anti-Thy 1.2	RAML
	%	%
I	88	8
II	<1	89

[a] BALB/cN spleen cells were labeled with fluoresceinated anti-Thy 1.2 and separated into Thy 1⁺ or Thy 1⁻ fractions on the FACS. The separated fractions were then stained with RAML followed by rhodaminated GARIG.

[b] Percentage of positive cells was determined by analysis on the FACS.

TABLE II

Reactivity of splenic B vs splenic T nylon wool fractions with RAML

Labeling Reagent[a]	% of Cells Labeled[b]		
	Whole spleen	Nylon wool column	
		Effluent	Eluate
	%	%	%
RAMIG (αIg)	43	<1	65
RAMT (αT)	41	98	25
Antigen antibody complexes	54	12	78
RAML	48	2	73
AgAb complex + RAML (double stained)	43	<1	68

[a] BALB/cN spleen cells were separated on nylon wool into T and B cell fractions. The fractions were stained with RAMIG, RAMT, or RAML followed by rhodaminated GARIG. Spleen cells were labeled with preformed fluorescein-conjugated antigen-antibody complexes as described in *Materials and Methods*.

[b] Percentage of positive lymphocytes are based on fluorescent microscope count of 100 to 400 nucleated cells.

TABLE III

Lack of interference of anti-Ig with labeling by RAML

Spleen Cell Treatment	% Cells Labeling[a] with RAML
None	44
RAMIG[b]	48
GαIgM[c]	45

[a] Cells were labeled and assayed as in Figure 2.

[b] Spleen cells were incubated 90 min at 37°C after being treated with RAMIG. Fewer than 10% of the cells had demonstrable Ig on their surface after such treatment.

[c] Spleen cells were treated with GαIgM 20 min at room temperature prior to exposure to RAML.

TABLE IV

Properties of thymocyte populations[a]

Cell Size	Large	Small	Medium
% of thymus	5–7	85–90	5–7
Anatomy	Subcapsular zone	Deeper cortex	Medulla
Life span	Rapidly turning over	Long-lived	Long-lived
Resistance to cortisone	No	No	Yes
Thy-1 (θ)	+++	++	+
TL	++	+	–
H-2	+	+	++
% Labeled by RAML[b]	94	56	3

[a] Separation of thymus subpopulations and characteristics of subpopulations taken from Weissman *et al.* (17) and Greaves *et al.* (3).

[b] BALB/cN thymocytes were labeled with fluoresceinated anti-Thy 1.2 and separated on the FACS into the three subpopulations described. Each subpopulation was treated with RAML, labeled with fluoresceinated GARIG and analyzed for percentage of labeled cells on the FACS.

on splenic B cells. Absorption of RAML with BALB/c liver, kidney, and brain did not alter its reactivity with either thymocytes or spleen cells.

DISCUSSION

Yutoku *et al.* (8) previously reported that antibodies raised against intact MOPC104E plasmacytoma cells were cytotoxic for plasma cells and a subpopulation of thymocytes. Utilizing a quantitative fluorescence assay employing the FACS, we have extended these findings to show that the antiserum is reactive with at least two determinants which we have tentatively designated Th-B and ML2. The ML2 determinant is present only on B lymphocytes insofar as a) exhaustive absorption of the antiserum with thymocytes does not remove the antibodies reactive with the spleen cell population, and b) no reactivity of the antiserum with splenic T cells could be demonstrated. The restriction of the ML2 determinant to B lymphocytes makes it analogous to the mouse B lymphocyte antigen (MBLA) described by Raff and Cantor (18). We have not confirmed that ML2 and MBLA are identical determinants. The Th-B determinant, on the other hand, is shared by splenic B lymphocytes and a large subpopulation of thymocytes, the latter of which express at least a 3-fold higher density of Th-B on their surface than do the B lymphocytes. None of the previously described heteroantigens has been shown to be shared by subpopulations of T and B lymphocytes (1, 3).

It is unlikely that the Th-B or ML2 determinants are either Thy 1.2, TL, or H-2 determinants since a) neither Th-B nor ML2 are found on medullary thymocytes or peripheral T cells, both of which express Thy 1.2 and H-2 determinants (2–4), b) Th-B and ML2 are not found on brain tissue, as is Thy 1.2 (3), c) Th-B and ML2 are both present on splenic B lymphocytes, whereas TL is restricted to thymocytes in normal TL^+ mice (3), and d) they are equally expressed in mouse strains bearing different H-2 determinants. Similarly, the determinants do not seem to be associated with either surface Ig or the Fc receptor since a) removal of the B lymphocyte's surface Ig does not impair reactivity with RAML and b) treatment of the cells

TABLE V

Strain specificity of RAML

Strain	% Cells Labeled[a]	
	Spleen	Thymus
12-week-old BALB/cN	43	60
12-week-old CSW	54	53
12-week-old NZB	39	49
12-week-old SJL	38	41

[a] Cells were labeled and assayed as described in Figure 2. Labeling with NRS was always less than 5%.

TABLE VI

In vitro absorption of RAML with spleen or thymus

RAML Absorbed with:[a]	% Cells Labeled[b] in BALB/cN	
	Spleen	Thymus
No cells	44% (35)[c]	58% (90)[c]
Thymus	39% (30)	2% (—)
Spleen	3% (—)	2% (—)

[a] RAML was exhaustively absorbed *in vitro* with either spleen or thymus.

[b] Fresh BALB/c spleen or thymus cells were labeled with unabsorbed or absorbed RAML as described in *Materials and Methods*. The cells were analyzed on the FACS for percentage of cells labeled and fluorescence intensity per cell.

[c] The numbers in parentheses represent the median fluorescence intensity of the labeled population in arbitrary fluorescence units per cell (fluorescence pulse height on the FACS).

with RAML does not impair their ability to bind antigen-antibody complexes.

The functional significance of the Th-B determinant is not known but available data suggest that it may represent a differentiation marker. Multipotential stem cells, derived from the embryonic yolk sac or adult bone marrow, enter the thymus and develop the distinctive characteristics of T cells (19). Weissman *et al.* (17) have reported that most immature thymocytes are localized in the subcapsular region of the thymus and that, as the thymocytes mature, they migrate through the cortex and into the thymic medulla. The observation that Th-B is expressed by some B lymphocytes and the cortical thymocytes but not by the medullary thymocytes or by peripheral T cells suggests that Th-B may be a stem cell determinant that is lost or masked upon maturation. Yutoku, Grossberg, and Pressman (unpublished observations) have found that thymus cells treated with RAML plus complement are still capable of cooperating with normal bone marrow cells to give an adoptive antibody response, confirming that functionally mature T cells do not bear the Th-B determinant. Recent experiments by one of us (R.D.S.) indicate that the percentage of B lymphocytes in spleen, lymph node, and bone marrow bearing only the Th-B determinant, as well as the amount of the Th-B determinant per positive cell, is higher in 3-week-old than in 15-week-old mice, and, in 15-week-old mice is higher in bone marrow than in spleen or lymph node. The possibility that Th-B may be a marker for immature T and B lymphocytes is currently under investigation.

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