Exclusion of Circulating T Cells from the Thymus Does Not Apply in the Neonatal Period

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Summary

Although T cells arise in the thymus, migration of mature postthymic T cells back to the thymus is very limited in adult mice and is restricted to activated cells. In neonates, by contrast, we present evidence that circulating $CD4^+$ and $CD8^+$ T cells with a naive/resting phenotype readily enter the thymus after intravenous injection and remain there for prolonged periods. The migration of resting T cells to the neonatal thymus is largely limited to an unusual subset of cells which lacks expression of the lymph node homing receptor, leukocyte-endothelial cell adhesion molecule 1 (LECAM-1) (MEL-14). Migration of mature T cells to the thymus in neonates may be important for self-tolerance induction.

T cells arise in the thymus by a complex process of positive and negative selection (1–4). Although the thymus consists predominantly of immature cortical T cells, about 15% of thymocytes have a mature phenotype. These cells reside in the medulla and closely resemble the subsets of recirculating $CD4^+$ and $CD8^+$ T cells found in the extrathymic environment. The immediate origin of mature thymocytes is unclear. Some of these cells are presumed to be the direct descendants of immature thymocytes undergoing selection in the cortex. However, a proportion of mature thymocytes might represent T cells that reentered the thymus from the peripheral lymphoid tissues. In fact, it has been suggested that thymic immigrants may account for the majority of the functional T cells found in thymocyte suspensions (5). Evidence against this possibility has come from the finding that entry of mature T cells to the adult thymus after intravenous injection is very limited and is restricted to activated T cells (6).

The finding that mature resting T cells fail to gain entry to the thymus of normal adult mice is surprising because intravenous injection of T cells, specifically the $CD8^+$ subset of T cells, is highly efficient at inducing intrathymic tolerance to Mls^a antigens in neonates (7). Such tolerance could reflect intrathymic entry of soluble Mls^a antigens released from the injected T cells. Conversely, the data may indicate that, in contrast to the adult thymus, the neonatal thymus is uniquely permeable to mature T cells. In support of this possibility we show here that T cells with a resting/naive ($CD45RB^{hi}$, $CD44^{lo}$) phenotype readily enter the thymus of newborn mice after intravenous injection. Such homing is largely restricted to a unique population of T cells that lacks expression of the LN homing receptor, leukocyte-endothelial cell adhesion molecule 1 (LECAM-1)[1] (MEL-14).

[1] *Abbreviations used in this paper:* HSA, heat-stable antigen; LECAM-1, leukocyte-endothelial cell adhesion molecule-1; NW, nylon wool.

Materials and Methods

Mice. C57BL/6 (B6), B6.PL Thy-1.1 (B6.PL), B10.BR/SgSnJ, AKR/J and (B6 × CBA/Ca)F_1 mice were obtained from The Scripps Research Institute breeding facility.

mAbs. The following mAbs to mouse antigens were used: 19E12 (anti-Thy 1.1) (8), Jlj (anti-Thy 1.2) (9), J11d (anti-heat stable antigen [HSA]) (9), GK1.5 (anti-CD4) (10), 1.M.7.8.1 (anti-CD44) (11), 23 (anti-CD45RB) (12), and MEL-14 (anti-LECAM-1) (13).

Preparation and Injection of T Cells. Resting mature T cells were prepared by passing pooled LN cells from cervical, axillary, mesenteric, and inguinal nodes over nylon wool (NW) columns (6). Blast T cells were prepared by injecting 2×10^7 B6.PL LN cells intravenously into (B6 × CBA/Ca)F_1 mice previously exposed to 900 rad (6). Thoracic duct cannulation was performed 3 d later and blast T cells were collected on ice over 16 h. For intravenous injection into neonatal mice (7-d-old or younger), donor T cells were injected into the anterior facial vein in a 100 μl volume using a 30-gauge needle. Older mice were injected through the tail vein in 200–1,000 μl volumes.

Immunohistochemistry on Cryostat Sections. Sections of frozen thymuses and spleens were cut at 5 μm in cryostat. The sections were dried overnight at 4°C and stained with biotinylated anti-Thy-1 Abs as described (6). Briefly, sections were incubated with biotinylated anti-Thy 1.2 (Jlj) mAb, washed, and incubated with horseradish peroxidase–conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). After further washing, the sections were developed with the substrate 3-amino-9-ethylcarbazole, washed, and lightly counterstained with hematoxylin.

FACS® Analysis. As described elsewhere (6, 14), thymocytes and splenocytes were stained for donor T cells using biotinylated anti-Thy 1.1 (19E12) or anti-Thy 1.2 (Jlj) mAbs. Cell suspensions were incubated with biotinylated anti-Thy-1 mAb, washed, and incubated with FITC-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and propidium iodide. To analyze T cell activation markers, three-color staining was performed as follows. Suspensions of cells were initially incubated either with normal rat IgG or with rat mAbs specific for CD44 (1.M.7.8.1), CD45RB (23G2), or LECAM-1 (MEL-14). After washing, the cells were in-

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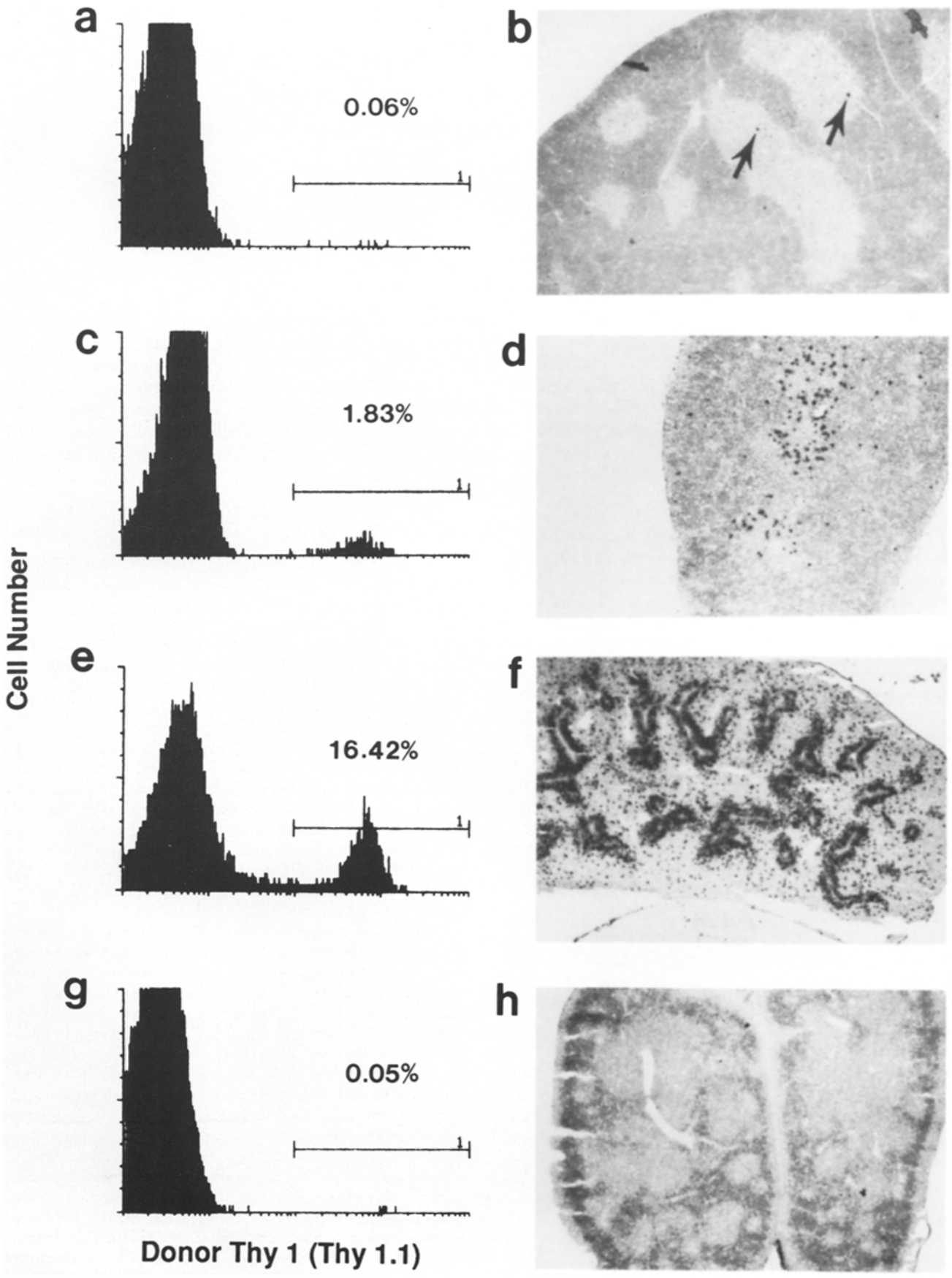
a
0.06%
1
b
c
1.83%
1
d
e
16.42%
1
f
g
0.05%
1
h
Cell Number
Donor Thy 1 (Thy 1.1)

Table 1. *Homing of Mature T Cells to the Thymus of Neonatal Mice after Intravenous Injection*

Expt.	Cells injected	Donor→1-d-old host	Time after adoptive transfer	Mean percent donor cells in: Thymus (HSA⁻)	Spleen
			d		
1	40 × 10^6 LN T (NW)	B6 → B6.PL (adult)	1	0.02 (0.12 × 10^4)*	4.0
	10 × 10^6 LN T (NW)	B6 → B6.PL	1	1.42 (1.55 × 10^4)	16.7
2	9 × 10^6 T blasts‡	B6.PL → B6	1	3.98 (3.50 × 10^4)	20.5
	9 × 10^6 LN T (NW)	B6.PL → B6	1	1.40 (1.34 × 10^4)	39.1
3	5 × 10^6 LN T (NW)	B6 → B6.PL	1	2.31	17.1
4	10 × 10^6 LN T (NW)	B6 → B6.PL	1	4.98	27.2
			5	1.58	10.1
5	5 × 10^6 LN T (NW)	B6.PL → B6	7	1.75	1.8
6	9 × 10^6 LN $CD4^+$	B6.PL → B6	7	2.48	ND
	9 × 10^6 LN $CD8^+$	B6.PL → B6	7	4.70	ND
	3 × 10^6 LN $CD8^+$	B6.PL → B6	7	1.11	ND
7	10 × 10^6 LN T (NW)	B6 → B6.PL	14	0.35 (0.77 × 10^4)	0.8
			28	0.36 (1.02 × 10^4)	0.8
			56	0.30 (1.81 × 10^4)	1.0
8	10 × 10^6 LN $CD8^+$	(B10.BR × AKR/J)F_1	7	2.2	ND
		→ B10.BR	14	1.3	ND
			21	0.6	ND

Newborn mice were injected intravenously within 24 h of birth with NW-passed LN T cells prepared from young adult mice or with in vivo-activated T blast cells as described in Materials and Methods. The donor and recipient mice differed at the Thy-1 locus. The host mice were killed at various times after injection and the donor T cells in the thymus and spleen were stained for donor Thy-1 and analyzed by FACScan® (see Materials and Methods). Thymocytes were treated with anti-HSA (Jlld mAb) plus C' and passed through low ionic strength buffer columns before staining to enrich for mature T cells. Nonspecific staining was determined for each experiment by staining HSA^- thymocytes from uninjected age-matched littermates. The level of background staining was ≤0.05 (see Fig. 1 *g*). The background value has been subtracted from the data shown.

* Mean percent of cells expressing the donor Thy-1 marker from two to four mice. The total number of donor cells in the thymus suspensions is shown in parentheses.

‡ Blast T cells were collected from thoracic duct lymph of irradiated (B6 × CBA/Ca)F_1 mice previously exposed to 900 rad and injected with B6.PL LN cells 3 d before (Materials and Methods). The lymph-borne cells consisted of a mixture of $CD4^+$ cells (55%) and $CD8^+$ cells (45%), and >95% of the cells were large blast cells.

cubated with γ chain–specific FITC-conjugated mouse anti-rat IgG mAb (Jackson ImmunoResearch Laboratories). After further washing, the cells were blocked with rat serum, incubated with biotinylated anti-Thy 1.1 mAb (19E12) and PE-conjugated anti-CD4 mAb (GK1.5; Becton Dickinson & Co., Sunnyvale, CA), washed, and incubated with R613-conjugated streptavidin (Gibco MRL, Grand Island, NY). Cells were analyzed on a FACScan® flow cytometer (Becton Dickinson & Co.). Before staining, thymocytes were depleted of immature T cells by treating with anti-HSA (J11d) mAb plus C followed by passage through low ionic salt buffer columns (15).

Results

Experimental Approach. To study T cell homing to the neonatal thymus, purified B6 ($H\text{-}2^b$, Thy 1.2) T cells prepared from LN were injected intravenously into groups of

Figure 1. Migration of Thy-1–disparate donor T cells to the neonatal thymus detected by FACS® analysis and staining of cryostat sections. NW-passed B6 (Thy 1.2) LN T cells were transferred intravenously to B6.PL (Thy 1.1) hosts, and the donor T cells reaching the host thymus 1 d later were detected with biotinylated anti-Thy 1.2 mAb (see Table 1). Thymocytes were stained after removal of HSA^+ cells. Cryostat sections were stained with anti-Thy 1.2 mAb and then lightly counterstained with hematoxylin (see Materials and Methods). (*a* and *b*) Thymus of an adult mouse injected with 40 × 10^6 T cells. Stained cells are very rare in thymus suspensions (the value for background staining has not been subtracted; see Fig. 1 *g*) and are difficult to detect (*arrows*) in sections. (*c* and *d*) Thymus of a neonatal mouse injected with 10 × 10^6 T cells. Stained cells are clearly detectable in cell suspensions and, in sections, are scattered throughout the medulla. (*e* and *f*) Spleen of mouse analyzed in *c* and *d*. Stained cells are conspicuous in cell suspensions and, in sections, form dense aggregates in the white pulp. (*g* and *h*) Thymus of a neonatal mouse not injected with T cells. Background staining with the anti-Thy 1.2 mAb is very low. ×40.

Thy 1-marked neonatal B6.PL (H-2^b, Thy 1.1) mice, or vice versa. In some experiments (B10.BR × AKR/J)F_1 (H-2^k, Thy 1.2 × Thy 1.1) cells were transferred to B10.BR (H-2^k, Thy 1.2) mice. Before injection, LN cells were passed through NW columns to enrich for resting T cells. The host mice were killed at various intervals and cell suspensions were prepared from spleen and thymus to search for donor-derived T cells using FACS® analysis. For thymus suspensions, thymocytes were pretreated with anti-HSA antibody and C′ to deplete immature T cells, thereby amplifying the detection of immigrant mature donor T cells.

T Cell Migration to the Neonatal Thymus. In confirmation of previous findings (6), intravenous transfer of large numbers of NW-passed T cells into adult mice led to negligible entry of the donor T cells into the host thymus (Table 1, Expt. 1; Fig. 1, *a* and *b*). In marked contrast, T cell entry to the thymus was easily detectable in neonatal (1-d-old) hosts. Thus, with intravenous injection of 5–10 × 10^6 cells, donor Thy 1-marked T cells accounted for 1.5–5% of the HSA$^-$ component of host thymocytes (compared with a yield of 0.02% donor T cells for the thymuses of adult mice injected with 4 × 10^7 T cells). In tissue sections, the injected T cells were localized almost entirely in the medulla of the neonatal thymus (Fig. 1, *c* and *d*). The donor T cells were prominent in the host thymus as early as 3 h after transfer (data not shown) and were detectable for up to 8 wk (Table 1). In terms of total cell numbers, the yields of donor T cells in the host thymus showed little change between 1 d and 8 wk after injection (see numbers in parentheses in Table 1). T cell homing to the neonatal thymus was proportional to the number of T cells injected and applied to both CD4$^+$ and CD8$^+$ cells (Table 1, Expts. 6 and 8; see also Fig. 3).

Age Dependency of Thymic Homing. To determine at what stage the thymus becomes impermeable to circulating T cells, LN T cells were injected into mice of various ages. To take into consideration the difference in body weight between young and adult mice, the dose of T cells was normalized to 4 × 10^6 cells/g body weight (a 1-d-old mouse weighs about 1 g). Donor T cells in the recipients were quantitated at 1 d after transfer. As a percentage of HSA$^-$ thymocytes, levels of donor T cells declined sharply between 1 and 7 d and then fell to near-undetectable levels by 2 wk (Fig. 2 *a*). Because the size of the thymus (and the number of HSA$^-$ thymocytes) increases markedly after birth, thereby diluting the donor T cells, it is more appropriate to consider the data in terms of the percentage of injected cells that reached the host thymus. Expressed in these terms, thymic homing was equivalent in 1- and 7-d-old mice (0.14% total recovery of the injected T cells in the thymus at both ages) and then declined sharply to reach adult levels in 2-wk-old hosts (0.01–0.02% recovery) (Fig. 2 *b*). With regard to other organs, homing to the spleen was maximal in 1-d-old mice, i.e., 25% total recovery of injected T cells, and then declined by 2–3-fold in adult mice (Fig. 2 *b*). This decline in adults is attributed to compensatory homing of T cells to LNs, which are almost undetectable in neonates. The specificity of T cell homing to the neonatal thymus is apparent from the finding that the ratio of the percent recovery of the total number of donor T cells in thymus vs. spleen declined abruptly after the neonatal period (Fig. 2 *c*).

Surface Phenotype of Thymic Immigrants. To investigate the possibility that the T cells entering the neonatal thymus are a minor subset of blast cells, 1-d-old mice were injected with NW-passed LN T cells vs. in vivo-activated T blast cells. In terms of percent localization, homing of blast cells to the neonatal thymus was more prominent than with NW-passed cells, but only by a factor of 2–3-fold (Table 1, Expt. 2). To study the surface phenotype of T cells entering the neonatal thymus, HSA$^-$ thymocytes taken from T cell–injected hosts at 1 d after transfer were stained for expression of CD44 (Pgp-1), CD45RB, and MEL-14 (LECAM-1); using three-color immunofluorescence, the cells were also stained for the donor Thy-1 marker and for CD4. As expected, most of the donor T cells recovered from the thymus of neonates injected with T blast cells expressed the typical CD44^{hi}, CD45RB^{lo}, MEL-14^{lo} phenotype of activated T cells (Fig. 3, *d*, *h*, *l*, and *p*). By contrast, with injection of NW-passed LN cells the vast majority of T cells reaching the host thymus were CD44^{lo}, CD45RB^{hi} (Fig. 3, *c*, *g*, and *k*) and thus resembled normal naive/resting cells. These cells also predominated in the host spleen (Fig. 3, *b*, *f*, and *j*). It is interesting that in marked contrast to cells reaching the spleen, most of the donor T

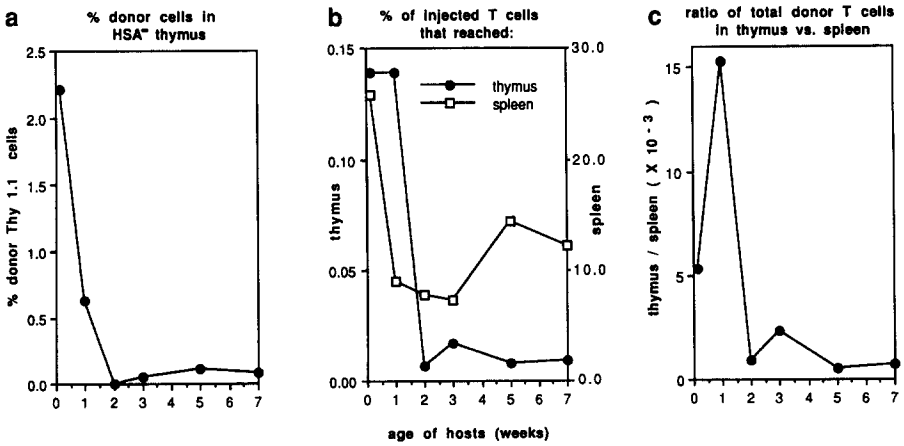

Figure 2. Decreased T cell migration to the thymus with age. B6 (Thy 1.2) mice of different ages were injected intravenously with 4 × 10^6 B6.PL (Thy 1.1) T cells/g body weight (e.g., 5 × 10^6 cells for 1-d-old neonates of 1.25 g) and killed 1 d later for detection of the donor T cells in thymus (HSA$^-$ thymocytes) and spleen. The data are expressed as the mean of the results from two separate experiments with three to four mice for each time point (tested on individual mice). The mice were aged 1 d, 1, 2, and 7 wk for one experiment, and 1 d, 1, 3, and 5 wk for the other.

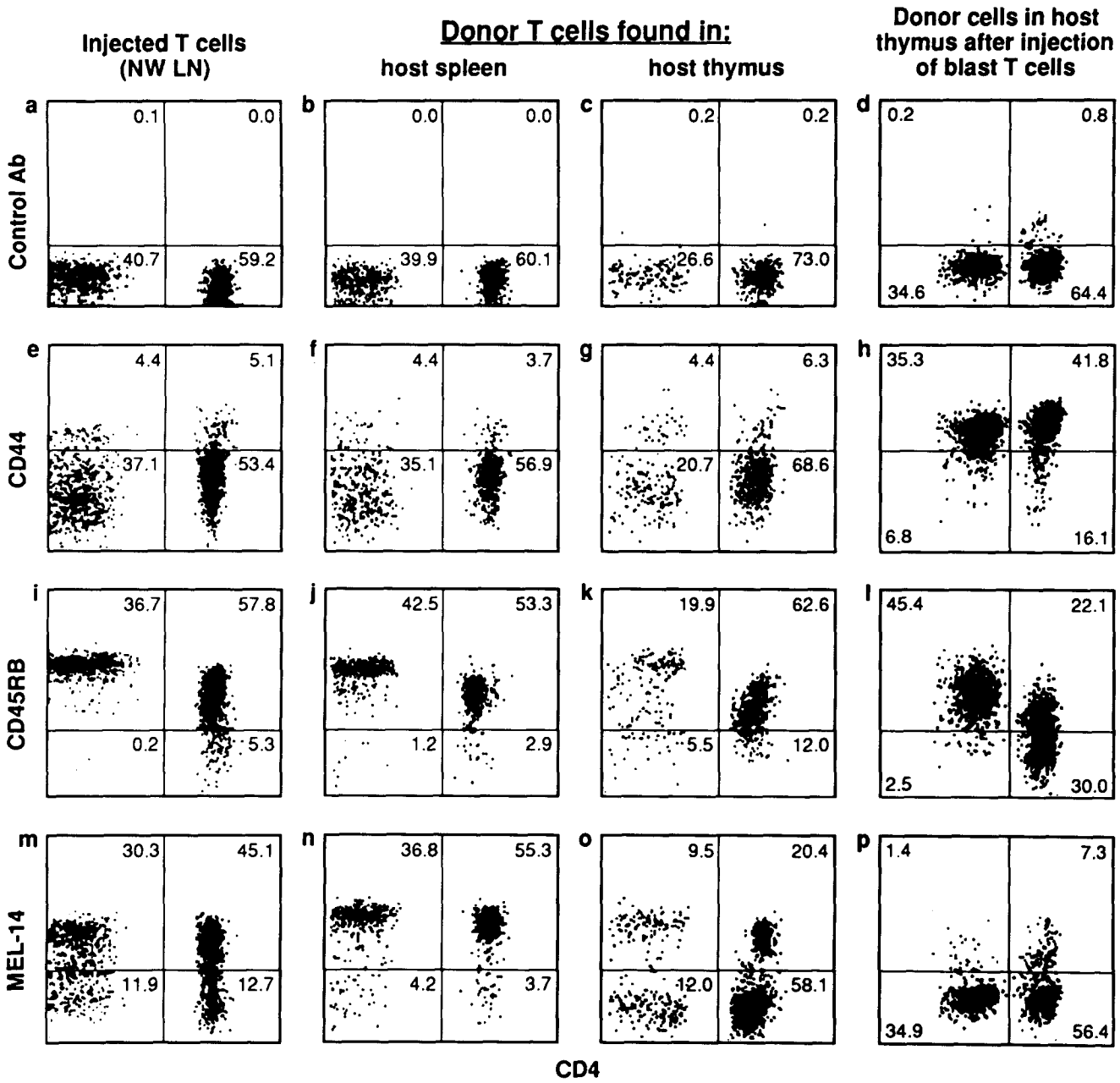

Figure 3. Surface markers on T cells homing to the neonatal thymus. Neonatal B6 mice were injected within 24 h of birth with a dose of 10^7 (B6.PL) NW-passed LN T cells or in vivo-activated T blast cells (see Table 1). The recipients were killed 16 h later and cell suspensions pooled from four to five mice were prepared from thymus (HSA^-) and spleen. Cells were stained for expression of CD4, donor Thy-1.1, and either CD44, CD45RB, or MEL-14 using three-color immunofluorescence and analyzed on a FACScan® (see Materials and Methods). Donor Thy 1.1^+ cells were gated and at least 5,000 events collected. In the case of resting T cells, the phenotype of the injected T cells (*a*, *e*, *i*, and *m*) is compared with the donor cells recovered from the host spleen (*b*, *f*, *j*, and *n*) and thymus (*c*, *g*, *k*, and *o*). The data are representative of three separate experiments. For the blast cells, the phenotype of the cells recovered from the host thymus (*d*, *h*, *l*, and *p*) was quite similar to the injected cells (not shown). With regard to CD45RB expression on the activated T cells, it should be noted that expression on the $CD4^-$ ($CD8^+$) subset of cells (*l*) is substantially lower than the (very high) expression on normal resting $CD4^-$ T cells (*i*).

cells in the thymus lacked expression of MEL-14, i.e., the molecule controlling homing to peripheral LN (13) (Fig. 3, *n* vs. *o*). For $CD4^+$ cells, the proportion of $MEL\text{-}14^-$ cells in the thymic immigrants was far higher than in the injected T cells (Fig. 3, *m* vs. *o*) or the donor T cells recovered from the spleen (Fig. 3, *n* vs. *o*); similarly, the donor $CD4^-$ cells in the thymus were also enriched for $MEL\text{-}14^-$ cells.

Discussion

Collectively, the data indicate that circulating resting T cells are excluded from the adult thymus, but readily enter the thymus of neonatal mice, where the cells lodge selectively in the medulla and remain for prolonged periods. The unusual $CD44^{lo}$, $CD45RB^{hi}$, $MEL\text{-}14^{lo}$ phenotype of the im-

migrant T cells implies that entry to the neonatal thymus is a highly specific process and presumably reflects T cell contact with certain vascular addressins expressed on thymic blood vessels (16, 17). The nature of these addressins and the type of T cell homing receptors used for recognizing these molecules, however, are unknown. In contrast to the normal adult thymus, it is interesting that circulating T cells readily enter the thymus of adult SCID mice (14), and the preneoplastic thymus of old AKR mice (18). Whether these thymuses express a fetal pattern of vascular addressins is unknown.

One might object that homing of T cells to the neonatal thymus is an artifact reflecting the high concentration of T cells in the bloodstream after intravenous injection. This seems unlikely because the dose of T cells injected into neonatal vs. adult mice was normalized for body weight. More importantly, the unique phenotype of the thymic immigrants is difficult to explain in terms of artifactual homing. It is also worth mentioning that, in contrast to T cells, resting B cells do not enter the neonatal thymus after intravenous injection (unpublished data of the authors). Preliminary studies suggest that homing of B cells to the neonatal thymus is restricted to activated B cells.

The biological significance of T cell homing to the neonatal thymus is a matter for speculation. There would appear to be two broad possibilities, which are not mutually exclusive. First, migration of T cells to neonatal thymus could be an epiphenomenon, the biologically significant finding being that resting T cells are excluded from the adult thymus. Excluding T cells from the adult thymus may be critical for preventing overcrowding of the medulla and/or be a reflection of a homeostatic mechanism. Second, reentry of T cells to the thymus during the neonatal period may be a device to promote self-tolerance induction, e.g., to differentiation antigens expressed selectively by fully mature T cells. The extreme potency of T cells ($CD8^+$ cells) in inducing intrathymic tolerance to Mls^a antigens in neonates (as few as 2×10^4 T cells produce strong tolerance [7]) is in line with this notion.

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