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## Long-term thymic reconstitution by peripheral CD4 and CD8 single-positive lymphocytes

Significant immigration of peripheral T cells into SCID thymus was observed following reconstitution with normal Peyer's patch, mesenteric lymph node or peripheral lymph node cells. Immunohistologic and flow cytometric analyses reveal that T cells from these tissues are found in the thymus for as long as 177 days and can account for up to 67% of intrathymic cells. The returning cells express the CD3/T cell receptor  $\alpha\beta$  complex, indicative of mature cells, and are equally divided among helper (CD4<sup>+</sup>CD8<sup>-</sup>) and cytotoxic (CD4<sup>-</sup>CD8<sup>+</sup>) phenotypes. The immigration of peripheral T cells is not accompanied by the appearance of immature, double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes as seen in similar reconstitutions using bone marrow. Taken together, these results suggest that peripheral T cells from a variety of lymphoid organs may regularly re-enter the thymus and, thus, possibly play a role in normal thymic development.

### 1 Introduction

The T cell-associated molecules CD4 and CD8 have been used extensively to define the cell surface phenotypes of peripheral and intrathymic T cell populations. In the periphery, essentially all T cells are either CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup>. Within the thymus, the major population consists of immature, double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) cells with normally about 20% bearing the mature CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> phenotypes. The phenotypic similarities between intrathymic and peripheral single-positive T cells raises the question whether thymic cells expressing these phenotypes represent recently derived virgin T cells awaiting emigration to the periphery, and/or mature peripheral cells that have returned to the thymus from secondary lymphoid tissues. Studies of T cell ontogeny have demonstrated that some intra-thymic, mature T cells are recently derived from precursors which entered the thymus via the bone marrow (BM). However, the presence of immigrating, peripheral T cells has been far more controversial. A number of reports [1-6] have failed to find evidence for immigration of such cells. However, other analyses using chromosomal or allelic markers have indicated that small numbers of mature, peripheral T cells from untreated lymph node (LN) [7-11], antigen-stimulated LN [5, 6, 12, 13], or LN-derived, antigen-specific T cell lines [14, 15] can re-enter the thymus. Thymic homing of peripheral T cells has also been observed in autoimmune mice [16] and pre-leukemic AKR mice [17]. Taken together, these studies suggest that some T cells can immigrate to the thymus; however, due to the low frequency of cells re-entering the thymus, and the presence of overwhelming numbers of endogenous, immature thymocytes, it has been difficult to

obtain detailed phenotypic analyses of thymic immigrants. Moreover, it remains unclear whether thymic re-entry is restricted to LN-derived T cells or, whether T cells from a variety of lymphoid tissues can also return to the thymus. In an effort to define further the ability of peripheral lymphocytes to re-enter the thymus, we have examined thymic tissue from SCID mice reconstituted with cells from various secondary lymphoid tissues.

### 2 Materials and methods

#### 2.1 Mice

Female DBA/2NCR, BALB/cANNCR, and C.B17ICR mice were obtained from Frederick Cancer Research Facility (Fort Detrick, Frederick, MD). C.B-17ICR SCID/SCID breeding pairs were initially provided by Dr. Nancy Jenkins (FCRF, Frederick, MD). All mice were housed in an NCI specific-pathogen-free animal facility (Fort Detrick, MD) under barrier conditions.

#### 2.2 Cell and tissue preparations

Peyer's patches (PP), mesenteric lymph node (MLN), peripheral lymph nodes (PLN), and BM were removed from 7-10-week-old female DBA/2 mice, placed in RPMI 1640 containing 5% FCS, 2 mM L-glutamine and 40  $\mu$ g/ml gentamycin. Individual tissues were teased apart and passed through a sterile stainless steel mesh to remove particulate matter. Cells were washed once and resuspended at 10<sup>7</sup> cells/ml in RPMI 1640 containing 5% FCS, 2 mM L-glutamine, 40  $\mu$ g/ml gentamycin, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and fungizone for 5 min after which they were washed an additional three times and resuspended at 5  $\times$  10<sup>7</sup> cells/ml in RPMI 1640 without FCS. SCID recipients received a single, tail vein injection of 10<sup>7</sup> cells in a volume of 0.2 ml.

#### 2.3 FCM analyses

The cell surface phenotype of thymocytes from normal controls and reconstituted SCID mice were determined by

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**Abbreviations:** PP: Peyer's patches MLN: Mesenteric lymph node PLN: Peripheral lymph node HRP: Horseradish peroxidase

**Key words:** Thymic immigration / *In vivo* lymphocytes homing / Peyer's patches / Flow cytometry

FCM. Analyses of control mice were performed on individual animals, while cells from two to five-PP or LN-reconstituted mice were pooled to obtain sufficient cells for phenotyping. Cell suspensions were prepared and analyzed as previously described [18] using the following reagents: FITC monoclonal rat anti-mouse IgM (Zymed, San Francisco, CA); FITC or PE monoclonal rat anti-mouse CD45(B220), clone RA3-6B2 [19]; biotinylated monoclonal 1-9-9 mouse anti-mouse Qa-2 [20]; APC-avidin (Molecular Probes Inc. Eugene, OR); FITC-avidin (Zymed); FITC anti-Thy-1.2 (Becton Dickinson, Mountain View, CA); FITC anti-mouse CD8 (Becton Dickinson); PE rat anti-mouse CD4, clone GK1.5 (Becton Dickinson) [21]; biotinylated monoclonal rat anti-mouse CD3, clone 500-A2 [22]; FITC monoclonal rat anti-mouse IL-2R, clone 7D4 [23]; FITC or PE monoclonal anti-mouse  $\alpha/\beta$  TcR (PharMingen); and FITC monoclonal anti-mouse  $\gamma/\delta$  TcR (PharMingen). To prevent nonspecific FcR-mediated binding of antibodies, unlabeled mAb 2.4G2 specific for mouse Fc $\gamma$ RII (CD32) [24] was added prior to staining with labeled mAb. Cells were analyzed on a Becton Dickinson FACStar Plus equipped with argon and argon/dye lasers. Nonviable cells and RBC were excluded by forward angle scatter and/or uptake of propidium iodide. All analyses were collected as list mode data and reanalyzed using Consort 40/VAX software (Becton Dickinson).

**2.4 Tissue sections**

At various times post-reconstitution recipient mice were killed and portions of thymus were frozen in OCT compound (Miles Inc. Elkhart, IN) for sectioning and staining as previously described [25]. To ensure an unbiased representation, each tissue block was sectioned at three levels.

Immunohistologic staining was performed using biotinylated anti-CD4 and anti-CD8 (Becton Dickinson). Sections were developed using horseradish peroxidase-biotin-avidin complexes (ABC Kit, Vector Laboratories), and the diam-

inobenzidine tetrahydrochloride reaction products were enhanced by addition of NiCl<sub>2</sub>.

**3 Results**

**3.1 Experimental design**

For the following experiments, SCID mice were employed as recipients due to the absence of intrathymic T cell precursors and peripheral lymphocytes [26, 27] which, we reasoned, would facilitate detection and analysis of donor cells. A series of reconstitution experiments were performed using either syngeneic or marked donor cells to assess quantitative and qualitative aspects of donor cell immigration over an extended period of time. Furthermore, since differential *in vivo* migratory patterns have been ascribed to lymphocytes from mucosal vs. peripheral lymphoid tissues ([28-30], reviewed in [31]), and since cells from these tissues express organ-specific homing receptors [32-35], donor lymphocytes from both types of tissue were employed in the succeeding analysis.

**3.2 Thymic homing of syngeneic PP cells**

Initial experiments were designed to test the ability of syngeneic cells from mucosal tissue to repopulate the SCID thymus, with PP cells chosen as a source of prototypical mucosal lymphocytes. Accordingly, thymic homing was assessed in SCID mice reconstituted with syngeneic (C.B17) PP cells (approximately  $2 \times 10^6$  T cells in a total inoculum of  $10^7$  cells) at various times after cell transfer. As shown in Fig. 1, normal SCID and reconstituted thymi contain a predominant population of CD4<sup>+</sup>CD8<sup>-</sup> cells which, upon further analysis, was found to be Thy-1<sup>+</sup>, IL-2R<sup>+</sup> (data not shown). This phenotype has previously been associated with virtually all endogenous SCID thymocytes [36].

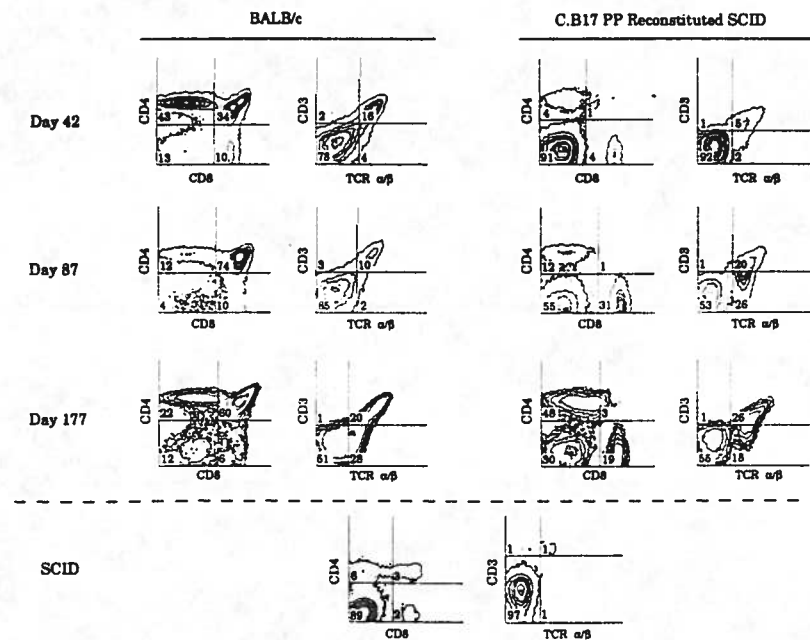


Figure 1. Cell surface phenotyping of intrathymic T cells obtained from normal BALB/c, SCID, and C.B17 PP-reconstituted SCID mice. The cell surface phenotype of normal BALB/c thymic cells was compared to a pool of cells taken from the thymi of PP-reconstituted SCID mice. The normal SCID profile (bottom) was obtained from a pool of ten thymi from 4-6-week-old mice. Analyses were performed 42, 87, and 177 days post reconstitution. The percent cells stained with a given reagent is noted in the lower left corner of each quadrant. Profiles were generated using the data collected on  $10^5$  cells.

Most notable among PP-reconstituted thymi is the absence at any time point of CD4<sup>+</sup>CD8<sup>+</sup> cells (which predominate in the normal controls) and the presence of significant numbers of CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> T cells. These single-positive cells represent 8% of intrathymic cells by day 42 and increase with time, reaching 67% of total cells by day 177. In contrast to the few single-positive thymocytes detected in untreated SCID thymus, approximately 50% of the thymic immigrants in PP-reconstituted mice express both CD3 and TCR  $\alpha/\beta$ , suggesting they are of donor origin. Similar analyses of normal BALB/c thymus identify comparable proportions of single-positive cells expressing CD3 and TCR  $\alpha/\beta$ . However, in terms of absolute numbers, normal thymus contains approximately tenfold more single-positive cells than PP-reconstituted thymus, although it remains unclear what proportion of these cells are recent peripheral immigrants as opposed to newly arisen cells in the process of emigration.

### 3.3 Thymic homing of phenotypically marked PP cells

While the above experiments strongly suggest that syngeneic PP T cells re-enter the thymus of SCID mice, it remains formally possible that the intrathymic, single-positive cells observed in these mice could be SCID-derived cells that have been induced to express normal peripheral phenotypes as a consequence of the transfer of C.B17 cells. To eliminate this possibility unambiguously, a series of reconstitutions were performed with marked donor cells. DBA/2 was selected as the donor source because it is MHC compatible with C.B17, but differs in its expression of Qa-2 [20], making donor cells readily identifiable.

Analyses of thymi from DBA/2 PP-reconstituted SCID mice were performed at various times following cell transfer as shown in Fig. 2. CD4 and CD8 single-positive cells expressing the Qa-2 donor marker were detected by day 25.

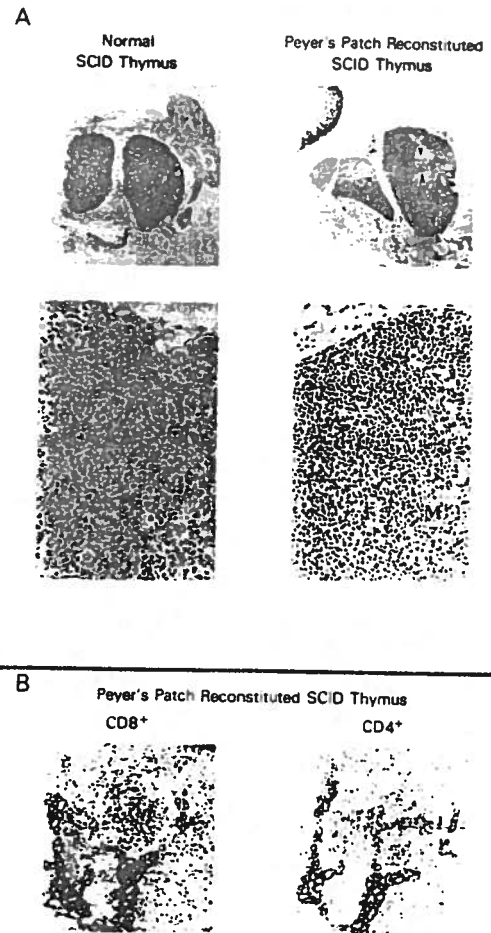


Figure 3. Histologic analyses of thymic sections from normal SCID and DBA/2 PP-reconstituted SCID mice 63 days post cell transfer. Cryostat sections from individual thymi were stained with H&E (panel A), anti-Cd8 (panel B, left) or anti-CD4 (panel B, right).

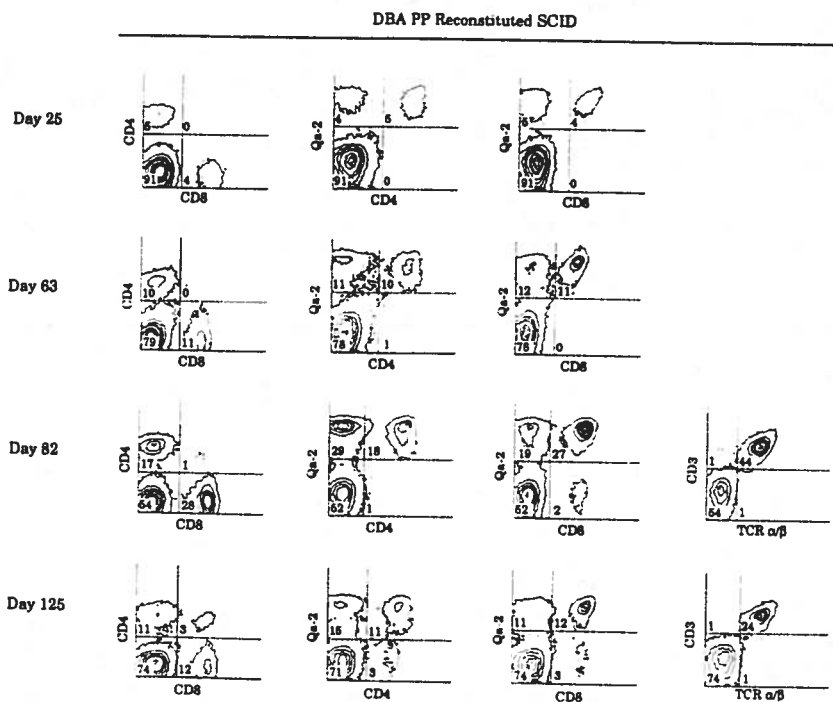


Figure 2. Cell surface phenotyping of intrathymic T cells obtained from DBA PP-reconstituted SCID mice at various times following cell transfer. At each time point, a pool of five thymi from reconstituted mice was analyzed. Profiles were generated as described in Fig. 1.

These populations were present in roughly equal proportions accounting for 9, 21, 45, and 23% of thymic cells at days 25, 63, 82, and 125 post reconstitution, respectively. At each time point, few if any (0-3%) double-positive thymocytes were detected (as was also the case in the transfer of syngeneic PP cells), suggesting that the intrathymic, single-positive cells did not arise from the co-transfer of T cell precursors contained within the original donor cell population. Moreover, the co-expression of Qa-2, CD3 and TcR  $\alpha/\beta$  on virtually all single-positive cells demonstrates that mature, peripheral T cells taken from mucosal tissues can undergo thymic immigration.

The results obtained by FCM were confirmed by immunohistologic analyses. Tissue sections from PP-reconstituted animals reveal moderate cellularity with readily demarcated cortical and medullary regions as compared to untreated SCID thymus (Fig. 3, top and middle, see arrows). Immunohistochemical staining of serial sections for CD4 and CD8 reveals apparently non-overlapping populations, both of which are located primarily in medullary regions.

### 3.4 Thymic homing of LN cells

To compare the ability of T cells from other lymphoid tissues to re-enter the SCID thymus, similar experiments were performed using mice reconstituted with DBA/2 MLN or PLN cells. Results indicate that, irrespective of the source of cells used, single-positive T cells of donor origin were found in reconstituted thymi at all time points analyzed. MLN reconstitutions (Fig. 3) result in 13-22% single-positive cells, all expressing the Qa-2 donor marker. The CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> phenotypes are equally represented and, as shown for days 67 and 91 post reconstitution, virtually all single-positive cells are CD3<sup>+</sup> and TcR  $\alpha/\beta$ <sup>+</sup>. The proportion of single-positive cells in MLN-reconstituted mice is comparable to that observed in

normal DBA thymus (Fig. 3, bottom). As shown in Fig. 4, similar results were obtained from thymi of PLN-reconstituted mice, in which 12-26% single-positive intrathymic cells were identified. Taken together, these experiments demonstrate that T cells from virtually any peripheral lymphoid tissue are capable of thymic immigration. A consistent finding in both PP- and LN-reconstituted mice has been the absence of intrathymic, double-positive cells (0-3%) compared to normal DBA/2 mice with the exception of the day 91 MLN analysis, where 16% of the cells were double positive (CD4<sup>+</sup>CD8<sup>+</sup>). This is the only instance in 14 such experiments where greater than 3% double-positive cells were detected. The origin of these cells is unclear, but several possibilities can be suggested. First, they may be derived from infrequent contaminating stem cells in the donor population that have undergone normal differentiation in the SCID thymus. Second, they may represent SCID thymocytes induced to differentiate by the presence of mature peripheral donor cells. These two alternatives can not be distinguished, as double-positive cells from normal DBA/2 thymus do not express the Qa-2 antigen. A third possibility is that one thymus in the day 91 pool may be from a "leaky" SCID, although, if this were the case, these double-positive cells do not give rise to single-positive cells as all of the single-positive cells express the donor Qa-2 marker.

The results above demonstrate that normal peripheral T cells from a variety of lymphoid tissues can return to the SCID thymus. However, it remains possible that the re-entry of peripheral cells may simply reflect an abnormal SCID thymic microenvironment. To test this possibility, thymi from SCID mice reconstituted with DBA/2 BM were analyzed (Fig. 5). The reconstituted thymi were found to be indistinguishable from normal DBA/2 thymus with respect to the various subpopulations expressing CD4, CD8, Qa2, CD3, and TcR  $\alpha/\beta$ . This finding is consistent with previous reports describing thymic reconstitution in SCID mice following BM transfer [36, 37].

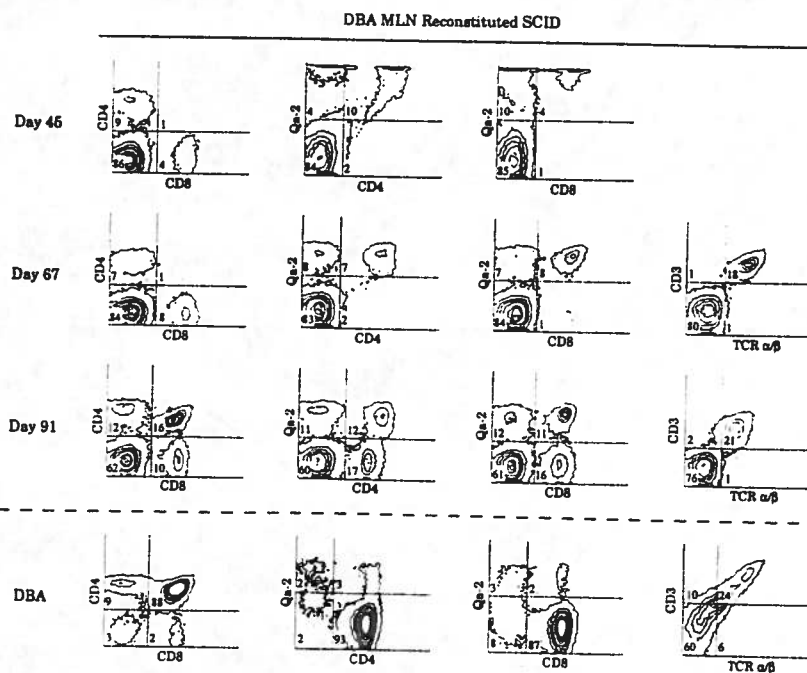


Figure 4. Cell surface phenotyping of intrathymic T cells obtained from DBA MLN-reconstituted SCID mice at various times following cell transfer. At each time point, a pool of five thymi from reconstituted mice was analyzed. Analyses of DBA/2 thymic cells (bottom) was performed on cells taken from an individual mouse. Profiles were generated as described in Fig. 1.

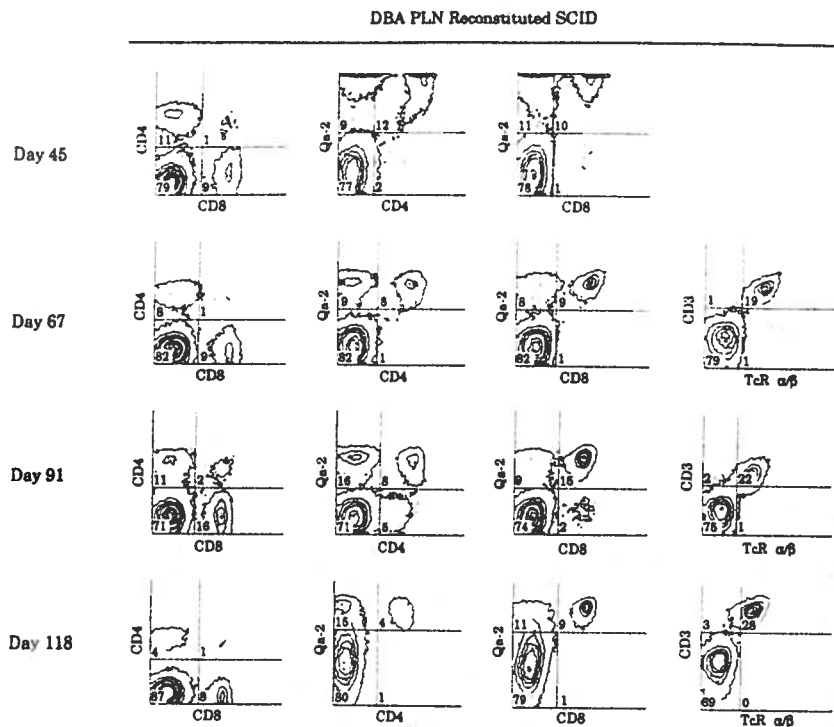


Figure 5. Cell surface phenotyping of intrathymic T cells obtained from DBA PLN-reconstituted SCID mice at various times following cell transfer. At each time point, a pool of five thymi from reconstituted mice was analyzed. Profiles were generated as described in Fig. 1.

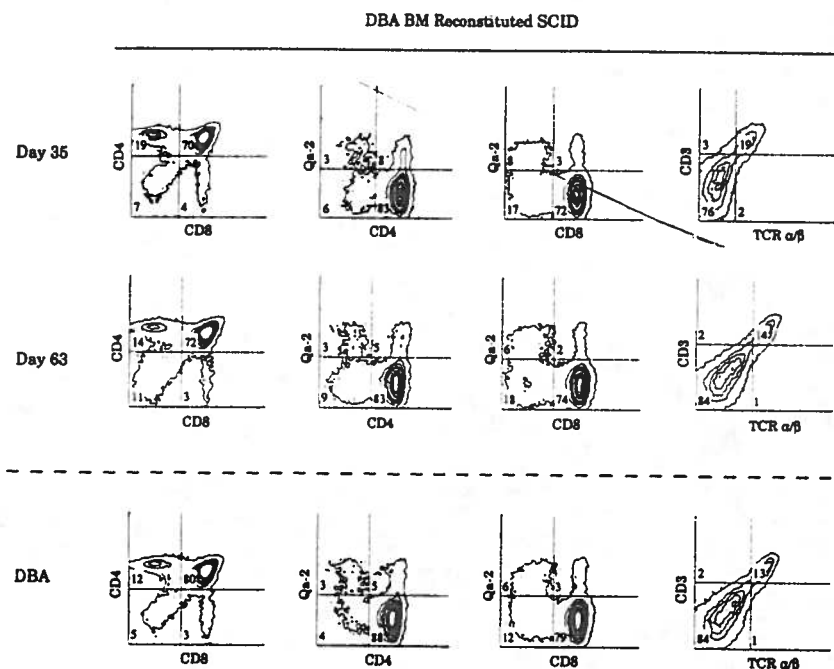


Figure 6. Cell surface phenotyping of intrathymic T cells obtained from normal DBA/2 and DBA/2 BM-reconstituted SCID mice at 35, and 63 days after cell transfer. At the times indicated, individual thymi from BM-reconstituted mice were analyzed. Profiles were generated as described in Fig. 1.

#### 4 Discussion

The present series of experiments was performed to assess long-term thymic immigration and reconstitution by mature T cell from various lymphoid tissues. The question of whether the SCID thymus is capable of supporting normal thymic development was addressed by reconstitution studies with normal BM (Fig. 5) which demonstrated that BM-reconstituted thymi are indistinguishable from those of normal controls with the predominant population consisting of double-positive ( $CD4^+CD8^+$ ) thymocytes

that are  $CD3^-$  and  $\alpha/\beta^-$ . This result indicates that the SCID thymic epithelium and other structural elements are fully capable of supporting normal thymic development.

Reconstitution experiments using lymphocytes derived from mucosal tissue (PP), MLN and PLN demonstrate significant thymic immigration of single-positive T cells of donor origin. These cells routinely account for 25% of total thymic cells and are found primarily in medullary regions. In contrast to BM-reconstituted thymus, animals reconstituted with mucosal or peripheral lymphocytes did not

develop double-positive cells, and endogenous IL-2R<sup>+</sup> SCID thymocytes were not replaced. Several additional features of the observed thymic reconstitution are worth noting. First, although numerous reports have suggested that PP cells display distinct homing properties from those of LN [28-31], our data reveal that the kinetics and extent of thymic immigration by PP and LN T cells are indistinguishable. Second, the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> intrathymic T cells (approximately 1:1) does not reflect that of the donor PP (3:1), MLN (4:1) or PLN (3:1) cells (data not shown). The mechanism by which CD8-bearing cells are disproportionately represented remains unclear, but may reflect preferential homing or selective expansion of CD8<sup>+</sup> cells.

In the present studies, the proportion of intrathymic, single-positive donor cells, as well as the absolute number of cells, far exceeds that observed in other systems where immigration to normal thymus has been examined. Agus et al. [5] have reported that fewer than 0.001% donor cells are found in thymi of Thy-1 congenic recipients following transfer of unactivated T cells. Similarly, Michie et al. [9] described a maximum 0.3% of medullary cells to be of donor origin. Taken together, these results suggest that fewer than 10<sup>4</sup> unactivated T cells re-enter the thymus, a number approximately tenfold less than that observed in a single reconstituted SCID thymus (1 × 10<sup>5</sup>-3 × 10<sup>5</sup>) in the present study. This number of cells appears more similar to that described [5, 6] for activated T cells, wherein approximately 2% donor cells could be enumerated following *in vivo* activation across a class I or class II MHC difference. Although the activation state of our donor cells was not assessed, it is, in fact, unlikely that a significant proportion of cells were activated (especially in syngeneic transfers), as all animals were maintained in a specific-pathogen-free colony under barrier conditions. Moreover, comparable thymic homing was observed in mice receiving either syngeneic (C.B17) (Fig. 1), or semi-syngeneic (DBA/2) cells (Figs. 2-4), suggesting that the minor MHC disparities and allelic differences between SCID and DBA/2 are not responsible for the presence of donor-derived, intrathymic T cells. Our data are consistent with that obtained by Surh et al. [11] in an analysis of short-term thymic homing also using SCID mice. These experiments demonstrate that 1 day post reconstitution less than 1% of intrathymic cells are of donor origin, a proportion consistent with other studies performed in normal adult mice [5, 6, 9]. This proportion could be increased to levels comparable to those in the present report by injecting new born SCID mice with 2 × 10<sup>7</sup> T cells followed by three weekly injections of 2 × 10<sup>7</sup>-5 × 10<sup>7</sup> T cells. The cumulative T cell inoculum received by these animals is approximately 100-fold greater than the present experiments, in which comparable thymic immigration is detected with only 10<sup>7</sup> total PP cells (2 × 10<sup>6</sup> T cells) in adult mice by 25 days post reconstitution. Thus, our data demonstrate that mature peripheral T cells continue to accumulate or expand in the adult SCID thymus over a 1-6-month period until they represent ~50% of total thymic cells. It remains possible that thymic homing in SCID mice is significantly more efficient due to a lack of competition from endogenous, immature thymocytes. This possibility is consistent with a recent report [6] suggesting that circulating resting T cells readily enter the thymus of normal neonates during the first 2 weeks following birth. Thus, in both the neonatal and SCID thymus, the paucity of

developing thymocytes may facilitate thymic immigration of mature T cells.

The biologic significance of thymic immigration of mature T cells in any of the above systems remains unclear, however, it is possible that this phenomenon represents a feed-back mechanism by which peripheral responsiveness and tolerance could be imposed on the developing T cell repertoire prior to emigration to the periphery. Such tolerance has been suggested for Mls<sup>d</sup> determinants [38] and MHC class II-bearing transgenic mice [39]. The ability to introduce significant numbers of peripheral T cells into the SCID thymus makes this system an ideal model for assessing the biologic role of peripheral T cell homing to the thymus.

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