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Generation of Intestinal Mucosal Lymphocytes in SCID Mice Reconstituted with Mature, Thymus-Derived T Cells[1]

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Transfer of peripheral lymph node lymphocytes to SCID mice leads to the long term establishment of mucosal T lymphocytes within the epithelium and lamina propria of the small and large intestines. Analysis of engrafted intraepithelial lymphocytes (IEL) showed that they had acquired a surface phenotype that in several respects is typical of IEL. In addition, the functional profile of engrafted IEL derived from lymph node T cells was similar to that of normal IEL; as the donor-derived T cells exhibited a strong cytolytic activity, a poor proliferative response to mitogenic stimuli, and a tendency to home and expand specifically in the intestine upon transfer to secondary SCID recipients. Optimal engraftment of intestinal T cells required bacterial flora, as the number of lymphocytes was greatly reduced in SCID recipients with a reduced flora. These results demonstrate that mature, thymus-derived T cells can migrate to the intestine and become functionally specialized to the intestinal milieu. The acquisition of phenotypic markers characteristic of the intestinal microenvironment by engrafted cells suggests that T cell migration of lymphocytes to the SCID intestine is not aberrant, but it may reflect processes that are ongoing in immunocompetent mice. Furthermore, these data suggest that the homing and/or expansion of typical, thymus-derived T cells in the intestine may be driven by luminal Ags such as those derived from bacterial flora. *The Journal of Immunology,* **1998, 160: 2608–2618.**

T lymphocytes dispersed within the epithelial layer and lamina propria of the large and small intestines represent the majority of T cells found within the gut-associated lymphoid tissue (GALT)[6] (1, 2). These intestinal T cells are, as a group, notably distinct from T cells resident in nonmucosal sites (3, 4). These differences are most striking when small intestinal intraepithelial lymphocytes (SI-IEL) are compared with T cells found in the spleen or peripheral lymph node. For example, in mice, SI-IEL as a group constitutively express activation Ags such as CD69 (4, 5) and a unique $\alpha_E\beta_7$ integrin (6, 7), and they lack expression of molecules such as CD2, which characterize most other T cell populations (4, 8). Unlike T cells derived from spleen or lymph node, SI-IEL proliferate poorly in response to mitogenic stimuli (9, 10), but ex vivo they demonstrate brisk cytolytic activity in redirected lysis assays (10–12), and they have a constitutively activated MAP-2 kinase (10). In addition, SI-IEL are enriched for TCR $\gamma\delta^+$ cells (13), and subpopulations of IEL express coreceptors that are almost never found in other sites, including lymphocytes that are single positive for the homodimeric form of CD8 ($CD4^-$, $CD8\alpha\alpha^+$) and double positive ($CD4^+$, $CD8\alpha\alpha^+$) lymphocytes (3, 14). T lymphocytes found in other locations in the intestine, including those in the large intestinal epithelium (4, 15–17) and lamina propria lymphocytes (LPL) of the small and large intestines (18, 19) (our unpublished observations), share some of the unique features of SI-IEL, although there clearly are regional differences within the intestinal mucosal immune system. The phenotypic and functional specialization of intestinal mucosal T lymphocytes probably reflects their close association with both the external environment and epithelial cells, but the precise role of these lymphocytes in host defense and immune regulation remains to be determined.

Results from a number of studies suggest that intestinal mucosal T lymphocytes, particularly SI-IEL, constitute a separate lineage of T lymphocytes. First, there is evidence that many or most IEL are derived from a thymus-independent developmental pathway (20, 21), although the degree of thymus influence and the subpopulations that are thymus independent remain controversial issues (22, 23). It is widely believed that the thymus-independent T cells arise from bone marrow precursors that migrate directly to the intestine for selection (14, 20), although direct proof for intraintestinal T cell selection is lacking. Second, studies of parabiotic mice indicate that there is relatively little recirculation of SI-IEL (24). Third, in a transfer model in which IEL suspensions are transferred to SCID recipients, we found that IEL preferentially populated intestinal as opposed to peripheral lymphoid tissue (25). Collectively, these data suggest that IEL are a distinct population of T lymphocytes, some of which arise from precursors in the intestine, and the majority of which tend to remain at that site. In addition, the data from parabiotic mice suggest that mature thymus-derived T

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[6] Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; SI-IEL, small intestinal intraepithelial lymphocytes; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; high, high level; SPF, specific pathogen free; RF, reduced flora; PE, phycoerythrin; LI-IEL, large intestine intraepithelial lymphocytes.

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cells from the recirculating pool rarely enter the intestinal compartments.

Despite data suggesting that IEL are a distinct and separate lineage from other T cells, results from previous studies indicate that IEL can be derived from mucosal T cell populations found in either mesenteric lymph node or Peyer's patches (26, 27). By contrast, the ability of typical, thymus-derived T cells found in peripheral lymph node and spleen to home to intestinal tissues is controversial, but recent studies have shown that adoptively transferred T cells from such peripheral lymphoid tissues can home to the intestinal epithelium of SCID mice (28–31). The data from this transfer model therefore suggest that nonmucosal, thymus-derived T cells are capable of establishing populations of intestinal T cells. The majority of studies on spleen or peripheral lymph node have involved transfer of the $CD4^+$, $CD45RB^{high}$ subpopulation of T lymphocytes, a condition that leads to colitis in the SCID host (28, 30, 31). It remains possible, therefore, that the homing of lymph node T cells to the intestine is an aberrant process that could be associated with the induction of inflammatory disease.

In this report we have characterized the phenotypic and functional properties of the lymph node-derived T cells that home to the SCID intestine in the absence of colitis. We found that the cells resident in the intestine of the SCID host are markedly different from the donor population. These T cells are not typical of lymph node T lymphocytes, but instead, they are more similar to normal IEL and LPL with regard to the expression of a variety of cell surface molecules, cytolytic and proliferative activities, and their propensity to home to the intestine upon retransfer. The data, therefore, demonstrate that the process of intestinal homing in the SCID host does not lead to the presence of phenotypically abnormal T lymphocyte populations in the intestine. This suggests that a qualitatively similar process could take place in immune-competent hosts, thereby providing a potential source of mature, thymus-derived T cells in IEL and LPL populations. Furthermore, we demonstrate that a normal bacterial flora is required for the intestinal homing and/or expansion of the donor lymph node T cells in this model, suggesting that bacteria or bacterial products may play a critical role in the in vivo expansion and/or homing of mucosal T lymphocytes to the intestine.

Materials and Methods

Mice

C.B-17($H\text{-}2^d$) SCID mice homozygous for the *scid* mutation were bred from stocks obtained from the SCID Mouse Core Facility at University of California-Los Angeles. These specific pathogen-free (SPF) SCID mice were housed in cages held in enclosed racks with filtered air and were given autoclaved bedding and food and acidified water ad libitum. SPF SCID mice are negative for a variety of bacterial, viral, and fungal pathogens for which they have been screened, but they have a normal extent of aerobic and anaerobic flora (W. McBride, unpublished observations). By contrast, reduced flora (RF) C.B-17 SCID mice were derived by cesarean section delivery of SPF fetal mice, with transfer to RF foster mothers in a separate facility maintained by the Department of Radiation Oncology, University of California-Los Angeles. Mice in this colony, including the rederived C.B-17 SCID mice, are devoid of intestinal aerobes. They have been reassociated with two nonpathogenic, anaerobic (clostridial) bacterial species (W. McBride, unpublished). This was performed to optimize the health and fecundity of these otherwise germfree mice. We, therefore, do not call these mice germfree, but refer to them as having RF. They are maintained as described above for SPF SCID mice in enclosed cage racks with filtered air and autoclaved food, bedding, and water. Donor lymphocyte populations were prepared from the external inguinal lymph nodes, although in some cases a mixture of axial, inguinal, and popliteal lymph nodes of 6- to 8-wk-old SPF male or female mice. BALB/c × C57BL/6 F_1 ($CB6F_1$) mice, which have the $H\text{-}2^d/H\text{-}2^b$ haplotype, were used for transfer into C.B-17 SCID mice. When possible, donor mice were matched by sex with recipients. Unless stated in *Results*, SCID mice no older than 8 to 10 wk were used in all cell transfer experiments.

Preparation of donor lymphocyte populations

Cell suspensions in RPMI with 5% FCS (Life Technology, Grand Island, NY) were prepared from the peripheral lymph node or spleen from 6- to 8-wk-old mice. Cell suspensions were cleared of debris, counted, and resuspended in PBS. Cells ($2–5 \times 10^5$) in 0.2 ml of PBS were injected into the peritoneum of recipient SCID mice. In some cases, before transfer the cells were sorted on a FACStar instrument (Becton Dickinson, Mountain View, CA) located in the University of California-Los Angeles Flow Cytometry Core Facility (Jonsson Comprehensive Cancer Center) for $CD4^+$ cells with FITC-conjugated anti-CD4 clone GK1.5 or for $CD8^+$ cells with anti CD8α clone 53-6.7. Resultant T cell populations were >98% enriched for either $CD4^+$ or $CD8^+$ T cells, were >99% viable, and represented a 50 to 70% recovery from the presorted population. For secondary transfers, 5 to 8×10^5 SI-IEL prepared from SCID mice transplanted with lymph node cells 8 wk earlier were transferred to a group of secondary SCID recipients by i.p. injection. SI-IEL from individual recipient mice were not pooled, but were transferred separately.

Preparation of IEL and LPL

Intestinal mucosal lymphocytes were prepared from individual or pools of control $CB6F_1$ mice or from individual SCID mice 6 to 10 wk after transfer of donor cells, using a modification of a previously described procedure (32). Cells isolated from engrafted SCID mice were never pooled for experimental analysis. Briefly, the small and large intestines were removed from their mesentery, and the Peyer's patches or lymphoid aggregates were excised. The intestines were then cut longitudinally, the contents were removed, and the segments were washed before cutting them into 0.5-cm-long pieces. IEL were prepared using mechanical and chemical disruption of the epithelial layer in Ca^{2+}- and Mg^{2+}-free HBSS (Life Technology) supplemented with 1 mM DTT (Sigma Chemical Co., St. Louis, MO). Intestinal pieces were shaken at 37°C three times for 20 min each time at 250 rpm. Cells were collected after each shake, pooled, and isolated on a discontinuous 40/70% Percoll (Pharmacia Biotechnology, Inc., Piscataway, NJ) gradient at $900 \times g$ for 20 min. Following removal of the epithelial layer, LPL were released from finely chopped intestinal segments by incubation in 1.5 mg/ml of dispase (Sigma) in RPMI with 5% FCS for 60 min at 37°C. LPL cell suspensions were filtered through nylon mesh, and mononuclear cells were isolated by discontinuous Percoll gradient centrifugation as described above. Purified cells were >98% viable by exclusion of trypan blue. Cells prepared in this way are predominantly lymphocytes, although IEL preparations contain significant numbers of intestinal epithelial cells, as judged by light microscopy, light scatter properties in the flow cytometer, and staining with specific mAb. Final T lymphocyte cell numbers were determined from the total cell yield, which was corrected for the percentage of $CD3^+$, donor haplotype cells as determined by flow cytometry.

Flow cytometric analysis of lymphocyte populations

Following purification of T cells from engrafted SCID mice or from donor mice, cells were resuspended at a concentration of at least 1×10^5 cells/ml in PBS staining buffer containing 2% BSA (w/v) and 0.02% NaN_3 (w/v). Pretitrated mAb, either unconjugated or directly conjugated to FITC, phycoerythrin (PE), or biotin, were added to cell suspensions at 4°C and incubated for 20 to 30 min. All directly conjugated mAb to various surface Ags were purchased from PharMingen (San Diego, CA), while streptavidin Tricolor was purchased from CalTag (South San Francisco, CA). The mAb used for these studies included FITC-labeled or biotinylated anti-CD3ϵ (145-2C11) (33), FITC- or PE-labeled anti-CD4 (GK1.5) (34), FITC- or PE-labeled or biotinylated anti-CD8α (53-6.7) (35), PE-labeled anti-CD8β (53-5.8) (35), PE-labeled CD45R/B220 (RA3-6.32) (36), biotinylated anti-L-selectin (CD62L; MEL-14) (37), biotinylated anti-CD-69 (H1.2F3) (38), FITC-labeled anti-TCR $\alpha\beta$ (H57-597) (39), PE-labeled anti-mouse integrin α_{IEL} (M290) (40), and biotinylated anti-K^b (AF6-88.5) or anti-K^d (SF1-1.1). Cells were incubated in Ab staining buffer at 4°C for 20 min with the primary mAb, washed twice, and then incubated with the secondary reagent for an additional 20 min at 4°C. At the completion of the staining reaction, cells were washed as described above and resuspended in fixative (PBS buffer with 0.02% NaN_3 (w/v) and 1% (w/v) paraformaldehyde) until analysis by flow cytometry. The samples were run on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) in the University of California-Los Angeles Flow Cytometry Core Facility. IEL were distinguished from epithelial cells based on their size and granularity as they appeared in the forward angle and side angle light scatter

setting. Between 1500 and 5000 events in the lymphocyte gate were acquired.

Histologic and immunocytochemical studies

Segments of large and small intestines were excised and cleaned as described in the previous section. One- to two-centimeter sections of intestine were either fixed in 10% formalin (Sigma Chemical Co.) or immersed in Optimal Cutting Temperature Compound (Miles, Inc., Elkhart, IN) and snap-frozen in liquid nitrogen. Formalin-fixed tissue was embedded in paraffin, and 5-μm sections were prepared and stained with hematoxylin and eosin by the Core Pathology Laboratory at University of California-Los Angeles or by the Pathology Associates Laboratory (Los Angeles, CA). Frozen sections were cut at 5-μm thickness on a cryostat microtome and placed on glass slides. These sections were air-dried and fixed in acetone. Before mAb staining, frozen tissue sections were rehydrated with PBS and then blocked with 20% normal rabbit serum (Vector Laboratories, Inc., Burlingame, CA) in PBS at 4°C for 20 min before incubation with pretitrated CD8α mAb 53-6.7 in PBS supplemented with 2% normal rabbit serum for 60 min at 4°C. The sections were rinsed twice in PBS at 4°C and incubated with pretitrated biotinylated rabbit anti-rat IgG (Vector Laboratories) for 30 min at 4°C. Following incubation, samples were washed and incubated with avidin-horseradish peroxidase and then substrate as described by the manufacturer (Vectastain Elite, ABC Kit, Vector Laboratories). Before analysis, frozen sections were counterstained with Coomassie blue (Sigma Chemical Co.). Stained tissue sections were photographed through a Nikon Labophot microscope with attached camera (Nikon, Melville, NY).

Proliferation assays

Microtiter plates were precoated with a solution of protein G column-purified CD3ϵ mAb (145-2C11; a gift from Dr. H. Holcombe, Yale University, New Haven, CT) in PBS. The plates were incubated at 37°C for 2 h. The wells were subsequently washed three times with RPMI 1640 (Tissue Culture Media Center, University of California-Los Angeles). To measure proliferation, freshly isolated IEL, lymph node, or spleen cells from normal mice or engrafted SCID mice were plated at a density of 2.0×10^5 cells/well in flat-bottom microtiter plates (Corning, Corning, NY). The cultures, with or without CD3 mAb precoating, were conducted in 200 μl of complete RPMI with 10% FCS and gentamicin (20 μg/ml; Sigma Chemical Co.). Cells were incubated at 37°C with 5% (v/v) CO_2 for 48 h, and then pulsed for 20 h, with the addition of 1 μCi/well of [^{3}H]TdR (New England Nuclear-DuPont, Wilmington, DE). The cultures were harvested with an automated cell harvester, and [^{3}H]TdR incorporation measured by liquid scintillation counting.

Redirected lysis assays

Fc receptor-positive P815 target cells (DBA/2, H-2^d) were prepared for use by incubating 1×10^6 cells with 200 μCi of $Na^{51}Cr_2O_3$ (New England Nuclear-DuPont) for 4 h at 37°C with 5% (v/v) CO_2. Labeled target cells were washed four times and plated at 1×10^4 cells/well in U-bottom 96-well microtiter plates (Costar, Cambridge, MA). SI-IEL prepared from lymph node-transplanted mice or normal lymph node spleen cells prepared from (C57BL/6 × BALB/c) F_1 mice were titrated in separate wells to give E:T cell ratios ranging from 0.6:1 to 46:1, depending on the experiment, in a total volume of 200 μl. Where indicated, CD3 mAb was added at the same time as effector cells to give a final concentration of 10 μg/ml. The assays were incubated at 37°C with 5% (v/v) CO_2. After 6 h, 100 μl of supernatant was collected and counted in a gamma counter. The percent specific killing was calculated as the sample release (in counts per minute) minus the spontaneous release (in counts per minute) divided by the total release (in counts per minute) minus the spontaneous release (in counts per minute). Spontaneous release was measured in supernatants collected from labeled target cells incubated with medium alone, while total release was measured in supernatants collected from labeled target cells after treatment with 2% (v/v) Triton X-100 (Sigma Chemical Co.). Spontaneous release was <10% of maximal release in all experiments.

Results

Donor T cells from lymphoid organs populate the intestines of SCID mice

We and others have developed a model system to study mucosal T cell engraftment following injection of lymph node or spleen cells into SCID mice (27–31). This model permits the introduction of exogenous lymphocytes without the need for irradiation or surgery, and the use of semisyngeneic, F_1 donors permits the quantitation of donor-derived cells without the risk of a graft-vs-host reaction. By injecting as few as 2 to 5×10^5 T cells from semisyngeneic $CB6F_1$ mice obtained from either the inguinal lymph nodes or a mixture of peripheral lymph nodes excluding the mesenteric lymph nodes into the peritoneum of C.B-17 SCID mice, we were able to detect donor T cell engraftment in the SCID intestine. This engraftment plateaus at 6 to 8 wk postinjection and lasts for at least 20 wk, which was the longest time elapsed before the analysis of a SCID recipient. A representative two-color immunofluorescence analysis of cells from IEL and LPL of the small and large intestines from an individual mouse (Fig. 1) demonstrated that nearly all the CD3$^+$ lymphocytes in the intestinal mucosa of the SCID recipient expressed the donor parental MHC haplotype (K^b), and that nearly all the donor-derived cells in the intestine were CD3$^+$ T cells. The preparations also contained CD3$^-$, K^{b-} cells that were host derived; some of these may have been immature T cell precursors that may arise in the intestine and cannot differentiate further in the SCID host (41). In some hosts we observed an increase in the number of such SCID host, CD3$^-$ cells following lymphocyte transfer (data not shown). This may occur as a result of T cell-derived cytokines.

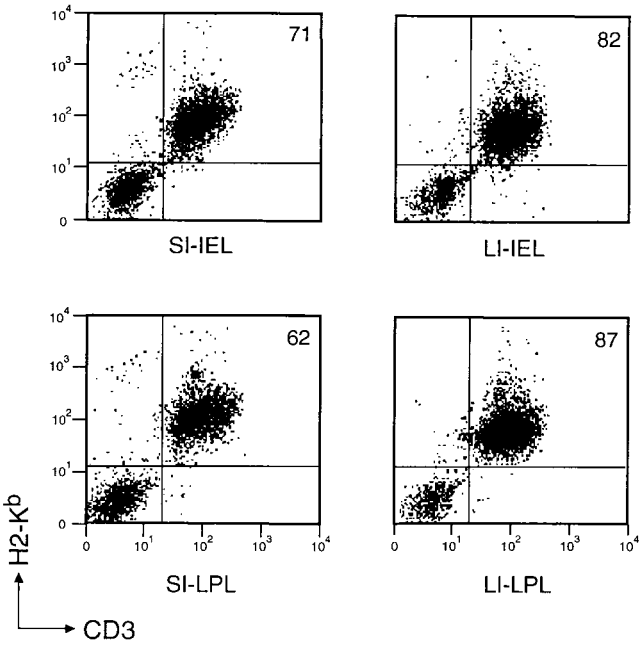

FIGURE 1. Lymph node T cells are engrafted throughout the intestinal mucosa of SCID mice. IEL and LPL were prepared from the small and large intestines of an individual recipient C.B-17 SCID mouse 6 wk after transfer of unfractionated $CB6F_1$ lymph node cells. Isolated mucosal lymphocyte populations were stained with mAb anti-CD3ϵ-FITC and anti-H2-K^b biotin followed by streptavidin tricolor to identify donor T cells. Representative data are shown from 1 of >50 similar analyses.

Histologic analysis of hematoxylin- and eosin-stained sections of the small and large intestines from SCID mice (Fig. 2*A*) confirmed the presence of increased numbers of mononuclear cells in the engrafted SCID intestine. This increase is most apparent in the lamina propria of the lymph node cell-engrafted SCID mice (*right panel*) compared with the unmanipulated control SCID intestine (*left panel*). IEL are somewhat more difficult to visualize by this method. Sections, therefore, were stained with CD4 and CD8 mAbs to more clearly detect and define T lymphocytes present in the SCID recipients. Numerous CD8$^+$ cells

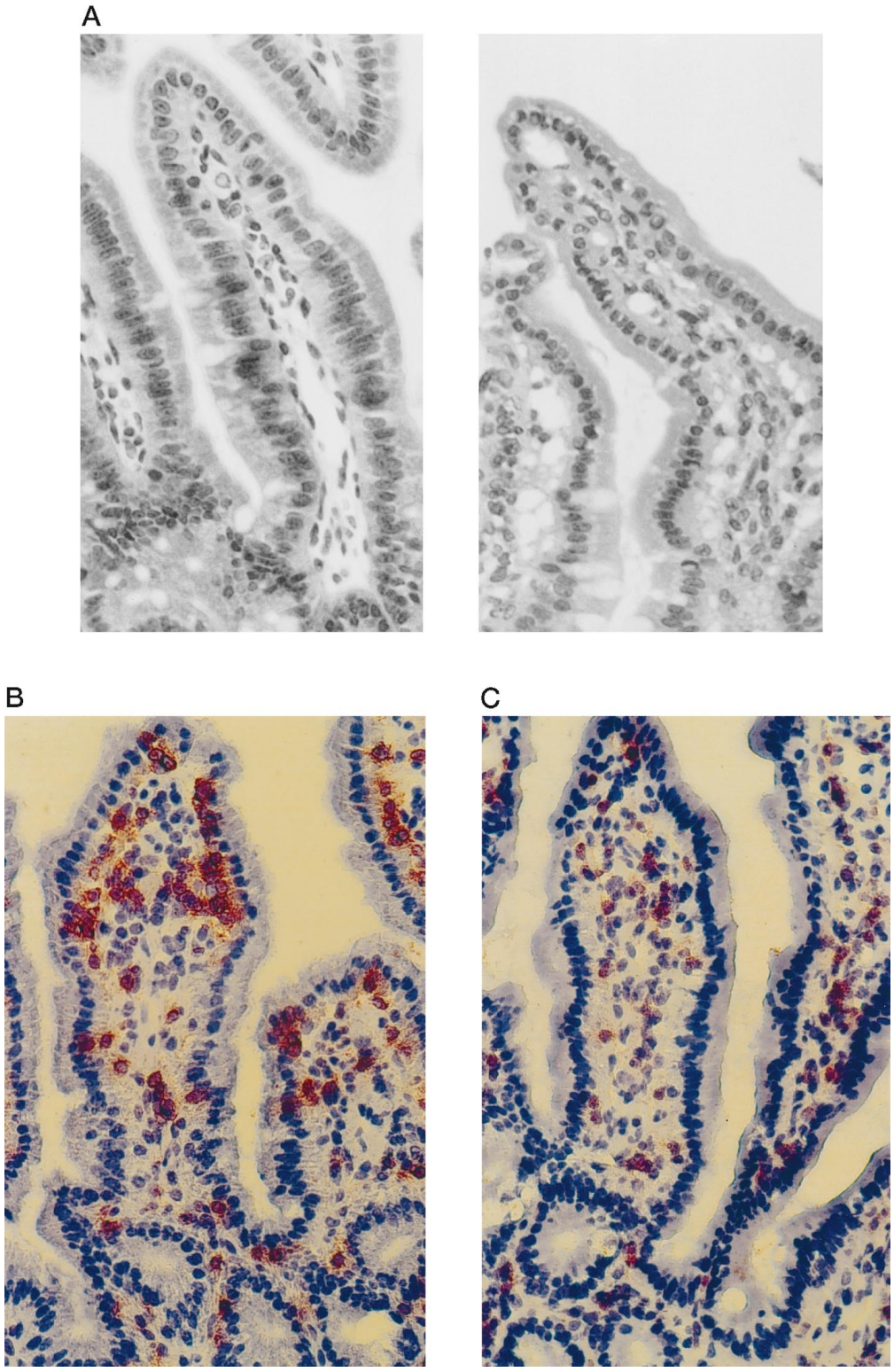

FIGURE 2. Histologic and immunohistochemical analysis of T cells engrafted in the SCID intestine. *A*, Analysis of hematoxylin- and eosin-stained sections. The *left panel* shows the intestine of an unmanipulated SCID mouse. There is a paucity of mononuclear cells. The *right panel* shows the intestine of a lymph node-engrafted SCID mouse with mononuclear cells particularly evident in the lamina propria. *B*, Immunostaining of a section of lymph node-engrafted small intestine from a SCID mouse 8 wk after transfer demonstrates $CD8^+$ cells present in the epithelium and lamina propria. *C*, CD4 immunostaining of the small intestine from the same SCID recipient as that in *B* reveals $CD4^+$ cells located primarily in the lamina propria. Original magnification, ×400. Control staining of unmanipulated SCID mice and staining with isotypic control Abs were negative (data not shown).

could be detected within the intestinal epithelium and the lamina propria of reconstituted mice (Fig. 2*B*). $CD4^+$ cells were most numerous within the lamina propria (Fig. 2*C*), consistent with the compartmental distribution of T cells in engrafted SCID mice as determined by flow cytometry (Table I and see below). $CD3^+$ and $CD4^+$ cells were not detectable in unmanipulated control SCID mice, while $CD8^+$ cells were infrequent (data not shown).

Table I. *Differential expansion of CD4$^+$ and CD8$^+$ cells in different sites in SCID recipients following transfer of lymph node T cells*

Source	CD4$^+$	CD8$^+$	CD4$^+$/CD8$^+$	Ratio CD4:CD8	*n*
Lymph node	45 ± 10	25 ± 4	0 ± 0	1.80	11
Reconstituted SCID					
SI-IEL	17 ± 9	53 ± 19	11 ± 1	0.32	12
LI-IEL	27 ± 9	32 ± 10	6 ± 4	0.84	10
SI-LPL	30 ± 11	34 ± 14	6 ± 5	0.88	10
LI-LPL	33 ± 13	32 ± 15	5 ± 4	1.03	11
Spleen	20 ± 9	7 ± 6	1 ± 1	2.86	11

Mature T cells expand in vivo following transfer

All intestinal mucosal compartments were highly populated with T lymphocytes in transplanted SCID mice, and the transferred lymph node-derived T cells were capable of expanding in vivo. A quantitative analysis of IEL prepared from the small intestines of individual, lymph node- or spleen cell-engrafted mice is presented in Figure 3. The recipient SCID mice achieved at least normal IEL cellularity by 6 to 8 wk post-transfer and were in fact, on the average, slightly hypercellular. While nearly 1×10^6 T cells were purified, on the average, from the small intestinal epithelium of $CB6F_1$ control mice, individual lymph node-engrafted SCID mice gave rise to an average of 2.8×10^6 SI-IEL (Fig. 3). This increase was statistically significant, but despite the increased numbers of cells, the recipients did not show any signs of intestinal inflammation. The large intestinal epithelium of the SCID recipients contained approximately 2-fold more T cells than were recovered from $CB6F_1$ control mice (Fig. 3), also indicative of a significant repopulation, although this increase was not statistically significant. These data clearly show that nonmucosal T cells efficiently engraft and expand to normal levels, or greater than normal levels, in the host. Including the lamina propria and the intestinal epithelium, the total number of T cells purified from all intestinal mucosal compartments in SCID mice was approximately 7- to 10-fold greater than that in the injected population, demonstrating that the donor cells expand in vivo. This expansion may occur primarily in the intestine or elsewhere. In contrast, the spleen was relatively hypocellular, containing nearly 10-fold fewer T cells than were recovered from control $CB6F_1$ mice (Fig. 3). It is not known why repopulation of the intestine is more efficient than repopulation of the spleen. CD3$^-$, B220$^-$ cells in the spleen of SCID recipients could be either immature T or B lymphocytes, which are known to be present in SCID mice (42). Peyer's patches and lymph nodes were macroscopically not detectable in the recipient SCID mice at any time point, including points earlier than the 6 wk required for maximal colonization of the host intestine.

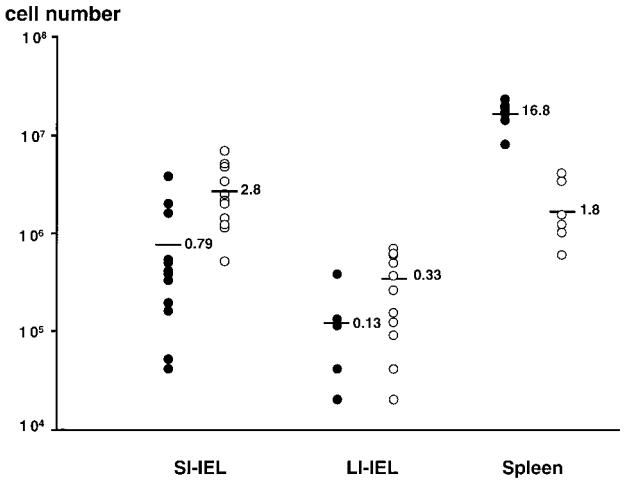

FIGURE 3. Donor lymph node T cells expand within all mucosal compartments of transplanted SCID mice. CD3$^+$, H2-K^{b+} $CB6F_1$ donor cells were enumerated after isolation from the epithelium of the large and small intestines of individual recipient C.B-17 SCID mice. Lymphocytes were obtained 6 to 8 wk following injection with lymph node lymphocytes, and the numbers obtained were compared with the numbers of CD3$^+$ T lymphocytes isolated from the same compartments of control, immune-competent $CB6F_1$ mice. Open circles indicate lymph node-engrafted SCID mice; closed circles indicate unmanipulated control $CB6F_1$, immune-competent mice. Data from a single series of experiments conducted at approximately the same time are shown, with the mean T lymphocyte numbers ($\times 10^6$) indicated. The data were analyzed using Wilcoxon scores (rank sums). $p = 0.0016$ for the increased SI-IEL in SCID recipients compared with that in normal mice, $p = 0.20$ comparing LI-IEL in SCID recipients to the number in normal mice, and $p = 0.0014$ for the decrease in spleen cells in the SCID recipients compared with the number in normal mice.

Lymph node T cells resident in the SCID intestine have a mucosal phenotype

The efficiency with which peripheral lymph node T cells established intestinal lymphocyte compartments in engrafted SCID mice prompted us to ask whether engrafted populations displayed surface Ags characteristic of the T cells in this location. We found that engrafted SI-IEL, like normal SI-IEL, expressed the mucosa-specific integrin, α_{IEL} (also known as α_E), and the activation Ag, CD69, and showed concurrent loss of L-selectin (CD62L), the lymph node-homing receptor. Data from a representative experiment are shown in Figure 4. Nearly all splenic and lymph node lymphocytes from donor $CB6F_1$ mice expressed CD62L, while only 2% had α_{IEL} and 12% expressed the activation Ag CD69 (Fig. 4, *top row*). By contrast, in this representative experiment depicted in the *middle row* of Figure 4, the progeny of the donor population that localized in the small intestinal epithelium were 89% α_{IEL} positive and 78% CD69 positive, with only 5% expressing CD62L. This profile is very similar to that of normal IEL (Fig. 4, *bottom row*). In addition, similar to normal IEL, CD2 levels were decreased or absent in the engrafted SI-IEL, and the engrafted CD8$^+$ IEL were CD28 negative. The donor-derived IEL were nearly all Thy-1$^+$ (data not shown), which is consistent with their being thymus derived or activated (4, 8). Spleen cells in the engrafted SCID mice had a phenotype intermediate between those of normal spleen and engrafted SI-IEL. They expressed high levels of CD2 and Thy-1, up to 30% were CD69 and α_{IEL} positive, but they were mostly CD62L negative (data not shown). As CD69 and α_{IEL} can be acquired by activated T cells (43), while CD62L is down-regulated, this suggests that donor T cells in the spleen of SCID recipients are comprised partially of activated or memory cells, and perhaps that only those T cells are capable of long term survival in the SCID hosts.

Although the data indicate that the cell populations present in the different intestinal compartments of the lymphocyte-engrafted SCID mice generally are representative of the T cells normally found there, they do not precisely recapitulate the normal situation. For example, TCR $\gamma\delta^+$ lymphocytes are very infrequent, and TCR $\alpha\beta^+$ and CD8$\alpha\alpha^+$ single-positive cells are essentially undetectable (data not shown). The low frequency of TCR $\gamma\delta^+$ and the absence of TCR $\alpha\beta^+$, CD8$\alpha\alpha$ single-positive cells in the SCID host intestine probably reflect the absence of these T cell subsets in lymph nodes, as these subpopulations are well represented in the SCID intestine following transfer of unfractionated SI-IEL populations (B. Sydora et al., manuscript in preparation).

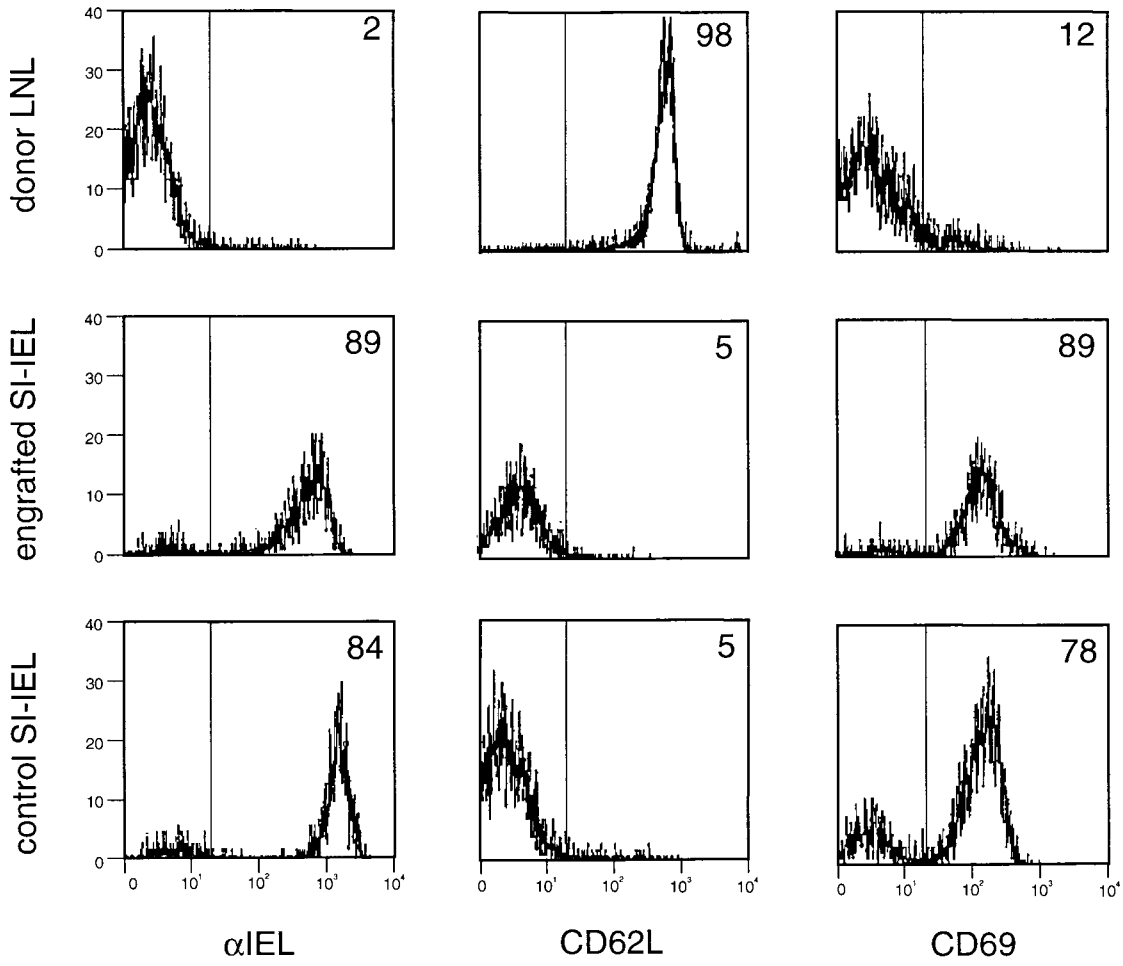

FIGURE 4. Donor lymph node T cells acquire a phenotype similar to that of IEL once engrafted within the intestinal epithelium of SCID mice. The expression of α_{IEL}, CD62L, and CD69 on unfractionated, $CB6F_1$ donor lymph node lymphocytes (LNL; *top row*) was compared with the expression of these three Ags on SI-IEL isolated from an individual representative C.B-17 SCID mouse following transfer of unfractionated lymph node cells (*middle row*) and from SI-IEL prepared from a control $CB6F_1$ mouse (*bottom row*). Cells were prepared and stained as described in *Materials and Methods*. The percentages refer to the fraction of total cells staining positive for each Ag, with the line denoting background fluorescence with control Ab. Representative results are shown from one of many experiments.

Regional specialization of lymphocytes engrafted in the SCID host intestine

A degree of regional specificity of T cell homing and expansion in SCID mice is suggested by the $CD4^+$ and $CD8^+$ composition of the T cell populations isolated from the intestinal epithelium, lamina propria, and spleen. The data are summarized in Table I. In this analysis, any contaminating TCR-$CD8^+$ host-derived T cells were excluded by gating on the K^{b+} donor-derived population. The average ratio of CD4 to CD8 T cells in the donor lymph node population was 1.8:1. Engrafted LPL display, on the average, equal proportions of $CD4^+$ and $CD8^+$ T cells in both small and large intestines (ratios of 0.88 and 1.03, respectively; Table I). Donor T cells purified from the SCID spleen favored $CD4^+$ lymphocytes with an average ratio of 2.9 (Table I). In contrast to these results, $CD8^+$ T cells were selectively enriched in SI-IEL, with an average CD4:CD8 ratio of 0.32 (Table I). The LI-IEL showed a more equal ratio of CD4 and CD8 cells (ratio = 0.84), similar to the LPL populations. In addition, as in the intestine of normal mice (3, 14), a distinct population of $CD4^+/CD8^+$ double-positive T cells was evident among the IEL of engrafted SCID mice. These cells were infrequent among engrafted SCID LPL, and they were largely absent from the engrafted spleen and the original donor population (Table I). In summary, these results demonstrate that in the engrafted SCID mice, homing and expansion of lymphocytes favor those T cells with the CD8 phenotype in mucosal tissues compared with spleen, with the greatest selection for $CD8^+$ lymphocytes in SI-IEL. These results are qualitatively similar to the distribution of CD4 and $CD8^+$ cells observed in normal mice (4, 15–17).

Lymphocytes engrafted in the intestine re-home specifically to the intestine

We have reported previously that IEL re-home preferentially to the intestine when transferred into SCID mice (25). To determine whether the T lymphocytes engrafted in the intestine of SCID mice behave like normal intestinal mucosal lymphocytes with regard to intestine-specific homing, we performed secondary transfers. SI-IEL were isolated from SCID mice that were given lymph node cell suspensions 8 to 10 wk earlier and were transferred to secondary SCID recipients. Individual primary and secondary recipient SCID mice were analyzed, and the results obtained are summarized in Figure 5. As noted above, a primary transfer of lymph node T cells led to the establishment of T lymphocytes in both the epithelium of the small intestine and the spleen of the SCID recipient (Figs. 3 and 5). The epithelium in these primary SCID

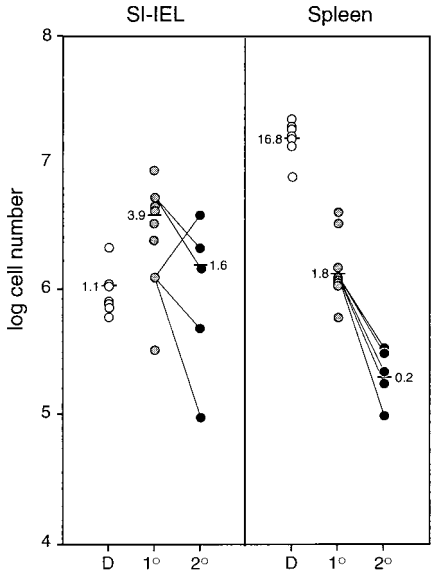

FIGURE 5. Lymph node-derived T cells resident in the SCID intestine re-home preferentially to the intestine. Lymph node lymphocytes prepared from $CB6F_1$ mice were transferred to C.B-17 SCID mice. Eight to ten weeks post-transfer, SI-IEL were prepared from individual SCID mice (primary recipients) and counted, and SI-IEL from two of the primary recipients were transferred to a total of five secondary SCID recipients. SI-IEL and splenocytes were isolated from individual mice and stained with mAb anti-CD3ϵ and H2-K^b to identify donor-derived T cells. Open circles indicate T cell numbers from control $CB6F_1$ mice (D = donors), gray circles indicate T cell numbers from primary SCID recipients of lymph node cells (1° = primary), and filled circles indicate T cell numbers obtained in secondary SCID recipients following transfer of SI-IEL from two of the primary recipients (2° = secondary). The lines connect the individual primary donors with the individual secondary recipients who received their SI-IEL. Data were analyzed using Wilcoxon scores (rank sums). The number of SI-IEL in a secondary SCID recipient was not significantly different from that in control mice ($p = 0.74$) or that in a primary recipient of lymph node donor cells ($p = 0.12$). In contrast, the average number of splenic T cells was significantly reduced compared with either the number in a normal, control mouse ($p = 0.006$) or the number in a primary recipient ($p = 0.004$).

recipients was relatively hypercellular with respect to lymphocytes, yielding an average of 3.9×10^6 T cells (Fig. 5), which is approximately 3.5 times as many SI-IEL as were found in a normal, CB6 F_1 control, immune-competent mouse in this series of experiments. The spleen, however, was relatively hypocellular with respect to T lymphocytes, with an average of only 1.8×10^6 donor T cells present. This is about 1/10th the number of T cells found in normal mice. When transfer of SI-IEL from two primary SCID recipients to a set of secondary SCID recipients was conducted, there was only a small reduction in the donor-derived SI-IEL cell number in these secondary recipients, to an average of 1.6×10^6 T cells. The difference in the average number of IEL in these secondary recipients is not statistically significant compared with the average numbers in the control mice or in the primary recipients. By contrast, nearly 10-fold fewer donor-derived T cells were present in the spleen of the secondary recipients compared with those in the primary recipients, with an average of only 2×10^5 cells (Fig. 5). This decrease in average cell number in the secondary recipients was highly statistically significant. It therefore appears that T cells purified from nonmucosal sites undergo a functional differentiation, and that they are subsequently limited in their ability to re-establish nonmucosal populations in the spleen. The relatively intestine-specific homing pattern of these lymph node-derived T cells in SCID mice is similar to the pattern obtained following transfer of intestinal mucosal T cells from normal mice to SCID recipients (25) (B. Sydora et al., manuscript in preparation).

Lymphocytes engrafted in the intestine proliferate poorly in response to mitogenic stimulation

It has been demonstrated in a number of studies that mucosal lymphocytes proliferate poorly in response to mitogenic stimuli or stimulation with polyvalent anti-CD3 mAbs (9, 10, 44). Some subpopulations of mouse SI-IEL, such as those that express CD2 or those that express Thy-1, retain a somewhat greater proliferative capacity than the bulk of the population of IEL (8, 45). Like IEL from control mice, IEL populations isolated from SCID mice engrafted with lymph node T cells proliferated poorly in response to TCR stimulation with CD3 mAb compared either with the donor population or with donor-derived T cells located in the spleen (Fig. 6). The donor-derived cells that located to the SCID recipient spleen produced twofold fewer counts per minute than normal splenocytes, but this difference was not statistically significant.

Lymphocytes engrafted in the intestine exhibit ex vivo cytotoxic activity

The presence of cytoplasmic granules in populations of SI-IEL (12, 46) and their de novo cytolytic activity when these cells were

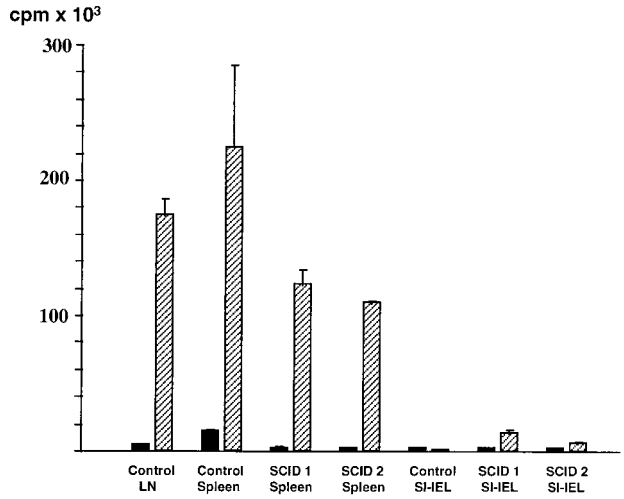

FIGURE 6. IEL prepared from lymph node-engrafted SCID mice proliferate poorly. SI-IEL and splenocytes isolated and prepared in parallel from two individual lymph node-engrafted C.B-17 SCID mice (SCID 1 and SCID 2) were plated in triplicate in wells coated with anti-CD3ϵ mAb. [^{3}H]TdR incorporation was compared with that in lymphocytes taken from the lymph node (LN), spleen, and SI-IEL of individual control $CB6F_1$ mice assayed under the same conditions. Filled bars indicate no CD3 stimulation; hatched bars indicate CD3 stimulation. Data from one representative experiment of three are shown. The SEM is noted by the error bars, and the data were analyzed using Student's two-tailed *t* test. Spleen cells from SCID recipients proliferated significantly in response to CD3 cross-linking compared with those cultured in medium ($p = 0.005$ and $p = 0.009$ for mouse 1 and mouse 2, respectively). The proliferation of normal spleen cells in response to CD3 was not statistically significant compared with the proliferation of spleen cells from SCID recipients in response to CD3 ($p = 0.11$ and $p = 0.09$ for mouse 1 and mouse 2, respectively). IEL did not respond well to CD3; the proliferation of IEL from the reconstituted SCID mice following culture with CD3 mAb was only significantly above the background for one of the two recipient mice. In the recipient SCID mice, the difference between CD3-induced proliferation in spleen cells and that in IEL was statistically significant ($p = 0.003$ and $p = 0.0003$ for mouse 1 and mouse 2, respectively).

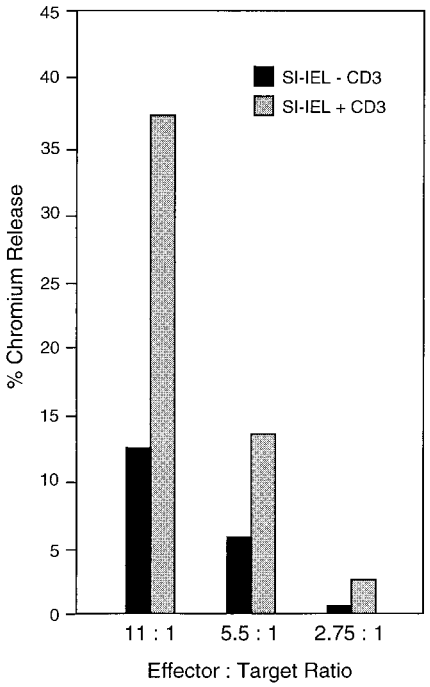

FIGURE 7. Cytolytic activity of SI-IEL prepared from lymph node-transplanted SCID mice. Freshly isolated SI-IEL were prepared from a single $CB6F_1$ lymph node-transplanted C.B-17 SCID mouse 8 wk after transfer. SI-IEL were mixed with ^{51}Cr-labeled P815 target cells at the E:T cell ratios noted on the abscissa. Effectors are corrected for the total $CD3^+$ $H\text{-}2K^{b+}$ cells in the preparation, determined as described in *Materials and Methods*. The data are expressed as the percent specific lysis calculated as noted in *Materials and Methods*. These experimental data are representative of three different experiments.

freshly isolated (10, 45, 47) suggest that SI-IEL are cytolytic effector cells in vivo. This cytotoxic activity could be measured in a redirected lysis assay, in which polyclonal stimulation of cytotoxic effectors was achieved by binding anti-TCR mAbs to the Fc receptor of ^{51}Cr-labeled targets (48). Unlike SI-IEL, $CD8^+$ T cells isolated from the lymph node and spleen did not exhibit cytotoxic activity in a redirected lysis assay, unless they had undergone a prior Ag stimulation. To determine whether peripheral lymph node T cells that had populated the small intestine of a SCID host were functionally similar to resident SI-IEL, these engrafted lymphocytes were tested in a redirected lysis assay. Killing of CD3 mAb-coated P815 targets by lymph node donor-derived SI-IEL was obtained at E:T cell ratios as low as 5.5:1 (Fig. 7). In contrast, spleen or lymph node from control CB6 F_1 mice or IEL prepared from the large intestine or spleen cells from engrafted SCID mice showed no chromium release, with or without CD3 mAb, at all E:T cell ratios tested (data not shown).

Optimal engraftment of mucosal lymphocytes requires bacterial flora

To define factors that may be responsible for the migration of lymph node-derived T lymphocytes to intestinal tissue, we have examined the role of bacterial colonization in the engraftment of intestinal T cells in SCID mice. Lymph node cells were transferred to SPF SCID mice, which had not been infected with any of a set of known mouse pathogens but had normal intestinal flora, and to RF SCID mice, which are close to being germfree (see *Materials and Methods*). The recipients were analyzed 6 to 10 wk later for the presence of $CD3^+$, K^{b+} T lymphocytes in the intestine. The results from a representative flow cytometric analysis of individual SCID recipients are presented in Figure 8*A*. The two RF SCID mice shown had only 23 and 32% of $CD3^+$, $H\text{-}2K^{b+}$ cells within the SI-IEL preparation compared with 86 and 82% from two individual SPF SCID recipients. This representation understates the true difference, as the total cell yields were greatly reduced when the preparations were obtained from RF SCID mice. Quantitative analysis of cell yields of IEL and LPL purified from the large and small intestines of the two types of engrafted SCID mice showed that the average cell numbers were reduced 45-fold among SI-IEL in RF compared with those in SPF recipients, while LI-IEL were, on the average, 3-fold lower, SI-LPL were 20-fold lower, and LI-LPL were 10-fold lower than those in SPF controls (Fig. 8*B*). The overall difference in cell number between RF and SPF SCID mice

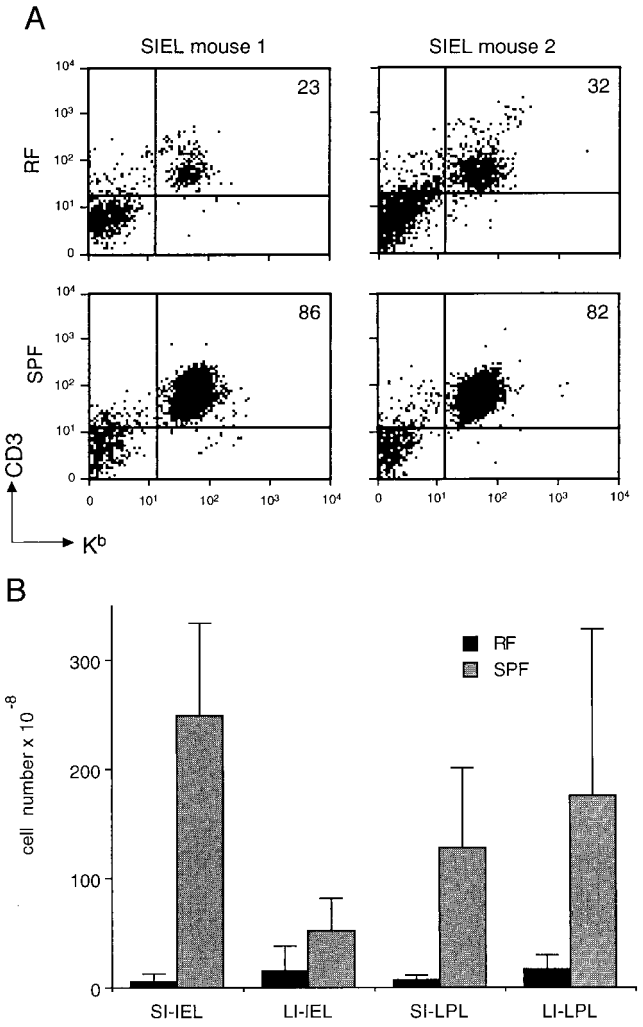

FIGURE 8. Bacterial flora enhance engraftment of T cells within the intestinal mucosa of SCID mice. *A*, Representative flow cytometric analysis of SI-IEL from two RF C.B-17 SCID compared with two SPF C.B-17 SCID that were engrafted with $CB6F_1$ lymph node lymphocytes 8 wk earlier. The cells recovered from the intestine were stained with anti-CD3ϵ-FITC and anti-$H\text{-}2K^b$-biotin followed by incubation with streptavidin tricolor. *B*, Quantitative analysis of the donor-derived, $CD3^+$, $H\text{-}2K^{b+}$ lymphocytes, recovered from the small and large intestinal epithelia and the small and large intestinal lamina propria of engrafted RF SCID mice (solid bars) compared with those of engrafted SPF SCID mice (gray bars). Each bar represents the average number of lymphocytes isolated from four mice that were analyzed individually with SEM indicated by the error bars. A three-way analysis of variance (ANOVA) was performed on log-transformed data. The difference between RF and SPF recipients was significant ($p = 0.0001$) by this method.

was statistically significant ($p = 0.0001$). In summary, these results show that optimal engraftment of T cells into intestinal mucosal compartments is influenced by bacterial flora. The primary residence of diverse, anaerobic flora within the small intestine (49) and their absence in the RF colony suggest that this class of bacterial flora plays a major role in the establishment of SI-IEL following lymphocyte transfer to SCID mice, although other mucosal T cell populations also were greatly reduced in the RF SCID recipients.

Discussion

Previous experiments have indicated that T cells derived from Peyer's patch and mesenteric lymph node can give rise to IEL and LPL upon adoptive transfer into SCID mice (26, 27, 46), although the degree to which these cells resemble normal IEL and LPL has not been fully characterized. Outside of cases where inflammation is induced by cell transfer, it is less certain whether T cells derived from peripheral lymph node or spleen can faithfully establish IEL and LPL populations following transfer (reviewed in Ref. 50). Furthermore, the degree to which resident IEL and LPL in intact mice are derived from the typical, post-thymic, recirculating T cells remains to be determined.

We show here that lymphocytes derived from the peripheral lymph node can home to the intestinal epithelium and lamina propria in SCID mice, where they provide a long term reconstitution of mucosal T cells. Although the data reported resulted from transfer of peripheral lymph node cells, transfer of spleen cells gave essentially similar results. T lymphocytes are capable of populating the SCID host intestine, but, in the doses used, B cells are not. The selective reconstitution of the lymphoid system together with the results from phenotypic analysis of the donor-derived T cells in the SCID intestine indicate that the ability to populate the intestine is a function of mature, CD4 single-positive and CD8$\alpha\beta$ single-positive T cells. This is supported by the results obtained following transfer of either sorted CD4 or sorted CD8 single-positive T cells in our studies and those of others (28–31).

The population of the SCID host with donor-derived T cells is more efficient in the intestine than in the spleen. The intestine shows increased numbers of T cells compared with those in normal, control mice, while the spleens of the SCID recipients have approximately 10-fold fewer T cells than those found in control spleens. We speculate that the population of long term surviving cells in the SCID host may be enriched for memory cells, and that some of these cells may localize preferentially to the intestine on account of exposure to intestinal flora.

Results from previous experiments have shown clearly that cells activated in the Peyer's patch of normal mice home to the intestinal epithelium (46, 51). In addition, we have found that transfer of Peyer's patch lymphocytes to SCID mice does lead to the generation of IEL and LPL populations in the recipients, although this process was not particularly efficient (data not shown). The absence of detectable Peyer's patches and lymph nodes in the SCID recipients suggests that this Peyer's patch to intestine migration pathway is not obligatory for the long term reconstitution of IEL and LPL. The presence of intestinal lymphocytes in *aly* mutant mice lacking Peyer's patches (52) also is consistent with the possibility that IEL need not be derived from activated Peyer's patch lymphocytes. Although we could not detect visible Peyer's patches at any time point, we cannot exclude the possibility that small numbers of lymphocytes reside there transiently before going on to the lamina propria and epithelium. This is consistent with results from short term assays showing binding of peripheral T cells to Peyer's patch high endothelial venules (53).

The factors that prevent IEL from efficiently re-entering the peripheral T cell pool following secondary SCID transfer (25) (Fig. 5) are not known. It is unlikely that SI-IEL are restricted due to their lack of L-selectin, as we found L-selectin-negative T cells in the spleen of engrafted mice, and residence within the spleen was enhanced in L-selectin knockout mice (54) and in mice treated with the anti-L-selectin mAb MEL-14 (55). The marked reduction of SI-IEL and LPL in β_7-deficient mice (56) suggests that integrins containing β_7, paired with either α_4 or α_{IEL}, facilitate entry of T cells into the epithelial layer (57) or may function to tether cells in this location (58). This is in agreement with the enrichment of β_7-positive T cells in the SCID host intestine following lymph node cell transfer. Additional integrins may be involved in intestinal localization, as suggested by recent studies of β_2 integrin and intercellular adhesion molecule-1-deficient mice (59).

Several experimental results support an important role for T cell activation in mucosal colonization of the SCID host. First, we found that the majority of lymph node-derived T cells in the intestinal mucosa constitutively express the activation Ag CD69. Second, we demonstrated that bacterial flora were required for the efficient colonization of the SCID host intestine. It remains possible that a decrease in mucus or other factors in the intestine of RF mice could have effected the cell yields obtained following mucosal T cell preparation rather than the actual cell number present. This is not likely, however, to explain the >10-fold decrease in cells colonizing most compartments of the intestine in the RF recipients, particularly LPL, which are separated from the mucus. Bacterial flora might provide a source of Ag for the activation and expansion of T lymphocytes. The requirement for diverse bacterial flora for the establishment of cells within the intestine of engrafted SCID mice is similar to what has been observed in normal, intact mice, particularly for TCR $\alpha\beta^+$ SI-IEL (60, 61).

It is not known whether the T cell expansion observed in the SCID host occurs systemically or primarily in the intestine. Several earlier observations are consistent with a systemic expansion. First, intestinal inflammation is a major complication of graft-vs-host disease (46, 62), and recirculating T cell blasts activated by MHC-encoded alloantigen have been shown to traffic to the intestine (63). Second, systemic infection with lymphocytic choriomeningitis virus leads to the presence of lymphocytic choriomeningitis virus-specific CTL in SI-IEL, although viral Ag could not be detected in the intestinal epithelium (64). On the other hand, studies comparing oral and systemic administrations of intestinal viral pathogens, reovirus, and rotavirus showed that oral administration of either virus was more efficient in the development of virus-specific CTL among Peyer's patches or IEL (51, 65) and in the inflammation model in which SCID recipients develop colitis, most T cell proliferation occurred in the intestine (66).

By a variety of criteria, the T lymphocytes that populate the SCID intestine are similar to the T cells normally found there. These criteria include the pattern of expression of cell surface proteins, the preferential re-homing of the donor-derived T cells to the intestine, and the functional consequences of TCR cross-linking, notably the acquisition of cytolytic activity. There are two possible explanations for the differences between the donor population and the T cells that ultimately populate the SCID intestine. First, factors in the SCID host might lead to marked phenotypic and functional changes in the donor T lymphocytes found in the intestine. Second, a very few T cells with a mucosal phenotype, i.e., CD69$^+$, $\alpha_{IEL}{}^+$, and CD62L$^-$, might already be present in the lymph node, and they might be selectively expanded in the intestine following transfer. Because some of the populations found in the intestine, such as CD4, CD8$\alpha\alpha$ double-positive IEL, are not detectable among lymph node cells, we consider expansion of a pre-existing,

infrequent T cell subset to be a less likely possibility. Consistent with a possible phenotypic change in vivo, it has been demonstrated that CD3 cross-linking in vitro in the presence of TGF-β leads to the strong induction of α_{IEL} expression (43).

Because the lymph node T cells that home to the SCID intestine are similar to the resident mucosal lymphocytes in normal mice, we believe that a similar homing process may occur continuously following T cell activation in immune-competent organisms. This would provide a source of thymus-selected T cells in the intestinal mucosa. It remains possible, however, that even those IEL and LPL with a phenotype similar to that of thymus-derived T cells, such as TCR $\alpha\beta^+$ CD4$^+$ and CD8$\alpha\beta^+$ T lymphocytes, may be derived from an extrathymic lineage. Even if a fraction of activated T cells normally homed to the intestine, the increase in T cell numbers in the lymphocyte-engrafted SCID intestine suggests that the homing of thymus-derived T cells to the intestine may be regulated differently in immune-deficient hosts. For example, resident T cells within the epithelium (such as TCR $\gamma\delta^+$ IEL) or lamina propria could exert feedback mechanisms limiting the expansion of thymus-derived T cells in the intestine (67).

In contrast to the results we have obtained following transfer of lymph node T cells, data from parabiotic mice analyzed over a period of several weeks indicated that SI-IEL rarely contained T cells derived from the other parabiont, unlike those of the peripheral T cell pool (24). These data imply that the migration of thymus-derived, peripheral T cells to the intestinal epithelium can account quantitatively for only a small percentage of IEL in immune-competent mice. It remains possible, however, that the conditions used in these experiments, such as the state of the bacterial flora, did not promote intestinal homing and/or T cell expansion. Given the data from graft vs host disease and virus-infected mice, we hypothesize that the presence of thymus-derived, peripheral T cells in the intestine could be most significant in cases of infection or acute intestinal inflammation.

The factors, other than activation, that regulate the homing and/or expansion of peripheral T lymphocytes to the intestine remain to be defined. These considerations are likely to be critical for a general understanding of host defense against mucosal infection. They also may be critical for understanding the pathogenesis of inflammatory bowel diseases, as an excess of activated, CD4$^+$, T lymphocytes in the intestine is characteristic of conditions such as Crohn's disease (68). Recently, a number of mouse models of inflammatory bowel diseases have been described, and in nearly every model, where tested, pathogenesis is greatly attenuated when the bacterial flora are reduced, such as when either RF or gnotobiotic animals are compared with SPF animals (Refs. 31, 69, and 70, and references cited therein). This suggests that the bacterial flora may be important in the homing and activation of pathogenic lymphocytes to the intestine, similar to the important role it plays in the homing of nonpathogenic T cells in the SCID transfer model described here.

Acknowledgments

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