# Identical T Cell Clones Are Located within the Mouse Gut Epithelium and Lamina Propria and Circulate in the Thoracic Duct Lymph

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## Abstract

Murine gut intraepithelial (IEL) T cell receptor (TCR)-$\alpha/\beta^+$ lymphocytes bearing CD8$\alpha/\beta$ or CD8$\alpha/\alpha$ coreceptors have been shown previously to express different oligoclonal TCR $\beta$ chain repertoires in the same mouse, in agreement with other evidence indicating that these two populations belong to different ontogenic lineages, with only CD8$\alpha/\beta^+$ IELs being fully thymus dependent. CD8$\alpha/\beta^+$, but not CD8$\alpha/\alpha^+$, T lymphocytes are also present in the lamina propria. Here, we show that CD8$\alpha/\beta^+$ lymphocytes from the lamina propria and the epithelium are both oligoclonal, and that they share the same TCR-$\beta$ clonotypes in the same mouse, as is also the case for CD4$^+$ T cells. Furthermore, identical T cell clones were detected among CD8$\alpha/\beta^+$ IELs and CD8$\alpha/\beta^+$ blasts circulating into the thoracic duct (TD) lymph of the same mouse, whereas TD small lymphocytes are polyclonal. These findings must be considered in light of previous observations showing that T blasts, but not small T lymphocytes, circulating in the TD lymph have the capacity of homing into the gut epithelium and lamina propria. These combined observations have interesting implications for our understanding of the recirculation of gut thymus-dependent lymphocytes and their precursors, and of the events leading up to the selection of their restricted TCR repertoire.

Key words: gut lymphocyte • TCR-$\beta$ repertoire • lymphocyte circulation • thoracic duct lymphocyte

## Introduction

Gut intraepithelial lymphocytes (IELs)[1] consist of two main subpopulations. One bears either CD4 or CD8$\alpha/\beta$ molecules and TCR-$\alpha/\beta$, and expresses a TCR repertoire that displays the features of the negative selection characteristic of T lymphocytes processed through the main thymic pathway. These cells are lacking in athymic nude mice, hence we refer to them as thymus dependent (for review, see reference 1). The other bears CD8$\alpha/\alpha$ molecules and either TCR-$\gamma/\delta$ or -$\alpha/\beta$, and is present in the absence of a thymus, although in decreased amounts and altered proportions of TCR-$\gamma/\delta^+$ and -$\alpha/\beta^+$ cells. These thymus-independent IELs have the features of an NK T cell lineage peculiar to the gut, as they display both T and NK cell markers and cytotoxic abilities (2, 3), possess CD3-transducing molecules distinct from those of thymus-derived cells, and have an ontogenic origin probably largely localized to the gut wall (for a review, see reference 1).

We previously studied the TCR $\beta$ chain repertoire expressed by CD8$\alpha/\beta^+$ and CD8$\alpha/\alpha^+$ IELs (4). This was achieved by use of the Immunoscope® (PE Biosystems) technique (5), which consists of detecting the respective amounts of all rearranged $\beta$ chain transcripts present in a

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[1]*Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; LT, lymphotoxin; TD, thoracic duct.

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given lymphocyte population (on the basis of their CDR3 length), followed by sequencing of expanded populations to ascertain their clonal nature. We found that the CD8α/β and CD8α/α IEL repertoires were strikingly oligoclonal, yet different, with no TCR-β clone being shared by the two populations in the same mouse. These observations argue strongly for a difference in ontogenic pathway between these two populations.

In contrast to CD8α/α T cells, which are located mainly within the gut epithelium, $CD4^+$ and $CD8\alpha/\beta^+$ T cells are present in the gut mucosa both as IELs and as lymphocytes located in the lamina propria (LPLs). $CD4^+$ and $CD8\alpha/\beta^+$ T cells, but not $CD8\alpha/\alpha^+$ cells, are also found circulating in the thoracic duct (TD) lymph. The TD drains lymph from the gut lymphatic vessels and from the mesenteric lymph nodes, and releases this lymph into the blood at the level of the left subclavian vein (6). In addition to large numbers of small lymphocytes, the TD lymph contains a small percentage of lymphoblasts. It was shown more than 30 years ago, by the use of cell transfer experiments, that TD blasts selectively home into the gut wall and its associated lymphoid structures, in contrast to TD small lymphocytes, which disseminate in all lymphoid organs (7–9). Subsequently, we have shown that $CD4^+$ as well as $CD8\alpha/\beta^+$ TD T blasts, when injected intravenously, can be recovered in part as IELs and LPLs in the gut wall (10, 11).

These observations suggested that thymus-dependent T cells from the lamina propria and the epithelium might share a common origin: progeny derived from the blasts circulating in the TD lymph. In this work, again using the Immunoscope technique, we studied the TCR β chain repertoires of both $CD4^+$ and CD8α/β IELs and LPLs from the same mouse. We also compared $CD8\alpha/\beta^+$ IELs and TD blasts from the same mouse. All of these populations were found to be oligoclonal, in contrast to TD small lymphocytes, which are highly polyclonal. Furthermore, some identical clones were found in these three localizations. Combined with the observations discussed above concerning cell traffic, these findings have interesting implications in the understanding of the recirculation of the gut mucosal thymus-dependent lymphocytes and their precursors, and of the events leading up to the selection of their restricted TCR repertoire.

## Materials and Methods

*Animals.* 6–10-mo-old $(C3H/DBA_2)F_1$ mice were raised in standard conditions in the Animal House at Hôpital Necker-Enfants Malades.

*Cell Isolation and Sorting.* IELs from the small bowel were isolated as described previously (10). In brief, Peyer's patches were removed and, after flushing with PBS, the gut was opened on a wet linen square. The mucosa was scraped with a scalpel, then dissociated by stirring in 50 ml of medium 199 containing 10% newborn calf serum and dithioerythritol (1 mM) for 15 min at room temperature. After centrifugation, the pellet was vortexed for 3 min in PBS containing 10% newborn calf serum, and 40 ml was rapidly passed through a glass wool column, buffered previously (1.6 g packed in a 20-ml syringe; Fisher Scientific). IELs were further purified on a Ficoll/Isopaque gradient (Nyco-Prep™ 1.077A; NycoMed).

To isolate gut LPLs, Peyer's patches were removed, and the epithelium was eliminated by stirring, first twice for 10 min in 60 ml of PBS containing 3 mM EDTA at 37°C, then twice for 15 min in 30 ml of Ca-free RPMI containing 1% dialyzed FCS, 1 mM EGTA, and 1.5 mM $MgCl_2$. The pellet was vortexed for 2 min. Gut pieces were collected, cut into 2-mm samples, and stirred at 37°C for 90 min in 30 ml RPMI containing 20% FCS, 100 U/ml collagenase (C2139; Sigma-Aldrich Corp.), and 5 U/ml DNase 1 (Sigma-Aldrich Corp.). At the middle and at the end of the incubation, the suspension was dissociated by multiple aspirations through a syringe for 2 min. The pellet was washed, and LPLs were purified on a Ficoll/Isopaque gradient.

To isolate IELs and LPLs from the same mouse, the duodenum, jejunum, and ileum were separated into two pieces.

Cells were collected overnight from the TD lymph as described previously (12), using a hot curved polythene tube (inner diameter 0.58 mm; Portex).

Isolated cells were sorted with a FACStar™ flow cytometer (Becton Dickinson) after double labeling. The following mAbs were used in combination: PE-labeled anti-CD4, PE- or FITC-labeled anti-CD8β and anti-CD11c, biotinylated anti-CD8β revealed with tetramethyl rhodamine isothiocyanate (TRIC) and, after fixation and permeabilization, FITC-labeled anti–5-bromo-2′-deoxyuridine (BrdU; PharMingen).

*Transfer Experiments of TD Cells.* $(C3H/DBA_2)F_1$ mice were perfused intravenously with 20 ml of PBS containing 1 mg/ml BrdU (Sigma-Aldrich Corp.) throughout the canulation procedure used to collect TD lymph (30 h). TD lymphocytes were pooled from six canulated mice and kept at 4°C. After labeling with anti-CD4 mAb (TIB207; American Type Culture Collection), $CD4^+$ lymphocytes and B lymphocytes were removed by 1-h incubation on Optilux dishes (Falcon) coated with anti–mouse Ig (Jackson ImmunoResearch Laboratories). The nonadherent cells were injected intravenously into a syngeneic recipient that was killed 20 h later. Cryosections of the recipient small bowel were stained with PE–anti-BrdU mAb (PharMingen) as described (13).

*RNA Extraction and cDNA Synthesis.* Total RNA was isolated from cells using Trizol reagent (GIBCO BRL) according to the manufacturer's guidelines. P815 cells were used as a carrier. cDNA was prepared using dT primer (final concentration 5 μM) and avian myoblastosis virus reverse transcriptase (Boehringer Mannheim) in the presence of RNasin® (Promega).

*Immunoscope Analyses of TCR-β Repertoires.* PCR was conducted in a volume of 40 μl on 1/23 of the cDNA with 1 U Taq polymerase (Goldstar; Eurogentec) in the buffer provided by the manufacturer. A set of 23 BV-specific primers (5) and an antisense primer (5′-GCCCATGGAACTGCACTTGGC), designed to hybridize in the Cβ gene, were used. Each PCR product was then used as a template for an elongation (run-off) reaction. The run-off products were analyzed on a sequencing gel using an automatic sequencer (PE Biosystems) equipped with a computer program allowing the determination of the fluorescence intensity of each band as well as its size (Immunoscope; PE Biosystems [5]). The results are expressed as peaks corresponding to the size and amount of the product. If peaks of the same size were observed among IELs and LPLs or IELs and cells isolated from lymph of the same mouse, the corresponding BVs were further subjected to BV-BJ analysis. The BV-BC PCR

product was subjected to 5 cycles of elongation with 12 different BJ primers (5), and was analyzed using the Immunoscope software. The area of the peak, indicating the intensity of the corresponding band, correlates with the number of cells bearing that particular BV-BJ rearrangement and CDR3 length.

*Sequencing of Selected BV-BJ Rearrangements.* After BV-BJ Immunoscope analysis, each BV-BJ rearrangement was compared with the same BV-BJ rearrangement of the other sample. Some of the peaks with the same BV-BJ combination and the same CDR3 size were randomly selected for sequencing.

A further amplification using the BV-BJ primer combination was performed with the BV-BC PCR product as template. If only one peak was observed in the BV-BJ rearrangement, the PCR product was sequenced directly. Otherwise, the peak of interest was separated on an 8% acrylamide gel and stained using the DNA Silver Staining System (Promega) in accordance with the manufacturer's instructions. The excised piece of gel was then used as template for further amplification, and direct sequencing of the PCR product was performed. In both cases, if the sequence obtained was unintelligible or contained multiple signals at the same position within the CDR3, the products were cloned and sequenced again. The PCR products were cloned to the pCR2.1 vector using the TOPO™ TA Cloning® kit (Invitrogen), and the insert was amplified by using the M13-40 and reverse primers. Excess primers and nucleotides were inactivated with 0.25 U shrimp alkaline phosphatase (Nycomed Amersham plc) and 2.5 U exonuclease (Nycomed Amersham plc) for 40 min at 37°C, after which the samples were incubated for 20 min at 80°C to denature the enzymes. Sequencing reactions were performed using the ABI Prism™ Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and the M13-20 primer. Samples were run on a DNA sequencer (model 377; PE Biosystems) and were later analyzed using software developed specifically for this purpose.

*Statistical Analysis.* *P* values were calculated using the $\chi^2$ test.

## Results

*Comparison of Gut Thymus-dependent IEL and LPL TCR β Chain Repertoires.* $15 \times 10^6$ IELs and $8 \times 10^6$ LPLs were harvested from the small intestine of a C3H/DBA$_2$ mouse and labeled with either anti-CD4 or anti-CD8β mAb (Fig. 1). $4 \times 10^5$ CD4$^+$ IELs, $4 \times 10^5$ CD8α/β$^+$ IELs, $6 \times 10^5$ CD4$^+$ LPLs, and $2 \times 10^5$ CD8α/β$^+$ LPLs were obtained by FACS®, RNAs were extracted, and cDNAs were prepared from each sample and then analyzed by the Immunoscope technique (5).

To compare the repertoire of CD4$^+$ cells, the cDNAs from the CD4$^+$ IELs and LPLs were used as a template for 23 PCR reactions, each specific for a different BV-BC combination, allowing the analysis of the total mouse BV repertoire. Immunoscope analyses of the PCR products showed that both populations were oligoclonal, as each BV-BC combination displayed an irregular profile with few peaks (for comparison with the profile of a polyclonal population, which is characterized by multiple peaks with a Gaussian-like height distribution, see Fig. 8, bottom). In nine of the BV-BC combinations, we observed several peaks of the same CDR3 length in the two populations, suggesting that CD4$^+$ IELs and LPLs partially shared a common repertoire (Fig. 2; eight samples are shown).

To compare more accurately the repertoires of both populations, the 9 BV-BC PCR products with peaks of identical CDR3 length were analyzed by performing PCR with 12 different BJ gene–specific primers. For the most part, the detected rearrangements in these two separate gut populations were of the same size (Fig. 3; five comparative analyses are shown). For example, for the BV14-BC product submitted to the PCR with the BJ primers, 29 different sizes of CDR3 rearrangements were found in the CD4$^+$ IELs, and 31 in the LPLs. 19 of these were of identical size (Fig. 3), a degree of similarity that has a *P* value of $<0.001$ for occurring by chance. Finally, two peaks in BV2-BJ1.3 and BV17-BJ1.3 rearrangements, which were shared by the CD4$^+$ IELs and the CD4$^+$ LPLs, were randomly selected for sequencing. In both of

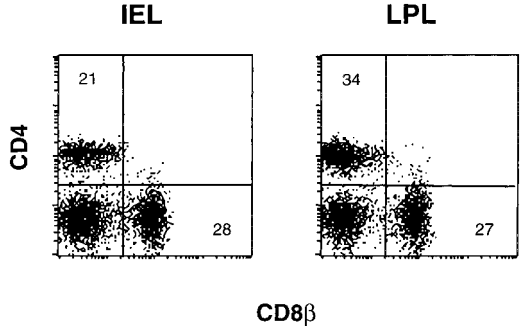


**Figure 1.** CD4$^+$ and CD8α/β$^+$ LPLs and IELs. Cells that were sorted from the same mouse for further analyses are shown. In this mouse, the percentage of CD4$^+$ IELs, which varies somewhat between different animals, is especially high, as is occasionally seen in old mice maintained in standard conditions. This percentage was lower in the second set of experiments, described below.

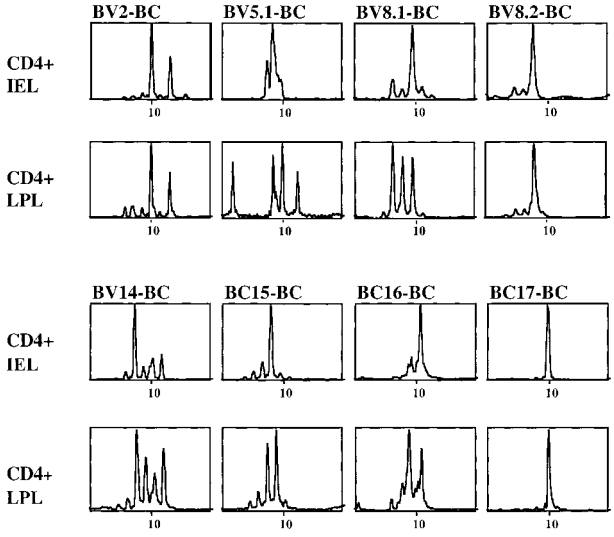


**Figure 2.** Comparison of TCR-β diversity between CD4$^+$ IELs and LPLs, showing BV-BC segment analyses. Few peaks are seen in each panel, strongly suggesting that the populations are oligoclonal. BV genes containing rearrangements of the same size are found in both populations. In each profile, a CDR3 length of 10 amino acids is indicated; the peaks are spaced by 3 nucleotides.

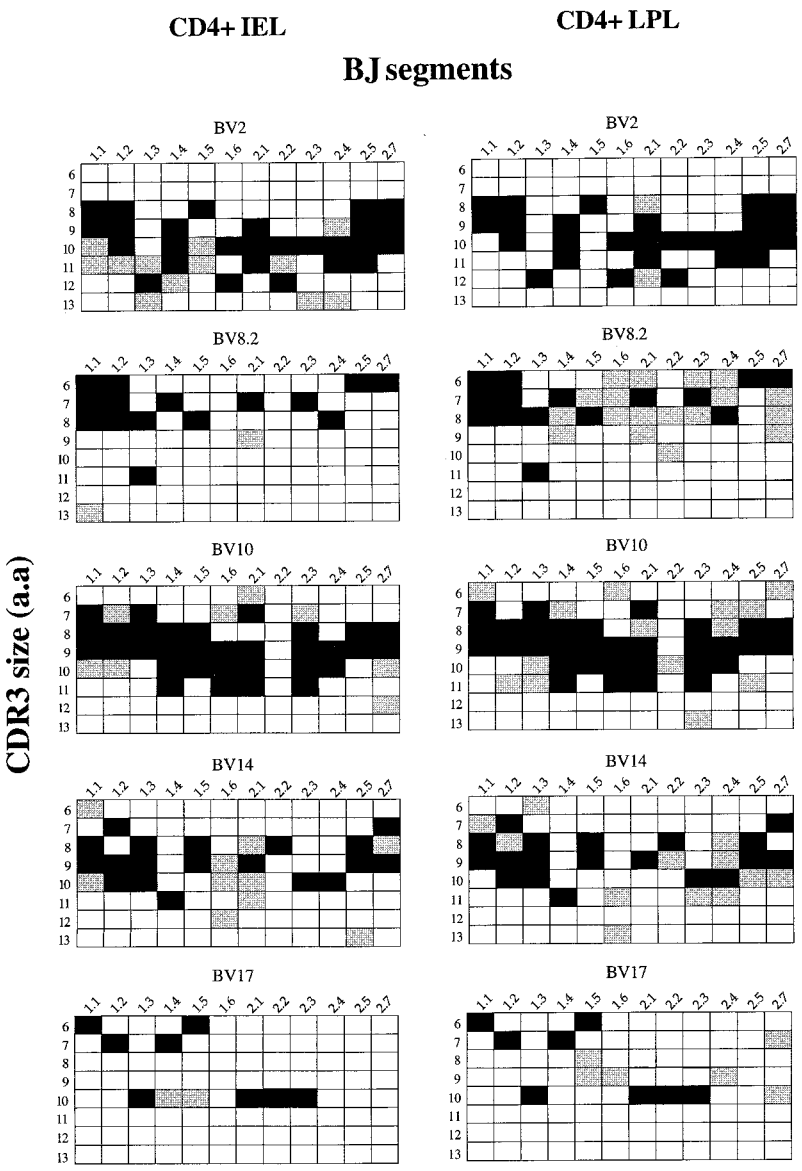


**Figure 3.** Comparison of TCR-β diversity between $CD4^+$ IELs and LPLs for selected BV genes, showing VB-JB segment analyses. Rearrangements shared between these populations are indicated by black squares, and other rearrangements by gray squares. White squares indicate that no measurable peak corresponding to that BV-BJ combination and CDR3 length was observed. a.a, amino acid(s).

these rearrangements, we found identical TCR-β sequences in the two locations (Table I, top).

These results provide a minimum estimate for β chain diversity in the $CD4^+$ IELs and LPLs. Both populations were, as noted, oligoclonal. By counting the average number of different rearrangements for BV-BJ segments and multiplying by the number of different Vβ genes, we calculated that there is a minimum of ~650 clones of $CD4^+$ IELs and $CD4^+$ LPLs.

We then performed a similar analysis of the $CD8\alpha/\beta^+$ IEL and LPL repertoires. The repertoires of the $CD8\alpha/\beta^+$ IELs and the $CD8\beta^+$ LPLs were highly oligoclonal, as described previously (4). Furthermore, both populations displayed peaks of the same size for 16 of the 23 BV genes analyzed (Fig. 4), which were then subjected to BV-BJ analysis. As for $CD4^+$ cells, a large proportion of the CDR3 rearrangements in these two separate gut populations was of the same size (Fig. 5; five samples are shown). For instance, when the BV5.2-BC PCR product was analyzed by using BJ-specific primers, the $CD8\alpha/\beta^+$ IEL population contained 12, and the LPL population 33 CDR3s of various sizes, with 10 of each being the same size in both populations (Fig. 5; $P < 0.001$). Three BV-BJ PCR products with the same size in both populations were randomly selected for sequencing, namely, BV5.2-BJ2.4, BV5.2-BJ2.5, and BV8.3-BJ2.7. In each case, the TCR-β sequences were identical in the two populations (Table I, top).

The minimum clonal size of β chain diversity among the $CD8\alpha/\beta^+$ cells, as calculated above, was estimated to be 250 clones for IELs and 350 for LPLs. This estimate is comparable to that reported previously for the IEL population (14).

In a second set of experiments, analyzing the BV-BC repertoire of CD4 and $CD8\alpha/\beta$ LPLs and IELs from another mouse, we confirmed the oligoclonality of these

**Table I.** *TCR-β Sequences of CD4⁺ and CD8β⁺ IELs and LPLs, and CD8β⁺ IELS and CD8β⁺CD11c⁺ Lymphocytes from the TD Lymph*

| | IELs | LPLs |
|---|---|---|
| CD4⁺ | | |
| BV2-BJ1.3 | SAAATSGNTL (11) | SAAATSGNTL (17) |
| | SADPDSGNTL (1) | |
| | | SPGTGVGNTL (1) |
| | | SAAATSGNTL (1) |
| BV17-BJ1.3 | SLYLQNSGNTL (4) | SLYQNSGNTL (6) |
| CD8β⁺ | | |
| BV5.2-BJ2.4* | SQCSQNTL | SQCSQNTL |
| BV5.2-BJ2.5* | SLHWILTRHQ | SLHWILTRHQ |
| BV8.3-JB2.7* | KGRLGGSNEQ | KGRLGGSNEQ |
| | IELs | Lymph |
| CD8β⁺ | | |
| BV1-BJ1.6* | SQDNSPL | SQDNSPL |
| BV10-BJ2.7 | SQDWGGYEQ (14) | SQDWGGYEQ (1) |
| | | SFRDWGYEQ (2) |
| | | SSGQGAYEQ (2) |
| | | SFPYWGYEQ (1) |
| BV4-BJ2.3 | SQDWTTSAETL (4) | SQDWTPSAETL (5) |
| | SQGWTTSAETL (1) | |
| BV13-BJ1.2 | RRQGDSDY (23) | SLDTNSDY (23) |
| BV15-BJ1.2* | RQGAXDY | RDRGSDY |

As described in Materials and Methods, the BV-BJ amplification products were first sequenced directly. If the sequence thus obtained was unintelligible, or if overlapping signals were observed, the products were cloned and sequenced. For the cloned rearrangements, the number of clones with the particular sequence is shown after the sequence.
*Indicates directly sequenced rearrangements.

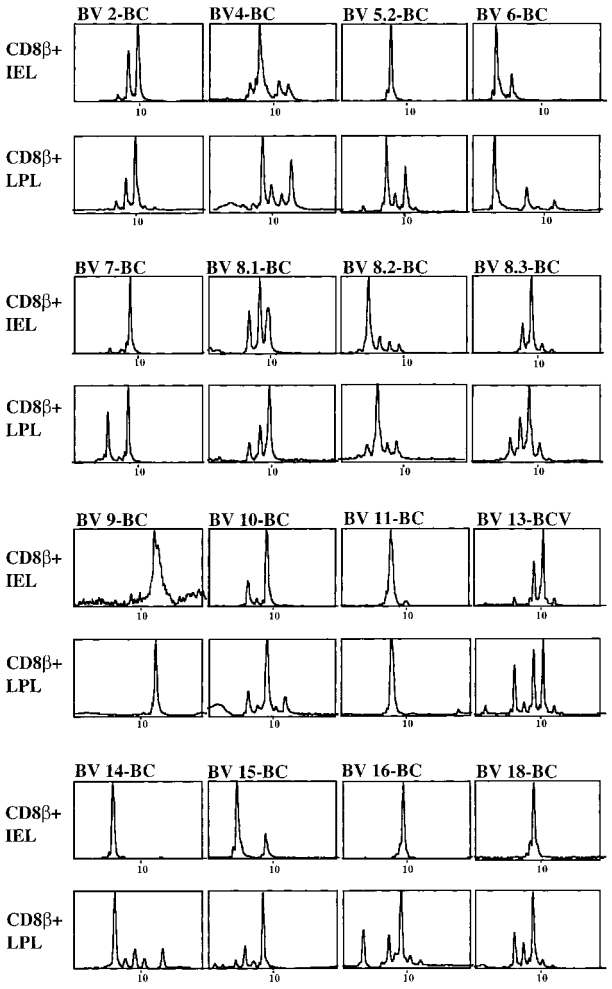


**Figure 4.** Comparison of TCR-β diversity of CD8α/β⁺ IELs and LPLs, showing BV-BC segment analyses. Few peaks are seen in each panel, strongly suggesting that the populations are oligoclonal. BV genes containing rearrangements of the same size are found in both populations. In each profile, a CDR3 length of 10 amino acids is indicated; the peaks are spaced by 3 nucleotides.

populations. The CD4 repertoire from both sources contained CDR3 of the same size in 6 of the 23 genes studied, and the CD8 repertoire in 15. In addition, we analyzed four TCR-α rearrangements in CD8α/β⁺ LPLs and IELs. Again, few peaks were observed and peaks of the same size were found in both populations (data not shown).

*Comparison of the Repertoire of CD8α/β⁺ IELs and of CD8α/β⁺CD11c⁺ Lymphocytes Isolated from the TD Lymph of the Same Mouse.* TD lymph contains mostly small nondividing lymphocytes, and a few (<1%) rapidly dividing large cells (7, 8, 10, 15). Because of the difficulty of selecting these latter cells on the basis of their size, we took advantage of an observation of Huleatt and Lefrançois (16), that the molecule CD11c is expressed by activated CD8α/β⁺ lymphocytes. We found that the TD lymphocytes indeed contain a very small subpopulation of CD8α/β⁺CD11c⁺ cells (0.4%), which consists in part of cells larger than the bulk of CD8α/β⁺ lymphocytes (11%) as shown by forward light scatter analysis (see below). We sorted both CD8α/β⁺CD11c⁺ and CD8α/β⁺CD11c⁻ populations from TD lymph, as well as CD8α/β⁺ IELs isolated at the end of the canulation procedure for repertoire analyses.

To ensure that CD8α/β⁺CD11c⁺ cells are indeed blasts capable of homing to the gut wall, as observed in previous experiments (7–11), we performed transfer experiments of CD8α/β⁺ TD cells obtained under continuous perfusion of BrdU to label cycling cells. Since it is not possible to inject cells coated with mAbs into recipients to explore their physiological tissue homing, we used pools of negatively selected TD cells depleted in CD4⁺ and B cells (see Materials and Methods). By three-color analysis of the transferred cells, the vast majority (90%) were CD8β⁺, as was also the case of virtually all BrdU⁺ cells (2.6% of the selected population;

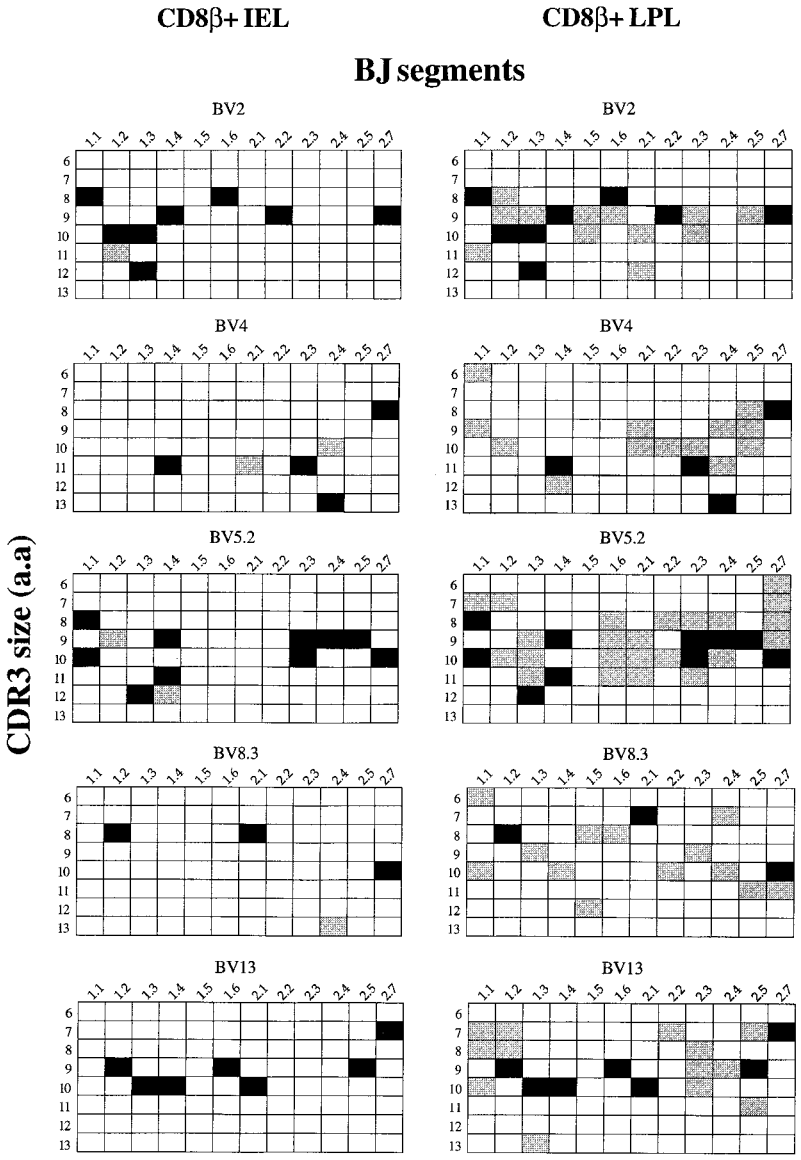


**Figure 5.** Comparison of TCR-β diversity between CD8α/β$^+$ IELs and LPLs with selected BV genes, showing VB-JB segment analyses. Rearrangements shared between these populations are indicated by black squares, and other rearrangements by gray squares. White squares indicate that no measurable peak corresponding to that BV-BJ combination and CDR3 length was observed. a.a, amino acid(s).

Fig. 6 A); 4.3% of CD8β$^+$ cells were CD11c$^+$ (in agreement with the percentage observed with CD8α/β$^+$CD11c$^+$ selected by sorting for repertoire analyses), and all CD11c$^+$ cells were CD8β$^+$ (Fig. 6 B). About 50% BrdU$^+$ cells were CD11c$^+$, and there was a clear correlation between the level of CD11c expression and the intensity of BrdU labeling of BrdU$^+$ cells, although CD11c$^+$ cells also contain a sizable fraction of BrdU$^-$ cells (Fig. 6 D). This last observation was consistent with the results of forward scatter analyses of CD11c$^+$ and CD11c$^-$ cells, showing that the CD11c$^+$ population contains larger lymphocytes than the bulk of CD11c$^-$ lymphocytes (Fig. 6 C). 24 h after transfer of the negatively selected population just described, BrdU$^+$ cells were detected in all sections of the recipient's gut (where their density ratio versus the spleen was comparable to that described previously [reference 15; Fig. 7]).

Three conclusions can be derived from this transfer experiment and from the three-color analysis of the CD8α/β$^+$CD11c$^+$ cells: (a) since the well-labeled BrdU$^+$ cells observed in tissue sections are likely to correspond to the brightest BrdU$^+$ cells detected by FACS® analysis, which are CD11c$^+$, the selected CD8α/β$^+$CD11c$^+$ cells prepared for repertoire analysis indeed appear to contain gut-homing blasts; (b) this latter selected population also contains noncycling lymphocytes, which may somewhat blur the repertoire explored because these last cells are expected to display the highly polyclonal repertoire characteristic of small T lymphocytes (see below); and (c) the population of CD8α/β$^+$CD11c$^-$ TD cells used for repertoire study also contain a subpopulation of cycling cells, which represent only a small minority whose repertoire, if oligoclonal, is thus unlikely to be detected in the background polyclonal repertoire of the noncycling cells present in a large majority.

Immunoscope analyses of BV-BC PCR products obtained from CD8α/β$^+$CD11c$^-$ TD lymphocytes dis-

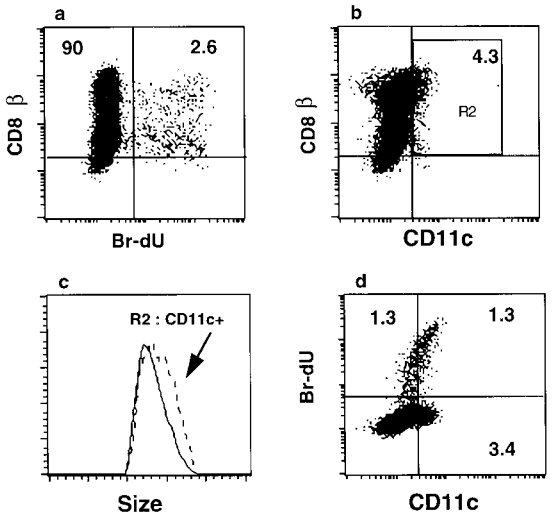


**Figure 6.** FACS® analysis of TD lymphocytes used in transfer experiments. TD lymphocytes, strongly enriched for $CD8^+$ lymphocytes (a and b; see Materials and Methods), were tripled stained with anti-CD8β, anti-CD11c, and, after permeabilization, with anti-BrdU. Cells strongly stained with BrdU show high expression of CD11c (d). The $CD11c^+$ population contains larger cells (c) and 20-fold more $BrdU^+$ cells than the $CD11c^-$ population (not shown). (Note that permeabilization blurs surface fluorescence, but does not change the percentage of fluorescent cells.)

played the usual Gaussian-like distribution of size peaks characteristic of polyclonal populations (Fig. 8, bottom rows). In contrast, for $CD8\alpha/\beta^+CD11c^+$ lymphocytes the profiles were more irregular, and the presence of prominent peaks suggested that some populations were expanded (Fig. 8, middle rows). Peaks of the same size were observed for $CD8\alpha/\beta^+$ IELs (Fig. 8, top rows). The repertoire of the sorted $CD8\alpha/\beta^+CD11c^+$ TD lymphocytes is less restricted than that of the $CD8\alpha/\beta^+$ IELs, in agreement with the fact that this population also contains noncycling lymphocytes, as discussed above. Analyses of BV-BJ rearrangements (Fig. 9) confirmed that the repertoire of $CD8\alpha/\beta^+CD11c^+$ cells from the lymph was oligoclonal (for comparison with a polyclonal repertoire, see right column), and showed that rearrangements with identical CDR3 size were present among $CD8\alpha/\beta^+CD11c^+$ TD lymphocytes and $CD8\alpha/\beta^+$ IELs ($P < 0.001$).

We then sequenced five rearrangements that showed the same CDR3 size in both $CD11c^+$ TD lymphocytes and IELs. Two of these, BV1-BJ1.6 and BV10-BJ2.7, had identical sequences, whereas the other three were different. However, one of them, BV4-BJ2.3, differed only by one base, which could result from PCR error (Table I, bottom). Thus, in the TD lymph, large $CD8\alpha/\beta^+$ lymphocytes display a restricted repertoire compared with the polyclonal repertoire of the much more numerous small $CD8\alpha/\beta^+$ lymphocytes, and some express the same TCR β chains as IEL clones. Given the oligoclonality of both the activated TD lymphocytes and IELs, and since the expanded $CD8\alpha/\beta^+$ intraepithelial clones differ from one mouse to another (4), it is highly unlikely that we should have found identical sequences by chance.

## Discussion

It has been shown previously in both rodents and humans that TCR-$\alpha/\beta^+CD8\alpha/\beta^+$ IELs express an oligoclonal repertoire (4, 14, 17–22), and that clones of cells expressing the same TCR-β are found at different levels of the gut in the same mouse (4). This work extends these observations on thymus-dependent gut lymphocytes by showing that (a) $CD4^+$ as well as $CD8\alpha/\beta^+$ IELs are oligoclonal; (b) $CD4^+$ and $CD8\alpha/\beta^+$ LPLs are also oligoclonal; and (c) identical clones can be found in $CD4^+$ IELs and LPLs, and in $CD8\alpha/\beta^+$ IELs and LPLs. This last observation establishes that IELs and LPLs ($CD4^+$ or $CD8\alpha/\beta^+$) are the progeny of common precursors that have proliferated under the same antigenic stimulation. Since most of these gut mucosal lymphocytes are not proliferating in situ (only a small proportion of them is labeled after a single in vivo pulse of $^3$H-TdR [10]), and since their development requires antigenic stimulation (23), two questions then arise: how do the precursors of LPLs and IELs of thymic origin reach the gut wall, and where have they been antigenically stimulated?

The TD lymph contains a vast majority of small lymphocytes and a small percentage of blasts (which become labeled after brief incubation in vitro with $^3$H-TdR or in vivo with BrdU, allowing the study of their fate in cell transfer experiments [15, 24; this study]). After intravenous transfer to normal recipients, TD blasts home to the gut wall, where some of them mature into IgA plasma cells (15, 25) and others can be recovered as $CD4^+$ or $CD8\alpha/\beta^+$ IELs and LPLs. Some also migrate to the Peyer's patches, the mesenteric lymph nodes, and the spleen, but few migrate to the peripheral lymph nodes (10, 15, 24). This is in striking contrast to the TD small lymphocytes, which disseminate evenly to all peripheral lymphoid organs. Cell transfer experiments showed that this selective homing to the gut mucosa and its associated lymphoid structures are properties shared by T blasts obtained from mesenteric lymph nodes, which drain intestinal lymph and release their lymph into the TD. In contrast, T blasts obtained from peripheral lymph nodes migrate back to peripheral lymph nodes and to the spleen, but not at all to the gut mucosa or its associated lymphoid structures (10, 15, 24). These observations led to the proposal that thymus-dependent gut mucosal lymphocytes, rather than resulting from the local stimulation of randomly distributed small lymphocytes, are mainly the progeny of blasts arising in the gut-associated lymphoid tissues and circulating in the TD lymph, from which they reach the blood to seed the entire length of the gut mucosa in addition to returning into gut-associated lymphoid structures.

To isolate T blasts present in the TD lymph, we used an observation of Huleatt and Lefrançois (16), that $CD8\alpha/\beta^+$ (but not $CD4^+$) T cells express CD11c molecules under in vivo antigenic stimulation. We were able to select among TD lymph cells a small subset of $CD8\alpha/\beta^+CD11c^+$ cells that contains, besides small lymphocytes, large cells and a high proportion of dividing cells (as detected by BrdU labeling). After transfer into a normal recipient, $CD8\beta^+$ $BrdU^+$ cells were observed along the whole gut wall, both

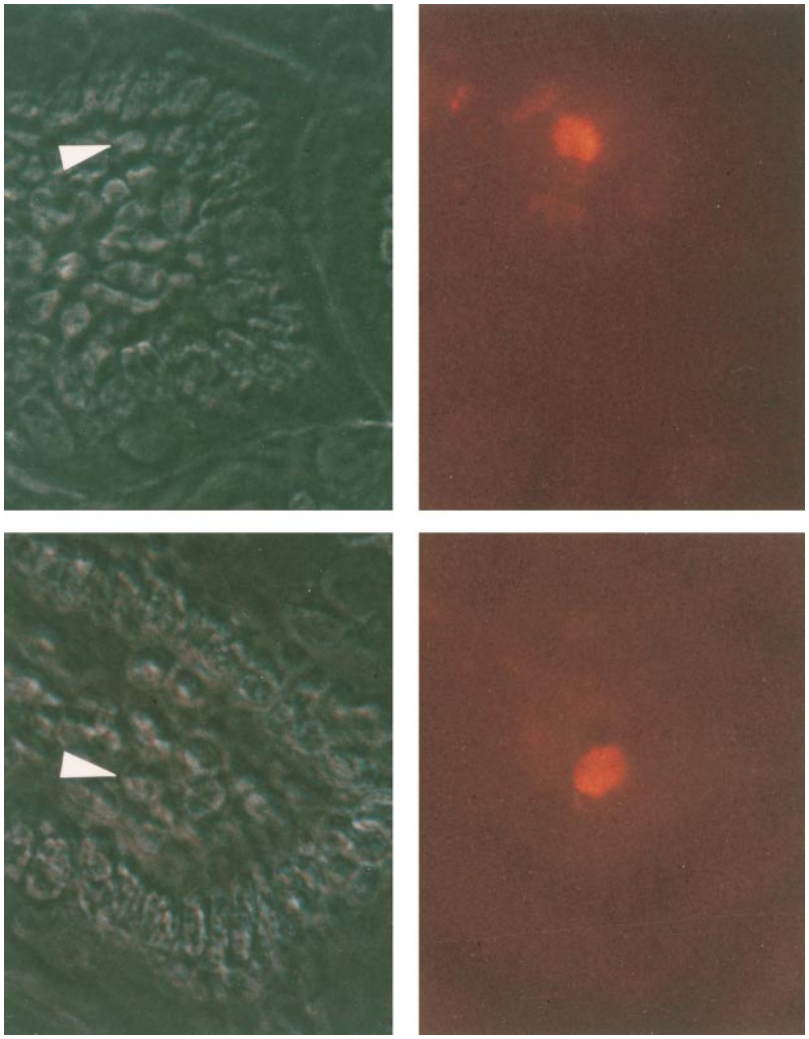

**Figure 7.** Gut mucosa of a mouse recipient of the BrdU-labeled TD cells shown in Fig. 6. Gut sections displaying two BrdU-labeled cells localized (right) or as seen by phase–contrast microscopy (left) in the epithelium (top) and in the lamina propria (bottom).

within the epithelium and in the lamina propria. Thus, this population corresponds to the circulating CD8β T blasts with gut-homing properties that we had described previously (8, 10, 11, 15). We then compared the repertoire of these circulating $CD8\alpha/\beta^+CD11c^+$ TD lymphocytes with that of the $CD8\alpha/\beta^+$ IELs obtained from the same mouse at the end of a 30-h TD drainage. TD small lymphocytes showed a polyclonal TCR-β repertoire, whereas the population enriched in $CD8\alpha/\beta^+$ T blasts was oligoclonal, and, moreover, shared clones with the $CD8\alpha/\beta^+$ IELs from the same mouse, as shown by the same CDR3 size and identical nucleotide sequences.

The presence of identical clones among IELs and TD blasts establishes a link between these two lymphocyte populations in the same animal, and is consistent with the concept of a circuit through the TD lymph and the blood allowing dissemination of antigenically stimulated thymic-derived T blasts to the whole length of the gut wall, as discussed above, as well as with the previously reported observation that identical clones of $CD8\alpha/\beta^+$ IELs can be found in the same mouse in widely distant segments of the gut (4).

Where are the TD T blasts first antigenically stimulated? We previously presented evidence that thymus-derived T cells, when stimulated in the gut wall, tend to migrate into the lymph rather than expanding and differentiating locally. Widespread antigenic stimulation of Peyer's patch T lymphocytes, such as occurs, for instance, in conditions of graft-versus-host reactivity, leads to a massive increase in blasts of Peyer's patch origin into the TD lymph followed by a marked increase in T lymphocytes into the gut mucosa (which can be prevented by TD drainage [10, 11]). Concomitantly, the Peyer's patches decrease in size, and contain few T blasts or small cells (24). This has led to the suggestion that the antigenic stimulation and proliferation of IEL precursors first occur mainly in the Peyer's patches, followed by their rapid emigration into the mesenteric lymph nodes and the TD lymph (10, 11, 24). Indeed, the Peyer's patches are lymphoid structures that are well adapted to respond to antigenic stimulation arising from the gut content, as they are covered by a peculiar variety of epithelial cells, the M cells. Under these cells, which are specialized in the transport of particles present in the gut lumen (26), are localized immature dendritic cells capable of processing antigen and presenting stimu-

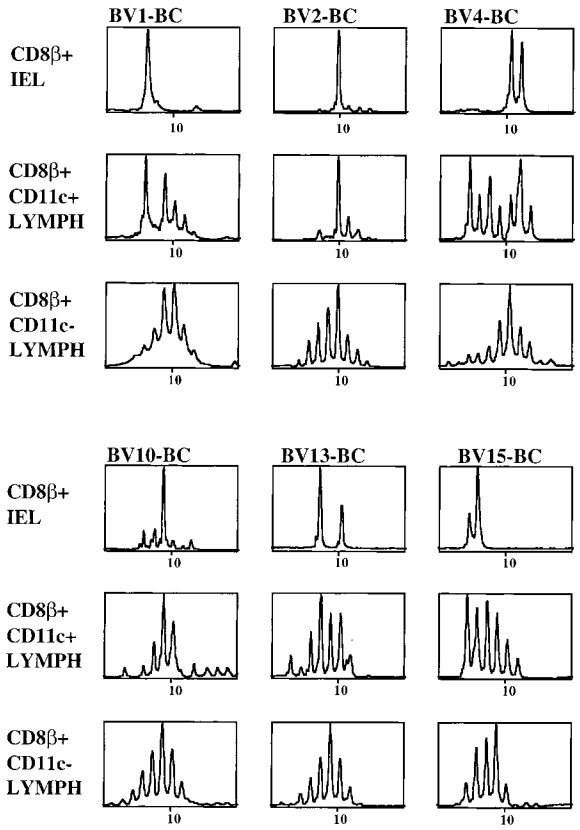


**Figure 8.** Comparison of TCR-β diversity between CD8α/β$^+$ IELs and CD8α/β$^+$CD11c$^+$ TD lymphocytes showing BV-BC segment analyses. Few peaks are seen in each panel, strongly suggesting that the populations are oligoclonal. BV genes containing rearrangements of the same size are found in both populations. TD lymphocytes appear less oligoclonal than IELs; however, the peak size is irregular, contrasting with that displayed by CD8αβ$^+$CD11c$^-$ TD cells (bottom rows), which show the characteristic regular Gaussian-like profile of a polyclonal population. In each profile, a CDR3 length of 10 amino acids is indicated; the peaks are spaced by 3 nucleotides.

lating peptides to the lymphocytes clustered in the patches (27, 28). However, antigenic stimulation does not need to be restricted to Peyer's patches, as dendritic cells are also found disseminated in the lamina propria and circulating in the TD lymph, from which they can reach the spleen (29; Guy-Grand, D., unpublished observations). Thus, gut-derived antigens can be presented by dendritic cells at all levels of the gut, and even at a distance, i.e., in the mesenteric lymph nodes and the spleen. This probably explains why mice lacking Peyer's patches and mesenteric lymph nodes as a result of the *aly* mutation (30), or of targeted mutations of the lymphotoxin (LT)β receptor locus (31) or the TNF/LTα locus (32), have normal numbers of CD4$^+$ and CD8α/β$^+$ T lymphocytes in the gut mucosa (30; Guy-Grand, D., unpublished observations). In these special situations, most of the initial priming of gut-homing T blasts may have occurred in the spleen, as dendritic cells are present in the TD lymph of TNF/LTα$^{-/-}$ mice in increased numbers (Guy-Grand, D., unpublished observations). It should be noted in this respect that, even in normal mice, spleen T blasts in transfer experiments show some tendency to home to the gut (24).

Study of the TD lymph of TNF/LTα$^{-/-}$ mutant mice provides another observation of interest. The lymph from these mice contains very few small lymphocytes, in agreement with the total lack of peripheral lymphoid organs in these animals and consequently with the absence of small lymphocyte recirculation through the postcapillary venules; in contrast, the TD lymph of TNF/LTα$^{-/-}$ mutant mice contains absolute numbers of CD4$^+$ and CD8α/β$^+$ T blasts similar to those found in the TD lymph of normal animals (Guy-Grand, D., unpublished observations). The presence of normal amounts of T blasts in the TD lymph of mice devoid of all secondary lymphoid organs (except the spleen) is not compatible with an antigenic stimulation of the progenitors of these blasts occurring mainly in the spleen: this organ is located downstream of the TD lymph, and TD blasts have little or no tendency to recirculate, that is, to reach the TD lymph again after their release in the blood (7). One is led to conclude that these blasts may result from in situ antigenic restimulation of gut mucosal lymphocytes present in normal numbers in these mice, as mentioned above, and which in this situation are the only lymphocytes located upstream of the TD lymph. This hypothesis has important implications as well for understanding the progressive generation of gut mucosal thymus-dependent T lymphocytes in normal mice.

The following general scheme may be proposed (Fig. 10). The first circulating T blasts, endowed with gut-homing properties as the result of stimulation of their precursors by dendritic cells presenting antigens from gut origin, arise in normal mice in the Peyer's patches, the mesenteric lymph nodes, and the spleen by order of decreasing frequency, and arise in mutant mice described above, in the spleen. The progeny of these circulating blasts colonize the gut mucosa, and in normal mice colonize the Peyer's patches and the mesenteric lymph nodes as well, and, to a much lesser extent, the spleen. A process of repeated antigenic stimulation within the mucosa would result in successive waves of migrating T blasts undergoing new rounds of TD lymph blood traffic, progressively expanding a population of gut thymus-dependent T lymphocytes and shaping up its repertoire. Repeated identical antigenic stimulations progressively narrow the repertoire of the T cells bearing the corresponding TCR (33–35). Furthermore, recent observations on the emergence of T cell clones have shown that identical antigenic peptides may stimulate a diversity of TCRs (36, 37), and that the time of the encounter determines the variety of the expanded clones (38). This latter finding might explain the otherwise puzzling observation that the oligoclonal repertoires of CD8α/β$^+$ IELs are different between mice of the same litter raised in the same cage, and even between germ-free mice of the same litter (14), which have about sixfold fewer IELs than normal mice (3). All of these mice are probably stimulated by common antigenic peptides, as are those resulting from the bacterial flora shared by all non–germ-free mice, from food, and from autoantigens peculiar to the gut.

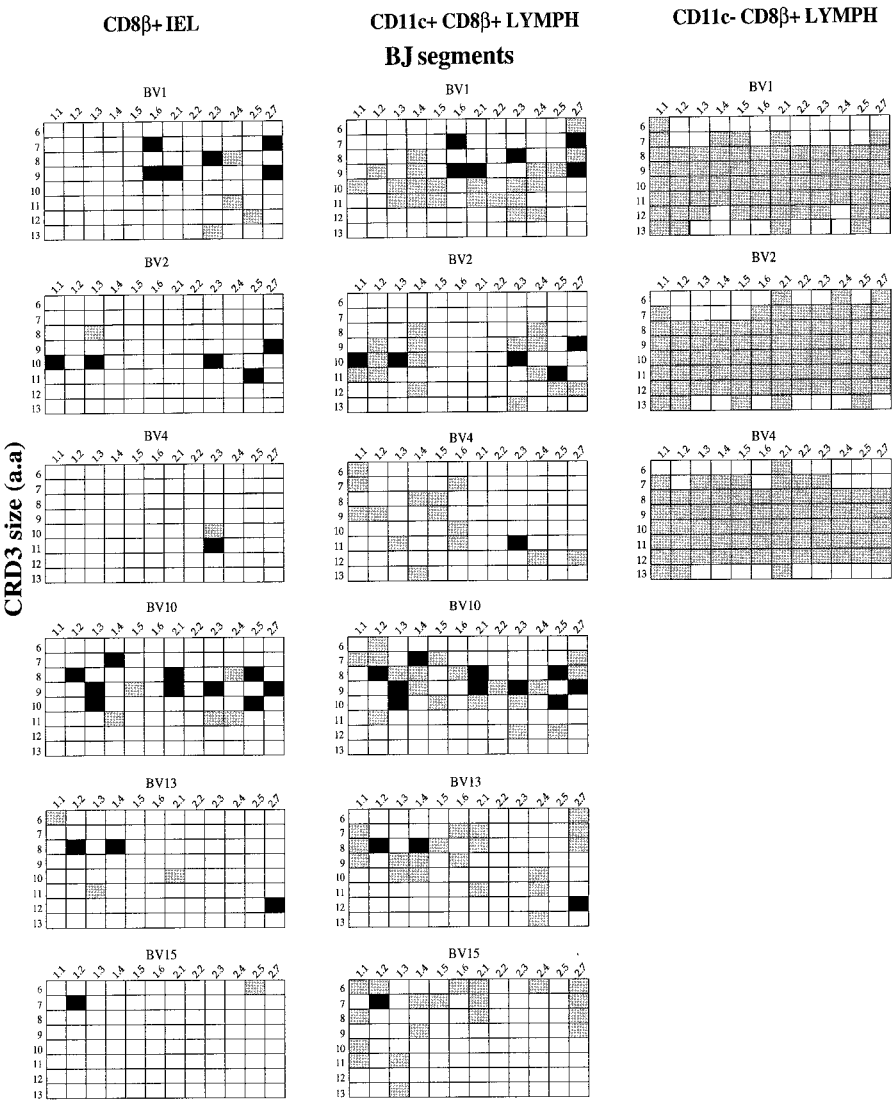


**Figure 9.** Comparison of TCR-β diversity between CD8α/β$^+$ IELs and CD8α/β$^+$CD11c$^+$ TD lymphocytes with selected BV genes, showing BV-BJ segment analyses. Rearrangements shared between these populations are indicated by black squares, and other rearrangements by gray squares. White squares indicate that no measurable peak corresponding to that BV-BJ combination and CDR3 length was observed. In addition, and for comparison, three examples of the polyclonal repertoire pattern of the CD8α/β$^+$CD11c$^-$ TD lymphocytes are shown (right). a.a, amino acid(s).

In conclusion, it is possible to combine the observations made on the repertoire of thymus-dependent IELs, LPLs, and TD blasts showing their oligoclonality and the presence in the same animal of some identical clones in these three localizations and at different levels of the gut, together with the observations concerning cell traffic in the gut and the respective cell composition of TD lymph and gut mucosa in various conditions. On this basis, we propose the following ontogenic pathway. First, thymus-dependent IELs and LPLs are mostly the progeny of T blasts circulating in the TD lymph and reaching the blood, from which they seed the whole length of the small bowel wall. Second, the original antigenic stimulation of the circulating blast progenitors occurs, probably by order of decreasing frequency, in the Peyer's patches, the mesenteric lymph nodes, and occasionally the spleen. T blasts originating in these locations rapidly migrate into the lymph and the blood, rather than proliferating extensively locally, resulting in the colonization in primed lymphocytes not only of the gut mucosa but also of the Peyer's patches and the mesenteric lymph nodes. Third, thymus-dependent gut mucosal lymphocytes, as well as primed T lymphocytes from Peyer's patches and mesenteric lymph nodes, upon meeting their cognate peptides in situ may undergo further stimulations to divide, resulting in migrating blasts following new rounds of lymph blood traffic, thus progressively shaping up a clonally restricted population which, with little or no division in situ, extends to the whole mucosa.

Finally, it must be stressed that this ontogenic scheme applies only to the thymus-dependent CD8α/β$^+$ or CD4$^+$ TCR-α/β$^+$ populations of gut lymphocytes. CD8α/α$^+$ IELs, TCR-α/β$^+$ or γδ$^+$, appear to differentiate locally, and no cells with the corresponding phenotypes have yet been described in the TD.

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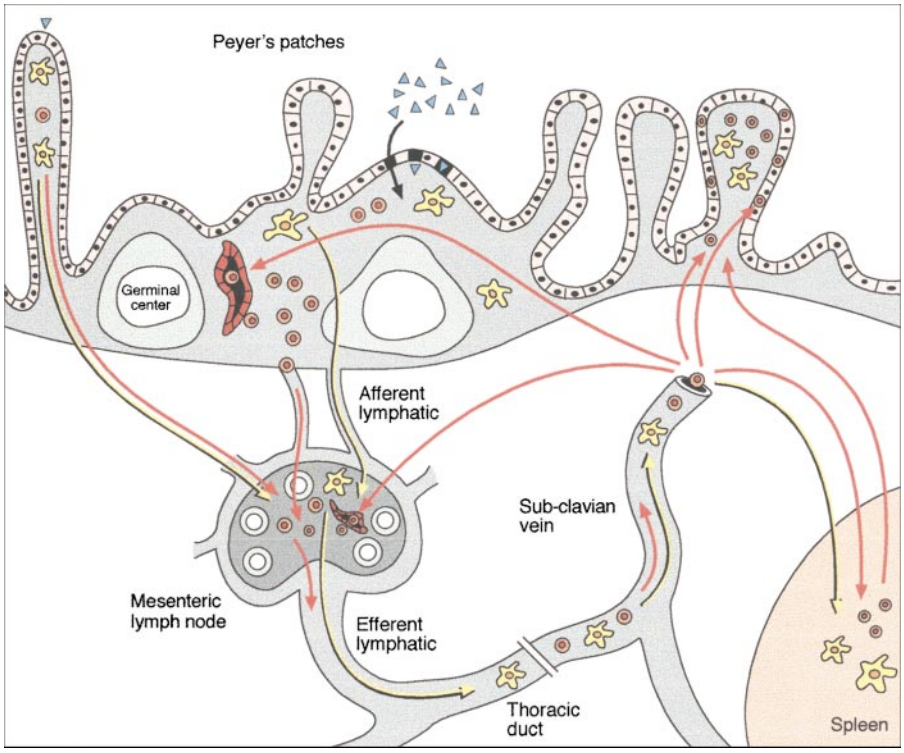


**Figure 10.** Schema of the lymph to blood circulation of T blasts and dendritic cells antigenically elicited in the gut wall or its associated lymphoid structures (see text for details). Red, T lymphocytes; yellow, dendritic cells; red, postcapillary venules.

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