Heterogeneity and Biased T Cell Receptor \( \alpha/\beta \) Repertoire of Mucosal CD8+ Cells from Murine Large Intestine: Implications for Functional State

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Summary

Up to 90% of CD8+ intraepithelial lymphocytes (IEL) of the murine large intestine (LI) belong to the \( \alpha/\beta \) T cell lineage and consist of two subsets. One subset expresses both \( \alpha \) and \( \beta \) subunits of the CD8 coreceptor, and is uniformly Thy1+, CD5+, B220-, CD2+, CD28+. The CD8\( \alpha^+\beta^+ \) LI-IEL exclude self-reacting V\( \beta \) structures, and readily proliferate in vivo in response to T cell receptor-mediated stimuli. The CD8\( \alpha^+\beta^- \) subset of TCR-\( \alpha/\beta^- \) LI-IEL is Thy1+/-, CD5-, B220+, CD2+/-, and CD28-. It contains cells with potentially self-reacting V\( \beta \) structures and is responsive in vivo to high doses of anti-TCR-\( \alpha/\beta \) monoclonal antibody (mAb), but not to bacterial superantigens. Both subsets are abundant in LI-IEL of old nude mice, and CD8\( \alpha^+\beta^+ \) LI-IEL in nude mice undergo the same V\( \beta \) deletions as in euthymic mice of the same background. Both subsets express the intestinal T cell-specific integrin \( \alpha_{M}290\beta^7 \), known to be a homing receptor for IEL. Unusually high proportions of CD69+ cells within both subsets indicate chronic activation. The proportions of CD69+ and otm9037+ cells within the CD8\( \alpha^+\beta^- \) subset increase with age, probably due to constant antigenic challenge. We propose that CD8\( \alpha^+\beta^+ \) and CD8\( \alpha^+\beta^- \) subsets of LI-IEL permanently reside in LI and represent a lineage different from spleen and lymph node CD8+ T cells. The CD8\( \alpha^+\beta^- \) undergoes negative selection, and is responsive to TCR-mediated stimuli. The CD8\( \alpha^+\beta^- \) subset of LI-IEL is a subject of distinct selection mechanisms, and has low responsiveness to TCR-mediated stimuli.

The T cell lymphocyte populations of the murine small intestine (SI), including its intraepithelial lymphocyte (IEL) compartment, has been thoroughly investigated during the last few years. Some of the functional properties of the SI-IEL (1, 2), and the origin and composition of their subsets are fairly well characterized (3-7). CD8+ SI-IEL consist of two major subsets, one of them expressing both the \( \alpha \) and \( \beta \) subunits of the CD8 coreceptor, and the other expressing only the CD8\( \alpha \) subunit (5). A major fraction of CD8\( \alpha^+\beta^- \) SI-IEL bear TCR-\( \gamma/\delta \) (3-5), and the rest belong to the \( \alpha/\beta \) T cell lineage (5). TCR-\( \alpha/\beta^+ \) CD8\( \alpha^+\beta^- \) SI-IEL are partly CD2- (8), and CD28- (9), which is in good agreement with their in vitro unresponsiveness to TCR-mediated signals both in terms of proliferation (7-9) and lymphokine production (9, 10).

When expression of "forbidden" V\( \beta \) genes was revealed within the CD8\( \alpha^+\beta^- \), but not within the CD8\( \alpha^+\beta^- \) subsets of SI-IEL (6), it was suggested that the former are extrathymic and the latter are thymic-dependent T cells (5, 6).

Later, both subsets appeared to be extrathymically derived (7), and differences in their TCR repertoires were explained by the unresponsiveness of the CD8\( \alpha^+\beta^- \) SI-IEL, rather than by different origins (7). To make the picture more complicated, recent data suggest that these presumably unresponsive extrathymic SI-IEL are in fact responsive to an MHC-mediated positive stimulation (11), though they are refractory to negative selection by self-Ag (12).

IEL from the murine large intestine (LI) have attracted much less attention. While the present study was in progress, the first flow cytometry data on the LI-IEL were published (13). In that study, LI-IEL were shown to contain a higher proportion of TCR-\( \alpha/\beta^+ \) and CD4+ cells compared with SI-IEL. As has been observed with SI-IEL, CD8+ LI-IEL appeared to contain CD8\( \alpha^+\beta^+ \) and CD8\( \alpha^+\beta^- \) subsets. A substantial fraction of LI-IEL was shown to express intestinal T cell–specific integrin \( \alpha_{M}290\beta^7 \), and a minor fraction were CD2- (13).

Our present experiments were concentrated on a detailed analysis of the CD8+ LI-IEL. We found that the general population structure of the CD8+ LI-IEL is similar to that of CD8+ SI-IEL, but that CD8+ LI-IEL contain relatively more T cells with a "conventional" phenotype (TCR-\( \alpha/\beta^+ \), CD8\( \beta^+ \), Thy1+, CD2-).
In addition, we found that within the TCR-α/β⁺ LI-IEL the CD8α⁺β⁺, and CD8α⁻β⁻ subsets have two additional markers in common: (a) intestinal T cell homing receptor α5β1 (14), and (b) CD69, an Ag expressed by freshly or chronically activated T cells (15, 16). Furthermore, we found two markers that clearly discriminate between the CD8⁺ subsets: the CD8α⁺β⁺ LI-IEL are almost 100% CD5⁺, B220⁻, whereas the CD8α⁻β⁻ are CD5⁻, B220⁺. The CD8α⁺β⁺ LI-IEL do not express self-reacting Vβs and are sensitive to in vivo TCR-mediated signals. The CD8α⁻β⁻ LI-IEL express self-reacting Vβs and have a lower responsiveness to stimulation via the TCR. On the basis of our data we propose that both CD8α⁺β⁺ and CD8α⁻β⁻ LI-IEL represent a separate lineage from splenic and LN CD8⁺ T cells. CD8α⁻β⁻ LI-IEL are functionally competent and undergo negative selection, whereas CD8α⁺β⁺ LI-IEL are less responsive to TCR-mediated signals and appear to escape negative selection.

Materials and Methods

Animals and In Vivo Treatment. C57BL/6, AKR/J, CBA/J, and MRL/MpJ +/- female mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Euthymic BALB/c +/- female mice and athymic BALB/c nu/nu female mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) and housed in the animal facility at the University of Iowa. Mice were injected with 15 μg i.p. of Staphylococcal enterotoxin A (SEA) (Sigma Chemical Co., St. Louis, MO) or with 30 μg of Staphylococcal enterotoxin B (SEB) (Sigma Chemical Co.), or with 500 μg of purified anti-α/β mAb H57.597. Age-matched control mice were injected with PBS.

Cell Preparations. LI including feces and colon were dissected free from connective tissue and lymphoid aggregates, and the fecal contents were flushed with ice cold PBS containing 5 mM EDTA and 5 mM dithiothreitol. LI were opened longitudinally, gently rinsed in several changes of the same ice cold buffer, cut into small pieces, placed in 50-ml plastic tubes, and incubated in this buffer for 30 min at 37°C with constant shaking. After that, the tubes were vortexed for 30 s, supernatants were collected, and the tissue pellets were vortexed for another 30 s in a fresh portion of buffer. The procedure was repeated, and all the supernatants were combined. Dissociated cells were filtered through Pyrex wool (Corning Glass Inc., Corning, NY), plated on plastic dishes to remove adherent cells and cell aggregates, and IEL were separated using discontinuous 40/70% Percoll gradient centrifugation for 20 min at 900 g. Cells from three to six animals were pooled in each experimental group. This protocol yields 6–10 x 10⁶ single cells/adult animal. Preparations from LI of aged nude mice give similar results. This protocol yields 6–10 x 10⁶ single cells/adult animal. Preparations from LI of aged nude mice give similar results.

mAbs and Other Reagents. FITC-labeled mAbs 16A (anti-CD45RB), RR4-7 (anti-CD6), MR5-2 (anti-Vβ6.1+2), RR3-15 (anti-Vβ11), 14-2 (anti-Vβ4), 53.5.8 (anti-CD8β), biotin (Bi)-labeled mAbs RM2-5 (anti-CD2), 37.51 (anti-CD28), 53.5.8 (anti-CD8β), H1.2F3 (anti-CD69), and unlabeled mAb GL3 (anti-TCR-γ/δ) were purchased from PharMingen (San Diego, CA). Cyanin (Cy)-labeled mAb M298 (anti-β7 integrin subunit), supernatant containing mAb M295 (anti-α5β1 integrin subunit) and PE-labeled mAb Mel-14 (anti-L-selectin) were kindly provided by Cheng-Chi Chao and Dr. Morris O. Dailey (University of Iowa). Hybridoma clones H57.597 (anti-TCR-α/β), GKL.5 (anti-CD4), 434 CD8 T Cell Heterogeneity in Murine Large Intestine

Results

IEL of Large Intestine Contain Two Populations of TCR-α/β⁺ CD8α⁺ T Cells. In adult normal mice of all the strains tested (BALB/c, AKR/J, C57BL/6, MRL/MpJ, CBA/J) LI-IEL contained two distinct populations of CD8⁺ cells: CD8α⁺β⁺ and CD8α⁻β⁻ (Fig. 1 A). The proportion of CD8α⁺β⁺ cells within the entire CD8α⁺ population gradually increased with age reaching over 50% in 40+ -wk-old mice (Fig. 1 B). Three-color flow cytometry analysis showed that in contrast to SI-IEL, TCR-γ/δ⁺ cells from LI-IEL accounted for a minor portion of CD8α⁻β⁻ cells never exceeding 10–15% of the latter in young mice (Fig. 1 C) and decreasing to 4–7% in 20+ -wk-old mice (not shown). Accordingly, the vast majority of CD8α⁻β⁻ cells and the entire CD8α⁺β⁺ population bore TCR-α/β (Fig. 1 C). In our experiments CD4⁺ CD8α⁺ LI-IEL never accounted for more than 1.5% of total CD8α⁺ LI-IEL (up to 3% according to Camerini et al. [13]), in older mice being consistently below 1% (not shown). The proportion of Ig⁻ cells within CD8α⁺ LI-IEL was <0.5% in all age groups (not shown).

Besides heterogeneity in TCR composition, CD8α⁻β⁻ cells were considerably different from CD8α⁺β⁺ counterparts by the pattern of Thy1 expression. Whereas CD8α⁺β⁺ cells were uniformly Thy1⁺, the density of Thy1 on CD8α⁻β⁻ cells varied from high to completely negative (Fig. 1 C, lower panel), thus making it not a suitable marker to discriminate between CD8α⁺β⁺ and CD8α⁻β⁻ subsets.

We found two markers, CD5 and B220, to be differentially expressed in these two subsets. All CD8α⁺β⁺ LI-IEL
express CD5, a common marker of peripheral T cells (17), and are devoid of B220, the B cell–specific form of lymphocyte transmembrane phosphatase CD45 (18). In contrast, CD8α+β- LI-IEL are B220+ and CD5− (Fig. 2, A and B). It is important to note, that in contrast to SI-IEL, CD5−, B220+, and Thy1− CD8α+ LI-IEL are mostly TCR-α/β+, rather than TCR-γ/δ+.

The phenotypic differences seen between the CD8α+β+ and CD8α+β− LI-IEL subsets described above suggest that these subsets belong to different lineages and could differ functionally. To address this possibility we looked for the expression of a panel of markers which define certain stages of T cell development and which appear to be connected with their functional properties.

Expression of the Activation Markers and Adhesion Molecules by the CD8 Subsets. We studied LI-IEL CD8+ cells for their expression of CD2, an adhesion and costimulatory molecule common to all mouse cells of T lineage (19, 20). CD2 expression by the CD8+ LI-IEL appeared to be biphasic with 25–30% of CD8α+β− cells being CD2−, whereas the rest of them and all the CD8α+β+ cells were CD2+ (Fig. 3 A). Absence of CD2 from a portion of CD8+ cells is noteworthy, since CD2− T cells from normal SI-IEL and from spleen and lymph nodes of lpr/lpr mice are known to exhibit a block in signal transduction (21, 8). Another costimulatory molecule uniformly expressed by mature peripheral T cells is CD28 (22). In all the mouse strains tested there were no LI-IEL CD8+ cells that expressed CD28 at the high level found on CD8+ cells in the spleen and the LN, even though 100% of CD8α+β− cells were CD28+ (Fig. 3 B). The CD8α+β− LI-IEL were mostly CD28−, or CD28low (Fig. 3 B).
Figure 2. Differential expression of CD5 and B220 Ag by CD8α+β+ and CD8α+β− LI-IEL. LI-IEL from BALB/c mice were processed in pool, as described in Materials and Methods. Distribution of CD5 and B220 markers is shown for CD8α+β+ and CD8α+β− LI-IEL. LI-IEL shown were gated for CD8α+ cells.

Figure 3. Expression of accessory and adhesion molecules by CD8α+β− LI-IEL. LI-IEL from four C57BL/6 mice were processed in pool, as described in Materials and Methods. Distribution of CD2 (A), CD28 (B), L-selectin (C), and the β7 subunit of the M290 integrin (D) is shown for CD8α+β− LI-IEL. LI-IEL shown were gated for CD8α+ cells.

Less than 2% of CD8α+β− cells and up to 70% of CD8α+β+ cells from BALB/c LI-IEL expressed L-selectin (Fig. 3 C), an adhesion molecule for lymph node homing T cells, which is downregulated in peripheral T cells upon activation (23).

To look for “intestine-specific” homing receptors we stained LI-IEL CD8+ cells for the expression of both subunits of αM290β7 integrin. This integrin is expressed by SI-IEL (14, 24). We found that anti-αM290 and anti-β7 mAbs, each labeled with different fluorochrome, uniformly produced completely overlapping stainings (not shown), suggesting that all the β7 subunits were complexed with αM290 subunits on CD8+ LI-IEL. Interestingly, in all ages the majority of CD8α+β− cells were αM290β7+ (Fig. 3 D), but the CD8α+β− population showed a biphasic pattern of αM290β7 expression (Fig. 3 D), which shifted with age to higher expression of αM290β7 (not shown).

To further elucidate the functional status of LI-IEL CD8+ cells, we assessed their expression of IL-2R, CD44, and CD69 molecules, since IL-2R is upregulated in activated T cells, CD44 is an adhesion molecule permanently expressed by activated/memory peripheral T cells (25), and CD69 is a marker of freshly or chronically activated T cells (15, 16). None of the CD8 subsets of LI-IEL in normal mice expressed significant amounts of IL-2R (not shown).

As shown in Fig. 4 B, CD8α+β+ cells are uniformly CD44+. CD8α+β− cells are CD44variable, the majority of them being CD44+. Surprisingly, presumably anergic CD2-CD8α+ cells are also CD44+ (Fig. 4 D). Expression of CD44 implies previous activation, so the presence of CD44 on CD2− CD8+ cells suggests that either these cells could be activated even though they do not express CD2, or these cells have lost CD2 subsequent to being activated.

Another unexpected result was our finding with CD69 expression. The proportion of CD69+ cells within the entire CD8α+ LI-IEL population was found to be 80−90%. To the best of our knowledge such a high proportion of CD69+ cells has never been reported for any T cell subset in normal murine tissues. CD69 is very early and transiently expressed by activated T cells (15). In all the strains and all the age groups tested almost all CD8α+β− cells from LI-IEL were CD69+ (Fig. 4 A). CD8α+β− appeared to be CD69variable, the majority of them converting into CD69+ with age, as shown in Fig. 4 A for 26-wk-old BALB/c mice. Almost 100% of the CD2− CD8α+ cells are CD69+ (Fig. 4 C). According to these data, and in agreement with the CD44 expression data, “abnormal” CD8α+β−CD5−B220+ CD28− LI-IEL including the CD2− and Thy1− cells have the phenotypic features of activated T cells.

The nu Mutation Does Not Affect Development of the CD8α+β+ and CD8α+β− LI-IEL. Since the two CD8+ LI-IEL subsets differ significantly in their phenotype, we addressed the question of their differences in the developmental
requirements. We isolated and phenotypically characterized LI-IEL from genetically athymic nu/nu BALB/c mice. This recessive mutation is known to heavily affect the development of all the thymus dependent T cells. 1-yr-old mice were used, since in nu/nu mice T cells are known to accumulate slowly with age both in systemic tissues (26) and in mucosa-associated tissues of lung and female genital tract (Ibraghimov, A., and R. G. Lynch, unpublished observations).

As presented in Table 1 and Fig. 5 A, both subsets of CD8+ LI-IEL appeared to be abundant in old nu/nu mice. Besides lower proportion of TCR-α/β+ cells within both subsets (Fig. 5, E and F) and correspondingly higher proportion of TCR-γ/δ+ cells (not shown), each subset was phenotypically similar to its counterpart in euthymic mice. These similarities included characteristic patterns of expression of accessory molecules and activation markers (Table 1 and Fig. 5, B–D). Thus, we did not find any differential influence of the nu mutation on the two CD8+ LI-IEL subsets. Both subsets might develop extrathymically, though the extrathymic development of the T cells accumulating in nu/nu mice remains controversial. Still, normal yield and phenotype of CD8α+β+ and CD8α-β- LI-IEL in nu/nu mice strongly suggest that these cells represent a lineage different from splenic and lymph node CD8+ TCR-α/β+ cells that accumulate in the periphery of nude mice very slowly and never reach normal levels (26).

**Based TCR V\_β Repertoires of the CD8+ LI-IEL Subsets.** Assuming that different subsets of CD8 LI-IEL could have different functional properties and thus experience differential selection pressures it might be expected that the subsets would express different TCR V\_β repertoires. We analyzed expression of different V\_β structures by CD8α+ cells in combination with each of the following markers: Thyl, CD2, CD5, B220, and CD8β. This three-color analysis was performed with LI-IEL freshly isolated from BALB/c, CBA/J, C57BL/6, and AKR/J mice employing mAbs against V\_β6, V\_β8.1+2, V\_β11, and V\_β14 structures of the TCR-α/β. The frequencies of certain V\_β structures within CD8β- and CD5- subsets of CD8α+ LI-IEL were corrected to the proportion of TCR-α/β+ cells. The results of these experiments with CBA/J LI-IEL CD8α+ cells are shown in Fig. 6. Several interesting points can be made based on these data: (a) the V\_β repertoire expressed by the CD5– subset closely parallels that of CD8β– subset, whereas the repertoire expressed by the CD5+ subset is very similar to the repertoire of CD8β+ subset; (b) there are very significant differences between the CD5– CD8β– (B220+) and the CD5+ CD8β+ (B220–) subsets; (c) differences in the repertoires of Thyl+ and Thyl– subsets are modest; (d) there are almost no differences in V\_β usage between CD2+ and CD2– subsets of CD8α+ cells; (e) the CD5+ CD8β+ (B220–) subset expresses V\_β14 almost 10 times more frequently than the CD5– CD8β– (B220+) subset; and (f) the CD5– CD8β+ (B220–) subset contains the highest and the CD5+ CD8β+ (B220–) subset contains the lowest proportions of cells expressing V\_β6 (not shown) and V\_β11 (Fig. 6). V\_β6+ and V\_β11+ cells are potentially self-

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**Table 1. The Composition of CD8+ LI-IEL in +/+ and nu/nu BALB/c Mice**

| Proportion of LI-IEL | +/+ | nu/nu |
|----------------------|-----|-------|
|                      | %   | %     |
| Total CD3+LI-IEL (x 10^-6) | 7 ± 0.6 | 6.8; 9.4 |
| TCR-α/β+/CD8+       | 94 ± 6 | 87; 79 |
| CD8α+/CD8α+         | 51 ± 4 | 52; 57 |
| CD8α+/CD5+          | 56 ± 3 | 55; 61 |
| CD8α+/TCR-α/β+      | 98 ± 1 | 92; 95 |
| CD8α+/CD8α–         | 93 ± 2 | 34; 41 |
| CD8α+/Thy1–         | 96 ± 2 | 98; 100 |
| CD8α–/Thy1–       | 83 ± 5 | 91; 88 |
| CD8α+/CD69+        | 94 ± 3 | 96; 97 |

* Experiment was performed three times and the mean and SD are represented.
* Experiment was performed two times and both values are shown.

LI-IEL were isolated from 1-yr-old BALB/c +/+ and nu/nu female mice, stained with cocktails of appropriate mAbs conjugated to FITC, Cy, or Bi, and analyzed by three-color flow cytometry as described in Materials and Methods.
Figure 5. Flow cytometry analysis of CD8+ LI-IEL from nu/nu BALB/c mice. LI-IEL from 1-yr-old nude BALB/c mice were processed in pool as described in Materials and Methods. Distribution of CD8α and CD8β subunits (A) and distribution of CD8α and CD69 markers (B) are shown for total LI-IEL from old nude mice. Distribution of Thy1 (C) and CD28 (D) markers vs. CD83 subunit is shown for the same preparation of LI-IEL gated for CD8α+ cells. Distribution of TCR-α/β vs. CD8β in LI-IEL from +/+ (E) and nu/nu (F) mice is shown for preparations gated for CD8α+.

reacting in Mls-1+ and I-E+ mice, respectively (27, 28), and thus are deleted during intrathymic development in Mls-1+,
I-E+ CBA/J mice.

The percentages of CD8 cells expressing certain Vβ structures differed between strains, but significant differences between the CD5-CD8β- B220+ and CD5+CD8β- B220- subsets, and almost no differences between the CD2+ and CD2- subsets were observed within each strain. The biased repertoires of the CD8β- vs. CD8β+ cells from LI-IEL of AKR/J mice are shown in Fig. 7. Distributions of Vβ11+ cells between the CD5+ and CD5- CD8 cells in four mouse strains are shown in Table 2. In all the three I-E+ strains where Vβ11+ cells are deleted intrathymically, Vβ11 is overrepresented in the CD5-CD8α+ LI-IEL but not in the CD5+CD8α+ LI-IEL. Another important observation is that the CD5+CD8α+ LI-IEL from athymic nude mice are also devoid of Vβ11 TCR. This suggests that: (a) CD8α+β+CD5- LI-IEL, if extrathymic, undergo negative selection in the periphery, and (b) the CD8α+β-CD5- LI-IEL are either nonfunctional, or subject to distinct selection mechanisms.

CD8α+β- and CD8α+β+ Subsets in LI-IEL Are Different in Their Responses to In Vivo TCR-mediated Signals. To study the responsiveness of CD8α+β- and CD8α+β+ LI-IEL in vivo, we injected C57BL/6 mice with SEA or SEB. SEA is a bacterial superantigen selectively stimulating T cells expressing certain Vβ structures, including Vβ11 (29). 48 h after injection of 15 μg i.p. of SEA we found, in agreement with what was previously reported, that the proportion of Vβ11+ cells within CD8α+ splenocytes increased fourfold compared with control mice, but we made the novel observation that the percentage of Vβ11+ CD8α+ LI-IEL decreased by 30-40% (Fig. 8, A and B). Three-color staining of LI-IEL showed that within the CD8α+Vβ11+...
Figure 6. Expression of Vα11 and Vα14 TCR by different subpopulations of CD8+ LI-IEL in CBA/J mice. (A) Percentages of Vα11+ cells among CD8α+ splenocytes and different subpopulations of CD8α+ LI-IEL. (B) Percentages of Vα14+ cells among CD8α+ splenocytes and different subpopulations of CD8α+ LI-IEL. Splenocytes of two CBA/J mice were pooled and stained with anti-CD8α-Cy and anti-Vα11-FITC or anti-Vα14-FITC mAbs. LI-IEL from three to five CBA/J mice were stained with anti-CD8α-Cy, anti-Vα11-FITC, or anti-Vα14-FITC, and anti-Thy1-Bi, anti-CD2-Bi, anti-CD5-Bi, or anti-CD8α-Bi mAbs. These stainings were followed by avidin-PE in the second step. Each variant of staining was performed in three different experiments with the mice of similar age, and the mean and SD are represented. Stainings for CD4, TCR-α/β and TCR-γ/δ were performed in each preparation, and the frequency of CD4-CD8α+/β LI-IEL expressing certain Vα was corrected for the proportion of TCR-α/β+ cells.

population only CD5+CD8β+ cells were deleted, whereas CD5−CD8β− cells were not affected (Fig. 8, C and D). These data suggest that: (a) LI-IEL CD8 cells with conventional phenotype (CD8α+/β+CD5+) differ functionally from their splenic counterparts, undergoing deletion under the same conditions that cause splenic cells to proliferate and (b) CD8α+/β−CD5− cells from LI-IEL are less (if at all) responsive to the in vivo TCR-mediated stimuli caused by SEA.

In the experiments using SEB, which stimulates the majority of Vβ8-expressing T cells as well as some others (30), we found that in the spleen the Vβ8.1+2+CD8+ cells increased from 18.1 ± 3.5% to 41.7 ± 1.4% and that their CD5+ B220−CD8α+β+ counterparts in the LI-IEL also increased from 10.7 ± 1.5% to 17.8 ± 0.3%, whereas Vβ8.1+2+ CD5−B220+CD8α−β− LI-IEL did not increase (not shown). Thus in both instances, SEA and SEB, the conventional CD8α+β+ cells in the spleens responded by proliferation and their counterparts (CD8α−β+) in LI-IEL also responded, but with SEA the response led to decreased expression of the relevant Vβ-region, whereas in the case of SEB, the response led to an increased expression of the relevant Vβ-region.

To extend these data to all TCR-α/β structures and to see if conditions could be identified in which CD8α+β+ cells responded to TCR-mediated signals, we modified the in vivo experiment by using anti-TCR-α/β mAb. A single injection of 500 μg i.p. of purified anti-TCR-α/β mAb produced somewhat similar results on both splenic and LI-IEL BALB/c CD8α+ cells: 72 h postinjection their TCR-α/β was downregulated on 97% of splenic CD8+ cells and on 97% of CD8+ LI-IEL (Fig. 9, A–D) with no significant increase in TCR-γ/δ+ compartment (not shown). In contrast to the spleen, where the relative amount of CD8+ cells increased 60% (Fig. 9, A and B) (with an even more significant absolute increase since the spleens of injected mice were enlarged), the relative amount of CD8− LI-IEL was 30% lower in treated mice (Fig. 9, C and D). We conclude that both CD8+ LI-IEL subsets were affected, since anti-TCR-α/β mAb did not cause a significant shift in CD8β+/CD8α− ratio (Fig. 9 E).

Thus, the entire CD8α+β− LI-IEL subset appear to be sensitive to the in vivo treatment by a massive dose of anti-TCR-α/β mAb: (a) a significant proportion of them is eliminated or downregulate CD8 coreceptor; and (b) all the rest stay CD8+ and downregulate their TCR in response to the signal.

Figure 7. Biased Vβ repertoire of CD8α+β+ and CD8α−β− LI-IEL from AKR/J mice. LI-IEL from three to four AKR/J mice were processed as described in Materials and Methods and stained with anti-CD8α-Cy, anti-CD8β-Bi, and either anti-Vβ6-FITC, anti-Vβ11-FITC, or anti-Vβ14-FITC mAbs. These stainings were followed by avidin-PE in the second step. Distributions of Vβ6, Vβ11, or Vβ14 TCR and the CD8β subunit, and percentages of Vβ+ cells are shown for LI-IEL gated for CD8α+ cells. Similar results were produced in three separate experiments. The frequency of CD4+ CD8α+β− LI-IEL expressing Vα6, -11, or -14 was corrected for the proportion of TCR-α/β+ cells.
Table 2. Expression of $\text{V}_{\beta}11^+$ TCR by CD8$\alpha^+$ Splenocytes and LI-IEL from Different Strains of Mice

| Strain of mice | I-E | Spleen | CD5$^-$ LI-IEL | CD5$^+$ LI-IEL |
|----------------|-----|--------|----------------|----------------|
| AKR/J          | +   | 1.8 ± 0.2$^*$ | 4.5 ± 0.2       | 1.5 ± 0.1       |
| CBA/J          | +   | 1.9;1.6$^t$  | 3.6;3.9$^t$    | 0.4;0.7$^t$    |
| BALB/c +/+     | +   | 1.3 ± 0.1    | 4.8 ± 0.3       | 1.1 ± 0.2       |
| BALB/c nu/nu   | +   | ND$^5$       | 4.1;3.7$^t$    | 0.0;0.4$^t$    |
| C57BL/6        | -   | 4.2 ± 0.1    | 5.6 ± 0.1       | 6.0 ± 0.2       |

* Experiment was performed three or more times, and the mean and SD are represented.
$t$ Experiment was performed twice, and both values are shown.
$^5$ Not done.

LI-IEL were isolated from mice of different strains as described in Materials and Methods. In each experiment IL-IEL from two to four mice were pooled and stained with anti-CD8$\alpha$-Cy, anti-$\text{V}_{\beta}11$-FITC, and anti-CD5-Bi mAb, followed by avidin-PE in the second step. Staining with anti-CD8$\alpha$-Cy was used for positive gating, and staining with anti-CD5-Bi was used either for positive or negative gating, so that the percentages of $\text{V}_{\beta}11^+$ cells were evaluated within CD8$\alpha^+$, CD5$^-$, and within CD8$\alpha^+$, CD5$^+$ LI-IEL. The frequencies of $\text{V}_{\beta}11^+$ CD8$\alpha^+$-LI-IEL were corrected for the proportion of TCR-$\alpha/\beta^+$ cells which was evaluated in each preparation.

It is important to note that the $\text{V}_{\beta}11^+$ TCRs, which are forbidden in the periphery in BALB/c mice, but which remain in the nonconventional CD8$\alpha^+$-LI-IEL subset, were downregulated in anti-TCR-$\alpha/\beta$-treated mice as efficiently as other TCRs (Fig. 9, F and G). The data generated in experiments using SEA, SEB, and anti-TCR-$\alpha/\beta$ mAb show that CD8$\alpha^+$-LI-IEL including those expressing forbidden $\text{V}_\beta$s, are not completely unresponsive, though they appear significantly less sensitive to TCR-mediated signals.
Discussion

Our studies of CD8⁺ LI-IEL revealed their similarity to CD8⁺ SI-IEL, but some differences between the CD8⁺ IEL in these two locations are pronounced. CD8⁺ LI-IEL are characterized by: (a) a higher proportion of CD8α⁺β⁺ cells in adult mice; (b) a lower proportion of α/β T cells; (c) a lower proportion of Thy1⁻ cells; and (d) a lower proportion of CD2⁻ cells. These differences suggest a greater fraction of a conventional CD8⁺ T cells within LI-IEL than within SI-IEL. This "normalization" of phenotype is probably driven by the antigenic challenge of local microflora that is much more abundant in LI as compared with SI.

The results presented here demonstrate the existence of two distinct TCR-α/β⁺ CD8⁺ subsets within murine LI-IEL. One subset expresses both α and β subunits of the CD8 coreceptor, is phenotypically similar to mature peripheral T cells (CD5⁺, Thy1⁺, CD2⁺, CD28⁺, B220⁻), and expresses functional TCR complexes. The features making this LI-IEL subset different from conventional CD8⁺ cells are: (a) the intestine-specific integrin αMβ7; (b) an unusually high proportion of CD69⁺ cells which increases with age; (c) a different sensitivity to TCR-mediated signals; and (d) a high frequency of TCR Vβ14⁺. The second subset has only the α subunits of the CD8 complex, is almost exclusively CD5⁻B220⁺CD28⁻CD69⁻Ly-2⁻, and includes a proportion of Thy1⁻ and CD2⁻ cells. It also shares the unique phenotypic features with CD8α⁺β⁺ LI-IEL, being nearly 100% CD69⁺ and αMβ7⁺.

The similarities of the two subsets might be of functional significance. Expression of the αMβ7 integrin suggests that both subsets reside in, rather than pass through, mucosal epithelium of the large intestine, since this integrin is unique for T cells of intestinal mucosa and conserved between different
species (24, 31). Moreover, this integrin has been directly proven to recognize epithelial cells (32).

The extraordinary high frequency of expression of CD69 by CD8+ LI-IEL is noteworthy. In normal mice CD69 is present on very few splenic and lymph node T cells. It is also not common for resident T cells from other mucosa-associated tissues (lung, female genital tract, liver sinuses) (Ibraghimov, A., and R. G. Lynch, unpublished observations). This antigen is characterized by very early and transient expression upon T cell activation (15), and its constant presence suggests chronic activation (16). In this work we present the first evidence that CD69 is expressed by the vast majority of CD8+ LI-IEL, and in other studies we have found similar levels of CD69 expression on SI-IEL (Ibraghimov, A., and R. G. Lynch, unpublished observations). The reasonable explanation is that in both sites TCR-α/β+CD8+ cells are constantly stimulated by Ag from the lumen which would include dietary and the local microflora.

One more important feature common to both CD8+ LI-IEL subsets is that they belong to a lineage distinct from conventional splenic and lymph node CD8+ T cells: the two LI-IEL subsets are abundant in genetically athymic mnu/mnu BALB/c mice, and have phenotypes closely resembling those of their counterparts in euthymic mice, while the development of splenic and lymph node T cells in mnu/mnu mice is heavily affected. The only clear difference we found between CD8+ LI-IEL from mnu/mnu mice as compared with CD8+ LI-IEL from euthymic mice is the higher proportion of TCR-γ/δ+ cells.

The phenotypic differences seen between the two subsets suggest different functional properties. Several lines of evidence support this view. It is established that the Thy1 molecule is involved in T cell activation (33). It is also known that CD5 is a T cell–specific, activation-inducible serine kinase (34) associated with CD2, CD4, and CD8 coreceptors and with the TCR complex (35), and provides accessory signals for T lymphocyte activation and proliferation (36).

CD2 is a pan-T surface adhesion molecule participating in a multimolecular complex consisting of the TCR/CD3, CD4/8, CD5, and p56 lk and P59kn kinases (35, 37). CD2 augments TCR-mediated signals (20) and mediates cell-cell adhesion in case of CD8+ cytotoxic lymphocytes (38). Known exceptions to the pan-T distribution are the CD2− T cells that accumulate in the periphery of mutant lpr/lpr (39) mice, and the CD2− SI-IEL in normal mice (8). In both cases CD2− T cells are unresponsive to TCR-mediated signals by the criteria of proliferation and cytokine production (8, 21).

CD28 is yet another pan-T accessory molecule augmenting signal transduction via the TCR/CD3 complex, which normally leads to T cell proliferation and lymphokine production (22). CD28+ T cells were found previously in SI-IEL, constituting up to 95% of CD8α+β+ SI-IEL (9). In SI-IEL CD28+ phenotype correlated with unresponsiveness to TCR-mediated stimuli (9).

Thus, the differences revealed between CD8α+β+ and CD8α+β− LI-IEL suggest that the former are functional T cells, while the latter are either nonfunctional, or employ unconventional pathways of transduction of the TCR-mediated signals. It might be true at least in the case of CD2− LI-IEL, which are uniformly CD44+, CD69+ (thus expressing markers of committed T cells), and do not differ in their Vβ repertoire from CD2+ CD8α−β− LI-IEL.

Responsiveness to the TCR-induced signals of the CD8α−β+ LI-IEL deduced from their phenotype is supported by several lines of evidence: (a) in all the strains tested including nu/nu BALB/c, this subset is devoid of TCRs using forbidden Vβ genes, suggesting its ability to undergo negative selection; (b) in vivo injections of superantigens SEA or SEB produced selective effects on the CD8α+β+ LI-IEL expressing appropriate Vβ genes; (c) this subset increases with age, probably due to proliferation driven by constant antigenic stimulation; and (d) this subset shows ontogenetic changes consistent with their ability to respond to ongoing antigenic challenge: accumulation of CD69+ and αM μ2β7hi cells. We consider upregulation of this integrin to be activation dependent, since in the in vivo experiment SEB-induced T cell activation was accompanied by the shift of a proportion of these cells from αM μ2β7lo to αM μ2β7hi (not shown).

The unresponsiveness of the CD8α−β− LI-IEL subset implied by some of their phenotypic features (CD5−, CD28−, CD2+−) is in agreement with the following observations: (a) the age-dependent decrease in their relative amount (probably, due to overgrowth by functional LI-IEL); (b) the lack of negative selection, resulting in expression of forbidden Vβ genes; and (c) the inability to respond to in vivo administrated bacterial superantigens.

Interestingly, there are some phenotypic and functional properties of the CD8α−β− LI-IEL subset that argue against unresponsiveness of this subset. These include expression of CD44 and CD69, markers of previous and recent (chronic) activation, respectively, and the ability of this subset to respond in vivo to a massive dose of anti-TCR-α/β mAb. CD44 expression suggests that CD8α−β− LI-IEL once experienced activation. Moreover, according to CD69 expression, the entire subset might be constantly restimulated. A possible scenario might be that an initial activation event (reflected by the CD44 expression) occurred employing a conventional pathway of TCR signal transduction, but while the subset originally bore the needed accessory molecules, it lost them upon activation, and thus became anergized. Similar events take place in Peyer’s patches, where about 15% of CD4+ cells permanently lose expression of the Thy1 molecule and become anergic upon activation (40).

In this regard it is also important to note that whereas unresponsiveness of CD2− and CD28− SI-IEL, including CD8α+β−, was very well documented in vitro (8, 9), there was a 97% decrease in the amount of CD8+ TCR-α/β+ SI-IEL in MHC class I-deficient mice (11). This suggests that these euthymic T cells, insensitive to negative selection, still require an MHC-mediated positive stimulation to complete their development. Such putative behavior of CD8α−β− SI-IEL gets direct experimental proof in mice transgenic for TCR-α/β specific for male antigen (H-Y).
presented by class I MHC molecules: CD8α+/β- SI-IEL are not deleted in male intestine by the self-antigen, but rather positively selected to differentiate; in the female intestine in the absence of the specific peptide they are not positively selected (12, 41). Still, these once positively selected CD8α+/β- IEL are refractive to stimulation by H-Y+ cells in vitro (41). It is tempting to speculate that CD8α+/β- LI-IEL under study also display this unusual combination of features: requirement for positive selection (their phenotype reflecting this past activation event), and unresponsiveness to negative selection by the self Ags (as reflected by their Vβ repertoire), as well as to experimental TCR-mediated stimuli. Moreover, CD8α+/β- LI-IEL might even be positively selected for the forbidden Vβ8 since very recent studies have demonstrated the expression of Mls-1+ products by the intestinal epithelium, and the ability of these epithelial cells to stimulate Vβ6+ T cells (42). Thus, the CD8α+/β- subset of LI-IEL appears to be a subject of unconventional selection mechanisms rather than a completely unresponsive T cell population. In agreement with this, in our in vivo experiments, CD8α+/β- LI-IEL responded to anti-TCR treatment. Selective responsiveness was also revealed in SI-IEL. They respond poorly to mitogens and anti-TCR Abs, but are very effectively stimulated by the heat-shock protein of Mycobacterium tuberculosis to proliferate and secrete lymphokines (43).

In summary, there are two subsets of TCR-α/β+ CD8+ LI-IEL: the CD8α+/β+ and the CD8α+/β- subsets. Both subsets are different from conventional CD8+ cells of the periphery by the expression of intestinal T cell homing receptor αM290β7, and by an unusually high frequency of chronically activated cells, as reflected by expression of the early activation marker CD69. Moreover, the CD8α+/β+ LI-IEL differ from splenic counterparts by 1.5-2-fold higher frequency of Vβ14+ cells, suggesting selection by an intestine-specific Ag. The CD5-CD2+CD28+Thy1+B220+CD8α+/β- LI-IEL undergo negative selection in the periphery, and readily respond in vivo to TCR-mediated stimuli. The CD5-CD2+CD28+Thy1+B220+CD8α+/β- subset of LI-IEL is subjected to distinct selection mechanisms and has a low responsiveness to TCR-mediated stimuli.

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