Selection and Expansion of CD8α/α⁺ T Cell Receptor α/β⁺ Intestinal Intraepithelial Lymphocytes in the Absence of Both Classical Major Histocompatibility Complex Class I and Nonclassical CD1 Molecules

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Summary

Intestinal intraepithelial lymphocytes (IELs) in mice include two main subsets of TCR-α/β⁺ cells which differ functionally and ontogenetically from each other. One expresses the CD8α/α homodimer, whereas the other expresses the CD8α/β heterodimer. Although the presence of all CD8⁺ TCR-α/β⁺ IELs is dependent on β2-microglobulin molecules, the nature of the major histocompatibility complex (MHC) class I molecules recognized by the CD8α/α and the CD8α/β⁺ subsets has remained elusive. Using mutant mice lacking the expression of both H-2K⁺ and H-2D⁺, we show that the CD8α/β⁺ TCR-α/β⁺ subset is dependent on K or D molecules, whereas the CD8α/α⁺ TCR-α/β⁺ subset is independent of classical MHC class I molecules. Furthermore, the CD8α/α⁺ cells are conserved in mice lacking expression of CD1, a nonclassical MHC class I-like molecule previously proposed to be a potential ligand for IELs. Using transporter associated with antigen processing (TAP)-deficient mice, this cell population can be further separated into a TAP-dependent and a TAP-independent subset, suggesting either the recognition of two nonclassical MHC-like molecules, only one of which is TAP-dependent, or the involvement of a single nonclassical MHC-like molecule that is only partially TAP-dependent. These findings demonstrate that CD8α/β⁺ TCR-α/β⁺ IELs are restricted by H-2K and H-2D molecules, whereas the unusual subset of CD8α/α⁺ TCR-α/β⁺ resident IELs recognize nonclassical MHC class I-like molecules that are distinct from CD1.

Key words: major histocompatibility complex • CD1 • intestinal intraepithelial lymphocytes • CD8 • gene-targeted mouse

A large contingent of lymphocytes is located in the epithelial barriers that are exposed to the outside environment. Although a few of these lymphocytes are related to peripheral lymphocytes, most of them appear to be resident cells that are only encountered inside epithelial barriers. The ontogeny and functions of these resident cells appear to be unique. For example, sequential waves of TCR-γ/δ⁺ T cells expressing distinct TCR gene families seed the epithelia of the skin, reproductive tract, and intestine (1). Their antigen specificity and their function remain elusive, although it is increasingly recognized that they participate in the stress response of epithelia and are involved in the wound healing process (2, 3).

Intestinal intraepithelial lymphocytes (IELs) include prominent populations of both TCR-γ/δ⁺ and TCR-α/β⁺ cells (4–6). Among the TCR-α/β⁺ IELs, there exist two subsets according to the expression of the CD8α/β heterodimer or the CD8α/α homodimer (which is also expressed on most of the TCR-γ/δ⁺ IELs). The CD8α/β⁺ subset can participate in conventional T cell responses against microbes as well as food antigens (7–13). It includes cells that were primed in the Peyer’s patches, and have circulated back to seed the whole length of the intestinal epithelium (14, 15). In contrast, the CD8α/α⁺ subset remains mysterious, as it appears to be a resident subset that has not yet been associated with antigen-specific responses but might instead play local functions of regulation (16, 17). However, the detection of oligoclonal ex-
pansions of CD8α/α⁺TCR-α/β⁺ IELs and their activated phenotype do suggest that, like CD8α/β⁺ cells, they may be actively involved in ongoing immune responses (18–20).

Although the MHC or MHC-like molecules contributing to the selection or activation/expansion of the TCR-α/β⁺ IEL subsets have not been identified, they are known to be dependent on β2-microglobulin (β2m), because all CD8⁺ TCR-α/β⁺ IELs were absent in β2m-deficient mice (21). In transporter associated with antigen processing (TAP)-deficient mice, the CD8α/β subset is absent while some CD8α/α cells are conserved (22), suggesting that at least some of the CD8α/α IELs might recognize nonclassical MHC-like molecules such as CD1, which is TAP-independent and has been reported to be expressed by the intestinal epithelium of mice and humans and recognized by CD8 clones derived from human IELs (23).

In this paper, we used the recently generated CD1-deficient, H-2K⁺/D⁺ double-deficient, and K⁺/D⁺/CD1 triple-deficient mice to investigate the nature of the MHC and MHC-like ligands associated with intestinal TCR-α/β⁺ lymphocytes. Our results demonstrate that all CD8α/β⁺ TCR-α/β⁺ IELs are dependent on the classical H-2K and H-2D MHC class I molecules, whereas CD8α/α⁺ TCR-α/β⁺ IELs are independent of both the classical H-2 and the nonclassical CD1 molecules. Thus, the CD8α/α⁺ TCR-α/β⁺ IELs must recognize nonclassical, non-CD1, as yet undefined MHC class I-like molecules. These results show that the phenotypic expression of CD8α/α versus CD8α/β among TCR-α/β⁺ IELs reflects a fundamental dichotomy with respect to antigen specificity and function.

Materials and Methods

Mice. Mice were housed at Princeton University under specific pathogen-free conditions and were 10–14 wk old at the time of study. Mutant mice (deficient in TAP-1 [24], K⁺/D⁺ [25], CD1.1 [Park, S.-H., and A. Bendelac, manuscript in preparation], CD1.1/1.2 [26], or β2m [27]) were generated from embryonic stem cells of 129 origin and used after 6–10 backcrosses to C57BL/6. K⁺/D⁺/CD1-deficient mice were generated by crossing CD1.1- with K⁺/D⁺-deficient mice. In all cases, +/+ or −/− littermates were used as controls, except for β2m- and CD1.1/CD1.2-deficient mice, which were compared with age- and sex-matched C57BL/6 mice.

Histology. For histological study, duodenal fragments of 1 cm taken 3 cm below the pylorus were properly oriented on filter paper and fixed in Carnoy’s fluid for 24–48 h. Paraffin-embedded sections were prepared and stained with periodic acid-Schiff (PAS), and the numbers of IELs per 100 epithelial cells (ECs) were counted under the microscope.

Lymphocyte Isolation. The small intestine was separated from the small intestine was separated from Peyer’s patches and mesenteric lymph nodes, cut longitudinally, and washed in PBS. Fragments of 0.5–1 cm were incubated for 30 min at 37°C in RPMI 1640 (GIBCO BRL) containing 1% dialyzed FCS (Biowhittaker), 1.5 mM MgCl₂, and 1 mM EGTA and supplemented with 1 mM dithiothreitol (Sigma Chemical Co.), under constant shaking. The IELs contained in the supernatant were collected, washed three times in PBS/5% FCS, and passed through a nylon mesh. IELs were further purified by centrifugation over ficoll-paque™ (Amersham Pharmacia Biotech).

Flow Cytometry. Lymphocytes were first incubated for 30 min with 2.4G2 anti-Fc antibodies in order to block Fc receptors. For triple membrane staining with directly conjugated antibodies, cells were labeled with a combination of antibodies conjugated to PE, FITC, Cy-Chrome, or biotin. Biotinylated antibodies were revealed with Streptavidin Tricolor (Caltag Laboratories).

Anti-TCR-α/β, TCR-γ/δ, CD8α, CD8b, CD4, CD19, CD44, CD69, B220, CD103, and TCR-Vβ were purchased from PharMingen.

Fluorescence was analyzed on a FACScan™ (Becton Dickinson). The live gate for acquisition contained >95% CD103⁺ cells, no CD19⁺ cells, and <10% CD4⁺ cells in all cases.

Results

Persistence of CD8⁺ TCR-α/β⁺ cells in the intestinal epithelium of K⁺/D⁺ double-deficient mice. Fig. 1 shows a flow cytometry analysis of splenocytes and intestinal IELs from K⁺/D⁺-deficient mice and littermate controls. Unlike CD8⁺ TCR-α/β⁺ cells in the spleen, which were virtually all dependent on K⁺ or D⁺ expression, the percentage of CD8⁺ TCR-α/β⁺ IELs was unaffected by the loss of the classical MHC molecules. This striking observation was confirmed on a total of nine K⁺/D⁺-deficient mice (33 ± 9% CD8⁺ TCR-α/β⁺ IELs) and eight littermate controls (38 ± 9% CD8⁺ TCR-α/β⁺ IELs). Furthermore, because cell recoveries after isolation of IELs may vary from one sample or one experiment to another, we counted the number of IELs per 100 ECs on histological sections of duodenal samples, and found that the numbers of IELs/100 ECs were comparable in K⁺/D⁺-deficient mice (15 ± 3 IELs/100 ECs) and littermate controls (15 ± 3 IELs/100 ECs) (Table I). Thus, the absolute number of CD8⁺ TCR-α/β⁺ IELs seemed to be conserved.

In addition, no change in the frequency of TCR-γ/δ⁺ or CD4⁺ TCR-α/β⁺ IELs could be detected in K⁺/D⁺-deficient mice (Fig. 1, and Table I).
CD8α/α⁺ TCR-α/β⁺ IELs Are Independent of Kᵇ/Dᵇ, Whereas CD8α/β⁺ TCR-α/β⁺ IELs Are Dependent. To further investigate the observation that CD8α⁺ TCR-α/β⁺ IELs seemed unaffected in Kᵇ/Dᵇ double-deficient mice, we analyzed the CD8α⁺/α⁺ and CD8α⁺/β⁺ subsets of TCR-α/β⁺ cells separately. These two subsets differ with respect to their ontogeny, recirculation, and function, suggesting that they might exhibit different requirements for MHC ligands as well (4–6). Fig. 2 and Table I show that the CD8α⁺/β⁺ subset was virtually entirely absent in Kᵇ/Dᵇ double-deficient mice, whereas the CD8α⁺/α⁺ cells were conserved or even increased. As previously noted, there were no significant differences between the absolute numbers of IELs/100 ECs found in the various genetically modified mice used in this study and in their littermate controls. Thus, the frequency of various subsets among total IELs directly reflected their absolute number (Fig. 2, and Table I).

These results demonstrate a fundamental difference in the MHC ligands used by the two subsets of CD8⁻ TCR-α/β⁺ IELs. Most CD8α⁺/α⁺ cells are independent of classical MHC class I molecules, whereas most CD8α⁺/β⁺ cells are dependent on Kᵇ/Dᵇ.

The CD8α⁺/α⁺ TCR-α/β⁺ Population in Kᵇ/Dᵇ-deficient Mice Has a Normal Phenotype. To further investigate the possibility that the development and/or expansion of a CD8α⁺/α⁺ TCR-α/β⁺ cell population was dependent in any way on classical Kᵇ and Dᵇ molecules, we examined their surface phenotype in the Kᵇ/Dᵇ-deficient mice. We found that they expressed the same degree of activation as their littermate controls, as judged by the expression of the activation markers CD44, CD69, and B220 (Fig. 3, and data not shown). We also examined the rare subset of CD8⁻/CD4⁻ double-negative TCR-α/β⁺ IELs, as it might include precursors of the CD8α⁺/α⁺ lineage. Again, no difference could be detected between Kᵇ/Dᵇ-deficient mice and littermate controls (data not shown).

We next analyzed the Vβ repertoire of the CD8α⁺/α⁺ population of Kᵇ/Dᵇ-deficient mice. Table II shows that CD8α⁺/α⁺ cells expressed a diverse TCR Vβ repertoire, but that the frequency of various Vβs varied considerably from mouse to mouse. This pattern was also found in Kᵇ/Dᵇ+/⁺ littermates, and most likely results from the presence of oligoclonal expansions, as reported previously (19, 20).

### Table I. IEL Subsets of Normal and Mutant Mice

| Mice                  | No. IELs/100 ECs | CD8α⁺/α⁺ | CD8α⁺/β⁺ | CD4⁻/β⁺ | TCR-γ/δ |
|-----------------------|------------------|----------|----------|---------|---------|
| Controls (n = 27)*    | 14 (3)           | 20 (10)  | 7 (4)    | 2 (2)   | 59 (8)  |
| Kᵇ/Dᵇ KO (n = 9)    | 14 (3)           | 28 (10)  | 0.6 (0.4)| 2 (1)   | 60 (4)  |
| CD1 KO (n = 6)       | 13 (2)           | 17 (5)   | 12 (8)   | 1 (1)   | 51 (9)  |
| TAP KO (n = 6)       | 13 (3)           | 10 (7)   | 0.5 (0.4)| 2 (1)   | 80 (7)  |
| β2m KO (n = 7)       | 10 (3)           | 1.4 (1.1)| 0.1 (0.1)| 1.1 (0.3)| 90 (3)  |

*Controls include +/- or +/+ littermates of Kᵇ/Dᵇ, CD1-, and TAP-deficient mice and age/sex-matched C57BL/6 mice for β2m-deficient mice. KO, knockout.

†Total no. of IELs per 100 ECs (mean value, with SD in parentheses).

‡Percentage of total IELs (mean value, with SD in parentheses).

![Figure 2](https://example.com/fig2.png)

**Figure 2.** CD8α⁺/α⁺ and CD8α⁺/β⁺ IEL subsets in mutant mice. IELs of control and mutant mice were stained with anti-TCR-α/β, anti-CD4, anti-CD8α, and anti-CD8β. The left panels display the CD8α versus TCR-α/β profiles of gated CD4⁻/CD8β⁻ negative IELs, and the right panels display the CD8β versus TCR-α/β profiles of gated CD4⁻/negative IELs. Thus, in the left panels the upper right quadrants of the dot plots correspond to the CD8α⁺/α⁺ TCR-α/β⁺ cells, whereas in the right panels they correspond to the CD8α⁺/β⁺ TCR-α/β⁺ cells. TCR-γ/δ⁺ cells are seen in the left quadrants as TCR-α/β⁻/negative CD8β⁻/negative cells (most TCR-γ/δ⁻ IELs are CD8α⁺/+).
Thus, the CD8α/α+ TCR-α/β+ IEL subset of Kβ/Dβ- deficient mice appears to conserve the activated phenotype and the pattern of oligoclonal expansions that are characteristic of normal IELs, further indicating that CD8α/α+ TCR-α/β+ IELs persist unaltered in Kβ/Dβ-deficient mice.

Kβ/Dβ-independent, CD8α/α+ TCR-α/β+ IELs can be divided into subsets of TAP-dependent and TAP-independent populations. Although it was previously reported that all of the CD8α/β+ IELs were dependent on the presence of TAP, some CD8α/α+ TCR-α/β+ IELs seemed to be TAP independent (21, 22). We confirmed these results, showing that TAP-deficient mice retained about half the number of CD8α/α+ TCR-α/β+ IELs in their littermate controls (10 ± 7 vs. 19 ± 10%; see Fig. 2 and Table I). In contrast, as reported previously (21), all CD8α/α+ IELs as well as CD8α/β+ IELs depended on β2m. These results suggest two possibilities. Either CD8α/α+ TCR-α/β+ cells recognize two distinct nonclassical MHC class I-like molecules, only one of which is TAP dependent, or they recognize one nonclassical MHC class I-like molecule that is partially TAP dependent.

CD8α/α+ TCR-α/β+ IELs are conserved in CD1-deficient mice. The conservation of the nonclassical MHC class I-like molecule CD1d in mammals, and the reports that it is expressed by intestinal epithelial cells of both mice and humans and that CD8+ T cell clones isolated from human IELs were CD1d reactive in a TAP-independent fashion, made CD1d an attractive candidate as the ligand of a subset of the CD8α/α+ TCR-α/β+ IELs (23). Mice have two CD1 genes that are 95% identical, both belonging to the CD1d family (28, 29). However, the CD1.2 gene has a frameshift mutation in the B6 strain that is predicted to abolish cell surface expression and in other strains CD1.2 also seems to be poorly, if at all, expressed on the cell surface (30, 31). We analyzed the IEL population of CD1.1 as well as CD1.1/CD1.2 double-deficient mice. Fig. 2 shows that both the CD8α/α+ and CD8α/β+ TCR-α/β+ IELs were conserved in CD1.1/CD1.2-deficient mice. Results obtained from CD1.1- and CD1.1/CD1.2-deficient mice are comparable and are pooled in Table I. Altogether, these results show that neither the CD8α/α+ nor the CD8α/β subset is dependent on CD1, and suggest that the CD8α/β+ subset requires an unknown nonclassical MHC-like molecule that is β2m dependent and partially TAP independent.

Table II. TCR Vβ Repertoire of CD8α/α+ TCR-α/β+ IELs in KβDβ+/− and KβDβ−/− Mice

| Mouse | Vβ2 | Vβ3 | Vβ5.1 + 5.2 | Vβ6 | Vβ7 | Vβ8.1 + 8.2 | Vβ8.3 | Vβ9 | Vβ10 | Vβ11 | Vβ12 | Vβ13 | Vβ14 |
|-------|-----|-----|-------------|-----|-----|-------------|-------|-----|------|------|-----|-----|-----|-----|
| KβDβ+/− | 1.7* | 0.6 | 2.4 | 10.9 | 3.7 | 0.6 | 12.4 | 9.8 | 3.2 | 3.1 | 7.2 | 0.2 | 35.1 | 1.1 |
| KβDβ+/+ | 5.3 | ND | 4.1 | 3.6 | 8.2 | ND | 15.7 | 9.1 | ND | 15.6 | 12.2 | ND | 12.2 | 2.1 |
| KβDβ−/− | 1.4 | 0.4 | 5.4 | 4.6 | 2.9 | 0.6 | 8.4 | 15.8 | 3.7 | 5.3 | 18.2 | 0.4 | 12.1 | 1.1 |
| KβDβ−/+ | 6.3 | ND | 4.4 | 8.2 | 0.8 | ND | 9.2 | 20.3 | ND | 1.4 | 18.9 | ND | 13.5 | 1.3 |
| KβDβ−/− | 2.9 | ND | 4.5 | 4.8 | 4.4 | ND | 11.1 | 14.0 | ND | 3.1 | 9.0 | ND | 2.2 | 1.0 |

IELs of KβDβ−/− deficient and littermate control mice were stained with anti-CD8α, anti-Vβs, and a cocktail of anti-TCR-α/β/CD4/CD8β. The percentages of cells expressing indicated Vβs among TCR-α/β/CD4/CD8β-negative, CD8α+ IELs (corresponding to the CD8α/α+ TCR-α/β+ subset) are represented.

* Frequencies of Vβ usage were determined from at least 5,000 CD8α/α+ TCR-α/β+ IELs.
CD8α/α+TCR-α/β+ IELs in Kb/Db/b1 CD1 triple-deficient mice. IELs were triple-stained with anti-CD4/CD8β, anti-CD8α, and anti-TCR-α/β. CD8α versus TCR-α/β profiles of gated CD4/CD8β-negative IELs are displayed. The upper right quadrants of the dot plots correspond to the CD8α/α+TCR-α/β+ cells.

Figure 4. Persistence of CD8 α/α+TCR-α/β+ IELs in Kb/Db/b1 CD1 triple-deficient mice. IELs were triple-stained with anti-CD4/CD8β, anti-CD8α, and anti-TCR-α/β. CD8α versus TCR-α/β profiles of gated CD4/CD8β-negative IELs are displayed. The upper right quadrants of the dot plots correspond to the CD8α/α+TCR-α/β+ cells.

Discussion

Although CD8α/α+TCR-α/β+ IELs could be detected in transgenic mice expressing TCR-α/β with defined MHC class I/peptide specificities (32–34), normal non-transgenic CD8α/α+TCR-α/β+ IELs have not been associated with antigen-specific, classical MHC class I-restricted function. The pattern of expression of CD8α/α+TCR-α/β+ IELs in β2m-, TAP-, Kb/Db-, and CD1-deficient mice, as reported in this study, clearly demonstrates that most CD8α/α+TCR-α/β+ IELs do not recognize classical MHC class I molecules, and points to nonclassical MHC class I-like molecules that are β2m-dependent and partially TAP independent. Since CD1 could be ruled out by the study of CD1-deficient mice, candidate ligands include, but may not be restricted to, TL, which is expressed on intestinal epithelial cells (35), and Qa1 (36, 37), both nonclassical MHC class I molecules that can function in the absence of TAP.

There is increasing recognition that several body tissues, especially barrier epithelia of the skin and intestine, and the liver, have specialized immune systems that contain prominent populations of resident T lymphocytes with original antigen specificity and functions. In recent years, two previously orphan families of nonclassical MHC class I-like molecules have become associated with such populations, including MICA/MICB for human intestinal TCR-γ/δ+ IELs and CD1 for liver NKT cells (3, 31). Thus, the emerging pattern suggests that nonclassical MHC-like molecules with specialized functions are critical in specialized tissue environments. Our results now clearly demonstrate that another major subset of mouse intestinal IELs, the CD8α/α+TCR-α/β+ cells, recognize a nonclassical, non-CD1 type of MHC class I-like molecule. Further studies are warranted to identify the ligand(s) of CD8α/α+TCR-α/β+ intestinal IELs, a key for our understanding of local immunity in the intestine.

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In summary, the intestinal intraepithelial lymphocytes (IELs) play a crucial role in the defense against intestinal infections. They are a heterogeneous population of T cells that respond to a variety of antigens and have distinct functions. The development and survival of these cells are influenced by interactions with the epithelial cells and the microenvironment of the gut. Further studies are needed to fully understand the complex regulation of IELs and their role in the immune response to intestinal infections.