

Selection and Expansion of CD8 α / α ⁺ T Cell Receptor α / β ⁺ Intestinal Intraepithelial Lymphocytes in the Absence of Both Classical Major Histocompatibility Complex Class I and Nonclassical CD1 Molecules

By Se-Ho Park,* Delphine Guy-Grand,[†] François A. Lemonnier,[§] Chyung-Ru Wang,^{||} Albert Bendelac,* and Bana Jabri*

From the *Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544; the [†]Hôpital Necker Enfants Malades, INSERM U429, 75015 Paris, France; the [§]Institut Pasteur, Unité d'Immunité Cellulaire Antivirale, 75015 Paris, France; and the ^{||}Department of Pathology, Gwenn Knapp Center for Lupus and Immunological Research, University of Chicago, Chicago, Illinois 60637

Summary

Intestinal intraepithelial lymphocytes (IELs) in mice include two main subsets of TCR- α / β ⁺ cells which differ functionally and ontogenically from each other. One expresses the CD8 α / α homodimer, whereas the other expresses the CD8 α / β heterodimer. Although the presence of all CD8⁺TCR- α / β ⁺ IELs is dependent on β 2-microglobulin molecules, the nature of the major histocompatibility complex (MHC) class I molecules recognized by the CD8 α / α and the CD8 α / β ⁺ subsets has remained elusive. Using mutant mice lacking the expression of both H2-K^b and H2-D^b, we show that the CD8 α / β ⁺TCR- α / β ⁺ subset is dependent on K or D molecules, whereas the CD8 α / α ⁺TCR- α / β ⁺ subset is independent of classical MHC class I molecules. Furthermore, the CD8 α / 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 CD8 α / β ⁺TCR- α / β ⁺ IELs are restricted by H-2K and H-2D molecules, whereas the unusual subset of CD8 α / 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 CD8 α / β heterodimer or the CD8 α / α homodimer (which is also expressed on most of the TCR- γ / δ ⁺ IELs). The CD8 α / 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 CD8 α / 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 CD8 α / α ⁺TCR- α / β ⁺ IELs and their activated phenotype do suggest that, like CD8 α / 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 β 2-microglobulin (β 2m), because all CD8⁺TCR- α / β ⁺ IELs were absent in β 2m-deficient mice (21). In transporter associated with antigen processing (TAP)-deficient mice, the CD8 α / β subset is absent while some CD8 α / α cells are conserved (22), suggesting that at least some of the CD8 α / 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^b/D^b double-deficient, and K^b/D^b/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 CD8 α / β ⁺TCR- α / β ⁺ IELs are dependent on the classical H-2K and H-2D MHC class I molecules, whereas CD8 α / α ⁺TCR- α / β ⁺ IELs are independent of both the classical H-2 and the nonclassical CD1 molecules. Thus, the CD8 α / 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 CD8 α / α versus CD8 α / 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^b/D^b [25], CD1.1 [Park, S.-H., and A. Bendelac, manuscript in preparation], CD1.1/1.2 [26], or β 2m [27]) were generated from embryonic stem cells of 129 origin and used after 6–10 backcrosses to C57BL/6. K^b/D^b/CD1-deficient mice were generated by crossing CD1.1- with K^b/D^b-deficient mice. In all cases, +/+ or +/- littermates were used as controls, except for β 2m- 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 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 (Biofluids), 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 , CD8 β CD8 α , CD4, CD19, CD44, CD69, B220, CD103, and TCR V β 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^b/D^b Double-deficient Mice. Fig. 1 shows a flow cytometry analysis of splenocytes and intestinal IELs from K^b/D^b-deficient mice and littermate controls. Unlike CD8⁺TCR- α / β ⁺ cells in the spleen, which were virtually all dependent on K^b or D^b 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^b/D^b-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^b/D^b-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^b/D^b-deficient mice (Fig. 1, and Table I).

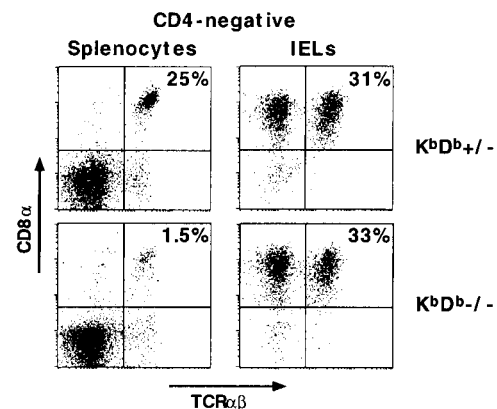


Figure 1. The CD8⁺TCR- α / β ⁺ IEL subset is present in K^b/D^b-deficient mice. Splenocytes and IELs were triple-stained with anti-TCR- α / β , anti-CD4, and anti-CD8 α . The dot plots display the CD8 α versus TCR- α / β profiles of gated CD4-negative cells.

Table I. IEL Subsets of Normal and Mutant Mice

Mice	No. IELs/100 ECs	TCR- α/β			TCR- γ/δ
		CD8 α/α	CD8 α/β	CD4 $^+8^-$	
Controls ($n = 27$)*	14 (3) [‡]	20 (10) [§]	7 (4)	2 (2)	59 (8)
K ^b D ^b 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)
β 2m KO ($n = 7$)	10 (3)	1.4 (1.1)	0.1 (0.1)	1.1 (0.3)	90 (3)

*Controls include +/- or +/+ littermates of K^bD^b-, CD1-, and TAP-deficient mice and age/sex-matched C57BL/6 mice for β 2m-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).

CD8 α/α ⁺TCR- α/β ⁺ IELs Are Independent of K^b/D^b, Whereas CD8 α/β ⁺TCR- α/β ⁺ IELs Are Dependent. To further investigate the observation that CD8⁺TCR- α/β ⁺ IELs seemed unaffected in K^b/D^b double-deficient mice, we analyzed the CD8 α/α ⁺ and CD8 α/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 CD8 α/β ⁺ subset was virtually entirely absent in K^b/D^b double-deficient mice, whereas the CD8 α/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 CD8 α/α ⁺ cells are independent of classical MHC class I molecules, whereas most CD8 α/β ⁺ cells are dependent on K^b/D^b.

The CD8 α/α ⁺TCR- α/β ⁺ Population in K^b/D^b-deficient Mice Has a Normal Phenotype. To further investigate the possibility that the development and/or expansion of a CD8 α/α ⁺TCR- α/β ⁺ cell population was dependent in any way on classical K^b and D^b molecules, we examined their surface phenotype in the K^b/D^b-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 CD8 α/α ⁺ lineage. Again, no difference could be detected between K^b/D^b-deficient mice and littermate controls (data not shown).

We next analyzed the V β repertoire of the CD8 α/α ⁺ population of K^b/D^b-deficient mice. Table II shows that CD8 α/α ⁺ cells expressed a diverse TCR V β repertoire, but

that the frequency of various V β s varied considerably from mouse to mouse. This pattern was also found in K^b/D^b +/- littermates, and most likely results from the presence of oligoclonal expansions, as reported previously (19, 20).

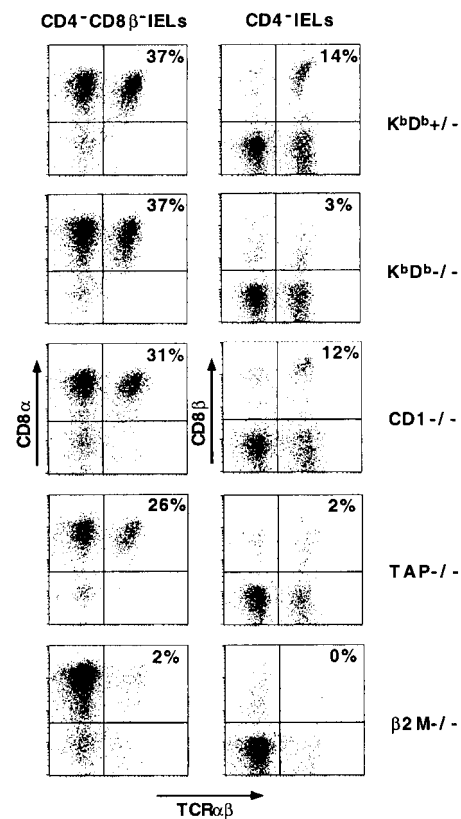


Figure 2. CD8 α/α ⁺ and CD8 α/β ⁺ IEL subsets in mutant mice. IELs of control and mutant mice were stained with anti-TCR- α/β , anti-CD4, anti-CD8 α , and anti-CD8 β . The left panels display the CD8 α versus TCR- α/β profiles of gated CD4/CD8 β -negative IELs, and the right panels display the CD8 β 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 CD8 α/α ⁺TCR- α/β ⁺ cells, whereas in the right panels they correspond to the CD8 α/β ⁺TCR- α/β ⁺ cells. TCR- γ/δ ⁺ cells are seen in the left quadrants as TCR- α/β -negative CD8 β -negative cells (most TCR- γ/δ ⁺ IELs are CD8 α/α ⁺).

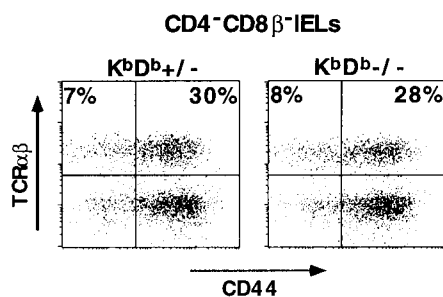


Figure 3. Unaltered phenotype of CD8 α/α^+ TCR- α/β^+ IELs in K^b/D^b -deficient mice. IELs were stained with anti-CD4/CD8 β , anti-CD44, and anti-TCR- α/β . CD44 versus TCR- α/β profiles of gated CD4/CD8 β -negative IELs are displayed.

Thus, the CD8 α/α^+ TCR- α/β^+ IEL subset of K^b/D^b -deficient mice appears to conserve the activated phenotype and the pattern of oligoclonal expansions that are characteristic of normal IELs, further indicating that CD8 α/α^+ TCR- α/β^+ IELs persist unaltered in K^b/D^b -deficient mice.

K^b/D^b -independent, CD8 α/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 CD8 α/β^+ IELs were dependent on the presence of TAP, some CD8 α/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 CD8 α/α^+ TCR- α/β^+ IELs in their littermate controls (10 ± 7 vs. $19 \pm 10\%$; see Fig. 2 and Table I). In contrast, as reported previously (21), all CD8 α/α^+ IELs as well as CD8 α/β^+ IELs depended on $\beta 2m$. These results suggest two possibilities. Either CD8 α/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.

CD8 α/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 CD8 α/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 CD8 α/α^+ and CD8 α/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 were comparable and are pooled in Table I. Altogether, these results show that neither the CD8 α/α nor the CD8 α/β subset is dependent on CD1, and suggest that the CD8 α/α subset requires an unknown nonclassical MHC-like molecule that is $\beta 2m$ dependent and partially TAP independent.

CD8 α/α^+ TCR- α/β^+ IELs Are Conserved in K^b/D^b /CD1 Triple-deficient Mice. Because of the compensatory expansion of IEL subsets often observed in the various mutant mice (see Table I), there remained the possibility that CD8 α/α^+ TCR- α/β^+ IELs were a heterogeneous population made of a TAP-dependent subset restricted by K^b/D^b and another TAP-independent subset restricted by CD1, but that the ablation of one subset in the corresponding mutant mouse was masked by the expansion of the other. To formally address this possibility, we generated K^b/D^b /CD1 triple-deficient mice. Fig. 4 shows that the IELs isolated from K^b/D^b /CD1 triple-deficient mice contained similar proportions of CD8 α/α^+ TCR- α/β^+ cells as CD1-deficient or K^b/D^b -deficient control littermates. These results suggest that neither CD1 nor K^b/D^b are ligands of CD8 α/α^+ TCR- α/β^+ cells, unambiguously demonstrating the existence of a large population of intestinal IELs that is dependent on a nonclassical, non-CD1 MHC class I-like molecule.

Table II. TCR $V\beta$ Repertoire of CD8 α/α^+ TCR- α/β^+ IELs in $K^bD^b +/-$ and $K^bD^b -/-$ Mice

Mouse	V β 2	V β 3	V β 4	V β 5.1 + 5.2	V β 6	V β 7	V β 8.1 + 8.2	V β 8.3	V β 9	V β 10	V β 11	V β 12	V β 13	V β 14
$K^bD^b +/-$	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
$K^bD^b +/-$	5.3	ND	4.1	3.6	8.2	ND	15.7	9.1	ND	15.6	12.2	ND	12.2	2.1
$K^bD^b -/-$	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
$K^bD^b -/-$	6.3	ND	4.4	8.2	0.8	ND	9.2	20.3	ND	1.4	18.9	ND	13.5	1.3
$K^bD^b -/-$	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 K^bD^b -deficient and littermate control mice were stained with anti-CD8 α , anti-V β s, and a cocktail of anti-TCR- γ/δ /CD4/CD8 β . The percentages of cells expressing indicated V β s among TCR- γ/δ /CD4/CD8 β -negative, CD8 α^+ IELs (corresponding to the CD8 α/α^+ TCR- α/β^+ subset) are represented.

*Frequencies of V β usage were determined from at least 5,000 CD8 α/α^+ TCR- α/β^+ IELs.

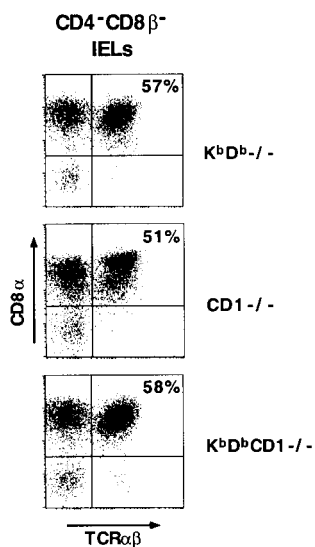


Figure 4. Persistence of CD8 α/α^+ TCR- α/β^+ IELs in K^b/D^b/CD1 triple-deficient mice. IELs were triple-stained with anti-CD4/CD8 β , anti-CD8 α , and anti-TCR- α/β . CD8 α versus TCR- α/β profiles of gated CD4/CD8 β -negative IELs are displayed. The upper right quadrants of the dot plots correspond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

Discussion

Although CD8 α/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 CD8 α/α^+ TCR- α/β^+ IELs have not been associated with antigen-specific, classical MHC class I-restricted function. The pattern of expression of CD8 α/α^+ TCR- α/β^+ IELs in β 2m⁻, TAP⁻, K^b/D^b⁻, and CD1-deficient mice, as

reported in this study, clearly demonstrates that most CD8 α/α^+ TCR- α/β^+ IELs do not recognize classical MHC class I molecules, and points to nonclassical MHC class I-like molecules that are β 2m 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 non-classical 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, yet poorly understood, 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 CD8 α/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 CD8 α/α^+ TCR- α/β^+ intestinal IELs, a key for our understanding of local immunity in the intestine.

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Address correspondence to Bana Jabri, Department of Molecular Biology, Princeton University, Princeton, NJ 08544. Phone: 609-258-5351; Fax: 609-258-2205; E-mail: bjabri@molbio.princeton.edu

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