Reduced Thymic Maturation but Normal Effector Function of CD8+ T Cells in CD8β Gene-targeted Mice

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Summary

CD8 is a cell surface glycoprotein on major histocompatibility complex class I-restricted T cells. Thymocytes and most peripheral T cells express CD8 as heterodimers of CD8α and CD8β. The intestinal intraepithelial lymphocytes (IEL), which have been suggested to be generated extrathymically, express CD8 predominantly as homodimers of CD8α. We have generated CD8β gene-targeted mice. CD8α+ T cell population in the thymus and in most peripheral lymphoid organs was reduced to 20–30% of that in wild-type littermates. CD8α expression on thymocytes and peripheral T cells also decreased to 44 and 53% of the normal levels, respectively. In contrast, neither the population size nor the CD8α expression level of CD8α+ IEL was reduced. This finding indicates that CD8β is important only for thymic-derived CD8+ T cells. The lack of CD8β reduces but does not completely abolish thymic maturation of CD8+ T cells. Our result also reveals the role of CD8β in regulating CD8α expression on thymic derived T cells. Peripheral T cells in these mice were efficient in cytotoxic activity against lymphocytic choriomeningitis virus and vesicular stomatitis virus, suggesting that CD8β is not essential for the effector function of CD8+ T cells.

CD8 is expressed on MHC class I-restricted T cells as a disulfide-linked heterodimer of α and β subunits (1–3). The exception are the intestinal intraepithelial lymphocytes (IEL)1 that express CD8 predominantly as homodimers of CD8α (4). Cell surface expression of CD8β requires the coupling with CD8α as heterodimers, whereas CD8α can be expressed on the cell surface as homodimers (5–8). CD8 has been suggested to have dual functions. It serves as an adhesion molecule to enhance the affinity of T cells to their target cells (9–11). It also is a coreceptor to the same MHC class I molecule (12, 13, 15–17), and the association of CD8 with protein tyrosine kinase p56lck (10, 11, 14). The extracellular portion of CD8α binds to the α3 nonpolymorphic domain of MHC class I molecules (12, 13, 18–20), and the cytoplasmic portion of CD8α is associated with the protein tyrosine kinase p56lck (21).

CD8 is crucial for thymic maturation of cytotoxic T cells (22–24). Mice bearing a null mutation at the CD8α gene by gene targeting lack MHC class I-restricted T cells and are defective in cytotoxic effector function (24). T cell response can be synergized by transfected CD8α (9, 10, 13, 14, 15, 25, 26). However, CD8αβ heterodimers have been reported to be more effective than CD8α homodimers in enhancing T cell response (27, 28). The role of CD8β in T cell ontogeny and function is still largely unknown. A regulatory role of CD8β in altering the structure of CD8 complex upon T cell activation has been suggested (29). A recent report on mice chimerized with CD8β gene-targeted embryonic stem (ES) cells suggested that CD8β is necessary for thymic maturation of CD8+ T cells (30). We have simultaneously generated CD8β gene-targeted mice. Our study showed that a significant peripheral CD8+ T cell population was still present in these mice. We also compared the CD8+ T cells in the peripheral lymphoid organs and in the intestinal epithelium. The result demonstrates that CD8β is important only for CD8+ T cells from the thymus, but not for IEL. We further examined the cytotoxic effector function of peripheral T cells against lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infection.

1 Abbreviations used in this paper: ES cells, embryonic stem cells; IEL, intraepithelial lymphocytes; LCMV, lymphocytic choriomeningitis virus; pfu, plaque-forming unit; VSV, vesicular stomatitis virus.
Materials and Methods

Disruption of the CD8β Gene in Mice by Homologous Recombination. A 2.5-kb mouse genomic DNA fragment covering the first exon of the CD8β gene, 0.4 kb of the 5' flanking region and 2 kb of the intron region was used in the gene-targeting construct (Fig. 1A). The neomycin resistance gene pMCneoA (Stratagene, La Jolla, CA) was inserted into the NcoI site within exon 1 of the gene. The gene-targeting construct was electroporated into D3 ES cells as described previously (24). Transfected cells were selected by G418 and the targeted gene was detected by PCR using primers specific for the neomycin resistance gene and the CD8β gene. The targeted CD8β gene in cells was eventually confirmed by genomic hybridization. ES cells with the targeted CD8β gene were injected into blastocysts to generate chimeric mice. Mice heterozygous for the targeted CD8β gene (CD8β +/- mice) were obtained from chimeric mice by germline transmission. Mice homozygous (-/-) for the disrupted CD8β gene were obtained by crossbreeding of CD8β +/+ mice.

Flow Cytometric Analysis of Lymphocytes. Single cell suspensions from thymus, spleen, and lymph nodes of 6-8-wk-old mice were prepared. IEL were purified from the intestine according to the method described by van der Heijden and Stock (31). 10⁶ cells were stained with mAb for 30 min at 4°C in 100 μl of PBS containing 2% FCS and 0.1% sodium azide. Cells were then washed and analyzed for single-, double-, and triple-color flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA). mAb used were specific for CD8α, CD8β, CD4, and CD3 (PharMingen, San Diego, CA). Anti-Thy-1 and anti-B cell specific CD45 mAb (PharMingen) were used for detecting T and B cells, respectively.

LCMV-induced Footpad Swelling Reaction. Mice were infected with 500 plaque forming units (pfu) of LCMV-Armstrong (32) by intradermal injection into hind footpads. Footpad thickness was measured daily with a spring-loaded caliper. Footpad swelling is calculated as (actual thickness - thickness before infection)/(thickness before infection).

Primary Ex Vivo Cytotoxicity against LCMV Infection. Mice were immunized by intradermal injection of 30 μl LCMV-Armstrong (500 pfu). On day 8, spleen cells were tested for LCMV-specific

Figure 1. Disruption of the CD8β gene by homologous recombination. (A) The 5' region of the mouse CD8β gene within the 2.5-kb HindIII-digested DNA fragment was used in the gene-targeting construct. The neomycin resistance gene was inserted in reverse orientation into the NcoI site within exon 1 of the CD8β gene. The restriction maps of the CD8β gene before and after homologous recombination are shown. The intact CD8β gene after EcoRI digestion was hybridized by the 5' and 3' probes as well as the neo probe used in genomic hybridization are shown. Solid squares, exons; E, EcoRI; H, HindIII; B, BamHI; N, NcoI. (B) CD8β +/-, +/−, and −/− mice were confirmed by genomic hybridization. The intact CD8β gene after EcoRI digestion was hybridized by the 5' and 3' probe as an 11.5-kb DNA fragment, whereas the disrupted CD8β gene was split into two 6.5-kb DNA fragments. The disrupted CD8β gene was also identified by the neo probe as a 3.5-kb DNA fragment after HindIII digestion.
cytotoxicity in a 4-h 51Cr release assay. Target cells were EL-4 cells (H-2b) that had been incubated with or without the relevant MHC class I-binding LCMV glycoprotein peptide (amino acid residues 33-42, 50 mM) for 1 h at 37°C (33, 34). 51Cr release was measured in duplicate and mean values are shown. Spontaneous 51Cr release was <20% for all target cells. Specific 51Cr release was calculated as (measured 51Cr release - spontaneous 51Cr release)/(total 51Cr release - spontaneous 51Cr release).

Primary Ex Vivo Cytotoxicity against VSV Infection. Mice were immunized intravenously with VSV (2 × 10^6 pfu serotype Indiana in 200 μl) on day 0. On day 6, spleen cells were tested for cytotoxicity in a 4-h 51Cr release assay. The EL-4 target cells were either uninfected or infected with VSV (15 pfu/cell) for 3 h at 37°C. 51Cr release was measured in duplicate and mean values are shown. Spontaneous 51Cr release was <20% for all target cells. Specific 51Cr release was calculated as described above.

LCMV-specific Cytotoxicity after In Vitro Restimulation. Mice were immunized with LCMV-Armstrong as described in the above section. On day 8, spleen cells were restimulated in vitro with irradiated peritoneal macrophages that had been infected with LCMV, as described in detail previously (35). Spleen cells were harvested after 5 d and tested for cytotoxicity on LCMV glycoprotein peptide–labeled EL-4 target cells as described above. Spontaneous 51Cr release was <22% for all target cells. Specific 51Cr release was calculated as described above.

VSV-specific Cytotoxicity after In Vitro Restimulation. Mice were immunized intravenously with VSV as described above. On day 6, spleen cells were restimulated with irradiated syngeneic C57BL/6 spleen cells that had been cultured with UV inactivated VSV (15 pfu/cell) for 1 h at 37°C. 3 × 10^6 responder spleen cells were restimulated with 2 × 10^6 stimulator cells in 24-well plates. After 5 d of culture, spleen cells were tested for cytotoxicity on VSV-infected EL-4 target cells as described above. Spontaneous 51Cr release was <16% for all target cells studied.

### Results

Generation of the CD8β Gene-targeted Mice. To understand the role of the CD8β in T cell ontogeny and function, we generated mice with the CD8β gene disrupted by homologous recombination. The CD8β gene was mutated in exon 1 by an insertion of the neomycin resistance gene (Fig. 1A). The site of insertion is 7 nucleotides downstream from the translation start site, which would abolish translation of the CD8β protein. Mice chimerized with CD8β gene–targeted ES cells were generated from embryo injection. CD8β+/− mice were obtained from germline transmission of chimeric mice and CD8β−/− mice were from cross-breeding of CD8β+/− mice. Disruption of the CD8β gene in mice was confirmed by genomic Southern hybridization (Fig. 1B). The CD8β−/− mice were fertile and appeared to be healthy. Cell numbers recovered from thymi, lymph nodes, spleens, and intestinal epithelia of the CD8β−/− mice were similar to those from wild-type littermates (Table 1). The percentages of the T cell and B cell subsets in different peripheral lymphoid organs of CD8β+/− and −/− mice were within normal ranges (Table 2).

Reduction in Population Size and in CD8α Expression of Thymic-derived CD8+ T Cells. CD8β was not detected on peripheral T cells or thymocytes from CD8β−/− mice (Figs. 2, 3, and 4). However, CD8α+ T cells were present in the peripheral lymphoid organs of CD8β−/− mice (Figs. 2, 3, and Table 3). CD8α+ T cells in CD8β−/− mice had a normal level of TCR expression (data not shown), but the population size was smaller than that in wild-type littermates. In lymph nodes, CD8α+ T cells were reduced to 30% of

| Table 1. Cell Numbers Recovered from Different Lymphoid Organs of the CD8β +/+, +/-, and −/− mice. |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Mouse genotype                  | Thymus                          | Lymph nodes                      | Spleen                          |
| CD8β +/+                        | 10.0 × 10⁷                      | 0.9 × 10⁷                        | 4.5 × 10⁷                       |
| CD8β +/-                        | 7.1 × 10⁷                       | 6.0 × 10⁷                        | 5.4 × 10⁷                       |
| CD8β −/−                        | 11.5 × 10⁷                      | 1.0 × 10⁷                        | 10.2 × 10⁷                      |
|                                 |                                 |                                  |                                  |
| Intestinal                      | Intestinal                      | Intestinal                      | Intestinal                      |
| IEL                              |                                 |                                  |                                  |
| CD8β +/+                        | 1.2 × 10⁶                       |                                  |                                  |
| CD8β +/-                        | 3.0 × 10⁶                       |                                  |                                  |
| CD8β −/−                        | 1.4 × 10⁶                       |                                  |                                  |

| Table 2. The Percentages of B Cell and T Cell Populations in Lymphocytes from Different Lymphoid Organs of the CD8β +/+, +/-, and −/− mice |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Mouse genotype                  | Lymph nodes                     | Spleen                          | Blood                           |
|                                 | B cell                          | T cell                          | B cell                          |
| CD8β +/+                        | 26%                             | 74%                             | 49%                             |
| CD8β +/-                        | 22%                             | 78%                             | 54%                             |
| CD8β −/−                        | 24%                             | 76%                             | 58%                             |

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that in wild-type mice (Fig. 3). Ratios of CD4+ to CD8α+ T cells in the peripheral lymphoid organs of CD8β−/− mice were about 10:1 in repeated experiments, as opposed to the ratio of about 1.6:1 in wild-type littermates (Fig. 3, Table 3). Immature thymocytes in CD8β−/− mice were CD4+ CD8α+ double positive and were normal in population size and in CD3 expression (Fig. 4). Consistent with the observation in the periphery, the subset of CD8α+ mature thymocytes in CD8β−/− mice was reduced to 20% of that in wild-type mice (Fig. 4). In contrast, the CD8α+ IEL population in CD8β−/− mice was comparable with that in wild-type mice (Fig. 5). The IEL cell number harvested from the intestinal epithelia of CD8β−/− mice was also in a normal range (Table 1).

A significant reduction of CD8α surface expression was
Table 3. The Ratio of CD4+ T Cell to CD8+ T Cell Subsets in Different Lymphoid Organs of the CD8β +/+, +/-, and −/− Mice

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Thymus</th>
<th>Lymph nodes</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8β +/+</td>
<td>3.7:1</td>
<td>1.6:1</td>
<td>1.8:1</td>
<td>1.1:1</td>
</tr>
<tr>
<td>CD8β +/-</td>
<td>3.3:1</td>
<td>1.7:1</td>
<td>2.6:1</td>
<td>3.6:1</td>
</tr>
<tr>
<td>CD8β −/−</td>
<td>14:1</td>
<td>8.7:1</td>
<td>9.5:1</td>
<td>11.5:1</td>
</tr>
</tbody>
</table>

found on peripheral T cells from CD8β −/− mice (53% of the level in wild-type control mice) (Fig. 2). CD8α expression levels on immature thymocytes from CD8β −/− mice were also low (44% of normal levels) and spanned a wide range (Fig. 4). Low CD8α expression was particularly obvious for early stage thymocytes which display low levels of CD3 expression. In contrast, CD8α expression levels on IEL from CD8β −/− mice were unchanged (Fig. 5).

In conclusion, the absence of CD8β only affects thymic derived T cells but has no impact on IEL. CD8β is needed for optimal CD8α expression and efficient thymic maturation of CD8+ T cells.

CD8+ T Cells without CD8β Are Efficient in Cytotoxic Effector Function against In Vivo Viral Infection. CD8β −/− mice provide a well-defined animal model for studying the in vivo immune response and effector function of the CD8+ T cells that lack CD8β. Cytotoxic T cell responses were studied in CD8β −/− mice after infection with LCMV and VSV. LCMV-specific CTL function in vivo was assessed by monitoring footpad swelling after intradermal injection of LCMV. Due to extensive local virus replication, the T cell response against LCMV causes an immunopathological swelling reaction of the footpad. The early phase of the swelling reaction (starting on day 8) is mediated exclusively by CD8+ cytotoxic T cells (36–38). This swelling reaction was normal in CD8β −/− mice (Fig. 6 A), demonstrating that an efficient in vivo cytotoxic T cell activity can be triggered in these mice.

Primary ex vivo cytotoxic T cell function was assessed with 51Cr release assays. After 8 d of LCMV infection in CD8β −/− mice, peripheral T cells showed a virus-specific cytotoxicity comparable to that in wild-type controls (Fig. 6 B). Similarly, efficient VSV-specific cytotoxic activity was observed in CD8β −/− mice after VSV infection (Fig. 6 C).
or T cells must have occurred, because the frequency of antigen demonstration of primary ex vivo cytotoxicity in CD83 -/- proliferation of effector cells during the restimulation. As mice. The question as to whether this reduction resulted from mice were shown to be efficient in cytotoxic effector func-
mice is not reduced in population size or decreased in CD8α expression. IEL are distributed at the intestinal epithelium and are heterogeneous populations dominated by CD8 + T cells with a TCR-α/β or γ/δ (40-42). It has been suggested that IEL are generated extrathymically (42-45). T cells with autoreactive TCR are deleted in the thymus during maturation (46-51), whereas IEL with the forbidden TCR are still present in the intestinal epithelium (44, 45). The observation that IEL are not affected by the absence of CD8β in these mice further supports the notion that IEL are derived extrathymically.

The finding of a low CD8α expression on thymic-derived T cells is unexpected, because IEL do not express CD8β and still retain normal levels of CD8α expression. There are two possible explanations for this observation. It could be a consequence of preferential thymic selection for CD8α+ T cells. Alternatively, CD8 as α/α homodimers could be less stable than α/β heterodimers and therefore are expressed less efficiently on the cell surface. The first explanation does not seem to be plausible, because low CD8α expression was found on thymocytes at different stages of maturation. In particular, CD8α on mature T cells is not lower than that on immature thymocytes, which argues against an enrichment for CD8α+ T cells after thymic selection. The second explanation is possible but only applies to thymic-derived T cells, because the lack of CD8β has no impact on CD8α expression on IEL. In fact, the specific decrease of CD8α expression on thymic-derived T cells reveals a novel regulatory role of CD8β in adjusting the CD8α expression level on thymic-derived T cells.

We have shown in a previous report that mice heterozygous for the disrupted CD8α gene (CD8α +/−) have a reduced cell surface expression of both CD8α and CD8β (50% of the normal level) (24). Despite low CD8 levels, thymic ontogeny of CD8+ T cells in these mice appears to be normal (24). On the other hand, the lack of CD8β in CD8β -/- mice causes a similar decrease in CD8α expression, but results in decreased thymic ontogeny of CD8+ T cells. Thus, an overall decrease in CD8 level may not be critical, but complete ablation of CD8β results in impaired thymic ontogeny.

How does CD8β affect the function of CD8 in thymic ontogeny? The role of CD8β could be quantitative in enhancing the overall CD8 expression, or qualitative in modifying CD8 functions. In CD8α -/- mice carrying different transgenic TCR, we have shown previously that CD8 is strictly needed in positive selection, but differentially required in negative selection of T cells (52). The reduced CD8+ T cell population in CD8β -/- mice could result from an increased negative selection, or a decreased positive selection of thymocytes. Further examination of CD8β -/- mice

**Figure 7.** Efficient effector function of cytotoxic T cells in CD8β gene-targeted mice. (A) Spleen cells from mice infected with LCMV were restimulated in vitro with LCMV-infected irradiated peritoneal macrophages, as described in Materials and Methods. Cytotoxicity of spleen cells on LCMV glycoprotein peptide labeled EL-4 target cells are shown. Open triangles, CD8β -/- mice; solid triangles, CD8β +/+ mice; solid circles, unprimed C57BL/6 control mice. Spontaneous 51Cr release was <22% for all targets shown. (B) Spleen cells from VSV-infected mice were restimulated with irradiated syngeneic C57BL/6 spleen cells that had been cultured with UV-inactivated VSV, as described in Methods and Materials. Cytotoxicity of spleen cells on VSV-infected EL-4 target cells was studied. Open triangles, CD8β -/- mice; solid triangles, CD8β +/+ mice; solid circles, unprimed C57BL/6 control mice. Spontaneous 51Cr release was <16% for all targets shown.

**Discussion**

We have demonstrated in CD8β -/- mice that thymic ontogeny of CD8+ T cells is reduced but not completely abolished in the absence of CD8β. This is evidenced by a significant number of CD8+ T cells remaining in the periphery and by a normal CTL activity found in these mice in response to viral infection. The thymic origin of the peripheral CD8+ T cells in these mice is also suggested by the corresponding decrease in CD8α expression on thymocytes and peripheral T cells. The result suggests that CD8α alone as a homodimer is inefficient for thymic maturation of CD8+ T cells.

It is interesting to note that CD8+ IEL in CD8β -/- mice is not reduced in population size or decreased in CD8α expression. IEL are distributed at the intestinal epithelium and are heterogeneous populations dominated by CD8 + T cells with a TCR-α/β or γ/δ (40-42). It has been suggested that IEL are generated extrathymically (42-45). T cells with autoreactive TCR are deleted in the thymus during maturation (46-51), whereas IEL with the forbidden TCR are still present in the intestinal epithelium (44, 45). The observation that IEL are not affected by the absence of CD8β in these mice further supports the notion that IEL are derived extrathymically.

In conclusion, peripheral CD8+ T cells in CD83 -/− mice must have occurred, because the frequency of antigen specific CTL must be in the range of 10^-5 to allow detectable primary ex vivo cytotoxicity (35, 39).

Although primary ex vivo cytotoxicity against LCMV and VSV in the CD8β -/- mouse was observed, a slight reduction was noticed when compared with those in wild-type mice. The question as to whether this reduction resulted from a limited number of CD8+ T cells or a decreased effector function of the CD8+ T cells is clarified by performing 51Cr release assays after in vitro restimulation. The difference in numbers of effector cells is evened out by further in vitro proliferation of effector cells during the restimulation. As demonstrated in both LCMV- and VSV-specific responses, the cytotoxic activities of the restimulated effector cells from CD8β -/- mice were as effective as those from wild-type littermate controls (Fig. 7).

In conclusion, peripheral CD8+ T cells in CD8β -/- mice were shown to be efficient in cytotoxic effector function against LCMV and VSV infection.

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bearing transgenic TCR should allow us to dissect the distinct role of CD8β in positive and negative selection.

The role of CD8β in cytotoxic function was examined by infecting CD8β−/− mice with LCMV or VSV. Cytotoxic T cell activity as assessed by primary ex vivo 31Cr release assays was slightly less than that in wild-type control mice, apparently due to the reduced CD8+ T cell population. After secondary in vitro stimulation with the same viral antigens, cytotoxicity generated in CD8β−/− mice was efficient and comparable with wild-type controls. Therefore on a cell-to-cell basis the CD8+ T cells that lack CD8β are as efficient in cytotoxic function as the CD8+ T cells in wild-type littermate controls. Taken together, the antigen induction, the in vivo proliferation, the differentiation into lytic effector cells and the cytolytic effector function of the CD8+ T cells in the periphery of the CD8fl−/− mice were shown to be normal. The demonstration of CTL functions against the above two nominal antigens in CD8β−/− mice also suggests that the TCR repertoire of CD8+ T cells in these mice is comparable with that in wild-type mice.

The results obtained from our CD8β−/− mice are different from a recent report on chimeric mice (30). We have provided phenotypic and functional data to suggest that a significant population of thymic-derived CD8+ T cells is still present in CD8β−/− mice. The previous report on chimeric mice showed only a minute CD8+ T cell population in the periphery that, in contrast to our study, was suggested to be derived extrathymically (30). In addition, the change in CD8α expression was not reported for the chimeric mice. These discrepancies probably arise from the limitation of the chimeric model as compared with CD8β−/− mice. The cells to be analyzed in the chimeric mice represented only a fraction of the total population. Furthermore, the population size of ES cell-derived CD8+ T cells could be affected by other factors, such as competition between defective ES cell-derived cells and normal host cells during thymic maturation of CD8+ T cells.

In summary, the phenotypic and functional studies in our CD8β−/− mice show that the absence of CD8β only reduces thymic maturation of functional CD8+ T cells. The result also reveals a role of CD8β in regulating CD8α cell surface expression on thymic derived T cells. Despite the lack of CD8β expression, these CD8+ T cells are functionally comparable to those in wild-type mice, suggesting that CD8β is not crucial for the antigenic response and the effector function of CD8+ T cells.

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