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## Positive selection of MHC class Ib–restricted CD8<sup>+</sup> T cells on hematopoietic cells

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### Abstract

Unlike conventional CD8<sup>+</sup> T cells, major histocompatibility complex (MHC) class Ib–restricted CD8<sup>+</sup> T cells show an activated phenotype in uninfected mice and respond rapidly to foreign invaders. The underlying factors that contribute to these differences are not well understood. We show here that the activated phenotype of MHC class Ib–restricted CD8<sup>+</sup> T cells was partially acquired as a result of interactions in the thymus and reflected an increased capacity to be selected *via* interactions with MHC molecules on hematopoietic cells. Using bone marrow–chimeric mice, we have shown that MHC class Ib–restricted, but not MHC class Ia–restricted, CD8<sup>+</sup> T cells specific for *Listeria monocytogenes* were efficiently selected when MHC class I was expressed only on hematopoietic cells. Thus, the distinct functional properties of MHC class Ib–restricted *versus* MHC class Ia–restricted CD8<sup>+</sup> T cells may result, at least in part, from the different ways in which they are positively selected in the thymus.

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Major histocompatibility complex (MHC) class Ib molecules—including H2-M3, Qa-1, Qa-2 and CD1d in mice and HLA-E, HLA-F, HLA-G and CD1a to CD1d in humans—differ from MHC class Ia molecules primarily because they have limited polymorphism<sup>1</sup>. Nevertheless, many MHC class Ib molecules, like MHC class Ia molecules, associate with  $\beta_2$ -microglobulin ( $\beta_2$ M) and present antigens to CD8<sup>+</sup> T cells. Insight into a distinct role played by MHC class Ib was obtained with the discovery that murine H2-M3 contains a hydrophobic peptide-binding groove with a specific preference for binding *N*-formylated peptides<sup>2</sup>. Because bacteria initiate protein synthesis with *N*-formyl methionine and eukaryotic hosts (except for a few mitochondrial proteins) do not, it was hypothesized that MHC class Ib molecules have evolved to bind distinct microbial products for presentation to T cells<sup>3</sup>. Indeed, CD8<sup>+</sup> T cells recognizing bacteria-derived *N*-formylated peptides presented by H2-M3 are important mediators of protective immunity against *Listeria monocytogenes*<sup>4–6</sup>.

MHC class Ib–restricted CD8<sup>+</sup> T cells specific for the antigens of a number of pathogens have been identified<sup>7–10</sup>. For example, mice infected with *Mycobacterium tuberculosis* contain H2-M3–restricted CD8<sup>+</sup> T cells<sup>7</sup>, whereas humans infected with *M. tuberculosis* contain both HLA-E–<sup>8</sup> and CD1–restricted cells<sup>9,10</sup>. Qa-1–restricted CD8<sup>+</sup> T cells are abundant in *Salmonella typhimurium*–infected mice<sup>11</sup> and have also been reported in *L. monocytogenes*–

infected mice<sup>12</sup>. MHC class Ib–restricted CD8<sup>+</sup> T cells differ from MHC class Ia–restricted T cells in at least four ways. First, most MHC class Ib–restricted T cells have an activated CD44<sup>hi</sup> phenotype, even in uninfected mice<sup>13</sup>. Second, these cells are capable of rapidly producing interferon- $\gamma$  (IFN- $\gamma$ ) after *in vivo* stimulation with anti-CD3 and have, therefore, been proposed to be an early source of IFN- $\gamma$  that promotes T helper type 1 (T<sub>H</sub>1) priming<sup>14</sup>. Third, MHC class Ib–restricted CD8<sup>+</sup> T cells show a peak immune response that precedes the peak response of MHC class Ia–restricted CD8<sup>+</sup> T cells after *L. monocytogenes* infection<sup>13, 15</sup>. Fourth, these cells participate minimally in the memory response associated with a secondary challenge with *L. monocytogenes*<sup>13, 15</sup>. Thus, in many ways MHC class Ib–restricted CD8<sup>+</sup> T cells behave like innate, rather than adaptive, effector cells.

Using K<sup>b-/-</sup>D<sup>b-/-</sup> mice that lack MHC class Ia, but express MHC class Ib, we extended the above observations and showed that MHC class Ib–restricted CD8<sup>+</sup> T cells had an activated phenotype that was distinct from MHC class Ia–restricted CD8<sup>+</sup> T cells. We also examined a small population of CD8<sup>+</sup> T cells in lethally irradiated  $\beta_2M^{-/-}$  mice, which lack both MHC class Ia and Ib, reconstituted with wild-type bone marrow (B6  $\rightarrow$   $\beta_2M^{-/-}$  chimeras). The CD8<sup>+</sup> T cells in these chimeric mice result from positive selection, in the thymus, on MHC class I molecules expressed on hematopoietic cells<sup>16</sup>, whereas most CD8<sup>+</sup> T cells require selection on thymic epithelial cells<sup>17</sup>. We observed that these unusual CD8<sup>+</sup> T cells had a similar activated phenotype to MHC class Ib–restricted T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice. These observations led us to hypothesize that MHC class Ib–restricted CD8<sup>+</sup> T cells might be positively selected on hematopoietic cells.

We showed here that mature CD8<sup>+</sup> thymocytes in K<sup>b-/-</sup>D<sup>b-/-</sup> mice began to express an activated phenotype immediately before they left the thymus, suggesting that this phenotype was acquired as a result of interactions in the thymus, rather than peripheral activation. Using bone marrow chimeras, we also showed that H2-M3–restricted CD8<sup>+</sup> T cells that recognize a *Listeria*-derived *N*-formylated peptide were selected efficiently on hematopoietic cells, whereas MHC class Ia–restricted T cells were selected poorly. The ability of MHC class Ib–restricted CD8<sup>+</sup> T cells to respond quickly to antigen may be a consequence of this unusual pattern of positive selection.

## Results

### MHC class Ib–restricted T cells

The majority of peripheral MHC class Ib–restricted T cells express an activated CD44<sup>hi</sup> phenotype, even in naïve K<sup>b-/-</sup>D<sup>b-/-</sup> mice; this confirmed published data<sup>13</sup> (Fig. 1a). In contrast, most CD8<sup>+</sup> T cells (primarily MHC class Ia–restricted) in C57BL/6 mice have a naïve CD44<sup>int</sup> phenotype (Fig. 1a). To investigate this difference in more detail, we examined a broad range of activation markers and showed that most peripheral MHC class Ib–restricted CD8<sup>+</sup> T cells were  $\beta_1$  integrin<sup>hi</sup> $\beta_7$  integrin<sup>lo</sup>CD11a<sup>hi</sup>CD122<sup>hi</sup>Ly6C<sup>hi</sup> (Fig. 1a). In contrast, most MHC class Ia–restricted CD8<sup>+</sup> T cells were  $\beta_1$  integrin<sup>int</sup> $\beta_7$  integrin<sup>hi</sup>CD11a<sup>int</sup>CD122<sup>lo</sup>Ly6C<sup>lo</sup> (Fig. 1a). However, MHC class Ib– and MHC class Ia–restricted CD8<sup>+</sup> T cells expressed identical high amounts of CD62L (data not shown). In addition, CD4<sup>+</sup> T cells in C57BL/6 and K<sup>b-/-</sup>D<sup>b-/-</sup> mice showed no marked differences in any of these activation markers (data not shown).

### T cells selected on hematopoietic cells

We constructed radiation bone marrow–chimeric mice in which either C57BL/6 or  $\beta_2M^{-/-}$  mice were lethally irradiated and reconstituted with C57BL/6 bone marrow (B6  $\rightarrow$  B6 or B6  $\rightarrow$   $\beta_2M^{-/-}$  chimeras, respectively). We observed that the small population of CD8<sup>+</sup> T cells that developed in B6  $\rightarrow$   $\beta_2M^{-/-}$  chimeras had an activated phenotype that was almost identical

(Fig. 1b) to the one expressed by MHC class Ib–restricted CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice (Fig. 1a); the majority of these T cells were CD44<sup>hi</sup>β<sub>1</sub> integrin<sup>hi</sup>β<sub>7</sub> integrin<sup>lo</sup>CD11a<sup>hi</sup>CD122<sup>hi</sup>Ly6C<sup>hi</sup>. This activated phenotype was not an artifact of irradiation, as CD8<sup>+</sup> T cells in B6 → B6 chimeras expressed a phenotype that resembled the pattern seen in unirradiated C57BL/6 mice (Fig. 1b). This unusual population of CD8<sup>+</sup> T cells in B6 → β<sub>2</sub>M<sup>-/-</sup> chimeras is selected in the thymus by interacting with MHC class I molecules on hematopoietic cells, instead of epithelial cells<sup>16</sup>. Thus, similarities between these CD8<sup>+</sup> T cells and CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice led us to hypothesize that MHC class Ib–restricted CD8<sup>+</sup> T cells are also positively selected on hematopoietic cells.

### MHC class Ib–restricted CD8<sup>+</sup> T cells in the thymus

If the activated phenotype of peripheral CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice reflected the nature of their intrathymic selection, we considered the possibility that this phenotype might be expressed by CD4<sup>-</sup>CD8<sup>+</sup> T cells during development in the thymus. Therefore, we examined mature CD8<sup>+</sup> thymocytes—defined by their lack of expression of CD24<sup>18</sup>—in K<sup>b-/-</sup>D<sup>b-/-</sup> mice before they left the thymus. In agreement with published reports<sup>18</sup>, none of the CD24<sup>-</sup>CD8<sup>+</sup> thymocytes expressed CD4 (data not shown). Some of these mature CD4<sup>-</sup>CD8<sup>+</sup> thymocytes were already expressing the activated phenotype CD44<sup>hi</sup>β<sub>7</sub> integrin<sup>lo</sup>CD11a<sup>hi</sup>CD122<sup>hi</sup> (Fig. 2a), which suggested that this phenotype resulted from intrathymic interactions. This experiment was repeated four times, and age-matched mice that ranged from 5–13 weeks old were used. CD24<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup> thymocytes in K<sup>b-/-</sup>D<sup>b-/-</sup> mice consistently contained a population of activated cells in all experiments; however, the percentage of cells within this population was higher in older mice. As expected, mature CD4<sup>-</sup>CD8<sup>+</sup> thymocytes in C57BL/6 mice expressed a naïve resting phenotype: CD44<sup>int</sup>β<sub>7</sub> integrin<sup>hi</sup>CD11a<sup>int</sup>CD122<sup>lo</sup> (Fig. 2b).

In contrast to these differences, most mature CD8<sup>+</sup> thymocytes in both K<sup>b-/-</sup>D<sup>b-/-</sup> and C57BL/6 mice were Ly6C<sup>lo</sup> (Fig. 2) and CD45RB<sup>lo</sup> (data not shown). Our observation that the majority of lymph node CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice expressed high amounts of Ly6C (Fig. 1a) and CD45RB (data not shown) suggested that the CD8<sup>+</sup> cells found in the thymi of K<sup>b-/-</sup>D<sup>b-/-</sup> mice were not simply mature CD8<sup>+</sup> T cells that were activated in the periphery and migrated back to the thymus. Instead, these data support the idea that the distinct activated phenotype of MHC class Ib–restricted T cells is due, in part, to a developmental program acquired as a consequence of their intrathymic selection.

### T cell selection on hematopoietic cells

We hypothesized that the activated phenotype of mature CD4<sup>-</sup>CD8<sup>+</sup> thymocytes in K<sup>b-/-</sup>D<sup>b-/-</sup> mice might reflect their capacity to be positively selected on hematopoietic cells. To test this idea, we constructed bone marrow–chimeric mice that expressed MHC class Ib only on hematopoietic cells (K<sup>b-/-</sup>D<sup>b-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> chimeras) or only on nonhematopoietic cells (β<sub>2</sub>M<sup>-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> chimeras) and compared the CD8<sup>+</sup> T cells in the spleens of each set of chimeric mice. Spleens from control K<sup>b-/-</sup>D<sup>b-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> chimeras contained a population of CD8<sup>+</sup> T cells (1%, Fig. 3a) that was similar in size to the population observed in unirradiated K<sup>b-/-</sup>D<sup>b-/-</sup> mice (data not shown). Slightly smaller populations of CD8<sup>+</sup> T cells were present in both K<sup>b-/-</sup>D<sup>b-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> and β<sub>2</sub>M<sup>-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> chimeras (0.4% and 0.5%, respectively, Fig. 3a). In comparison, CD8<sup>+</sup> T cells comprised only 0.1% of spleen cells in β<sub>2</sub>M<sup>-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> chimeras (data not shown). Thus, although MHC class Ib–restricted CD8<sup>+</sup> T cells seemed to develop most efficiently in mice expressing MHC class Ib on both hematopoietic cells and nonhematopoietic cells, CD8<sup>+</sup> T cells could also be selected in chimeras that expressed MHC class Ib only on hematopoietic cells and in chimeras that expressed these molecules only on nonhematopoietic cells.

Next, we compared the cell surface phenotypes of MHC class Ib–restricted CD8<sup>+</sup> T cells in each of these chimeras. Like  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  controls, the majority of MHC class Ib–restricted CD8<sup>+</sup> T cells in both  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  and  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras were CD44<sup>hi</sup> (Fig. 3a), although the percentage of CD44<sup>+</sup> cells in  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras was always slightly smaller than the percentage in chimeras expressing MHC class Ib molecules on hematopoietic cells. When we examined integrin expression, we found that most CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  chimeras, like CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  controls, were  $\beta_1$  integrin<sup>hi</sup> $\beta_7$  integrin<sup>lo</sup>CD11a<sup>hi</sup>. In contrast, most CD8<sup>+</sup> T cells in  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras were  $\beta_1$  integrin<sup>int</sup> $\beta_7$  integrin<sup>hi</sup>CD11a<sup>int</sup> (Fig. 3a). CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  and  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  chimeras also expressed similar amounts of CD122 that were higher than the amounts expressed by CD8<sup>+</sup> T cells in  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras (Fig. 3a). In contrast to these differences, CD8<sup>+</sup> T cells in both  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  and  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras expressed low amounts of Ly6C, whereas CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras expressed high amounts (data not shown). Overall, however, MHC class Ib–restricted CD8<sup>+</sup> T cells that were positively selected on hematopoietic cells had a more activated phenotype than MHC class Ib–restricted CD8<sup>+</sup> T cells selected on nonhematopoietic cells. These results supported the idea that the activated phenotype of MHC class Ib–restricted CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-}$  mice was associated with positive selection on hematopoietic cells.

To further investigate this possibility, we asked whether MHC class Ib–restricted CD8<sup>+</sup> T cells that recognized a *L. monocytogenes*–derived peptide, f-MIGWII, presented by H2-M3<sup>5</sup> could be positively selected on hematopoietic cells. We infected either  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  or  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  chimeric mice with *L. monocytogenes* and analyzed their spleen cells 6 days later using M3–f-MIGWII tetramers.  $K^{b-/-}D^{b-/-} \rightarrow \beta_2M^{-/-}$  chimeras showed a marked expansion of M3–f-MIGWII–reactive CD8<sup>+</sup> T cells after infection (0.6% of CD8<sup>+</sup> T cells in uninfected *versus* 25% in infected mice, Fig. 3b). Expansion was also seen in  $K^{b-/-}D^{b-/-} \rightarrow K^{b-/-}D^{b-/-}$  controls, but the magnitude was less marked (0.6% in uninfected *versus* 6.1% in infected mice).  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  and  $\beta_2M^{-/-} \rightarrow \beta_2M^{-/-}$  chimeras were also immunized with *Listeria*, as controls; neither showed any M3–f-MIGWII tetramer staining after infection (data not shown). Our inability to detect M3–f-MIGWII–specific CD8<sup>+</sup> T cells in  $\beta_2M^{-/-} \rightarrow K^{b-/-}D^{b-/-}$  chimeras, however, did not exclude the possibility that these cells might be selected on thymic epithelial cells. This is because expansion of these cells to detectable amounts after infection may require the presence of MHC class Ib on hematopoietic antigen-presenting cells. Nevertheless, these results showed that H2-M3–restricted CD8<sup>+</sup> T cells were efficiently positively selected in chimeric mice that expressed MHC class Ib only on hematopoietic cells.

We observed that the majority of CD8<sup>+</sup> T cells in  $B6 \rightarrow \beta_2M^{-/-}$  chimeras shared a similar, activated phenotype with CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-}$  mice (Fig. 1). Thus, we further hypothesized that MHC class Ib–restricted T cells must have a substantial advantage over MHC class Ia–restricted T cells when positive selection is forced to occur on hematopoietic cells. To directly test this idea, we compared the selection of MHC class Ia– *versus* MHC class Ib–restricted CD8<sup>+</sup> T cells involved in the immune response against *L. monocytogenes*. Because naturally occurring MHC class Ia–restricted epitopes in H-2<sup>b</sup> mice are not well characterized, a recombinant *L. monocytogenes* strain<sup>19</sup> expressing secreted ovalbumin (rLM-OVA) was used. Thus, the MHC class Ia–restricted response against an OVA-derived peptide, SIINFEKL, presented by K<sup>b</sup> (ref. 20) could be monitored. For comparison, we again followed the response of CD8<sup>+</sup> T cells specific for f-MIGWII presented by H2-M3. Using this system, we compared the frequency of MHC class Ia– *versus* MHC class Ib–restricted CD8<sup>+</sup> T cells in  $B6 \rightarrow B6$  or  $B6 \rightarrow \beta_2M^{-/-}$  chimeric mice after infection with rLM-OVA. The response of OVA-specific CD8<sup>+</sup> T cells was dominant in  $B6 \rightarrow B6$  mice compared to the f-MIGWII–specific response at day 6 after infection (Fig. 4a). The percentage of CD8<sup>+</sup> T cells detected with K<sup>b</sup>-OVA tetramers was consistently three- to fivefold higher than the percentage detected with M3–f-

MIGWII tetramers. In contrast, OVA-specific CD8<sup>+</sup> T cells were rare (Fig. 4a) or undetectable (data not shown) in B6 →  $\beta_2M^{-/-}$  chimeras, whereas f-MIGWII-specific CD8<sup>+</sup> T cells were abundant. CD8<sup>+</sup> T cells specific for f-MIGWII were reproducibly five- to tenfold more frequent than OVA-specific CD8<sup>+</sup> T cells in chimeras expressing MHC class I molecules only on hematopoietic cells.

Functional studies were done to determine whether CD8<sup>+</sup> T cells selected on hematopoietic cells were capable of producing IFN- $\gamma$  and mediating cytolysis. Consistent with the frequencies observed with the use of tetramers, CD8<sup>+</sup> T cells producing IFN- $\gamma$  in response to OVA peptide were 20-fold more abundant in infected B6 → B6 chimeras than T cells responding to f-MIGWII peptide (Fig. 4b). In contrast, B6 →  $\beta_2M^{-/-}$  chimeras contained at least fivefold more f-MIGWII-reactive than OVA peptide-reactive CD8<sup>+</sup> T cells. Similarly, CD8<sup>+</sup> T cells from infected B6 → B6 chimeras showed strong cytotoxicity against OVA peptide-loaded target cells and weak cytotoxicity against f-MIGWII-loaded targets (Fig. 4c), whereas CD8<sup>+</sup> T cells from B6 →  $\beta_2M^{-/-}$  chimeras showed the opposite pattern: strong cytotoxicity against f-MIGWII-loaded cells and weak cytotoxicity against OVA-peptide-loaded cells. Thus, whereas most MHC class Ia-restricted thymocytes required interactions with MHC class Ia molecules on thymic epithelial cells to mature, our findings showed that functional MHC class Ib-restricted T cells were preferentially selected when MHC class I molecules were expressed only on hematopoietic cells.

## Discussion

The results we present here show events that mediate the selection of MHC class Ib-restricted CD8<sup>+</sup> T cells differ from those that mediate the selection of MHC class Ia-restricted CD8<sup>+</sup> T cells. H2-M3-restricted CD8<sup>+</sup> T cells are positively selected on H2-M3 molecules expressed in the thymus<sup>21,22</sup>, but whether this occurs on epithelial cells or hematopoietic cells had not been examined. We show here that H2-M3-restricted CD8<sup>+</sup> T cells are efficiently selected on hematopoietic cells, whereas MHC class Ia-restricted CD8<sup>+</sup> T cells are poorly selected.

It was shown previously that a small population of CD8<sup>+</sup> T cells can be selected on hematopoietic cells in the thymus, and if these T cells are primed *in vivo* and boosted *in vitro* with minor histocompatibility antigens, an MHC class Ia-restricted response can be generated<sup>16</sup>. This seemed to suggest that CD8<sup>+</sup> T cells selected on hematopoietic cells were not a distinct population, but represented a small subset of MHC class Ia-restricted T cells, perhaps those with relatively high affinities. However, the same rules do not seem to apply to CD4<sup>+</sup> T cells, as selection of MHC class II-restricted T cells on hematopoietic cells has not been observed<sup>23,24</sup>. In addition, some MHC class Ia-restricted CD8<sup>+</sup> T cells in T cell receptor (TCR)-transgenic mice are unable to be positively selected on hematopoietic cells<sup>25,26</sup>. Nevertheless, an example of an MHC class Ia-restricted T cell (2C, bearing a K<sup>b</sup>-restricted TCR) that can be selected on hematopoietic cells has been demonstrated<sup>26</sup>. Initially, this finding seemed to support the idea that a subset of CD8<sup>+</sup> T cells could be selected *via* interactions with MHC class Ia molecules expressed on hematopoietic cells. An alternative possibility was suggested by the subsequent finding that the 2C TCR, in addition to recognizing K<sup>b</sup>, also recognizes and can be positively selected by an MHC class Ib molecule<sup>27</sup>. Thus, it is possible that most or all positive selection mediated by hematopoietic cells occurs *via* interactions with MHC class Ib molecules, but some of these T cells, like 2C, may have dual specificities for MHC class Ia molecules. The observation that positive selection of 2C can occur in lethally irradiated  $\beta_2M^{-/-}$  mice reconstituted with K<sup>b</sup>-D<sup>b</sup> bone marrow<sup>26</sup> suggests the latter possibility, at least for 2C T cells.

Our finding that *Listeria*-specific H2-M3-restricted CD8<sup>+</sup> T cells developed efficiently in B6 →  $\beta_2M^{-/-}$  chimeras, but MHC class Ia-restricted T cells did not, supported the idea that MHC

class Ib-restricted T cells were preferentially selected on hematopoietic cells. We could not exclude the possibility that a small number of CD8<sup>+</sup> T cells could also be positively selected by interacting with MHC class Ia molecules on hematopoietic cells. For example, in most B6 → β<sub>2</sub>M<sup>-/-</sup> chimeric mice, a small number of K<sup>b</sup>-OVA-specific T cells were usually detected. It is possible that this small population of CD8<sup>+</sup> T cells was selected *via* interactions with MHC class Ia molecules on hematopoietic cells. Alternatively, these K<sup>b</sup>-OVA-reactive T cells may actually have been selected on MHC class Ib molecules, but coincidentally have a dual specificity for K<sup>b</sup>-OVA. Our experiments did not distinguish between these possibilities, but clearly indicated that positive selection on hematopoietic cells favored MHC class Ib-restricted T cells.

The experiments we present here were done to try to explain our finding that CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice and B6 → β<sub>2</sub>M<sup>-/-</sup> chimeric mice show similar activated phenotypes. One possibility that we initially considered was that these small populations of CD8<sup>+</sup> T cells may become activated simply as a result of proliferation that can occur in lymphopenic hosts. This explanation seemed unlikely, however, as neither population of CD8<sup>+</sup> T cells showed much expansion, but remained at low numbers (1–3% of total spleen cells) for the lifetime of the mice. The finding that large numbers of CD4<sup>+</sup> T cells—as are present in both K<sup>b-/-</sup>D<sup>b-/-</sup> mice and B6 → β<sub>2</sub>M<sup>-/-</sup> chimeras—inhibit, at least partially, the expansion of CD8<sup>+</sup> T cells in hosts with small numbers of CD8<sup>+</sup> T cells<sup>28,29</sup> may help to explain this lack of expansion. In addition, our observation that CD4<sup>-</sup>CD8<sup>+</sup> thymocytes began to express the activated phenotype in K<sup>b-/-</sup>D<sup>b-/-</sup> mice suggested that this phenotype resulted, in part, from interactions that occurred during development in the thymus, instead of expansion in the periphery.

We considered an alternative possibility, that CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> and B6 → β<sub>2</sub>M<sup>-/-</sup> mice might be similar because they contained many of the same cells. We hypothesized that this would be true if MHC class Ib-restricted T cells were preferentially selected on hematopoietic cells. The presence of a population of CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> chimeric mice showed that MHC class Ib-restricted CD8<sup>+</sup> T cells could be selected on hematopoietic cells; however, the presence of CD8<sup>+</sup> T cells in β<sub>2</sub>M<sup>-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> chimeras showed that positive selection of MHC class Ib-restricted CD8<sup>+</sup> T cells could also occur on nonhematopoietic cells, most likely thymic epithelial cells. The observation that CD8<sup>+</sup> T cells in both K<sup>b-/-</sup>D<sup>b-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> and β<sub>2</sub>M<sup>-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> mice were CD44<sup>hi</sup> indicated that some components of the activated phenotype were intrinsic to MHC class Ib-restricted T cells, regardless of whether they were positively selected on hematopoietic or epithelial cells. In contrast, other markers of the activated phenotype—including expression patterns of β<sub>1</sub> integrin, β<sub>7</sub> integrin, CD11a and CD122—seemed to be correlated with positive selection on hematopoietic cells. Whether T cells selected on epithelial *versus* hematopoietic cells represented distinct subsets or whether some T cells had the capacity to be selected on either—but expressed different surface phenotypes depending upon how they were selected—was not directly addressed here. However, the finding that most CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice expressed the phenotype correlated with selection on hematopoietic cells suggests that this process is important for a large number of MHC class Ib-restricted CD8<sup>+</sup> T cells.

We found that MHC class Ib-restricted CD8<sup>+</sup> T cells selected on hematopoietic cells (in K<sup>b-/-</sup>D<sup>b-/-</sup> → β<sub>2</sub>M<sup>-/-</sup> chimeras) expressed a different set of activation markers compared to MHC class Ib-restricted CD8<sup>+</sup> T cells that were selected on epithelial cells (in β<sub>2</sub>M<sup>-/-</sup> → K<sup>b-/-</sup>D<sup>b-/-</sup> chimeras). These differences were particularly marked for integrin expression, despite that fact that most CD8<sup>+</sup> T cells in both populations expressed an activated CD44<sup>hi</sup> phenotype. It has been hypothesized that MHC class Ib-restricted CD8<sup>+</sup> T cells may be a distinct subset of effector cells that functions in an immune-regulatory role<sup>30,31</sup>. It seems possible that the differences in integrin expression conferred by positive selection on hematopoietic cells may alter the trafficking of these cells through lymphoid and nonlymphoid

tissues, positioning them uniquely for these roles. In addition, the possibility that positive selection on hematopoietic cells functions to give MHC class Ib–restricted T cells many of their innate effector properties also needs to be addressed in future studies.

Why are MHC class Ib–restricted T cells efficiently selected on hematopoietic cells, but not MHC class Ia–restricted T cells? It is likely that the selecting ligands of many MHC class Ia–restricted T cells are expressed on hematopoietic cells and yet selection does not occur. Epithelial cells are generally believed to be specially equipped with cell-surface molecules, or cytokines, that promote T cell development, which makes them the most efficient mediators of positive selection<sup>17</sup>. It is possible that developing T cells with relatively high affinities for self-MHC molecules have a reduced requirement for these specialized signals. In support of this idea, CD1d-restricted natural killer T (NKT) cells—the only other T cells known to be routinely selected on hematopoietic cells<sup>32–36</sup>—are self-reactive and are believed to have higher affinities for self-MHC ligands than conventional T cells<sup>37</sup>. Because MHC class Ib–restricted T cells in  $K^{b-/-}D^{b-/-}$  have an activated phenotype in naïve mice, it seems likely that they also have relatively high affinities for self-MHC–peptide complexes. This idea is supported by the finding that a physiological ligand for positive selection of H2-M3–restricted CD8<sup>+</sup> T cells can function as a weak agonist for the activation of the same CD8<sup>+</sup> T cells when they are mature<sup>38</sup>.

As discussed above, NKT cells are the only other T cells known to be routinely selected on hematopoietic cells in the thymus<sup>32–36</sup>. NKT cells show limited TCR diversity, recognize a nonpolymorphic MHC class Ib–related molecule (CD1d), have an activated phenotype in naïve mice and participate in the early “innate” response to foreign invaders<sup>37</sup>. These “innate” T cells have been postulated to be evolutionary precursors of conventional T cells involved in adaptive immunity<sup>37</sup>, which, in contrast, have broad TCR diversity and recognize polymorphic MHC molecules. It is unclear where MHC class Ib–restricted CD8<sup>+</sup> T cells fit in the spectrum between “innate” NKT cells and conventional “adaptive” T cells. MHC class Ib–restricted CD8<sup>+</sup> T cells, like NKT cells, have an activated phenotype in naïve mice and participate in early immune responses to pathogens. However, despite recognizing MHC molecules with limited polymorphism, MHC class Ib–restricted CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-}$  mice have diversity in their  $V_{\alpha}$  and  $V_{\beta}$  regions that equals the diversity seen in conventional CD8<sup>+</sup> T cells<sup>39</sup>. However, MHC class Ib–restricted CD8<sup>+</sup> T cells are not a pure population, but represent a mixture of cells with specificities for a variety of MHC class Ib molecules, for example, H2-M3, Qa-1, Qa-2 and probably several others. It is possible that if T cells that react with each individual MHC class Ib molecule could be analyzed separately, each population would have limited TCR diversity.

We have demonstrated that H2-M3–restricted CD8<sup>+</sup> T cells, like CD1d-restricted NKT cells, have the capacity to be positively selected on hematopoietic cells. In addition, the finding that many CD8<sup>+</sup> T cells in  $K^{b-/-}D^{b-/-}$  mice have an activated phenotype that is similar to T cells selected on hematopoietic cells suggests that this may prove to be a general characteristic in the development of many MHC class Ib–restricted T cells. Our results raise the possibility that the distinct properties of MHC class Ib–restricted T cells may result from a different developmental program imprinted by the distinct way in which they are positively selected.

## Methods

### Mice

C57BL/6 and  $\beta_2M^{-/-}$  (backcrossed 11 times to C57BL/6 mice) mice were from Jackson Laboratories (Bar Harbor, ME).  $K^{b-/-}D^{b-/-}$  breeders<sup>40</sup> (backcrossed six times to mice) were from J. Forman (Texas Southwestern Medical Center, Dallas, TX) and a colony was maintained

at our specific pathogen-free animal facility at the University of Washington. Mice were used at 5–13 weeks of age; all experiments were done according to institutional guidelines.

### Bone marrow chimeras

Bone marrow chimeras were prepared by  $\gamma$ -irradiating (1000 rad) 6–8-week-old C57BL/6,  $\beta_2M^{-/-}$  or  $K^b^{-/-}D^b^{-/-}$  recipient mice with a  $^{137}\text{Cs}$  source followed by intravenous injection of  $10^7$  T-depleted bone marrow cells from C57BL/6,  $\beta_2M^{-/-}$  or  $K^b^{-/-}D^b^{-/-}$  donors. Before injection, T cell depletion was done with CD4 (RL-172<sup>41</sup>), CD8 (3.168<sup>42</sup>), Thy1.2 (30-H12, American Type Culture Collection, Manassas, VA) monoclonal antibodies (mAbs) and Low-Tox M rabbit complement (Cedarlane, Hornby, Canada) and was >95% effective. Chimeras were analyzed or infected with *L. monocytogenes* 8–15 weeks later.

### Bacteria and infections

The wild-type *L. monocytogenes* strain 10403s was from D. Portnoy (University of California at Berkeley, Berkeley, CA) and was grown in brain-heart infusion (BHI) broth. The recombinant *L. monocytogenes* strain (rLM-OVA), which secretes chicken OVA and contains an erythromycin-resistance marker was from H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA)<sup>19</sup>. The rLM-OVA was grown in BHI broth supplemented with 5  $\mu\text{g}/\text{ml}$  of erythromycin. Bacteria culture samples were measured by optical density and, at the mid-log growth phase, were diluted in PBS for injection. Chimeric mice were infected with 2000 *L. monocytogenes* by tail-vein injection. Injected numbers (colony forming units) were more accurately determined by spreading bacterial samples on BHI plates and incubating them overnight at 37 °C. Splenocytes were collected on day 6 or 7 after infection for analysis.

### Peptides and tetramers

H-2K<sup>b</sup> tetramers bound to the OVA-derived peptide SIIN-FEKL were generated as described<sup>43,44</sup>. H2-M3 tetramer bound to the Listeria-derived peptide f-MIGWII was a gift of E. Pamer (Sloan-Ketterling, New York, NY). Negative control peptides for both MHC tetramers were prepared to determine the degree of nonspecific background staining, as described<sup>15</sup>. Splenocytes were resuspended in PBS with fetal calf serum (5%) and  $\text{NaN}_3$  (0.1%) at a concentration of  $10^7$  cells/ml and stained with phycoerythrin (PE)-tetramer, allophycocyanin-anti-CD8 (clone 53.6.7, BD PharMingen, San Diego, CA) and fluorescein isothiocyanate (FITC)-anti-CD44 (clone IM7, BD PharMingen) for 1 h at 4 °C.

### Flow cytometry

Single-cell suspensions from spleens, lymph nodes (pooled axillary, brachial, inguinal and mesenteric) or thymi were obtained and red cells were lysed with a hypotonic buffer. The following mAbs were used for flow cytometry: FITC-anti-CD11a (clone 2D7), FITC-anti-CD24 (clone M1/69), FITC-anti-CD44, FITC-anti-CD62L (clone MEL-14), FITC-anti-CD122 (clone TM- $\beta$ 1), FITC-anti-Ly6C (clone AL-21), anti-TCR $\beta$  (clone H37-597), FITC-anti-CD8 $\beta$ .2 (clone 53-5.8), PE-anti- $\beta$ 7 integrin (clone M293), biotin-anti-CD29 ( $\beta$ 1 integrin, clone Ha2/5), biotin-anti-CD49d (clone R1-2), cychrome-anti-CD4 (clone RM4-5) and allophycocyanin-anti-CD8 $\alpha$  (all from BD Pharmingen). FITC-streptavidin (Vector Laboratories, Burlingame, CA) was used as a secondary reagent for biotin-labeled mAb. Flow cytometry was done on a FACSCalibur and analyzed with CELLQuest software (Becton Dickinson, Mountain View, CA).

### Intracellular IFN- $\gamma$ staining

Intracellular IFN- $\gamma$  staining was done with a kit, as instructed by the manufacturer (PharMingen). Briefly, in a 24-well plate,  $4 \times 10^6$  spleen cells per well were stimulated with media alone or  $10^{-8}$  M of either OVA peptide (SIINFEKL) or f-MIGWII for 6 h in complete



RPMI (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 0.5  $\mu$ M 2-mercaptoethanol, 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin) in the presence of monensin. Cells were washed, stained with allophycocyanin-anti-CD8 $\alpha$ , resuspended in permeabilization-fixation buffer and stained with FITC-anti-IFN- $\gamma$  (clone XMG1.2, Pharmingen). Labeled cells were washed in permeabilization buffer, resuspended in fix buffer and analyzed on a FACSCalibur.

### CTL assay

Splenocytes from all experimental mice were assayed *ex vivo* for their ability to kill peptide-loaded target cells. Serial dilutions (1:3) of effector splenocytes were done in triplicate in 96-well round-bottomed plates. Target cells (a C57BL/6-derived Rauscher leukemia virus (MuLV)-induced T cell lymphoma cell line, RMA) were labeled with <sup>51</sup>Cr and cultured with 10  $\mu$ M OVA or f-MIGWII peptide for 1 h at 37 °C. Negative control target cells were cultured without peptide. Targets were then washed and added to effector cells at 7500 cells/well. Effector cells from each mouse were assayed separately with both peptide-loaded targets. After a 5 h incubation at 37 °C, the plates were centrifuged at 1500 rpm for 5 min and cell supernatants collected. Supernatant samples (100  $\mu$ l) were counted on a 1470 Wizard automatic  $\gamma$  counter (Perkin Elmer, Shelton, CT) to determine amount of <sup>51</sup>Cr release. The percent specific lysis was calculated as  $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$ .

### Acknowledgments

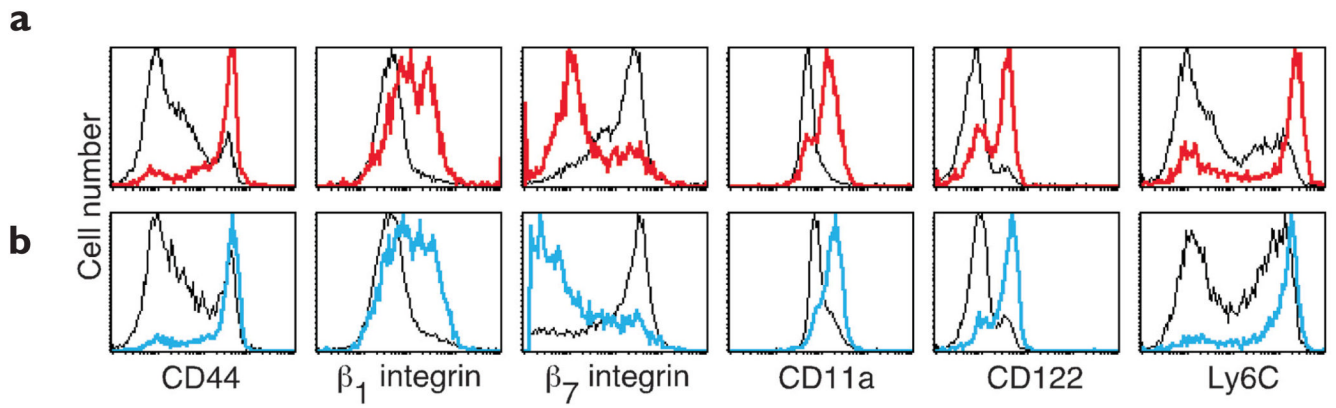
We thank X. C. Pan for animal care; B. Dere for tetramer synthesis; J. Yamagiwa for assistance with manuscript preparation; and A. Gallegos and M. Gavin for critical review of the manuscript. Supported by NIH grant K12-HD00850 from the Pediatric Scientist Development Program (to K. B. U.), the Howard Hughes Medical Institute and NIH grant AI19335 (to M. J. B.).

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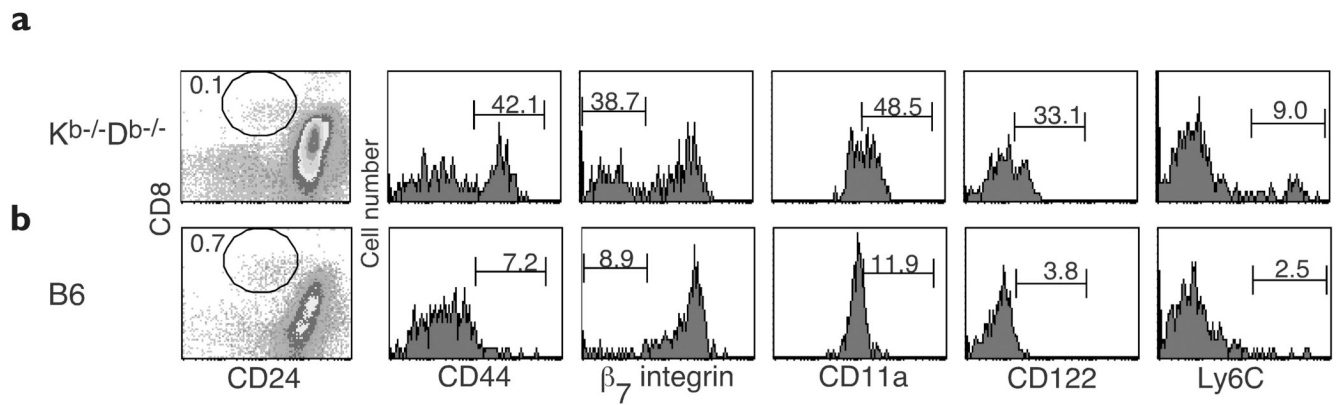
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**Figure 1. Phenotype of CD8<sup>+</sup> T cells in  $K^b^{-/-}D^b^{-/-}$  and  $B6 \rightarrow \beta_2M^{-/-}$  chimeric mice**

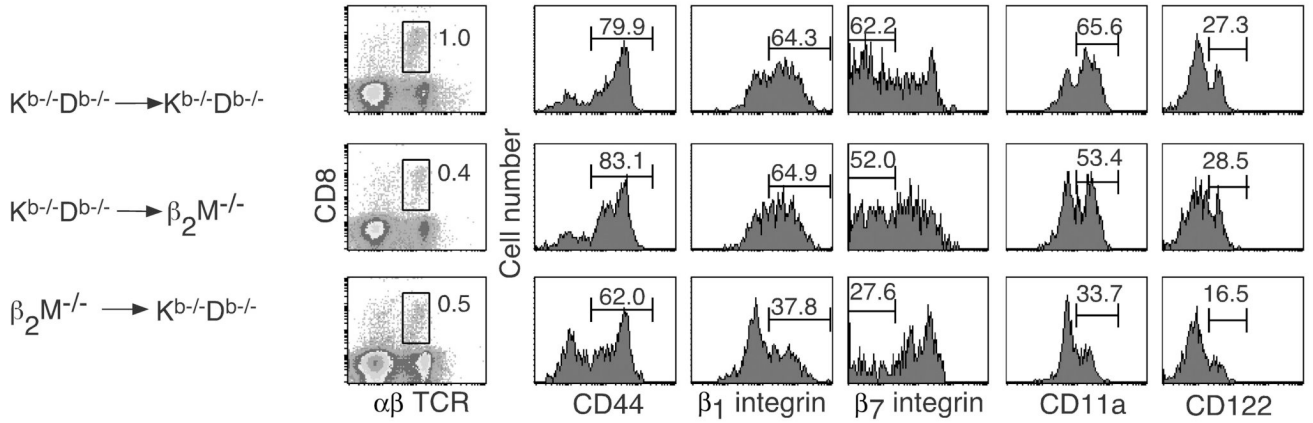
Surface expression of activation markers, gated on CD8<sup>+</sup> T cells from lymph nodes. **(a)** Flow cytometry histograms show CD8<sup>+</sup> T cells from B6 (thin black lines) versus  $K^b^{-/-}D^b^{-/-}$  mice (red lines). Mice were aged 13 weeks. **(b)** B6  $\rightarrow$  B6 (thin black lines) versus B6  $\rightarrow$   $\beta_2M^{-/-}$  chimeras (blue lines). Mice were examined 88 days after irradiation.



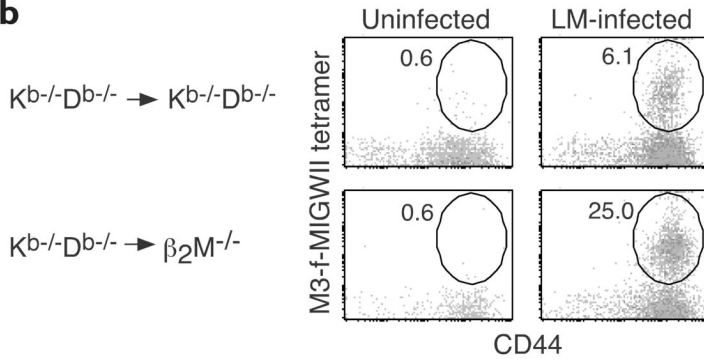
**Figure 2. Phenotype of thymic CD8<sup>+</sup> T cells in K<sup>b-/-</sup>D<sup>b-/-</sup> mice**

Surface expression of activation markers gated on CD24<sup>-</sup>CD8<sup>+</sup> thymocytes in (a) K<sup>b-/-</sup>D<sup>b-/-</sup> and (b) C57BL/6 mice. The percentages of each CD24<sup>-</sup>CD8<sup>+</sup> population within total thymus cells and the percentages of CD24<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup> cells displaying each activation marker are shown. Mice were aged 13 weeks.

**a**

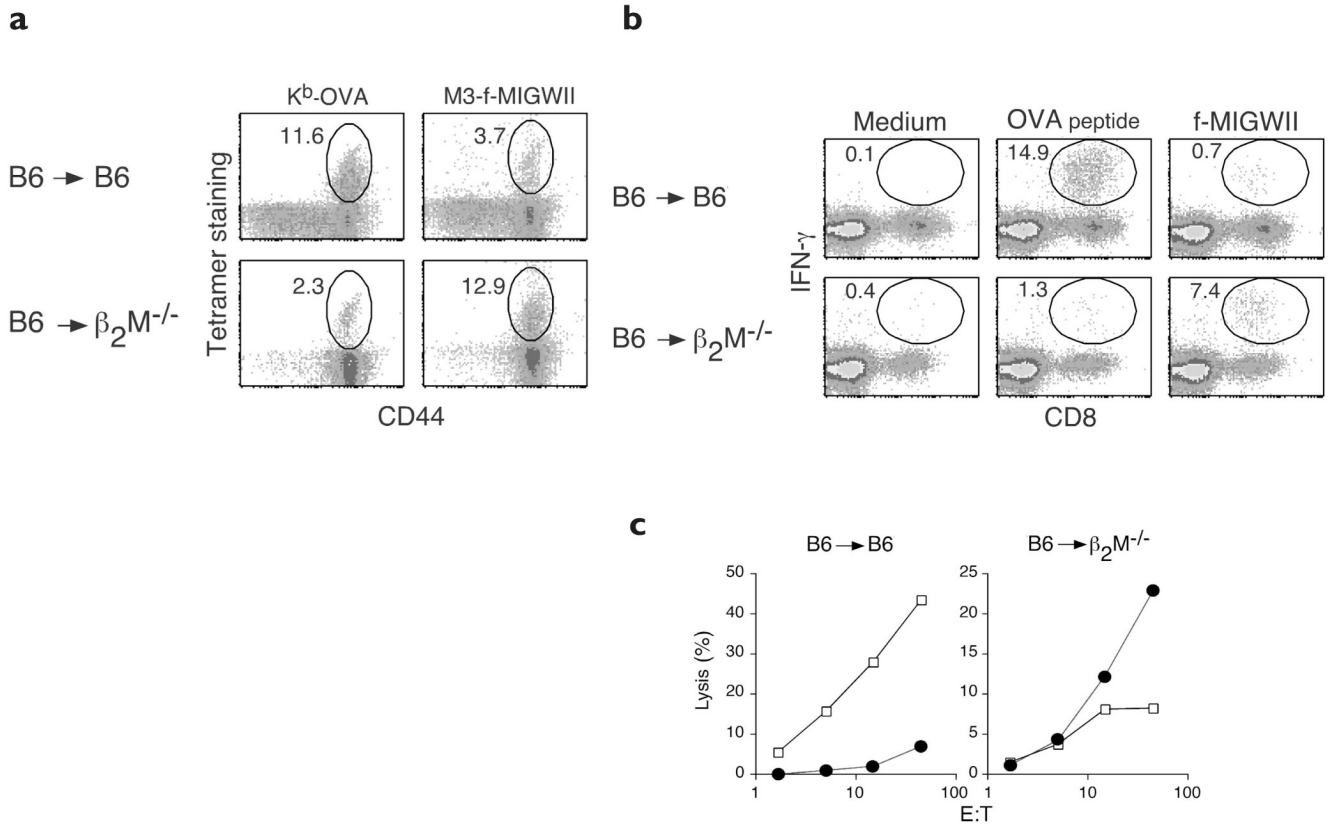


**b**



**Figure 3. Selection of MHC class Ib–restricted CD8<sup>+</sup> T cells on hematopoietic cells**

(a) Surface expression of activation markers gated on TCRβ<sup>+</sup>CD8<sup>+</sup> spleen cells in  $K^b\text{-}D^b\text{-} \rightarrow K^b\text{-}D^b\text{-}$ ,  $K^b\text{-}D^b\text{-} \rightarrow \beta_2M\text{-}$  and  $\beta_2M\text{-} \rightarrow K^b\text{-}D^b\text{-}$  chimeric mice. The percentages of the TCRβ<sup>+</sup>CD8<sup>+</sup> populations within total spleen cells and the percentages of TCRβ<sup>+</sup>CD8<sup>+</sup> cells displaying each activation marker are shown. Chimeric mice represented age-matched mice analyzed 15 weeks after irradiation. (b)  $K^b\text{-}D^b\text{-} \rightarrow K^b\text{-}D^b\text{-}$  or  $K^b\text{-}D^b\text{-} \rightarrow \beta_2M\text{-}$  chimeric mice were infected intravenously with 2000 CFU *L. monocytogenes* strain 10403S or were mock infected. Spleen cells were analyzed 6 days after infection. The percentages of CD44<sup>+</sup>tetramer<sup>+</sup> cells within the total CD8<sup>+</sup> population are shown. Results are representative of two mice per group.



**Figure 4. Preferential selection of H2-M3-restricted T cells on hematopoietic cells**  
**(a)** Tetramer staining: B6 → B6 or B6 → β<sub>2</sub>M<sup>-/-</sup> chimeric mice were infected intravenously with 2000 rLM-OVA and spleen cells were analyzed 6 days after infection. The percentages of CD44<sup>+</sup>, K<sup>b</sup>-OVA or M3-f-MIGWII tetramer<sup>+</sup> cells within the total CD8<sup>+</sup> population are shown. **(b)** Intracellular IFN-γ analysis: spleen cells were stimulated with medium, OVA peptide or f-MIGWII and intracellular IFN-γ was analyzed by flow cytometry. The percentages of IFN-γ-expressing cells within the CD8<sup>+</sup> population are shown. **(c)** Cytolysis assay: specific killing of OVA peptide-loaded (open squares) or f-MIGWII-loaded (filled circles) RMA target cells are shown. Analyses in **a–c** were done at the same time with spleen cells from the same mice. Results are representative of two separate experiments; two mice were used per group in each experiment.