# The CD8+ T Cell Repertoire in $\beta_2$-microglobulin-deficient Mice Is Biased towards Reactivity Against Self-Major Histocompatibility Class I

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## Summary

$\beta_2$-Microglobulin-deficient ($\beta_2$m −/−) mice are reported to lack cell surface expression of major histocompatibility complex (MHC) class I molecules, CD8+ T cells, and the ability to mount MHC class I-specific T cell responses. We have observed that $\beta_2$m −/− mice possess CD8+ T cells that can be induced to perform strong allospecific cytotoxic responses against nonself-MHC class I by in vivo priming. We report that these $\beta_2$m −/− cytotoxic T lymphocyte (CTL) differ from those induced in $\beta_2$m-positive littermates in that they cross-react and kill cells expressing self-MHC class I at normal ligand density with $\beta_2$m. $\beta_2$m −/− CTL could even be induced in primary mixed lymphocyte culture by self-MHC class I expressing stimulator cells, whereas allogeneic stimulator cells failed to elicit a response under similar conditions. Cells with a reduced cell surface MHC class I expression were less sensitive, while syngeneic $\beta_2$m −/− cells were resistant to the $\beta_2$m −/− CTL. This antiself-MHC reactivity could not be induced when $\beta_2$m −/− T cells matured in an environment with normal MHC class I expression in bone marrow chimeric mice. Antiself-MHC reactivity was also observed against human peptide loading-deficient cells expressing the appropriate murine class I molecules, suggesting that affinity to self-MHC class I may occur irrespective of peptide content. The results fit with a model where positive and negative selection of CD8+ T cells in $\beta_2$m −/− mice is mediated by low levels of MHC class I free heavy chains. In this model, low ligand density on selecting cells leads to positive selection of rare T cells that bind to low levels of MHC class I free heavy chains, resulting in a very small peripheral CD8+ compartment. Due to low density of the selecting ligand, negative selection does not remove T cells recognizing $\beta_2$m-positive cells expressing self-MHC class I at normal ligand density, which generates a T cell repertoire that would be autoreactive in a $\beta_2$m-positive littermate. The first "MHC deficient" animals thus paradoxically provide a tool for direct demonstration and analysis of self MHC bias in the T cell repertoire.

MHC class I molecules are expressed on virtually all nucleated mammalian cells (1). $\beta_2$m, the membrane-anchored heavy chain, and a short peptide are thought to assemble in the endoplasmatic reticulum where they form a complex that is transported to the cell surface (2). All three subunits are necessary for efficient transport and surface expression of functional MHC class I molecules. Cell lines deficient in $\beta_2$m express little if any MHC class I molecules detectable by mAbs at the cell surface (3–5). Cells deficient in genes coding for transporter associated with antigen processing (TAP)[1] products involved in peptide loading also have a low surface expression of MHC class I (2, 6, 7). However, several recent reports have indicated cell surface expression of free MHC class I heavy chains without $\beta_2$m. Such free heavy chains can bind $\beta_2$m (5), present exogenously added peptides to CTL (8), and mediate positive selection in fetal thymic organ cultures (9). In all these studies $\beta_2$m had to be added exogenously for effects to be seen.

Recent studies by Bix and Raulet (10), as well as by ourselves (11), indicate that strong MHC class I allospecific CTL responses can be induced against $\beta_2$m-deficient ($\beta_2$m −/−) cells in the absence of exogenous $\beta_2$m. CTL could be induced also against minor histocompatibility antigens, suggesting that free MHC class I heavy chains not only reach the cell surface in sufficient amounts to be recognized by CTL, but that they are capable of presenting peptides (11).

[1] *Abbreviation used in this paper:* TAP, transporter associated with antigen processing.


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Mice lacking $\beta_2$m gene expression are virtually devoid of normally conformed MHC class I molecules at the cell surface and they have a drastically reduced number of CD8$^+$ T cells in the periphery (12, 13). This is regarded as a consequence of impaired positive selection in the absence of MHC class I molecules. In spite of this deficiency in CD8$^+$ T cells, the mice reject grafts and resist many virus infections surprisingly well (14–17). In view of this, as well as the recent observations that CD8$^+$ T cells can recognize $\beta_2$m −/− cells in an MHC-specific or -restricted manner, we decided to investigate CD8$^+$ T cells and their specificity in $\beta_2$m −/− mice. We reasoned that if free MHC class I heavy chains can reach the cell surface and be recognized by CTL, they may also play a role during selection of CD8$^+$ T cells. If indeed $\beta_2$m −/− mice would be able to positively select a pool of CD8$^+$ T cells, it would be important to investigate tolerance, with respect to MHC class I of the self genotype, in order to assess the efficiency of negative selection. We observed strong allospecific CD8$^+$ CTL responses in $\beta_2$m −/− mice, as recently also shown by Apasov et al. (18). Our study demonstrates that such CTL specifically recognize MHC class I molecules not only of the appropriate allogeneic type used in the priming step, but also the self type when these are expressed with $\beta_2$m at normal ligand density. Our results provide evidence for a functional selection process of CD8$^+$ T cells in $\beta_2$m −/− mice resulting in a skewed repertoire with respect to tolerance to syngeneic MHC class I products.

## Materials and Methods

*Mice.* All mice were bred and maintained at the Department of Tumor Biology, Karolinska Institutet. The generation of the $\beta_2$m −/− mice has been described previously (19). We denote $\beta_2$m −/− and $\beta_2$m +/− for mice being homozygous or heterozygous for the $\beta_2$m mutation. The $\beta_2$m −/− and the $\beta_2$m +/− mice used were (B6 × 129)$F_2$–$F_5$, and are of the H-2$^b$ (K$^b$, D$^b$) haplotype. C57Bl/6 (B6), BALB/c, DBA, and AKR mice are of the H-2$^b$ (K$^b$, D$^b$), H-2$^d$ (K$^d$, D$^d$, L$^d$), H-2$^d$, and H-2$^k$ (K$^k$, D$^k$) haplotypes, respectively. A/Sn carries the H-2$^a$ (K$^k$, D$^d$, L$^d$) haplotype. A.BY, A.CA, and A.SW are MHC congeneic mice of A/Sn background, and are of the H-2$^b$, H-2$^f$, and H-2$^s$ haplotypes, respectively. C57Bl/10 (B10) is a substrain of B6. B10.D2 (K$^d$, D$^d$, L$^d$) and B10.A(2R) (K$^k$, D$^b$) are MHC congenic B10 mice. Bone marrow chimeras were derived by 950-rad irradiation of the recipient mice and subsequent infusion of 5 × 10$^6$ i.v. donor bone marrow cells. The recipient mice were pretreated with 0.2 ml of ascites fluid of the NK1.1 CPK 136 hybridoma 1 d before infusion of donor bone marrow.

*Cell Lines.* P815 is a mastocytoma cell line derived from DBA (H-2$^d$), and P1HTR is a subline of P815. These two cell lines gave similar results, and both are, for reasons of simplicity, denoted P815 in the tables of this paper. P1HTR-K$^b$, P1HTR-KKA, and P1HTR-AAK are transfectants of P1HTR (20), and were the kind gift from Hans Stauss (University College, London, UK). KKA is an exon-shuffled construct containing the $\alpha1/\alpha2$ domains of H-2K$^b$, and the $\alpha3$ domain of HLA-A2. AAK is a construct containing the $\alpha1/\alpha2$ domains of HLA-A2 and the $\alpha3$ domain of H-2K$^b$. P1HTR-D$^b$ and P1HTR-K$^b$-D$^b$ are H-2D$^b$ and H-2K$^b$, D$^b$ transfectants of P1HTR, respectively. EL-4 is a benzopyrene-induced lymphoma derived from B6, and EL-4D$^d$ is an H-2D$^d$ transfectant of EL-4. C4.4-25$^-$ is a $\beta_2$m −/− variant of EL-4, and E50.16$^+$ is a $\beta_2$m transfectant of C4.4-25$^-$ (21). C4.4-25$^-$D$^d$ is an H-2D$^d$ transfectant of C4.4-25$^-$. RMA is a subline of the Raucher Virus–induced B6 lymphoma RBL-5, and RMA-S is a TAP2 loss mutant of RMA (2, 22–24). T2 is a hybrid between the two human cell lines .174 and CEM, and has an antigen processing deficiency due to a deletion in the MHC class II region, including the TAP1 and TAP2 genes (6, 7). T2-K$^b$, T2-D$^b$, T2-D$^d$, and T2-D$^p$ are MHC class I transfectants of T2, kindly provided by Peter Cresswell (Yale University School of Medicine, New Haven, CT). T1 is the wild type of T2. T1-K$^b$ and T1-D$^d$ are MHC class I transfectants of T1. T1-K$^b$ was kindly provided by Frank Momburg and Gunther Hämmerling (Deutsche Krebsforschungszentrum DKFZ, Heidelberg, Germany). The generation of Con A–activated T cell blasts has been described (11). Briefly, 15 × 10$^6$ erythrocyte-depleted spleen cells were plated in each well in 12-well plates, in RPMI supplemented with 5 $\mu$g/ml Con A and 10% FCS. After 2–3 d the blasts were used as targets in a $^{51}$Cr release assay.

*Transfection.* 5–10 × 10$^6$ cells were suspended in 500 $\mu$l of PBS. P1HTR and P1HTR-K$^b$ were electroporated in with 10–20 $\mu$g of pSV2gptD$^b$ plasmid (kindly provided by Alain Townsend, Oxford University, England) at 250 V and 960 $\mu$F in a Genepulser (Bio-Rad Laboratories, Richmond, CA). For selection of D$^b$-expressing transfectants, cells were stained with MHC class I–specific mAbs and subsequently positively selected using anti–mouse Ig–coated Dynabeads (Nycomed, Oslo, Norway). EL-4, C4.4-25$^-$ and T1 were electroporated with 10–20 $\mu$g of the pSV2neoD$^d$ plasmid (kindly provided by Peter Robinson, Medical Research Council, London, UK) using 960 $\mu$F and 250, 250, and 210 V, respectively. Before positive selection as described above, transformants were selected by growth in medium containing 0.5 mg/ml G418 (Sigma, Stockholm, Sweden).

*Effector Cells and Mixed Lymphocyte Culture (MLC).* Mice were immunized two to three times with 10 × 10$^6$ i.p. irradiated tumor or spleen cells. 60–70 × 10$^6$ spleen cells were restimulated in vitro with 5–8 × 10$^6$ tumor cells or 20–30 × 10$^6$ spleen cells (irradiated with 10,000 rad), in RPMI supplemented with 10% FCS and 5 × 10$^{-5}$ M 2-ME. After 5 d the cells were used as effector cells in a standard $^{51}$Cr release assay.

*mAbs, FACS® Analysis, and Complement-mediated Depletion of Effector Cell Populations.* For flow cytometry, FITC-conjugated mAbs directed against CD4 (YTS 191.1; Sera-Lab, Crawley Down, Sussex, England), CD8$\alpha$ (169.4); TCR-$\alpha/\beta$ (H57-597), or H-2K$^b$ (53-6-7 and AF6-88.5; all from PharMingen, San Diego, CA) were used. Before FACS® analysis (on a FACS IV®; Becton Dickinson & Co., Mountain View, CA), cells were incubated with 1–2 $\mu$g of antibody (diluted in PBS to 100 $\mu$l) for 30–60 min at 4°C. For complement-mediated effector cell depletion, the cells were first incubated in 100 $\mu$l/10$^6$ cells using a 100-$\mu$g/ml mixture of two different mAbs (for CD4 depletion, 191.1 and 3.1.2.; and for CD8 depletion, 169.4 and 156.7.7). (These antibodies were kindly provided by Herman Waldman, University of Cambridge, Cambridge, UK). The cells were then washed once and incubated with rabbit complement (Pel-Freeze Biologicals, Brown Deer, WI) diluted 1:8 for 75–90 min at 37°C. Hybridoma cells producing a mAb against H-2K$^b$, D$^b$ (28-8-6S; HB51) were obtained from the American Type Culture Collection (Rockville, MD). For depletion of H-2K$^b$, D$^b$–expressing cells, 100 $\mu$l/10$^6$ cells of protein A–purified antibody diluted to 20 $\mu$g/ml were used for the first incubation, followed by incubation with complement as described above.

*Synthetic Peptides.* All peptides were purchased from Chiron Mimotopes (Melbourne, Australia). The H-2K$^b$ and H-2D$^b$ bind-

ing sequences were derived from the immunodominant epitopes of Sendai and A/PR/8/34 influenza viruses and were: FAPGNYPAL and ASNENMETM (in single letter amino acid code). $NH_2$ and COOH terminals were free in both peptides.

*Cold Target Competition Assay.* Effector cells and cold (unlabeled) target cells were mixed and preincubated in 37°C for 60 min before addition of hot ($^{51}$Cr-labeled) target cells. Tumor cells or Con A blasts were used as cold targets. The cold Con A blasts used as cold targets were not cytotoxic to any of the targets used (data not shown).

*Generation of Fibrosarcomas from $\beta_2$m −/− and $\beta_2$m +/− Mice.* Mice were injected intramuscularly with 0.05 mg of methylcholantrene dissolved in 0.1 ml olive oil. Fibrosarcomas developed after a period of 3–5 mo. The tumors were adapted to in vitro culture and typed by Southern blot analysis for the $\beta_2$m genotype.

## Results

*Generation of Allospecific CD8$^+$ CTL from $\beta_2$m −/− Mice.* After in vivo priming and in vitro restimulation with BALB/c (H-2$^d$) or A/Sn (H-2$^a$) spleen cells, $\beta_2$m −/− (H-2$^b$) responder mice developed strong cytotoxic responses against the allospecific H-2$^d$ (or H-2D$^d$ only)-expressing targets (Tables 1 and 2). The strength of the response was in the same range as that of effector cells derived from $\beta_2$m +/− or B6 control mice. The response was not dependent on epithelial cells or professional antigen-presenting cells among the allogeneic splenocytes used for priming and restimulation, as these could be substituted by the H-2$^d$-expressing tumor P815, a CD4$^-$CD8$^-$ mastocytoma (Tables 1–3). However, the response was dependent on in vivo priming, since it was poor when unprimed spleen cells were used, as observed previously (data not shown, and reference 13). The CTL responses of $\beta_2$m −/− mice have been observed in >30 consecutive experiments.

The in vivo priming of $\beta_2$m −/− mice resulted in a small but consistent increase in the number of CD8$^+$ cells in the spleen so that these represented up to 3% of total spleen cells (not immunized: mean 1.1% CD8$^+$ cells, range 0.2–1.6; immunized: mean 2.8% CD8$^+$ cells, range 1.7–5.3). It should be noted that in absolute numbers the figures correspond to 2–5 × 10$^6$ CD8$^+$ cells and an increase of approximately twofold of the whole CD8$^+$ population as a response to in vivo priming. A more dramatic effect was noted at the end of the in vitro restimulation, when $\beta_2$m −/− CD8$^+$ cells had expanded 4–10-fold to represent up to 34% of total cells in the culture (Fig. 1). The CD8$^+$ cells in this population were also TCR-$\alpha/\beta^+$, and the majority of the CD8$^-$TCR-$\alpha/\beta^+$ were CD4$^+$ (Fig. 1 and data not shown). The cytotoxic $\beta_2$m −/− effector cells were CD4$^-$CD8$^+$, as determined by antibody and complement depletion of effector cells generated in the MLC (Table 1), and MHC class I allospecific since they killed human T1 or T2 cells transfected with H-2D$^d$, but not untransfected cells (Tables 2 and 4). CD4$^+$ cells also expanded upon secondary in vitro allostimulation of $\beta_2$m −/− spleen cells, which contrasted to the decreased proportion of CD4$^+$ cells in the corresponding cultures of spleen cells from in vivo primed $\beta_2$m +/− mice (data not shown). However, depletion of CD4$^+$ cells had no effect on allospecific cytotoxicity in this experimental system. $\beta_2$m −/− CTL generated by allogeneic stimulation thus behaved as expected from classical MHC-specific alloreactive CTL, with respect to phenotype and MHC class I–oriented allospecificity.

*CTL Generated from $\beta_2$m −/− (H-2$^b$) Mice Cross-react on Cells Expressing H-2$^b$.* In contrast to the effector cells generated from the $\beta_2$m +/− littermates, anti-H-2$^d$-primed $\beta_2$m −/− CTL also killed target cells with cell surface expression of H-2$^b$ (i.e., self) and $\beta_2$m at normal ligand density (H-2$^b/\beta_2$m) (Tables 1 and 2). This killing was dependent on a normal MHC class I expression since the $\beta_2$m −/− mutant of EL-4, C4.4-25$^-$, was resistant, while the $\beta_2$m transfectant of C4.4-25$^-$, with restored MHC class I expression, was sensitive (Table 2). Furthermore, T2-K$^b$ cells were killed whereas T2 and T2-D$^p$ were not (Table 2). When Con A blasts derived from MHC congeneic mice were used as targets, anti-H-2$^d$ $\beta_2$m −/− CTL showed a preference for Con A blasts expressing H-2$^b$ antigens, whereas the syngeneic $\beta_2$m −/− (H-2$^b$) Con A blasts were resistant (Table 2). The

**Table 1.** *Subset Depletion of $\beta_2$m +/− and $\beta_2$m −/− Effector Cells: Effector Cells from $\beta_2$m −/− Mice Are CD8$^+$ and Have a Broader Specificity*

| Effector cells* | | | Effector cell treatment and targets‡ | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | P815 | | | EL-4 | | |
| Responder mouse | Priming in vivo | Restimulation in vitro | C′ | CD4 + C′ | CD8 + C′ | C′ | CD4 + C′ | CD8 + C′ |
| $\beta_2$m +/− | A/Sn | A/Sn | 49, 30§ | 51, 42 | 3, 0 | 0, 0 | 0, 0 | 0, 0 |
| $\beta_2$m −/− | A/Sn | A/Sn | 34, 18 | 38, 21 | 2, 1 | 44, 32 | 44, 35 | 2, 2 |
| $\beta_2$m −/− | P815 | P815 | 77, 69 | 76, 59 | 17, 7 | 44, 34 | 37, 27 | 3, 3 |

* Effector cells were generated as described in Materials and Methods. A/Sn splenocytes or P815 tumor cells were used for priming.
‡ The effector cell population was depleted from various cellular subsets using antibody and complement treatment. The EL-4 and P815 targets are of H-2$^b$ and H-2$^d$ haplotype, respectively.
§ Percent specific lysis in a $^{51}$Cr release assay. E/T ratio: 60:1, 12:1.

**Table 2.** *Specificity of CTL Generated from $\beta_2$m +/− and $\beta_2$m −/− Mice*

| Exp. | Effector cells* Responder mice | Priming in vivo and restimulation in vitro | Target cells‡ | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | BALB/c H-2$^{d}$§ | EL-4 H-2$^{b}$ | C4.4-25$^{-}$ H-2$^{b}$, $\beta_2$m$^{-}$ | E50.16$^{+}$ H-2$^{b}$, $\beta_2$m$^{+}$ | | |
| 1 | $\beta_2$m +/− | BALB/c | 72, 62‖ | 5, 0 | 1, 0 | 0, 0 | | |
| | $\beta_2$m −/− | BALB/c | 65, 52 | 61, 46 | 4, 0 | 62, 36 | | |
| | | | P815 H-2$^{d}$ | T2 | T2-D$^{d}$ H-2D$^{d}$ | T2-K$^{b}$ H-2K$^{b}$ | T2-D$^{p}$ H-2D$^{p}$ | |
| 2 | $\beta_2$m +/− | P815 | 81, 60 | 0, 4 | 57, 26 | 9, 7 | 8, 1 | |
| | $\beta_2$m −/− | P815 | 80, 53 | 7, 8 | 41, 25 | 44, 23 | 19, 6 | |
| | | | BALB/c H-2$^{d}$ | B6 H-2$^{b}$ | $\beta_2$m −/− H-2$^{b}$ | A.BY H-2$^{b}$ | A.CA H-2$^{f}$ | A.SW H-2$^{s}$ |
| 3 | $\beta_2$m −/− | P815 | 49, 50 | 28, 35 | 10, 7 | 39, 42 | 6, 6 | 0, 2 |
| | $\beta_2$m −/− αCD8¶ | P815 | 0, 1 | 7, 4 | 1, 0 | ND | 0, 1 | 0, 0 |
| | | | P815 H-2$^{d}$ | RMA H-2$^{b}$ | B10.D2 H-2$^{d}$ | B10.A(2R) H-2K$^{k}$D$^{b}$ | B10.BR H-2$^{k}$ | |
| 4 | $\beta_2$m +/− | P815 | 75, 59 | 9, 0 | 69, 59 | 22, 0 | 34, 14 | |
| | $\beta_2$m −/− | P815 | 75, 69 | 84, 83 | 56, 61 | 45, 43 | 19, 3 | |

* Effectors were generated as described in Materials and Methods.
‡ Target cells in Exps. 1, 3, and 4 were tumor cells or Con A blasts generated from the mouse strains indicated. In Exp. 2 only tumor cell targets were used.
§ The murine MHC class I genotype is indicated below the name of the target cell.
‖ Percent specific lysis in a $^{51}$Cr release assay. E/T ratios were 60:1, 12:1 (Exp. 1); 40:1, 8:1 (Exp. 2); 10:1, 2:1 (Exp. 3); 8:1, 1.6:1 (Exp. 4).
¶ Effectors depleted in vitro with CD8 mAb and complement.

**Table 3.** *Sensitivity of RMA-S Cells to $\beta_2$m −/− CTL after Incubation in the Presence of Peptides or at Reduced Temperature*

| Exp. | Effector cells* Responder mice | Priming in vivo and restimulation in vitro | Target cells | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | P815 H-2$^{d}$‡ | EL-4 H-2$^{b}$ | C4.4-25$^{-}$ H-2$^{b}$, $\beta_2$m$^{-}$ | RMA H-2$^{b}$ | RMA-S H-2$^{b}$, TAP2$^{-}$ | RMA-S peptide§ | RMA-S 26°C‖ |
| 1 | $\beta_2$m +/− | P815 | 69, 42¶ | 6, 0 | 0, 0 | 3, 0 | 4, 4 | 8, 10 | n.t. |
| | $\beta_2$m −/− | P815 | 62, 53 | 61, 14 | 0, 0 | 63, 40 | 8, 6 | 54, 23 | n.t. |
| 2 | $\beta_2$m +/− | P815 | 82, 82 | 14, 6 | 1, 2 | 23, 15 | 3, 6 | n.t. | 10, 4 |
| | $\beta_2$m −/− | P815 | 79, 86 | 66, 59 | 0, 1 | 72, 73 | 38, 24 | n.t. | 83, 71 |

* Effector cells were generated as described in Materials and Methods.
‡ MHC genotype and mutant characteristics are indicated below the name of the target cell.
§ RMA-S was incubated at 37°C in a mixture 20 μM of Sendai (K$^{b}$ binding) and influenza (D$^{b}$ binding) peptides.
‖ RMA-S was cultured overnight at 26°C.
¶ Percent specific lysis in a Cr release assay. E/T ratio in both Exps. 1 and 2: 40:1, 8:1.

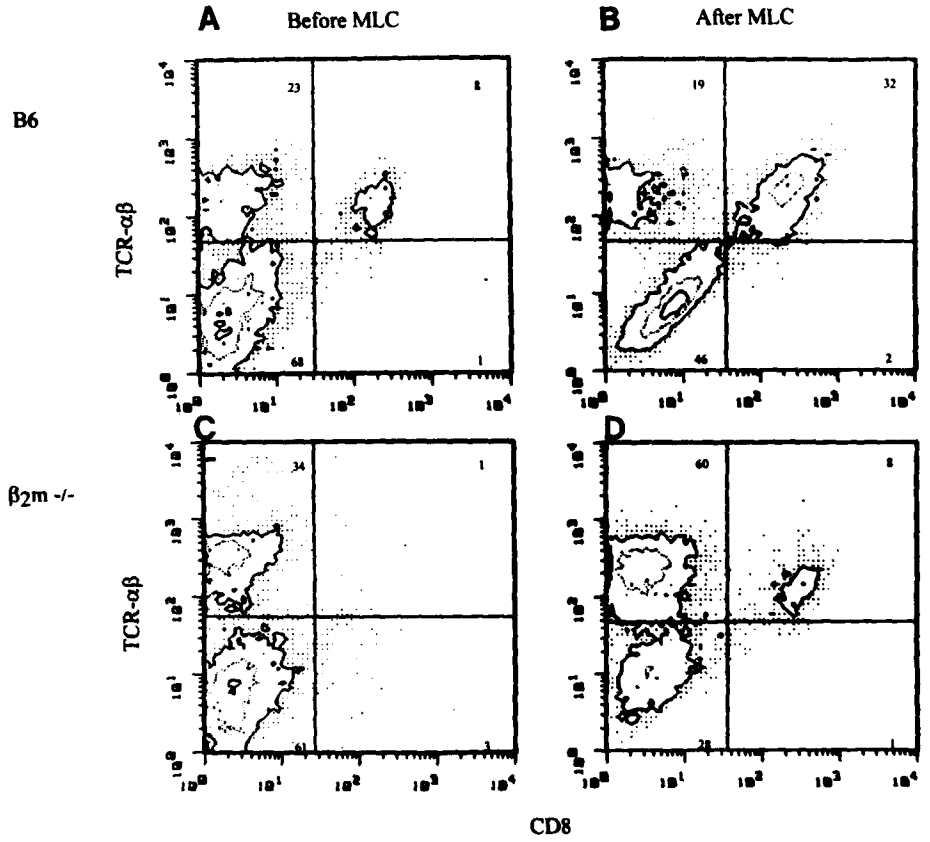


**Figure 1.** Expansion of CD8⁺TCR-$\alpha/\beta^+$ cells after allostimulation of in vivo primed $\beta_2$m −/− or B6 mice. $\beta_2$m −/− or B6 mice were primed in vivo with irradiated BALB/c (H-2$^d$) spleen cells, and subsequently responder cells were stained with FITC-conjugated anti-CD8 and anti-TCR-$\alpha/\beta$ mAb before and after in vitro restimulation. Both B6 (*A* and *B*) and $\beta_2$m −/− (*C* and *D*) spleen cells were assayed in parallel. The numbers in each quadrant indicate the percentage of cells in the quadrant.

killing of H-2$^b$-expressing targets was also mediated by CD4⁻CD8⁺ cells, as determined by complement-mediated depletion of the effector cell population (Table 1). To study whether this killing was due to a unique cross-reactivity between H-2$^b$ and H-2$^d$ (as discussed in reference 25) we also generated $\beta_2$m −/− CTL against AKR (H-2$^k$) or A.SW (H-2$^s$) spleen cells. The same pattern emerged: $\beta_2$m −/− (but not $\beta_2$m +/−) CTL killed targets expressing H-2$^b$/$\beta_2$m (data not shown).

*CTL Killing Induced by Peptide or Cold Temperature Treatment of RMA-S.* The peptide loading (TAP2)-deficient mutant RMA-S (H-2$^b$; references 2, 22–24) showed a low and somewhat variable sensitivity (range: 5–38% lysis) to the anti-H-2$^d$-stimulated $\beta_2$m −/− CTL. RMA-S was clearly less sensitive than the wild type RMA line (50–80% lysis; Table 3), but neither RMA nor RMA-S were sensitive to anti-H-2$^d$ $\beta_2$m +/− CTL (Table 3). Culture of RMA-S at 26°C or in the presence of H-2K$^b$ and H-2D$^b$ binding synthetic peptides significantly increased the sensitivity of RMA-S to the $\beta_2$m −/− CTL, but not to $\beta_2$m +/− CTL. Both of these treatments stabilize MHC class I molecules at the surface of RMA-S and increase their steady state expression (2, 26). The cell surface MHC class I levels induced by these treatments correspond to about half of what is expressed on wild type RMA cells. These observations suggest that $\beta_2$m −/− CTL can recognize H-2K$^b$ and H-2D$^b$ molecules independently of specific peptides loaded internally in the MHC class I peptide presentation pathway, and that ligand density of MHC class I molecules may be crucial when they recognize cells expressing H-2$^b$/$\beta_2$m.

*Specificity for Both H-2$^d$ and H-2$^b$ among Individual $\beta_2$m −/− (H-2$^b$) CTL.* We performed cold target competition experiments to test whether the killing of the allospecific H-2$^d$ and the self-H-2$^b$-expressing target was mediated by the same CTL. Bulk $\beta_2$m −/− CTL generated against P815 (H-2$^d$) killed EL-4 (H-2$^b$) as well as P815-labeled targets (Table 4). Using EL-4 as a hot target, we observed that both EL-4 and P815, but not the $\beta_2$m −/− C4.4-25⁻, competed for these $\beta_2$m −/− CTL (Table 4). This shows that the bulk population contained CTL able to recognize the self-MHC-matched as well as the allospecific target. Using P815 as hot target, competition was seen only with P815, whether $\beta_2$m −/− or $\beta_2$m +/− CTL were used (data not shown). Competition experiments were also made with $\beta_2$m −/− CTL generated against AKR (H-2$^k$) spleen cells. These killed A.BY (H-2$^b$) in addition to AKR Con A blasts of the allospecific H-2$^k$ type. Using A.BY as a hot target, both A.BY and AKR competed for these CTL, while third party A.CA (H-2$^f$) Con A blasts did not (Fig. 2 *A*). When AKR Con A blasts were used as hot targets, A.BY Con A blasts competed partially for $\beta_2$m −/− CTL while they completely failed to compete for $\beta_2$m +/− CTL generated under the same conditions (Fig. 2 *B* and data not shown). From the combined cold target competition experiments we conclude that anti-H-2$^d$-primed CTL bulk cultures generated from $\beta_2$m −/− H-2$^b$ mice contain CD8⁺ CTL that

**Table 4.** *$\beta_2$m −/− CTL Generated against Targets Lacking $\beta_2$m*

| Exp. | Effector cells* Responder mouse | Priming in vivo and restimulation in vitro | Target cells‡ EL-4 H-2$^{b}$§ | EL-4 D$^{d}$ H-2$^{b}$ + D$^{d}$ | C4.4-25$^{-}$ H-2$^{b}$, $\beta_2$m$^{-}$ | C4.4-25$^{-}$D$^{d}$ H-2$^{b}$, $\beta_2$m$^{-}$ + D$^{d}$ | T1 | T1-K$^{b}$ H-2K$^{b}$ | T1-D$^{d}$ H-2D$^{d}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | $\beta_2$m −/− | H-2 (b × d) F$_1$, $\beta_2$m −/− | 44, 25‖ | 39, 26 | 2, 2 | 5, 1 | 2, 0 | 5, 2 | 34, 7 |
| | $\beta_2$m −/− | H-2 (b × d) F$_1$, $\beta_2$m +/− | 45, 24 | 44, 25 | 2, 0 | 42, 15 | 7, 1 | 33, 14 | 46, 22 |
| | | | Target cells | | | | | | |
| 2 | | | EL-4 | C4.4-25$^{-}$ | C4.4-25$^{-}$D$^{d}$ | P815 | | | |
| | $\beta_2$m −/− | P815 | 59, 55 | 1, 7 | 51, 23 | 86, 76 | | | |
| | | | Cold target cells | | | | | | |
| | Cold target competition (Hot target: EL-4) | | EL-4 | C4.4-25$^{-}$ | C4.4-25$^{-}$D$^{d}$ | P815 | | | |
| | $\beta_2$m −/− | P815 | 0, 12, 58¶ | 0, 0, 0 | 0, 6, 46 | 24, 66, 96 | | | |

* Effector cells were generated as described in Materials and Methods.
‡ In both Exps. 1 and 2, EL-4, C4.4-25$^{-}$, and C4.4-25$^{-}$D$^{d}$ target cells had been grown in serum-free medium for a period of >2 mo and the assay was performed in serum-free medium. The effector cells had been cultured in serum-containing medium but were washed in PBS before the assay.
§ The murine MHC class I genotype is indicated below the name of the target cell.
‖ Percent specific lysis in a $^{51}$Cr release assay. E/T ratio: 40:1, 8:1.
¶ Percent inhibition of EL-4 lysis, compared with EL-4 lysis when no unlabeled targets were present. The E/T ratio was 4:1, and the killing of EL-4 without unlabeled targets was 33%.

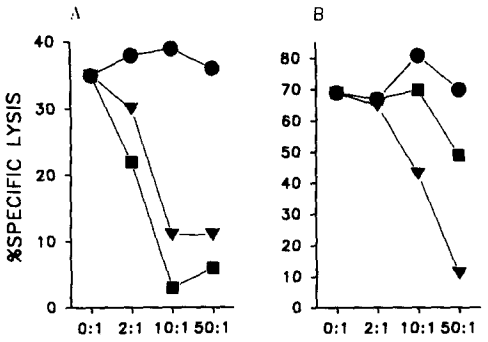


**Figure 2.** Cold target competition using A.BY (*A*) or AKR (*B*) as $^{51}$Cr-labeled targets. Effectors were $\beta_2$m −/− CTL generated against AKR by in vivo priming and in vitro restimulation and were used at an E/T ratio of 20:1. (*A*) Unlabeled A.BY (H-2$^{b}$) and AKR (H-2$^{k}$), but not A.CA (H-2$^{f}$), Con A blasts compete with $^{51}$Cr-labeled A.BY Con A blasts for anti-H-2$^{k}$ $\beta_2$m −/− CTL. (*B*) Unlabeled AKR and to a lesser extent A.BY, but not A.CA, Con A blasts compete with $^{51}$Cr-labeled AKR Con A blasts for anti-H-2$^{k}$ $\beta_2$m −/− CTL. The cold/hot target ratios are indicated. Cold targets in both *A* and *B* were: (■) A.BY, (●) A.CA, and (▼) AKR Con A blasts. The Con A blasts were not cytotoxic to either the hot target or to C4.4-25$^{-}$ $\beta_2$m −/− tumor cells. The experiment was made in medium containing FCS.

kill allospecific targets, of which some (but not all) cross-react on H-2$^{b}$/$\beta_2$m–expressing targets.

*Cross-reaction between H-2$^{b}$/$\beta_2$m Expressed at Normal Ligand Density and H-2D$^{d}$ Free MHC Class I Heavy Chains.* None of our $\beta_2$m −/− target cells were recognized by $\beta_2$m −/− CTL, and it was therefore important to test whether the $\beta_2$m −/− CTL could mediate cytotoxicity in a system devoid of $\beta_2$m, excluding the involvement of $\beta_2$m-derived peptides. To test this, we used $\beta_2$m −/− EL-4 cells transfected with H-2D$^{d}$ (i.e., C4.4-25$^{-}$D$^{d}$) as targets. When $\beta_2$m −/− (H-2$^{b}$) CTL were generated by in vivo priming and in vitro restimulation with $\beta_2$m$^{+}$ H-2$^{b\times d}$ spleen cells, these were able to kill C4.4-25$^{-}$ D$^{d}$, but not C4.4-25$^{-}$. The $\beta_2$m −/− CTL could thus perform H-2D$^{d}$ allospecific killing against a $\beta_2$m −/− target (Table 4). This demonstrates that (*a*) $\beta_2$m −/− CTL can recognize targets independently of $\beta_2$m as such, or of a $\beta_2$m-derived peptide, and (*b*) H-2D$^{d}$ expressed in cells with and without $\beta_2$m result in H-2D$^{d}$ sharing antigenic structures, since the spleen cells used for priming and restimulation expressed both H-2D$^{d}$ and $\beta_2$m, and the targets expressed H-2D$^{d}$ but no $\beta_2$m. Note that the H-2D$^{d}$-transfected as well as the nontransfected EL-4 cells were killed,

while the $\beta_2$m −/− C4.4-25$^-$ cells were killed only upon D$^d$ transfection. $\beta_2$m −/− CTL could thus distinguish between H-2$^b$ and H-2D$^d$ when expressed in EL-4 $\beta_2$m −/− cells, but not when expressed in $\beta_2$m-positive cells at normal ligand density. This was confirmed in cold target competition experiments using $\beta_2$m −/− CTL generated against P815 (H-2$^d$). Such CTL killed C4.4-25$^-$D$^d$ as well as EL-4, but not C4.4-25$^-$ cells (Table 4). A substantial number of these CTL killed both C4.4-25$^-$D$^d$ and EL-4, since unlabeled C4.4-25$^-$D$^d$ cells competed for labeled EL-4 cells as efficiently as EL-4 itself, whereas nontransfected C4.4-25$^-$ cells did not compete (Table 4).

*CTL Elicited by Priming $\beta_2$m −/− H-2$^b$ Mice with $\beta_2$m-positive H-2$^b$ Cells.* We immunized $\beta_2$m −/− (H-2$^b$) mice with B6 spleen cells with subsequent in vitro restimulation, to mount the response directly to H-2$^b$. This yielded effector cells capable of killing RMA as well as RMA-S and in addition a fibrosarcoma line from a $\beta_2$m +/− mouse, whereas three similar fibrosarcomas from $\beta_2$m −/− mice were resistant (Table 5, Exp. 1). MHC class I (K$^b$ or D$^b$) transfectants of the T2 cell line were sensitive but nontransfected T2 and T2-K$^k$ were resistant; the $\beta_2$m −/− CTL were thus MHC class I (H-2D$^b$ and H-2K$^b$) specific. The data further support the notion that $\beta_2$m −/− mice possess CTL precursors that can recognize H-2$^b$ MHC class I molecules independently of loaded antigen (Tables 2 and 5), since the murine MHC class I molecules in T2-D$^b$ and T2-K$^b$ are considered to be devoid of peptides (27). The data on the T2 transfectants, therefore, also argue against the possibility that the response was directed against minor histocompatibility antigens not shared by the $\beta_2$m −/− and B6 mice.

When B6-derived EL-4 tumor cells were used as immunogens in order to elicit an H-2$^b$–restricted CTL response in $\beta_2$m +/− mice, these CTL behaved differently from those induced in the same way from $\beta_2$m +/− mice. The $\beta_2$m +/− CTL killed EL-4 but not T1-K$^b$, presumably due to lack of tumor antigens (or other murine-specific peptides) expressed by EL-4 (Table 5, exp. 2). In contrast, $\beta_2$m −/− CTL killed both EL-4 and T1-K$^b$ as well as the antigen processing defective variant T2-K$^b$. This cytotoxicity was dependent on the $\alpha$1/$\alpha$2 domains of H-2K$^b$, as evident from testing P815 cells transfected with chimeric genes obtained by exon shuffling between HLA-A2 and H-2K$^b$ (Table 5, Exp. 2).

*Generation of H-2$^b$-specific $\beta_2$m −/− (H-2$^b$) CTL in a Primary MLC Using Syngeneic $\beta_2$m-positive Stimulator Cells.*

**Table 5.** *Specificity of CTL Generated from $\beta_2$m +/− and $\beta_2$m −/− Mice Immunized with H-2$^b$-expressing Cells*

| Exp. | Effector cells* Responder mouse | Effector cells* Stimulator cells | Target cells | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | T2 | T2-K$^b$ H-2K$^b$‡ | T2-D$^b$ H-2D$^b$ | T2-K$^k$ H-2K$^k$ | | |
| 1 | $\beta_2$m +/− | B6 | 2, 6§ | 0, 0 | 5, 3 | 0, 0 | | |
| | $\beta_2$m −/− | B6 | 3, 0 | 45, 25 | 26, 10 | 0, 0 | | |
| | | | MbetaC$^-$ H-2$^b$, $\beta_2$m$^-$ | MbetaD$^-$ H-2$^b$, $\beta_2$m$^-$ | MbetaJ$^+$ H-2$^b$, $\beta_2$m$^+$ | MbetaN− H-2$^b$, $\beta_2$m$^-$ | RMA H-2$^b$ | RMA-S H-2$^b$, TAP2$^-$ |
| | $\beta_2$m +/− | B6 | 8, 0 | 7, 2 | 17, 9 | 0, 0 | 35, 19 | 4, 2 |
| | $\beta_2$m −/− | B6 | 8, 0 | 3, 4 | 43, 35 | 0, 0 | 74, 64 | 60, 50 |
| | | | EL-4 H-2$^b$ | C4.4-25$^-$ H-2$^b$, $\beta_2$m$^-$ | E50.16$^+$ H-2$^b$, $\beta_2$m$^+$ | T1 | T1-K$^b$ H-2K$^b$ | T2-K$^b$ H-2K$^b$ |
| 2 | $\beta_2$m +/− | EL-4 | 52, 34 | 11, 8 | 47, 35 | 0, 0 | 3, 1 | 24, 12 |
| | $\beta_2$m −/− | EL-4 | 61, 57 | 3, 1 | 78, 69 | 11, 6 | 52, 30 | 55, 23 |
| | | | P815 H-2$^d$ | P815-AAK‖ H-2$^d$, A2/K$^b$ | P815-KKA‖ H-2$^d$, K$^b$/A2 | P815-K$^b$ H-2$^d$, K$^b$ | P815-D$^b$ H-2$^d$, D$^b$ | P815-K$^b$D$^b$ H-2$^d$, K$^b$, D$^b$ |
| | $\beta_2$m +/− | EL-4 | 2, 0 | 18, 2 | 19, 16 | 12, 2 | 11, 5 | 28, 6 |
| | $\beta_2$m −/− | EL-4 | 4, 0 | 16, 3 | 50, 20 | 39, 13 | 42, 12 | 71, 32 |

* Effectors were generated by in vivo priming, followed by in vitro restimulation.
‡ The murine MHC class I genotype of the target cell is indicated.
§ Percent specific lysis in a Cr release assay. E/T ratios were: 40:1, 8:1 (Exp. 1) and 1.6:1, 0.32:1 (Exp. 2).
‖ Chimeric H-2K$^b$/HLA-A2 molecule.

Given the fact that $\beta_2$m −/− (H-2$^b$) CTL cross-react on H-2$^b$/$\beta_2$m–expressing cells, one would suspect specificity for H-2$^b$ in the $\beta_2$m −/− CD8$^+$ spleen cell population in general. We tested if the $\beta_2$m −/− spleen cells would in fact respond in a primary MLC against $\beta_2$m-positive H-2$^b$ stimulator cells. For this we used $\beta_2$m −/− mice that had been backcrossed to B6 (H-2$^b$) for five generations ($\beta_2$m −/− B6 bc.5). $\beta_2$m −/− B6 bc.5 spleen cells generated almost no cytotoxic response when stimulated with irradiated DBA (H-2$^d$) spleen cells, which is in line with previously published data (13). However, a strong H-2$^b$–specific response developed when $\beta_2$m −/− B6 bc.5 spleen cells were stimulated with B6. This response was MHC class I specific since EL-4, but not C4.4-25$^-$, was killed. Furthermore, P815 (H-2$^d$) was resistant while P815-K$^b$D$^b$ was killed (Table 6). In reverse, normal B6 spleen cells generated a strong cytotoxic response to DBA but not to syngeneic B6 spleen cells. This indicates that the frequency of T cells in $\beta_2$m −/− mice with specificity for self-MHC class I/$\beta_2$m is higher than that for allogeneic MHC class I/$\beta_2$m, whereas the situation is reversed in the $\beta_2$m-expressing mice.

*Absence of H-2$^b$–specific Cross-reactivity among $\beta_2$m −/− CTL Maturing in a $\beta_2$m-positive Host.* We finally asked whether the cross-reactive response to self-MHC class I molecules was intrinsic to $\beta_2$m −/− CTL, or if it was influenced by other host cells. We derived bone marrow chimeras by reconstituting lethally irradiated B6 (H-2$^b$) mice with $\beta_2$m −/− bone marrow cells. Analysis of spleen cells from these chimeras revealed that <5% $\beta_2$m-positive cells were present (data not shown). The $\beta_2$m −/− bone marrow almost fully reconstituted the CD8 compartment, representing 8–10% of all spleen cells, compared with $\beta_2$m +/− mice that contain up to 15% CD8$^+$ spleen cells. This implies that normal MHC class I expression on CD8$^+$ T cells themselves is not needed for their development, as previously observed in reference 28. After 8 wk, these mice, as well as $\beta_2$m −/− and $\beta_2$m +/− control mice, were immunized with P815 (H-2$^d$), and tested as described above for $\beta_2$m −/− mice. In vitro restimulation of spleen cells from chimeric mice generated CTL that killed the specific target, P815, as did $\beta_2$m +/− CTL. The effector cells from the chimeric mice were H-2K$^b$ and H-2D$^b$ negative (Table 7). In contrast to CTL from $\beta_2$m −/− control mice, $\beta_2$m −/− CTL that had matured in $\beta_2$m-positive hosts did not kill EL-4 cells, suggesting that developmental events regulate the self-reactivity of $\beta_2$m −/− CTL.

## Discussion

Our results demonstrate that strong CTL responses can be induced in $\beta_2$m −/− mice by in vivo priming and subsequent in vitro restimulation with allogeneic cells. This is in line with a recent report (18) demonstrating that CTL can be induced in the peritoneal exudate of $\beta_2$m −/− mice by intraperitoneal inoculation of tumor cells. In addition, our results provide: (*a*) a clear demonstration, through the use of MHC class I–transfected (MHC class II negative) cells, that CTL in $\beta_2$m −/− mice can specifically recognize MHC class I molecules, and (*b*) evidence that some allospecific $\beta_2$m −/− CTL cross-react specifically on $\beta_2$m-positive cells expressing self-MHC class I.

There are at least three different explanations for the existence of functional CD8$^+$ CTL in $\beta_2$m −/− mice: (*a*) they do not arise from a CTL precursor pool present in the mice initially, but are rather induced from immature CD8$^+$CD4$^+$ cells by the priming procedure; (*b*) they represent CD8$^+$ cells that have matured and survived by chance, in a stochastic process independent of positive selection by MHC class I molecules; and (*c*) they represent a pool of CD8$^+$ CTL precursors selected on low ligand density of H-2$^b$–free heavy chains. We believe our data support the last explanation, for reasons outlined in the following discussion.

The current models of T cell selection are based on the idea that TCRs with too high affinity for self-MHC are negatively selected, while those with low to intermediate affinity are positively selected (29, 30). There is evidence for a role of MHC-bound peptides in positive as well as negative selection, even if these processes are not completely understood (31, 32). However, TCR affinity to MHC plus peptide is not the only determining factor; at the cellular level, total

**Table 6.** *Cytotoxic Response from $\beta_2$m −/− (H-2$^b$) Spleen Cells Stimulated with $\beta_2$m$^+$ (H-2$^b$) Cells without In Vivo Priming*

| Effector cells* | | Target cells | | | |
|---|---|---|---|---|---|
| Responder | In vitro stimulation | EL-4 H-2$^b$‡ | C4.4-25$^-$ H-2$^b$, $\beta_2$m$^-$ | P815 H-2$^d$ | P815-K$^b$D$^b$ H-2$^d$,K$^b$,D$^b$ |
| B6 | DBA | 14, 16§ | 0, 0 | 51, 41 | 66, 48 |
| $\beta_2$m −/− B6 bc.5‖ | DBA | 18, 10 | 0, 0 | 6, 4 | 20, 6 |
| $\beta_2$m −/− B6 bc.5 | B6 | 47, 35 | 0, 0 | 16, 6 | 51, 28 |

* 80 × 10$^6$ $\beta_2$m −/− B6 bc.5 or B6 spleen cells were stimulated in vitro for 5 d with 25 × 10$^6$ B6 or DBA spleen cells.
‡ The MHC class I genotype is indicated below the name of the target cell.
§ Percent specific lysis in a $^{51}$Cr release assay. E/T ratio: 80:1, 16:1.
‖ $\beta_2$m −/− (H-2$^b$) mice backcrossed five times to B6.

**Table 7.** *Specificity of Effectors from $\beta_2m^+$ Chimeric Mice Reconstituted with $\beta_2m$ −/− Bone Marrow*

| Effector cells* | | | Target cells and effector cell treatment‡ | | | |
|---|---|---|---|---|---|---|
| | | | P815 | | EL-4 | |
| Responder mouse | Priming in vivo | Restimulation in vitro | C'§ | HB51 + C' | C' | HB51 + C' |
| $\beta_2$m +/− | P815 | P815 | 73, 69‖ | 10, 4 | 0, 0 | 6, 2 |
| $\beta_2$m −/− | P815 | P815 | 58, 49 | 72, 57 | 27, 19 | 35, 19 |
| $\beta_2$m −/−: B6¶ | P815 | P815 | 46, 38 | 50, 32 | 0, 0 | 0, 0 |

* Effector cells generated as described in Materials and Methods.
‡ The effector cell population was depleted from MHC class I–expressing cells by HB51 and complement. The EL-4 and P815 target cells were of H-$2^b$ and H-$2^d$ haplotype, respectively.
§ Effector cell treatment.
‖ Percent specific lysis in a $^{51}$Cr release assay. E/T ratio: 40:1, 8:1.
¶ Bone marrow chimera generated by inoculation of 5 × $10^6$ $\beta_2$m bone marrow cells into 950-rad irradiated B6 mice.

avidity is also influenced by the density of the selecting ligand and by accessory molecules such as CD4 and CD8 (33). This predicts that a dramatic reduction in the density of ligand on the selecting cells should readjust the selection window such that there would be a reduced probability of finding T cell precursors with high enough avidity for positive selection. At the same time there would be a reduced probability for negative selection of T cells, including those expressing TCR with considerable affinity to self-MHC. These would escape elimination due to low total avidity. In other words, low MHC class I expression during T cell education would reduce the number of selected $CD8^+$ cells, with the concomitant possibility that the few cells that are selected might cross-react specifically on self-MHC class I when these molecules are expressed at normal ligand density. In contrast to the models based on total absence of $CD4^-CD8^+$ T cells, or a randomly leaky, nonselected $CD4^-CD8^+$ T cell repertoire in $\beta_2$m −/− mice (explanations *a* and *b* above), this model predicts a bias for self-MHC class I of the CTL repertoire in $\beta_2$m −/− mice. This is in line with what we observed in terms of H-$2^b$ cross-reactive responses. It must then be assumed that low levels of free MHC class I heavy chains are used in positive and negative selection processes in $\beta_2$m −/− mice. This is well in line with available data showing that low levels of MHC class I free heavy chains indeed can be expressed on cells of $\beta_2$m −/− mice (10, 11). It is important to remember that as little as 200 MHC class I/peptide complexes on the surface of a target cell may trigger CTL lysis (34), and it may be that comparable numbers of free MHC class I heavy chains can trigger selection signals. Note that the $CD8^+$ T cells of $\beta_2$m −/− mice were not generally cross-reactive, but showed a bias for H-$2^b$. In addition, this required MHC class I expression at normal levels with $\beta_2$m; the mice were tolerant to $\beta_2$m −/− cells of H-$2^b$ genotype, although these transport heavy chains to the cell surface that can be recognized by $CD8^+$ CTL with allospecificity for H-$2K^b$ or H-$2D^b$ (10, 11). These observations suggest that the small pool of $CD8^+$ T cells in these mice does not just represent random "leaking," but rather the consequence of negative and positive selection of TCR on low levels of free H-$2^b$ MHC class I heavy chains. The determination of CD4 vs. CD8 phenotype of the T cells may still be based on a partly stochastic and partly selective process, as discussed recently (35, 36).

We suggest that in relation to self-MHC class I expressed with $\beta_2$m at normal density, the skewed T cell selection window of $\beta_2$m −/− mice has neglected a lot of the "useful" clones, while it failed to eliminate and rather positively selected the "harmful" ones (37). The repertoire is however only harmful in relation to normal levels of class I molecules, and adequate in relation to the $\beta_2$m −/− self. $\beta_2$m −/− CTL could thus distinguish between allo and self-MHC class I in $\beta_2$m −/− cells, but not when expressed at normal ligand density in $\beta_2$m-positive cells. The importance of ligand density is illustrated by the reduced sensitivity of RMA-S compared with RMA and also by the enhanced sensitivity of RMA-S after incubation at reduced temperature or in the presence of H-2–binding peptides.

In addition to effects of low ligand density, there could also be qualitative effects due to the reduced ability of free MHC class I heavy chains on selecting cells in $\beta_2$m −/− to present and tolerize for specific self-peptides (including peptides derived from the $\beta_2$m molecule itself). $CD8^+$ T cells specific for self-peptides might thus escape elimination, which would contribute to the cross-reactivity on cells expressing H-$2^b$ class I molecules in association with $\beta_2$m and peptide. It has been reported that CTL reactive against self-peptides can be detected in the periphery of normal mice, only being reactive when target cells express an unphysiologically high density of a peptide/MHC complex, i.e., when they are supplied with large amounts of peptides exogenously (38). In our system, however, the reactivity of $\beta_2$m −/− CTL towards peptide loading–deficient RMA-S, T2-$K^b$, and T2-$D^b$ cells argues against this qualitative model as the only explanation for the data. The data rather suggest that TCR affinity for self-MHC class I of $\beta_2$m −/− CTL can be peptide independent. This is important for understanding of TCR–MHC class I interactions as well as the positively selected

T cell repertoire. However, even if the $\beta_2$m −/− CTL have TCRs capable of binding to empty H-2K$^b$ and H-2D$^b$ molecules, this does not necessarily mean that they have been selected on such molecules. If specific self-peptides are involved in positive selection, one would predict that these peptides should dramatically increase the killing of H-2$^b$ targets by the $\beta_2$m −/− CTL.

The observations on chimeric mice show that the anti-self-MHC crossreactivity is not a consequence of the $\beta_2$m/MHC class I–deficient status of the CTL themselves. After maturing in the presence of normal $\beta_2$m-positive cells, they behaved as normal CTL. This could be due to the influence of radioresistant nonhematopoetic cells, or of equally radioresistant bone marrow–derived cells of host type. Cells of the latter type are considered responsible for negative selection in chimeras studied within a couple of months of reconstitution (29).

How can these results and this model be reconciled with the initially reported absence of CD8$^+$ T cells and CTL responses in $\beta_2$m −/− mice? Scrutiny of the previously published data reveals CD8 staining of 1–2% of the spleen cells in such mice. This may be considered close to background, especially if a modest number of cells analyzed yield only a few uncertain dots in a FACS® plot. It may however represent 1–3 × $10^6$ cells in the spleen only, and a mere 10–25-fold reduction in number compared with wild type mice. This is a small reduction, considering that the frequency of precursors for H-2–restricted responses against viral or minor histocompatibility antigens are 20–200-fold lower than the frequency of allo H-2–specific precursors (39). Yet, the former give rise to strong in vivo responses capable of clearing virus infections and rejecting millions of tumor cells. They also yield strong cytotoxic responses in vitro, although these show a strict requirement for in vivo priming, just as the allospecific responses in the present study of $\beta_2$m −/− mice. We conclude that the search for CD8$^+$ cells and of CTL responses in immunodeficient mice should include FACS® analysis of a large number of cells as well as an in vivo priming step in functional studies. According to this view, the status of MHC class I–restricted cells in several gene knock out mice may still offer some surprises. There is for example an interesting possibility of a similar skewed repertoire selected on low levels of empty class I molecules in TAP1-deficient mice (40).

It is possible that the relatively "normal" phenotype and immune resistance of $\beta_2$m −/− mice partly depends on free MHC class I heavy chains substituting for complete MHC class I complexes, thus generating a skewed CTL repertoire whose responses may not always be detected in the conventional in vitro assays. Although we failed to generate $\beta_2$m −/− CTL using $\beta_2$m-deficient cells for priming in one combination, this needs further testing under conditions of adequate costimulation conditions, e.g., during the inflammatory response during a virus infection. Only CD4$^+$ CTL have been reported in $\beta_2$m −/− infected mice so far (17). Note that $\beta_2$m −/− CTL could kill $\beta_2$m-deficient targets when they had been induced by $\beta_2$m-positive allogeneic priming cells (Table 4).

One reflection emerging from the proposed model concerns the flexibility of the immune system as the T cell precursor repertoire meets the selection machinery of the host and adapts to perform optimally in this environment. Even if the latter is MHC class I deficient as perceived by FACS®, T cells apparently detect enough to go through the selection procedure, illustrating the fine tuning required when setting the limits of avidity for positive and negative selection windows. Finally, it is a common view that positive selection must bias the repertoire for binding to self-MHC irrespective of antigen, but this has been difficult to demonstrate. Even if conventional T cells can show a heteroclitic response on third party targets, they do not kill self (25). Our results provide a demonstration of T cell recognition of self–class I products, even when expressed in peptide loading–deficient cells. This system may serve as a tool to further analyze the self-MHC bias of the T cell repertoire, a somewhat unexpected development for the first "MHC deficient" mice.

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