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MHC class I expression and CD8$^+$ T cell development in TAP1/β_2-microglobulin double mutant mice

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Abstract

We have bred to homozygosity gene disruptions for the transporter associated with antigen processing 1 (TAP1) and β_2-microglobulin (β2m), each of which plays a distinct role in providing class I MHC subunits. Surface expression of H-2K^b or D^b on cells derived from TAP1/β2m –/– mice was undetectable by immunofluorescence or immunoprecipitation, unlike the situation observed for TAP1 –/– and β2m –/– single mutant mice. Yet, TAP1/β2m –/– cells were able to elicit a CD8$^+$ cytotoxic T cell (CTL) response in mice of different H-2 haplotypes and could be killed by anti-H-2^b specific CTL. Furthermore, TAP1/β2m –/– skin grafts were rejected by bm1 mutant mice. This suggests that very low levels of conformed class I heavy chains can reach the cell surface even in the complete absence of TAP1 and β2m gene products, and that these molecules may select a functional CD8$^+$ T cell repertoire. Indeed, CD4$^-$CD8$^+$ T cells were detected in TAP1/β2m –/– mice, but in numbers lower than in either of the single mutant mice. Nonetheless, it was possible to elicit a CD8$^+$ allospecific and H-2^b reactive CTL response in TAP1/β2m –/– mice. In line with this, TAP1/β2m –/– mice rapidly rejected TAP1/β2m +/– skin grafts. Our results suggest that some MHC class I heavy chains in TAP1/β2m –/– cells can reach the cell surface in a form that allows recognition by allospecific CTL and positive selection of CD8$^+$ T cells.

Introduction

Class I molecules of the MHC are expressed on the cell surface of almost all nucleated mammalian cells. Their function is to transport and present peptides, derived from intracellularly degraded proteins, to cytotoxic T cells (CTL) (1–3). They are also required for proper maturation and selection of CD8$^+$ T cells (4–6; for a review see 7).

MHC class I molecules consist of a highly polymorphic membrane-spanning heavy chain of ~45 kDa that is non-covalently associated with a light chain, β_2-microglobulin (β2m) (8). Class I molecules bind peptides, usually 8–11 amino acids in length. A majority of the class I bound peptides are generated in the cytosol (for a review see 3) and are subsequently translocated into the lumen of the endoplasmic reticulum (ER) through the ATP-dependent transporter associated with antigen processing 1/2 (TAP1/2) (9–11). Peptide as well as β2m are generally considered necessary for stable cell surface expression of the class I heavy chain. Cells lacking either TAP gene products or β2m are largely devoid

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of normal cell surface class I molecules (for a review see 12).

The generation and analysis of β2m −/− and TAP −/− mice have allowed studies of the biological effects of class I deficiency *in vivo*. However, neither a TAP deficiency nor a β2m deficit is sufficient to ablate expression of H-2K^b or D^b class I molecules at the cell surface. Cells devoid of β2m express a limited number of free H-2D^b class I heavy chains (6,13–15), at least some of which are occupied by peptide (15,16). Recent studies have also revealed expression of very low levels of free H-2K^b class I heavy chains on the cell surface of β2m −/− cells (16). Cells devoid of TAP gene products express reduced levels of class I heavy chains complexed with β2m, either devoid of peptide or occupied by peptides that bind to class I molecules independently of the TAP complex (17–20). Both TAP and β2m deficient cells are recognized by allospecific CTL (14,15,21–23).

As a consequence of reduced levels of class I molecules at the cell surface, and thus the absence of normal ligands involved in T cell selection, TAP1 −/− and β2m −/− mice have few but detectable levels of mature CD8$^+$ T cells (5,6,20,24–26). Nonetheless, in β2m −/− and TAP1 −/− mice these residual CD8$^+$ T cells respond vigorously to allogeneic lymphocytes and tumor cells (24–29). These results prompted us to explore whether a combined deficiency of TAP1 and β2m would lead to a more severe class I deficient phenotype than the one observed in either of the single mutant mice, in particular with respect to development of CD8$^+$ T cells as well as their ability to be recognized by CTL.

To assess class I expression and function in the absence of both TAP1 and β2m, we crossed TAP1 −/− mice with β2m −/− mice to generate TAP1/β2m double mutant mice. Surface expression of H-2K^b or D^b on cells derived from TAP1/β2m −/− mice was undetectable by immunofluorescence or immunoprecipitation. Nonetheless, CTL reactivity against cells devoid of both TAP1 and β2m expression was demonstrable. Our data indicate that CD8$^+$ T cells can be selected on low levels of conformed free class I heavy chains in the thymic microenvironment of TAP1/β2m −/− mice, notwithstanding the severe reduction in class I expression.

Methods

Mice

The generation of TAP1 −/− and β2m −/− mice (H-2^b background) has been described in detail (5,20). In order to generate TAP1/β2m double mutant mice (designated TAP1/β2m −/−), TAP1 −/− and β2m −/− mice were crossed, and offspring subsequently intercrossed, to produce mice with a homozygous mutation for both the TAP1 and β2m genes. Control mice heterozygous for the TAP1 and β2m gene mutations were generated from the same crosses or by mating TAP1/β2m −/− mice with C57BL/6 (B6) mice. Mice were typed by PCR and Southern blotting. B6 (H-2^b), 129/Sv (H-2^b), BALB/c (H-2^d), C3H (H-2^k), A.SW (H-2^s), B10.A(2R) (H-2K^{k}D^b), B10.A(4R) (H-2K^{k}D^b), B10.A(5R) (H-2K^{b}D^d) and B6.C-H-2^{bm1} (designated bm1, H-2K^{bm1}D^b) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). MHC class II mutant mice (30) were generously provided by Drs C. Benoist and D. Mathis (CNRS, INSERM, Strasbourg, France). β2m mutant mice were generously provided by Dr B. H. Koller (University of North Carolina, Durham, NC), via Dr D. Roopenian (Jackson Laboratory). All mice used were at the age of 4–10 weeks, usually littermates or otherwise age matched within 2 weeks in each experiment. All mice were maintained at the Animal Department, Center for Cancer Research, Massachusetts Institute of Technology (Cambridge, MA) except mice used for skin grafting which were maintained at the Transplantation Unit of the Surgical Services, and Department of Surgery and Pathology, Massachusetts General Hospital (Boston, MA). Animal care was in accordance with institutional guidelines.

Cell lines and generation of Con A activated T cell blasts

EL-4 is a thymoma line of C57BL/6 (H-2^b) origin. P815 is a mastocytoma of DBA/2 (H-2^d) origin. T2K^b is an H-2K^b transfectant of the human antigen processing defective mutant T2 (.174XCEM) (31,32). T2 and T2K^b cell lines were a kind gift from Dr P. Cresswell (Yale University School of Medicine, New Haven, CT). All cell lines were maintained in RPMI tissue culture medium supplemented with 10% FCS, penicillin–streptomycin, and L-glutamine (complete tissue culture medium). All target cell lines used in cytotoxic assays, except the cell lines described above, were Con A activated T cell blasts (Con A blasts). For generation of Con A blasts, single cell suspensions of splenocytes (~40×10^6 cells) were cultured in 30 ml complete tissue culture medium supplemented with 10% FCS, antibiotics and 2.5 μg Con A (Sigma, St Louis, MO)/ml for 48 h.

FACS analysis

For FACS analysis of CD4 and CD8 positive T cell subsets in peripheral blood, ~50 μl of tail vein blood was mixed with 10 μl 0.5 M EDTA (pH 8) and incubated with mAb for 30 min at room temperature. Then, 1 ml FACS brand lysis solution (Becton Dickinson, San Jose, CA), diluted 1:10 according to the manufacturer's protocol, was then added to the cells to lyse erythrocytes. Cells were then washed twice in PBS and 10,000 viable cells were analyzed using a FACScan flow cytometer (Becton Dickinson). Antibodies used for the flow cytometric analysis were: FITC-conjugated anti-CD8 (53-6.7; PharMingen, San Diego, CA) and phycoerythrin (PE)-conjugated anti-CD4 (RM4-5; PharMingen). Both antibodies were tested at a dilution of 1:200.

For FACS analysis of class I expression, 10^6 Con A blasts (generated in complete medium supplemented with 10% FCS, see below) were incubated with 100 μl of the following mAb: 28-14-8S, anti-D^b (α-3), tissue culture supernatant used in a 1:1 dilution, obtained from ATCC (Rockville, MD); B22.249, anti-D^b (α-1), tissue culture supernatant used in a 1:1 dilution, a kind gift from Dr G. J. Hämmerling (German Cancer Research Center, Heidelberg, Germany); KH95, anti-D^b, used in a 1:100 dilution, obtained from PharMingen; AF6-88.5, anti-K^b, used in a 1:100 dilution and obtained from PharMingen; Y3, anti-K^b (α-1 and -2), tissue culture supernatant used in a 1:1 dilution, a kind gift from Dr H. N. Eisen (Massachusetts Institute of Technology, Cambridge, MA); H141-31-10, anti-K^b, ascites fluid used in a 1:500 dilution, obtained from Accurate (Westbury, NY). Cells were then washed in PBS and directly analyzed by flow cytometry or incubated with appropriate secondary antibodies: FITC-labeled goat anti-

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mouse Ig (Southern Biotechnology Associates, Birmingham, AL) or streptavidin–PE (PharMingen) prior to FACS analysis. Dead cells were excluded from the analysis by staining with propidium iodide or based on the forward and sideways light scattering properties.

Cell surface iodination, immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF) analysis

Con A activated T cell blasts from TAP1/β2m +/−, β2m −/− and TAP1/β2m −/− splenocytes were prepared as described above. Prior to iodination, dead cells were removed by centrifugation with Lympholyte-M (Cedarlane, Hornby, Ontario, Canada) according to the manufacturer's protocol. Cells (10^7) were surface labeled with 1.0 mCi of $Na^{125}I$ (1 Ci = 37 GBq) in ice-cold PBS by lactoperoxidase-catalyzed iodination. Class I molecules were subsequently immunoprecipitated using a rabbit anti-mouse class I antiserum (a kind gift of Dr S. Nathenson, Albert Einstein College of Medicine, New York, NY), that preferentially reacts with H-2K^b on cells of the H-2^b haplotype. Immunoprecipitation and subsequent 1D-IEF was done essentially as described (33,34). Fluorography was accomplished by incubating the gels with 2,5-diphenyloxazol in DMSO prior to exposure to Kodak XAR-5 film.

Generation of CTL

Unless indicated otherwise, mice were immunized once i.p. with ~20×10^6 2500 rad irradiated spleen cells. At 1–2 weeks after immunization, responder spleen cells (50×10^6) were restimulated with 25×10^6 2500 rad irradiated spleen cells *in vitro* in complete tissue culture medium supplemented with 5×10^{-5}M 2-mercaptoethanol. After 5 days, the cells were used as effectors in a standard ^{51}Cr-release assay (see below).

Effector cell depletion

CD8$^+$ cells were depleted from effector cell populations by antibody and complement mediated cytolysis. Briefly, 1×10^7 effector cells were incubated on ice with 1 ml of a mixture of 20 μg/ml solutions of the anti-CD8 mAb YTS 156.7 and YTS 169.4, obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were then washed once and incubated with 2 ml of rabbit complement (Cedarlane), diluted 1:8 in complete tissue culture medium for 2 h at 37°C and 5% CO_2. Effective depletion of CD8 expressing cells was confirmed by FACS analysis, using anti-CD8 mAb (see above).

Cytotoxic assay

All cytotoxicity assays were performed in a standard 4 h ^{51}Cr-release assay. Titrated numbers of effector cells were tested against 10,000 ^{51}Cr-labeled target cells. Percent specific lysis was calculated according to the formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)]×100.

Skin grafting experiments

Skin grafting was performed essentially as described (35). Mice were anesthetized with chloral hydrate supplemented with ether and engrafted with donor trunk skin placed onto the thoracic area. Grafts were held in place for 7–9 days with vaseline gauze and plaster bandages. Rejection was scored when >90% destruction of the tissue had occurred. All groups consisted of between five and nine mice. When multiple experiments were performed, data from individual experiments were pooled.

Results

Generation of TAP1/β2m −/− mice

TAP1/β2m double mutant (−/−) mice (H-2^b background) were generated by crossing TAP1 −/− with β2m −/− mice, which generated TAP1/β2m heterozygous (+/−) litters. These were then intercrossed and the expected number of TAP1/β2m −/− offspring was obtained. TAP1/β2m −/− mice appear healthy and have no gross anatomical deficiencies. The mice are fertile and give rise to normal litter sizes.

MHC class I expression

Surface expression of either H-2K^b or D^b was undetectable when TAP1/β2m −/− cells were analyzed with a panel of well characterized anti-H-2K^b or D^b mAbs by FACS (Table 1). Notably, even epitopes detected on the cell surface of β2m −/− cells by the anti-D^b mAbs 28-14-8s (α-3 specific) and B22.249 (α-1 specific) were absent from TAP1/β2m −/− cells. This pattern applied whether fresh TAP1/β2m −/− splenocytes or Con A activated T cell blasts were analyzed. Preincubation of TAP1/β2m −/− Con A activated T cell blasts at 26°C for 24 h did not induce any detectable levels of class I expression with any of the anti-H-2K^b or D^b mAb tested (data not shown), while the same treatment dramatically increased surface class

Table 1. MHC class I expression on the cell surface of TAP1/β2m +/−, TAP1 −/−, β2m −/− and TAP1/β2m −/− cells

		Con A activated T cell blasts			
mAb	reactivity	TAP1/β2m +/−	TAP1 −/−	β2m −/−	TAP1/β2m −/−
28-14-8s	H-2D^b	161[a]	17	4	0
B22.249	H-2D^b	418	34	20	1
KH95	H-2D^b	167	9	0	0
AF6-88.5	H-2K^b	293	32	0	0
Y3	H-2K^b	103	34	0	0
H141-31-10	H-2K^b	207	16	0	0

[a]Mean fluorescence value as measured by FACS.

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I expression of TAP-deficient cells and increased class I expression on β2m −/− cells to some extent (16,20,36). We could also not detect any H-2K^b class I molecules on the cell surface of TAP1/β2m −/− splenocytes after cell surface iodination and subsequent immunoprecipitation using a polyclonal rabbit anti-mouse class I serum, that preferentially reacts with H-2K^b molecules on cells of the H-2^b haplotype. In contrast, such analysis revealed expression of H-2K^b on the cell surface of β2m −/− as well as on B6 or TAP1/β2m +/− control cells (Fig. 1, data not shown). This indicated that MHC class I expression on the cell surface of TAP1/β2m −/− cells was significantly below that of TAP1 −/− as well as β2m −/− cells.

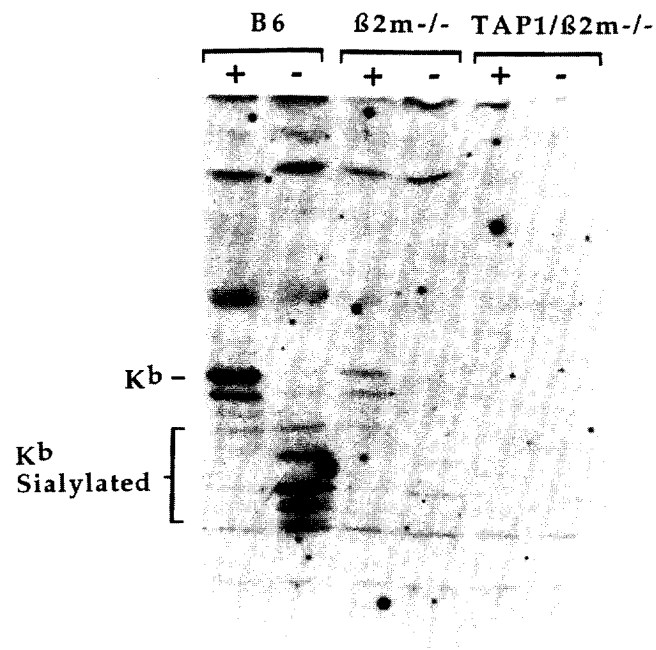

Fig. 1. TAP1/β2m −/− cells are deficient in cell surface H-2K^b expression. Con A activated splenocytes prepared from B6, β2m −/− and TAP1/β2m −/− cells were surface iodinated, followed by immunoprecipitation of H-2K^b. The immunoprecipitates were either digested with neuraminidase (+) or kept on ice (−) prior to analysis by 1D-IEF. The polypeptide close to the cathode, present in precipitates from B6 and β2m −/− mice but absent from TAP1/β2m −/− precipitates represents bovine β2m, which readily binds free class I heavy chains in the course of generation of the Con A activated T cell blasts cultured in the presence of FCS for 48 h (41).

Ability of TAP1/β2m −/− cells to elicit a CD8⁺ T cell response

Are TAP1/β2m −/− cells totally devoid of H-2K^b and D^b expression at the cell surface? Even though class I expression was undetectable on the cell surface utilizing either FACS or biochemical methods, results from functional assays strongly suggest that expression of class I molecules occurs in a fashion capable of being recognized by CD8⁺ T cells.

We immunized BALB/c mice (H-2^d) with splenocytes from TAP1/β2m −/− and control mice, and re-stimulated effectors in mixed lymphocyte cultures. Surprisingly, effector cells from BALB/c mice immunized with TAP1/β2m −/− cells elicited a weak response against EL-4 and TAP1/β2m +/− Con A blast targets. However, the response observed was significantly weaker than that obtained with effectors derived from BALB/c mice immunized with either TAP1 −/− or β2m −/− single mutant cells (Table 2). Nonetheless, this preliminary analysis suggested that TAP1/β2m −/− cells might be able to elicit a CTL response in allogeneic mice.

We therefore immunized an extended panel of mice of different H-2 haplotypes with TAP1/β2m −/− cells and B6 (or TAP1/β2m +/−) control cells to assess their ability to elicit a CTL response. Effector cells generated from BALB/c (H-2^d), C3H (H-2^k), A.SW (H-2^s) and B10.A(4R) (H-2K^{k}D^b) mice, immunized with TAP1/β2m −/− cells, were able to elicit a cytotoxic response against TAP1/β2m +/− target cells which in some experiments reached 50% specific lysis in the highest effector to target ratios (Table 3; for A.SW, data not shown). However, similar levels of lysis were obtained with effectors from the same strain of mice immunized with B6 splenocytes at effector to target cell ratios that were significantly lower (~100-fold lower). Effector cells generated from mice immunized with TAP1/β2m −/− cells were CD8⁺. Treatment of effector

Table 2. Ability of TAP1/β2m +/−, TAP1 −/−, β2m −/− and TAP1/β2m −/− cells to elicit a CTL response in BALB/c mice

Targets	E:T ratio	Effectors			
		BALB/c anti-TAP1/β2m +/− (%)	BALB/c anti-TAP1 −/− (%)	BALB/c anti-β2m −/− (%)	BALB/c anti-TAP1/β2m −/− (%)
EL-4	100:1	73[a]	67	31	14
	33:1	77	56	19	11
	11:1	74	40	9	7
	4:1	69	18	4	3
	1:1	47	7	1	2
	0.4:1	26	3	1	2
TAP1/β2m +/−	100:1	81	67	24	6
	33:1	85	53	13	3
	11:1	83	28	5	5
	4:1	66	12	0	4
	1:1	46	4	0	4
	0.4:1	24	0	0	0

[a]Percent specific lysis.

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Table 3. Ability of TAP1/β2m −/− cells to elicit an immune response in different allogeneic and recombinant inbred mice

Targets	E:T ratio	Effectors BALB/c TAP1/β2m −/− (%)	anti-B6 (%)	C3H TAP1/β2m −/− (%)	anti-B6 (%)	B10.A(4R) TAP1/β2m (%)	anti-B6 (%)
TAP1/β2m +/−	33:1	53	80	49	79	40	78
	11:1	41	75	38	82	32	74
	4:1	26	61	24	80	12	68
	1:1	10	50	15	69	6	50
	0.4:1	8	19	13	51	9	42
TAP1/β2m −/−	33:1	27	4	39	20	21	14
	11:1	18	0	36	19	16	16
	4:1	13	0	25	15	10	12
	1:1	10	0	15	13	0	0
	0.4:1	7	0	10	0	0	0

cells with anti-CD8 plus complement completely abrogated the cytotoxic response, whereas complement alone had no effect (Fig. 2).

Taken together, these results suggest that some MHC class I heavy chains are able to reach the cell surface of TAP1/β2m −/− cells, where they can elicit a class I specific response mediated by $CD8^+$ CTL.

Ability of TAP1/β2m −/− cells to function as targets for allospecific CTL

Given the fact that TAP1/β2m −/− splenocytes were able to elicit a $CD8^-$ CTL response in mice, we asked whether such cells could function as targets for allospecific CTL *in vitro*. CTL from BALB/c, C3H, B10.A(4R) and B10.A(5R) (H-$2K^bD^d$) mice, immunized with B6 or TAP1/β2m −/− cells, were tested against TAP1/β2m −/− derived Con A activated T cell blasts. When BALB/c mice were primed and effectors re-stimulated with B6 cells, they only elicited a very weak response against TAP1/β2m −/− targets (Table 3). Similar results were observed with effectors from B10.A(5R) mice (data not shown). However, effectors derived from C3H and B10.A(4R) mice immunized and re-stimulated with B6 cells generally elicited stronger responses against TAP1/β2m −/− targets (Table 3). Even when significant levels of lysis were observed against TAP1/β2m −/− targets, such targets were killed less efficiently than TAP1/β2m +/− targets. Interestingly, when mice were immunized, and effector cells re-stimulated with TAP1/β2m −/− cells, they usually elicited a stronger response towards the TAP1/β2m −/− Con A blasts than did effectors derived from mice immunized and re-stimulated with B6 cells (Table 3). Targets of haplotypes other than H-2^b were not killed under these conditions (data not shown).

Taken together, these results demonstrate that TAP1/β2m −/− cells can serve as targets for allospecific CTL, and that CTL primed and re-stimulated with TAP1/β2m −/− cells elicit a stronger response against TAP1/β2m −/− targets than do CTL generated against B6 cells. The observation that different strains of mice differ in their capacity to elicit CTL responses against TAP1/β2m −/− cells was not investigated further. It is possible that the phenomenon is related to the fact that the TAP1/β2m −/− mice are not inbred. Blocking studies with anti-class I specific antibodies may shed more light into the target structures that are being recognized on the TAP1/β2m −/− cells.

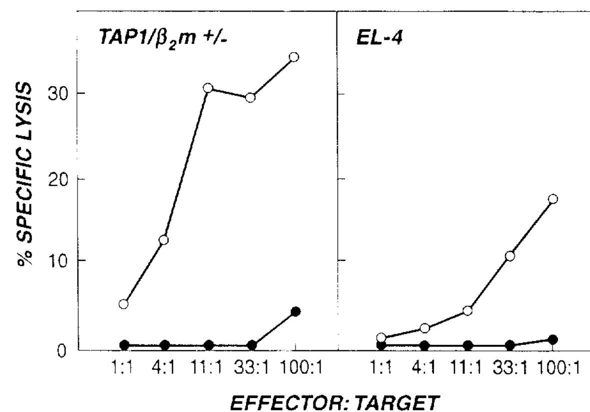

Fig. 2. CTL elicited in C3H mice after immunization with TAP1/β2m −/− cells are $CD8^+$. Effector cells from C3H anti-TAP1/β2m −/− mixed lymphocyte cultures were treated with C′ alone (open circles) or anti-CD8 mAb and C′ (filled circles) and tested against TAP1/β2m +/− and EL-4 target cells.

Table 4. Number of $CD4^-CD8^+$ and $CD4^+CD8^-$ T cells in peripheral blood of TAP1/β2m +/−, TAP1 −/−, β2m −/− and TAP1/β2m −/− mice

Mice	Percent CD4 and CD8 positive T cells $CD4^-CD8^+$	$CD4^+CD8^-$
TAP1/β2m +/−	11.2[a] (1.00)	22.5 (3.12)
TAP1 −/−	0.83 (0.24)	41.0 (12.6)
β2m −/−	0.20 (0.07)	38.3 (6.97)
TAP1/β2m −/−	0.15 (0.05)	38.3 (5.58)

[a]Mean percentage of positive cells in peripheral blood (n = 10; for TAP1 −/− mice n = 20). Figures in parentheses are SDs.

Development and function of $CD8^+$ T cells in TAP1/β2m −/− mice

The inference that TAP1/β2m −/− cells must express some conformed class I heavy chains at the cell surface raises the

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Table 5. Reactivity of anti-H-2^d allospecific T cells from TAP1/β2m −/− mice with H-2^d and H-2^b expressing cells

Targets	E:T ratio	Effectors	
		TAP1/β2m +/− anti-BALB/c (%)	TAP1/β2m −/− anti-BALB/c (%)
Experiment 1			
P815	40:1	82	85
	20:1	82	73
	10:1	76	59
	5:1	61	40
	2.5:1	52	31
	1.2:1	30	13
BALB/c	160:1	81	85
	80:1	79	61
	40:1	60	51
	20:1	51	30
	10:1	39	19
	5:1	29	2
Experiment 2			
P815	100:1	98	89
	33:1	84	68
	11:1	93	34
	4:1	83	15
	1:1	59	7
TAP1/β2m +/−	100:1	0	50
	33:1	0	48
	11:1	0	23
	4:1	0	8
	1:1	0	0
TAP1/β2m −/−	100:1	0	0
	33:1	0	0
	11:1	0	0
	4:1	3	0
	1:1	0	1

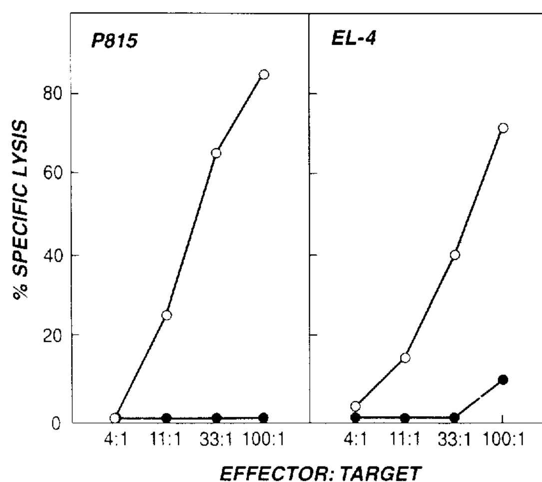

Fig. 3. Allospecific (anti-H-2^d) and anti-H-2^b reactive CTL in TAP1/β2m −/− mice are CD8⁻. Left panel: effector cells from TAP1/β2m −/− anti-BALB/c mixed lymphocyte cultures were treated with C′ alone (open circles) or anti-CD8 mAb and C′ (filled circles) and tested against P815 (H-2^d) target cells. Right panel: effector cells from TAP1/β2m −/− anti-B6 mixed lymphocyte cultures were treated with C′ alone (open circles) or anti-CD8 mAb and C′ (filled circles) and tested against EL-4 (H-2^b) target cells.

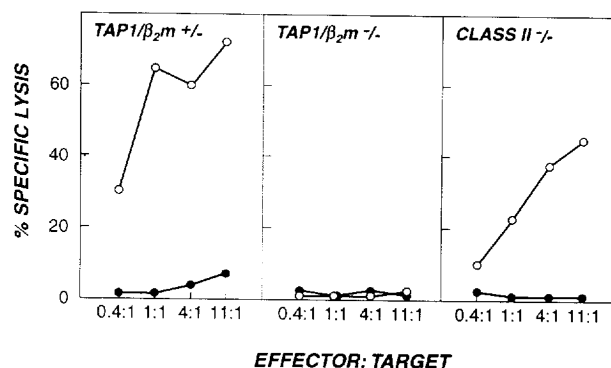

Fig. 4. Generation and target cell specificity of anti-H-2^b reactive CTL derived from TAP1/β2m −/− mice. Effector cells were from TAP1/β2m −/− (open circles) or control TAP1/β2m +/− (filled circles) anti-TAP1/β2m +/− mixed lymphocyte cultures. Effector cells from TAP1/β2m −/− mice readily killed class I H-2^b expressing TAP1/β2m +/− and class II −/− targets, but did not react with class I H-2^b deficient TAP1/β2m −/− targets.

possibility that some CD8$^+$ T cells may be selected in the thymus of TAP1/β2m −/− mice. Peripheral blood lymphocytes from TAP1/β2m −/− mice had very low numbers of CD8$^+$ T cells (Table 4). Levels were lower than those observed in either TAP1 −/− or β2m −/− mice. Nonetheless, TAP1/β2m −/− mice generated a surprisingly strong anti-H-2^d specific CTL response when mice were immunized, and effector cells re-stimulated with allogeneic BALB/c splenocytes (Table 5). The response was mediated by CD8$^+$ T cells. Depletion of CD8$^+$ T cells from TAP1/β2m −/− anti-BALB/c effectors by anti-CD8 mAb and complement abrogated all cytotoxic responses against H-2^d expressing cells (Fig. 3, left panel).

As has been observed for alloreactive CD8$^+$ T cells from the TAP1 −/− and β2m −/− single mutant mice (24,25), alloreactive TAP1/β2m −/− anti-BALB/c CD8$^+$ T cells cross-reacted with target cells expressing normal levels of H-2^b class I molecules (TAP1/β2m +/− targets), but not with cells devoid of normal class I H-2^b expression (TAP1/β2m −/− targets) (Table 5).

When immunized and re-stimulated with H-2^b expressing cells, TAP1/β2m −/− effectors also readily killed H-2^b expressing targets (B6, TAP1/β2m +/− or EL-4), while class I deficient (TAP1/β2m −/−) targets were not lysed (Fig. 4; see Discussion). All CTL activity was mediated by CD8$^+$ T cells (Fig. 3, right panel). Notably, the anti-H-2^b specific response generated in TAP1/β2m −/− mice was significantly stronger than that in TAP1 −/− mice and usually stronger than in β2m −/− mice (Table 6). Strong cytotoxicity was also observed against MHC class II −/− targets expressing normal levels of class I H-2^b molecules (Fig. 4), as well as against human T2 cells transfected with H-2K^b but not against non-transfected T2 cells (Fig. 5), demonstrating that the response was mainly directed against class I molecules. The latter experiment in particular implies that the target for CTL recognition is H-2K^b itself, unless it is assumed that a fragment of the K^b chain is presented by a structure that allows cross-reaction of the

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Table 6. Reactivity of anti-H-2^b specific T cells from TAP1/β2m −/− mice; comparison with T cells generated in TAP1 −/− and β2m −/− mice

Target	E:T ratio	Effectors			
		TAP1/β2m +/− anti-B6 (%)	TAP1 −/− anti-B6 (%)	β2m −/− anti-B6 (%)	TAP1/β2m −/− anti-B6 (%)
Experiment 1					
EL-4	33:1	6	59	80	81
	11:1	3	33	75	82
	4:1	0	15	64	68
	1:1	0	6	43	40
	0.4:1	0	2	19	14
	0.1:1	0	1	8	3
Experiment 2					
EL-4	11:1	7	21	86	99
	4:1	1	8	62	71
	1:1	0	2	27	41
	0.4:1	0	0	7	16
	0.1:1	0	0	0	2

murine CTL with targets of human and murine origin, a possibility we consider unlikely.

Skin grafting experiments

The *in vitro* studies predicted that TAP1/β2m −/− mice should be able to reject TAP1/β2m +/− skin grafts, and that class I positive mice of H-2 haplotypes other than H-2^b should be able to reject TAP1/β2m −/− skin grafts.

TAP1/β2m +/− male skin grafts were rapidly rejected by TAP1/β2m −/− mice. Complete rejection was observed within 14 days in all mice grafted, irrespective of the sex of recipient mice (Fig. 6A and B). This rejection course is consistent with the *in vitro* data showing that CTL from TAP1/β2m −/− mice reacted against TAP1/β2m +/− targets (Fig. 4) and with the rejection times of wild-type B6 skin by β2m −/− mice (37). In contrast, similar grafts were accepted by male TAP1/β2m +/− recipients (no rejection within 5 weeks), notwithstanding the fact that the mice ($[B6\times129]F_{5-10}$) had not been backcrossed to homogeneous background with respect to non-MHC genes.

Grafting male skin onto female recipients allowed the study of rejection responses by TAP1/β2m +/− mice to the H-Y minor histocompatibility antigen presented by either TAP1/β2m −/− or TAP1/β2m +/− mice (Fig. 6B). Rejection of TAP1/β2m +/− male skin by TAP1/β2m +/− female recipients occurred within 5 weeks with a mean survival time of 28 days, consistent with H-Y rejection responses by wild-type B6 mice (38). In contrast, TAP1/β2m −/− male skin was not rejected by TAP1/β2m +/− female recipients, when monitored over a period of 5 weeks. This indicated that, compared with TAP1/β2m +/− skin, TAP1/β2m −/− skin is less capable of eliciting an immune response in TAP1/β2m +/− female mice to the H-Y antigen.

While TAP1/β2m −/− male skin is accepted by minor antigen-disparate recipients such as TAP1/β2m +/− female mice, it is readily rejected by male bm1 mutant mice (K^b mutant but identical for H-$2D^b$ and class II MHC) (Fig. 6C). These data again support the *in vitro* cytotoxicity data, demonstrating alloreactive CTL may be able to respond to class I heavy chains expressed on the cell surface of TAP1/β2m −/− cells (Table 3), and are consistent with the rejection times seen in the rejection of class I disparate skin grafts (38,39).

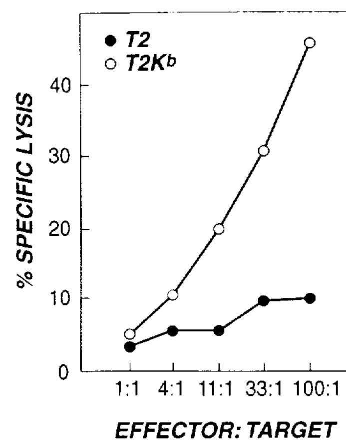

Fig. 5. Anti-H-2^b reactive CTL derived from TAP1/β2m −/− mice kill $T2K^b$ but not T2 cells. Effector cells were from TAP1/β2m −/− anti-B6 mixed lymphocyte cultures.

Discussion

The state of class I deficiency in the TAP1/β2m −/− mice is the most profound described thus far. In contrast to cells derived from either TAP1 −/− or β2m −/− mice, we could not detect expression of cell surface H-$2K^b$ or D^b on cells derived from TAP1/β2m −/− mice by immunofluorescence or biochemistry. Nonetheless, our results indicate that minute amounts of MHC class I heavy chains, with or without TAP1 independent peptides, are expressed on the cell surface of TAP1/β2m −/− cells. These cells elicited a $CD8^+$ T cell response in allogeneic mice and served as targets for allo-specific CTL *in vitro* and *in vivo*. Furthermore, TAP1/β2m mice were able to select some functional $CD8^+$ T cells. We therefore conclude that small numbers of properly conformed class I

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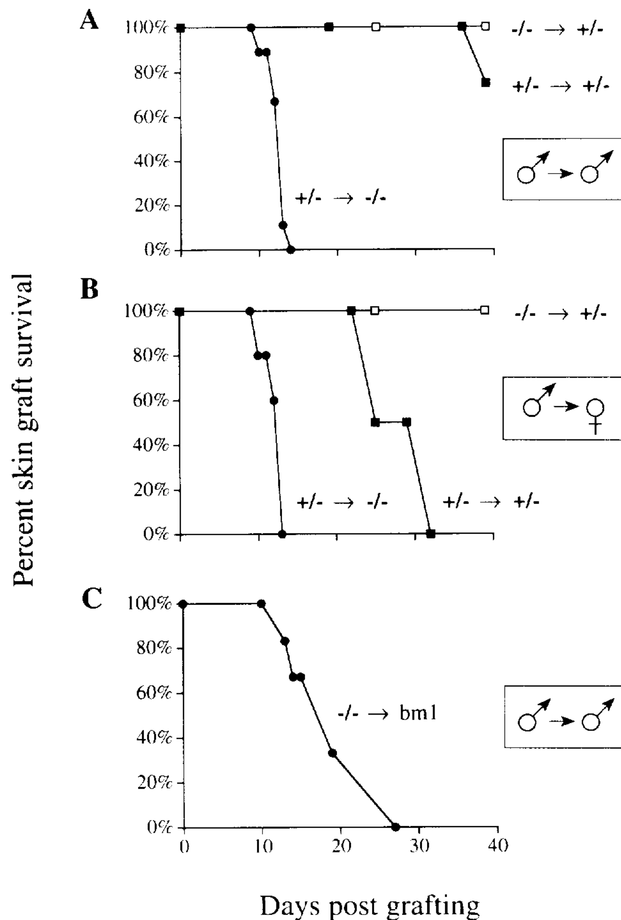

Fig. 6. Skin grafting experiments using TAP1/β2m +/− and TAP1/β2m −/− mice. (A) Male to male skin grafts. (B) Male to female skin grafts. (C) TAP1/β2m −/− male to bm1 male skin grafts. +/− represents TAP1/β2m +/− donors or recipients, −/− represents TAP1/β2m −/− donors or recipients.

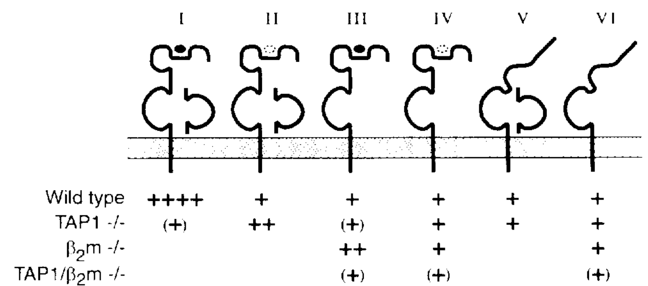

Fig. 7. Possible forms of MHC class I molecules on the cell surface of TAP1/β2m +/−, TAP1 −/−, β2m −/− and TAP1/β2m −/− cells. The grading (++++, ++, +, (+)) represents rough estimates of the relative levels of expression that may be expected for each genotype. The membrane-embedded heavy chain is represented in forms I–VI. β2m is present only in forms I, II and V. Forms I and III contain peptides (solid ellipsoid). Forms II and IV may either be empty or contain low affinity peptides (open ellipsoid) that can be easily replaced by high affinity peptides—the absence or presence of such peptides is not easily established experimentally (36). Properly conformed class I heavy chains are found in forms I–IV. The figure is meant to illustrate and partly simplify the reasoning in the discussion (adapted from 44).

chains are most likely responsible for the effects observed (Fig. 7; conformed heavy chains refers to forms I–IV, of which III and IV may exist on the cell surface of TAP1/β2m −/− cells).

It is not impossible that intracellular transport of free class I heavy chains is dependent on molecules other than β2m and/or TAP dependent peptides. For example, in T2 cells the interaction of H-2D^b molecules with human invariant chain is sufficiently stable to survive immunoprecipitation conditions (39). While we do not wish to suggest that murine invariant chain might be involved in surface expression of free class I chains, it is not far fetched to suggest that other membrane proteins serve as chaperones to transport free K^b or D^b chains to the cell surface. We also cannot exclude that (some of the) class I heavy chains may have bound exogenous β2m or peptide at the cell surface when mice were immunized with TAP1/β2m cells or when such cells were used as targets in cytotoxicity assays *in vitro*. Most cells produce β2m in excess over class I heavy chains and secrete the surplus. However, previous studies have demonstrated that β2m −/− targets can be killed by allospecific and class I restricted CTL even in the total absence of exogenous β2m (14), suggesting that class I heavy chains devoid of β2m may function as a recognition unit for CTL *per se*.

The ability of CTL, elicited in allogeneic mice by immunization with TAP1/β2m −/− cells, to kill targets expressing normal levels of H-2^b (TAP1/β2m +/− and EL-4) raises the possibility that these target cells may express conformed free heavy chains similar to those expressed on TAP1/β2m −/− cells (14,40,41). Alternatively, the CTL elicited against TAP1/β2m −/− cells may cross-react with properly conformed and assembled class I heavy chain–β2m complexes.

The indication that functional CD8⁺ T cells can be selected in TAP1/β2m double mutant mice suggests that free heavy chains, with or without TAP1 independent peptides, may be able to interact with the TCR and positively select CD8⁺ T cells in an environment totally devoid of β2m and TAP1-dependent peptides. Some of these CD8⁺ T cells in TAP1/β2m −/− mice might have been selected on I-A^b (class II) or non-classical class I molecules whose surface expression is less affected by TAP1 and/or β2m mutations. However, as has recently been discussed for T cell selection in TAP1 −/− or β2m −/− mice (24–26), several observations support the notion that selection occurs on class I H-2^b molecules as such. The number of CD8⁺ T cells correlates with levels of cell surface expressed class I molecules in TAP1 −/−, β2m −/− and TAP1/β2m −/− mice, while expression of I-A^b is not affected (unpublished results). Alloreactive CTL from TAP1 −/−, β2m −/− and TAP1/β2m −/− mice show a strong bias towards reactivity with H-2^b class I. Class II −/− target cells of the H-2^b haplotype are efficiently killed by such anti-H-2^b reactive CTL. Likewise, T2K^b but not T2 (both class II negative) cells are killed by such cells. On the other hand, it remains quite possible that positive selection of some CD8⁺ T cells is mediated by non-classical class I molecules in TAP1/β2m −/− mice.

The ability of residual CD8⁺ T cells in TAP1/β2m −/− mice to react with class I H-2^b molecules on normal H-2^b expressing

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cells, indicates that cells that harbor this reactivity have not been negatively selected (24,25). This could be a consequence of low ligand density of the negatively selecting molecules, as well as restrictions in the repertoire of peptides presented by class I molecules. This proposed lack of negative selection is particularly pronounced in TAP1/β2m −/− mice, as judged by their strong ability to react with H-2^b expressing cells (Table 6). One explanation for this may be that these mice have lower levels of class I molecules than compared with either of the single mutant mice. Yet, this low expression of class I molecules is apparently sufficient to positively select a limited pool of CD8$^+$ T cells that account for the cytotoxic reactivity observed. These results support the differential avidity model of T cell selection (42,43).

The results described in this report illustrate the potential of minute quantities of class I molecules exerting immunologically relevant functions. The possibility that free MHC class I heavy chains can serve as recognition structures may be relevant not only for CTL recognition, but also for the target cell recognition by natural killer cells.

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Abbreviations

b2m	β_2-microglobulin
B6	C57BL/6
C′	complement
ER	endoplasmic reticulum
1D-IEF	one-dimensional isoelectric focusing
PE	phycoerythrin
TAP	transporter associated with antigen presentation

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