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Immunology

Positive selection of self- and alloreactive CD8$^+$ T cells in *Tap-1* mutant mice

(cytotoxic T lymphocytes/major histocompatibility complex/peptide transporters)

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ABSTRACT **Mice with a homozygous deletion in their *Tap-1* gene (−/− mice) express very low levels of cell membrane major histocompatibility complex class I molecules and have <1% peripheral CD8$^+$ T cells. We show that these −/− mice but not their +/− littermates display strong primary syngeneic anti-H-2K^b and -D^b-specific responses mediated by CD8$^+$ T cells. These responses are augmented by *in vivo* priming. Further, −/− mice primed *in vivo* with H-2^d alloantigens generate an anti-H-2^d response which appears nearly as strong as that found in +/− littermates. Both −/− anti-H-2^b and anti-H-2^d T cells do not recognize target cells from *Tap-1* −/− animals or Tap-2-deficient RMA-S cells. Thus, some CD8$^+$ anti-self and alloreactive T cells can be selected in the absence of Tap proteins.**

Tap-1 and -2 proteins control the transport of antigenic peptides from the cytosol to the endoplasmic reticulum, where they bind to newly synthesized major histocompatibility complex (MHC) class I molecules, hereafter referred to as class I (1–6). Peptide-bound class I is subsequently expressed on the cell membrane, where it can be recognized by antigen-specific CD8$^+$ T cells. Defects in either of the Tap proteins result in low expression of cell membrane class I molecules, most of which are unstable at 37°C (7). Recently, mice with a homozygous deletion in their *Tap-1* gene were produced; they express very low levels of cell membrane class I and have <1% peripheral CD8$^+$ T cells (8, 9). Cells from these mice fail to present cytoplasmically loaded ovalbumin (OVA) to OVA-specific cytotoxic T lymphocytes (CTL) (8).

The repertoire of CD4$^-$CD8$^+$ T cells is shaped by both positive and negative selection that takes place in the thymus. Both of these cellular selection processes involve the recognition of self peptide/class I complexes by the T-cell antigen receptor (TCR) expressed on double-positive (CD4$^+$CD8$^+$) thymocytes (9–14). A recent study suggests that the key parameter dictating the fate of thymocytes is the number of ligand-engaged TCRs; if this number is higher than a minimum threshold and in an appropriate range, positive selection occurs, and when it is higher than this range negative selection takes place (12). Since *Tap-1* −/− mice express low levels of class I, it would be expected that both positive and negative selection would be abnormal and could account for the low numbers of CD8$^+$ T cells in these animals. However, class I molecules independent of Tap should be expressed in *Tap-1* −/− mice (15–23), which could allow for the generation of some CD8$^+$ T cells. In this report, we evaluate the anti-self and allogeneic class I repertoire in *Tap-1* −/− mice.

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MATERIALS AND METHODS

Animals. The *Tap-1* −/− mice have been described (8). F_2 or F_3 animals, homozygous or heterozygous for the mutation, were used in the present study. Mice with disrupted β_2-microglobulin genes (*B2m* −/−) were kindly provided by B. Koller (University of North Carolina, Chapel Hill). RMA and RMA-S cells were obtained from K. Kärre (Karolinska Institute, Stockholm).

CTL Assay. Animals were primed with 30×10^6 spleen cells i.p. For CTL assays, 5×10^6 responder spleen cells were cocultured with an equal number of irradiated splenocytes in RPMI 1640 medium with 10% fetal bovine serum for 6 days. The effector cells were harvested and tested against 5×10^3 ^{51}Cr-labeled target cells in a standard 4-hr assay. Percent specific release is calculated by the formula [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Flow Cytometry. The percentage positive cells was determined by using flow cytometry (FACScan, Becton Dickinson). Anti-CD4 antibodies were purchased from either PharMingen or the American Type Culture Collection (GK1.5). Anti-CD8 was from either PharMingen or YTS169.4 hybridoma.

RESULTS AND DISCUSSION

***Tap-1* −/− Animals React Against Syngeneic H-2^b Class I Antigens.** *Tap-1* −/− mice on an *H-2^b* background express very low levels of class I and have <1% peripheral CD8+ T cells (8). We cultured cells from *Tap-1* −/− and *Tap-1* +/− animals, or animals previously primed *in vivo* against C57BL/6 (B6) (H-2^b) splenocytes, with irradiated B6 cells for 6 days *in vitro* and tested the resulting effector cells for CTL activity (Fig. 1). *Tap-1* −/− animals mount a strong response against lymphoblast target cells which express H-2^b antigens, including those from B6, 129/Sv (129), and *Tap-1* +/− littermates (Fig. 1*a*). This response is augmented by priming such that lytic activity is ≈2–7 fold greater than that seen in animals that had not been previously sensitized *in vivo* (Fig. 1 *a* and *c*). In contrast, priming *Tap-1* +/− littermates generated no detectable anti-H-2^b reactivity (Fig. 1 *b* and *d*). *Tap-1* −/− mice express very low levels of class I molecules. Therefore, it might be expected that such animals are not tolerant of most H-2^b molecules, and this could account for the anti-H2^b reactivity. It is likely that *Tap-1* −/− mice

Abbreviation: CTL, cytotoxic T lymphocyte(s).

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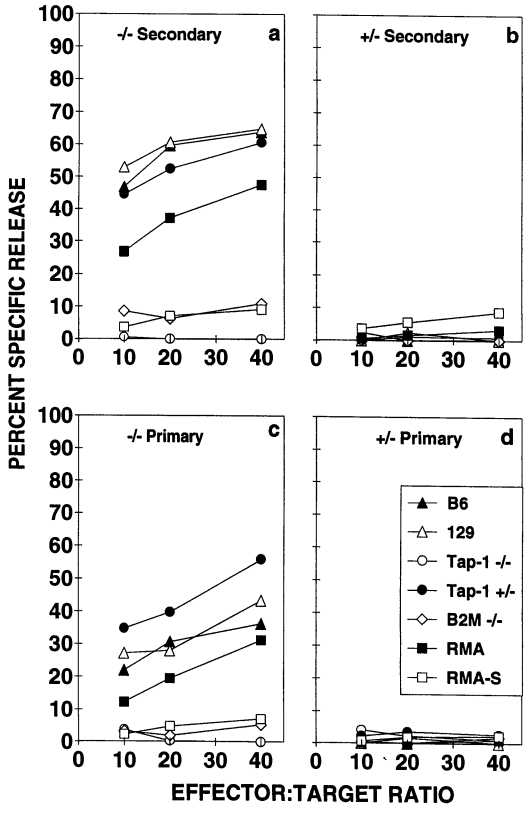

FIG. 1. *Tap-1* −/− animals generate CTL activity vs. syngeneic class I, H-2^b. Spleen cells from −/− (*a*, *c*) or +/− (*b*, *d*) mice unprimed (*c*, *d*) or primed with B6 spleen cells (*a*, *b*) 7–10 days earlier were cocultured *in vitro* with B6 splenocytes and assayed for lytic activity. Concanavalin A (Con A)-stimulated lymphoblast target cells from B6 (▲), 129 (H-2^b) (△), *Tap-1* −/− (○), *Tap-1* +/− (●), or *B2m* −/− (◇) animals, or RMA (■) and RMA-S (□) tumor cells were used in a standard ^{51}Cr release assay. One of three experiments is shown.

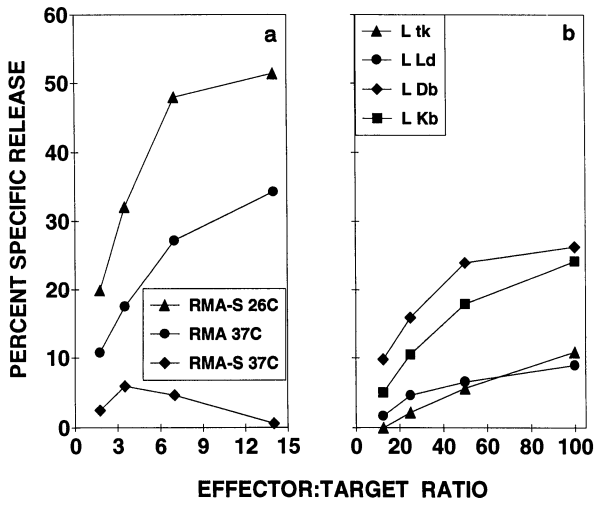

FIG. 2. Specificity of *Tap-1* −/− CTL. Spleen cells from −/− animals previously primed *in vivo* i.p. with 30 × 10^6 B6 spleen cells were cultured with irradiated B6 splenocytes for 6 days and tested for lytic activity in a standard ^{51}Cr release assay. (*a*) Target cells were RMA cells cultured overnight at 37°C (●) or RMA-S cells cultured overnight at 26°C (▲) or 37°C (◆). (*b*) Target cells were L cells transfected with genes encoding thymidine kinase (▲), L^d (●), D^b (◆), or K^b (■). One of two experiments is shown.

display some class I molecules which bind peptides independent of Tap-1/Tap-2, as has been described (15–23). Consistent with this, alloreactive CTL can recognize cells from −/− mice, though to a much lesser extent than those of +/− cells (ref. 8; data not shown). Accordingly, anti-H-2^b T-cell activity in −/− should be directed against the class I molecules whose expression is dependent on Tap proteins and not against the class I molecules that might be expressed independent of Tap-1/Tap-2. Therefore, we tested whether such −/− effector cells could recognize class I on −/− (Tap-1-defective) or RMA-S (Tap-2-defective) cells (24–28). As shown in Fig. 1 *a* and *c*, −/− anti-H-2^b CTL display no cytotoxic activity against *Tap-1* −/− or RMA-S target cells, while control +/− and RMA cells are readily lysed. Incubation of RMA-S cells at room temperature allows for increased expression of class I on the cell surface (7). This increase is thought to be due to transport of both "empty" molecules and peptide-bearing class I, since peptides can be eluted from RMA-S cells that have been cultured at room temperature (29, 30). Such cells incubated at 26°C are susceptible to lysis by −/− anti-H-2^b effectors (Fig. 2*a*). This result is consistent with the notion that CD8$^+$ cells from −/− mice recognize peptide-bound class I on RMA-S whose expression is facilitated by culture at room temperature. To further establish the class I specificity of these cells, we tested whether they would recognize target cells from animals that have had their β_2-microglobulin genes disrupted (*B2m*−/−) and thus express very low levels of class I (31, 32). Such effector cells fail to recognize target cells from these animals (Fig. 1 *a* and *c*). We also tested the ability of −/− cells to recognize L cells expressing either K^b or D^b. Both targets were readily recognized, indicating that there is no preference in selection of T cells directed against either of these antigens and confirming that the response is class I-specific (Fig. 2*b*).

***Tap-1* −/− Animals React Against Allogeneic H-2^d Class I Antigens.** The above experiments demonstrate that *Tap-1* −/− mice react against syngeneic class I antigens. Therefore, we next determined whether these mice could also generate alloreactive CTL directed against H-2^d antigens. Here the activity detected in a primary response by −/− CTL was weak (Fig. 3*c*), while −/− mice previously primed *in vivo* with BALB/c splenocytes generated a strong response against H-2^d antigens (Fig. 3*a*). In fact, the lytic activity generated is often indistinguishable from that of the +/− animals (Fig. 3 *a* vs. *b*). Their gross specificity resembles that seen from +/− mice in that H-2^d lymphoblasts are lysed while target cells from H-2^k or H-2^s haplotypes are not, and *Tap-1* −/− effectors generated against H-2^f are specific for these antigens and not H-2^d, H-2^k, or H-2^s target cells (data not shown). However, unlike effector cells from +/− animals, −/− anti-H-2^d effectors crossreact on self H-2^b class I, albeit not very strongly (Fig. 3 *a* vs. *b*). Similar to −/− cells sensitized to H-2^b, these CTL recognize H-2^b alloantigens only on +/−, B6, and RMA cells; −/− or RMA-S cells are not recognized unless the latter are cultured at 26°C (data not shown). They also fail to recognize class I on cells from *B2m* −/− animals (Fig. 3 *a* and *c*).

Phenotype of Cells from *Tap-1* −/− Mice Primed Against Self or Alloantigens. To determine the phenotype of the CTL responsible for the lytic activity, we treated the effector cells with anti-CD8 antibody plus complement. As a result, the activity in both the anti-H-2^b and -H-2^d responses was eliminated (data not shown). −/− animals have on average 0.8% CD8$^+$ cells in their spleen (Table 1). After *in vivo* priming with H-2^b or H-2^d splenocytes, the number of CD8$^+$ cells in the spleen increases slightly. Following *in vitro* culture with H-2^b stimulator cells, this number increases dramatically (50.2%), which could readily account for the strong lytic activity observed. Surprisingly, −/− mice primed *in vivo* and restimulated *in vitro* with H-2^d cells have small numbers of CD8$^+$ cells (2.2%) compared with +/−

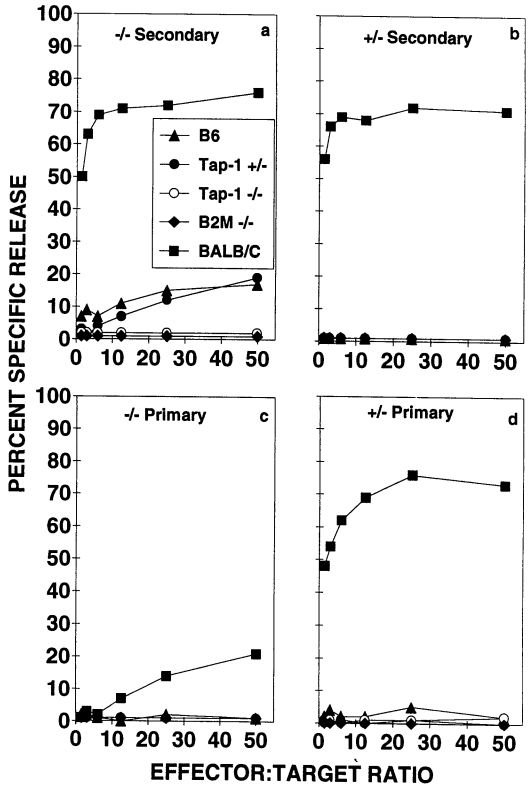

FIG. 3. *Tap-1* −/− animals generate CTL activity vs. allogeneic class I, H-2^d. Spleen cells from −/− (*a*, *c*) or +/− (*b*, *d*) mice unprimed (*c*, *d*) or primed with BALB/c spleen cells (*a*, *b*) 10 days earlier were cocultured *in vitro* with BALB/c splenocytes and assayed for lytic activity. Target cells were Con A-stimulated lymphoblasts from B6 (▲), *Tap-1* −/− (○), *Tap-1* +/− (●), *B2m* −/− (◆), or BALB/c (■) mice. One of three experiments is shown.

controls even though these cultures display strong anti-H-2^d activity. The low number of CD8 cells may be due in part to the fact that these cells are responding to both class I and II antigens, while the response to H-2^b stimulators involves a class I difference only.

Table 1. Percentage of CD4$^+$ and CD8$^+$ splenocytes in *Tap-1* −/− and +/− animals primed against H-2^b or H-2^d antigens

Tap-1 strain	Primed *in vivo**	Primed *in vitro*†	% of splenocytes‡ CD4$^+$	% of splenocytes‡ CD8$^+$
+/−	—	—	25.5 ± 2.7	9.7 ± 1.9
−/−	—	—	29.0 ± 2.8	0.8 ± 0.5
+/−	H-2^b	—	18.0 ± 2.3	9.3 ± 3.6
+/−	H-2^b	H-2^b	ND	ND
+/−	—	H-2^b	19.7, 38.5	11.3, 10.6
−/−	H-2^b	—	25.0 ± 5.8	1.3 ± 0.5
−/−	H-2^b	H-2^b	15.4 ± 2.3	59.1 ± 18.8
−/−	—	H-2^b	33.9 ± 9.8	9.4 ± 6.1
+/−	H-2^d	—	15.7, 23.2	7.0, 10.1
+/−	H-2^d	H-2^d	69.9, 48.5	25.0, 46.8
+/−	—	H-2^d	42.4 ± 6.0	14.4 ± 1.3
−/−	H-2^d	—	24.9, 25.4	1.5, 1.7
−/−	H-2^d	H-2^d	90.6 ± 3.7	2.2 ± 1.2
−/−	—	H-2^d	60.9 ± 8.5	0.7 ± 0.7

*Mice were primed with 30 × 10^6 spleen cells and analyzed on day 10–14.
†Cells were cultured with indicated cells and analyzed on day 5.
‡Mean ± SD percentage of total splenocytes from individual experiments. In some cases, values from two individual experiments are given. ND, not done.

Previous studies examining positive selection of T cells indicate that peptide has a role in determining the repertoire of specificities exhibited (9–12, 33–36). The extent of development of single-positive CD8$^+$ cells in the thymus varies with the complexity of peptides bound to self-class I (9, 10). What is striking here is that −/− mice appear to generate strong anti-H-2^b and -H-2^d responses by selection on very low levels of class I molecules that presumably display a limited array of peptides relative to +/− animals. We expect that the class I molecules expressed are occupied by peptides such as those derived from signal sequences (15, 23, 37) or whose expression is reported to be independent of Tap-1/Tap-2 (16–22). While these molecules permit positive selection, the specificity of the cells generated is not directed toward class I molecules filled with Tap-independent peptides, since −/− effector cells do not recognize cells from *Tap-1* −/− mice or RMA-S targets. However, because the T cells generated are not negatively selected against Tap-1/Tap-2-dependent class I molecules, they display reactivity against self-H-2^b. The crossreactivity of *Tap-1* −/− anti-H-2^d alloreactive CTL with H-2^b may also result from a lack of negative selection.

Mice with a disruption in their *B2m* gene also express very low levels of class I and CD8$^+$ cells (31, 32). We (data not shown) and others have noted that these animals mount a strong allogeneic and anti-self response (38–40). This suggests that selection of a functional T-cell repertoire can also occur on surface class I molecules in the absence of β_2-microglobulin (β_2m). Although the cells generated in mice lacking either Tap-dependent peptides or β_2m molecules appear similar in reactivity, the peptides associated with the selecting molecules are likely to be dissimilar, since in the former case many of the peptides may be represented by leader sequences (15, 23), while in the latter the peptides probably represent a different pool (41, 42).

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