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Immunology

Altered natural killer cell repertoire in *Tap-1* mutant mice

(major histocompatibility complex/peptide transporters)

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ABSTRACT **We have analyzed the specificity and function of natural killer (NK) cells in mice with a homozygous deletion of the major histocompatibility complex (MHC)-encoded transporter gene associated with MHC class I-restricted antigen presentation (*Tap-1*). These mice express very low levels of class I molecules at the cell surface, and these molecules are either devoid of peptide or occupied only by TAP-independent peptides. NK cells in *Tap-1* −/− mice, though normal in number, appeared tolerant toward autologous *Tap-1* −/− Con A-activated blasts, *Tap-1* −/− as well as allogeneic BALB/c bone marrow cells, and RMA-S tumor cell grafts. In contrast, they killed YAC-1 cells as efficiently as did NK cells from wild-type mice. Defective *Tap-1* expression was sufficient to render nontransformed target cells sensitive to NK cell-mediated lysis. It is concluded that proper expression of TAP molecules is necessary for normal development of NK cells, as well as for rendering target cells resistant to NK cell-mediated lysis. These results support the hypothesis that class I molecules of the MHC influence the sensitivity of target cells to lysis by NK cells, as well as the development of the NK cell repertoire.**

Major histocompatibility complex (MHC) class I molecules consist of a glycoprotein heavy chain (HC) non-covalently associated with β_2-microglobulin (β_2m) (1). Their function is to present antigen in the form of short peptides, derived from degraded intracellular proteins, to $CD8^+$ cytotoxic T lymphocytes (CTL) (2, 3).

A direct role for MHC class I molecules in interaction with natural killer (NK) cells has also been suggested (4–7). NK cells represent a significant proportion of lymphocytes in peripheral blood and spleen. They are spontaneously cytotoxic against a variety of target cells, including certain tumor cell lines and virus-infected cells as well as allogeneic bone marrow and lymphoid cells (8). Their cytotoxic action is not dependent on prior immunization, expression of the α, β, or δ T-cell receptor (TCR) genes or *Rag-1/2*-dependent gene rearrangement (refs. 8 and 9; H.-G.L., P. Mombaerts, and S.T., unpublished data). It is well established that several aspects of NK cell function are influenced by MHC-linked genes (9). More recently, several NK-linked phenomena have been linked to the class I molecules themselves (4–7).

However, MHC class I gene products influence NK cells and T cells differently. The expression of class I molecules on the target cell protects it from NK cell lysis (reviewed in refs. 5–7), and this protection has been linked to the peptide binding α-1/2 domains of the HC (10, 11). Peptides presented by class I molecules may also be directly or indirectly involved in interactions of NK cells and their targets (12-16). However, the actual form in which NK cells do or could recognize MHC class I products is not known (ref. 17; see *Discussion*).

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The *Tap-1* gene encodes a subunit of a transporter molecule that delivers short fragments of degraded proteins from the cytosol to the endoplasmic reticulum for association with class I molecules (18–20). *Tap-1* mutant mice are deficient in peptide transport, and consequently also in normal MHC class I-restricted antigen presentation. Cells from *Tap-1* mutant mice express class I molecules at the cell surface, but expression is severely reduced and the class I molecules expressed are devoid of TAP-dependent peptides. In the periphery, these animals have very few $CD4^-8^+$ CTL, presumably due to impaired positive selection during thymic development (21).

Given the association between class I expression and the ability to be recognized by NK cells, we have asked here if the generation of the NK cell repertoire as well as target cell sensitivity is affected by disruption of the *Tap-1* gene. The present data demonstrate that the target cell sensitivity as well as the NK cell repertoire in *Tap-1* mutant mice are altered. The present results are discussed in relation to recent studies with *B2m* (the gene encoding β_2m) mutant mice as well as mutant cell lines.

MATERIALS AND METHODS

Cell Lines. The RMA and RMA-S cell lines (kindly provided by Klas Kärre, Karolinska Institutet, Stockholm) were derived from the Rauscher virus-induced lymphoma line RBL-5 of C57BL/6 mouse origin. The generation and characterization of the cell lines have been described elsewhere (4, 22). In RMA-S cells, the class I HCs and β_2m fail to assemble (23, 24), due to a point mutation in the *Tap-2* gene (25). YAC-1 is a Moloney murine leukemia virus-induced line of A/Sn origin. For generation of Con A-activated T-cell blasts, single spleen cell suspensions (approximately 50×10^6 cells) were cultured in 40 ml of Dulbecco's modified Eagle's tissue culture medium (DMEM) supplemented with 10% fetal calf serum, antibiotics, and 2.5 μg of Con A (Sigma) per ml for 48 hr.

Mice. The generation of *Tap-1* −/− mice has been described in detail elsewhere (21). F_2 and F_3 *Tap-1* mice, homozygous or heterozygous for the mutation, were used in the present study. C57BL/6 (B6) and 129/Sv (129) control mice were from The Jackson Laboratory. All mice used were at the age of 6–10 weeks, usually littermates or otherwise age matched within 2 weeks in each experiment. All mice were bred and maintained at the Animal Department, Center for Cancer Research, Massachusetts Institute of Technology. Animal care was in accordance with institutional guidelines.

Flow Cytometric Analyses. For fluorescence-activated cell sorter (FACS) analysis, approximately 50 μl of tail vein blood

Abbreviations: β_2m, β_2-microglobulin; HC, heavy chain; CTL, cytotoxic T lymphocytes; NK, natural killer; TAP, transporter associated with antigen presentation; IdUrd, 5-iodo-2′-deoxyuridine.

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was mixed with 10 μl of 0.5 M EDTA (pH 8) and incubated with monoclonal antibodies for 30 min at room temperature. Then 1 ml of FACS lysis solution (Becton Dickinson; diluted 1:10) was added to the cells and the erythrocytes were lysed for 5 min. Cells were then washed twice in phosphate-buffered saline (PBS) and 10,000 viable cells were analyzed by using a FACScan flow cytometer (Becton Dickinson). Antibodies used for the flow cytometric analysis were R-phycoerythrin-conjugated anti-NK1.1 (PK136; PharMingen), final dilution in PBS 1:100, and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (53-6.7; PharMingen), final dilution 1:200.

***In Vitro* Cytotoxicity Assays.** Before suspensions were used as targets in a standard ^{51}Cr release cytotoxic assay, dead cells were removed by centrifugation with Lympholyte-M (Cedarlane Laboratories) according to the supplier's protocol. Single cell suspensions of splenocytes depleted of erythrocytes by osmotic lysis were used as effector cells. The interferon inducer Tilorone (T-8014, Sigma), which augments NK cell activity, was administered *per os* to the mice (0.2 ml of a 10 mg/ml solution per mouse) 24 hr before sacrifice and removal of the spleen.

Rapid Elimination Assay. For radiolabeling of tumor cells, 50 μg of 5-fluoro-2'-deoxyuridine (from stock solution of 250 μg/ml; Sigma) was added to 8×10^6 RMA-S tumor cells resuspended in 2 ml of DMEM supplemented with 10% fetal calf serum and antibiotics giving a final concentration of 25 mg/ml. After 15 min, 5 μCi (1 Ci = 37 GBq) of ^{125}I-labeled 5-iodo-2'-deoxyuridine ([^{125}I]IdUrd; Amersham) was added. After 2 hr, cells were washed three times in a large volume of PBS and resuspended in PBS at a final concentration of 1.6×10^6 cells per ml. Then 3×10^5 cells (0.2 ml) were inoculated intravenously (i.v.) into the retroorbital plexus of Metofane (methoxyflurane; Pitman-Moore, Mundelein, IL) anesthetized untreated or NK1.1-depleted mice. Mice were anesthetized by inhalation according to the supplier's description. Experiments were done on groups of four to five animals. Labeling according to the present protocol usually gives an incorporation corresponding to approximately 9×10^5 cpm per 0.2 ml of cell suspension (the inoculation volume). Approximately 18 hr after inoculation, mice were sacrificed and the lungs, liver, spleen, and kidneys were removed. The remaining radioactivity in each (pair of) organs(s) was measured by γ counting. Results are expressed as percentage of total radioactivity inoculated with SEM indicated. B6 mice were depleted of NK cells by intraperitoneal (i.p.) inoculation of 0.2 ml of ascites fluid containing anti-NK1.1 (PK136) antibodies 24 hr before tumor cell inoculation.

Tumor Outgrowth. Tumor cells, suspended in PBS, were inoculated subcutaneously (s.c.) into the flanks of untreated mice in 0.1 ml. Tumor growth was followed by palpation and was registered at least twice weekly. Tumors always appeared at the site of inoculation. Mice were killed when tumors reached 15 mm in diameter, and no signs of regression were seen. Mice without tumor growth were kept for observation for at least 6 weeks after inoculation.

Bone Marrow Transplantation. Bone marrow cells were obtained by flushing the lumen of tibia and femur with PBS under aseptic conditions. Suspensions of 1×10^6 bone marrow cells in PBS were grafted i.v. (see procedure for rapid elimination assay) into groups of four to six irradiated [800 rad (8 gray)] recipients. On day 5, 3 μCi of [^{125}I]IdUrd (see above) in 0.2 ml of PBS was inoculated i.p. Twenty-four hours later, mice were sacrificed and the radioactivity in the spleen was measured by a γ counter. Log^{10} values were calculated from the cpm of each individual spleen and arithmetic mean values and SEMs were calculated for each group. Data represent pooled results from two independent experiments, except +/− → B6 and −/− → B6 NK1.1 (one experiment) (→ indicates inoculated into).

Statistical Analysis. The difference between control and test groups was evaluated by a Student's *t* test.

RESULTS

NK Cells in *Tap-1* −/− Mice. The NK1.1 allele is expressed in mice of B6 but not 129 origin (8, 9) and can thus be used as a marker for NK cells in a majority of *Tap-1* −/− (B6 × 129)F_2 or F_3 mice. NK cells, as defined by the NK1.1 expression, were found to be normal in number in peripheral blood or spleen from *Tap-1* −/− mice as compared with *Tap-1* +/− control mice (Fig. 1, data not shown). On average, 6.0% (SD 2.8%; $n = 7$) of *Tap-1* −/− and 4.8% (SD 1.2%; $n = 11$) of *Tap-1* +/− peripheral blood leukocytes were NK1.1$^+$. The somewhat higher relative numbers of NK cells from *Tap-1* −/− mice may be due to the severe reduction in number of CD8$^+$ T cells in these mice (ref. 21; Fig. 1). *Tap-1* −/− mice homozygous or heterozygous for the NK1.1 allele as distinguished by the fluorescence intensity after NK1.1 staining did not differ in actual number of NK1.1$^+$ cells (data not shown).

Target Cell Specificity of *Tap-1* −/− NK Cells. Killing of the classical NK target cell line YAC-1 by *Tap-1* −/− NK cells was unimpaired compared with *Tap-1* +/− NK cells (Table 1), regardless of prior activation *in vivo* by the interferon-inducing agent Tilorone (data not shown).

In contrast, NK cells from *Tap-1* −/− mice showed a selective tolerance against *Tap-1* −/− Con A-activated blasts *in vitro* (Table 1), whereas the same targets were readily killed by NK cells from *Tap-1* +/− mice. The ability of *Tap-1* +/−, and the inability of *Tap-1* −/− NK cells, to kill *Tap-1* −/− Con A blasts *in vitro* did not correlate with NK1.1 expression of the effector cells (data not shown). Regardless of NK1.1 expression, a similar specificity of the effector cells was seen.

A similar pattern was seen when the *Tap-2*-defective RMA-S tumor cell line was used as target (Table 1), though occasional experiments revealed killing of RMA-S cells even by *Tap-1* −/− effectors. Though interesting, this matter was not investigated further.

Sensitivity of *Tap-1* −/− Cells to NK Cell-Mediated Lysis. Con A-activated blasts from *Tap-1* −/− mice were killed by B6, 129/Sv, and *Tap-1* +/− NK cells (Table 1, data not shown), while *Tap-1* +/− Con A blasts were completely resistant to the same effectors (data not shown). In this respect, the results obtained with *Tap-1* −/− and +/− Con A blasts as targets resembled the cytotoxicity patterns observed for the RMA-S and RMA tumor cell lines, respectively (Table 1; refs. 4 and 26).

Impaired Rapid Elimination and Tumor Formation of RMA-S Cells in *Tap-1* −/− Mice. RMA-S tumor cells are rapidly eliminated by NK cells when inoculated i.v. into

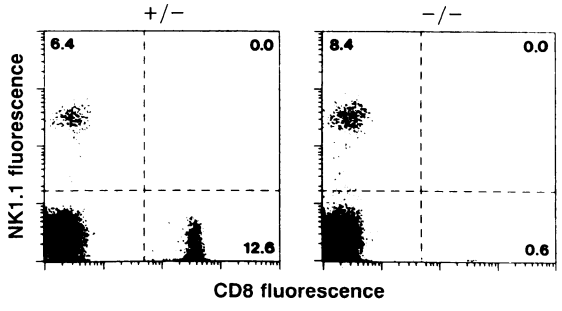

FIG. 1. *Tap-1* −/− mice have normal numbers of NK cells. Peripheral blood from *Tap-1* −/− and +/− mice was depleted of erythrocytes, stained for NK1.1- and CD8-expressing cells, and subjected to flow cytometric analysis. The numbers indicate the percentage of cells in each quadrant.

Table 1. NK cells from *Tap-1* −/− mice are tolerant to *Tap-1* −/− Con A-activated blasts and RMA-S cells

Effectors	E:T* ratio	% specific lysis of target cells YAC-1	RMA-S	*Tap-1* −/− Con A blasts
B6	200:1	48	23	22
	100:1	46	19	19
	50:1	39	13	9
	25:1	27	9	8
B6	200:1	55	19	20
	100:1	52	23	22
	50:1	38	17	18
	25:1	30	12	10
Tap-1 +/−	200:1	55	38	21
	100:1	53	31	22
	50:1	43	22	13
	25:1	33	14	10
Tap-1 +/−	200:1	56	43	32
	100:1	47	32	21
	50:1	36	25	14
	25:1	22	15	14
Tap-1 −/−	200:1	46	4	0
	100:1	41	4	2
	50:1	31	2	0
	25:1	24	2	0
Tap-1 −/−	200:1	56	7	2
	100:1	47	6	2
	50:1	39	5	2
	25:1	26	5	1

One representative experiment is shown. NK cells from two individual mice (B6, *Tap-1* +/− or −/−) were tested against the indicated target cells.
*Effector to target.

syngeneic B6 mice (22, 27) and, consequently, they also fail to form tumors *in vivo* (4, 22, 27). In contrast, in *Tap-1* −/− mice, RMA-S cells were spared from elimination (Fig. 2). When the number of surviving cells, measured by the levels of remaining radioactivity in the lungs, was compared between control *Tap-1* +/− and −/− mice inoculated with RMA-S cells, the differences were almost 10-fold (Fig. 2). Clear differences, though smaller, were also seen in the kidneys, liver, and spleen (data not shown).

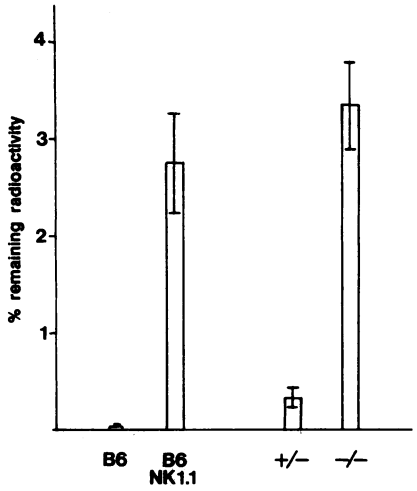

FIG. 2. *Tap-1* −/− mice do not eliminate radiolabeled RMA-S cells. Elimination was measured by survival of [^{125}I]IdUrd-labeled RMA-S cells in the lungs of B6, NK1.1-depleted B6, and *Tap-1* +/− and −/− mice. Percentage remaining radioactivity (arithmetic mean and SEM indicated) of total amount inoculated indicates the amount of surviving cells. RMA-S → −/− vs. RMA-S → +/−, $P < 0.01$.

In *Tap-1* −/− mice, RMA-S cells formed tumors in 3/5 mice when 2×10^4 cells were inoculated s.c. In contrast, 0/5 *Tap-1* +/− mice developed tumors under identical conditions. Thus, though only a limited number of mice were tested, the present results indicate that a single genetic defect in the host—i.e., the absence of a functional *Tap-1* gene—is sufficient to render RMA-S cells tumorigenic.

Impaired Rejection of *Tap-1* −/− and Allogeneic Bone Marrow in *Tap-1* −/− Mice. *Tap-1* −/− mice readily accepted *Tap-1* −/− bone marrow grafts (Fig. 3). However, *Tap-1* −/− but not *Tap-1* +/− bone marrow cells were rejected in an NK cell-dependent manner when grafted to B6 mice (Fig. 3). NK cells are also known to be the effector cells responsible for the rejection of allogeneic bone marrow grafts by lethally irradiated mice (9, 28). However, while B6 mice rejected allogeneic (BALB/c) bone marrow grafts, our current data indicate that such grafts were accepted by *Tap-1* −/− mice (Fig. 3). A more detailed study will reveal whether the *Tap-1* −/− mice generally accept allogeneic bone marrow grafts that are rejected by heterozygous littermates.

DISCUSSION

Disruption of the *Tap-1* gene in mice by homologous recombination in ES cells alters the development of CD8$^+$ T cells (21) as well as NK cells (this study), while CD4$^+$ T cells are not affected (21). Further, Con A-activated blasts from *Tap-1* −/− mice show drastic alterations in susceptibility to both MHC class I-restricted CTL (21) and to NK cell lysis (this study). While susceptibility for lysis by CD8$^+$ class I-restricted CTL is lost, Con A blasts from *Tap-1* −/− mice become susceptible to lysis by NK cells from normal mice. Thus, normal *Tap-1* gene function is crucial not only for the development of CD8$^+$ T cells but also for the development of a normal NK cell repertoire.

The specific killing of normal (nontransformed) *Tap-1* mutant cells by NK cells implies that class I molecules, or molecules that interact with class I molecules, act as ligands for receptors on (subsets of) NK cells. How do NK cells detect the "absence" of normal class I/peptide complexes? A direct inhibitory signal could be transmitted by the class I/peptide complex to the NK cell (5). The discovery of a

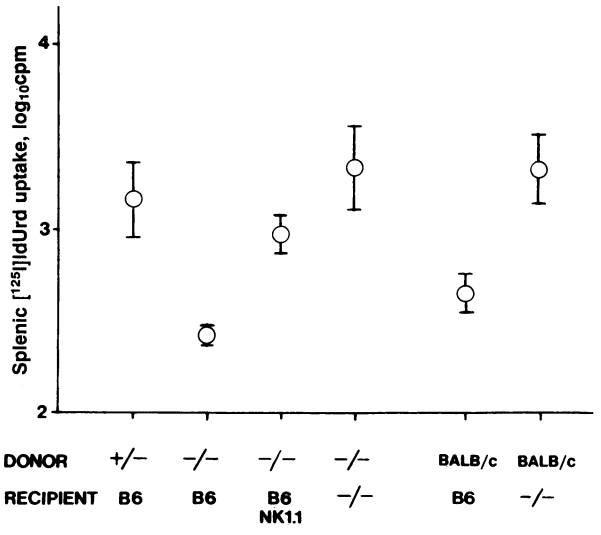

FIG. 3. *Tap-1* −/− mice accept *Tap-1* −/− as well as allogeneic bone marrow grafts. Engraftment measured by [^{125}I]IdUrd incorporation of proliferating cells in the spleen of lethally irradiated and bone marrow grafted mice (arithmetic mean and SD of $\log_{10}$ values indicated). −/− → B6 vs. +/− → B6, $P < 0.05$; −/− → B6 vs. −/− → B6 NK1.1, $P < 0.001$; −/− → B6 vs. −/− → −/−, $P < 0.01$; BALB/c → B6 vs. BALB/c → −/−, $P < 0.01$.

family of NK-related receptors (reviewed in ref. 29), such as the Ly-49 molecule (30), which upon engagement with target cell MHC class I molecules may deliver inhibitory signals, is interesting in relation to this model. Alternatively, class I/peptide complexes could eclipse or modulate a triggering ligand on the target.

The selection of the NK cell repertoire is not understood, but gene rearrangement is not necessary for generating NK cell specificity (ref. 31; H.-G.L., P. Mombaerts, and S.T., unpublished results). The normal number of NK1.1$^+$ cells in *Tap-1* −/− mice argues against the deletion of a major subset of NK cells in these mice. Because NK cells from *Tap-1* −/− mice are tolerant to *Tap-1* −/− targets, while they readily kill YAC-1, it is possible that down-regulation of a specific (set of) receptor(s) on (a subset of) NK cells has occurred while other functions of NK cells remain intact. Note that NK cells, in contrast to CTL, may be capable of killing target cells through a variety of mechanisms including antibody-dependent cytotoxicity mediated by the Fc receptor for immunoglobulin (32).

Tap-1 −/− and *B2m* −/− NK cells bear many similarities (this study; refs. 33–35). NK cells are normal in number and kill the standard NK cell target cell line YAC-1 with virtually unaltered efficacy compared with wild-type mice, though poor YAC-1 killing was initially reported in *B2m* mutant mice (34). In contrast to wild-type mice, NK cells from mutant mice (either *Tap-1* −/− or *B2m* −/−) are tolerant toward autologous Con A-activated blasts. However, it is important to point out that NK cells generated in *Tap-1* −/− versus *B2m* −/− hosts may not be identical with regard to target cell specificity. Differences could be caused by the selection of an NK repertoire on different forms of class I molecules, and a direct comparison of the two mutant mice may very well reveal (subtle) differences in this regard. Depending on the expression of β_2m or provision of peptides by the TAP1/2 transporter, the class I molecules may be displayed in distinct forms at the cell surface (schematically illustrated in Fig. 4). Cells devoid of β_2m are likely to express a limited number of class I HCs occupied by peptides (36, 37). Cells devoid of TAP-1 may express a limited number of class I HCs complexed with β_2m, either devoid of peptide or occupied by peptides that are transported across the endoplasmic reticulum (ER) membrane independently of the TAP complex (38, 39) or degraded within the ER lumen (40, 41). All cells, whether wild type or mutant (β_2m or TAP-1) are also likely to express free HCs and various degradation products of class I molecules. It follows that TAP and β_2m mutant cells share several features, the most prominent being the absence of an abundant expression of properly folded class I/β_2m/peptide complexes. This may account for many of the similarities observed with regard to the development of the NK cell repertoire in these mice. On the other hand, different forms of class I molecules expressed at the cell surface in these mice might lead to differences in target cell specificity of NK cells.

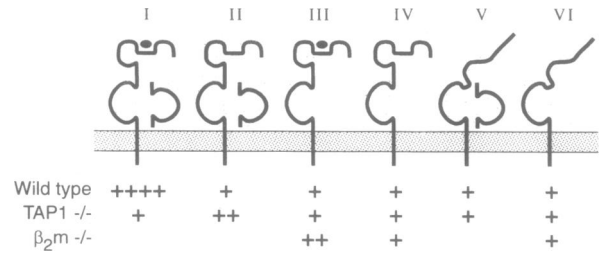

FIG. 4. Schematic representation of possible forms of class I molecules on the cell surface of wild-type as well as TAP-1 and β_2m mutant cells. The grading (++++, ++, or +) does not represent actual, but rather relative, levels of expression that may be expected within each group of mice. The figure is meant to illustrate and partly simplify the reasoning in the discussion. The membrane-embedded heavy chain is present in forms I–VI; β_2m is present only in forms I, II, and V. Forms I and III contain peptide (objects in peptide-binding groove).

Tap-1-defective mice may be a valuable tool in studies of how MHC class I molecules, and in particular the MHC class I-bound peptide repertoire, affect generation of the NK cell repertoire.

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