Expression and Functional Analysis of Human Leukocyte Antigen Class I Antigen-Processing Machinery in Medulloblastoma

Lizzia Raffaghello, Paolo Nozza, Fabio Morandi, Marta Camoriano, Xinhui Wang, Maria Luisa Garrè, Armando Cama, Giuseppe Basso, Soldano Ferrone, Claudio Gambini, and Vito Pistoia

Abstract

Defects in the expression and/or function of the human leukocyte antigen (HLA) class I antigen-processing machinery (APM) components are found in many tumor types. These abnormalities may have a negative impact on the interactions of tumor cells with host’s immune system and on the outcome of T cell–based immunotherapy. To the best of our knowledge, no information is available about APM component expression and functional characteristics in human medulloblastoma cells (Mb). Therefore, in the present study, we have initially compared the expression of APM components in Mb, an embryonal pediatric brain tumor with a poor prognosis, with that in noninfiltrating astrocytic pediatric tumors, a group of differentiated brain malignancies with favorable prognosis. LMP2, LMP7, calnexin, β2-microglobulin–free heavy chain (HC) and β2-microglobulin were down-regulated or undetectable in Mb lesions, but not in astrocytic tumors or normal fetal cerebellum. Two Mb cell lines (DAOI and D283) displayed similar but not superimposable defects in APM component expression as compared with primary tumors. To assess the functional implications of HLA class I APM component down-regulation in Mb cell lines, we tested their recognition by HLA class I antigen-restricted, tumor antigen (TA)–specific CTL, generated by stimulations with dendritic cells that had been transfected with Mb mRNA. The Mb cell lines were lysed by TA-specific CTL in a HLA-restricted manner. Thus, defective expression of HLA class I–related APM components in Mb cells does not impair their ability to present TA to TA-specific CTL. In conclusion, these results can contribute to optimize T cell–based immunotherapeutic strategies for Mb treatment.

Note: L. Raffaghello and P. Nozza contributed equally to this work.

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Materials and Methods

Patients. A total of 10 primary classic Mb and 11 primary noninfiltrating astrocytoma lesions, resected at disease onset, were retrieved from the files of the Department of Neurosurgery at the Giannina Gaslini Institute (IGG), Genoa, Italy. The noninfiltrating astrocytoma lesions that included eight pilocytic astrocytomas (PA), two pleomorphic xantoastrocytomas (PXA), and one subependimal giant cell astrocytoma (SEGA), will be referred to as "astrocytic tumors." The main Mb patient characteristics, including age, gender, anatomic site, and stage of the tumors, are summarized in Table 1. The age of patients with astrocytic tumors ranged from 2 to 11 months; nine of them were males, and two were females. All patients underwent surgery...
before any other therapy. No patient was immunocompromised; all were HIV negative. Tissue sections from a human fetal brain (gestational age: 28 weeks) were stained with APM component–specific monoclonal antibody (mAb) as normal counterpart.

Histopathologic diagnoses were made by routine light microscopic evaluation of formalin-fixed, paraffin-embedded tissue sections stained with H&E and silver impregnation for reticulin. Immunohistochemical stainings for synaptophysin, vimentin, neuron-specific enolase, glial fibrillary acid protein, S-100 protein, epithelial membrane antigen, CD34, and Mib-1 were also done for diagnostic purposes (10, 13).

**Cell lines.** The Mb cell lines, DAOI and D283, and the Raji Burkitt lymphoma cell line were maintained in RPMI 1640 (Euroclone) supplemented with 10% fetal bovine serum (FBS, Life Technologies Invitrogen), HEPES buffer, nonessential amino acids, L-glutamine and penicillin/streptomycin (Cambrex Bio Science Verviers). Before being used as targets in ELISPOT and cytotoxicity assays, the DAOI and D283 cell lines were cultured for 48 h in the presence human rIFN-γ (Boehringer Ingelheim Italia) at the final concentration of 1,000 IU/mL that, in previous experiments, proved to be optimal for HLA-ABC up-regulation on neuroblastoma cell lines (14).

**Antibodies.** The mAb HC 10, which recognizes a determinant expressed on all β2-microglobulin–free HLA-B HC and on β2-microglobulin–free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and HLA-A33 HC (15, 16); the anti-β2-microglobulin–specific mAb L368 (17) and the 2-microglobulin–specific mAb L368 (17) and the

**Table 1. Main characteristics at diagnosis of 10 children with primary pediatric medulloblastoma**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (y, mo)</th>
<th>Gender</th>
<th>Site</th>
<th>WHO stage</th>
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<td>M</td>
<td>Cerebellum</td>
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<td>2</td>
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<td>3</td>
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<td>9, 7</td>
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<td>Cerebellum</td>
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<td>5</td>
<td>9, 2</td>
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<td>Cerebellum</td>
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<td>6</td>
<td>9, 8</td>
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<td>7</td>
<td>8, 3</td>
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<td>8</td>
<td>12, 11</td>
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<td>Cerebellum</td>
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<td>9</td>
<td>5, 1</td>
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<td>Cerebellum</td>
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<td>10</td>
<td>2, 3</td>
<td>F</td>
<td>Cerebellum</td>
<td>G 4</td>
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**Table 2. HLA class I antigen-processing molecule expression in primary pediatric medulloblastoma**

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA-ABC</th>
<th>Heavy chain</th>
<th>β2-Microglobulin</th>
<th>Tapasin</th>
<th>Calnexin</th>
<th>Calreticulin</th>
<th>Erp57</th>
<th>LMP2</th>
<th>LMP7</th>
<th>LMP10</th>
<th>MB-1</th>
<th>Zeta</th>
<th>Delta</th>
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**NOTE:** Semiquantitative analysis of neuroblastoma cells as assessed by immunohistochemistry according to the following score: −, <25%; +, 25% to 75%; ++, >75% cells occupying the microscopic area.

Abbreviation: ND, not done.
The percentage of stained tumor cells in each lesion was evaluated independently by two investigators. The variation between the results obtained by the two investigators was <10%. Results were scored as negative, heterogeneous, and positive, when the percentage of stained tumor cells in each microscopic area was <25%, between 25% and 75%, and more than 75%, respectively (7). The assignment of each tumor sample to one of the above scores was based on the score of the microscopic area containing the highest number of stained tumor cells.

Figure 1. APM components expression in medulloblastoma primary tumors. A, MB-1, delta, zeta, LMP10, ERp57, and TAP2 expression in primary medulloblastoma tumors. Immunoperoxidase staining of formalin-fixed, paraffin-embedded primary Mb tumors. One representative staining for each mAb is shown: anti–MB-1 (a), anti-delta (b), anti-zeta (c), anti-LMP10 (d), anti-ERp57 (e), and anti-TAP2 (f) mAb. Arrows, tumor cells stained by the above mAb. Original magnification, ×40. B, calnexin, calreticulin, and tapasin expression in primary Mb tumors. One representative example for each mAb is shown: anti-calnexin (a), anti-calreticulin (b), anti-tapasin (c) mAb. Arrows, tumor cells stained by the above mAb. Original magnification, ×40. C, α2-microglobulin–free HC, α2-microglobulin, LMP2, and LMP7 expression in astrocytic tumors. One representative case of pilocytic astrocytoma stained with each mAb is shown: anti–α2-microglobulin–free HC (a), anti–α2-microglobulin (b), anti-LMP2 (c), and anti-LMP7 (d). Arrows, tumor cells stained by the above mAb. Original magnification, ×40.
Flow-cytometry analysis of cell lines. The intracellular staining of cell lines with mAb was done as described (21). Briefly, cells were fixed with 2% paraformaldehyde at room temperature for 20 min, washed, and resuspended at 5 × 10^5/mL in PBS containing 0.5% BSA for microwave treatment at 200 W power for 45 s. Cells were then chilled on ice for 10 min, washed twice with staining buffer, and incubated in permeabilization buffer treatment at 200 W power for 45 s. Cells were then chilled on ice for 10 min, and the MFI of cells stained with isotype-matched mouse Ig was expressed as mean relative fluorescence intensity (MRFI), i.e., the ratio between the amount of primary mAb and with an optimal amount of FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG antibodies at room temperature for 30 min. Cells were finally washed twice in permeabilization buffer and resuspended in staining buffer before being analyzed by flow cytometry using a FACSscan instrument (BD Biosciences).

For surface staining, cells were sequentially incubated with an optimal amount of primary mAb and with an optimal amount of FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG antibodies. Stained cells were analyzed by flow cytometry. Isotype- and subclass-matched mouse Ig were used as negative controls in all the experiments. Cell Quest software (BD Biosciences) was used for data analysis. The results of flow cytometry experiments are expressed as mean relative fluorescence intensity (MFI), i.e., the ratio between the mean fluorescence intensity (MFI) of cells stained with the selected mAb and the MFI of cells stained with isotype-matched mouse Ig.

Mb cell mRNA extraction and DC transfection. mRNA was extracted from DAOI and D283 Mb cell lines using the mRNA Isolation Kit (Roche Diagnostics) according to the manufacturer’s protocol and stored at −80°C until use. DC were generated from peripheral blood mononuclear cells as described (22). DC transfection was done using a nonlipid cationic reagent (Transfectam reagent; Promega) as described (22). Mature DC were collected, washed twice in PBS (Sigma), and resuspended in X-VIVO medium. DC (1 × 10^5/500 μL) were then added to the transfection mixture. Following 45 min incubation at 37°C, DC were washed twice in PBS, resuspended in the maturation cocktail, and cultured for an additional 24 h before being used.

CTL generation. CD8+ T-cell suspensions were purified (>90% purity) from peripheral blood mononuclear cells (PBMC) of normal donors by immunomagnetic enrichment with CD8 MicroBeads (Miltenyi Biotec GmbH). CD8+ T cells (70,000 cells per well) were cultured in 96-well plates (Corning Incorporated) at a 10:1 ratio with autologous transfected DC in RPMI 1640 supplemented with 10% heat-inactivated human AB serum and 0.1% saponin (Sigma) at room temperature for 30 min. Cells washed twice with staining buffer, and incubated in permeabilization buffer, and incubated with FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG antibodies at room temperature for 30 min. Cells were then chilled on ice for 10 min, washed twice in permeabilization buffer and resuspended in staining buffer before being analyzed by flow cytometry using a FACSscan instrument (BD Biosciences).

Table 3. Expression of HLA class I antigen-processing molecules in pediatric Mb versus noninfiltrating astrocytic tumors

<table>
<thead>
<tr>
<th>Lesions</th>
<th>HLA-ABC*</th>
<th>Heavy chain (%)</th>
<th>β2-Microglobulin (%)</th>
<th>Tapasin (%)</th>
<th>Calnexin (%)</th>
<th>Calreticulin (%)</th>
<th>Erp57 (%)</th>
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<tbody>
<tr>
<td>Medulloblastoma</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td>20 (2/10)</td>
<td>30 (3/10)</td>
<td>20 (2/10)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>Noninfiltrating</td>
<td>9 (1/11)</td>
<td>91 (10/11)</td>
<td>70 (7/10)</td>
<td>55 (5/9)</td>
<td>91 (10/11)</td>
<td>55 (6/11)</td>
<td>91 (10/11)</td>
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</table>

*Results are expressed as percent positivity. Values in brackets indicate the number of tumors positive for each antigen-processing molecule in relation to the total number of cases tested.

Results

HLA class I APM component expression in pediatric Mb tumors. Table 2 summarizes the results obtained by immunoperoxidase staining of 10 primary pediatric Mb lesions with the panel of APM component-specific mAb. Microscopic areas containing more than 90% tumor cells were selected for the assessment of APM component expression. The delta, MB-1, and zeta housekeeping proteasomal subunits, the LMP10 immunoproteasomal subunit, the ATP-dependent peptide transporter TAP2, and the chaperone molecule ERp57 were detected in the majority of the Mb (Fig. 1A; Tables 2 and 3), as well as astrocytic tumor (Tables 2 and 3), lesions.

Comparison of calnexin, calreticulin, and tapasin expression in Mb and astrocytic tumors showed that only calnexin had a significantly lower expression in the former than in the latter tumors (χ^2 test, P = 0.001; Fig. 1B; Tables 2 and 3).

The immunoproteasomal subunits LMP2 and LMP7, β2-microglobulin–free HC, and β2-microglobulin were not detected in any of the Mb lesion tested, but were intensely expressed in astrocytic tumors (Fig. 1C; Tables 2 and 3).

Finally, HLA-ABC molecules were not detected either in Mb or astrocytic tumors (Tables 2 and 3). Endothelial cells in each tissue section were stained by all the mAb tested, thus serving as positive internal controls (data not shown).

APM component expression in a normal fetal cerebellum. To define the APM component expression profile in a postulated
normal counterpart of Mb, fetal cerebellum was stained with APM component-specific mAb in the immunoperoxidase reaction.

Figure 2A–D shows the staining patterns of the cerebellar cortex (composed of four layers, external granular, molecular, middle Purkinje, and internal granular) obtained with mAb recognizing HLA-ABC, β2-microglobulin–free HC, β2-microglobulin, tapasin, calnexin, calreticulin chaperons, MB-1, delta, zeta housekeeping proteasomal subunits, LMP2, LMP7, LMP10 immunoproteasomal components, ERp57 thiol-reductase, and TAP2 subunit. The staining intensity of most cerebellar cortex cells was strong for calnexin (Fig. 2B-b), calreticulin (Fig. 2B-c), MB-1 (Fig. 2C-a, delta (Fig. 2C-b), zeta (Fig. 2C-c), LMP2 (Fig. 2C-d), LMP7 (Fig. 2C-e), ERp57 (Fig. 2D-a), TAP2 (Fig. 2D-b), β2-microglobulin–free HC (Fig. 2D-a), tapasin (Fig. 2D-a), and LMP10 (Fig. 2D-f) were expressed with variable intensity only in some Purkinje cells.

Surface HLA-ABC (Fig. 2A-a) and β2-microglobulin (Fig. 2A-c) were not detected in any cerebellar cortex cell. In contrast, endothelial cells were consistently stained by anti–HLA-ABC surface (Fig. 2A-c), and β2-microglobulin (Fig. 2 inset A-c) mAb.

These results suggest that the absence of β2-microglobulin–free HC, tapasin, calnexin, calreticulin, LMP2, LMP7, and TAP2 in Mb lesions are related to malignant transformation.

Expression of HLA class I–related APM components in Mb cell lines. APM component expression was next investigated in the human Mb cell lines DAOI and D283, differing in cell surface expression of HLA-ABC molecules, that are detected in DAOI, but not of D283 Mb cell lines. Figure 3A shows the mean ± SE of the MRFI values obtained by cytofluorometric analysis of DAOI and D283 Mb cells stained with APM component-specific mAb.

Delta, calnexin, calreticulin, TAP2, β2-microglobulin–free HC, and β2-microglobulin were expressed in DAOI cells, with MRFI values ranging from 2.5 to 16 (Fig. 3A). MB-1, zeta, LMP2, LMP7, LMP10, tapasin, and ERp57 expression was low to virtually undetectable, with MRFI values ranging from 1 to 2.5 (Fig. 3A). Similar results were obtained from the analysis of D283 Mb cell line with the exception of β2-microglobulin–free HC, β2-microglobulin, zeta, and TAP2 in which expression was low to undetectable (MRFI values ranging from 1 to 2.5; Fig. 3A).

To determine whether the expression of APM components in the two Mb cell lines was modulated by IFNγ, cells were incubated with IFNγ (1,000 IU/mL) for 48 h at 37°C (14). In DAOI cells, up-regulation of β2-microglobulin–free HC, β2-microglobulin, zeta, TAP2, and surface HLA-ABC molecules was consistently detected (Fig. 3A).

IFNγ-treated D283 cells did not show up-regulation of any APM component, with the exception of calnexin (Fig. 3A). In addition, de novo induction of surface HLA class I expression was observed in the same cells (Fig. 3A).

Finally, DAOI and D283 cells tested negative for CD40, CD80, and CD86 costimulatory molecules (data not shown).

Antigen-presenting cell functions of Mb cell lines. CD8+ T-cell populations were purified from PBMC of normal donors. For CTL priming, CD8+ lymphocytes underwent four weekly cycles of stimulation with autologous DC transfected with pooled DAOI and D283 Mb cell line mRNA and were then expanded in medium containing human rIL-15 before being characterized.

The ability of in vitro expanded CTL to recognize DAOI and D283 cells was investigated by IFNγ ELISPOT and 51Cr release cytotoxic assays using IFNγ-treated or untreated Mb cell lines as targets. Figure 3B shows that CD8+ T cells from an HLA-A2+ normal subject contained tumor-specific T cells secreting IFNγ in response to HLA-matched DAOI and D283 cells. The frequency of specific spots in the CTL populations shown in Fig. 3B ranged from 58 to 60 IFNγ spots per 30,000 blasts against DAOI cell line, 50 to 53 IFNγ spots per 30,000 blasts against D283 cell line, 89 to 93 IFNγ spots per 30,000 blasts against IFNγ-treated DAOI cell line, and 67 to 70 IFNγ spots per 30,000 blasts against IFNγ-treated D283 cell line. IFNγ secretion by CTL was significantly down-regulated when target cells were preincubated with HLA class I antigen-specific mAb (TP25.99), but not with an isotype-matched irrelevant mAb, before being tested in the ELISPOT assay (Fig. 3B).

CTL lysed HLA-matched IFNγ-treated or untreated DAOI Mb cells at different E/T ratios (Fig. 3C). As expected, pretreatment of DAOI cell line with IFNγ enhanced the specific lysis of target cells, especially at low E/T ratios (from 25:1 to 3:1; Fig. 3C). Cell lysis was significantly reduced by the addition of HLA class I–specific mAb, but not of an isotype-matched irrelevant mAb (Fig. 3D). Likewise, D283 cells were lysed by Mb-specific CTL only after IFNγ treatment, but with lower efficiency (data not shown).

Taken together, these results indicate that Mb-reactive CTL can be generated in vitro from normal subjects upon incubation with autologous DC transfected with tumor cell–derived mRNA. Tumor cell recognition by CTL implies that endogenous tumor-associated antigen (TAA)–derived peptides are presented as HLA class I antigen-peptide complexes on Mb cell surface.

Discussion

This is the first report on the expression of HLA class I–related APM in pediatric Mb. Abnormalities of APM component expression have been described in many malignant tumor cells such as melanoma, ovarian carcinoma, squamous head and neck carcinoma, and in three embryonal tumors, i.e., neuroblastoma, embryonal carcinoma, and invasive retinoblastoma (3, 7–9, 14, 23).

Here, we show that multiple defects in the expression of HLA class I–related APM components are present in an embryonal malignancy of the central nervous system (CNS), i.e., pediatric Mb, but not in pediatric noninfiltrating astrocytic tumors, tested as a model of well-differentiated CNS neoplasia. Thus, the LMP2 and LMP7 immunoproteasomal components, the calnexin chaperon, β2-microglobulin–free HC, and β2-microglobulin were down-regulated or undetectable

Table 3. Expression of HLA class I antigen-processing molecules in pediatric Mb versus noninfiltrating astrocytic tumors (Cont’d)

<table>
<thead>
<tr>
<th>LMP2 (%)</th>
<th>LMP7 (%)</th>
<th>LMP10 (%)</th>
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<th>Zeta (%)</th>
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Figure 2. APM components expression in a normal cerebellum. Immunoperoxidase staining of a formalin-fixed, paraffin-embedded normal cerebellum. One representative staining for each mAb is shown. A, HLA-ABC (a), ß2-microglobulin-free HC (b), and ß2-microglobulin (c). B, tapasin (a), calnexin (b), and calreticulin (c). C, MB-1 (a), delta (b), zeta (c), LMP2 (d), LMP7 (e), and LMP10 (f). D, ERp57 (a) and TAP2 (b). Arrows, cerebellum cells stained by the above mAb. Positive staining for the anti-HLA-ABC (A-a) and ß2-microglobulin (inset A-c) mAbs is detected in endothelial cells (arrowheads), but not in cerebellum cells (arrows). Original magnification, ×40. Inset, original magnification, ×63.
in Mb lesions, but were consistently expressed in astrocytic tumors. The latter finding has been already described in adult astrocytoma lesions, where HLA class I antigen down-regulation only correlated significantly with tumor grade (24). In other tumor models, defects of APM components expression have been correlated to tumor progression and/or clinical course (7–9, 25, 26). The differences in the expression of HLA class I APM components in Mb versus astrocytic tumors reported in the present study may be attributable to the highly immature stage of differentiation of the former tumor.

APM component expression was also investigated in normal fetal cerebellum, from which Mb is supposed to originate. These experiments showed that all APM components, but not HLA class I molecules, were expressed in fetal cerebellum, supporting the conclusion that the down-regulation of LMP2, LMP7, calnexin, β2-microglobulin–free HC, and β2-microglobulin in Mb lesions is associated with malignant transformation. Notably, in this respect, neurons and, to a lower extent, white matter astrocytes from normal mice were found to express LMP2 and LMP7 (27).

Staining of the DAOI and D283 Mb cell lines with APM component–specific mAb revealed some differences in comparison with Mb primary tumors. In particular, MB-1, LMP10, and ERp57 were detected in the latter tumors but not in cell lines. In addition, DAOI, but not D283 cells, expressed β2-microglobulin, β2-microglobulin–free HC, and surface HLA-ABC molecules that were never detected in primary tumor cells. These differences may be related to the selection of tumor subclones during the establishment of neuroblastoma cell lines and/or to changes in the antigenic profile caused by long-term culture. An alternative possibility is that immunohistochemical analysis done with primary tumors was less sensitive than flow cytometry and, therefore, unable to detect, for example, low-level HLA class I expression.

The poor prognosis of Mb patients has fostered the search for novel therapeutic approaches, among which immunotherapy...
has raised interest. Here, we investigated the role of DC, transfected with Mb-derived mRNA, in generating Mb-specific CTL. This strategy has been described in metastatic prostate carcinoma and neuroblastoma, in which tumor mRNA-transfected DC were found to stimulate TAA-specific CTL responses (22, 28).

Mb mRNA-transfected DC promoted the in vitro expansion of CTL that released IFNγ upon incubation with either Mb cell line and, most importantly, lysed the same cells in an HLA class I–restricted manner. Notably, inhibition of cytotoxicity by anti–HLA class I mAb, although significant, was less effective that that of IFN-γ release. This difference is likely related to intrinsic features of the two assays, but the possibility that CTL-mediated NK-like activity had a minor role in tumor cell killing cannot be completely excluded.

Our results show that surface HLA class I molecules on Mb cell lines are functional, and that the latter cells can behave as APC, presenting endogenous HLA class I–restricted peptides derived from TAA to CTL.

These findings suggest that the numerous defects in the expression of HLA class I–related APM components, detected in Mb cell lines, do not affect the generation and expression of HLA class I–peptide complexes on the cell surface required for the recognition of target cells by CTL (6).

Alternatively, as yet, poorly elucidated pathways of antigen processing and peptide generation may allow intracellular trafficking and surface expression of immunogenic HLA class I–peptide complexes. The latter possibility is supported by the results of other studies (29, 30) in which TAP-independent mechanisms of peptide loading on HLA class I molecules and presentation of peptide/HLA class I complexes to T cells have been characterized.

In summary, this study provides the first description of HLA class I–related APM component defects in pediatric Mb, leading to the unexpected conclusion that Mb cells can present tumor–associated antigens to CTL. These findings may pave the way to future development of T cell immunotherapy of Mb using autologous tumor-specific T cell.

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