NKG2D-Mediated Antitumor Activity by Tumor-Infiltrating Lymphocytes and Antigen-Specific T-Cell Clones Isolated from Melanoma Patients

Cristina Maccalli,1 Daisuke Nonaka,2 Adriano Piris,2 Daniela Pende,3 Licia Rivoltini,1 Chiara Castelli,1 and Giorgio Parmiani1

Abstract

Purpose: The role of NKG2D receptor in antitumor immnosurveillance has not been completely clarified. We addressed this issue by investigating the involvement of this receptor in tumor-specific immunologic response in melanoma patients.

Experimental Design: We determined the presence of NKG2D+ T cells among tumor-infiltrating lymphocytes (TIL) of 10 (one primary and 9 metastatic) melanoma samples and the expression of NKG2D ligands (NKG2DL) by these tumor cells. Moreover, the expression of NKG2D was assessed in a panel of antigen-specific T lymphocytes isolated from melanoma patients and the engagement of NKG2D in antitumor activity mediated by these T cells was determined.

Results: TILs located either in the periphery or within the tumor mass of melanoma samples expressed NKG2D and the expression of this receptor by T cells was retained after in vitro culture. However, NKG2DLs were weakly expressed, or not expressed, by most metastatic lesions with only the primary tumor being positive for all these molecules. In contrast, these ligands were expressed, although heterogeneously, by all in vitro established melanoma lines. Moreover, the engagement of NKG2D occurred in antitumor activity by both freshly isolated and in vitro cultured TILs. However, this receptor was involved to a different extent in the antitumor activity of antigen-specific T-cell clones.

Conclusions: These findings indicate that NKG2D+ T cells have a role in the immunologic response against tumor. Thus, new immunotherapeutic treatments for melanoma patients should be designed aimed at augmenting the NKG2D+ T lymphocyte–mediated immune response.

T lymphocytes recognize tumor-associated antigens (TAA) expressed as peptides in the context of human leukocyte antigen (HLA) molecules on the cell surface of tumor cells although costimulatory signals are also required to achieve their full activation state. The NKG2D receptor, expressed by natural killer (NK), α/β+ T, and γ/δ+ T lymphocytes (1–4), has been recently identified as a relevant component of this pathway and its ligands (NKG2DL), the stress-inducible MHC class I–related molecules (MICA, MICB, and ULBP), have been shown to be expressed by many human tumors (5–12). In mice, NKG2DL expression by tumor cell stimulates antitumor reactivity by NK or CD8+ T cells and ectopic expression of these molecules causes the in vivo rejection of tumors by NK and/or CD8+ T cells (13, 14). By engagement of NKG2D receptor, human NK and γ/δ+ T lymphocytes lyse tumor cells expressing NKG2DLs (4, 6–10, 12, 15–17); moreover, this receptor seems to play a relevant role as costimulatory molecule, after the triggering of T-cell receptor (TCR), in α/β+ T cells (17). The engagement of NKG2D can either elicit antitumor reactivity or strengthen TCR-mediated antigen stimulation by T-cell clones isolated from the peripheral blood of colon carcinoma patients (18). Moreover, the evidence that either CD3+ and/or CD56+ NKG2D+ cells were found in tumor-infiltrating lymphocytes (TIL) of human melanoma patients suggests that these cells could represent a relevant T-cell subpopulation for the immune response against cancer, although the antitumor effector function of these lymphocytes was not investigated (11).

Several studies also showed that in the blood of cancer patients whose tumor cells express NKG2DLs, soluble form of these molecules could be detected (19–21) that, in some cases, correlated with the prognosis of the disease (21–25). Moreover, a soluble form of NKG2DL can be released in vitro by tumor cells by a common cleavage process mediated by...
metalloproteases, and this represents one of the immune evasion mechanisms of tumor cells leading to the impairment of immunosurveillance by T and/or NK cells (19, 20, 26–28). Although in the last decade, a variety of studies aimed at the functional characterization of NKG2D have been undertaken, the role of this receptor as stimulatory or costimulatory molecule in T cell–mediated antitumor response is not established. The aim of the present study was to evaluate whether TILs from melanoma patients express NKG2D and whether this receptor can mediate antitumor reactivity. We observed that TILs from melanoma patients commonly expressed NKG2D and that the engagement of this receptor together with TCR occurred in tumor recognition mediated by these lymphocytes. However, a heterogeneous usage of NKG2D was observed in tumor-reactive T-cell clones, suggesting that a complex regulatory pathway governs the engagement of multiple activation receptors in antitumor immunosurveillance. In addition, we found that NKG2D+ T cells infiltrate melanoma lesions, inasmuch as only the primary lesion expressed MICA/B and ULBP1, ULBP2, ULBP3 molecules, suggesting that the selection of tumor cell variants failing to express NKG2D ligands can occur during the progression of the disease.

Materials and Methods

Tissues and cells. Tumor lesions and TILs were isolated from primary (n = 1) or stage III (n = 6) or stage IV (n = 3) melanoma patients admitted to the Istituto Nazionale Tumori, Milan, Italy. Peripheral blood mononuclear cells (PBMCs), TILs, and tumor cells were obtained from one additional stage III patient (15392). T lymphocytes were cultured in vitro in the presence of 100 or 300 IU/mL of recombinant human interleukin 2, in RPMI (Biowhittaker) plus 10% human serum. The melanoma-specific T-cell clones A1 and B1 (representing short-term in vitro cultured T-cell clones, “new clones”) were generated from CD8+ T cell enriched by CD4+ T-cell depletion and from TILs of patient 7 (HLA-A1, 30; HLA-B13, 35; HLA-CW04, 06; DR6*10701, 1035). T-cell clones were isolated by limiting dilution in the presence of allogeneic irradiated (50 Gy) PBMCs and 30 ng/mL of OKT3 monoclonal antibody (mAb) in RPMI 1640 plus 10% human serum. The melanoma-specific T-cell clones were cultured T-cell clones (representing long-term in vitro cultured T-cell clones, “old clones”) were also used: the gp100-specific TB254 and the PTPRK-specific TB515 T-cell clones were derived from TILs of the patient 15392 (29–31); the Melan-A/MART-1-specific A42 clone isolated from TIL (32); the gp100-specific D4F12 clone generated from TIL 620 (33); and the β-cat-kinin–specific G4G10 T-cell clone isolated from TIL 1541 (34). Tumor cell lines, EBV-transformed B cells line 1869 and 15392, and the HLA-A2 0201–deficient T2 lymphoma cell line (35) were cultured with RPMI 10% fetal bovine serum (Biowhittaker) at 37°C 5% CO2. The MHC class I and class II typing of the PBMCs and tumor lines used in this study was done by single-stranded oligonucleotide probe-PCR typing (36).

Immunohistochemical analysis. Immunohistochemical analysis of consecutive sections from frozen tissues was done as previously described (37). Sections were incubated with normal goat serum (1:50; DAKO Corp.) diluted in PBS containing 1% bovine serum albumin for 30 min. Primary antibody incubation was done overnight at 4°C with the following antibodies: anti-NKG2D M580, anti-MICA M673, anti-MICB M562, anti-ULBP1 M295, anti-ULBP2 M310, and anti-ULBP3 M551 (all from Amgen); anti-TCR ε/F1 (Endogen); and anti-CD3 Leu4 (Becton Dickinson) was used after 0.1% trypsin treatment for 5 min as described for antigen unmasking. Tissue sections subjected to the same treatment but without incubation with primary antibody were used as negative controls. Positive controls were represented by T-cell clones reactive for CD3, TCR α/β, and NKG2D (18) or by tumor cell lines expressing NKG2D (18). Stained sections were analyzed by Zeiss Axiostar 100 microscope. Lymphocytes in TILs were counted after acquiring digital images of 10 areas of tissue sections each including tumor tissue. Images of the corresponding fields stained with anti-CD3 or TCR α/β mAb in serial sections were used to count intratumoral and extratumoral CD3+ or CD3+ TCR α/β cells. NKG2D+ T cells were defined as no NKG2D signal (−, <5% of positive tumor cells), with weak signal (+, 5–50% of positive tumor cells), with moderate signal (+++, 50–95% of positive tumor cells), or with strong signal (+++, >95% of positive tumor cells).

Flow cytometry analysis. The expression of MICA, MICB, and ULBP molecules by the tumor lines was determined by flow cytometry and fluorescence-activated cell sorting (FACScan, Becton Dickinson) analysis using the following mAbs: anti-MICA BAM195 supernatant generated by one of us (D.P.) or the previously described purified antibodies provided by Amgen. The expression of NKG2D by antitumor T lymphocytes was similarly evaluated by using BAT221 mAb (10) supernatant. The expression of class I and class II HLA molecules was determined by the use of mAb W6/32 and L243 (BD Pharmingen), respectively. The phycoerythrin-conjugated goat anti-mouse IgGs (DAKO Corp.) were used for flowchrome staining of the mAbs used. The phenotype characterization of T-cell lines or of the antimelanoma T-cell clones was done by immunofluorescence and cytofluorimetric analysis with the FITC-conjugated anti-CD3, anti-CD4, anti-CD8 mAbs, or phycoerythrin-conjugated anti-CD16 and anti-CD56 mAbs (BD Pharmingen). Results are expressed as MRFI, representing the ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins (38).

Evaluation of antitumor activity by T cells. The reactivity of the T lymphocytes against tumor cell lines or defined TAAs was determined by IFN-γ secretion assay. T cells were incubated (5 × 10^4–5 × 10^5/well) in flat-bottomed 96-well plates in the presence of 5 × 10^5 to 5 × 10^6/well of tumor cell lines or either T2 cells or EBV-B cells preincubated with 10 μg/mL of TAA-derived peptide (MART1-127-29, or gp100) or purchased from NeoMPS; gp100 polypeptides provided by Dr. P.F. Robbins). After 18 h of incubation at 37°C and 5% CO2, the supernatants were collected and the IFN-γ released by T cells was evaluated by ELISA (anti-IFN-γ coating mAb, M700-A,E, and anti–IFN-γ biotinylated mAb M701-B, Endogen). The specificity of T lymphocytes was assessed by inhibition of IFN-γ release after preincubation of the target cells for 45 min with 10 μg/mL each of the anti-HLA class I mAb W6/32; the anti-HLA class II (DR) mAb L243; the anti-HLA A, B, C; M840.5 mAb; the anti-ULBP1, 2, 3; mAbs M295, M310, and M551; or with 50 μL/well of the anti-MICA mAb BAM195. The inhibition of IFN-γ release was also evaluated following the preincubation of the T lymphocytes with 50 μL/well of the anti-NKG2D mAb BAT221 or with 10 μg/mL of anti-TCR α/β (TCR1043, Endogen). The assays were carried out in triplicates and the statistical analysis of differences between means for cytokine release assay was done using two-tailed t test.

Results

NKG2D+ T cells infiltrate melanoma lesions. The presence of NKG2D+ T lymphocytes in 10 melanoma lesions (one primary tumor and nine metastases) obtained from patients admitted to the Istituto Nazionale Tumori was evaluated by immunohistochemical technique on cryopreserved tissues. All the analyzed melanoma lesions were infiltrated by CD3+ T lymphocytes (data not shown) and, as shown in Table 1, these T cells strongly expressed TCR α/β. Moreover, TILs located either in
the periphery (out) or within the tumor mass (in) were commonly stained with the antibody specific for NKG2D. In four melanoma lesions, NKG2D+ T lymphocytes represented >50% of the infiltrating lymphocytes either from the “out” or “in” part of the tumor; whereas in six cases, T lymphocytes expressed NKG2D to a variable extent both in the “out” and in the “in” part of melanoma lesions. Of note, 5 of 10 TILs from the “in” part of the tumors expressed higher level of NKG2D than from the “out” part, whereas the opposite behavior was observed in two cases. Interestingly, the expression of NKG2D+ T cells was consistent both in the “in” and in the “out” part of two different fragments of the metastatic lesions deriving from the melanoma patient 263516-1 (263516-1 or 263516-1-2; see Table 1), indicating that TILs expressing NKG2D infiltrated

Table 1. Expression of NKG2D by TILs from melanoma lesions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Melanoma stage/metastasis</th>
<th>Anatomic site</th>
<th>Lymphocytes</th>
<th>TCRα/β</th>
<th>NKG2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>263487I</td>
<td>I</td>
<td>Primary tumor</td>
<td>Out</td>
<td>71</td>
<td>55</td>
</tr>
<tr>
<td>263109I</td>
<td>III/L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>65</td>
<td>39</td>
</tr>
<tr>
<td>263393I</td>
<td>IV/s.c., L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>82</td>
<td>13</td>
</tr>
<tr>
<td>263516I-1</td>
<td>III/L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>76</td>
<td>73</td>
</tr>
<tr>
<td>263516I-2</td>
<td>III/L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>76</td>
<td>63</td>
</tr>
<tr>
<td>263851I</td>
<td>IV/s.c., L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>96</td>
<td>64</td>
</tr>
<tr>
<td>321790I-8</td>
<td>III/s.c.</td>
<td>Metastatic s.c.</td>
<td>In</td>
<td>86</td>
<td>55</td>
</tr>
<tr>
<td>281219I-7</td>
<td>III/L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>291760I-9</td>
<td>IV/s.c., L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>96</td>
<td>28</td>
</tr>
<tr>
<td>23954I-10</td>
<td>III/L.N.</td>
<td>Metastatic L.N.</td>
<td>Out</td>
<td>77</td>
<td>62</td>
</tr>
</tbody>
</table>

NOTE: Melanoma samples 263516I-1 or 263516I-2 are two different fragments isolated from the same metastatic lesion.
Abbreviation: L.N., lymph node.
*Description of metastatic anatomic sites detected at the time of tissue collection for stage III or IV melanoma patients.
† Tumor lesion used for immunohistochemical analysis.
‡ Lymphocyte infiltration was divided into brisk (in) and nonbrisk (out) groups. Out, lymphocytes outside of (in the periphery of) tumor mass. In, lymphocytes within the tumor mass.
§ Results are expressed as percentage of positive cells.

Fig. 1. Expression of NKG2D by lymphocytes infiltrating melanoma lesions and of NKG2DLs by tumor cells. Immunohistochemical analysis of sections of the primary melanoma lesion. A, staining with mAbs to T-cell markers of TIL containing a brisk infiltrate of CD3+ TCRα/β+ and NKG2D+ lymphocytes. B, expression of MICA, MICB, and ULBP1, ULBP2, ULBP3 by melanoma cells (263487I). Each column of panels shows consecutive sections stained with the indicated markers. Original magnification, × 20.
homogeneously melanoma lesions. **Figure 1A** shows results of immunohistochemical staining of the primary melanoma lesions strongly infiltrated by CD3⁺, TCRα/β⁺, and NKG2D⁺ T lymphocytes; similar staining was obtained for metastatic lesions (data not shown).

On the contrary, no NKG2D⁺ CD3⁺ lymphocytes infiltrated the epidermis and the mesenchymal components of normal skin or tissue deriving from either primary or s.c. metastasis of melanoma (263487I and 321790I; data not shown). Similarly, NKG2D⁺ lymphocytes were detected only in the lymph node invaded by melanoma cells and not in the normal tissue, suggesting that a recruitment of T lymphocytes expressing NKG2D occurred at tumor site of melanoma patients (data not shown).

TILs were isolated from melanoma samples of patients 7, 9, and 10 and cultured in vitro for 3 days in the presence of low doses (100 IU) of recombinant IL-2. The phenotype evaluation (Table 2) done by immunofluorescence and cytofluorimetric analysis showed that TIL 7 included both CD4⁺ and CD8⁺ T cells and that significant expression of NKG2D was observed mostly in CD8⁺ cells. Similar results were obtained for TIL 9 and 10 (Table 2). However, few CD4⁺ T cells expressing NKG2D were found in TIL 9 and TIL 10 as determined by double-staining analysis. These results were confirmed by the evidence that the expression of NKG2D by long-term in vitro cultured TAA-specific T lymphocytes from one melanoma patient (15392 TILs and PBMCs) was mainly associated with CD8⁺ T cells. PBMCs from healthy donors (n = 3) have been used as control of NKG2D staining of T lymphocytes (Table 2). Taken together, these results indicate that melanoma lesions are commonly infiltrated by NKG2D⁺ T lymphocytes and that this receptor is mainly expressed by CD3⁺ and TCRα/β⁺ T-cell subpopulations. Furthermore, the expression of NKG2D by freshly in vitro isolated TILs or by a long-term in vitro cultured TAA-specific TILs or PBMCs was preferentially associated with CD8⁺ T lymphocytes.

Expression of NKG2D by antitumor T-cell clones from melanoma patients. The expression of NKG2D was assessed, as reported in Table 3, by immunofluorescence and cytofluorimetric analysis, in melanoma-specific T-cell clones isolated from TILs of tumor patients. Clone B1 was generated from TIL 7 and homogeneously expressed CD8 and NKG2D. Interestingly, clone A1 was CD4⁺ NKG2D⁺, deriving from few CD4⁺ T lymphocytes remaining in the in vitro cultured TIL 7 after immunomagnetic enrichment of CD8⁺ T cells; on the contrary, the CD4⁺ TB515 T-cell clone directed to the PTPRK melanoma-associated antigen (31) failed to express NKG2D. The expression of NKG2D was also analyzed in a panel of previously isolated antigen-specific CD8⁺ T-cell clones (29, 30, 32–34). Both the anti-gp100 TB254 and D4F12 clones, generated from

**Table 2. Expression of NKG2D by TILs or PBMCs isolated from melanoma patients**

<table>
<thead>
<tr>
<th>T cells</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4/NKG2D</th>
<th>CD8/NKG2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TILs pt. 7</td>
<td>16 (70)</td>
<td>11 (34)</td>
<td>26 (38)</td>
<td>1</td>
<td>6 (37)</td>
</tr>
<tr>
<td>TILs pt. 9</td>
<td>24 (71)</td>
<td>7 (32)</td>
<td>20 (26)</td>
<td>12 (10)</td>
<td>15 (21)</td>
</tr>
<tr>
<td>TILs pt. 10</td>
<td>28 (60)</td>
<td>10 (18)</td>
<td>12 (39)</td>
<td>2 (6)</td>
<td>4 (30)</td>
</tr>
<tr>
<td>TILs pt. 15392</td>
<td>18 (99)</td>
<td>14 (5)</td>
<td>37 (97)</td>
<td>1</td>
<td>3 (93)</td>
</tr>
<tr>
<td>PBMCs pt. 15392</td>
<td>17 (97)</td>
<td>22 (55)</td>
<td>21 (48)</td>
<td>1</td>
<td>4 (43)</td>
</tr>
<tr>
<td>PBMCs H.D.*</td>
<td>30 (99)</td>
<td>20 (38)</td>
<td>35 (62)</td>
<td>2 (5)</td>
<td>8 (59)</td>
</tr>
</tbody>
</table>

**NOTE:** Expression of T-cell markers was evaluated by immunofluorescence and cytofluorimetric analysis. Results are expressed as MRFI, representing the ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins; number in parentheses represents the percentage of positive cells.

*Mean of results obtained from three subjects has been represented.

**Table 3. Expression of NKG2D by melanoma-specific T-cell clones**

<table>
<thead>
<tr>
<th>T cells</th>
<th>Origin</th>
<th>Patient</th>
<th>TAA*</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>NKG2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>TILs</td>
<td>7</td>
<td>Unknown</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>6 (85)</td>
</tr>
<tr>
<td>A1</td>
<td>TILs</td>
<td>7</td>
<td>Unknown</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4 (63)</td>
</tr>
<tr>
<td>TB254</td>
<td>TILs</td>
<td>15392</td>
<td>gp100-71-78</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>5 (71)</td>
</tr>
<tr>
<td>D4F12</td>
<td>TILs</td>
<td>1200</td>
<td>gp100-209-217</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4 (57)</td>
</tr>
<tr>
<td>G4G10</td>
<td>TILs</td>
<td>888</td>
<td>bCat39-37mut</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>8 (80)</td>
</tr>
<tr>
<td>A42</td>
<td>TILs</td>
<td>501</td>
<td>MART-1-27-35</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>3 (33)</td>
</tr>
<tr>
<td>TB515</td>
<td>TILs</td>
<td>15392</td>
<td>PTPRK567-682</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

*TAAs recognized by T-cell clones isolated from melanoma patients.

† Expression of T-cell markers was evaluated by immunofluorescence and cytofluorimetric analysis. Results for CD3, CD4, and CD8 are defined as positive (+) or negative (−), whereas those for NKG2D are expressed as MFI, representing the ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins; number in parentheses represents the percentage of positive cells.
TIL 15392 and TIL 1200, respectively, were homogeneously CD8+ and mostly NKG2D+. Similarly, the anti-β-catenin G5G10 clone was positive for both CD8 and NKG2D, whereas the Melan-A/MART-1–specific clone A42 that homogeneously expressed CD8 was weakly positive for NKG2D. As expected, all these T-cell clones were homogeneously CD3 positive. These results indicate that antimelanoma CD8+ T-cell clones expressed NKG2D, although with different frequency, whereas only one (A1) of two CD4+ clones was NKG2D+.

Expression of NKG2DLs by melanoma lesions or in vitro established tumor cell lines. The expression of NKG2DLs by melanoma lesions was evaluated by immunohistochemistry using specific antibodies on cryopreserved tumor samples. As shown in Table 4, melanoma lesions expressed these molecules heterogeneously. ULBP2 was expressed with variable intensity by 5 of 11 melanoma samples; ULBP1 was detected in 3 of 11 melanoma samples, whereas two tumors expressed MICA. Only the primary melanoma expressed all the NKG2DLs, including MICB and ULBP3, as detailed in Table 4 and in Fig. 1B. Of note, no staining for NKG2DLs was observed by epidermal or mesenchymal cells of normal skin adjacent to primary melanoma lesion (data not shown). Similarly, normal tissue around s.c. metastasis failed to express NKG2DLs (data not shown), whereas these molecules were expressed by cutaneous tissues in the boundaries between neoplastic and normal regions of the s.c. lesions, although with preferential cytoplasmic pattern of staining. Along this line, NKG2DLs were not detected in area of metastatic lymph node not invaded by melanoma; moreover, positive staining for these molecules was limited to stromal components in the periphery of neoplastic

### Table 4. Expression of NKG2DLs by melanoma lesions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anatomic site*</th>
<th>NKG2DLs †</th>
<th>MICA</th>
<th>MICB</th>
<th>ULBP1</th>
<th>ULBP2</th>
<th>ULBP3</th>
</tr>
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<tr>
<td>263487I</td>
<td>Primary tumor</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>263109I</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>263393I</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>263595I</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>263516I-1</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>263516I-2</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>263851I</td>
<td>Metastatic L.N.</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>321790I-8mel</td>
<td>Metastatic s.c.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>281219I-7mel</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>291760I-9mel</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>239541I-10mel</td>
<td>Metastatic L.N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE:** Melanoma samples 263516I-1 or 2635161I-2 are two independent tissue fragments deriving from the same metastatic lesion.

†Tumor lesion used for immunohistochemical analysis.

### Table 5. Expression of NKG2DLs by tumor cell lines evaluated by immunofluorescence and cytofluorimetric analysis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MICA</th>
<th>MICB</th>
<th>ULBP1</th>
<th>ULBP2</th>
<th>ULBP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 mel</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5 (40)</td>
<td>1</td>
</tr>
<tr>
<td>9 mel</td>
<td>4 (98)</td>
<td>1</td>
<td>1</td>
<td>5 (25)</td>
<td>1</td>
</tr>
<tr>
<td>Me15392</td>
<td>10 (69)</td>
<td>1</td>
<td>1</td>
<td>5 (38)</td>
<td>1</td>
</tr>
<tr>
<td>501 mel</td>
<td>7 (90)</td>
<td>1</td>
<td>1</td>
<td>4 (13)</td>
<td>1</td>
</tr>
<tr>
<td>888 mel</td>
<td>14 (87)</td>
<td>1</td>
<td>1</td>
<td>7 (20)</td>
<td>7 (56)</td>
</tr>
<tr>
<td>1869 EBV-B†</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>15392 EBV-B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>K562</td>
<td>1</td>
<td>1</td>
<td>3 (15)</td>
<td>5 (43)</td>
<td>10 (66)</td>
</tr>
</tbody>
</table>

**NOTE:** 7 mel and 9 mel cell lines were in vitro established from melanoma lesions 281219I-7 and 291760I-9, respectively.

†The expression of MICA, MICB, ULBP1, ULBP2, and ULBP3 was evaluated by immunofluorescence and cytofluorimetric analysis with the following mAb, respectively: BAM195, M363, M295, M310, and M551 (see Materials and Methods). Values represent MRFI, that is the ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins; number in parentheses represents the percentage of positive cells.

† EBV-transformed B cells.
regions (data not shown). Interestingly, two samples (263516I-1 or 263516I-2, respectively) deriving from independent tissue fragments of one metastatic lymph node expressed similar levels of NKG2DLs (ULBP1 and ULBP2; Table 4), suggesting that tumor cells within melanoma lesions express these molecules homogeneously. Interestingly, ULBP2 was the NKG2DL more frequently detected in melanoma lesions (see Table 4) and, moreover, this molecule was expressed homogeneously at high level by melanoma cells (Fig. 1B).

The expression of NKG2DLs was analyzed by immunofluorescence and cytofluorimetric analysis in a panel of tumor cell lines (Table 5). The melanoma line 7 mel but not the corresponding tumor expressed ULBP2 (Table 4). The expression of ULBP2 and MICA was detected in the line 9 mel, deriving from a tumor lesion of patient 291760I, although the immunohistochemical analysis of this lesion (Table 4) showed significant expression only of ULBP2. These results suggest that the \textit{in vitro} culture conditions could promote the expansion of cells expressing NKG2D ligands and/or that immunohistochemistry could be less sensitive than the cytofluorimetric analysis in detecting markers not homogenously expressed by melanoma cells. The staining for NKG2DLs was also assessed in long-term \textit{in vitro} established cell lines (Table 5); three melanoma lines (Me15392, 501 mel, and 888 mel) expressed high levels of MICA and, although heterogeneously (38%, 13%, and 20% of positive cells, respectively), of ULBP2. Moreover, ULBP3 was expressed only by the melanoma line 888. EBV-transformed B-cell lines deriving from the melanoma patient 15392 or the colorectal cancer patient 1869, and the T2 cell line failed to express NKG2DLs. On the
contrary, expression of ULBP2, ULBP3, and, weakly, of ULBP1, but not of MICA/B was found in the NK target cell line K562. Taken together, these results suggest that NKG2D ligands were expressed heterogeneously by melanoma lesions and cell lines. Moreover, the expression of MICA and ULBP molecules was more frequently detected in \textit{in vitro} established melanoma cell lines than in \textit{in vivo} tumor lesions, suggesting that a negative selection of these molecules may occur \textit{in vivo}. This hypothesis is also supported by the evidence that only the primary tumor lesions expressed all the five NKG2D ligands, although these results need to be confirmed in a larger panel of primary melanoma samples. Furthermore, it should be noted that ULBP2 represents the NKG2DL detected at the highest level \textit{in vivo} and the most homogeneously expressed by cells within melanoma lesions. TILs and T-cell clones isolated from a melanoma patient can mediate an antitumor activity. The antitumor activity of TILs from patient 7 was evaluated by IFN-γ release following the incubation with autologous melanoma cells. As shown in Fig. 2A, significant cytokine release (9,000 IU/mL) by these T cells was observed and IFN-γ secretion was significantly inhibited after preincubation of target cells with the anti-HLA class I (W6/32), the anti-HLA class II (L243), or with the anti-NKG2DL (MICA or ULBP2) mAbs (see Material and Methods). T cells, as indicated, were preincubated in the presence of anti-NKG2D mAb (see Materials and Methods). The data shown represent averages of two independent experiments with SD of ±10%. Statistical analysis of differences between means of IFN-γ released by T cells have been done by two-tailed t test.

Fig. 3. TCR-specific and NKG2D-mediated recognition of the autologous melanoma by \textit{in vitro} cultured anti-gp100 T-cell lines isolated from patient 15392. T cells (2 × 10^5) were incubated with target cells (2 × 10^5) in a flat-bottomed 96-well plate in 250 μL final volume of AIMV plus 2% human serum. After 18 h, the supernatants were collected and the content of IFN-γ was evaluated by ELISA. Y axis, pg/mL of IFN-γ. Autologous Mel15392 and EBV-B cell lines were used as target cells. Where indicated, tumor cells were preincubated with the anti-HLA class I (W6/32), the anti-HLA class II (L243), or with the anti-NKG2DL (MICA or ULBP2) mAbs (see Material and Methods). T cells, as indicated, were preincubated in the presence of anti-NKG2D mAb (see Materials and Methods). The data shown represent averages of two independent experiments with SD of ±10%. Statistical analysis of differences between means of IFN-γ released by T cells have been done by two-tailed t test.
increased in the presence of anti-NKG2D mAb plus anti-L243 mAb or plus anti-TCR mAb, demonstrating that even in this case, specific tumor recognition was achieved by cooperation of NKG2D and TCR activation. This conclusion was reinforced by the lack of cytokine secretion by T cells following stimulation with allogeneic 888 melanoma, SW480, and K562 cell lines expressing NKG2DLs but not sharing class I HLA with patient 7. In addition, 7 mel recognition was affected by preincubation of tumor cells with anti-ULBP2 mAb and not with anti-MICA or with anti-ULBP3 mAbs, confirming that antigen-specific stimulation of NKG2D, consistent with the expression of NKG2DLs by melanoma cells, has occurred.

Taken together, these results show the functional role of NKG2D in antitumor activity by freshly isolated TILs or their T-cell clones and that cooperation of this receptor with the αβ TCR is necessary to achieve antitumor reactivity.

**Heterogeneous NKG2D engagement of long-term in vitro cultured TILs, PBMCs, or CD8+ T-cell clones directed against melanoma-associated antigens.** The objective of this part of our study was to see whether NKG2D triggering occurred in antitumor activity of long-term in vitro cultured T lymphocytes isolated from melanoma patients. Figure 3 shows the reactivity pattern of the anti–gp100-specific oligoclonal TILs (A) and of PBMCs stimulated in vitro with the gp100\textsubscript{71-78} peptide (B) from patient 15392 (29, 30). These T cells, expressing NKG2D (Table 2), recognized the autologous tumor by engagement of both TCR and NKG2D. In fact, IFN-γ was significantly released (727 or 1,500 pg/mL, respectively) after incubation of T cells with the autologous tumor and the reactivity was completely or partially inhibited after preincubation of target cells with the anti-HLA class I W6/32 or anti-NKG2D mAbs, respectively. Moreover, NKG2D-specific stimulation occurred in tumor recognition as confirmed by significant inhibition of IFN-γ secretion after preincubation of Me15392 cells with anti-MICA mAb. On the contrary, although the autologous melanoma line expressed also ULBP2 (see Table 5), no inhibition of cytokine secretion was found in the presence of the mAb specific for this molecule, indicating that only MICA induced functional NKG2D stimulation in the reactivity of 15392 TILs or PBMCs against the autologous tumor. As negative control, tumor cells were incubated with anti-HLA class II, anti-ULBP1, or anti-ULBP3 mAbs; in addition, these T cells failed to recognize the autologous EBV-B cells not expressing gp100 or NKG2DLs. It is noteworthy that TCR engagement alone by 15392 PBMCs (Fig. 3B) was sufficient to achieve specific recognition when the antigen was exogenously pulsed on EBV-B cells, whereas tumor recognition was mediated by both TCR and NKG2D, suggesting that a complex regulator pathway involving multiple receptors can govern the recognition by T lymphocytes of TAAs endogenously processed by melanoma cells.

Surprisingly, as described in Supplementary Fig. S1, the reactivity against the autologous tumor by the gp100-specific TB254 and D4F12 clones, isolated from in vitro cultured TILs of patients 15392 (29, 30) or from TIL 620 of the melanoma patient 1200 (33), respectively, was independent from NKG2D. In addition, this pattern of tumor-specific reactivity was observed for the Melan-A/MART-1–specific CD8+ T-cell clone A24 (data not shown), indicating that in the case of some “old” in vitro antigen–specific established T-cell clones, probably characterized by TCR with high affinity/avidity for TAAs, the cooperation of NKG2D with TCR does not represent an essential requirement for antitumor activity.

It is noteworthy that the reactivity against melanoma cells of the β-catenin–specific CD8+ T-cell clone G5G10 (34), as shown in Fig. 4, was HLA class I restricted and elicited by recognition of tumor cell line 888 and the allogeneic HLA-A24+ EBV-B cell line. As indicated, EBV-B cells were used as target cells were the autologous melanoma line 888 and the allogeneic HLA-A24+ EBV-B 1869 cell line. As indicated, EBV-B cells were preincubated, or not preincubated, with 10 μg/mL of β-catenin\textsubscript{29-37} peptide. Target cells were preincubated, as indicated, with the anti-HLA class I (W6/32) or the anti-NKG2DLs mAbs. When indicated, T cells were preincubated at 37°C for 45 min in the presence of anti-TCR and/or anti-NKG2D mAbs. Averages of two independent experiments with SD of ≤10%. Statistical analysis of differences between means of IFN-γ released by T cells have been done by two-tailed t test.
G5G10, suggesting that, in this model, engagement of both TCR and NKG2D could lead to more efficient reactivity.

Thus, these results indicate that TCR- and NKG2D-mediated tumor recognition occurred in both TILs and PBMCs isolated from one melanoma patients, whereas engagement of NKG2D, besides TCR, occurred heterogeneously in tumor reactivity of TAA-specific clones (Fig. 3; Supplementary Fig. S1).

**Discussion**

The present study shows that NKG2D+ T lymphocytes infiltrated all the analyzed (n = 10) melanoma samples, although to a variable extent. These TILs, although expressing NKG2D heterogeneously (Table 1), have been detected either in the periphery and within the tumor mass. Thus, our results indicate that NKG2D+ T lymphocytes are commonly recruited at melanoma sites where they could exert antitumor activity. The efficient expression of NKG2D was confirmed by in vitro phenotypic analysis of TILs isolated from melanoma specimens (patients 7, 9, and 10). Moreover, a high expression of this receptor was detectable on long-term melanoma specimens (patients 7, 9, and 10). Of note, NKG2D expression was commonly found in association with the CD8+ T subset (Table 2), whereas NKG2D+CD4+ T cells were represented with low frequency among TILs or PBMCs of melanoma patients, as described previously (1, 3, 6, 18).

With the aim to assess whether NKG2D+ T cells could actually exert antitumor activity, we examined four CD8+ T-cell clones (see Tables 1 and 2). Only the Melan-A/MART-1–specific CD8+ T-cell clone (A42), characterized by long-term in vitro culture, showed a weak expression of NKG2D, suggesting that the engagement of this molecule could no longer be necessary for the recognition of antigens with strong immunogenic potency (39). Of note, one of two CD4+ T-cell clones was NKG2D+, similarly to what has been observed in colorectal cancer (18), indicating that this subtype of lymphocytes, although low, represented in vitro (1–3, 5), can be selected in vitro. The analysis of melanoma cells highlighted that the in vitro expression of NKG2D+ was heterogeneous. Only the primary lesion expressed a significant level of all the analyzed molecules (Table 4). Moreover, only ULBP2 was strongly and homogeneously expressed by most melanoma cells within tumor lesions. These results suggest but do not prove that, along with melanoma progression, decreasing expression of NKG2D+ by tumor cells could occur. This conclusion is supported by the previous demonstration that soluble form of NKG2D+ can be detected in the blood of cancer patients whose tumor cells expressed these molecules (19–27) correlating, in some cases, with the prognosis of the disease (21, 25–27). Thus, this phenomenon could represent a mechanism of tumor evasion from immunosurveillance by T lymphocytes or NK cells. With the aim of evaluating this hypothesis, the expression of NKG2D+ will be further analyzed in a larger panel of primary melanoma lesions, that, unfortunately, was not available in the form of frozen sections while performing this study. Heterogeneous expression of NKG2D+ was also detected by in vitro established melanoma cell lines, although more common and clear staining of these molecules was evident in these tumor cells. Furthermore, significant expression of MICA or ULBP2 was observed in two melanoma lines (7 mel and 9 mel) established in vitro from tumor samples that were negative or weakly positive by immunohistochemical analysis. This suggests that in vitro culture conditions could favor the growth and expansion of tumor cells expressing, although at low level, NKG2D+ and/or that the cytometric analysis could be more sensitive than immunohistochemistry in detecting the expression of these markers. Moreover, a significant diversity in promoter regions of NKG2D genes that can lead to differential regulation of the expression of these molecules by cells has been documented (40).

A relevant finding of our study is the evidence that NKG2D can be engaged, besides TCR, in antitumor activity exerted by TILs or antigen-specific PBMCs of melanoma patients (Figs. 2 and 3). Of note is the observation that short-term in vitro isolated TILs from one melanoma lesion exerted antitumor activity through engagement of both TCR and NKG2D, indicating that, although this issue could be addressed only for one patient, NKG2D+ T lymphocytes commonly infiltrating melanoma lesions indeed may play a crucial role in the immunologic response against cancer. Moreover, both CD8+ and CD4+ T-cell clones isolated from this TIL population showed NKG2D-mediated recognition of the autologous tumor. The complexity in the regulation of the engagement of NKG2D in antitumor activity is documented by the evidence that heterogeneous usage of this receptor occurred even with antigen-specific T-cell clones, suggesting that the activation state of lymphocytes or the affinity/avidity of TCR for the MHC/peptide complexes could affect the role of multiple receptors in T cell–mediated antitumor activity. Along this line, the amount of antigen expression and/or the efficiency of the presentation by MHC molecules of immunogenic epitopes could represent the factor that will determine whether to engage costimulatory receptors, such as NKG2D, in the immune response.

In conclusion, our study shows that NKG2D+ T lymphocytes infiltrate tumor site and represent T-cell subset potentially exerting antimelanoma activity. Hitherto, new immunotherapeutic treatments, either active vaccination or adoptive cell transfer, for cancer patients should be designed aimed at augmenting and sustaining the NKG2D+ T lymphocyte–mediated immune response in early-stage melanoma patients. Our observations also indicate the need to further dissect the complexity that governs the engagement of multiple stimulation receptors in T cell–mediated antitumor activity.

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References

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