TRANCE- and CD40 Ligand-matured Dendritic Cells Reveal MHC Class I-restricted T Cells Specific for Autologous Tumor in Late-Stage Ovarian Cancer Patients

Katia Schlienger, Bruce L. Levine, Richard G. Carroll, and Carl H. June

ABSTRACT

Purpose: The use of mature dendritic cells (DCs) presenting tumor-associated antigens (TAA) to trigger tumor-specific T cells in vivo or in vitro represents a promising approach for cancer immunotherapy. We hypothesized that tumor antigens, mostly unidentified, are present on ovarian tumor cells and that mature DCs could be used to generate tumor-specific responses in unprimed patients. We also sought to measure preexisting antitumor immunity in patients with advanced ovarian cancer.

Experimental Design: Autologous DCs from 10 patients with ovarian cancer were pulsed with killed autologous primary tumors as a source of TAA. DCs were then matured in the presence of tumor necrosis factor α + TRANCE (tumor necrosis factor-related activation-induced cytokine) to induce maturation. Mature TAA-pulsed DCs were used in vitro to stimulate tumor-specific peripheral blood T cells.

Results: TRANCE and CD40 ligand were effective at maturing DCs. T-cell lines were generated in vitro that were capable of secreting IFN-γ in response to autologous tumor. These tumor-specific T cells were MHC class I restricted. The frequency of tumor-specific T cells in uncultured cells from malignant ascites fluid and peripheral blood was measured in the same patients.

Conclusions: IFN-γ-secreting tumor-specific T cells were demonstrated at baseline in uncultured T cells from some unvaccinated ovarian cancer patients; however, the T cells could not kill autologous tumor. These data demonstrate that mature DCs presenting tumor antigens from engulfed autologous tumors can be used to augment antitumor immunity in vitro in patients with epithelial ovarian cancer. The results support the feasibility of therapeutic vaccination of ovarian cancer patients.

INTRODUCTION

In cancer patients, tumor immunity is generally impaired. This could be due to defective processing and presentation of TAA by DCs (1) or impairment of effector functions through mechanisms such as central deletion, peripheral tolerance, ignorance, or defective homing of the effector lymphocytes (2–5). There is evidence, however, that tumor immunity may occur in vivo. Spontaneous remissions have been reported in some patients (6–11). In addition, circulating tumor-specific antibodies and CTLs have been identified in a few cancer patients (12, 13), suggesting that effective antitumor responses may be elicited through immunotherapeutic strategies. In recent years, several clinical trials have proved the safety and feasibility of using DCs to present microbial or tumor Ags in vivo (14–21). DC-based vaccines thus represent an attractive approach for cancer immunotherapy and ultimately for cancer relapse prevention (22).

In ovarian cancer, only a few TAA capable of inducing T-cell responses have been described. These include MUC-1 (23), Her-2/neu (24), epidermal growth factor receptor (25, 26), CDR2 (13), and CA-125 (27). These defined tumor Ags may not be optimal therapeutic choices for eliciting tumor-protective immunity in ovarian cancer. For example, DC processing of the glycoproteins Her-2/neu and MUC-1 is impaired, leading to defective MHC class II Ag presentation and T-cell activation (28, 29). In addition, MHC class I-restricted CTLs generated to an immunodominant Her-2/neu peptide fail to recognize Her-2/neu-expressing tumors in some instances (30, 31).

An alternative approach for ovarian cancer immunotherapy

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The abbreviations used are: TAA, tumor-associated antigen; Ag, antigen; DC, dendritic cell; CD40LT, CD40 ligand trimer; DPBS, Delbucco’s phosphate buffered saline; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; ELISPOT, enzyme-linked immunospot; MLR, mixed leucocyte reaction; IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony stimulating factor; ILA, human leukocyte antigen; mAb, monoclonal antibody; PE, phycoerythrin; PI, propidium iodide.
is the use of unfractionated tumor material as a source of TAAs, such as tumor-derived RNA (32), apoptotic tumor cells (33), tumor exosomes (34), or DC-tumor heterokaryons (35). In previous studies, dying allogeneic tumor cell lines were used as a source of TAAs to prime in vitro autologous tumor-specific CTLs (36–38). In this study, we demonstrate that human immature DCs fed with autologous apoptotic/necrotic tumor cells and matured by TNF-α + TRANCE can induce tumor-specific T cells in vitro from ovarian cancer patients. These T cells are capable of secreting IFN-γ in the presence of autologous tumor in a MHC class I-restricted manner. In addition, this assay permitted the demonstration of baseline antitumor immunity in some unvaccinated patients.

MATERIALS AND METHODS

Patient Specimens

Appropriate informed consent for blood and tissue donation under an institutional review board-approved protocol was obtained from patients with previously diagnosed or suspected ovarian cancer. Peripheral blood, as well as solid and/or ascites tumor specimens, was collected at the time of surgery from 10 ovarian cancer patients. These T cells are primary peritoneal serous adenocarcinoma. Peripheral blood, as well as solid and/or ascites tumor specimens, was collected at the time of surgery from 10 ovarian cancer patients. These T cells are capable of secreting IFN-γ in the presence of autologous tumor in a MHC class I-restricted manner. In addition, this assay permitted the demonstration of baseline antitumor immunity in some unvaccinated patients.

Patient Specimens

Appropriate informed consent for blood and tissue donation under an institutional review board-approved protocol was obtained from patients with previously diagnosed or suspected ovarian cancer. Peripheral blood, as well as solid and/or ascites tumor specimens, was collected at the time of surgery from 10 chemotherapy- and radiotherapy-naïve patients (Table 1). Peripheral blood was also obtained from healthy donors after obtaining informed consent under an institutional review board-approved protocol.

Reagents, Culture Media, and Tumor Cell Lines

DC culture medium consisted of AIM-V (Invitrogen, Carlsbad, CA) supplemented with IL-4 (R&D Systems, Minneapolis, MN) and GM-CSF (ImmuneX, Seattle, WA). T-cell culture medium consisted of AIM-V supplemented with heat-inactivated human AB serum (BioWhittaker/Cambrex, East Rutherford, NJ). Other recombinant human cytokines used in DC or T-cell culture included TNF-α, IL-6, IL-12 (R&D Systems), TRANCE/RANK-L, IL-7, IL-15 (Peprotech, Rocky Hill, NJ), IL-2 (Chiron, Emeryville, CA), and soluble CD40L (kindly provided by Elaine K. Thomas; ImmuneX). All reagents and media were endotoxin free as determined by a quantitative chromogenic limulus amebocyte lysate assay (BioWhittaker/Cambrex). LPS was obtained from Sigma (St. Louis, MO).

Tumor digestion medium consisted of RPMI 1640 (Invitrogen) containing 500 μg/ml collagenase type IV, 15 IU/ml DNase type I, 50 μg/ml hyaluronidase type V (Sigma), 2 mM L-glutamine, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml Fungizone (BioWhittaker/Cambrex), and 50 μg/ml gentamicin (Invitrogen).

Three types of medium were used to culture primary ovarian tumors. R10 medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. A3 medium consisted of AIM-V medium supplemented with 3% human AB serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. EOC-E medium consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 10 IU/ml penicillin, 10 μg/ml streptomycin, 1× ITS (insulin, transferrin, ethanolamine, and selenium), 0.1 mM triiodo-L-thyronine, 1 ng/ml recombinant human epidermal growth factor, 50 mM hydrocortisone (all from BioWhittaker/Cambrex), and 0.1 mM MEM nonessential amino acids (Invitrogen). Tumor cryopreservation medium consisted of RPMI 1640 containing 10% DMSO (Sigma) and 40% human AB serum.

The human epithelial ovarian adenocarcinoma cell line SKOV-3 (American Type Culture Collection, Manassas, VA) was cultured in R10. The transformed BALB/c mouse fibroblast cell line EJ-6-2-Bam-6A (Ei6; American Type Culture Collection) was grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin.

Cell Isolation and Separation

PBMCs. PBMCs were separated by density gradient centrifugation of 45 ml of heparinized whole blood using lymphocyte separation media (BioWhittaker/Cambrex) and plated in AIM-V for 2 h at 37°C. Nonadherent cells were removed and frozen for use as a source of T cells.

Processing of Tumor Specimens. Solid tumors were initially processed mechanically by mincing with scalpels until the tumor was reduced to pieces of approximately 1 mm³. A subset of the tumors (indicated in Table 2) were further processed in tumor digestion media overnight at room temperature. After passing processed tumors through a fine mesh sterile sieve (E-C Apparatus Corp., Holbrook, NY) to exclude large pieces, density gradient centrifugation was used. Tumor cells from ascites were isolated by density gradient centrifugation. Gradient-purified cells were washed in HBSS (BioWhittaker/Cambrex) and either plated in tumor culture media or frozen in tumor cryopreservation medium.

Table 1 Patient clinical characteristics

<table>
<thead>
<tr>
<th>Patient*</th>
<th>Age (yrs)</th>
<th>Stage</th>
<th>Grade</th>
<th>Histology</th>
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</thead>
<tbody>
<tr>
<td>OV22</td>
<td>61</td>
<td>IIIC</td>
<td>3</td>
<td>Primary peritoneal serous adenocarcinoma</td>
</tr>
<tr>
<td>OV23</td>
<td>55</td>
<td>IIIC</td>
<td>2–3</td>
<td>Adenocarcinoma of ovary</td>
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<tr>
<td>OV24</td>
<td>76</td>
<td>IIIB</td>
<td>3</td>
<td>Primary peritoneal papillary adenocarcinoma</td>
</tr>
<tr>
<td>OV26</td>
<td>69</td>
<td>IIIC</td>
<td>3</td>
<td>Primary peritoneal papillary serous adenocarcinoma</td>
</tr>
<tr>
<td>OV27</td>
<td>65</td>
<td>IIIC</td>
<td>3</td>
<td>Adenocarcinoma of ovary</td>
</tr>
<tr>
<td>OV28</td>
<td>51</td>
<td>IIIC</td>
<td>2–3</td>
<td>Adenocarcinoma of ovary with prominent endometrioid pattern</td>
</tr>
<tr>
<td>OV29</td>
<td>33</td>
<td>IIIC</td>
<td>3</td>
<td>Papillary serous adenocarcinoma of ovary</td>
</tr>
<tr>
<td>OV31</td>
<td>58</td>
<td>IIIC</td>
<td>2–3</td>
<td>Primary peritoneal papillary serous adenocarcinoma</td>
</tr>
<tr>
<td>OV32</td>
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<td>IV</td>
<td>3</td>
<td>Primary peritoneal papillary serous adenocarcinoma</td>
</tr>
<tr>
<td>OV34</td>
<td>58</td>
<td></td>
<td>3</td>
<td>Mucinous adenocarcinoma, unknown primary</td>
</tr>
</tbody>
</table>

* None of these patients had received chemotherapy before tissue collection.
Table 2  Processing and culture of primary tumor cells for 10 patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Digestion enzymes</th>
<th>Medium/serum</th>
<th>Tumor cell source</th>
<th>γ-Irradiation before pulsing on DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>OV22</td>
<td>+</td>
<td>R10</td>
<td>Tumor/cultured</td>
<td></td>
</tr>
<tr>
<td>OV23</td>
<td>+</td>
<td>A3</td>
<td>Tumor/thawed</td>
<td></td>
</tr>
<tr>
<td>OV24</td>
<td>+</td>
<td>A3</td>
<td>Tumor/thawed</td>
<td></td>
</tr>
<tr>
<td>OV26</td>
<td>+</td>
<td>A3</td>
<td>Tumor/thawed</td>
<td></td>
</tr>
<tr>
<td>OV27</td>
<td>+</td>
<td>A3</td>
<td>Tumor/thawed</td>
<td></td>
</tr>
<tr>
<td>OV28</td>
<td>+</td>
<td>A3</td>
<td>Tumor/thawed</td>
<td></td>
</tr>
<tr>
<td>OV29</td>
<td>+</td>
<td>A3</td>
<td>Tumor/cultured</td>
<td>+</td>
</tr>
<tr>
<td>OV31</td>
<td>–</td>
<td>EOC-E</td>
<td>Tumor/cultured</td>
<td>+</td>
</tr>
<tr>
<td>OV32</td>
<td>–</td>
<td>EOC-E</td>
<td>Tumor/cultured</td>
<td>+</td>
</tr>
<tr>
<td>OV34</td>
<td>–</td>
<td>EOC-E</td>
<td>Tumor/cultured</td>
<td>+</td>
</tr>
</tbody>
</table>

**Induction of Tumor Cell Death**

For apoptosis induction, cells (PBMCs, subconfluent cultures of primary tumors, or established cell lines) were placed in DPBS (BioWhittaker/Cambrex) and irradiated for 5 min with a medium wave UVB lamp (Spectronics Corp., Westbury, NY) calibrated to deliver 1900 μW/cm². The DPBS was then aspirated and replaced with complete medium. Alternatively, apoptosis was induced by either γ-irradiation (100 Gy from a 137Cs source) or a 2-h exposure to cisplatin (25 μg/ml; Sigma). Cells were then replated in complete medium and harvested after 24–48 h of culture at 37°C in 5% CO2. Early apoptosis was measured by flow cytometry analysis of cells stained with annexin V-FITC and PI (apoptosis detection kit; R&D Systems) according to the manufacturer’s instructions.

**DC Cultures**

Human DCs were generated by culturing adherent PBMCs from peripheral blood or ascites fluid macrophages with 800 IU/ml GM-CSF and 500 IU/ml IL-4 for 7 days in AIM-V medium (18). On day 7, immature DCs were incubated at 4°C with DPBS for 10 min to detach the adherent cells, collected by pipetting, and counted by trypsin blue exclusion. The immature DCs were either replated at 5 x 10⁵ cells/ml in AIM-V media supplemented with 1000 IU/ml GM-CSF, 1000 IU/ml IL-4, 20 ng/ml TNF-α, and 1 μg/ml TRANCE or frozen. The culture medium was supplemented in a similar fashion on day 9. At day 11, DCs were harvested, counted by trypsin blue exclusion, evaluated for their size on a Coulter counter (Coulter, Miami, FL), and used for phenotypic and functional analysis. As a control, immature DCs were also harvested at day 7, replated for 2 days in IL-4 and GM-CSF, and matured with either CD40LT (3 μg/ml) or LPS (1 μg/ml) at day 9 for 2 additional days.

**Flow Cytometric Analysis and Antibodies**

Fluorochrome-conjugated antibodies used were HLA-DR, CD3, CD14, CD16, CD19, CD45, CD80, IgG1, IgG2a (Becton Dickinson, San Jose, CA), CD32, CD40, CD86 (PharMingen, San Diego, CA), CD83 (ImmunoTech Inc., Westbrook, ME), and IgG2b (Southern Biotechnology Associates, Birmingham, AL). The CCR-7/HLA-DR stain was performed in a two-step procedure using purified anti-CCR7 mAb (clone 2H4; Becton Dickinson), followed by a PE-conjugated goat antimouse IgM mAb (Southern Biotechnology) and by FITC-HLA-DR (IgG2a). PI (Sigma) was used for cell viability determination. Analysis was performed on a FACSCalibur flow cytometer with CellQuest version 3.2.1f1 software (Becton Dickinson).

**Allogeneic MLR**

After 11 days of culture, DCs were harvested, irradiated (30 Gy from a 137Cs source), and seeded in triplicate in 96-well plates at various E:T ratios.Responder allogeneic CD4⁺ T cells from healthy donors were added at 10⁵ cells/well in AIM-V supplemented with 3% heat-inactivated human AB serum. Proliferation was measured 6 days later with [3H]thymidine (1 μCi/well; NEN Life Science, Boston, MA) added for the last 18 h of culture. Plates were harvested using a Tomtec Mach III harvester, and thymidine incorporation was measured using a Microbeta TriLux (Perkin-Elmer Wallac, Inc., Gaithersburg, MD).

**Measurement of Apoptotic Body Uptake by DCs**

To verify DC uptake of apoptotic tumor cells, SKOV-3 cells were stained with PKH26 red fluorescent cell linker (Sigma) and replated in their culture medium. After a 2-h adherence, apoptosis was induced by UVB irradiation. After 36 h, allogeneic immature DCs from healthy donors were thawed and pulsed with apoptotic SKOV-3 cells at 4°C and 37°C for 2 h in AIM-V medium. The cells were then stained with antibodies to HLA-DR-FITC and Her-2/neu-Ag-presenting cell for flow cytometric analysis.

**TAA-presenting DCs**

For preparation of TAA-presenting DCs, autologous tumor cells were harvested, irradiated with UVB, and counted in the presence of trypan blue 30 h later, as described above. For the first stimulation of tumor-specific T cells, freshly cultured immature DCs (PBMC-derived or ascites-derived) were used and plated for 20 h with autologous dying tumor cells at a 1:1 or 1:2 DC:tumor ratio in AIM-V supplemented with IL-4 and GM-CSF. TNF-α and TRANCE were then added, and the culture medium was supplemented with IL-4, GM-CSF, TNF-α, and TRANCE at day 10 instead of day 9. For subsequent stimulations of tumor-specific T cells, thawed immature DCs were used. At day 11, DCs were harvested, washed, and counted by trypan blue exclusion for T-cell stimulation. For patients OV31, OV32, and OV34, UVB-treated autologous tumor cells were γ-irradiated (100 Gy from a 137Cs source) before pulsing immature DCs (Table 2).
Table 3 Phenotypic analysis of mature DCs*

<table>
<thead>
<tr>
<th>Maturation agent</th>
<th>Size (μm²)</th>
<th>HLA-DR</th>
<th>CD14</th>
<th>CD40</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1477±12</td>
<td>95(488)</td>
<td>6(45)</td>
<td>11(25)</td>
<td>36(100)</td>
<td>33(50)</td>
<td>89(621)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1825</td>
<td>100(781)</td>
<td>2(151)</td>
<td>55(41)</td>
<td>86(112)</td>
<td>82(56)</td>
<td>99(1202)</td>
</tr>
<tr>
<td>TNF-α + TRANCE</td>
<td>2263</td>
<td>100(831)</td>
<td>5(72)</td>
<td>66(42)</td>
<td>91(214)</td>
<td>88(68)</td>
<td>99(1499)</td>
</tr>
<tr>
<td>CD40LT</td>
<td>2261</td>
<td>100(967)</td>
<td>1(305)</td>
<td>12(38)</td>
<td>96(283)</td>
<td>94(94)</td>
<td>100(1883)</td>
</tr>
</tbody>
</table>

* DCs were induced to undergo maturation for 4 days as described in Fig. 1. Values are representative of three experiments with three different healthy donors.
* DC volume as measured with a Coulter counter after 11 days of culture.

Generation of Ag-specific T-cell Lines

For the initial stimulation cycle, 10⁷ autologous nonadherent lymphocytes were thawed and cultured with apoptotic tumor-pulsed DCs at a 10:1 ratio in AIM-V medium supplemented with 3% human AB serum, IL-6 (1000 IU/ml), and IL-12 (10 ng/ml). The culture medium and cytokines were renewed on days 3 and 6. On day 8, T cells were restimulated as described above in the presence of only IL-7 at 10 ng/ml. On days 3 and 6 of the second stimulation, the cells were counted and replated at 10⁶ cells/ml in AIM-V medium supplemented with 3% human AB serum. IL-7 (10 ng/ml), IL-2 (20 IU/ml), and IL-15 (50 ng/ml). On day 14, T cells were collected, washed, and counted for functional analysis.

ELISPOT Assay for IFN-γ Release from Single Ag-specific T Cells

Tumor-specific T-cell lines were tested for cytokine release against various stimulators: DCs pulsed with apoptotic autologous tumor; DCs pulsed with apoptotic EJ62 cells; DCs pulsed with apoptotic autologous PBMCs cultured in the presence of FCS; unpulsed DCs; viable autologous tumor; autologous lymphoblasts or EBV lymphoblastic B-cell lines; and viable EJ62 cells. All apoptotic tumor cells were harvested 36 h after exposure to UVB for DC phagocytosis as described above. PBMCs underwent UVB-induced apoptosis at a faster rate and were harvested 24 h after irradiation for DC phagocytosis.

Plates (MultiScreen-IP; Millipore) were coated overnight at 4°C with mAb antihuman IFN-γ (50 μl/well; Endogen, Woburn, MA) at 10 μg/ml in sodium carbonate buffer (2.93 g of sodium bicarbonate, 1.59 g of sodium carbonate, and 0.2 g of sodium azide, in a final volume of 1 liter of distilled water). The plates were washed three times in sterile PBS and then blocked with AIM-V and 3% AB serum for 1 h at room temperature. T-cell lines generated from ovarian cancer patients were washed three times in AIM-V, resuspended in AIM-V and 3% AB serum at 4×10⁵ or 2×10⁵ T cells/ml with stimulus cells at a ratio of 10:1, and plated in triplicate at 100 μl/well. When blocking was performed, anti-HLA-DR, anti-MHC class I, or isotype control mAbs were preincubated at 10 μg/ml with stimulator cells for 15 min at room temperature. After 20 h at 37°C in 5% CO₂, cells were removed by washing with PBST (PBS containing 0.1% Tween 20). Antihuman IFN-γ biotinylated detection mAb (1 μg/ml; Endogen) was added to each well for 2 h in PBST containing 0.5% human serum albumin. After additional washing, extravidin:alkaline phosphatase at a 1:10,000 dilution in PBS (Sigma) was added and incubated for 1 h at room temperature. After washing, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for 20–30 min at room temperature in the dark. The reaction was stopped by adding 100 μl/well of a 1 M sodium phosphate solution.

The spots were scanned and counted by computer-assisted ELISPOT image analysis (Hitech Instruments, Edgemont, PA). Digitized images were analyzed for the presence of areas in which color density, spot size, and circularity exceed background by a factor set on the basis of the comparison of control wells. The ELISPOT assay was highly reproducible as measured by IFN-γ secretion in response to recall Ags such as tetanus toxoid and herpes simplex virus with aliquots of frozen PBMCs from healthy donors (data not shown).

RESULTS

TNF-α + TRANCE/RANK-L Induces Human DC Maturation. TAAAs are predominantly self-Ags also expressed by normal cells (39). A critical issue for the development of cancer immunotherapy is how best to induce T-cell immunity to “self” tumor Ags. The T-cell precursor frequency against these tumor Ags is low, comparable with that observed for neo-Ags (40). We have previously shown that a 4-day exposure to TNF-α confers on monocyte-derived DCs the ability to prime naïve CD4⁺ T cells to neo-Ag in vitro (41), although priming of Ag-specific MHC class I-restricted CD8⁺ T cells was less efficient (data not shown). To optimize induction of class I-restricted tumor-specific T cells, we tested various DC maturation factors that could be obtained in a good manufacturing practice form for potential application in human clinical trials. TRANCE, or RANK-L, a member of the TNF family, confers on monocyte-derived DCs the ability to prime naïve CD4⁺ T cells to neo-Ag in vitro (41), although priming of Ag-specific MHC class I-restricted CD8⁺ T cells was less efficient (data not shown). To optimize induction of class I-restricted tumor-specific T cells, we tested various DC maturation factors that could be obtained in a good manufacturing practice form for potential application in human clinical trials. TRANCE, or RANK-L, a member of the TNF family, is expressed on activated T cells and has been shown to enhance DC survival and maturation (42, 43). We thus compared monocyte-derived DCs from healthy donors cultured in the presence of TRANCE, TNF-α plus TRANCE, CD40LT, or LPS for up-regulation of DC maturation markers and for their capacity to stimulate allogeneic T cells. We found that TNF-α + TRANCE induced DC maturation almost as efficiently as CD40LT or LPS, as evidenced by membrane expression of typical DC maturation markers such as HLA-DR, CD80, CD83, and CD86 (Table 3). In addition, we found that DC size was proportional to DC maturation (Table 3). Similarly, in an allo-
Immature DCs Phagocytose Apoptotic Tumor Cells.

Immature DCs have been shown to endocytose and process Ag (51). We thus determined the capacity of immature DCs to uptake dying tumor cells by measuring capture of UVB-irradiated PKH26 red-labeled SKOV-3 tumor cells by monocyte-derived DCs from healthy donors. After a 2-h coculture, the percentage of HLA-DR<sup>+</sup> DCs that become positive for PKH26 was measured by flow cytometry. As shown in Fig. 3, 17% of DCs were able to phagocytose dying tumor cells at 37°C, as evidenced by DCs double-positive for PKH26 and HLA-DR<sup>+</sup>. In contrast, uptake of tumor cells was poor at 4°C (Fig. 3). The phagocytosis of dying tumor cells was also evident when SKOV-3 cells were labeled with annexin V after UVB irradiation, thus demonstrating that tumor cells undergoing apoptosis were uptaken by DCs. Phagocytosis was observed in this system with UVB-irradiated primary tumor cells from epithelial ovarian cancer, non-small cell lung cancer, and non-Hodgkin’s lymphoma (data not shown). These results demonstrate that immature DCs have the ability to uptake dying tumor cells, independently of the pathology of the tumor and of tissue-specific marker.

UVB Irradiation Induces Early Apoptosis of Epithelial Ovarian Tumors. Cells undergoing early apoptosis have been shown to serve as an essential trigger for the cross-presentation of epitopes derived from tissue-restricted Ags by MHC class I molecules on DCs (48). In contrast to apoptotic cells, necrotic cells can induce DC maturation and may be responsible for inducing CD4<sup>+</sup> T-cell responses (49, 50). Several groups have shown that DCs from patients with melanoma or prostate cancer pulsed with dying allogeneic tumor cell lines can induce in vitro CTLs capable of killing autologous tumor cells (36–38). Here, we used dying primary ovarian tumor cells, including both apoptotic and necrotic cells, as a source of TAA to pulse DCs from patients with ovarian cancer. As a model, we first compared UVB irradiation, γ-irradiation, and chemotherapy agents used in epithelial ovarian cancer such as cisplatin and paclitaxel, for their ability to kill SKOV-3 ovarian tumor cells. After exposure to these agents, SKOV-3 cells were washed and replated in their medium for 20–72 h to allow apoptosis to occur. Cell death was measured by staining with FITC-labeled annexin V (which binds to externalized phosphatidylinerine) and PI to discriminate between live cells (annexin V<sup>−</sup> and PI<sup>−</sup>, cells in early apoptosis (annexin V<sup>+</sup> and PI<sup>−</sup>), and cells in late apoptosis (annexin V<sup>+</sup> and PI<sup>+</sup>). As shown in Fig. 2, A and B, cisplatin, paclitaxel, and γ-irradiation, alone or in combination, inefficiently induced tumor cell death. In contrast, a brief exposure to UVB irradiation for 5 min triggered apoptosis in 97% of the SKOV-3 cells (Fig. 2C). In addition, primary tumor cells such as epithelial ovarian tumors (Fig. 2D) and primary non-small cell lung tumors (data not shown) were equally as sensitive to UVB exposure as immortalized cell lines. In fact, UVB irradiation induced more than 88% of the primary tumor cells to undergo apoptosis, with 42% in early apoptosis (Fig. 2C), thus favoring cross-presentation of tumor Ags by DCs (48).

genic MLR, DCs generated in the presence of TNF-α + TRANCE, CD40LT, or LPS all appeared to be functionally equivalent in their ability to stimulate T cells. In contrast, cells matured in the presence of TNF-α stimulated T cells less efficiently (Fig. 1A). Interestingly, DCs cultured in the presence of TRANCE alone showed a greater ability to activate T cells than immature DCs but were less efficient than DCs cultured with TNF-α (Fig. 1A). For a more sensitive determination of DC maturation, we measured the level of CCR-7 expression on DCs cultured for 4 days in the presence of CD40LT or of TNF-α + TRANCE (Fig. 1B). CCR-7 is a chemokine receptor associated with DC maturation and DC ability to migrate to secondary lymphoid organs, where SLC, the ligand for CCR-7, is expressed (44–47). On immature DCs, the CCR-7 molecule was present on 6% of HLA-DR<sup>+</sup> cells, whereas in the presence of TNF-α, 48% of DCs were positive for CCR-7 (Fig. 1B). The addition of TRANCE induced 61% of DCs to express CCR-7, essentially equivalent to the percentage of CCR-7<sup>+</sup> cells observed after CD40LT exposure (65%). Thus, DCs generated with TNF-α + TRANCE were essentially equivalent in function and cell surface phenotype to DCs generated with CD40LT, and we therefore used TNF-α + TRANCE as the DC maturation signal for subsequent generation of tumor-specific CD8<sup>+</sup> T cells.

UVB Irradiation Induces Early Apoptosis of Epithelial Ovarian Tumors. Cells undergoing early apoptosis have been shown to serve as an essential trigger for the cross-presentation of epitopes derived from tissue-restricted Ags by MHC class I molecules on DCs (48). In contrast to apoptotic cells, necrotic cells can induce DC maturation and may be responsible for inducing CD4<sup>+</sup> T-cell responses (49, 50). Several groups have shown that DCs from patients with melanoma or prostate cancer pulsed with dying allogeneic tumor cell lines can induce in vitro CTLs capable of killing autologous tumor cells (36–38). Here, we used dying primary ovarian tumor cells, including both apoptotic and necrotic cells, as a source of TAA to pulse DCs from patients with ovarian cancer. As a model, we first compared UVB irradiation, γ-irradiation, and chemotherapy agents used in epithelial ovarian cancer such as cisplatin and paclitaxel, for their ability to kill SKOV-3 ovarian tumor cells. After exposure to these agents, SKOV-3 cells were washed and replated in their medium for 20–72 h to allow apoptosis to occur. Cell death was measured by staining with FITC-labeled annexin V (which binds to externalized phosphatidylinerine) and PI to discriminate between live cells (annexin V<sup>−</sup> and PI<sup>−</sup>, cells in early apoptosis (annexin V<sup>+</sup> and PI<sup>−</sup>), and cells in late apoptosis (annexin V<sup>+</sup> and PI<sup>+</sup>). As shown in Fig. 2, A and B, cisplatin, paclitaxel, and γ-irradiation, alone or in combination, inefficiently induced tumor cell death. In contrast, a brief exposure to UVB irradiation for 5 min triggered apoptosis in 97% of the SKOV-3 cells (Fig. 2C). In addition, primary tumor cells such as epithelial ovarian tumors (Fig. 2D) and primary non-small cell lung tumors (data not shown) were equally as sensitive to UVB exposure as immortalized cell lines. In fact, UVB irradiation induced more than 88% of the primary tumor cells to undergo apoptosis, with 42% in early apoptosis (Fig. 2C), thus favoring cross-presentation of tumor Ags by DCs (48).
DCs Can Stimulate Tumor-specific T Cells on Phagocytosis of Dying Tumor Cells. We next used UVB-treated primary tumor cells from each patient as a source of autologous tumor Ags for presentation by DCs. In a first series of experiments, we used TNF-α and TRANCE as a maturation factor for DCs generated from ovarian cancer patients. However, in five of six patients, we were unable to generate specific T-cell responses to autologous tumors (data not shown). In a second series of experiments, DCs matured with TNF-α and TRANCE were used. T cells from 10 patients were stimulated two (OV22 to OV29) or three times (OV31 to OV34) with autologous DCs pulsed with apoptotic primary tumor and matured with TNF-α + TRANCE. Tumor specificity was evaluated in an overnight IFN-γ ELISPOT assay with either DCs pulsed with autologous apoptotic tumors or untreated live autologous tumor cells as stimulators. In three of six patients, 2 week T-cell lines specifically secreted IFN-γ when stimulated with DCs pulsed with autologous dying tumor (Fig. 4; Table 4). To differentiate between CD8+ T-cell and CD4+ T-cell responses, interactions with stimulator cells were blocked with mAbs against MHC class I or class II, respectively. Tumor-specific responses were blocked by anti-MHC class I mAb but not by anti-MHC class II mAb, although class II-dependent blocking of tetanus toxoid-specific T-cell responses from healthy donors was demonstrated as a positive control (data not shown). In some patients, strong nonspecific secretion of IFN-γ was observed in the presence of both unpulsed DCs and DCs pulsed with dying autologous PBLs (OV23, OV26, OV27, and OV28). This response could be blocked by anti-MHC class I mAb (OV26 and OV27), suggesting that in some patients, shared tumor self-Ag could be presented spontaneously by DCs (data not shown). Interestingly, in one patient (OV26), unstimulated T cells from peripheral blood (D0 PBLs) and ascites (D0 ascites) secreted IFN-γ in the presence of autologous unpulsed DCs (data not shown), suggesting again the presence of shared Ags between autologous DCs and tumor cells.

T Cells Stimulated in Vitro with DCs Pulsed with Dying Autologous Tumor Can Secrete IFN-γ in Response to Live Autologous Tumor. We next sought to determine whether T-cell lines that secreted IFN-γ in the presence of DCs pulsed with autologous tumor could also recognize autologous live tumor cells in an ELISPOT assay. For 5 of 10 patients, T-cell lines secreted IFN-γ in the presence of autologous tumor cells (Table 4). This response was partially blocked by anti-MHC class I mAbs (Fig. 5; Table 4). Normal ovarian epithelial cells from these patients were not available to be used as controls.
cells in the assay. We therefore tested MHC-matched controls, such as autologous EBV-transformed B cells, irradiated phytohemagglutinin-stimulated T cells, or murine epithelial tumor cells (EJ62) to control for natural killer cell activity.

These data demonstrate that in 5 of 10 nonvaccinated patients, tumor-specific T cells functionally capable of secreting IFN-γ against autologous live tumor could be amplified from circulating T-cell precursors by stimulation with DCs pulsed with dying autologous tumor cells (Table 4).

**Table 4** Precursor frequency of tumor-specific T cells in blood and ascites of 10 patients with ovarian cancer

<table>
<thead>
<tr>
<th>Patients</th>
<th>Day 0 PBLs</th>
<th>Day 0 Ascites</th>
<th>PBL-T cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DC/tumor</td>
<td>Tumor</td>
<td>DC/tumor</td>
</tr>
<tr>
<td>OV22</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>645</td>
</tr>
<tr>
<td>OV23</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>37</td>
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<tr>
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<td>&lt;1</td>
<td>152</td>
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<tr>
<td>OV34</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

- Precursor frequency of tumor antigen-specific T cells per 10⁶ T cells. Calculated by [(average spot-forming cells (SFCs) in the presence of DCs pulsed with dying tumor − average SFCs in the presence of DCs pulsed with dying control cells)/number of cells per well] × 10³. The precursor frequency of tumor antigen-specific T cells per 10⁶ T cells was considered to be increased if the number of IFN-γ SFCs generated in the presence of tumor antigens exceeded by 2.5-fold that of SFCs generated with control conditions.

- Precursor frequency of tumor antigen-specific T cells per 10⁶ T cells. Calculated by [(average SFCs in the presence of autologous tumor − average SFCs in the presence of control cells)/number of cells per well] × 10³. The precursor frequency of tumor antigen-specific T cells per 10⁶ T cells was considered to be increased if the number of IFN-γ SFCs generated in the presence of tumor antigens exceeded by 2.5-fold that of SFCs generated with control conditions.

- Precursor frequency of tumor antigen-specific T cells per 10⁶ T cells. Calculated by [(average SFCs in the presence of DCs pulsed with tumor − average SFCs in the presence of DCs pulsed with control cells)/number of cells per well] × 10³. The precursor frequency of tumor antigen-specific T cells per 10⁶ T cells was considered to be increased if the number of IFN-γ SFCs generated in the presence of tumor antigens exceeded by 2.5-fold that of SFCs generated with control conditions.

- Precursor frequency of tumor antigen-specific T cells per 10⁶ T cells. Calculated by [(average SFCs in the presence of autologous tumor − average SFCs in the presence of control cells)/number of cells per well] × 10³. The precursor frequency of tumor antigen-specific T cells per 10⁶ T cells was considered to be increased if the number of IFN-γ SFCs generated in the presence of tumor antigens exceeded by 2.5-fold that of SFCs generated with control conditions.

**Fig. 4** Tumor-specific T-cell lines from ovarian cancer patients secrete IFN-γ on stimulation with TAA-presenting DCs and are MHC class I restricted. A, digitized images of IFN-γ secretion by TAA-stimulated T cells. T-cell lines generated from ovarian cancer patients were mixed with autologous DCs previously pulsed with apoptotic autologous tumor cells or with apoptotic autologous PBMCs at a T:DC ratio of 10:1. Wells containing 2 × 10⁴ T cells and 2 × 10³ DCs, pulsed or unpulsed, are shown. From left to right: DCs unpulsed; DCs pulsed with autologous apoptotic PBMCs; DCs pulsed with autologous apoptotic primary tumor (for these three series in the presence of an IgG2a control mAb); and DCs pulsed with autologous apoptotic primary tumor in the presence of a blocking anti-MHC class I mAb. B, number of IFN-γ spots obtained after computerized assisted analysis, on stimulation of 2 × 10⁴ T cells, as described in A. Results are from triplicate wells. Results from one patient (OV22) are representative of three (Table 4).
Ovarian Tumor-specific T Cells

Our study of primary ovarian tumors demonstrates that DCs pulsed with dying autologous tumor cells can stimulate MHC class I-restricted T cells capable of secreting IFN-γ in response to autologous tumors. Furthermore, the use of autologous primary tumors in an ELISPOT assay permitted the measurement of baseline T-cell responses to autologous TAAs, in both the peripheral blood and the local tumor environment, in 10 unvaccinated patients with ovarian cancer. This assay was independent of HLA phenotype and did not require identification of TAAs expressed by each tumor. We provide evidence for natural priming of T cells to autologous tumor in uncultured peripheral blood and in malignant ascites. However, the baseline activity against autologous tumor appears to be extremely low and may not be biologically significant in patients with advanced ovarian carcinoma.

In contrast to melanoma, few TAAs have been defined for ovarian cancer. We hypothesized that unknown tumor Ags are present on ovarian tumor cells, which may be expressed by both tumor and normal tissue (52). We used dying primary tumor cells as a source of bulk TAAs for pulsing onto autologous DCs. This approach bypasses the need to identify tumor Ags, is not restricted to a specific HLA molecule, and therefore tests for T-cell responses simultaneously against multiple possible Ags with multiple restriction elements. A multiepitope approach against cancer is ultimately thought to be a keystone in reducing the well-described risk of immune escape mutants seen in single-Ag immunotherapeutics. To stimulate the observed low frequency of tumor-specific T cells, we used mature DCs to amplify Ag presentation, optimize activation of tumor-specific precursors, and potentially overcome tumor tolerance.

One critical issue in our investigation was the ability to optimize DC maturation and activation ex vivo after loading of dying tumors. Decreased numbers of DCs can be present in peripheral blood, in draining lymph nodes, and at the tumor site (53). The few DCs that are present are often immature (54, 55) and thus incapable of providing adequate Ag presentation to T lymphocytes (53) to elicit effective T-cell immunity. Furthermore, regulatory T cells have been found in tumors from patients with ovarian cancer (56). Recently, it was shown that immature DCs may contribute to the induction of regulatory T cells both ex vivo and in vivo, thereby leading to tolerance (57, 58). Thus, the use of mature DCs may be critical to reverse the immune dysfunction observed in cancer patients. In our studies, the use of TNF-α was not sufficient to induce a level of DC activation compatible with CD8+ T-cell activation. We thus used a combination of TNF-α + TRANCE. TRANCE, like CD40 ligand, has been shown to promote DC activation and survival during CD4+ T-cell licensing of DCs (43, 59). Indeed, using DCs pulsed and matured in the presence of TNF-α and TRANCE, we could generate tumor-specific CD8+ T cells.

In this study, we found that in about half of the patients, we could elicit MHC class I-restricted T cells against the autologous primary tumors. Indeed, it was striking that after only two stimulations, the tumor-specific T-cell precursor frequency increased to more than 1 of 1000 T cells. However, killing activity against autologous tumors was not detected after two stimulations (data not shown). Interestingly, MHC class II-restricted responses could not be generated with this approach. This could be due to the in vivo impairment of CD4+ T-cell precursors in ovarian cancer patients because we have recently demonstrated that CD4+ regulatory T cells are present in the ovarian tumor microenvironment (56).

**DISCUSSION**

malignant ascites were already primed to autologous tumors. Nonstimulated cells from peripheral blood (D0 PBLs) and malignant ascites (D0 ascites) from the same patients were tested when available (Table 4; Fig. 5). In 3 of 10 patients, peripheral blood or ascites T cells specifically secreted IFN-γ in the presence of autologous tumors (Table 4). Interestingly, in the two patients with specific D0 PBLs, a tumor-specific T-cell line could be generated (Table 4). In another patient (OV26), D0 T cells from blood and ascites responded nonspecifically to both unpulsed autologous DCs and living tumor cells, whereas D0 ascites T cells from another patient (OV29) secreted a low level of IFN-γ spontaneously in the absence of stimulator cells (data not shown). Taken together, these data suggest that in some patients, tumor-primed T cells may already be present at a detectable frequency in both blood and malignant ascites, confirming that tumor-specific T-cell responses may occur spontaneously in vivo in ovarian cancer patients.
Our study further indicates that tumor-specific T cells could be generated using autologous primary tumor cells as a source of Ags. Although powerful, the use of primary tumor cells raises several issues, including the concern that the culture not be contaminated by other normal tissues such as fibroblasts. Indeed, the specific composition of culture medium was crucial to provide a growth advantage to tumor cells. In addition, whereas additives such as digestion enzymes and FCS are commonly used to process and grow tumor cell lines from patients, we modified our protocol to exclude their use. We found that, for example, collagenase was contaminated by endotoxins. If the purity of the primary tumor culture system can be assured, the use of primary autologous tumors as a source of TAAs is particularly attractive to elicit an individualized anti-tumor response for each patient. Although the procedure is work-intensive, it avoids the introduction of dominant alloge-neic Ags, which may elicit irrelevant immune responses.

The ELISPOT assay permits the quantification of functional IFN-γ-secreting T-cell precursors in the blood. Mature DCs pulsed with melanoma peptides or infected by poxvirus encoding TAAs have been used in ELISPOT assays to quantify the number of TAA-specific T cells in unvaccinated patients with melanoma (60). In ovarian cancer, few TAA-derived pep-
tides are available for such quantification. For example, Her-2/ neu peptides restricted to MHC class I and II have been used to evaluate the precursor frequency of tumor-specific T cells in patients’ blood (61, 62). Indeed, the use of peptides limits the feasibility of the assay to patients with particular HLA types whose tumors overexpress known Ags such as the 30% of patients with ovarian cancer that express Her2/neu (24). We hypothesized that the use of whole tumors as a source of Ag would enable the routine detection of tumor-specific T-cell responses in ovarian cancer patients. We found that in eight of the nine patients tested, IFN-γ was detectable by ELISPOT in uncultured T cells from blood or ascites, although in some patients this was not specific. This contrasts with other studies in which scarce proliferative responses (5 of 45 patients) and no IFN-γ secretion (0 of 9 patients) were detected in uncultured T cells stimulated by Her2/neu (62). In conclusion, our study demonstrates that preexisting tumor immune responses can be amplified using mature DCs pulsed with dying autologous tu-
tors, providing the rationale to consider immunotherapy in relapsing ovarian cancer patients.

ACKNOWLEDGMENTS

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Ovarian Tumor-specific T Cells


TRANCE- and CD40 Ligand-matured Dendritic Cells Reveal MHC Class I-restricted T Cells Specific for Autologous Tumor in Late-Stage Ovarian Cancer Patients

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