Oxidation of Ovarian Epithelial Cancer Cells by Hypochlorous Acid Enhances Immunogenicity and Stimulates T Cells that Recognize Autologous Primary Tumor

Cheryl L-L. Chiang,[1] Jonathan A. Ledermann,[2] Egla Aitkens,[4] Elizabeth Benjamin,[3] David R. Katz,[1] and Benjamin M. Chain[1]

Abstract

Purpose: Hypochlorous acid, a product of neutrophil myeloperoxidase, is a powerful enhancer of antigen processing and presentation. In this study, we examine whether ovarian epithelial cells (SK-OV-3) exposed to hypochlorous acid can stimulate T cells from patients with ovarian epithelial cancer that recognize common tumor antigens as well as autologous tumor.

Experimental Design: T cells from human leukocyte antigen (HLA)-$A2^+$ and HLA-$A2^-$ patients or healthy controls were stimulated with autologous dendritic cells cocultured with the generic ovarian tumor line SK-OV-3, previously exposed to hypochlorous acid.

Results: Hypochlorous acid–treated SK-OV-3 cells drove expansion of $CD8^+$ T cells from HLA-$A2^+$ individuals, which recognized the HLA-A2–restricted tumor antigen epitopes of HER-2/neu (E75 and GP2) and MUC1 (M1.1 and M1.2). Up to 4.1% of the T cells were positive for the HER-2/neu KIFGSLAFL epitope using pentamer staining. Dendritic cells loaded with oxidized SK-OV-3 cells and further matured with CD40 agonistic antibody or monophosphoryl lipid A additionally induced $CD4^+$ class II–restricted responses. Critically, T cells stimulated with mature oxidized SK-OV-3 (but not a control oxidized melanoma cell line) directly recognized autologous tumor cells isolated from patient ascites.

Conclusions: Immunization with mature dendritic cells loaded with a generic oxidized tumor cell line stimulates a polyclonal antitumor response that recognizes autologous tumor. These findings suggest a new immunotherapeutic strategy to extend remission in ovarian cancer.

Dendritic cells are the key antigen-presenting cells of the immune system, which prime and activate naïve T cells. Because of this, many groups are exploring the ability of dendritic cells to stimulate therapeutic antitumor T-cell responses. Some therapeutic responses have been achieved (1–6); however, overall, the response rate remains low and further optimization will be required before the approach can yield significant clinical benefit. One key variable in this regard is the optimum source of antigen with which to load dendritic cells. Synthetic peptides offer the simplest alternative but suffer from the disadvantage that they are usually human leukocyte antigen (HLA) specific, and the limited breadth of response may drive the rapid emergence of tumor escape variants (7, 8). Thus, alternative strategies using whole-cell lysates, or mixed RNA isolated from tumor, are being used (9–13).

Successful immunotherapeutic strategies must overcome existing immune tolerance to tumor in order to be effective. It is therefore interesting that oxidation enhances the immunogenicity of many antigens (14–17), including autoantigens and tumor cell antigens (18, 19). Because oxidative species, including hypochlorous acid and hydrogen peroxide, are typically produced by inflammatory cells, this provides another example of cooperation between innate and adaptive immunity. Oxidation of tumor cells "as antigens" might therefore be a useful strategy for cancer immunotherapy, but this has not previously been tested in a cancer setting. A strategy that favors cross-presentation of a generic cell line rather than having to rely on autologous tumor antigen isolated in a patient specific manner is particularly attractive. The critical question that remains to be addressed, however, is whether this approach allows the expansion of tumor-specific T cells from patients whose immune system is exposed to high levels of cancer antigens over prolonged periods but is not able to eradicate the tumor. Strong T cell cross-reaction between the generic line used as antigen and individual tumors is another obvious requirement for any potential immunotherapeutic approach, but needs to be balanced by the possibility of overwhelming cross-reactive autoimmune responses to ubiquitously shared antigens.

In this study, we have tested these questions in the setting of ovarian cancer. The clinical characteristics of this disease offer

Authors' Affiliations: [1]Division of Infection and Immunity, and Departments of [2]Oncology and [3]Histopathology, University College London; and [4]Department of Oncology, University College London Hospitals, London, United Kingdom

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Requests for reprints: Benjamin M. Chain, Division of Infection and Immunity, University College London, Windeyer Building, 46 Cleveland Street, London W1T 4JF, United Kingdom. Phone: 44-207-6799402; Fax: 44-207-6799301; E-mail: b.chain@ucl.ac.uk.

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some important methodologic advantages. Many patients enter periods of clinical remission (20), during which immunologic function seems to return to normal. Most patients relapse subsequently, due to the persistence of microscopic and undetected tumors, and these tumors offer an attractive target for immunotherapeutic approaches. A further advantage is that tumor cells can frequently be obtained directly from ascitic fluid as a single-cell suspension, thus minimizing the need for tissue disruption and manipulation and facilitating *in vitro* restimulation assays. Finally, studies of the immune microenvironment in ovarian tumors show convincing evidence for a "natural" immunoprotective response, which may be capable of enhancement by appropriate vaccination strategies (21). Using this combination of clinical findings as background, in the present study, we have examined responses to the well-characterized ovarian epithelial cell line SK-OV-3, treated with the oxidizing agent hypochlorous acid. We propose that the results of this study suggest a generic method to stimulate an antitumor T cell response that will be capable of targeting autologous tumor in patients with ovarian cancer.

Materials and Methods

This study was approved by the University College London Hospitals Ethics Committee (project 03/0241).

Ovarian cancer patient ascites samples. Samples were collected from patients after informed consent had been obtained. Details of the patients used in the study are shown in Table 1. Ascites samples were collected under sterile conditions from patients 23 and 26 who had confirmed epithelial carcinoma of the ovary with peritoneal involvement. Tumor cells, collected at diagnosis and before chemotherapy, were isolated from ascites by density gradient centrifugation (300 × *g*); washed twice in HBSS; and plated in M199 medium (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Cancer Research UK, Lincoln's Inn Fields, London, United Kingdom; complete M199). The ovarian tumor cell clumps would adhere to the culture plate surface and were refed with fresh medium after 3 d of culture. At least 10^8 cells from each ascites sample were frozen in 10% DMSO plus complete M199 on the day of collection for use as targets in IFN-γ ELISpot.

Antibodies. The following antibodies were used: CD1a: supernatant mouse monoclonal antibody NA1/34, IgG2a, a gift from Prof. A. McMichael (John Radcliffe Hospital, Oxford, United Kingdom); CD2: mouse monoclonal antibody MAS 593, IgG2B (Harlan Sera-Lab); CD3: supernatant mouse monoclonal antibody UCH T1, IgG1; CD8: clone UCHT-4, mouse monoclonal antibody IgG2a; CD14: supernatant mouse monoclonal antibody HB246, IgG2b; HLA-DR, supernatant mouse monoclonal antibody L243, IgG2a (CD3, CD8, CD14, and HLA-DR were all gifts from Prof. P.C.L. Beverley, The Edward Jenner Institute for Vaccine Research, Berkshire, United Kingdom); CD19: supernatant mouse monoclonal antibody BU12, IgG1 (a gift from D. Hardie, Birmingham Medical School, Birmingham, United Kingdom); HLA-ABC: mouse monoclonal antibody W6/32 IgG2a (SeroTec); phycoerythrin-conjugated CD83: clone HB15e, mouse IgG1; phycoerythrin-conjugated CD40: clone 82111, mouse IgG2b; phycoerythrin-conjugated CD86: cClone 37301, mouse IgG1. All the above phycoerythrin-conjugated antibodies were from R&D Systems; phycoerythrin-conjugated mouse anti–HLA-DR was from *e*Bioscience; phycoerythrin-conjugated CD3: clone UCHT1, mouse IgG1 (R&D Systems). HER-2/neu expression in tissue sections was detected using a polyclonal antibody, A0485, directed to the internal domain of the HER 2 receptor (DAKO). For Western blotting, monoclonal antibodies against HER-2/neu (Santa Cruz Biotechnology) and HMFG2 anti-MUC1 (reactive with Asp-Thr-Arg; a kind gift from Dr. Joyce Taylor-Papadimitriou, Cancer-Research UK, GKT School of Medicine, London, United Kingdom) were used. The secondary antibody for flow cytometry was rabbit anti-mouse FITC-conjugated IgG2a (Dako Cytomation) at a 1:20 dilution of a 0.3 mg/mL stock to give a final concentration of 1.5 μg antibody/10^6 cells/100 μL.

Synthetic peptides. The HLA-A*0201–restricted peptides derived from HER-2/neu (E75: KIFGSLAFL, GP2: IISAVVGIL; refs. 22, 23), MUC1 (M1.1: STPPVHNV, M1.2: LLLLTVLTV; refs. 24, 25), and melanoma (MART-1: ELAGIGILTV; ref. 26) were purchased from Alpha Diagnostic International. The MHC class II T helper peptides derived from HER-2/neu (H369: KIFGSLAFLPESFDGDPA; H776: GVGSPYVSRLLGICL; refs. 27, 28) were synthesized at Cancer Research UK Protein and Peptide Chemistry Laboratory (Lincoln's Inn Fields, London, United Kingdom). These latter peptides are promiscuous, binding to a wide variety (but not all) HLA-DR haplotypes (27, 28). All peptides were >79% pure as indicated by reversed phase high-performance liquid chromatography and identities were validated by mass spectrometry. The lyophilized peptides were dissolved in at least 50% DMSO plus HBSS to a concentration of 1 mmol/L, filter-sterilized with 0.2-μm filter, and stored at -80°C until required.

Cell lines. The ovarian carcinoma cell line SK-OV-3 (which is HLA-$A3^+/A28^+$, HER-$2/neu^+$, $MUC1^+$; a gift from Dr. M. O'Hare, Ludwig Institute of Cancer Research, London, United Kingdom) was maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (all from Cancer Research UK). The melanoma cell line MEL-11 (HLA-$A24^+/A25^+$; B18 Cw5, HER-$2/neu^-$, $MUC1^-$, MART-1^+, MAGE-1^+; ref. 29; a gift from Dr. L. Lopes, Department of Immunology and Molecular Pathology, University College London, London, United Kingdom) was maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin. All cell lines were tested regularly for *Mycoplasma* and were found to be negative.

Western blot. Cryopreserved ascites samples from ovarian cancer patients 23 and 26 were thawed rapidly at 37°C, washed twice with HBSS, and lysed in sample buffer [0.15 mol/L Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol]. The cell lysates were boiled for 8 min at 100°C. Tumor cells (10^6 per sample) were resolved on 7.5% SDS-PAGE electrophoresis and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The presence of cellular HER-2/neu and MUC1 glycoproteins was shown using monoclonal antibodies detailed above. The Amersham Biosciences enhanced chemiluminescence protocol supplied by the manufacturer was used as a detection system.

Immunohistochemistry of HER-2/neu and MUC1 expression in primary ovarian tumors. HER-2/neu immunostaining was carried out using the Dako Hercep Test Kit. Paraffin sections were dewaxed, rehydrated, and heated in Epitope Retrieval Solution in a water bath at 98°C for 40 min. Controls included three breast cancer cell lines scoring 3+, 2+, 1+, and 0, and positive and negative tissue control sections. Slides were scored based on the staining pattern. 0: No staining at all or very slight partial membrane staining in <10% of tumor cells. 1+: Faint barely perceptible membrane staining in >10% of tumor cells or the cells are stained in only part of their membrane. 2+: Weak to moderate complete membrane staining in >10% of tumor cells. 3+: Strong complete membrane staining in >10% of tumor cells. For MUC1 immunostaining, paraffin sections were dewaxed, rehydrated, and heated in retrieval solution (ERI) for 20 min at 100°C. Monoclonal MUC1 antibody (HMFG2) was used at a dilution of 1:10 for 20 min. Immunostaining was assessed on the basis of intensity of staining (1+ to 3+) and distribution (membrane and cytoplasmic).

Dendritic cell, T cell, and peripheral blood mononuclear cell preparation. Fresh whole blood (120 mL) was collected from each patient in remission following surgery and chemotherapy for advanced ovarian cancer. Monocyte-derived dendritic cells were prepared as described previously (30). HLA-$A2^+$ samples were identified by flow cytometry (clone BB7.2, IgG2b; a gift from Dr. L. Lopes).

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Table 1. Details of patients used in the study

Patient no.	HLA-A2 status	Age	Tumor histology	Tumor grade	MUC-1 staining		HER-2/neu staining
					Pattern	Intensity	
1	Positive	70	Serous	3	Cyto, mem	3+	0
6	Positive	58	Serous	3	NA	NA	NA
7	Positive	70	Serous	2	Cyto, mem	3+	3+*
8	Negative	64	Serous	2	Cyto, mem	3+	1+
9	Positive	52	Clear cell	NA	NA	NA	NA
12	Positive	62	Serous	2	Cyto, mem	3+	0
13	Negative	77	Endometrioid	2	Cyto, mem	3+	3+*
14	Negative	52	Endometrioid	2	Cyto, mem	2+	3+*
15	Negative	63	Serous	3	Cyto, mem	3+	0
16	Not done	68	Endometrioid	3	NA	NA	NA
17	Negative	63	Serous	2	Cyto, mem	3+	2+
18	Negative	67	Serous	3	Cyto, mem	2+	3+*
19	Negative	67	Endometrioid	2	Cyto	3+	0
20	Negative	60	Serous	2	Cyto, mem	3+	1+
21	Not done	58	Endometrioid	2	Cyto, mem	3+	1+
22	Not done	48	Serous	3	Cyto, mem	3+	0
23 †	Negative	70	Serous	3	Cyto, mem	3+	0
24	Not done	46	Serous	3	NA	NA	NA
26 †	Positive	72	Endometrioid	2	Cyto, mem	3+	1+
27	Positive	60	Serous	3	Cyto, mem	3+	2+

NOTE: Patients 3, 4, 5, 6, and 10 were not shown in the table as they were used for another study, whereas patients 2, 11, and 25 gave insufficient number of cells for experiments.
Abbreviations: Cyto, cytoplasm; mem, membrane; Endometrioid, endometrioid carcinoma; Serous, serous carcinoma; Clear cell, clear cell carcinoma; NA, no tissue sample available for these patients.
*HER-2/neu 3+ interpreted as a clear positive.
† Ascites also available and tested.

Peripheral blood mononuclear cells. At least 10^7 of the peripheral blood mononuclear cells (PBMC) were cryopreserved in 90% FCS plus 10% DMSO as antigen-presenting cells for T cell restimulation and ELISpot assays.

T cells. The remaining cells were allowed to adhere for 2 h and the nonadherent cells were removed and cryopreserved for isolation of T cells as described below.

Dendritic cells. The adherent cells were cultured for 7 d in AIM-V supplemented with 0.075% sodium bicarbonate, 0.05 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (AIM-V CM) containing 100 ng/mL recombinant human granulocyte-macrophage colony stimulating factor and 50 ng/mL interleukin-4 (gifts from Schering-Plough Research Institute, Kenilworth, NJ). Lymphocytes and $CD14^+$ cells were removed on day 4 of culture as described (30). On day 7, these dendritic cells were >95% pure (as judged by the absence of CD3- or CD19-expressing lymphocytes and expression of CD1a). The cells had an "immature" phenotype characterized by absence of CD83; low levels of CD86; and moderate levels of HLA-DR, HLA-ABC, and CD40. They showed a typical dendritic cell appearance by light microscopy.

Dendritic cell activation and phenotyping. Dendritic cell maturation was induced by using either CD40 agonistic antibodies (mouse IgG1, clone mAb89, Immunotech), or a lipopolysaccharide A analogue (31, 32), monophosphoryl lipid A (MPL: detoxified lipid A derived from *Salmonella*; Avanti Polar Lipids, Inc.), chosen because both reagents have previously been approved for use in clinical trials (31–35). Dendritic cells were cocultured with oxidized SK-OV-3 and then treated with CD40 antibody (100-1,000 ng/mL) or MPL (50-200 ng/mL) for 24 h at 37°C/5%CO_2 AIM-V CM. As a negative control, dendritic cells were cultured in AIM-V CM. After activation, dendritic cell phenotyping was done on harvested cells; cells were resuspended in cold staining buffer (HBSS, 10% rabbit serum, 0.1% sodium azide); blocked for 15 min on ice; washed; and then incubated with phycoerythrin-conjugated HLA-DR, CD83, CD86, and CD40 antibodies for a further 30 min on ice. The cells were washed thrice in cold HBSS, fixed in 3.8% formaldehyde, and collected the same day on a FACScan flow cytometer (Becton Dickinson). The data were analyzed using CellQuest software.

In vitro *stimulation of T cells.* Our previous experiments showed that oxidation of SK-OV-3 cells with 60 μmol/L HOCl induced >99% tumor cell death (14). Dendritic cells (2×10^6) were therefore pulsed with 2×10^6 60 μmol/L HOCl-oxidized SK-OV-3 cells (prepared as described in ref. 14) for 24 h, 37°C/5% CO_2, in AIM-V CM. In some experiments, SK-OV-3 cells were killed by heat treatment (56°C, 30 min) as described (14). In some experiments, 2×10^6 of 60 μmol/L HOCl-oxidized MEL-11 tumor cells were used as an alternative. In others, dendritic cells were washed and cocultured for a further 24 h with CD40 antibody (500 ng/mL) or MPL (100 ng/mL). These concentrations were chosen after initial flow cytometry optimization as described above.

Dendritic cells were then washed and 2×10^5 of these cells were cocultured with 2×10^6 purified autologous T cells or purified CD4 T cells in AIM-V CM. T cells were prepared from the nonadherent population of PBMCs cryopreserved on day 1 of dendritic cell culture as described above. Total T cells were purified by immunomagnetic bead depletion with antibodies to HLA-DR, CD19, CD20, and CD14, resulting in a population that was >95% $CD3^+$. In some experiments, $CD4^+$ T cells rather than total T cells were isolated by adding a CD8 antibody to this cocktail. The isolated $CD4^+$ T cells were >95% pure as judged by flow cytometry. After 7 d of coculture with dendritic cell antigen, viable T cells were purified of necrotic debris by separation on Ficoll/Paque. T cells (1×10^6-3×10^6/mL) were restimulated with irradiated autologous PBMCs (20 Gy) and oxidized tumor cells at a ratio of T cells/PBMCs/tumor cells of 10:1:1. In certain experiments, the T cells were repurified by Ficoll Lymphoprep 4 d after restimulation and cultured in fresh medium without antigen for further 3 d. Following this, viable T cells were harvested for IFN-γ

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ELISpot assay or restimulated for a 3rd week with HER-2/neu peptide (E75).

Pentamer staining. T cells were stimulated with dendritic cells preloaded with 60 μmol/L HOCl-oxidized SK-OV-3 tumor cells as described above. Viable T cells were harvested and restimulated for a 3rd week with HER-2/neu peptide (E75) or MUC1 peptide (M1.1; at a final concentration of 1 μmol/L) and interleukin-2 (5 ng/mL). After this, viable T cells were obtained by Ficoll Lymphoprep, and 5×10^5 T cells per group were washed once with staining buffer (PBS with 1% FCS and 0.1% sodium azide) and stained with a phycoerythrin-conjugated HLA-A*0201–restricted HER-2/neu pentamer specific for KIFGSLAFL (abbreviated p-HER-2/$neu_{369-377}$; ProImmune) for 20 min at 37°C. Cells were counterstained with CD8-FITC (clone LT8; ProImmune) for 30 min on ice. After washing twice with PBS containing 0.1% sodium azide, the cells were fixed with 3.8% formaldehyde and analyzed by flow cytometry, gating on $CD8^+$ cells. T cells that were double positive for CD8 and HER-2/neu pentamer were expressed as a percentage of the total number of $CD8^+$ T cells gated.

IFN-γ ELISpot. IFN-γ ELISpot assays were done according to the manufacturer's recommendations, as described previously (18). T cells (10^5) were added per well.

Statistics. Means for different experimental groups were analyzed from a minimum of three independent experiments (i.e., PBMCs from

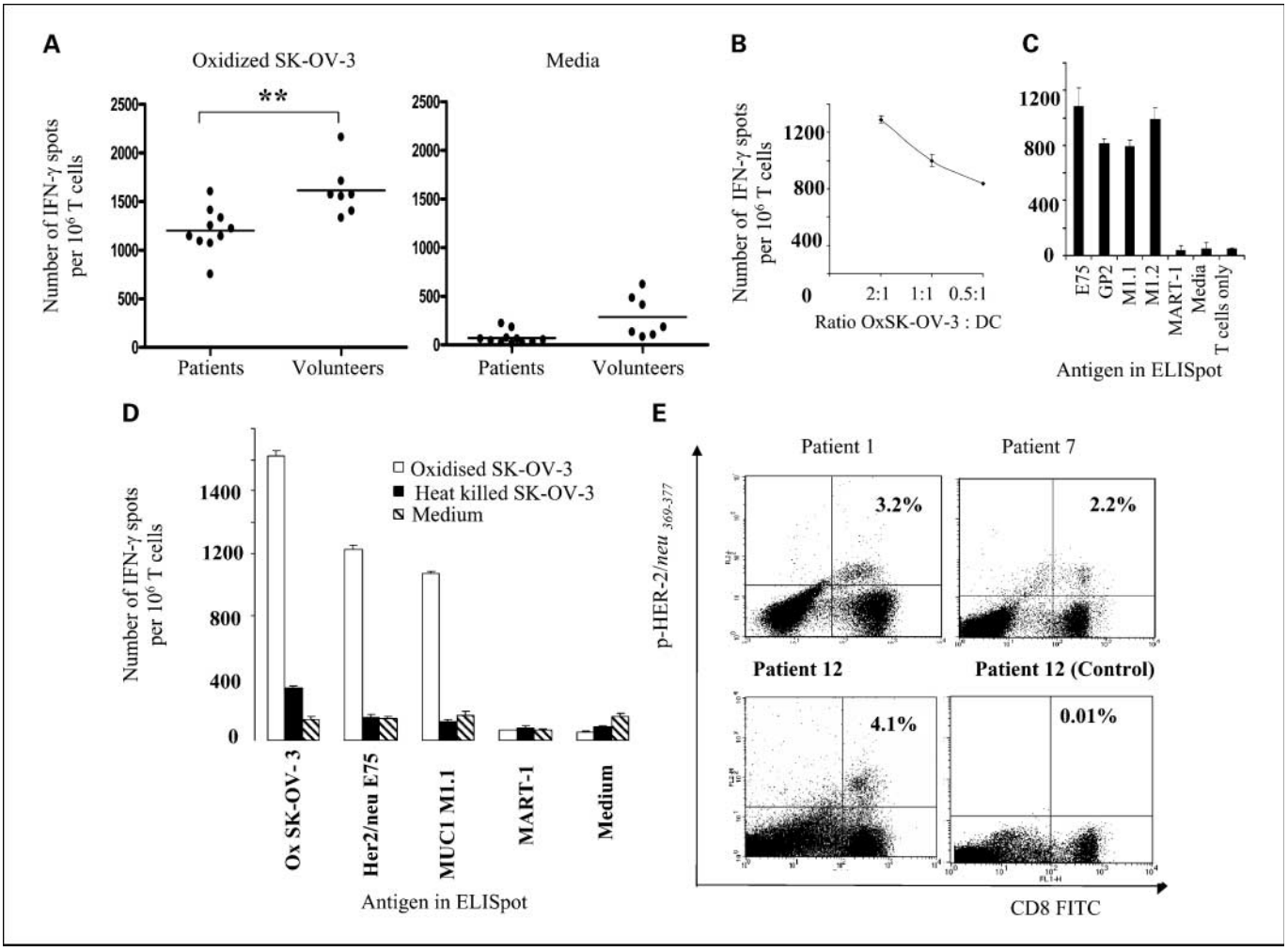

Fig. 1. T cells from patients with ovarian cancer respond to autologous dendritic cells loaded with oxidized SK-OV-3 tumor cells. *A,* IFN-γ T-cell responses using T cells and dendritic cells isolated from 10 patients and 7 healthy volunteers. Dendritic cells were cocultured with oxidized SK-OV-3 (*left*) or medium (*right*) and then with autologous T cells as described in Materials and Methods. After a 2-wk expansion period, T cells were collected and restimulated with oxidized SK-OV-3 cells (ratio of 1 T cell to 1 tumor cell) and PBMCs in ELISpot assays. Points, average of triplicate cultures from a different individual; line, median response. The two groups were compared using the Mann-Whitney test. The difference between the responses of patients' and volunteers' T cells to oxidized SK-OV-3 was statistically significant (**, $P < 0.01$; Mann-Whitney), and both these responses were significantly greater than to the medium (**, $P < 0.01$; Mann-Whitney). *B,* T cells from a HLA-$A2^-$ patient were expanded with oxidized SK-OV-3–loaded dendritic cells as in *A* and restimulated with PBMCs and different ratios of oxidized SK-OV-3 cells as shown. Points, average of triplicate cultures from 1 representative individual out of 10; bars, SE. *C,* T cells from HLA-$A2^+$ patients were expanded with oxidized SK-OV-3–loaded dendritic cells as in *A* and restimulated with PBMCs and HER-2/neu (E75 and GP2), MUC1 (M1.1 and M1.2), MART-1, or medium control in ELISpot assays (all peptides were used at a final concentration of 1 μmol/L). The last column shows the response of T cells in the absence of antigen or PBMC. Columns, mean of responses from four individuals; bars, SE. The mean differences between the responses of T cells to the peptides (i.e., E75, GP2, M1.1, M1.2) compared with the medium were statistically significant (**, $P < 0.01$; ANOVA with Dunnett's post hoc modification). *D,* T cells from an HLA-$A2^+$ patient were expanded with oxidized SK-OV-3 (*empty histograms*), heat-killed SK-OV-3 (*filled histograms*), or medium (*shaded histograms*)–loaded dendritic cells as in *A*, and restimulated with PBMCs and oxidized SK-OV-3 cells, HLA-A2–restricted peptides [HER-2/neu (E75), MUC1 (M1.1), MART-1], or medium control in ELISpot assays (all peptides used at a final concentration of 1 μmol/L). Columns, mean of triplicate wells; bars, SE. *E,* dendritic cells were cocultured with oxidized SK-OV-3 and then with autologous T cells as described in Materials and Methods. After a 2-wk expansion period, T cells were expanded for a further week in the presence of HER2/neu (E75) peptide (labeled patient 1, patient 7, and patient 12) or in the presence of MUC1 (M1.1) peptide (*Control*). T cells were then collected and stained with HER-2/neu (HLA-A2/KIFGSLAFL epitope) pentamers and CD8-FITC. The number in the top left panel indicates the percentage pentamer positive cells as a proportion of total $CD8^+$ cells. The figure shows results obtained from three individuals (patient numbers refer to Table 1).

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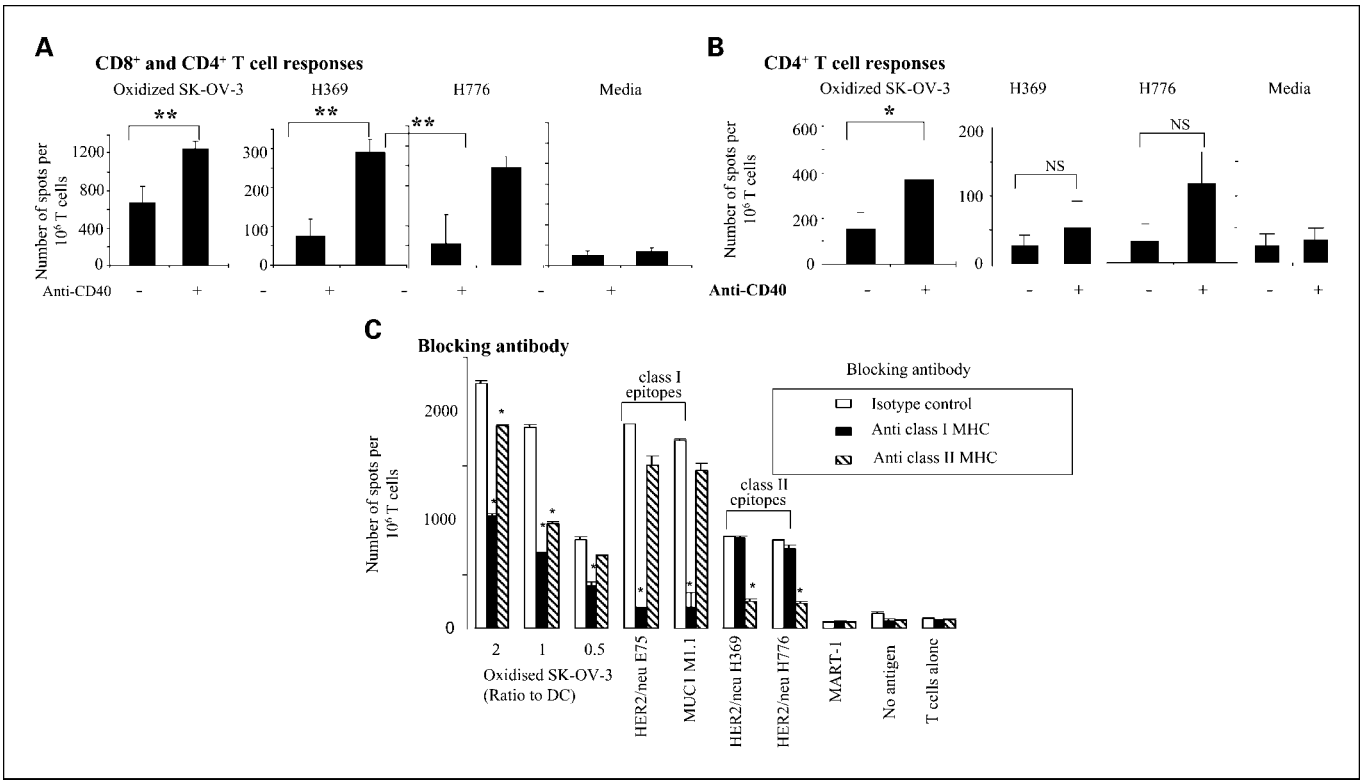

Fig. 2. Mature dendritic cells induce CD4 as well as CD8 tumor-specific T-cell responses. *A,* T cells isolated from patient PBMCs were cocultured with autologous dendritic cells that were loaded with oxidized SK-OV-3 and then either matured with 500 ng/mL CD40 agonistic antibody or cultured in medium alone. After a 2-wk expansion period, T cells were isolated and tested in IFN-γ ELISpot assays with PBMCs (1×10^4) and oxidized SK-OV-3 cells (ratio of 1 T cell to 1 tumor cell), or HER-2/neu class II MHC peptides H369 and H776 (all at a final concentration of 1 μmol/L), or medium only. The results show mean number of spots per 1 million T cells from four independent experiments (i.e., different patient PBMCs in each experiment). The T-cell responses to all antigens tested (i.e., oxidized SK-OV-3, H369, and H776) using anti-CD40 matured dendritic cells were statistically greater (**, $P < 0.01$, Student's paired *t* test) than those using dendritic cells without anti-CD40, and also significantly greater ($P < 0.05$; ANOVA with Dunnett's post hoc modification) than those to responses in the absence of antigen. *B,* T cells isolated from patient PBMCs were depleted of $CD8^+$ T cells by immunomagnetic bead selection as described in Materials and Methods. The residual T cells contained <5% $CD8^+$ cells. The purified $CD4^+$ cells were cocultured with autologous dendritic cells that were loaded with oxidized SK-OV-3 and then either matured with 100 ng/mL MPL or cultured in medium alone. After a 2-wk expansion period, viable cells were tested in IFN-γ ELISpot assays with PBMCs (1×10^4) and oxidized SK-OV-3 cells (ratio of 1 T cell to 1 tumor cell), or HER-2/neu class II MHC peptides H369 and H776 (all used at a final concentration of 1 μmol/L), or medium only. The results show mean number of spots per million T cells from three independent experiments (i.e., different patient PBMCs in each experiment). T-cell responses from the MPL treatment were statistically significant compared with that of no MPL treatment in the oxidized SK-OV-3 group (*, $P < 0.05$; Student's paired *t* test) but not for H369 and H776 groups (*NS,* not significant). The response to oxidized SK-OV-3 and H776 with MPL treatment was statistically significant ($P < 0.05$; ANOVA with Dunnett's post hoc modification) compared with MPL treatment and no antigen. *C,* T cells isolated from patient PBMCs were cocultured with autologous dendritic cells that were loaded with oxidized SK-OV-3 and then matured with 500 ng/mL CD40 agonistic antibody. After a 2-wk expansion period, T cells were isolated and tested in IFN-γ ELISpot assays in the presence of isotype control (*empty histograms*), anti-class I MHC antibody W6/32 (*filled histograms*), or anti-class II MHC antibody L243 (*shaded histograms*) with PBMCs (1×10^4) and antigen/oxidized SK-OV-3 cells (ratio as shown), HLA-A2–restricted peptides [HER-2/neu (E75), MUC1 (M1.1), MART-1], or HER-2/neu class II MHC peptides H369 and H776 (all at a final concentration of 1 μmol/L), or medium only. The last column shows the response to T cells without antigen or PBMC. Blocking antibodies were added as 50 μL undiluted monoclonal antibody tissue culture supernatants per 200 μL culture (i.e., 1:4 dilution). *, $P < 0.01$, significant difference from isotype within each group (Student's *t* test).

at least three different individuals). The analysis of significance was carried out using Mann-Whitney test, one-way ANOVA, Dunnett's post hoc modification, or paired Student's *t* test as detailed below.

Results

T cells from patients with ovarian cancer respond to autologous dendritic cells pulsed with oxidized SK-OV-3 tumor cells. We initially used dendritic cells loaded with oxidized SK-OV-3 and compared T-cell responses of seven healthy volunteers with the responses from a group of 10 patients with ovarian cancer (see Table 1 for patient details), using dendritic cells loaded with oxidized SK-OV-3 and autologous T cells (Fig. 1A). All 10 patients responded to the oxidized cells (>500 spots per million). The average number of spots was lower than in the volunteer cohort ($P < 0.01$, Mann-Whitney test), which may reflect differences in age distribution and general health. Indeed, the mean background (medium alone) response of the healthy volunteers was also slightly higher than that of the patients, although this difference was not statistically significant. A typical dose response to the oxidized SK-OV-3 cells in a representative patient is shown in Fig. 1B.

Four $HLA\text{-}A2^+$ individuals from this group were also tested using HLA-A2–restricted HER-2/neu and MUC1 peptides, and a control peptide derived from the melanoma antigen MART-1 (Fig. 1C). All four patients expressed high levels of tumor MUC1 as assessed by immunocytochemistry, and one (patient 27) was also positive for HER-2/neu (Table 1). Strong responses were seen to all four HER-2/neu and MUC1 peptides tested but there was no response to the MART-1 peptide. Because the SK-OV-3 line is $HLA\text{-}A2^-$, these experiments showed that oxidized SK-OV-3 cells are cross-presented by the dendritic cells and that the T cells recognized nonoxidized tumor-associated antigens. No responses were seen to either oxidized SK-OV-3 cells or peptides

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when PBMCs were tested directly from patients, without dendritic cell presentation/expansion, thus confirming that exposure to the tumor *in vivo* had not primed a significant response. Furthermore, only small responses were seen when using SK-OV-3 cells killed by heat treatment (56°C, 30 minutes), confirming our previous report that the enhancement of immunogenicity was due to oxidation and not simply to cell killing (Fig. 1D). Pentamer staining was also done on cells expanded from three patients, of which one was positive for HER-2/neu (see Table 1), using a HER-2/neu (E75; KIFGSLAFL)/HLA-A2 pentamer (Fig. 1E). After 3 weeks of *in vitro* stimulation (i.e., priming with oxidized SK-OV-3 loaded dendritic cells, and expansion with HER-2/neu peptide), between 2% and 4% of the $CD8^+$ T cells were specific for the HER-2/neu pentamer. Preexposure to HER-2/neu did not therefore either decrease or increase the ability of the oxidized SK-OV-3 to stimulate and expand the HER2/neu T cells, suggesting that priming/expansion occurred predominantly *in vitro*. Less than 0.1% pentamer-specific cells were detected on T cells expanded on a MUC1 peptide (Fig. 1E, *last panel*).

Ovarian cancer patients' dendritic cells loaded with oxidized SK-OV-3 and matured with MPL or CD40 agonist antibody induced both $CD4^+$ and $CD8^+$ responses. Oxidized SK-OV-3 cells induce only minimal maturation of dendritic cells. Therefore, in this study, dendritic cells were matured further by the addition of anti-CD40 antibody or the TLR4 agonist MPL. The optimum concentration was first determined by monitoring dendritic cell maturation by flow cytometry. Optimum up-regulation of dendritic cell maturation markers and costimulatory molecules—CD83, CD86, HLA-DR, and CD40—and minimal cell death were observed with in the presence of 100 ng/mL MPL or in the presence of 500 ng/mL CD40 antibody, and these concentrations were used for further experiments. The T-cell response to the dendritic cells treated with oxidized SK-OV-3 and CD40 antibody was examined first with unfractionated T cells (Fig. 2A). In these experiments, we used as antigens not only oxidized SK-OV-3 but also two HER-2/neu peptides, which have been shown to be promiscuous MHC class II epitopes, binding to a wide variety (although not all) of HLA-DR haplotypes (36). Unfractionated T cells stimulated by the SK-OV-3–loaded dendritic cells, and without additional maturation signal, responded to oxidized SK-OV-3 cells (as above) but failed to respond ($P > 0.5$, response not significantly above background) to either of the MHC class II peptides. However, stimulation with dendritic cells loaded with oxidized SK-OV-3 and then matured with CD40 antibody generated responses against both the class II HER-2/neu peptides tested, H360 and H776. The response against oxidized SK-OV-3 was also boosted ~2-fold by dendritic cell maturation.

The experiments shown in Fig. 2A examine the CD4 responses indirectly by using the two class II–restricted peptides as test antigens. To measure the CD4 response more directly, T cells were depleted of $CD8^+$ T cells before coculture with dendritic cells (Fig. 2B). The population tested contained <5% contaminating CD8 cells. The total $CD4^+$ population gave a smaller but significant response to oxidized SK-OV-3 cells, which was enhanced twice to thrice by using dendritic cells treated with oxidized SK-OV-3 followed by maturation with MPL. The MPL-matured dendritic cells also primed a response to H776.

To further confirm the presence of class I and class II MHC-restricted T cells, we used blocking antibodies (Fig. 2C). An antibody to class I MHC (W6/32) blocked the response to whole oxidized SK-OV-3 cells by more than 50%, and almost completely blocked the response to peptides containing class I epitopes. The response to peptides coding for the two class II–restricted epitopes was not blocked. In contrast, an antibody to class II MHC (L243) blocked the response to whole oxidized SK-OV-3 cells (albeit much less effectively) but completely blocked the response to peptides containing class II epitopes. The response to peptides coding for the two class I–restricted epitopes was not blocked. This experiment, therefore, is consistent with the data in Fig. 2A and B, which show that the response stimulated by oxidized SK-OV-3 contains a major class I (CD8) response and a smaller class II (CD4)–restricted response.

Dendritic cells pulsed with oxidized SK-OV-3–stimulated IFN-γ–producing T cells that efficiently recognize autologous ovarian tumor cells isolated from ascites. The key test for the strategy of using oxidized SK-OV-3 cells as a generic cell-based antigen is whether T cells primed in this way will recognize autologous tumor. For these experiments, PBMCs were taken from two patients in remission, from whom ascitic tumor cells had been collected and stored in advance. The expression of HER-2/neu and MUC1 in the tumor sample itself was analyzed by immunoblotting (Fig. 3A and B). Tumor MUC1 glycoproteins were detected as multiple glycosylated variants of ~250 kDa in the SK-OV-3 ovarian cell line and in both patients' ascites

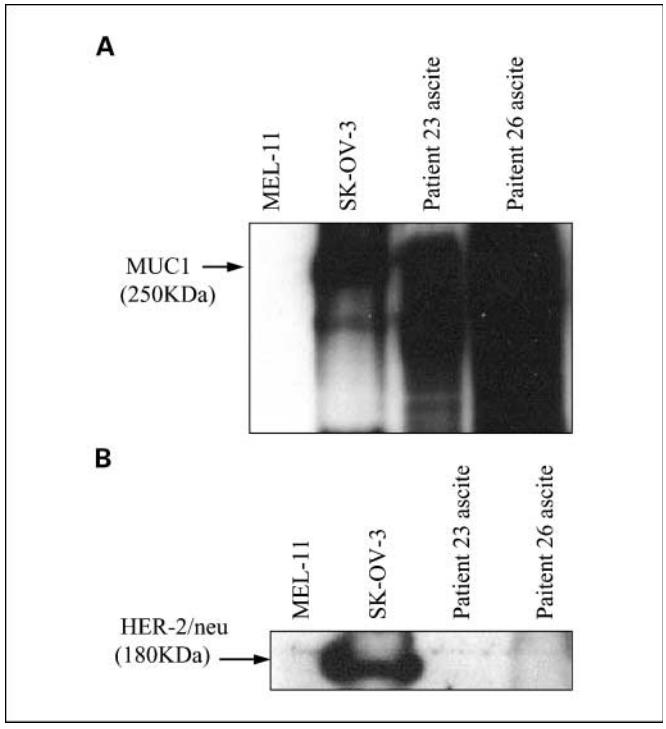

Fig. 3. Expression of MUC1 and HER-2/neu tumor antigens on ovarian cancer patients' ascites. Tumor cells (1×10^6) isolated from patients' ascites, or SK-OV-3 cells or MEL-11 cells, were lysed in sample buffer, resolved on 7.5% SDS-PAGE, and transferred to nitrocellulose membranes. MUC1 and HER-2/neu expression was detected by anti-MUC1 and anti – HER-2/neu monoclonal antibodies. *A,* MUC-1 glycoproteins were detected as multiple glycosylated variants of ~250 kDa on the ascites tumor cells and SK-OV-3. *B,* HER-2/neu appeared as a single band in the SK-OV-3 but was absent in the patients' ascites. MEL-11 was negative for both MUC1 and HER-2/neu.

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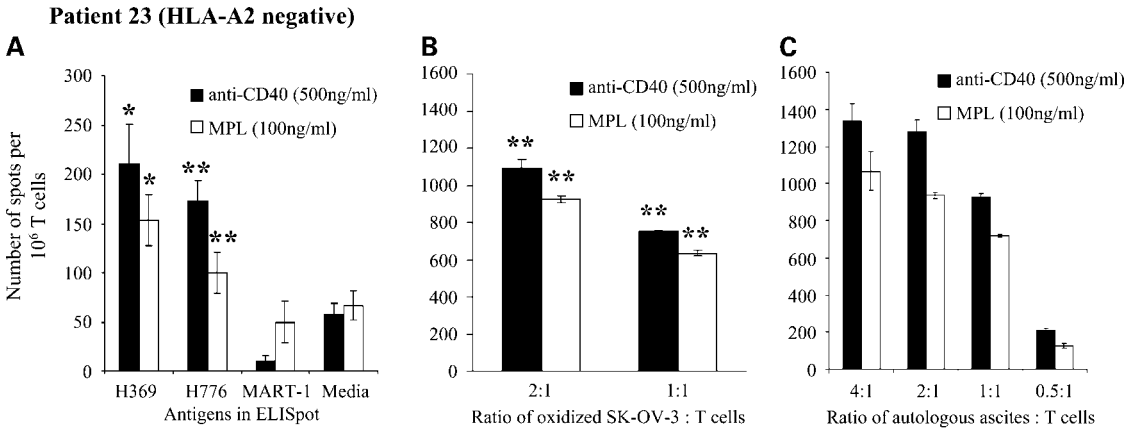

Fig. 4. Patient T cells stimulated with mature dendritic cells pulsed with oxidized SK-OV-3 efficiently recognize autologous ascites – derived tumor cells. T cells from a HLA-A2 – negative patient (patient 23) were cocultured with autologous dendritic cells that were loaded with oxidized SK-OV-3 and then matured with CD40 agonistic antibody (500 ng/mL, *filled histograms*) or MPL (100 ng/mL, *empty histograms*). After a 2-wk expansion period, T cells were isolated and tested in IFN-γ ELISpot assays. *A,* T cells were cocultured with PBMCs (1×10^4) and HER-2/neu class II MHC peptides H369 and H776, or control peptide MART-1 (all at a final concentration of 1 μmol/L), or medium only. *B,* T cells were cocultured with PBMCs (1×10^4) and oxidized SK-OV-3 cells (ratios of SK-OV-3 to T cells were 2:1 or 1:1 as shown). *C,* T cells were cocultured in the absence of PBMCs with autologous ascites-derived tumor cells at various ratios as shown. The results show mean number of spots per million T cells, from triplicate cultures. *P* value is significant if <0.05 (*) or <0.01 (**) when comparing the test groups to the medium control using ANOVA with Dunnett's post hoc modification. *C,* asterisks are not shown but all columns are significantly different from medium control ($P < 0.01$ or $P < 0.05$).

samples. HER-2/neu (molecular weight ~185 kDa) was detected as a single band in the SK-OV-3 line but was absent in both ascites samples. Both antigens were absent from the melanoma tumor line MEL-11.

For functional studies, on the basis of the data shown in Fig. 2, we used dendritic cells treated with oxidized SK-OV-3 and then cultured with either CD40 or MPL. The first patient (patient 23 in Table 1) was HLA-A2$^-$, and therefore was tested with the promiscuous class II MHC – restricted peptides, but not the HLA-A2 – restricted peptides (Fig. 4A). The T cells responded to both MHC class II peptides tested (Fig. 4A), as well as to the oxidized SK-OV-3 used for immunizing (Fig. 4B). Critically, the T cells primed with oxidized SK-OV-3 also showed responses to the autologous unmodified tumor cells over a range of tumor to T-cell ratios and in the absence of any exogenous antigen-presenting cells in the ELISpot assays (Fig. 4C).

Because more cells were available from the second patient (patient 26 in Table 1), an additional specificity control was added. Half the dendritic cells were loaded as above with oxidized SK-OV-3 cells, whereas half were loaded with an oxidized melanoma line, MEL-11 (also HLA-A2$^-$). Because the individual was HLA-A2$^+$, the T cells responded to the class I HER-2/neu peptide E75 as well as to the class II MHC HER-2/neu peptides tested (Fig. 5A). There was no response to the melanoma HLA-A2 – restricted peptide MART-1. The T cells stimulated with the oxidized SK-OV-3 cells also responded to oxidized SK-OV-3 cells but showed no response to oxidized MEL-11 cells (Fig. 5B). As with patient 23, the T cells also reacted to unmodified autologous tumor in the absence of exogenous antigen-presenting cells (Fig. 5C), demonstrating directly that they were not tolerant to the tumor cells. In contrast, the T cells stimulated *in vitro* with the oxidized MEL-11 cells showed little response to the autologous ovarian tumor cells (Fig. 5D). The cells responded strongly to the oxidized MEL-11 cells (the immunogen) and showed a significant response to the MART-1 peptide, but no response to any HER-2/neu peptide tested (Fig. 5E). Thus, the T cells stimulated by oxidized cells do not respond to common self-antigens shared between cells of different tissue origin, but predominantly respond to tissue-specific antigens expanded *in vitro* by dendritic cells loaded with SK-OV-3 or MEL-11, respectively.

Discussion

This study verifies the predictions of the hypothesis outlined in the Introduction that dendritic cell presentation of an established ovarian derived cell line, which has been oxidized by exposure to hypochlorous acid, induces a T-cell response that recognizes cross-reactive tumor antigens and autologous tumor cells isolated from ovarian cancer patients. Previous studies (18) had already shown that nonoxidized tumor cells, either live or killed by nonoxidative means, have very poor stimulatory activity in this system, and these antigens were therefore not explored further here. The responses seen included both MUC1 and HER-2/neu epitopes (although the intrinsic tumors overexpressed one or both these antigens) and were elicited from both CD8$^+$ and CD4$^+$ T cells. T-cell priming resulted from cross-presentation, because although SK-OV-3 is HLA-A2$^-$, HLA-A2$^+$ dendritic cells presenting oxidized SK-OV-3 cells stimulated T-cell responses to HLA-A2 – restricted peptides.

The model sheds light on some key questions regarding the tolerance/effector balance, which are fundamental both to our understanding of tumor immunology and to advancing realistic prospects for successful dendritic cell – driven adoptive immunotherapy. An important objective of the study was to show that the presentation of oxidized SK-OV-3 cells could stimulate T cells directed at bona fide "self" tumor antigens to which the immune system had previously been exposed *in vivo*. Oxidized SK-OV-3 cells contain a variety of non – self-antigens, including both SK-OV-3 – specific alloantigens (because this line is not syngeneic to the volunteers or the patients used in this study)

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and neo-epitopes formed by the oxidation process. Responses against bona fide tumor antigens were therefore documented in two ways. First, ovarian cancers often overexpress a variety of tumor-associated antigens, including the well-characterized HER-2/neu (37) and MUC1 (38) proteins, as confirmed in the responding individuals for this study (see Table 1). A panel of peptides encoding known MHC class I and MHC class II peptides from these antigens were recognized by the oxidized SK-OV-3 *in vitro* stimulated T cells. This implies processing and presentation of SK-OV-3 proteins by the dendritic cells via both class I and class II MHC, and establishes clearly that any prior exposure did not prevent the ability of the SK-OV-3–loaded dendritic cells to stimulate T-cell responses to defined tumor antigen epitopes. Second, T cells stimulated by the oxidized SK-OV-3 cells recognized and responded strongly to autologous unmodified tumor cells in the absence of any dendritic cells (Figs. 4C and 5C). Although we have thus far been able to collect matched PBMCs and ascites from only two individuals, these results suggest that, at least in a proportion of individuals, oxidized SK-OV-3 share sufficient antigens with primary ovarian tumors to act as a generic antigen for dendritic cell immunotherapy. Furthermore, presentation of the oxidized cells by dendritic cells is sufficient to break any existing tolerance to these primary ovarian cancer–related antigens.

Because the immune system is exposed to tumor antigens in large amounts *in vivo* (especially in the case of development of a large bulk of coelomic cavity metastatic tumor associated with ascites), although this does not prevent growth of tumor, it is clear that an efficient effector response does not occur in these patients. Many mechanisms may contribute to tumor escape, including failure to prime an effector response, active tolerance induction, and tumor evasion of effector mechanisms. We were unable to detect any *ex vivo* responses to tumor cells in patient PBMCs without prior *in vitro* stimulation (data not shown). Furthermore, the *in vitro* response stimulated by dendritic cells loaded with oxidized SK-OV-3 cells was not amplified in patients versus volunteers. Both these observations suggest that if there is any effector T-cell immune response stimulated *in vivo*, at least to MUC1 and HER-2/neu, then it is very limited.

A second important observation concerned the ability of oxidized cellular antigen to activate tumor antigen–specific T cells in a class II as well as a class I MHC-restricted fashion.

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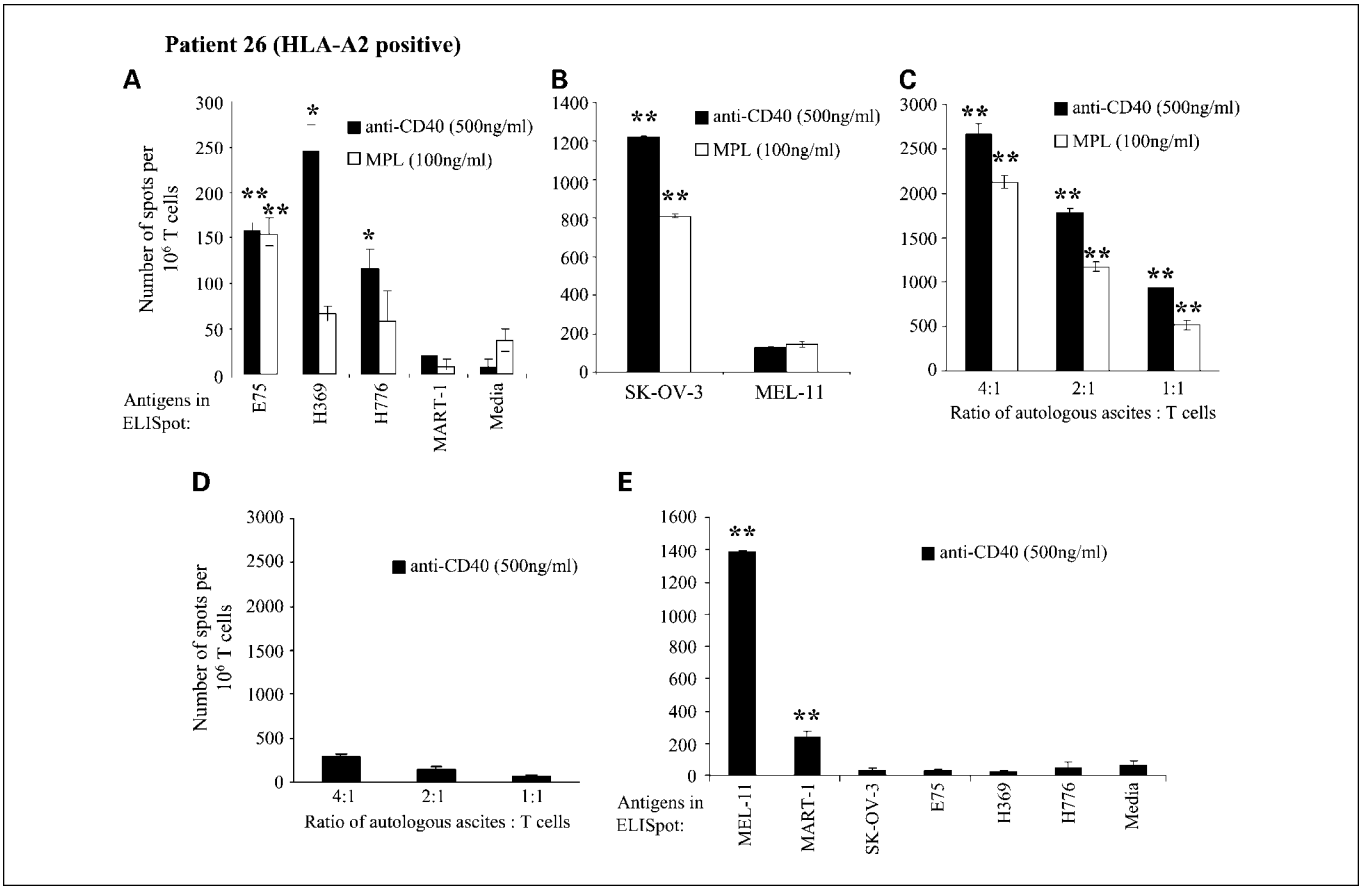

Fig. 5. Patients' T cells stimulated with mature dendritic cells pulsed with oxidized tumor cells respond with an immunogen-selected specificity. T cells from a HLA-A2$^+$ patient (patient 26) were cocultured with autologous dendritic cells that were loaded with either oxidized SK-OV-3 (*A-C*) or oxidized melanoma cells MEL-11 (*D-E*), and then all groups were matured with CD40 agonistic antibody (500 ng/mL) or MPL (100 ng/mL). After a 2-wk expansion period, T cells were isolated and tested in IFN-γ ELISpot assays. In ELISpot (*A*), T cells were cocultured with PBMCs (1×10^4) and HER-2/neu class I MHC (HLA-A2, E75) and class II MHC peptides H369 and H776, or control peptide MART-1 (all at a final concentration of 1 μmol/L) or medium only. In ELISpot (*B*), T cells were cocultured with PBMCs (1×10^4) and oxidized SK-OV-3 cells (ratios of SK-OV-3 to T cells were 2:1). In ELISpot assays (*C* and *D*), T cells were cocultured in the absence of PBMCs with autologous ascites-derived tumor cells at various ratios as shown. In ELISpot assay (*E*), T cells were cocultured with PBMCs (1×10^4) and oxidized MEL-11 or oxidized SK-OV-3 cells (ratio of 1 tumor cell to 1 T cell), or various peptides as detailed above. The results show mean number of spots per million T cells, from triplicate cultures. The *P* value is significant if <0.05 (*) or <0.01 (**) when comparing the test groups to the medium control using ANOVA with Dunnett's post hoc modification.

There is considerable evidence that suggests that $CD8^+$ T-cell responses primed in the absence of cognate help are impaired functionally in a variety of ways (39–42). Both by using peptide epitopes known to bind a broad range of class II MHC molecules, by direct depletion of $CD8^+$ T cells, and by the use of blocking antibodies, we showed that class II MHC–restricted $CD4^+$ T-cell responses are activated in these cultures. The response to the peptides is variable and may reflect differential binding of these peptides to different HLA alleles (note that we did not HLA type the individual patients other than to determine if they were HLA-A2 positive or negative). The overall magnitude of the response is much smaller (at least in terms of IFN-γ–producing cells per million T cells) than the class I response, a difference that is common for immune responses to many viral antigens (e.g., ref. 43). Furthermore, the response was more dependent on full maturation of the dendritic cells, perhaps reflecting the greater dependency of the $CD4^+$ T-cell response on costimulatory activity by the antigen-presenting cells, or a requirement for higher levels of MHC class II.

The third important observation in this study was the tumor specificity of the response. Given that the effectiveness of tumor therapy must depend on partial breaking of self-tolerance, a persistent concern has been that the immune system will recognize and kill cells other than the tumor and hence cause autoimmune disease. This concern is not simply hypothetical—a recent immunotherapy trial showed a close relationship between tumor response and the development of autoimmune pathology (44). This problem is particularly acute with the use of whole-cell immunogens because all cells share a large number of "common" proteins involved in housekeeping cellular metabolic functions and expressing smaller numbers of "tissue-specific" proteins. A striking result from our study was that these "common" proteins do not appear to break tolerance in these experiments because priming with oxidized ovarian-derived SK-OV-3 and with oxidized melanoma-derived MEL-11 cells generated T cells with minimum cross-reactivity with each other's priming antigen. Of course, the risk remains of some cross-reactivity with normal (untransformed) epithelial tissue, such as breast epithelium, which shares several tumor-associated antigens, or normal peritoneum. Further studies will be required to define the exact repertoire of the response and hence the potential danger of excess autoimmune reactions.

This study leaves some important questions for future study. The molecular mechanism by which oxidation enhances immunity, and why this can lead to a break in tolerance, remains unknown and is the subject of detailed examination in our laboratory. Furthermore, it is very likely that the responses generated could be amplified further, for example by blocking negative regulatory interactions between dendritic cells and T cells, such as those mediated by CTL-associated protein 4 (45, 46) or programmed death-ligand-1 (47–49). Nevertheless, the results presented here suggest that oxidized tumor cells will provide an excellent and novel starting option as a source of generic antigens for use in dendritic cell–based immunotherapy protocols. We are now further optimizing antigen loading and dendritic cell maturation in ovarian cancer patients with recurrent disease, as the combination of oxidized tumor and dendritic cells could provide a successful adjuvant therapeutic strategy for these patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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