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Tumor-Specific CD4⁺ T Lymphocytes from Cancer Patients Are Required for Optimal Induction of Cytotoxic T Cells Against the Autologous Tumor

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Tumor-Specific CD4⁺ T Lymphocytes from Cancer Patients Are Required for Optimal Induction of Cytotoxic T Cells Against the Autologous Tumor

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This study focuses on the specific CD4⁺ T cell requirement for optimal induction of cytotoxicity against MHC class II negative autologous tumors (AuTu) collected from patients with various types of cancer at advanced stages. CD4⁺ T cells were induced in cultures of cancer patients' malignant effusion-associated mononuclear cells with irradiated AuTu (mixed lymphocyte tumor cultures (MLTC)) in the presence of recombinant IL-2 and recombinant IL-7. Tumor-specific CD4⁺ T cells did not directly recognize the AuTu cells, but there was an MHC class II-restricted cross-priming by autologous dendritic cells (DCs), used as APC. CD8⁺ CTL, also induced during the MLTC, lysed specifically AuTu cells or DCs pulsed with AuTu peptide extracts (acid wash extracts (AWE)) in an MHC class I-restricted manner. Removal of CD4⁺ T cells or DCs from the MLTC drastically reduced the CD8⁺ CTL-mediated cytotoxic response against the AuTu. AWE-pulsed DCs preincubated with autologous CD4⁺ T cells were able, in the absence of CD4⁺ T cells, to stimulate CD8⁺ T cells to lyse autologous tumor targets. Such activated CD8⁺ T cells produced IL-2, IFN- γ , TNF- α , and GM-CSF. The process of the activation of AWE-pulsed DCs by CD4⁺ T cells could be inhibited with anti-CD40 ligand mAb. Moreover, the role of CD4⁺ T cells in activating AWE-pulsed DCs was undertaken by anti-CD40 mAb. Our data demonstrate for the first time in patients with metastatic cancer the essential role of CD4⁺ Th cell-activated DCs for optimal CD8⁺ T cell-mediated killing of autologous tumors and provide the basis for the design of novel protocols in cellular adoptive immunotherapy of cancer, utilizing synthetic peptides capable of inducing T cell help *in vivo*. *The Journal of Immunology*, 2000, 164: 3902–3912.

Adequate Th cell assistance is critical for the development of optimal immune reactions in the course of a cell-mediated immune response. During such responses, Ag is processed by professional APC and presented to CD4⁺ Th cells in the context of MHC class II molecules. The resulting activated CD4⁺ T cells subsequently provide additional help to Ag-specific CTL, mostly through the release of cytokines.

Although CD4⁺ Th cells possess a central role in regulating virtually all Ag-specific immune responses, their contribution in the initiation of antitumor immune responses has until recently been obscure. Strong evidence for the essential role of CD4⁺ T cells in antitumor immunity initially came from *in vivo* studies with murine tumor cell lines. After transfection with MHC class II genes, such tumor cells directly activated CD4⁺ T cells, resulting in tumor regression and induction of immunity against the wild-type MHC class II-negative tumors (1–4). Tumor-specific CD4⁺ T cells restricted by MHC class II gene products have also been reported in patients with melanoma, lymphoma, sarcoma, colon cancer, and breast cancer (5–7). Responses of CD4⁺ T cells against tumor-specific peptide sequences expressed in an idiotypic IgM of a B cell lymphoma (8), bcr-abl fusion proteins (9), HPV

type 16 E7 oncoprotein (10), and *k^{ras}* oncogene (11–13) have also been documented.

The essential role of CD4⁺ T cells providing help for maximal activation of tumor-specific CTL further emerged from cell-based vaccine models against MHC class II-negative murine tumors (14–16). Such studies indicated that tumor Ags released at the tumor site are taken up by macrophages, processed, and presented to CD4⁺ T cells, which in response produce and secrete lymphokines that activate tumor-specific CTL. In a recent report, Ossendorp et al. (17) demonstrated the induction of tumor-protective immunity by vaccination with a tumor-specific murine leukemia virus env-encoded Th peptide. In this way, strong protection was achieved against the highly aggressive MHC class II-negative RMA lymphoma line. These data provided indirect evidence for the cross-priming of tumor Ags by local APCs. In the same study as well as in others (18, 19), CD8⁺ T cells recognizing tumor Ags of viral origin were identified as the cytotoxic effector cells. Finally, in a murine model, Shoenberger et al. (20) demonstrated that *in vivo* generation of CTL specific for an adenovirus E18 protein-epitope requires cross-priming by host APC and is strictly helper dependent, because mice depleted of CD4⁺ Th cells were no longer capable of generating E18-specific CTL responses.

All these studies lead to the conclusion that induction of T cell help *in vivo* or cellular adoptive immunotherapy utilizing both tumor-specific helper and cytotoxic T cells are essential for improving clinical results in cancer immunotherapy. However, to apply such protocols in cancer treatment, the results obtained from the various experimental animal models must also be demonstrated in the human system. Herein, we report for the first time the absolute requirement of synergistic interactions accomplished by autologous tumor-specific CD4⁺ and CD8⁺ T cells and dendritic

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Table I. HLA serotypes of the patients included in experiments assessing specificity of CD4⁺ and CD8⁺ T cells^a

Patient ^b	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
1	2.1 ^c 3	35, 41	W4, W7	1, 13	6, 7
2	24, 32	18, 51	W4, W7	2, 3	5, 2
3	2.1, 19	35	W5	11, 14	5, 10
4	1, 32	35, 21	W6	5, 10	2, 3
5	2.1, 11	38	W8	4, 7	5, 7
6	24, 32	51	W1	1, 11	3
7	1, 28	27, 51	W2	9, 14	1, 9
8	1, 19	44, 60	W3	8, 12	1, 3
9	2.1, 3	18	W8	4, 13	5, 7
10	11, 28	18, 27	W2, W7	2, 5	4, 7

^a Results from specificity experiments are shown in Figs. 3 and 4 and presented in Tables II and III.

^b Patients are listed in the same order as in Figs. 3 and 4 and Tables II and III.

^c Expression of HLA-A2.1 subtype was determined with the BB7.2 mAb.

cells (DCs)² for optimal killing of autologous tumor cells in patients with various types of metastatic cancer. CD4⁺ T cells were cross-primed against the MHC class II-negative tumors by autologous DCs. Our data also demonstrate that an initial interaction of CD4⁺ T cells with acid wash extract (AWE)-pulsed autologous DCs is required for providing all necessary conditions to the latter to induce optimal CTL priming. Blockade of the interaction between CD4⁺ T cells and DCs by anti-CD40 ligand (CD40L) mAb greatly inhibited CTL priming, which could be bypassed by signaling through CD40.

Materials and Methods

Patients

The study included 19 patients (11 women, 8 men; medium age, 65 years; range, 45–79 years) with histologically proven malignant tumors. All had measurable metastatic disease and fulfilled the following criteria: Karnofsky performance status at least 80%; bilirubin concentrations <1.7 ng/dl; creatinine <2.2 ng/dl; leukocyte count >3000/μl; and platelet count >100,000/μl. They had not received any antineoplastic therapy during the 3 wk preceding the onset of the study. Patients' HLA serotypes, in the same order as they appear in Figs. 3 and 4 and in Tables II and III, are given in Table I. All patients were apprised of the study, and consents were obtained consistent with the policies of St. Savas Cancer Hospital. The clinical stage of disease of the participating patients was as follows: stage III, *n* = 5 and stage IV, *n* = 14. Tumor grade III was scored in all patients.

Monoclonal Abs

Anti-CD83 conjugated with PE mAb was obtained from Caltag Laboratories (Burlingame, CA). All other mAbs were purchased from Pharmingen (San Diego, CA). Anti-CD4, -CD8, -CD16, -CD20, -CD40, and -CD80 mAbs as well as mAb to monomorphic determinants of HLA class I and class II molecules were conjugated with FITC. Anti-CD3, -CD14 -CD54, -CD86, and HLA-DR mAbs were used conjugated with PE. For blocking experiments, purified anti-CD40 (IgG1, clone 5C3), anti-CD40L (CD154; IgG1, clone TRAP1), anti-HLA class I (IgG1, clone G46-2.6), anti-HLA class II (IgG2b, clone TŪ36) and isotype-matched mouse IgG1 (anti-trinitrophenol (TNP), clone 107.3), and mouse IgG2b (anti-TNP, clone 49.2) mAbs were used. The latter two mAbs conjugated with PE as well as FITC-conjugated anti-TNP mouse IgM (clone G155–228) mAb were also used as isotype-matched negative controls in immunostaining experiments. Anti-cytokine (IL-2, GM-CSF, TNF-α, IFN-γ, and IL-4) mAbs were used conjugated with PE. BB7.2 (anti-HLA-A2.1) mAb was kindly donated by Professor H.-G. Rammensee (Department of Immunology, University of Tübingen, Tübingen, Germany).

Preparation of effusion cells

Specimens of pleural effusions (300–500 ml) from 4 patients with lung adenocarcinoma and peritoneal effusions (1–2 l) from patients with mel-

noma (*n* = 1), breast cancer (*n* = 5), and ovarian cancer (*n* = 5) were centrifuged at 400 × *g* for 5 min to sediment cells. Malignant effusion-associated mononuclear cells (MEAMNC) and tumor cells were isolated from the cell pellet as previously described (21). In brief, 1 × 10⁸ cells in 4–5 ml X-VIVO 15 medium (BioWhittaker, Walkersville, MD) were placed on top of a 75–100% discontinuous Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 200 × *g* for 25 min. Tumor cells (>80%) were collected from the top of the 75% Ficoll-Hypaque, and MEAMNC (>90%) were collected from the interface of 75% and 100% Ficoll-Hypaque. Both layers were washed once and resuspended in X-VIVO 15 medium supplemented with 1% heat-inactivated autologous serum. In agreement with our previous report (22), freshly isolated MEAMNC consisted mainly of T cells (>70% CD3⁺ cells) with almost equal numbers of CD3⁺CD8⁺ (30–35%) and CD3⁺CD4⁺ (34–39%) T cells. A substantial number of monocytes (CD14⁺ cells) was also detected (10–16%). Total MEAMNC or CD4⁺ and CD8⁺ T cells were used thereof as responders in the MLTC, whereas CD14⁺ cells were used to generate DCs. Total MEAMNC were also used as APC for restimulations during the MLTC. In all cases examined, the number of MEAMNC isolated from the effusions ranged from 175 to 900 × 10⁶ and of tumor cells from 75 to 400 × 10⁶. Aliquots of MEAMNC and autologous tumor cells were frozen in liquid N₂. Phenotype analysis showed that all tumors expressed MHC class I molecules but were negative for MHC class II gene products.

Isolation of T cell subsets

CD4⁺ or CD8⁺ T cells were isolated from total MEAMNC using MACS CD4 or MACS CD8 Microbeads, respectively, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, 1 × 10⁷ MEAMNC were washed and resuspended in 80 μl PBS supplemented with 0.5% BSA and 2 mM EDTA (buffer A). To this, 20 μl MACS CD4 or MACS CD8 Microbeads were added, and the mixture was incubated for 15 min at 6–12°C. Cells were washed with buffer A, resuspended in 500 μl buffer A per 1 × 10⁸ cells, and separated on positive selection columns placed in the magnetic field of a MACS separator (Miltenyi Biotec). Negative cells were allowed to flow through the column, centrifuged, and resuspended in X-VIVO 15 medium. The column was further removed from the MACS separator and placed on a suitable tube. The CD4⁺ or CD8⁺ T cell fraction was eluted with 1–2 ml buffer A and collected using a plunger provided with the column. For the isolation of highly purified CD4⁺ T cells, total MEAMNC were initially monocyte-depleted by plastic adherence (2 h at 37°C) before incubation with MACS CD4 Microbeads. The purity of isolated CD4⁺ or CD8⁺ T cells was in all cases >97%, whereas the negative fractions (i.e., CD4⁺ or CD8⁺ T cell-depleted MEAMNC) were totally devoid of CD4⁺ or CD8⁺ T cells, respectively. Purity of the isolated cell populations was tested by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

Monocyte isolation

CD14⁺ cells were isolated from total MEAMNC using the Monocyte Isolation Kit (Miltenyi Biotec), comprising a mixture of CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs coupled to MACS Microbeads. The experimental procedure is identical with that described for CD4⁺ or CD8⁺ T cell isolation, the only difference being the addition of FcR blocking reagent before the addition of the Ab mixture. Monocytes (CD14⁺) thus isolated were highly pure (>98%), and no CD14⁺ cells could be detected in the MEAMNC-depleted fraction as analyzed by flow cytometry.

Preparation of AWE

This was performed as recently described (23) with modifications. Briefly, 2 × 10⁷ tumor cells were washed with HBSS (Life Technologies, Gaithersburg, MD), followed by homogenization in 1 ml homogenization buffer (23). Eluates from cells were titrated with 10% trifluoroacetic acid and clarified by two successive centrifugations at 2,500 × *g* and 80,000 × *g* for 30 min and 5 h, respectively. The peptides were processed immediately on a SepPak C₁₈ cartridge (Waters, Bedford, MA), equilibrated prior to use with 3 ml acetonitrile, followed by 3 ml deionized water. The eluate was allowed to flow through the cartridge by gravity, the column was washed with deionized water, and bound material was finally eluted with 2 ml 60% acetonitrile in deionized water and lyophilized in a Speed-Vac (Heto Lab Equipment, Allerød, Denmark). The dry product was reconstituted in HBSS and further processed on a Centricon centrifuge concentrator (Amicon, Beverly, MA) with a cutoff of 10 kDa by centrifugation at 2500 × *g* at 4°C for 2–3 h. The filtrate was aliquoted and stored at –20°C.

² Abbreviations used in this paper: DCs, dendritic cells; AWE, acid wash extracts; CD40L, CD40 ligand; MLTC, mixed lymphocyte tumor cultures; TNP, trinitrophenol; MEAMNC, malignant effusion-associated mononuclear cells; AuTu-AWE-DC, DCs pulsed with AWE from the autologous tumor.

Table II. *MLTC-derived CD4⁺ T cells proliferate in vitro in response to autologous DC pulsed with AWE from autologous but not allogeneic metastatic tumors*

CD4 ⁺ T Cell Donors ^a (patient no.)	AWE Donors (patient no.)									
	1	2	3	4	5	6	7	8	9	10
1	17.96^b	0.95	0.85	0.54	9.26	0.39	1.01	0.35	5.87	0.24
2	0.71	12.91	0.32	0.67	1.35	0.56	1.70	0.92	1.95	0.76
3	1.52	1.71	15.76	0.79	0.85	0.92	1.63	0.75	1.07	0.93
4	0.63	0.35	0.97	10.59	1.32	1.25	0.76	1.23	0.69	0.91
5	5.35	0.52	0.44	0.32	11.52	0.39	0.62	0.53	6.30	0.65
6	0.22	0.95	0.55	0.78	0.51	10.25	0.42	0.40	0.43	0.44
7	1.72	1.95	0.62	0.76	0.39	2.10	21.23	0.95	1.25	0.35
8	0.52	0.23	0.17	0.65	0.72	0.35	0.17	8.90	1.90	0.35
9	6.25	0.72	0.83	0.69	9.65	0.52	0.72	0.70	19.87	0.71
10	1.05	2.01	0.72	0.36	0.64	0.57	0.62	0.92	0.76	13.55

^a MLTC-activated CD4⁺ T cells were tested for proliferative responses in cultures with autologous DC pulsed with AWE from autologous or allogeneic metastatic tumors (see also *Materials and Methods*).

^b Mean values from triplicate cultures are given as cpm × 10⁻³. The SD was always <15% of the mean values and thus is omitted. Values in bold indicate statistically significant (*p* < 0.01) proliferative responses over background values (i.e., CD4⁺ T cells with nonpulsed DC, which never exceeded 1200 cpm).

Generation of DCs

DCs were prepared as recently described (24). Briefly, monocytes isolated from MEAMNC were grown for 7 days in X-VIVO 15 medium supplemented with 1% autologous serum, 800 U/ml rGM-CSF (Schering-Plough, Brinny, Innishannon, Ireland) and 500 U/ml rIL-4 (R&D Systems Europe, Abington, U.K.). rTNF-α (R&D Systems Europe) was added at 10 ng/ml for the last 24 h before culture termination. The percentage of DCs recorded was >50%, as based on the expression of a CD3⁻, CD14⁻, CD16⁻, CD20⁻, CD40⁺, CD80⁺, CD83⁺, CD86⁺, and MHC class II⁺ phenotype analyzed by flow cytometry.

Pulsing of DCs with AWE

AWE was added to 6-day cultured DCs along with rTNF-α. The amount of AWE (initially prepared from HLA-A2.1-positive patients) for pulsing DCs was estimated based on its capacity to induce maximal stabilization of HLA-A2.1 expression on T2 cells as detected with the BB7.2 mAb (25). Thus, AWE extracted from 5 × 10⁶ autologous tumor cells was added to 1 × 10⁶ DCs. After a 24 h incubation in CO₂ incubators, cells were irradiated (4000 rad), washed twice in medium, and used as AWE-pulsed DCs (AuTu-AWE-DC) in experiments. In the experiments shown in Fig. 3 and presented in Table II, pulsing of a single donor's DCs with AWE from various allogeneic tumors was performed as follows. DCs (1 × 10⁴) were placed in 100 μl X-VIVO 15 medium per well in V-bottom 96-well plates (Costar, Cambridge, MA) along with 100 μl X-VIVO 15 medium containing AWE extracted from 5 × 10⁴ cells of each allogeneic tumor. Every group consisted of triplicate cultures. Plates were incubated for 24 h in CO₂ incubators and thereafter were irradiated (4000 rad). AWE-pulsed, irradiated DCs were further gently washed twice (300 × g; 5 min), resuspended

in 100 μl fresh X-VIVO 15 medium, and coincubated with autologous CD4⁺ T cells for the proliferation assays (see below). A similar procedure was followed for the cytotoxicity assays shown in Fig. 4 and presented in Table III except 1) the DCs were first labeled with ⁵¹Cr and then incubated for 24 h with AWE and 2) the isotope-labeled and pulsed DCs were not irradiated but gently washed to remove excess of isotope before coincubation with the effectors for the cytotoxicity assays (see below).

Pulsing of MEAMNC with AWE

Thawed autologous MEAMNC (10 × 10⁶ cells/ml) were incubated for 24 h in CO₂ incubators with AWE extracted from an equal number of autologous tumor cells. Cells were irradiated (4000 rad), washed, and used as stimulators.

Mixed lymphocyte tumor culture

Responder MEAMNC (2 × 10⁶ cells/ml) were cocultured in 25-cm² flasks (Costar) with 2 × 10⁵ irradiated (10,000 rad) autologous tumor-stimulatory cells in a total volume of 5 ml X-VIVO 15 medium supplemented with 1% autologous serum, rIL-7 (20 ng/ml) (R&D Systems Europe), and 25 IU/ml rIL-2 (Cetus, Emeryville, CA) in CO₂ incubators. Five days later, one-half of the medium was replenished with fresh medium containing 40 ng/ml rIL-7 and 50 IU/ml rIL-2. After 10 days of culture (=stimulation phase), recovered responders were washed and restimulated with thawed irradiated (4000 rad) autologous MEAMNC used as APC and autologous irradiated tumor cells (10,000 rad) at a cell ratio of 10:1:1, respectively. Preliminary experiments showed that the responder-stimulator ratio of 10:1 was optimal for sensitizing both CD4⁺ and CD8⁺ T cell responses in the MLTC (almost equally effective to 5:1 but superior to 20:1; data not shown) and

Table III. *MLTC-derived CD8⁺ T cells lyse AuTu-AWE-DC or whole autologous tumor targets but not autologous DC pulsed with AWE from allogeneic tumors or whole allogeneic tumor targets*

CTL Donors ^a (patient no.)	AWE ^b /Whole Tumor Cell ^c Donors (Patient No.)									
	1	2	3	4	5	6	7	8	9	10
1	40^b/45^{c,d}	3/2	1/2	2/5	27/22	2/3	2/3	1/2	20/23	1/4
2	1/2	17/20	3/1	0.5/1	2/2	1/1	0.5/1	1/1	2/3	1/2
3	3/2	5/4	25/21	3/2	5/3	6/5	4/2	3/3	2/1	5/3
4	1/3	2/5	3/3	34/35	3/2	5/3	3/5	5/2	3/3	3/6
5	20/17	3/5	1/3	1/2	35/39	2/1	1/1	2/3	25/20	3/3
6	2/5	3/4	2/1	2/2	3/3	26/23	4/2	1/3	5/2	3/5
7	1/1	0.5/1	2/2	3/4	2/3	5/2	32/30	3/2	4/3	5/2
8	3/2	5/4	1/1	0.5/2	2/2	2/3	5/2	37/42	1/1	2/2
9	19/17	3/6	5/3	3/4	17/20	0.5/1	1/2	6/5	29/32	2/3
10	5/2	3/3	6/5	3/2	7/5	3/5	2/3	3/3	2/5	22/25

^a MLTC-activated CD8⁺ CTL were tested in cytotoxicity assays against autologous DCs pulsed with AWE from autologous or allogeneic tumors or against whole autologous or allogeneic tumor targets. Results are expressed as mean percent cytotoxicity from triplicate cultures. The SD never exceeded 25% of the means and thus is omitted.

^b Autologous or allogeneic tumors.

^c Whole autologous or allogeneic tumor targets.

^d Values in bold indicate statistically significant (*p* < 0.01) lytic responses over background values.

Table IV. Levels of adhesion, costimulatory and MHC class II molecules on treated DCs

Treatment ^a	CD54	CD80	CD86	HLA-DR
Plain medium	614	342	368	1064
CD4 ⁺ T cells	2069	808	1546	1854
Anti-CD40 mAb	2344	598	1435	1754
CD4 ⁺ T cells + anti-CD40L mAb	818	383	550	1006

^a DCs were treated for 48 h as described in *Materials and Methods*. Results show the median fluorescence intensity. Background values are subtracted.

therefore was used in all protocols. Fresh rIL-2 (25 IU/ml) was also added to the medium. Depending on the quality of each culture after the stimulation phase, viable responder cells were isolated over Ficoll-Hypaque. After two additional rounds of restimulation performed at 5-day intervals or as needed, cells were tested in proliferation and cytotoxicity assays.

To precisely analyze the role of CD4⁺ T cells as well as of DCs for the induction of cytotoxicity, MLTC were initiated with purified CD4⁺ and CD8⁺ T cells as responders (1×10^6 cells of each subset per ml) and irradiated (4000 rad) AWE-pulsed DCs (1×10^5 cells/ml) as stimulators. In this type of MLTC, control cultures consisted of (culture A) 1×10^6 CD8⁺ responder cells/ml and irradiated 1×10^5 /ml AWE-pulsed DCs or (culture B) 1×10^6 CD8⁺ T cells/ml and 1×10^6 CD4⁺ T cells/ml pulsed with AWE. Restimulations were performed with AWE-pulsed irradiated (4000 rad) autologous MEAMNC. In all restimulations, AWE-pulsed MEAMNC in control cultures (A) were depleted of CD4⁺ T cells and in culture B of CD14⁺ cells to ensure the absolute exclusion of CD4⁺ T cells in culture A and CD14⁺ cells in culture B in any activation process of the CD8⁺ T cells during the restimulation phase of the MLTC. The ratio of responder cells to AWE-pulsed MEAMNC during restimulations was always kept at 10:1. Cytokines were added throughout the MLTC as already described.

In another series of experiments, MLTC were set up with responder MEAMNC depleted of either CD4⁺ T (culture A) or CD14⁺ (culture B) cells. Such MLTC were stimulated with AWE-pulsed DCs at a cell ratio of 10:1 (culture A) or with AWE-pulsed CD4⁺ T cells at the same ratio (culture B). Addition of cytokines and restimulations in both groups of MLTC were performed as described above.

Interaction of DC with autologous CD4⁺ T cells

DCs were prepared from thawed MEAMNC and pulsed with AWE from the AuTu as previously described. One day before harvesting the AuTu-AWE-DCs, a new aliquot of autologous MEAMNC was thawed; cells were washed once and left overnight in a CO₂ incubator in X-VIVO 15 medium supplemented with 1% autologous serum. CD4⁺ T cells were isolated from MEAMNC the day after and incubated for 48 h with AuTu-AWE-DCs at a CD4⁺:DC ratio of 5:1, in the presence or absence of anti-CD40L mAb (10 μg/ml final concentration). In some groups, AuTu-AWE-DCs were incubated for 48 h with anti-CD40 mAb (10 μg/ml) or in plain medium. The time period of 48 h to induce activated DCs on incubation with CD4⁺ T cells or anti-CD40 mAb was chosen based on findings from others (26, 27) who measured high levels of IL-12 release and potent T cell-stimulatory capacity by similarly activated DCs. All incubations were performed in 24-well plates (Costar) at a final volume of 2 ml/well, in CO₂ incubators. Further, the supernatant including the nonadherent CD4⁺ T cells (with or without the mAbs) was decanted, and wells were washed twice with X-VIVO 15 medium to remove residual CD4⁺ T cells and/or excess mAbs. Treated DCs were then detached by vigorous pipetting and, if necessary, with a rubber policeman; thus, ~65–80% of the initially plated DCs could be recovered. An aliquot of DCs was subjected to immunofluorescence analysis (Table IV). MLTC were then initiated by culturing the variously treated DCs (see also Fig. 6) with autologous CD8⁺ T cells freshly isolated from thawed MEAMNC at a cell ratio of 1:10 in the presence of IL-7 and IL-2 at the aforementioned concentrations. Restimulations were performed with AWE-pulsed CD4⁺ T cell-depleted autologous irradiated (4000 rad) MEAMNC.

Cytotoxicity assays

These were performed as described (22). Briefly, MLTC-activated CD8⁺ effector cells (1×10^6 cells/ml) were placed in 100-μl aliquots into wells of 96-well V-bottom plates (Costar). As targets, whole tumor cells or DCs pulsed with AWE were labeled with sodium [⁵¹Cr]chromate (Radiochemical Centre, Amersham, U.K.; 100–200 μCi isotope per $1-2 \times 10^6$ target

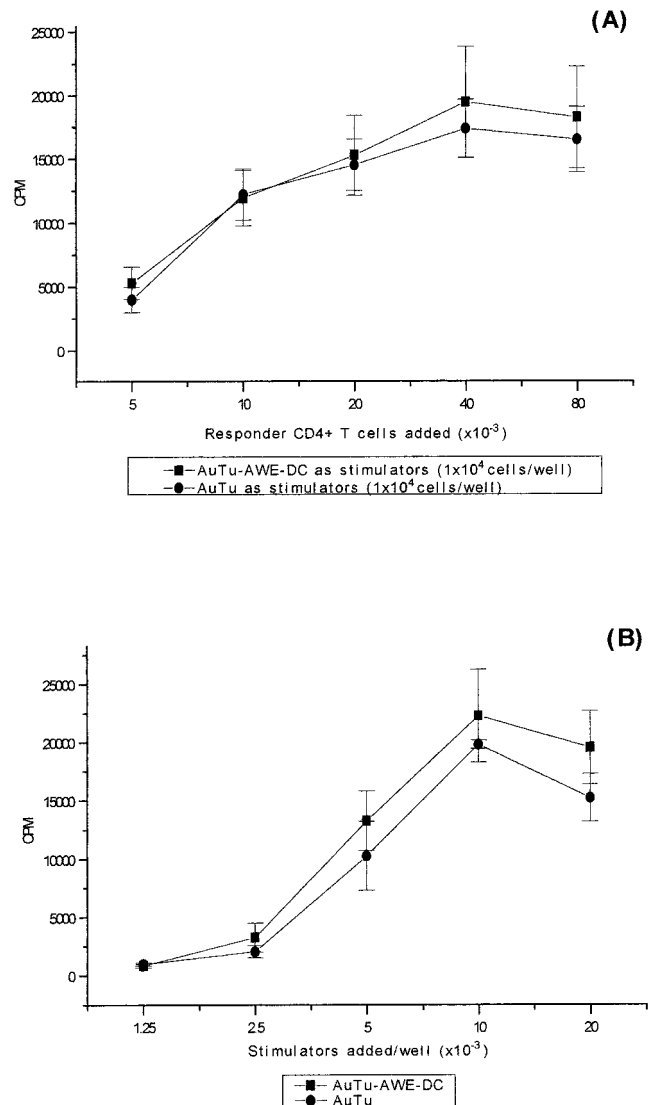


FIGURE 1. Titration of MLTC-derived responder CD4⁺ T cells (A) and stimulatory DCs pulsed with AWE from the autologous tumor (AuTu-AWE-DC) or whole autologous tumor cells (AuTu) in the proliferation assays (B). In B, responder CD4⁺ T cells were added at 4×10^4 cells/well. Values are mean \pm SD from two patients in each case. Details for the proliferation assay are given in *Materials and Methods*.

cells) and added to the effectors at an E:T ratio of 20 or as otherwise indicated. Incubation was performed for 6 h in CO₂ incubators. For blocking experiments, anti-MHC class I and isotype-matched control mAbs were added throughout the incubation period at a final concentration of 10 μg/ml. Supernatant (100 μl) was collected from each well, and the radioactivity was measured in a gamma counter (Packard, Downers Grove, IL).

Proliferation assays

CD4⁺ T cells isolated from 15–20-day MLTC by immunomagnetic separation from total MEAMNC were cultured for 5 days in X-VIVO 15 medium supplemented with 1% autologous serum in 96-well V-bottom plates (Costar) at 4×10^4 cells/well with 1×10^4 irradiated (4000 rad) autologous DCs pulsed with AWE or with 1×10^4 autologous DCs (4000 rad) and 1×10^4 autologous tumor cells (10,000 rad) at a final volume of 200 μl/well. The optimal numbers of cells used in the proliferation assays (i.e., responder CD4⁺ T cells and stimulatory AWE-pulsed DCs or tumor cells) were determined from titration experiments (Fig. 1). Anti-MHC class I or anti-MHC class II mAbs were added at a final concentration of 10 μg/ml for the entire incubation period. Anti-TNP mAb (isotype-matched to anti-MHC class II mAb) was also added at the same concentration as for a control. Fifty microliters ($=1 \mu\text{Ci}$) [³H]TdR (Amersham) were added per

well 24 h before culture termination. Incorporated [³H]TdR in the DNA of proliferating cells was measured in a beta counter (Packard). All cultures were performed in triplicates, and results are expressed as cpm.

Intracellular cytokine production

CD8⁺ T cells cultured with the variously treated autologous AWE-pulsed DC during the MLTC stimulation phase were analyzed for intracellular expression of IL-2, IFN- γ , GM-CSF, TNF- α , and IL-4. To enhance intracellular fluorescence, protein secretion was inhibited by the addition of 1 μ M brefeldin A (Sigma, St. Louis, MO) (28). Intracellular cytokine staining was then analyzed as described (29). In brief, cells were fixed with 4% paraformaldehyde for 10–15 min, and 0.1% saponin in PBS was used to permeabilize the cell membrane. The respective anti-cytokine mAbs were used at a concentration of 1 μ g/ml. To detect cytokine-positive CD8⁺ T cells, MLTC-recovered cells were stained for 30 min with a mixture of FITC-conjugated anti-CD8 mAb and peridin-chlorophyll conjugate (PerCP)-conjugated anti-CD3 mAb (Becton Dickinson, Mountain View, CA), followed by a further 30 min incubation with purified anti-cytokine mAbs conjugated with PE. All flow cytometry data were calculated with LYSYS II (Becton Dickinson) software.

Phenotype analysis

Flow cytometry was conducted on a FACScan (Becton Dickinson) flow cytometer. Direct single-color immunofluorescence assays were performed with FITC- or PE-conjugated mAbs. Isotype-matched mAbs conjugated with FITC or PE were used as background.

Quantitation of cytokines in culture supernatants

ELISA kits specific for IL-2, TNF- α , and IL-4 were obtained from R&D Systems Europe. IFN- γ and GM-CSF were quantitated with ELISA kits from Endogen (Boston, MA). Assays were performed according to the manufacturers' instructions.

Results

Generation of autologous tumor-specific CD4⁺ T cells during MLTC

Nineteen MLTC cultures were established from an equal number of cancer patients using total MEAMNC as responder cells and autologous tumor cells as stimulatory cells. On days 15–20 after culture initiation, CD4⁺ T cells were isolated by immunomagnetic separation and tested for proliferative responses against the autologous tumor cells in the presence of autologous DCs as APC (AuTu + DC) (Fig. 2A) or against autologous DCs pulsed with AWE from the autologous tumor (AuTu-AWE-DC) (Fig. 2B). CD4⁺ T cells proliferated vigorously in both cases with comparable results. The proliferative responses were blocked to a great extent by monomorphic mAb to MHC class II molecules but remained unaffected in the presence of an isotype-matched control mAb (anti-TNP) or anti-MHC class I mAb (Fig. 2). To check the specificity for the autologous tumor cells, MLTC-activated CD4⁺ T cells were tested for proliferation in response to autologous DCs pulsed with AWE from allogeneic metastatic tumors. The results from these experiments are shown in Fig. 3 and summarized in Table II. A typical immune response profile is shown with donor 6 (melanoma patient) whose CD4⁺ T cells proliferated only in response to AuTu-AWE-DC. Similar results were also obtained with donors 2, 3, 4, 7 (lung adenocarcinoma), 8 (breast cancer), and 10 (ovarian cancer) (Table II). An interesting situation appeared with donors 1 (breast cancer), 9, and 5 (both ovarian cancer), whose CD4⁺ T cells, in addition to the AuTu-AWE-DC, also recognized and proliferated in response to autologous DCs pulsed with AWE from allogeneic tumor cells from the other two patients (Fig. 3 and Table II). Such proliferative responses, although weaker compared with those stimulated by the AuTu-AWE-DC, still were significantly higher over background values (not exceeding 800 cpm) and could be inhibited with anti-MHC class II mAb (Fig. 3, B columns).

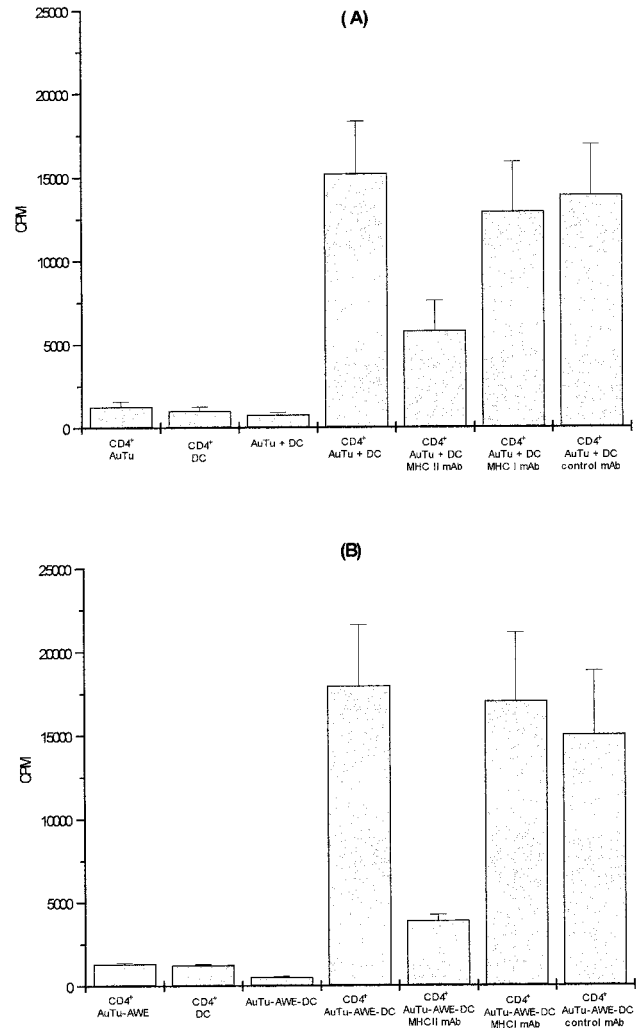


FIGURE 2. MLTC-activated CD4⁺ T cells proliferate in response to the stimulatory autologous tumor cells in the presence of autologous DCs as APC. CD4⁺ T cells were collected from MLTC and tested for proliferative responses in microculture plates in the presence of whole autologous tumor cells (AuTu) and autologous DCs (A) and autologous DCs pulsed with AWE from the autologous tumor cells (AuTu-AWE-DC) (B). All mAbs were added at 10 μ g/ml final concentration throughout the culture period. Bars represent mean values \pm SD from 19 experiments (all 19 patients were tested). Control anti-TNP mAb, isotype-matched to anti-MHC class II mAb, was included in four experiments.

Induction of CTL responses specific for the autologous tumor cells during MLTC

Using the same MLTC cultures as above, we succeeded, after three rounds of restimulation with autologous irradiated tumor cells and MEAMNC as APC, to generate in vitro cytotoxicity specific for the autologous tumor cells. As shown in Fig. 4, CD8⁺ T cells isolated by immunomagnetic separation from MLTC-activated MEAMNC of donor 6 lysed exclusively AuTu-AWE-DC (Fig. 4, column 6A). The cytotoxic response was to a great extent inhibited by monomorphic mAb to HLA class I molecules (Fig. 4, column 6B). Similar cytotoxicity patterns were observed with the other donors (Table III), with the exception of patients 1, 9, and 5 whose MLTC-activated CD8⁺ T cells showed significant cross-reactivity against autologous DCs pulsed with AWE from each other's tumors (Fig. 4 and Table III). In all cases, cytotoxicity was blocked with MHC class I mAb (Fig. 4, B columns). Similar cytotoxicity profiles were observed when whole tumor cells were used as targets (Table III).

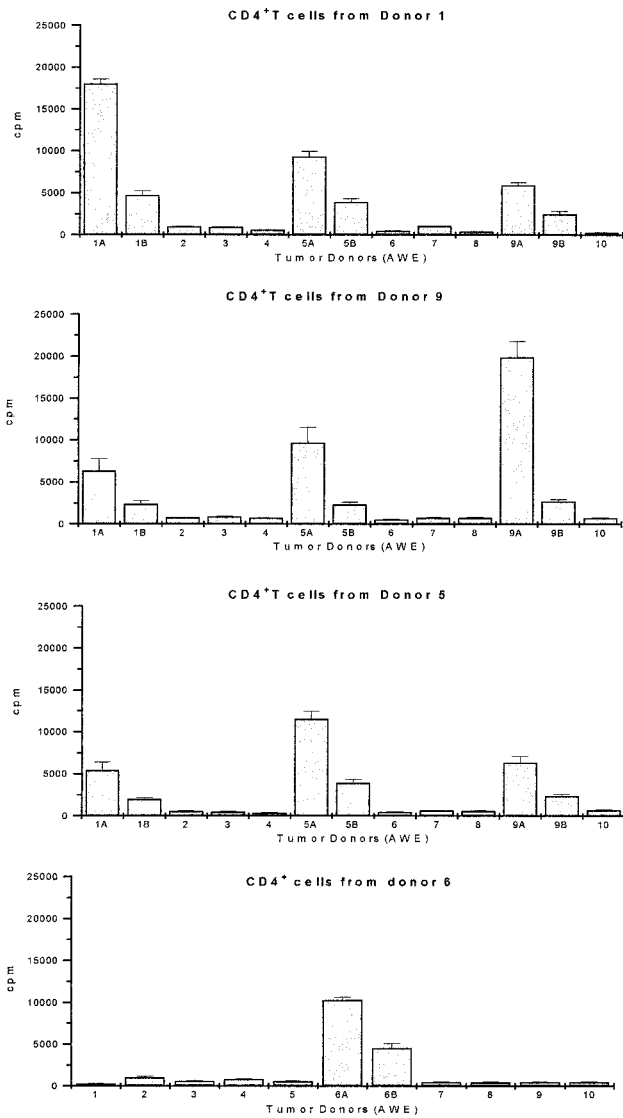


FIGURE 3. Specificity of CD4⁺ T cells activated during the MLTC. CD4⁺ T cells from patients 1, 9, 5, and 6 were tested in a proliferation assay against autologous DCs pulsed with AWE from autologous or allogeneic tumor cells. Columns indicate mean cpm ± SD from triplicate cultures. Pairs of columns indicate results from the same donor obtained with CD4⁺ T cells in parallel cultures set up in the absence (A) or presence (B) of anti-MHC-class II mAb.

Requirement of both CD4⁺ T cells and APC in MLTC for optimal killing of autologous tumors by CD8⁺ CTL effectors

To directly analyze the role of CD4⁺ T cells and APC in generating CD8⁺ T cell-mediated autologous tumor-specific cytotoxicity during the MLTC, we set up in vitro cultures with total MEAMNC or with MEAMNC depleted of either CD4⁺ or CD14⁺ cells. CD4⁺ T cell-depleted MEAMNC were stimulated with AuTu-AWE-DC and restimulated with the irradiated autologous tumor and autologous MEAMNC, which were also depleted of CD4⁺ T cells to ensure the absence of CD4⁺ T cells throughout the culture period. This was necessary to avoid any CD4⁺ T cell-mediated activation of the autologous CD8⁺ T cells later in culture, after the initial stimulation phase (i.e., after the first 10 days of culture). Similarly, MLTC initiated with MEAMNC depleted of CD14⁺ cells were stimulated with AWE-pulsed CD4⁺ T cells and restimulated with CD14⁺ cell-depleted MEAMNC to exclude any involvement of monocytes or monocyte-derived DCs in the late

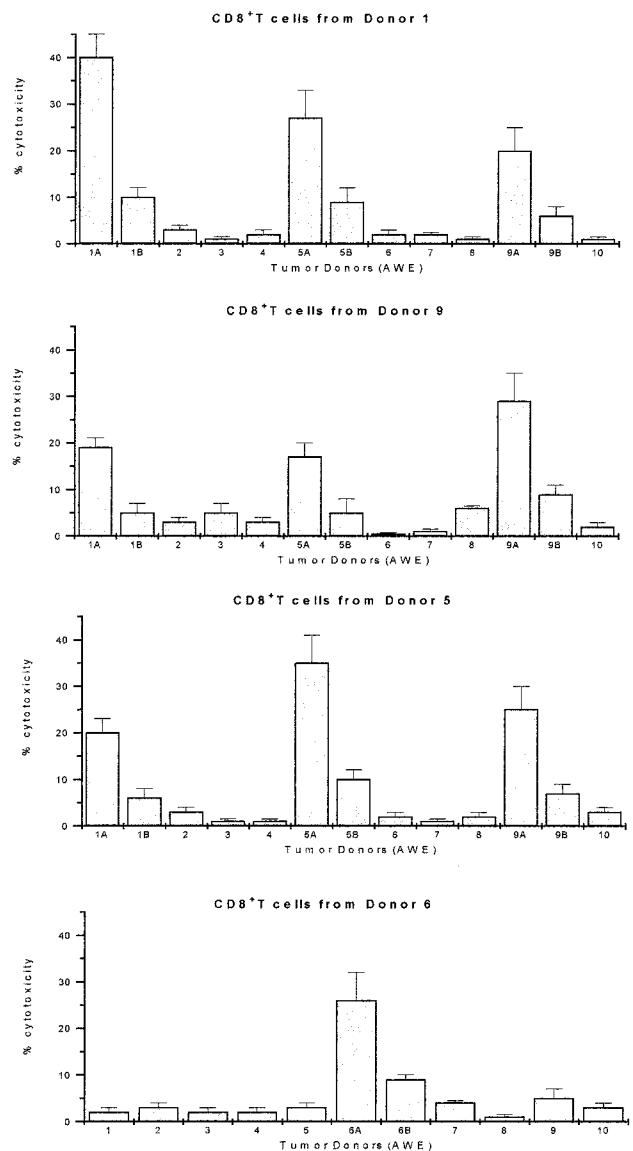


FIGURE 4. Specificity of CD8⁺ T cells activated during the MLTC. CD8⁺ T cells from patients 1, 9, 5, and 6 were tested in cytotoxicity assays for killing of the autologous DCs pulsed with AWE from autologous or allogeneic tumor cells. Columns indicate mean percent cytotoxicity from triplicates ± SD. Pairs of bars indicate results obtained with CD8⁺ T cells from the same patient in parallel cultures set up without (A) or with (B) anti-MHC-class I mAb.

activation phase of autologous CD8⁺ T cells. As shown in Fig. 5A, CD8⁺ T cells isolated from MLTC set up with unseparated MEAMNC lysed efficiently the autologous tumor targets in an E:T ratio-dependent manner. In contrast, significantly less killing was observed when CD4⁺ or CD14⁺ cells were eliminated from cultures. These results were confirmed in cultures from the same donors set up with highly purified cell populations (Fig. 5B). Thus, CD8⁺ T cells collected from MLTC cultures initiated with CD4⁺ and CD8⁺ T cells and AuTu-AWE-DC exhibited a high killing rate of the autologous tumor targets. However, when MLTC were initiated in the absence of CD4⁺ T cells, killing was reduced by ~53% at the highest E:T ratio. As already mentioned, such CD4⁺ T cell-depleted MLTC were restimulated with CD4⁺ T cell-depleted AWE-pulsed MEAMNC. An even higher decrease in cytotoxicity (62% reduction) against the autologous tumors was observed when the DCs were not included in the MLTC (Fig. 5B).

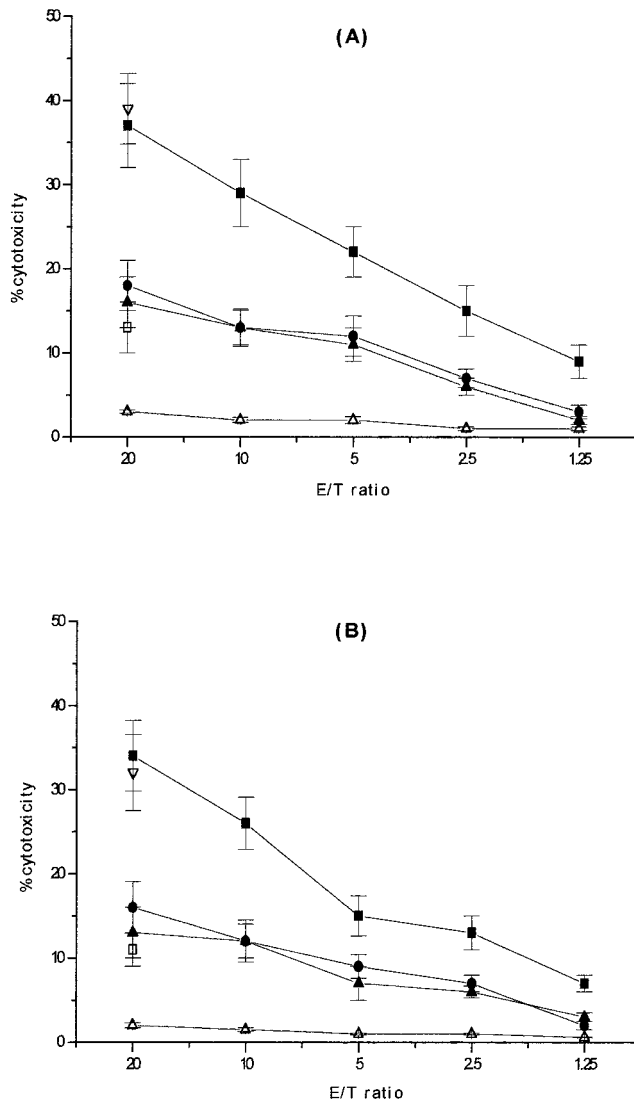


FIGURE 5. Cytotoxicity against autologous tumor targets mediated by CD8⁺ T cells collected from MLTC cultures set up using as responders whole MEAMNC (■); CD4⁺ T cell-depleted MEAMNC (▲); CD14⁺ cell-depleted MEAMNC (●) (A); or CD8⁺CD4⁺ T cells and AuTu-AWE-DC (■); CD8⁺ T cells and AuTu-AWE-DC (▲); CD8⁺ and CD4⁺ T cells pulsed with AWE (●) (B) (see text for details). Killing of K562 (△) was marginal in both A and B. □, Inhibition with anti-MHC class I mAb. Inhibition with isotype-matched to anti-MHC class I mAb at the same E:T ratio is also shown (▽). Results are given as mean percent cytotoxicity ± SD from four experiments conducted with an equal number of patients.

Also in this type of MLTC, restimulations were performed with CD14⁺ cell-depleted AWE-pulsed MEAMNC. CD8⁺ effectors collected from both types of MLTC lysed their targets in an MHC class I-restricted manner, as shown by the significant reduction of cytotoxicity at the highest E:T ratio tested with an anti-MHC class I mAb (Fig. 5). Cytotoxicity levels remained unaffected in the presence of a control isotype-matched mAb, and only marginal killing was observed against the control tumor cell line K562 (Fig. 5).

CD4⁺ T cells interact with AWE-pulsed DCs to enable the latter to efficiently activate CD8⁺ T cells for lysing autologous tumor cells

The data thus far suggested that lysis of metastatic tumor cells by autologous CD8⁺ CTL requires help provided by CD4⁺ T cells

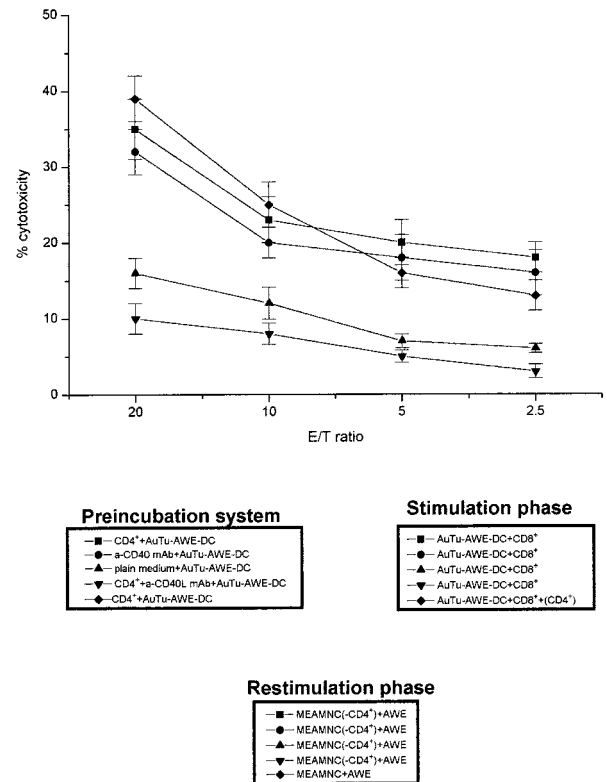


FIGURE 6. Activated, AuTu-AWE-DC are capable of stimulating CD8⁺ T cells to lyse autologous tumor targets in the absence of CD4⁺ T cells. AuTu-AWE-DC were treated for 48 h as indicated in "Preincubation system." Thereafter, CD4⁺ T cells and/or excess mAbs were removed (see also *Materials and Methods*) and the treated AuTu-AWE-DC were cocultured with autologous CD8⁺ T cells (stimulation phase). In one group (control group; ◆), CD4⁺ T cells from the preincubation system were added back to the culture during the stimulation phase. After 10 days of incubation, 3 rounds of restimulation were performed (restimulation phase) with MEAMNC depleted of CD4⁺ T cells and pulsed with AWE (MEAMNC (-CD4⁺) + AWE) except in group ◆, in which CD4⁺ T cells were included in AWE-pulsed MEAMNC. CD8⁺ T cells recovered from each group were then tested against autologous tumor targets and the results are expressed as mean percent cytotoxicity ± SD from five experiments.

and professional Ag-bearing APC (DCs). To better understand the nature of cell-to-cell interactions that take place after CD8⁺ T cell activation, we performed the following series of experiments. AWE-pulsed DCs were cultured for 48 h 1) with autologous CD4⁺ T cells, 2) with autologous CD4⁺ T cells plus anti-CD40L mAb, 3) with anti-CD40 mAb or 4) in plain medium. After this preincubation period, nonadherent CD4⁺ T cells were removed, treated DCs were washed to remove residual CD4⁺ T cells, and excess mAb (no mAb remained bound on DCs after 48 h; data not shown) and then added to autologous CD8⁺ T cells isolated from thawed autologous MEAMNC. Restimulations were performed with thawed CD4⁺ T cell-depleted MEAMNC pulsed with AWE as described in *Materials and Methods*. As for a positive control, CD4⁺ T cells from the preincubation period were added back to the stimulation phase, and restimulations were performed with total MEAMNC pulsed with AWE. As shown in Fig. 6, CD8⁺ T cells derived from CD4⁺ cell-depleted MLTC cultures that were stimulated with AuTu-AWE-DC preincubated with CD4⁺ T cells or with anti-CD40 mAb exhibited strong killing against the autologous tumor, comparable with that of the positive control. AuTu-AWE-DC cultured with CD4⁺ T cells plus anti-CD40L mAb or in

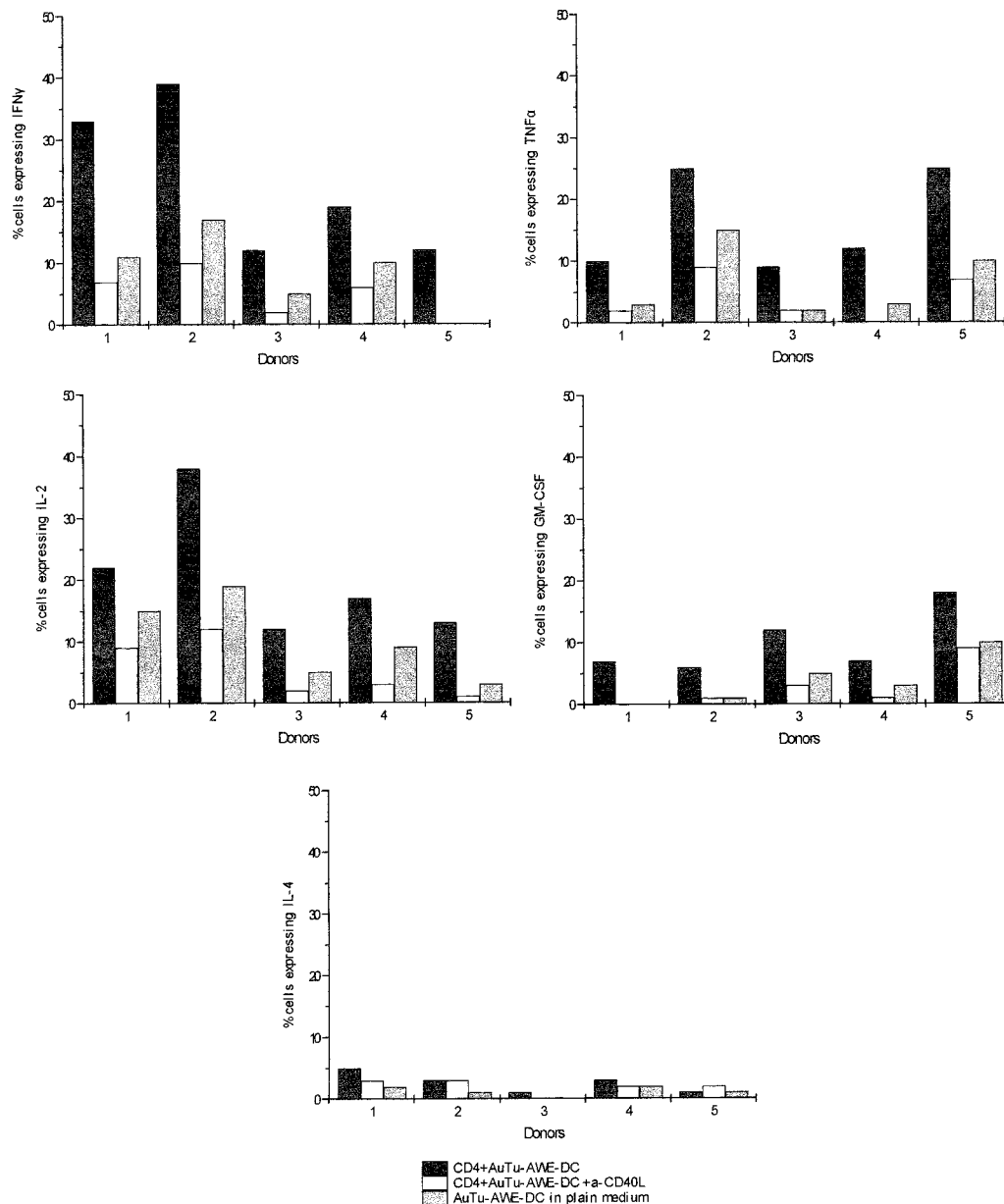


FIGURE 7. Intracellular cytokine production of CD8⁺ T cells. AuTu-AWE-DC were preincubated for 48 h with autologous CD4⁺ T cells in the presence (□) or absence (■) of anti-CD40L mAb or in plain medium (▨). Treated AuTu-AWE-DC were used to stimulate CD8⁺ T cells during the stimulation phase. After 10 days, cells were fixed with paraformaldehyde and permeabilized, followed by three-color staining with PE-labeled mAbs against IFN- γ , TNF- α , IL-2, GM-CSF, and IL-4 and with surface anti-CD8-FITC and anti-CD3-PerCP mAbs. Columns are the percentage of triple positive (CD3⁺CD8⁺cytokine⁺) cells.

plain medium failed to efficiently stimulate autologous CD8⁺ T cells during the initial MLTC phase, resulting in markedly reduced cytotoxic responses against the autologous tumor targets.

DCs can undergo phenotypic and functional changes after ligation of their CD40 with T cell CD40L (26, 27). It was therefore of interest to investigate whether the increased capacity of AuTu-AWE-DCs to trigger cytotoxic responses against the autologous tumor after preincubation with CD4⁺ T cells was associated with modulation of surface Ags. We thus measured the levels of adhesion, costimulatory, and HLA-DR Ags on DCs after 48 h preincubation under the same culture conditions as described above. Table IV shows that preincubation with CD4⁺ T cells or anti-CD40 mAb highly increased the expression of CD54, CD80, CD86, and HLA-DR molecules. In contrast, anti-CD40L mAb blocked to almost baseline levels the enhancement of Ag expres-

sion induced on preincubation with CD4⁺ T cells (Table IV). In both cases, DC preparations contained no CD4⁺ T cells (data not shown).

Cytokine production by CD8⁺ T cells stimulated by variously treated autologous DC

CD8⁺ T cells cultured with AuTu-AWE-DC, which were preincubated either with autologous CD4⁺ T cells or with CD4⁺ T cells and anti-CD40L mAb, were assessed by intracellular staining and FACS analysis for cytokine production, namely IFN- γ , TNF- α , IL-2, GM-CSF, and IL-4. None of the CD8⁺ T cells produced IL-4, whereas IL-2, IFN- γ , TNF- α , and GM-CSF were detected in a variable percentage of CD8⁺ T cells stimulated with CD4⁺ T cell-preincubated AuTu-AWE-DC (Fig. 7). Cytokine expression in CD8⁺ T cells was drastically reduced when preincubation of the

Table V. Concentrations of cytokines during the stimulation phase of CD4⁺ T cells with variously treated AWE-pulsed autologous DCs

Preculture System ^a	Culture System ^b		CD8 ⁺ T Cells + AuTu-AWE-DC	
	IFN- γ	TNF- α	IL-2	GM-CSF
1. CD4 ⁺ T cells + AuTu-AWE-DC	7–45 ^c	6–26	19–98	5–37
2. CD4 ⁺ T cells + AuTu-AWE-DC + anti-CD40L mAb	0.9–13	0.5–3	7–27	1–11
3. AuTu-AWE-DC	0.3–10	0.7–4	3–30	0.7–9

^a DCs pulsed with AWE from the autologous tumor (AuTu-AWE-DC) were incubated for 48 h with autologous CD4⁺ T cells or as otherwise indicated (for details see *Materials and Methods*).

^b CD8⁺ T cells were stimulated for 10 days with AuTu-AWE-DC, precultured as indicated in Column 1. Cytokines in culture supernatants were measured on day 10.

^c The range of concentrations (in nanograms per milliliter) from four patients tested are given.

AuTu-AWE-DC with CD4⁺ T cells included also anti-CD40L mAb or when AuTu-AWE-DC were left in plain medium (Fig. 7). Cytokine analysis in culture supernatants revealed similar results (Table V). With the use of commercially available ELISA kits, all four cytokines (i.e., IFN- γ , TNF- α , IL-2, GM-CSF) were detected in varying concentrations. In some instances, the concentrations were high (up to 100 ng/ml for IL-2). IL-4 levels were negligible (<1 ng/ml; data not shown).

Discussion

Taken together, the experiments presented in this study demonstrate that effective antitumor immunity is strictly dependent on CD4⁺ T cells, which are responsible for activating the autologous tumor Ag-bearing DCs. In particular, interactions between CD40 ligand and CD40 on CD4⁺ T cells and DCs, respectively, appear essential for the subsequent activation of the DCs to present Ags to and costimulate the priming of CD8⁺ CTL to lyse autologous tumor targets. Given that both mononuclear cells and autologous tumor cells derive from patients with metastatic cancer, our data show for the first time in humans that a critical pathway for delivery of help to CTLs is dependent on CD4⁺ T cells and uses APC as an intermediary.

In our system, CD8⁺ T cells generated during MLTC exhibited specific MHC class I-restricted recognition of the respective autologous tumors. CD8⁺ T cells from three patients (breast cancer, patient 1; ovarian cancer, patients 5 and 9) responded to each other's tumor cells and to unfractionated peptides (AWE) extracted from the same tumors (Fig. 4 and Table III), confirming data from other authors who demonstrated common tumor-associated Ags in breast and ovarian cancer (30). Most of the tumor-derived CTL peptide epitopes have been reported to be restricted by HLA-A2.1 (31). Because this particular allele is shared by all three patients (Table I), we could speculate that their CTL recognize common peptide(s) in its context. However, additional common CTL epitopes could be restricted also by other alleles, such as HLA-A3 (expressed in patients 1 and 9) or HLA-Cw8 (expressed in patients 5 and 9), both of which have been recently reported to present several tumor peptides to CTL (32–34). Our data provide also direct proof that such a common antigenic system between breast and ovarian tumors is recognized by tumor-specific CD4⁺ T cells in an MHC class II-restricted manner (Fig. 3 and Table II). The tumor-specific CD4⁺ T cells did not directly recognize the autologous or the cross-reactive allogeneic tumors (all of which are MHC class II negative) but required cross-priming of tumor Ags

by autologous APC (MEAMNC or DCs either pulsed with AWE or cocultured with intact irradiated tumor cells). Recognition of tumor peptides by CD4⁺ T cells has been shown to be restricted by various MHC class II alleles, including HLA-DR4 (35–37), HLA-DR1 (38), HLA-DR11 (39), HLA-DR15 (40), and HLA-DQ7 (41). Shared expression of some of these alleles in patients 1, 5, and 9 (e.g., all three patients share HLA-DQ7) may adequately explain recognition of common peptide(s) by each other's CD4⁺ T cells. The successful establishment of AWE-specific CD8⁺ CTL and CD4⁺ clones followed by HPLC fractionation of the tumor peptides included in AWE would be helpful to confirm the cross-recognition of single antigenic peptide(s) by the T cells and to identify the common antigenic system described herein.

The data presented also show that AWE-pulsed DCs on preincubation with CD4⁺ T cells or anti-CD40 mAb up-regulate adhesion and costimulatory molecules and are able to stimulate autologous CD8⁺ T cells directly, i.e., in the absence of CD4⁺ T cells. Such activated DCs are as stimulatory as those from MLTC cultures not devoid of CD4⁺ T cells. In other words, helper T cells need no longer communicate directly with the responding autologous CD8⁺ T cells. These results are consistent with recent reports demonstrating that ligation of CD40 on DCs with CD40L up-regulates the expression of ICAM-1, CD80, and CD86 molecules and also triggers the production of high levels of IL-12, resulting in the enhancement of their capacity to stimulate T cell-proliferative responses *in vitro* (26, 27) or to generate protective antitumor immunity *in vivo* (42). Binding of anti-MHC class II mAb on DCs also induces IL-12 release independently of CD40 ligation (26). Therefore, we did not attempt to block the CD4⁺ T cell-dependent activation of DC with anti-MHC class II mAb (as we did with anti-CD40L mAb), because this could possibly lead to a direct activation of DCs via ligation of MHC class II molecules.

Our data support the model of T cell help for CTLs proposed by others in various experimental animal models (14, 15, 20), extend this to the human system, and identify a series of cytokines (i.e., IFN- γ , IL-2, TNF- α , and GM-CSF) produced by the CTL as a mechanism via which activated AWE-pulsed DCs mediate CTL priming. The percentages of CD8⁺ CTL that produced these cytokines differed widely among the patients tested (almost 12–38% for IFN- γ , IL-2, and TNF- α and 6–17% for GM-CSF). A similar range of variation was also observed when the same cytokines were measured in culture supernatants. In both cases, cytokine production was largely inhibited when the interaction of AWE-pulsed DCs with CD4⁺ T cells was blocked by anti-CD40L mAb. The above mentioned cytokines have been shown to be produced by activated CTL (43–46) and also to be involved in cytolytic pathways as immunoregulatory (46, 47) or effector molecules (46, 48, 49). In addition, we have recently shown (50) that a synergism between all these cytokines (contained in culture supernatants from activated mononuclear cells) results in efficient lysis of both allogeneic and autologous tumor targets. Thus, such a cytokine mixture produced by the CD8⁺ CTL would provide the necessary conditions for optimal priming during the stimulation phase and for efficient activation during restimulations, leading ultimately to an effective antitumor immune response.

In this study, we have used unfractionated tumor-derived Ags present on intact tumor cells or extracted from them for T cell stimulation. The specificity of the immune responses for AWE preparations from the autologous tumor was demonstrated at the level of both CD4⁺ and CD8⁺ T cells in terms of proliferation and cytotoxicity, respectively. Thus, except for patients 1, 9, and 5, autologous tumor-specific CD4⁺ T cells proliferated *in vitro* in response to autologous DCs pulsed with AWE from the respective autologous, but not any allogeneic, metastatic tumors. Similarly,

CD8⁺ CTL activated during the MLTC lysed at similar levels autologous tumor cells and DCs pulsed with autologous tumor-derived AWE. In a recent report (23), it was shown that highly tumorigenic virus-induced T cell lymphoma cells pulsed with AWE from immunogenic tumor cells became also immunogenic and induced potent specific CTL responses in vivo and in vitro. Thus, it appears that peptide mixtures isolated from tumor cells bypass the need for characterizing tumor-specific Ags and allow the use of vaccination protocols to several types of cancer where tumor-specific peptides have not yet been identified.

We (21, 22) and others (51) have used ascitic fluids as a source of T lymphocytes to generate CTL with specificity for the autologous tumor. As a novel approach, we have used ascitic fluids from patients with ovarian and breast cancer and melanoma and pleural effusions from patients with lung cancer as a source of both CD4⁺ and CD8⁺ T cells, as well as of DCs, to generate improved CTL responses against autologous tumor cells. Thus, ascites and pleural effusions-derived T cells and DCs may be successfully used for triggering tumor-specific CTL to be used in protocols aiming at cancer immunotherapy.

Taken together, our data provide direct evidence that CD4⁺ T cells are crucial participants in the development of cytotoxic responses against autologous metastatic tumors. Furthermore, because metastatic tumor cells mostly do not express HLA class II molecules, CD4⁺ T cells gain specificity for the autologous tumor through cross-priming of tumor Ags by autologous APC. In this context, the presence of tumor specific CD4⁺ T cells is essential at the site of Ag presentation to the immune system (e.g., at tumor-draining lymph nodes), where they can help amplify the activated CTL population. The further identification of tumor peptides with specificity for helper T cell activation will be important for engineering peptide constructs consisting of covalently linked "helper" and "cytotoxic" epitopes. Such peptide constructs have been successfully used for priming of anti-HIV CD8⁺ CTL in vivo (52). Moreover, recent evidence indicates that tumor-specific CD4⁺ T cells, in addition to simply providing help for CD8⁺ T cells, also recruit other antitumor effector cells such as eosinophils and macrophages that produce both superoxide and nitric oxide (53). Thus, vaccination strategies aiming at the specific activation of CD4⁺ T cells in vivo should be considered as essential for the induction of multiple effector mechanisms that can cooperate in the most effective killing of tumor deposits.

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