Dendritic Cells Infiltrating Tumors Cotransduced with Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF) and CD40 Ligand Genes Take Up and Present Endogenous Tumor-associated Antigens, and Prime Naive Mice for a Cytotoxic T Lymphocyte Response

By Claudia Chiodoni,* Paola Paglia,* Antonella Stoppacciaro,‡ Monica Rodolfo,* Mariella Parenza,* and Mario P. Colombo*

*From the *Department of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy; and the ‡Department of Experimental Medicine and Pathology, University of Rome "La Sapienza," 00100 Rome, Italy*

Summary

We transduced BALB/c-derived C-26 colon carcinoma cells with granulocyte/macrophage colony-stimulating factor (GM-CSF) and CD40 ligand (CD40L) genes to favor interaction of these cells with host dendritic cells (DCs) and, therefore, cross-priming. Cotransduced cells showed reduced tumorigenicity, and tumor take was followed by regression in some mice. In vivo tumors were heavily infiltrated with DCs that were isolated, phenotyped, and tested in vitro for stimulation of tumor-specific cytotoxic T lymphocytes (CTLs). BALB/c C-26 carcinoma cells express the endogenous murine leukemia virus (MuLV) *env* gene as a tumor-associated antigen. This antigen is shared among solid tumors of BALB/c and C57BL/6 mice and contains two epitopes, AH-1 and KSP, recognized in the context of major histocompatibility complex class I molecules H-2L^d and H-2K^b, respectively. DCs isolated from C-26/GM/CD40L tumors grown in (BALB/c × C57BL/6)F1 mice (H-2$^{d\times b}$) stimulated interferon γ production by both anti–AH-1 and KSP CTLs, whereas tumor-infiltrating DCs (TIDCs) of BALB/c mice stimulated only anti–AH-1 CTLs. Furthermore, TIDCs primed naive mice for CTL activity as early as 2 d after injection into the footpad, whereas double-transduced tumor cells required at least 5 d for priming; this difference may reflect direct DC priming versus indirect tumor cell priming. Immunohistochemical staining indicated colocalization of DCs and apoptotic bodies in the tumors. These data indicate that DCs infiltrating tumors that produce GM-CSF and CD40L can capture cellular antigens, likely through uptake of apoptotic bodies, and mature in situ to a stage suitable for antigen presentation. Thus, tumor cell–based vaccines engineered to favor the interaction with host DCs can be considered.

Key words: dendritic cells • cross-priming • granulocyte/macrophage colony-stimulating factor • CD40 ligand • tumor antigens

Bone marrow–derived dendritic cells (DCs)[1] are professional APCs that are critical for the initiation of immune responses in vivo (1, 2). DCs take up antigens in the peripheral tissues, and process and transport them to lymphoid organs for presentation to T cells. The maturation and functions of DCs are tightly regulated by cytokines and costimulatory signals.

Solid tumors are frequently infiltrated with inflammatory cells, and the presence of DCs in the infiltrate has been associated with a better prognosis in some human cancers (3, 4). It has been demonstrated that tumor cells injected in vivo are subjected to cross-priming by host DCs (5) that capture, process, and present tumor-associated cellular antigens to naive T cells during their encounter within the draining lymph node. Despite significant improvements in the understanding of DC biology (2) and regulation of trafficking by chemokines and chemokine receptors (6, 7), the inability to isolate DCs from the tumors in sufficient number for in vitro exhaustive analyses has hindered efforts to

[1]*Abbreviations used in this paper:* DC, dendritic cell; MuLV, murine leukemia virus; TAA, tumor-associated antigen; TIDC, tumor-infiltrating dendritic cell; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling.

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define the precise events involved in DC local uptake of cellular antigens and in local DC maturation to an antigen-presenting stage. However, studies have implicated capture of apoptotic bodies (8–10) or endocytosis of heat shock protein–epitope complexes (11, 12) in DC uptake of cellular antigens.

We previously showed that cytokine-transduced C-26 colon carcinoma nodules are infiltrated by DCs, whose number and function depend on the transduced cytokine (13). Among the cytokines analyzed, IL-4 induced the recruitment of the highest number of DCs into the tumor mass, as detectable by immunohistochemistry. However, the number of tumor-infiltrating DCs (TIDCs) isolated from the tumor mass was not sufficient for extended in vitro investigation. Thus, attempts to correlate number and function were limited to a delayed-type hypersensitivity (DTH) assay showing that when injected into the ear pinna, TIDCs from tumors transduced with GM-CSF stimulated, whereas TIDCs from C-26/IL-4 tumors inhibited, DTH reaction to dinitrofluorobenzene (DNFB [13]). In the present study, we cotransduced C-26 tumor cells with the genes encoding soluble GM-CSF and a membrane-bound molecule, CD40L, based on the observation that soluble and cell–cell contact signals often synergize in cell activation (14).

GM-CSF induces proliferation and maturation of hematopoietic cells, and has been shown to stimulate DC accessory properties and to enhance the immune response initiated by these cells (15, 16).

The CD40/CD40L interaction plays a critical role in cell-mediated immunity (17) and in proliferation and activation of APCs, as shown for B cells (18), monocytes, and, more recently, DCs (19–21). Ligation of CD40 on monocytes and DCs results in the secretion of several cytokines, including IL-1, IL-6, IL-12, and TNF-α.

We show here that C-26/GM/CD40L tumors are heavily infiltrated by DCs that are able to capture and present endogenous tumor antigen(s) to specific CTL clones and to prime naive mice for a CTL response against the parental tumor. These findings point to the potential of tumor cell vaccines designed to exploit the DC functional bridge between T lymphocytes and the cell vaccine.

Materials and Methods

Mice and Tumors. 8–10-wk-old female BALB/c (H-2^d) and (BALB/c × C57BL/6)F1 (H-$2^{d\times b}$) hybrid mice were purchased from Charles River and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines.

C-26 is a murine colon adenocarcinoma cell line derived from BALB/c mice treated with *N*-nitroso-*N*-methylurethane. Tumor cells were cultured in DMEM (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL). Tumorigenic activity of control and transduced C-26 cells was assayed in mice injected subcutaneously in the left flank with 5×10^4 cells in 0.2 ml. Tumor growth and size were recorded twice each week.

Vector Construction and Retroviral Infection. The cDNAs for GM-CSF and CD40L were cloned by reverse transcription PCR: GM-CSF cDNA was obtained from Con A–stimulated murine blasts using GM-CSF–specific primer ends modified to include 5′ and 3′ EcoRI and BamHI sites, respectively. The resulting 517-bp insert was ligated into EcoRI and BamHI of retroviral vector LXSN (22) to obtain vector LmGMSN. CD40L cDNA was amplified from EL4 thymoma cells using specific primer ends containing 5′ and 3′ HpaI and XhoI sites, respectively, and the 817-bp insert was cloned into HpaI and XhoI sites of LXSH to obtain vector LmCD40LSH.

Retroviral vectors were transfected into the gp+E86 packaging cell line by standard calcium phosphate coprecipitation, and the 48-h culture supernatant was used to infect the amphotropic Am12 or PA317 packaging cell line. Infected Am12 and PA317 cells were selected with G418 and hygromycin, respectively, and used to generate helper-free virus–containing supernatants.

C-26 target cells were infected by four cycles of exposure to undiluted supernatant for 2 h in the presence of polybrene (8 mg/ml). At 48 h after infection, cells were diluted and selected in G418 or hygromycin. Bulk cultures and single resistant colonies were expanded and screened by ELISA for GM-CSF production, and by FACS® analysis for CD40L expression. Limiting dilution cloning was used to obtain optimal levels of CD40L expression.

Flow Cytometry. Expression of CD40L on transduced cell lines was assayed by flow cytometry after conventional staining with biotin-conjugated anti-CD40L mAb, clone MR1, followed by STREP-PE (PharMingen). The phenotype profile of tumor-infiltrating leukocytes was analyzed by double immunostaining using the following mAbs: FITC-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-CD11c, FITC-conjugated anti-B220, and isotype controls (all from PharMingen). Surface markers on TIDCs were detected using the following mAbs: M1/42, anti-MHC I; B21.2, anti-MHC II; 53.6.72, anti-CD8; SER4; 1G10, anti-B7.1; GL1, anti-B7.2; and isotype-matched mAbs of unrelated specificity as controls. Analysis was performed on a FACScan® (Becton Dickinson). Data were collected on 5,000–10,000 viable cells and analyzed using Lysis II software.

Morphological Analysis and Immunocytochemistry. Tumor fragments, tumor-draining lymph nodes, and spleens were embedded in OCT compound (Miles, Inc.), snap-frozen in liquid nitrogen, and stored at −80°C. Immunochemical analysis using the peroxidase-antiperoxidase (PAP) method was performed as described (23). In brief, 5-μm cryostat sections were fixed in acetone and immunostained with rat anti–mouse mAb against CD45 (M1/9.3.4.HL2 hybridoma, T200), CD8 (53.6.72 hybridoma, Lyt2), CD4 (GK1.5 hybridoma, L3T4), Mac-3 (M37/84 hybridoma, TIB168) (all from American Type Culture Collection), DEC-205 (NLDC-145; provided by Ralph Steinman, The Rockefeller University, New York), and GR-1 (RB6-8C5 hybridoma; PharMingen). Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit anti–rat IgG (Zymed Laboratories, Inc.), and rat PAP (Abbot Laboratories). Each incubation step lasted 30 min and was followed by a 10-min wash in TBS. Sections were then incubated with 0.03% H_2O_2 and 0.06% 3,3′-diaminobenzidine (BDH Chemicals) for 2–5 min, washed in tap water, and counterstained with hematoxylin. The number of immunostained cells was determined by light microscopy at 400× magnification in five fields on a 1-mm^2 grid, and is given as cells/mm^2 (mean ± SD).

For in situ TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) staining, tumor cryostat sections (5 μm) were fixed in acetone, conventionally immunoperoxidase stained with anti–DEC-205 using biotinylated secondary antibody/streptavidin-horseradish peroxidase, and developed in red by aminoethylcarbazole (AEC; Dakopatts). Sections were

then fixed with 4% paraformaldehyde for 20 min at room temperature, washed twice in PBS, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice, and washed twice with TBS. The labeling of 3′-OH fragmented DNA ends (TUNEL) was carried out using an in situ apoptosis detection kit (FragEL kit; Calbiochem). Labeled ends were detected with strepto-alkaline phosphatase (AP; Dakopatts). 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Dakopatts) was used as colorimetric substrate (black stain). Control tissue sections were prelabeled with isotype-matched irrelevant mAbs and treated for TUNEL staining without TdT.

Isolation of TIDCs. Tumor cells were implanted subcutaneously in the left flank at 5×10^4 cells/mouse, and tumors were surgically removed 13–25 d after implantation when they reached a mean diameter of 0.6–1.2 cm. Tumor masses were perfused with collagenase D (Boehringer Mannheim) solution (400 U/ml), gently minced into small pieces, and incubated in collagenase solution for 45–60 min at 37°C. After gentle pipetting, the fine suspension was washed several times with DMEM, and cells were seeded on 6-well plates at 0.7×10^6 cells/ml and incubated overnight to allow adherence of tumor cells to the plastic. The following day, nonadherent cells were collected and purified using $CD11c^+$ microbeads (MiniMacs®; Miltenyi Biotec GmbH) and used for subsequent experiments.

IFN-γ Production by C-26–specific CTL Clones. The generation and maintenance of anti-C26 CTL clone E/88 specific for murine leukemia virus (MuLV) *env*-derived peptide 423–431 SPSYVYHQF (24) has been described previously (25). CTL 9A/89 recognizes C-26–specific tumor-associated antigen (TAA) 9A in association with $H\text{-}2K^d$ (26). The CTL line TG905 was obtained from popliteal lymph node cells repeatedly restimulated in vitro with the MC38 ($H\text{-}2^b$) colon carcinoma cells. TG905 cells recognize MC38 cells and its *env*-derived peptide, 574–581 KSPWFTTL (27), but not C-26 cells or AH-1 peptide. CTL clones or lines (10^5 lymphocytes/well) were incubated with stimulating cells (10^5 cells/well or serial dilutions) or peptides (1 μg/ml) in 96-well plates for 24 h. Supernatants were assayed for IFN-γ content by specific ELISA (PharMingen).

Mixed Lymphocyte Tumor Culture and Cell-mediated Cytotoxicity. Mixed lymphocyte tumor culture (MLTC) was performed in RPMI 1640 medium (Hyclone) supplemented with 10% FCS (Hyclone). Lymphocytes from popliteal lymph nodes of mice injected into the right footpad with irradiated tumor cells or TIDCs were used as responder cells. Stimulators were C-26 cells inactivated with γ-irradiation (2,000 rad) or peptides (1 μg/ml). Responders and stimulators were suspended to 2.5×10^5 and 2.5×10^4 cells/ml, respectively, mixed in a total volume of 2 ml in 24-well plates (Costar Corp.), and cultures were incubated in a humidified atmosphere of 5% CO_2 in air. In cell-mediated cytotoxicity (CMC) assays, C-26 cells were the specific target and P815 plasmacytoma cells (DBA/2) were the negative control for C-26 tumor–specific lysis.

Results

In Vivo Tumorigenicity of GM-CSF– and CD40L-transduced C-26 Colon Carcinoma Cells. C-26 colon carcinoma cells were transduced with retroviral vectors carrying GM-CSF and CD40L genes together with selectable markers, neomycin and hygromycin, respectively.

Clones obtained by limiting dilution from antibiotic-resistant bulk cultures were screened for GM-CSF production by ELISA, and for expression of CD40L by FACS® analysis. Two clones producing the same amount of GM-CSF (10–15 ng/ml from 10^6 cells in 48 h) were selected among those obtained from single GM-CSF– and double GM-CSF/CD40L–transduced cells. Similarly, clones with the highest mean of CD40L expression were chosen from C-26/CD40L and C-26/GM/CD40L cells for subsequent experiments (data not shown).

To assess the effect of GM-CSF and/or CD40L transduction on tumor growth, BALB/c mice were injected subcutaneously with transduced or parental C-26 cells. Although all mice injected with C-26 or C-26/GM cells developed fast-growing tumors and were killed within 3 wk at the first sign of distress, 22 and 40% of animals injected with C-26/CD40L and C-26/GM/CD40L cells, respectively, remained tumor-free for the entire observation period (3 mo). Some of the double-transduced tumors regressed after an initial outgrowth, eventually resulting in 70% tumor-free mice (Fig. 1). Cell depletion by specific antibodies indicated that both $CD8^+$ T cells and $GR1^+$ PMNs were required for tumor rejection (data not shown).

Leukocyte Infiltration of GM-CSF– and CD40L-expressing Tumors. To investigate the effect of CD40L and GM-CSF transduction on DC recruitment, we examined tumor infiltrates by flow cytometry. Tumors injected subcutaneously and surgically removed 15–25 d later were perfused with collagenase D solution, and the resulting cell suspension was incubated overnight to let tumor cells adhere to the plastic. The following day, nonadherent cells were collected and stained for FACS® analysis. Although C-26 parental tumors were not infiltrated, GM-CSF–transduced tumors had numerous granulocytes (data not shown) and some (5–10%) $CD11c^+$ DCs. Some CD40L-expressing tumors were characterized by the presence of B cells ($CD11c^-B220^+$) and more numerous DCs ($CD11c^+B220^-$, range 7–20%; Fig. 2).

In the double-transduced tumors, the percentage of $CD11c^+$ cells ranged between 5 and 40%, but some tumors clearly showed that >20% of infiltrating cells stained with CD11c. Immunohistology with mAb to DEC-205 confirmed these data and revealed the highest percentage of

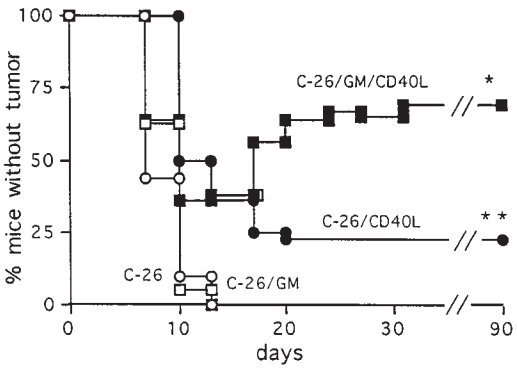

Figure 1. In vivo tumorigenicity of C-26 cells and genetically modified variants. Cells (5×10^4) were injected subcutaneously into the left flank of female BALB/c mice ($n = 35$). ○, C-26; □, C-26/GM; ●, C-26/CD40L; ■, C-26/GM/CD40L. *$P < 0.01$; **$P < 0.05$, as determined using the Mann-Whitney test to compare transduced cell lines with the parental C-26 cells.

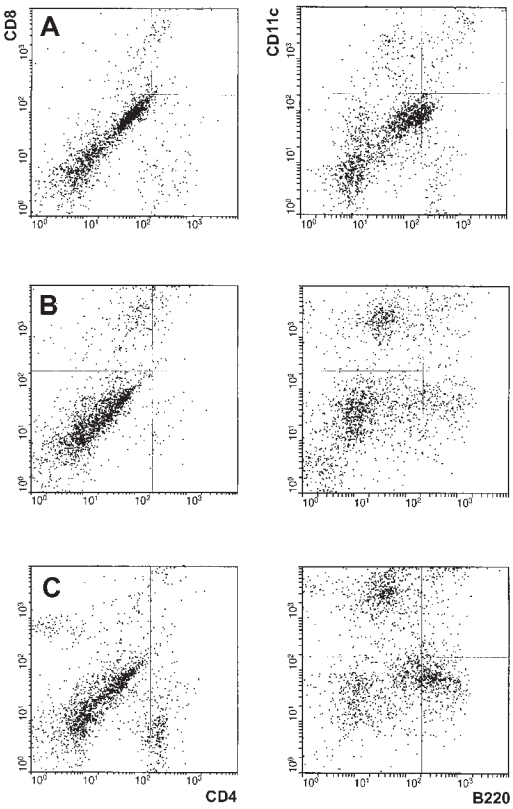

Figure 2. FACS® analysis of leukocyte infiltrate in tumors. (A) C-26/GM; (B) C-26/CD40L; (C) C-26/GM/CD40L. Tumor masses were surgically removed 15 d after implantation, then perfused with collagenase D solution, gently minced, and incubated in collagenase solution for 45–60 min at 37°C. After gentle pipetting and washing the suspension with DMEM several times, cells were seeded on 6-well plates at 0.7×10^6 cells/ml and incubated overnight to allow tumor cell adherence to the plastic. Nonadherent cells were collected, double-labeled with CD8-PE/CD4-FITC (left) or CD11c-PE/B220-FITC (right), and analyzed by flow cytometry. Quadrants were set using isotype-matched antibodies as controls (not shown).

DCs in the five double-transduced tumors positive for membrane CD40L expression (Table I), suggesting a role for local expression of CD40L in attracting or maintaining DCs in the tumor. Seven other double-transduced tumors lost CD40L expression, likely due to in vivo methylation and thus inactivation of the retroviral promoter (LTR) driving the transgene expression (28).

In the presence of both CD40L and GM-CSF, DCs represented between 20 and 40% of total $CD45^+$ leukocytes, thus allowing their in vitro isolation and purification using CD11c-conjugated beads.

GM-CSF expression by transduced tumors is reflected in spleen hypertrophy due to increased spleen hematopoiesis characterized by the presence of mixed colonies (megakaryocytes and granulocytes) throughout the white pulp. No such spleen hypertrophy was observed in mice injected with C-26/CD40L cells. In spleen of mice injected with GM-CSF– and CD40L-coexpressing tumor cells, the composition of the colonies showed a shift toward enrichment with eosinophils (data not shown).

Phenotypic Characterization of TIDCs. Expression of surface markers on TIDCs from C-26/GM/CD40L tumors was assessed by immunostaining and subsequent FACS® analysis. The $CD11c^+$ population showed a phenotypic profile characteristic of differentiated or mature DCs, i.e., high surface expression of MHC class I and class II proteins and substantial expression of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) (Fig. 3); such mature phenotype was confirmed by stimulatory activity of TIDCs in MLR (data not shown).

Prolonged in vitro culture of collagenase-digested C-26/GM/CD40L tumor cell suspension revealed aggregation of cells with DC morphology in characteristic clusters (Fig. 4 shows one such cluster at day 3) and survival of these cells on tumor layer for up to 7 d.

Functional Characterization of TIDCs; Antigen Uptake at the Tumor Site. The abundance of DCs infiltrating the C-26/GM/CD40L tumors enabled analyses to address whether such DCs take up and present tumor cellular antigens. The *env* gene of the endogenous ecotropic MuLV is expressed by C-26 colon carcinoma cells as an immunodominant TAA that contains the L^d-restricted peptide, AH-1 (amino acids 423–431; reference 24). In addition, C-26 cells express at least one additional, to date uncloned, TAA recognized by the CTL clone 9A/89 in an H-$2K^d$–restricted manner (26). The MuLV *env* is also expressed by MC38 (H-2^b) colon carcinoma and is recognized by the TG905 CTL line through the KSP peptide restricted in K^b (27).

Specific T cell clones and line were used to test whether TIDCs isolated from C-26/GM/CD40L tumors can present endogenous TAAs. CTL clones maintained in culture with IL-2 readily produce IFN-γ, without the need for APCs or costimulatory signals, when stimulated with tumor cells. To rule out the possibility that tumor cells contaminating the TIDC preparation were responsible for CTL stimulation, the experiment was performed in (BALB/c × C57BL/6)F1 hybrids (Table II).

CTL clones and line were cocultured overnight with either TIDCs from C-26/GM/CD40L grown into F1 mice, or splenic DCs from naive mice. As control for CTL restriction, C-26 (H-2^d) and MC38 (H-2^b) tumor cells were used. TIDCs (H-$2^{d/b}$) from F1 mice were able to stimulate both H-2^d (E/88 and 9A/89) and H-2^b (TG905) CTLs, whereas C-26 stimulated H-2^d but not H-2^b CTLs and MC38 stimulated TG905 cell line but not E/88 and 9A/89 CTL clones. Accordingly, TIDCs from C-26/GM/CD40L grown into BALB/c mice stimulated anti–AH-1 but not anti-KSP CTLs (not shown). The lower level of IFN-γ produced by stimulation with TIDCs compared with tumor cells (or the peptide alone) might rest in the fact that few TIDCs are likely to be loaded with the specific antigen, while all tumor cells express the TAAs. Thus, the C-26 MuLV *env*-derived antigens are taken up and processed by host DCs most probably at the tumor site.

Uptake of Apoptotic Cells. Immunohistological double staining for DCs (with anti–DEC-205 mAb) and apoptotic cells (TUNEL) revealed the presence of apoptotic bodies engulfed by DCs (Fig. 5) in the region outside necrotic areas.

Table I. *Immunohistology of C-26/GM/CD40L Double-transduced Tumors*

Mouse no.	Expression of CD40L on tumor	Necrosis	CD45	CD4	CD8	Mac-3	GR	DEC-205
IL2551#20		+	433 ± 91*	19 ± 6	3 ± 5	283 ± 30	85 ± 30	57 ± 16 (15%)‡
IL2551#30		+	511 ± 100	39 ± 19	19 ± 10	168 ± 35	239 ± 47	46 ± 16 (10%)
IL2552#3	+	+	519 ± 51	83 ± 17	55 ± 13	65 ± 12	334 ± 151	130 ± 68 (25%)
IL2552#10		++	630 ± 91	9 ± 5	2 ± 2	176 ± 35	389 ± 101	54 ± 10 (9%)
IL2553#3		+	429 ± 16	7 ± 5	1 ± 2	199 ± 17	ND	49 ± 19 (11%)
IL2550#2		++	382 ± 67	14 ± 6	3 ± 1	ND	180 ± 5	36 ± 9 (10%)
IL2550#10		++	428 ± 91	30 ± 23	17 ± 15	195 ± 15	131 ± 19	37 ± 20 (9%)
IL2550#20		+	414 ± 121	35 ± 15	6 ± 5	164 ± 35	149 ± 17	56 ± 13 (13%)
IL2551#0		−	765 ± 23	49 ± 5	11 ± 3	342 ± 83	230 ± 21	61 ± 30 (8%)
IL2552#2	+	+++	559 ± 17	71 ± 5	59 ± 11	100 ± 23	191 ± 30	105 ± 4 (19%)
IL2550#3		++	532 ± 45	26 ± 11	8 ± 5	114 ± 16	108 ± 18	61 ± 56 (12%)
IL2550#30		+	567 ± 110	80 ± 40	7 ± 3	225 ± 29	223 ± 20	23 ± 10 (4%)
IL2551#10	+	+++	525 ± 50	129 ± 18	121 ± 17	74 ± 12	118 ± 13	200 ± 30 (38%)
IL2551#2	+	++	452 ± 27	37 ± 17	31 ± 15	115 ± 27	169 ± 25	103 ± 78 (29%)
IL2553#20	+	Regressing	677 ± 55	156 ± 100	131 ± 53	191 ± 15	100 ± 36	140 ± 21 (21%)

*Mean no. of cells/mm^2 ± SD positive for immunostaining.
‡Percentage of DEC-205$^+$ cells among the CD45$^+$ leukocytes.

Although the tumor cell origin of such condensed nuclei remains to be proven, the micrographs together with the functional data strongly suggest that the uptake of apoptotic bodies most likely underlies cross-priming, consistent with results in a model of influenza virus–induced apoptosis (8, 10).

Rapid In Vivo CTL Priming of Naive Mice. To assess the ability of TIDCs to prime a specific CTL response in vivo, CD11c$^+$-enriched TIDCs or C-26/GM/CD40L tumor cells were injected in the footpad of naive BALB/c mice, popliteal lymph nodes were removed 5 d later, and lymphocytes were cultured in vitro with C-26 cells or AH-1–specific peptide and tested after 6 d for CTL activity. Lymphocytes from mice injected with TIDCs from the CD11c$^+$-enriched fraction primed mice to elicit specific CTLs against C-26 tumor cells or AH-1–pulsed P815 cells (Fig. 6, A and B). C-26/GM/CD40L tumor cells also induced a CTL response, although to a lesser extent than TIDCs. When the time allowed for in vivo priming was reduced from 5 to 2 d, lymphocytes collected from the nodes of mice primed with

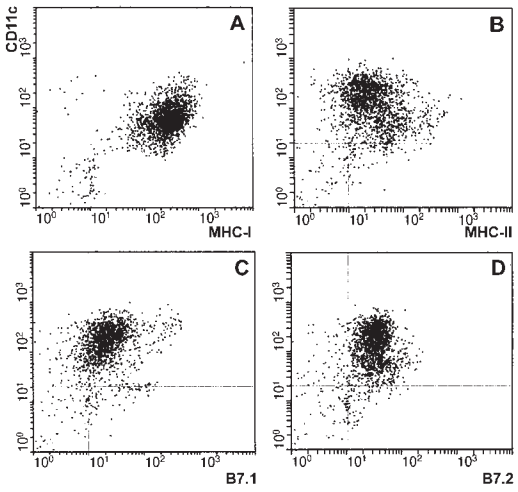

Figure 3. Phenotypic analysis of C-26/GM/CD40L TIDCs. DCs were purified using CD11c-conjugated microbeads. Sorted cells were double-stained for FACS® analysis as follows: CD11c-PE/biotinylated MHC I (A), CD11c-PE/MHC II (B), CD11c-PE/B7.1 (C), and CD11c-PE/B7.2 (D). Unlabeled mAbs were detected with mouse-FITC. Isotype-matched antibody controls were used to set quadrants (not shown).

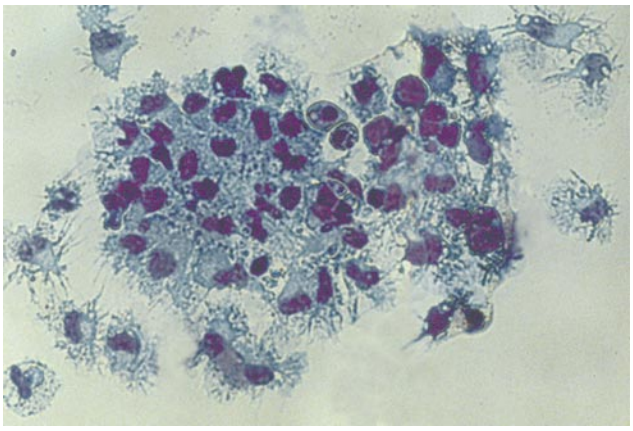

Figure 4. TIDC clusters after 3 d of in vitro culture on GM-CSF and CD40L cotransduced C-26 cells. After tumor mass digestion, the cellular suspension was seeded in 6-well plates at 0.7 × 10^6 cells/ml; at day 3, non-adherent cells were collected and gently cytospun onto glass slides to avoid cluster disaggregation, then stained with Neat Stain kit (International PBI) (original magnification: ×400).

Table II. *IFN-γ Production (ng/ml) from Anti-C26 CTL Clones and Anti-KSP CTL Line*

Stimulators (H-2 haplotype)	Cell no.	Experiment 1			Experiment 2	
		9A/89 (H-2K^d)	E/88* (H-2L^d)	TG905* (H-2K^b)	E/88* (H-2L^d)	TG905* (H-2K^b)
TIDCs (H-2$^{d/b}$)	10^5	13.57	17.64	16.61	19.95	26.93
	5×10^4	—	—	—	11.35	15.30
	2.5×10^4	—	—	—	4.60	11.35
	1.25×10^4	—	—	—	2.51	8.33
C-26 (H-2^d)	10^5	60.7	101	0	88.1	1.2
	5×10^4	—	—	—	31.12	0
	2.5×10^4	—	—	—	20.42	0
	1.25×10^4	—	—	—	16.47	0
MC38 (H-2^b)	10^5	—	0	78.6	0	87.2
	5×10^4	—	—	—	0	19.95
	2.5×10^4	—	—	—	0	16.63
	1.25×10^4	—	—	—	0	14.3
Spleen DCs‡	10^5	0.3	0.3	0	0	0

*E/88 and TG905 recognize different peptides (H-2^d and H-2^b restricted, respectively) of the same MuLV *env*-encoded antigen.
‡DCs purified from spleen of naive mice.

TIDCs 2 d earlier and restimulated in vitro with the specific AH-1 peptide showed a CTL response against C-26 cells, whereas those from mice primed with C-26/GM/CD40L did not (Fig. 6 B). A likely explanation for such difference is that TIDCs are able to migrate immediately to the draining lymph node upon injection, whereas tumor cells should be first destroyed and then antigen loaded onto host DCs to allow T cell priming.

Discussion

Tumor cells can be genetically modified to produce cytokines and/or costimulatory molecules that improve their immunogenicity, thus providing new testable cellular vaccines. These vaccines are usually designed to act directly on T cells, providing signals for the activation (e.g., IL-2) and/or costimulation (e.g., B7.1) of these cells. Based on this rationale, it should be possible to render the tumor cells "mock" APCs that directly interact with and activate tumor-specific

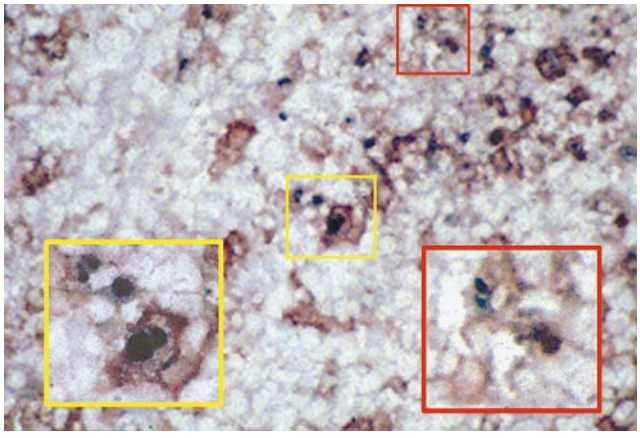

Figure 5. Apoptotic bodies within TIDCs. Double staining with DEC-205 and TUNEL reveals DCs engulfed with apoptotic bodies and blebs in C-26/GM/CD40L tumors (original magnifications: ×400; inserts: ×1,000).

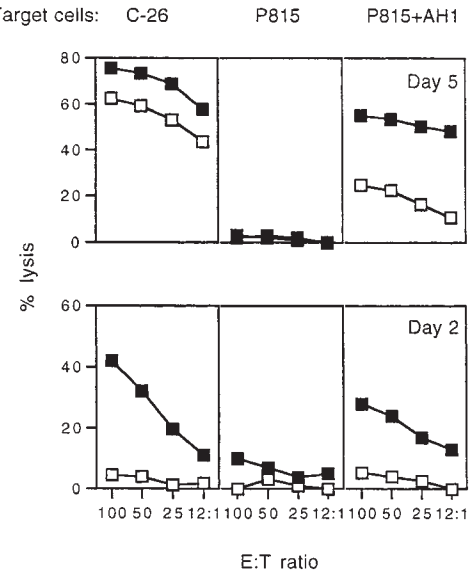

Figure 6. CTL activity after in vivo priming of BALB/c mice with CD11c$^+$-enriched fraction of TIDCs (■) or C-26/GM/CD40L control tumor cells (□). 10^5 cells were injected in the footpad of naive BALB/c mice; after 5 (top panels) or 2 d (bottom panels), popliteal lymph nodes were removed. Lymphocytes were cultured in vitro with the AH-1–specific peptide and tested 6 d later for CTL activity.

lymphocytes. Clinical application of such cellular vaccines requires an autologous setting or HLA-matched cell lines. While autologous application is difficult, since it requires tumor cell cultures from every patient for both gene transduction and immunological follow-up, the use of allogeneic cell lines provides the advantage of vaccines with well-characterized tumor antigen, MHC, and adhesion molecules, as well as a constant amount of cytokine released. These parameters may provide a standard reagent for clinical studies. Either syngeneic or allogeneic tumor cells expressing a common TAA are processed by host APCs such that TAA-derived peptides are presented in association with host MHC in both cases (5). In light of these considerations, we focused on the interaction of the cellular vaccine with professional APCs, i.e., DCs, in an effort to favor the in vivo cross-priming.

To design such a vaccine, we began by genetically modifying C-26 colon carcinoma cells to express GM-CSF and CD40L, two critical factors for DC maturation and activation.

GM-CSF has shown potent immunostimulatory activity that leads to long-lasting, specific antitumor immunity in some tumor models (29, 30) but not in others (31, 32), probably due to differences in tumor type or amount of cytokine released (33). Our preliminary experiments indicate that GM-CSF enhanced tumor immunogenicity, protecting mice from a subsequent challenge with live parental C-26 tumor cells (our unpublished results) without impairing growth of transduced cells. It seems likely that GM-CSF has no effect on the early immune response, but instead plays a crucial role in the T cell–mediated response by recruiting and activating APCs (1); GM-CSF can also substitute for IFN-γ in inducing late regression of C-26 cells transduced with IL-12 genes and injected into IFN-γ knockout mice (34).

The other component of our double-transduced cellular vaccine, CD40L, has been shown to play a central role in inducing an immune response through its interaction with CD40 (17, 21), although its antitumor effect has only recently been described in a few tumor models. Grossmann et al. (35) reported that constitutive expression of CD40L on a weakly immunogenic murine tumor (neuro-2a) delays the growth of coinjected parental cells and protects mice from subsequent challenge with parental neuro-2a cells. The antitumor effect was abrogated by in vivo depletion of $CD8^+$ cells. Those investigators also detected increased expression of markers such as CD25 and CD86 in splenocytes from mice injected with CD40L-transfected cells, but $B220^+$ cells were the most abundant subset. Similarly, we noted the overgrowth of $B220^+$ cells when CD40L- and GM-CSF/CD40L–transduced C-26 cells were each cocultured in vitro with naive splenocytes (not shown).

Transduction of CD40L into P815 tumor cells completely inhibited tumor take; there, CD40L activated macrophages to release IL-12, which in turn stimulated NK activity (36).

In C-26 cells transduced only with CD40L, tumor take was abrogated in a small fraction of injected mice (22%), whereas cotransduction of CD40L with GM-CSF led to at least an additive effect in inducing a late regression of incipient tumors. It is possible that CD40L activates an early mechanism of tumor killing, probably mediated by neutrophils, that counters tumor onset and thereby allows sufficient time for GM-CSF indirect activation, via DCs, of T cells. Tumor regression depended on $CD8^+$ T cells and granulocytes, but not on $CD4^+$ T cells. As suggested previously (37–41), it is likely that CD40L substitutes the need for CD4 Th cells in cross-priming.

In our model, GM-CSF and CD40L appear to be necessary and sufficient to promote the recruitment of DCs and to activate them at the tumor site. The phenotype of TIDCs is consistent with mature DCs, since they express high levels of MHC class I and class II, and of costimulatory molecules such as B7.1 and B7.2. This phenotype and the stimulatory activity in MLR correlate with antigen-presenting function; indeed, DCs isolated from C-26/GM/CD40L tumor tissues have taken up tumor antigens in vivo, and can present them to tumor-specific T cells and prime T lymphocytes upon injection into naive mice. GM/CD40L-transduced tumor cells are also able to induce a specific CTL response, although TIDCs do so more rapidly, suggesting a direct DC priming of T cells. However, in our transfer experiment, the question of whether TIDCs prime host T cells directly or are destroyed by host effector cells after which antigens are taken up and represented by host APCs (42) has not yet been investigated, and further experiments are needed to address this issue.

TIDCs purified from C-26/GM/CD40L tumors grown in BALB/c × C57BL/6 hybrid mice can present the entire known repertoire of C-26 cell TAAs (the L^d- and K^b-restricted epitopes from *env-1* and the K^d-restricted epitope of an unidentified cellular gene).

The functional data on in vivo cross-priming, together with immunohistology showing that TIDCs are engulfed with apoptotic bodies, point to the role of apoptosis as a source of cellular antigens in a tumor model (8, 9).

Unlike several other models that relied on the use of surrogate tumor antigens such as β-galactosidase (43), influenza nucleoprotein (5), or OVA (44) to prove presentation of exogenous antigens in the context of class I MHC, our model represents the first direct evidence that endogenous tumor antigens are taken up and processed by host DCs in an MHC I–restricted fashion directly at the tumor site.

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Address correspondence to Mario P. Colombo, Department of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-2390-252; Fax: 39-02-2390-630; E-mail: mcolombo@istitutotumori.mi.it

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