Tumor Progression Despite Efficient Tumor Antigen Cross-Presentation and Effective "Arming" of Tumor Antigen-Specific CTL[1]

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To determine whether APC function or "arming" of CTL for lytic function are the points at which Ags from a nonimmunogenic tumor fail to induce an effective immune response, we established a murine tumor model that expressed intracellular OVA and selected a clone (cOVA-9) that remained susceptible to lysis by specific CD8$^+$ T cells throughout tumor growth. Viable cOVA-9 tumor cells grew in normal mice at a rate similar to the parental tumor, and vaccination with irradiated cOVA-9 cells did not induce protection against itself or the parental line, confirming its nonimmunogenic status. In vivo evaluation during tumor growth demonstrated persisting tumor Ag cross-presentation accompanied by the generation of potent, specific CTL which were detectable when tumors were barely palpable. Despite the presence of highly active CTL in the tumor-draining lymph nodes, there was no apparent lysis of tumor-associated APC. These data show that tumor-draining APC are not dysfunctional with regard to two crucial processes, in vivo tumor Ag cross-presentation and specific CTL arming, and that failure to prevent tumor growth is not in the induction phase, but in the effector phase and occurs within the tumor itself before the tumor matrix is established. *The Journal of Immunology,* **2001, 166: 5557–5566.**

Most human tumors are thought to be poorly immunogenic, generating at best, a weak, ineffective, antitumor immune response. A number of underlying mechanisms have been proposed for this defect which, based on recent observations, can be broadly divided into two areas. First, some tumor models have shown that an antitumor immune response is induced but there may be 1) a kinetic disparity between exponentially increasing tumor cell numbers vs available or expanding specific effector CTL (1) or 2) a rapid attenuation of tumor-specific immune responses via a number of tolerizing mechanisms (2). Second, others have described a phenomenon referred to as "ignorance" in which tumor Ags are never presented to T cells within the lymphoid system and an antitumor response is simply not generated (3–6). These observations may all be ascribed to suboptimal tumor Ag presentation occurring within tumor-draining lymph nodes (DLN).[3]

It is now becoming clear that professional APC are the main cell type responsible for tumor Ag cross-presentation to host CD8 T cells (7–9). To generate an effective immune response, these tumor-draining APC must 1) efficiently cross-present tumor Ags in DLN and 2) effectively "arm" CD8$^+$ T cells to induce a potent and prolonged effector (cytotoxic) cell function. Failure of either process would produce an incompetent immune response and recent studies have suggested that APC in tumor-bearing animals may be dysfunctional (10–13). We established a murine model to analyze these processes. For this model to provide useful data, three key characteristics were required. First, the model had to be relevant to human cancers; therefore, we used a tumor known to be similar to human solid tumors which, importantly, has features that allow it to be described as nonimmunogenic; Lewis lung carcinoma (LL; Ref. 14). Second, the model had to express a tumor Ag to which specific immune responses could be monitored in vivo. We stably transfected OVA into LL cells as a marker neo tumor Ag. OVA was selected because it is well described in models of self (15, 16) and tumor Ags (17) and, importantly, because OVA-specific TCR mice are available for in vivo analysis of tumor recognition (18). Crucial to this model was the observation that the OVA transfectant grew in normal mice at the same rate as the parental cell line and that vaccination with irradiated OVA-expressing tumor cells did not alter LL's immunogenicity; i.e., our model remained nonimmunogenic.

Finally, few models exist which exclude other escape mechanisms employed by tumors. These mechanisms operate by protecting tumor cells from lysis by specific CTL and include down-regulation of class I molecules, insufficient peptide-MHC class I complex expression on the tumor cell surface, or the emergence of Ag loss variants (19–21). Therefore, the OVA Ag was located intracellularly, and a clone was selected that loaded the dominant peptide (SIINFEKL) into MHC class I molecules at sufficient levels that enabled effector CD8$^+$ CTL to lyse tumor cells throughout in vivo tumor growth. The experiments described here 1) assessed the location and duration of two key functions of host tumor-DLN APC, i.e., tumor Ag cross-presentation and CD8 arming using

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[3] Abbreviations used in this paper: DLN, draining lymph node; LL, Lewis lung carcinoma; LN, lymph node; cOVA, cytoplasmic form of OVA; CM, conditioned medium; COVA-9, cOVA clone 9; pCTL, precursor CTL; HA, hemagglutinin.

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recently developed in vivo assays; and 2) escape mechanisms that may be employed when a tumor is faced with a strong and persisting specific CTL response.

We show that APC draining the inoculation site of irradiated, or viable, growing tumor cells not only efficiently cross-present tumor Ag, but also arm endogenous tumor-specific CD8 cells (in the absence of artificial stimulation or adoptively transferred CD4-derived specific "help"), inducing high levels of lysis in vivo. These CTL were detectable when tumors were barely palpable, remained lytic in vivo throughout tumor growth, and were located in all secondary lymphoid compartments as well as in the tumor itself. Despite the presence of highly active and specific CTL within lymph nodes (LN) draining the tumor site, there was no apparent destruction of tumor peptide-bearing APC in these LN, as indicated by continuing tumor Ag cross-presentation and CTL activity. Nor was there any evidence of Ag loss variants or shifts in tumor Ag reactivities throughout tumor growth. These data suggest that nonimmunogenicity of solid tumors is not fundamentally due to a failure in the capacity of tumor-associated APC to cross-present tumor Ags to host CD8 cells to levels required to induce their activation and proliferation, nor in their ability to arm these CD8 cells for lytic activity.

Materials and Methods

Mice

Female C57BL/6 (H-2^b) mice between 6 and 8 wk of age were obtained from the Animal Resources Center (Murdoch, Western Australia) and maintained under standard housing conditions in the University Department of Medicine animal holding area. The OT-1 (H-2^b) TCR-transgenic mouse line, expressing a TCR recognizing the dominant H-2^b-restricted OVA epitope (SIINFEKL; Ref. 18), was kindly supplied by F. Carbone and W. Heath (University of Melbourne, Melbourne, Australia and Walter Eliza Hall Institute, Melbourne, Australia, respectively).

Murine tumor cell lines

The murine LL (LL2 or CRL-1642) and thymoma cell lines EL4 (TIB-39) and EG7 (CRL-2113) used in this study were all obtained from American Type Culture Collection (Manassas, VA). LL, EL4, and EG7 are H-2^b-restricted tumors derived from C57BL mice. EG7 yields secreted OVA and has been described previously (17).

Cell culture and maintenance

Murine tumor cell lines were maintained in conditioned medium (CM) consisting of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (CSL, Victoria, Australia), 20 mM HEPES (Life Technologies), pH 7.4, 48 mg/L gentamicin (Pharmacia and Upjohn, Western Australia, Australia), 60 mg/L benzylpenicillin (CSL), and 2 g/L $NaHCO_3$ (BDH, Victoria, Australia). Transfected tumor cell lines were maintained in CM supplemented with 400 μg/L of the neomycin analog G418 (Geneticin; Life Technologies). Cells were cultured at 37°C in a 5% CO_2 atmosphere and passaged when 70% confluent.

Plasmids and transfections

The cytoplasmic form of OVA (cOVA) was achieved by deleting aa 20–145 of the naturally occurring secreted OVA and constructed under the control of the CMV promoter in the mammalian expression plasmid pCI (kindly donated by Andrew Lew, Walter and Eliza Hall Institute) and has been described previously (22).

LL transfectants were developed by stably cotransfecting the LL parental cell line with cDNA encoding for cOVA and the neomycin selection marker using electroporation (1100 μF at 0.22kV). Positive clones were isolated via growth in culture medium containing G418. Single adherent colonies were picked and recloned by limiting dilution assay.

RT-PCR

RNA was prepared from tumor cells using RNAzol B according to the manufacturer's instructions (Biotecx Laboratories, Houston, TX). PCR were performed on cDNA samples after reverse transcription of 5 μg of RNA using a PTC-100 programmable thermal controller (M. J. Research, Watertown, MA). A 443-bp OVA amplicon was generated using the nucleotide primers (Life Technologies) OVA488f (5′-CTGAGCTAGCCAG AGAGCTCAATTCC-3′) and OVA931r (5′-CGGGATCCATCTTCATG CGAGGTAAGTA-3′). After 50 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, samples were visualized on a 1.5% agarose gel (Ameresco, Solon, OH) and stained with ethidium bromide (Sigma, St. Louis, MO) and PCR products.

In vivo tumor growth and immunological protection experiments

Mice were injected s.c. at day 0 with 5×10^5 or 10^6 tumor cells/mouse in 100 μl of PBS, and tumor development was monitored using microcalipers. The immunogenicity of selected tumor cell lines was determined by s.c. injecting mice with 10^6 irradiated (20,000 rad total dose) tumor cells in 100 μl of PBS. Fourteen to 21 days later, a secondary s.c. challenge with viable tumor cells (10^6 cells/mouse, 100 μl PBS) was given. Mice were regularly checked and sacrificed when tumor dimension reached 100 mm^2 as per the University of Western Australia ethics approval.

Peptides

SIINFEKL was manufactured by the Center for Cell and Molecular Biology (University of Western Australia, Perth) at a purity of 89%.

In vitro CTL assays

Effector cells were prepared from the spleens of either OT-I TCR mice (used to screen OVA-transfected clones) or pooled experimental groups of three C57BL/6-nontransgenic mice. The effector cells were expanded in vitro with a 1:1 ratio of whole splenic cells used as a source of normal APC taken from naive mice that were either osmotically loaded with whole native OVA or pulsed for 90 min with 10^{-6} M SIINFEKL peptide. APC were washed before addition to effector cells and incubated at 37°C for 5 days in CM in a humid environment.

Target cells which included our cOVA-transfected LL tumor clones, EG7 or EL4 cells pulsed with 10^{-6} M SIINFEKL, were labeled with 100 μCi of ^{51}Cr for 90 min and washed four times before use. Effector cells were added to corresponding targets at varying E:T cell ratios, incubated at 37°C for 4 h, 50 μl of each supernatant was harvested, and ^{51}Cr release was determined (gamma counter; Packard Top-Count, Zurich, Switzerland). The mean of duplicate samples was calculated and the percentage of specific ^{51}Cr release was determined as follows: percentage of specific lysis = (experimental ^{51}Cr release − control ^{51}Cr release)/(maximum ^{51}Cr release − control ^{51}Cr release) × 100. Experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from targets exposed to 5% Triton X-100.

In vivo analysis of tumor Ag cross-presentation

CFSE (Molecular Probes, Eugene, OR) labeling was performed as previously described (23). LN cells from TCR-transgenic OT-1 mice were resuspended in 20 ml of RPMI 1640 at 10^7cells/ml and incubated with 1 μl of CFSE stock solution (5 mM in DMSO) for 10 min at room temperature. Cells were washed through FCS four times and PBS twice, and 10^7 cells were i.v. injected into each recipient mouse. CFSE-labeled cells were recovered from secondary lymphoid organs 3 days after adoptive transfer and analyzed by FACS analysis.

In vivo analysis of CTL function (in vivo CTL)

Target cells for in vivo evaluation of cytotoxic activity were prepared as described elsewhere (24). Briefly, C57BL/6 LN cell suspensions were RBC lysed and the cells were washed and divided into two populations. One population was pulsed with 10^{-6} M SIINFEKL for 90 min at 37°C, washed in PBS, and labeled with a high concentration (5 μM) of CFSE. Control, uncoated target cells were labeled with a low concentration of CFSE (0.5 μM). Cells (10^7) of each population were mixed in 200 μl of PBS and i.v. injected into each recipient mouse. Specific in vivo cytotoxicity was determined by collecting the DLN, nondraining LN, spleens, and tumors from recipient mice 18 h after injection, and the number of cells in each target cell population was determined by flow cytometry. The ratio between the percentages of uncoated vs SIINFEKL-coated ($CFSE^{low}/CFSE^{high}$) was calculated to obtain a numerical value of cytotoxicity. Further controls included naive- and PBS-only-treated recipient mice. To normalize data allowing interexperimental comparisons, ratios were calculated between the percentages of peptide coated in control vs tumor-bearing mice.

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FACS analysis

LN or spleen cells were stained for two-color FACS analysis using the PE-conjugated mAb anti CD8 (clone 53-6.7; PharMingen, San Diego, CA). Analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest software. For analysis of CFSE-labeled cells, 100,000 events were collected and analyzed using the ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME).

Immunohistochemistry

Frozen sections (10 μm) of OCT-embedded tumors were fixed in cold ethanol, washed, and endogenous peroxidase blocked using 1% hydrogen peroxide. Primary Abs directed against murine CD4 and CD8 T cells (clones MT310 and MT25, respectively; Dako, Carpinteria, CA), MHC class II molecules (TIB-120 from P. Holt, Institute of Child Health Research, Perth, Australia), macrophages (F4/80), and CD11c (N418 expressed on dendritic cells; both Abs were kindly supplied by A. McWilliam, University of Western Australia, Perth), were applied for 45 min at room temperature and, after washing in PBS, sequentially linked to the secondary biotinylated Ab (sheep anti-mouse Ig; Jackson ImmunoResearch, West Grove, PA) and streptavidin-HRP (Dako, Glostrup, Denmark). Staining was visualized by precipitating 3,3′-diaminobenzidine (Sigma) and counterstaining with hematoxylin. Sections were mounted in Immunomount (Shandon, Pittsburgh, PA).

Results

Cytoplasmically located OVA loads MHC class I molecules with the dominant peptide (SIINFEKL) and exhibits unaltered growth kinetics in syngeneic mice

The murine LL cell line was transfected with cDNA coding for cytoplasmic expression of OVA. Thus, in LL tumor-bearing C57BL/6 mice, transfected OVA becomes a neo tumor Ag. Screening by an in vitro CTL assay, using effector T cells from OT-1 mice which recognize SIINFEKL (Fig. 1*A*) and RT-PCR analysis using OVA-specific primers (data not shown), revealed a number of clones expressing OVA at differing levels. One clone, referred to as cOVA clone 9, (cOVA-9) was effectively lysed by OT-1 T cells (indicated by percent lysis levels in Fig. 1*A*), demonstrating efficient production and MHC loading of SIINFEKL onto class I molecules, as well as recognition and lysis by OVA-specific effector CTL.

Inoculation of cOVA-9 tumor cells into normal, syngeneic mice revealed similar in vivo growth rates (Fig. 1*B*) and tumor frequencies (Fig. 1*C*) to the parental line. The in vitro growth rates of each cell line (determined by [^{3}H]thymidine incorporation) were also similar (data not shown). Both the parental cell line and cOVA-9 expressed low levels of MHC class I and no MHC class II, B7.1, or B7.2 (determined by FACS analysis; data not shown), implying that the tumor cells themselves cannot induce an immune response. Immunohistochemical staining of ex vivo tumors revealed a significant macrophage, as well as a small dendritic cell, infiltrate within the tumor milieu that was dispersed throughout the tumor and expressed class II, low levels of B7.2, and no B 7.1 (data not shown).

Expression of OVA does not alter the immunogenicity of the tumor cell line

To determine the immunogenicity of the parental vs cOVA-9 cell lines, mice were immunized with irradiated tumor cells and challenged 2 wk later with viable tumor cells. Cross-protection experiments determined whether the cOVA-9 cell line offered protection against itself or the parental cell line and vice versa. Regardless of which cell line was used to immunize mice, the growth rates (Fig.

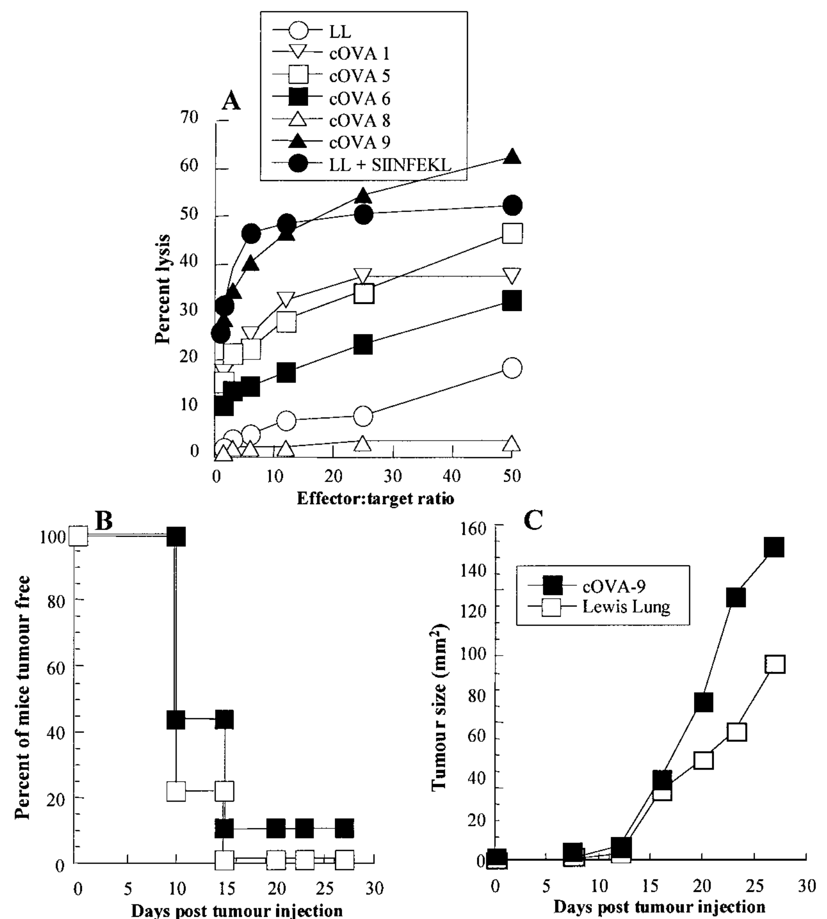

FIGURE 1. Characterizing the OVA-transfected murine tumor model. OVA-expressing transfectants were developed by stably cotransfecting the parental cell line (LL) with cDNAs coding for cytoplasmically targeted OVA and the neomycin selection marker. *A,* The resulting clones were screened using primed effector T cells from OT-1 mice in a 4-h ^{51}Cr release assay CTL assay and revealed a number of differentially recognized cOVA clones. We chose the clone most efficiently lysed by OT-1 T cells, referred to as cOVA-9. *B* and *C,* 5 × 10^5 viable cOVA-9 or LL cells were s.c. inoculated into naive C57BL/6 mice and their in vivo growth rates compared. *B,* The frequency of tumor development in mice given LL vs cOVA-9 was only marginally different. *C,* In mice with tumors, the growth kinetics of cOVA-9 was not significantly different than that seen for LL. These data are from a representative experiment (five mice per group) of at least three experiments.

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2*A*) and frequencies (100% of mice in all groups were tumor bearing 11 days after challenge) of both cell lines were identical, suggesting that no protection was generated, i.e., transfected OVA did not change the tumor into an immunogenic cell line.

OVA is recognized as target tumor Ag in vivo

To determine whether OVA retains its function as a CTL target in vivo, viable cOVA-9 tumor cells were s.c. inoculated into either OT-I mice or mice that had been immunized 2 wk earlier with high doses of OVA protein, with or without IFA. Both OVA alone and OVA/IFA provided partial protection; >50% of these mice remained tumor free (Fig. 2*B*), and of those in which tumors did grow, the rate was slow (Fig. 2*C*). However, if a high frequency of OVA-specific CD8 T cells was already present in host animals (OT-1 mice), complete protection was seen (Fig. 2, *D* and *E*). To confirm these results, CD8 T cells taken from OT-I TCR-transgenic mice were i.v. injected into normal C57BL/6J mice at log-fold concentrations ranging from 6×10^3 to 6×10^6 cells. Twenty-four hours later, mice were s.c. injected with 5×10^5 cOVA-9 cells. The incidence of tumor growth in mice was directly proportional to the number of transferred $CD8^+$ T cells (Fig. 3). Between 6×10^5 and 6×10^6 OVA-specific $CD8^+$ T cells provided a high degree of protection (60–80%). Decreasing the transferred OT-1 T cells number to below 6×10^5 resulted in a greater incidence of tumors.

These data show that despite expression of a potent Ag, cOVA-9 is unable to generate a protective immune response and may be regarded as a nonimmunogenic tumor. However, cOVA-9 tumors maintained their capacity to be CTL targets in vivo. Therefore, OVA is a rejection tumor Ag and $CD8^+$ T cells play a key role in protection against tumor growth. Nevertheless, protection was only seen when artificially high levels of tumor Ag-specific CTL were already present in host mice.

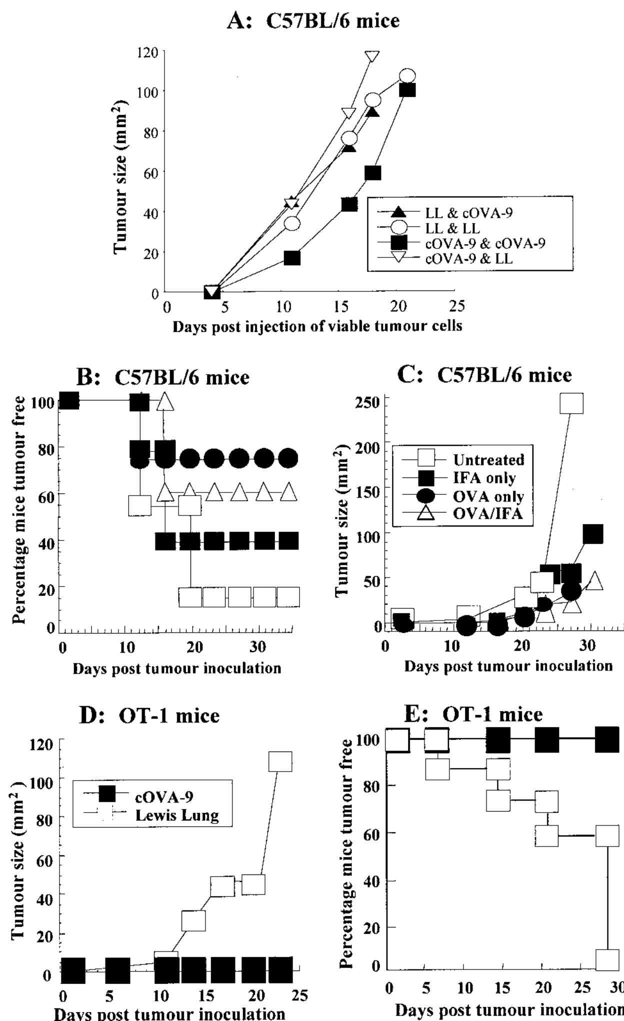

FIGURE 2. cOVA-9 is nonimmunogenic; however, OVA can be recognized and targeted by OVA-specific CTL in vivo. *A,* In cross-protection experiments, C57BL/6 mice were s.c. inoculated with 10^6 irradiated LL or cOVA-9 cells (20,000 rad) and challenged 2 wk later with 5×10^5 viable LL or cOVA-9 tumor cells. Neither cell line protected against tumor growth (five mice per group, two experiments), and both cell lines are therefore considered nonimmunogenic. *B–E,* A series of experiments were undertaken to see whether OVA remained a tumor rejection (target) Ag in vivo. *B* and *C,* C57BL/6 mice were s.c. immunized with OVA, with or without IFA, 2 wk before inoculation with viable cOVA-9 tumor cells. *B,* Tumor incidence shows that both OVA alone and OVA/IFA provided partial protection against cOVA-9 growth. *C,* Of those mice in which tumors did grow, the rate was slower in treated vs control mice. *D* and *E,* 5×10^5 LL or cOVA-9 cells were s.c. inoculated into OT-I mice. Complete protection was seen.

OVA is cross-presented by APC in LN draining the tumor

The data above suggest that either OVA was not presented to the immune system or that the levels of tumor-specific CTL generated after exposure to tumor cells were too low to be effective. This prompted an examination of the presence, location and duration of OVA cross-presentation during tumor growth. A kinetic study was undertaken in which CFSE-labeled OT-1 LN cells were adoptively transferred into recipient mice that had been s.c. inoculated with 10^6 viable tumor cells. Ag presentation, indicated by OT-I proliferation, was detected in the DLN 2 wk later (Fig. 4), when tumor size ranged from 20 to 30 mm^2. As the tumor progressed, the proliferative OT-1 response within the DLN continued, suggesting 1) ongoing tumor Ag transport and presentation to T cells within the DLN and 2) that tumor-draining APC are fully functional (as defined by induction of $CD8^+$ T cell proliferation) even when the tumor microenvironment is well established and tumor burden is large. No Ag presentation was seen at any time point in nondraining LN nor in the spleen (data not shown).

Irradiated tumor cells cross-present the dominant class I peptide earlier than progressing tumor

To determine whether an immune response could be induced after vaccination with irradiated tumor cells, mice were given

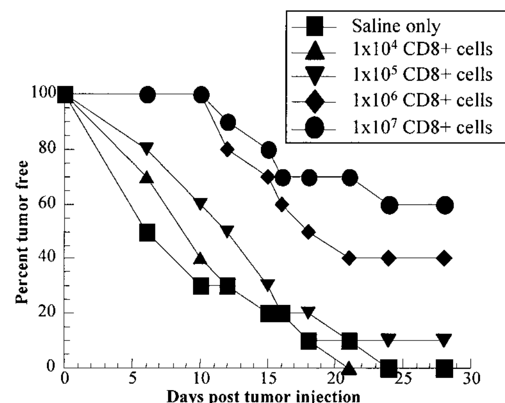

FIGURE 3. Specific $CD8^+$ cells can lyse tumor cells in vivo. LN T cells taken from OT-I TCR-transgenic mice were i.v. injected into normal mice at log-fold concentrations ranging from 1×10^4 to 1×10^7 cells (i.e., 6×10^3–6×10^6 SIINFEKL-specific $CD8^+$ T cells). Twenty-four hours later, mice were s.c. inoculated with 5×10^5 cOVA-9 cells and tumor growth was monitored. These experiments were repeated twice with five mice per group.

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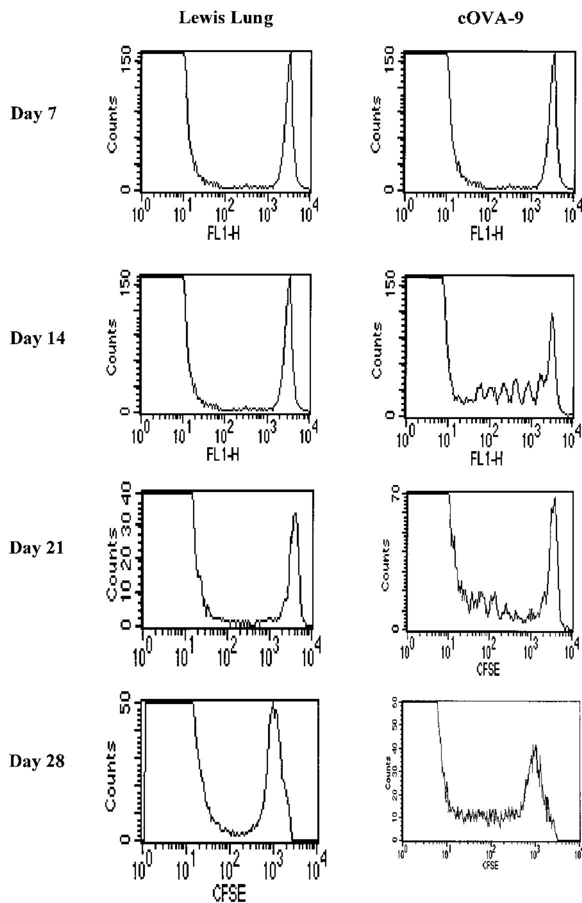

FIGURE 4. Tumor Ag cross-presentation is seen within the DLN during progressing tumor growth. CFSE-labeled class I-restricted OVA-specific T cells from OT-I mice were adoptively transferred at days 4, 11, 18, and 25 into mice s.c. inoculated on day 0 with 5×10^5 viable LL or cOVA-9 tumor cells. CFSE-labeled cells were reisolated from the DLN of mice 3 days after transfer, such that analysis by FACS was on days 7, 14, 21, and 28 after inoculation. All profiles obtained were gated on $CD8^+$ T cells. These results shown are from individual mice that are representative of at least three experiments (three mice per group).

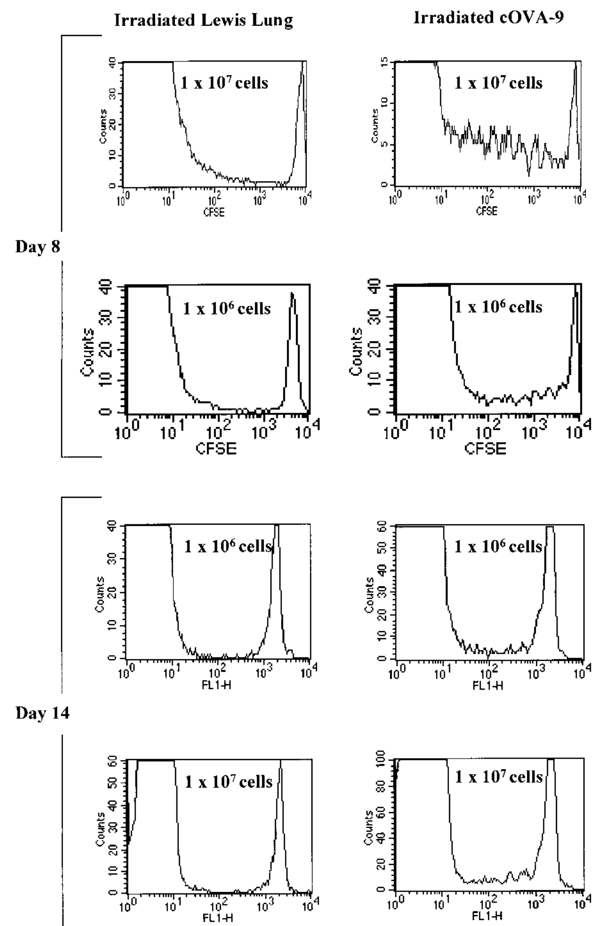

FIGURE 5. Tumor Ag presentation within the DLN after inoculation with irradiated tumor cells. CFSE-labeled T cells from OT-I mice were adoptively transferred into mice s.c. inoculated with 10^7 or 10^6 irradiated LL or cOVA-9 (20,000 rad) tumor cells. CFSE-labeled cells were reisolated from the DLN of mice 3 days after transfer, such that analysis by FACS was on days 8 and 14 after inoculation. All profiles obtained were gated on $CD8^+$ T cells. The results shown are from individual mice that are representative of three experiments (three mice per group).

varying doses of irradiated cOVA-9 tumor cells. A total of 10^6 irradiated cOVA-9 tumor cells gave a weak, but detectable OT-1 proliferative response in the DLN 8 days later (Fig. 5). In contrast, tumor Ag was clearly presented after inoculation with a log-fold higher concentration of irradiated tumor cells. Weak levels of Ag presentation were seen 2 wk after vaccination. No Ag presentation could be detected 4 wk after tumor vaccination (data not shown). Therefore, APC readily transport tumor Ag from growth-arrested irradiated tumor cells and from tumor cells that are either in the process of establishing or have established a tumor microenvironment.

OVA-specific effector CTL precursors can be expanded in vitro throughout tumor growth

As tumor Ag was consistently presented during tumor progression, we asked whether tumor-draining APC could arm CD8 cells to induce functional, tumor-specific, CTL. OVA-specific responses were initially measured using an in vitro CTL assay in which splenocytes from tumor-bearing mice were in vitro expanded with APC that were either osmotically loaded with whole native OVA or pulsed with SIINFEKL. Fig. 6*A* shows the development of SIINFEKL-specific CTL responses (using SIINFEKL-expanded T cells on SIINFEKL-pulsed targets) after mice were injected with viable cOVA-9 or the parental tumor cell line (LL), and tumors were allowed to progress to 100 mm^2 over 4 wk. Weak CTL responses were seen at day 7, which increased and plateaued 21 and 28 days after inoculation. Similar results were seen when cells expressing the whole OVA protein (EG7) were used as targets or when T cells were expanded in vitro with whole OVA-exposed APC (data not shown). OVA-specific CTL were also generated after host mice had been vaccinated with 5×10^5 irradiated cOVA-9 tumor cells; these CTL recognized and lysed both EG7 (Fig. 6*B*) and SIINEFKL-pulsed EL4 (Fig. 6*C*) targets. These data further support the notion that tumor-draining APC are fully functioning in vivo and generate precursor CTL (pCTL) after they have cross-presented a tumor Ag.

OVA-specific effector CTL are active in vivo in all secondary lymphoid compartments throughout tumor growth

The experiments described above clearly showed that pCTL could be detected in spleens taken from tumor-bearing mice, but did not tell us other sites where CTL may be located. Furthermore, although use of antigenic re-exposure in vitro gives an indication of the generation of peptide-specific pCTL, it may not be a true reflection of physiological events. Therefore, an in vivo CTL assay

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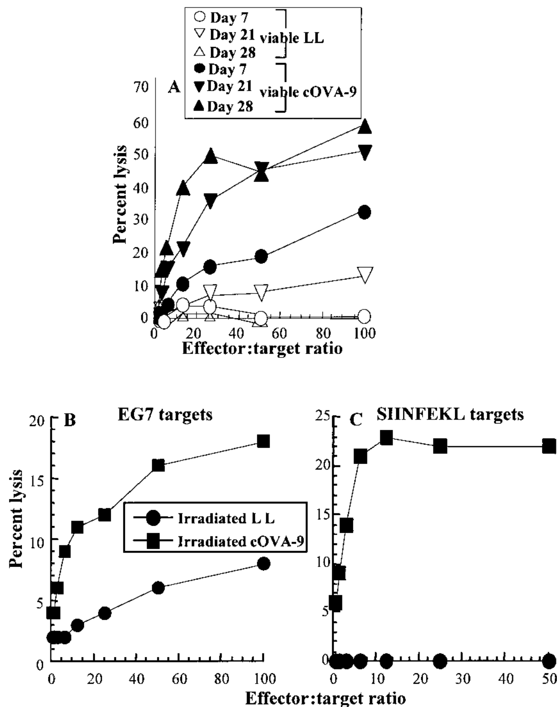

FIGURE 6. Tumor-specific CTL precursors can be detected during tumor growth and after vaccination with irradiated tumor cells. C57BL/6 mice were inoculated with viable (*A*) or irradiated (*B* and *C*) cOVA-9 or LL tumor cells and OVA-specific CTL responses were monitored. *A,* Splenocytes prepared, at varying days after tumor inoculation, from mice given viable LL (○, △, ▽) or cOVA-9 (●, ▲, ▼) were restimulated in vitro with SIINFEKL-pulsed APC. Five days later, a 4-h ^{51}Cr release CTL assay was performed on EL4 cells pulsed with SIINFEKL. These experiments were repeated three times and showed the same pattern. *B* and *C,* Vaccination with 10^6 irradiated tumor cells also led to the development of specific pCTL. Splenocytes taken 7 days after vaccination with irradiated cOVA-9 and cultured in vitro with SIINFEKL-pulsed APC were able to lyse OVA-expressing targets (EG7; *B*), as well as SIINFEKL-pulsed EL4 target cells (*C*) in a 4-h ^{51}Cr release in vitro CTL assay.

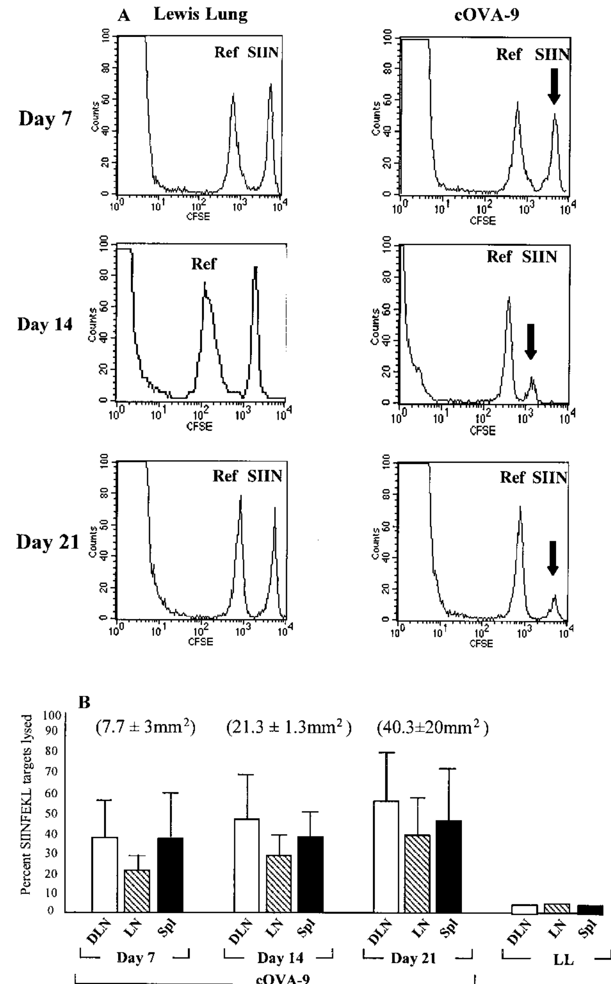

FIGURE 7. In vivo dominant epitope-specific effector CTL are located throughout the secondary lymphoid system during tumor growth. Target cells taken from the LN of naive syngeneic mice were divided into two populations. One population was pulsed with SIINFEKL and labeled with a high CFSE concentration (SIIN). Control uncoated target cells were labeled with a lower CFSE concentration (Ref). A total of 10^7 cells of each population was i.v. injected into recipient mice and specific cytotoxicity was determined 18 h later. *A,* FACS histograms are from the DLN of individual animals bearing either cOVA-9 or LL at each time point. *B,* The graph shows groups means (±SE) of the percentages of SIINFEKL-pulsed targets that had been lysed in the DLN, LN, and spleens (Spl) of tumor-bearing mice at varying days after tumor inoculation. Group means (±SE) of tumor size at each time point are shown in parentheses. Controls included LL-bearing mice (tumor sizes are not shown because data shown are pooled for days 7, 14, and 21). These experiments were repeated at least five times; the data shown are from one experiment.

was used to assess the location and duration of effector CTL in mice inoculated with the OVA transfectant or the parental cell line. It should be noted that the tumor cells themselves cannot readily be used as targets in vivo as 1) they may not migrate to all lymphoid sites and 2) a significant number of mice i.v. injected with 10^7 tumor cells did not survive overnight. Therefore, differentially CFSE-labeled SIINFEKL-pulsed or control target LN cells taken from naive mice were injected i.v. into tumor-bearing mice and lysis examined 24 h later. A potent SIINFEKL-specific response was seen in cOVA-9 tumor-bearing mice that was not only in the DLN (shown for one representative mouse in Fig. 7*A*; group means shown in Fig. 7*B*), but also throughout the secondary lymphoid system (Fig. 7*B*). This response steadily increased over time and in association with greater tumor burden (shown in parentheses in Fig. 7*B*). Tumor-specific CTL lytic levels remained consistently higher in the DLN, relative to the nondraining LN. In fact, each lymphoid site examined exhibited independent levels of SIINFEKL target killing (Fig. 7*B*), suggesting that killing was occurring within each site, independently of the other.

No Ag loss variants are seen with progressive tumor growth

The presence of persisting tumor-specific lytic CTL may place tumors under considerable selection pressure, resulting in Ag loss variants. We speculated that if Ag-loss variants had emerged during in vivo growth, then these tumor cells may expand when ex vivo tumors are cultured in vitro in the absence of selection pressure. Therefore, 100-mm^2 tumors were excised and re-established in vitro, with or without the neomycin selection marker (G418 and FCS, respectively). Ex vivo cOVA-9 tumor cells were susceptible to equivalent levels of CTL lysis by OT-I effector T cells, i.e., they retained expression and MHC loading of OVA (Fig. 8). RT-PCR also confirmed that ex vivo cOVA-9 tumor cells still expressed mRNA coding for OVA (data not shown).

Circulating T cells are able to penetrate the tumor microenvironment and retain their lytic capacity

The data described above show that despite the generation of significant levels of CTL the tumor continues to grow. Therefore, we

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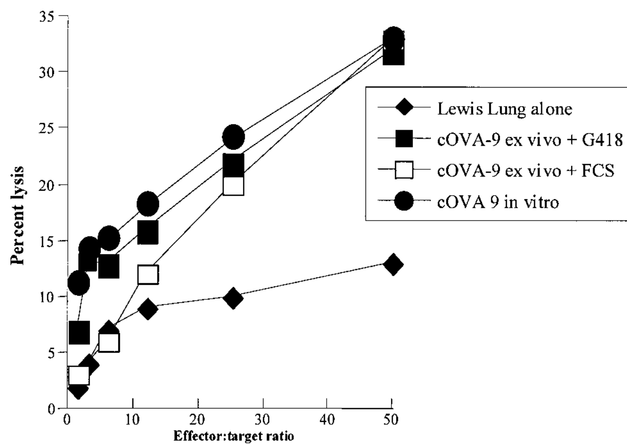

FIGURE 8. No Ag-loss variants are seen with progressive tumor growth. cOVA-9 tumors were allowed to grow to 100 mm^2, excised, and re-established in vitro in the presence or absence of selection medium (G418 or FCS, respectively). These tumor cells were used as target cells in a 4-h in vitro ^{51}Cr release assay CTL assay in which peptide-primed OT-1 T cells were effector cells. These data show equivalent levels of lysis, regardless of the presence or absence of selection (G418) pressure, to the in vitro-passaged cOVA-9 controls and confirms that expression of OVA is retained in tumor cells during in vivo growth.

used immunohistochemistry to examine whether or not there was a T cell infiltrate within the cOVA-9 tumor, compared with the parental cell line when these tumors reached 100 mm^2. Low numbers of CD8 and CD4 T cells were seen in both the parental tumor and cOVA-9 tumors (Fig. 9). Interestingly, low numbers of LN cells from syngeneic mice, used as targets for the in vivo CTL, were able to penetrate the tumor milieu of both parental and transfected tumors at all time points examined (Fig. 10). Killing of peptide-pulsed targets was seen in the transfected tumor, suggesting that active CTL were present within the tumor itself.

Discussion

To develop immunological strategies that will eradicate human nonimmunogenic tumors, an understanding of the ways such tumors interact, or fail to interact, with the host immune system is clearly required. To date, many murine models use immunogenic tumors that do not represent human cancers. Similarly, since the most common human tumors are solid tumors such as lung, breast, or bowel cancer, models using thymoma, leukemia, and mastocytoma tumor cell lines were not appropriate for our studies. Therefore, we established an animal model with a number of characteristics that made it relevant to human cancer and useful for analysis of the host antitumor response. That is, a solid nonimmunogenic class I^+, class II^- tumor that stably expressed a cytoplasmic Ag and was susceptible to lysis by tumor-specific CTL throughout tumor growth.

The Ag we chose was OVA because it is a well-characterized protein which has proven itself to be a useful model Ag in studies of immune responses to tissue Ags (15), and OVA-specific TCR mice can be used as a source of naive tumor-specific T cells. Because many tumor T cell Ags will be of intracellular origins, we used intracellular OVA to model a neo tumor Ag representing viral and mutated proteins. Use of the cytoplasmically located tumor Ag also avoided potential data interpretation problems that may occur when using OVA as a secreted tumor Ag, such as EG7 (17), when uptake of the secreted Ag could be independent of the fate of the tumor cell. The OVA-expressing tumor was not rejected in normal mice and grew in vivo at a rate similar to the parental cell line. A finding consistent with our previously published hemagglutinin (HA)-expressing tumor model (25). However, in contrast to the HA model, when used as a vaccine, the irradiated OVA transfectant did not induce protection against itself or the parental tumor cell line; i.e., it maintained the classical nonimmunogenic phenotype.

Tumor Ags are efficiently cross-presented in vivo

Most tumors, including ours, do not express class II or costimulatory molecules; therefore, in the absence of direct tumor Ag presentation by host tumor cells, cross-presentation must be occurring, and this has been confirmed by recent studies (7–9) including our own (26). Current concepts suggest that the failure of host CD8 cells to mount an effective response to tumor Ags resides largely in the function of tumor-draining APC. There are two main notions. The first is that tumor Ags are sequestered away from the immune system and are never cross-presented to host CD8 cells (3, 4, 6, 27). The second, more widely held view, is that tumor-derived signals are insufficient to condition APC to levels required to induce CD8 cells to differentiate into cytotoxic effector cells. There is evidence that tumor-associated APC function may be impaired via tumor-derived molecules such as TGF-β (28), IL-10 (29), and vascular endothelial growth factor (30). These APC may be unable to process tumor Ags (31, 32) or are defective in their Ag-presenting function (33) and inducible B7 expression (11), responses to maturation or migratory signals (10, 12, 13, 34), and undergo early cell death (10). Conversely, tumor-traversing APC may secrete molecules such as IL-10 that interfere with CTL priming (35). We assessed the in vivo location, duration, and intensity of tumor Ag cross-presentation and found that tumor-draining APC exhibited no obvious deficiency in either cross-presentation in the DLN or in generating potent specific CTL.

Ag presentation is a sophisticated multistep process involving tumor Ag uptake, processing, and peptide loading onto MHC class I molecules by APC within the tumor, their subsequent movement into DLN where they present tumor peptides to T cells, in association with costimulatory molecules, to a level required for T cell activation. We show cross-presentation occurring 2 wk following tumor injection. Cross-presentation was restricted to the DLN, probably reflecting APC trafficking from the tumor. The APC responsible for transporting and cross-presenting tumor Ag remains unknown; however, there are many macrophages and a small number of dendritic cells within the tumor and it is likely that Ag is carried to LN via these cells.

We used animals vaccinated with irradiated tumor cells to examine whether the failure to protect from tumor rechallenge was due to the lack of an immune response and to evaluate APC function when presenting the same tumor Ag in the absence of a tumor mass. However, irradiated tumor cells are used in animal studies and in a variety of human vaccination clinical trials. Our data demonstrated dose-responsive tumor Ag cross-presentation after inoculation with irradiated cells, which was detectable a week earlier than inoculation with similar numbers of viable tumor cells. Thus, irradiation-induced apoptosis may be an effective source of tumor Ags for cross-presentation by APC (36). These observations may provide useful information when determining clinical regimens using irradiated tumor cells.

Specific antitumor CTL are active in vivo throughout the period of tumor growth

Recent studies have shown that T cell activation is not an automatic outcome of tissue Ag cross-presentation. The possible subsequent immune events are tolerance (15), limited T cell proliferation without CTL generation (37, 38), or the generation of active cytotoxic effector cells including CTL (7, 39). Our previous studies

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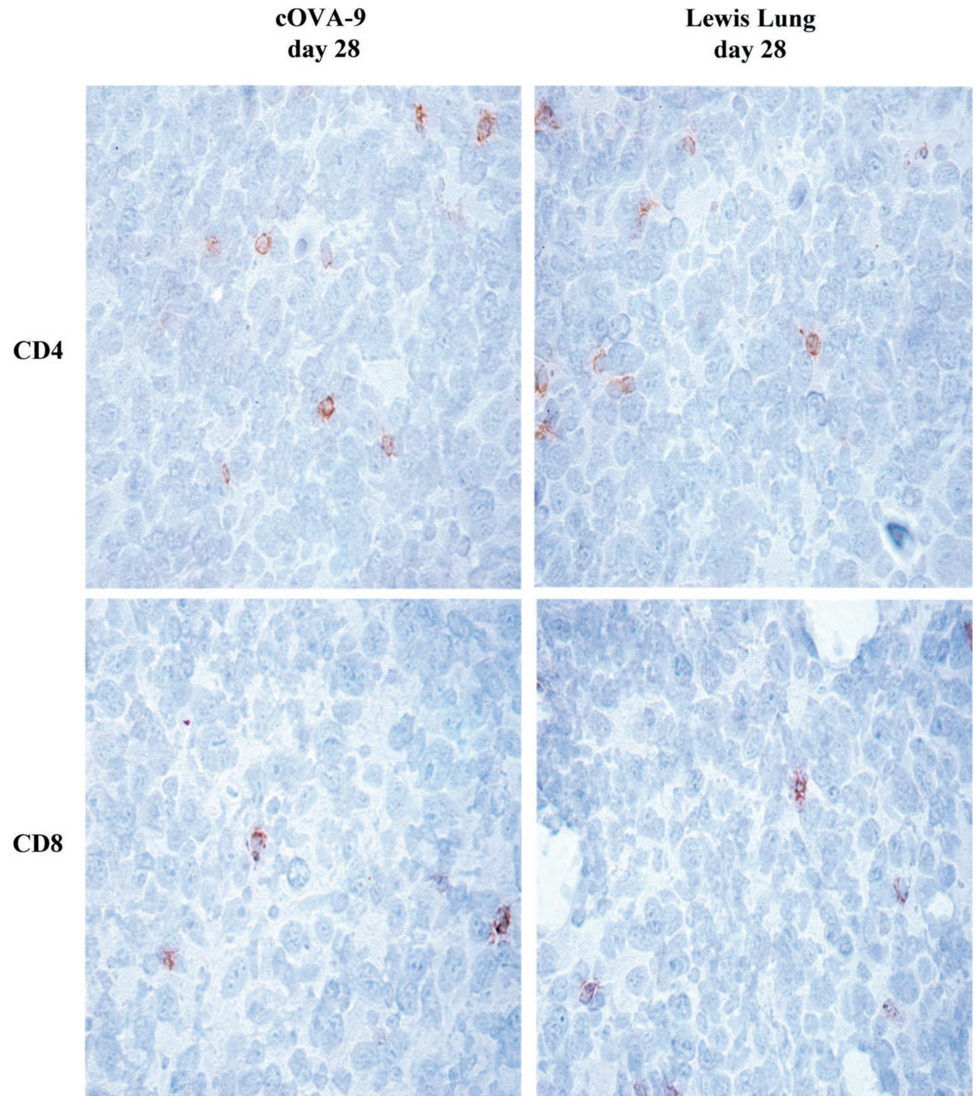

FIGURE 9. Infiltrating $CD8^+$ and $CD4^+$ T cells can be seen in cOVA-9 tumors. Tumors were removed once they had grown to 100 mm^2 in normal mice, frozen in embedding medium, and 10-μm frozen sections stained using immunohistochemistry to detect tumor-infiltrating $CD4^+$ and $CD8^+$ T cells.

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using an HA-expressing immunogenic tumor model have shown that a weak and transient tumor-specific CTL response can be detected despite tumor Ag cross-presentation continuing throughout the period of tumor growth (26). This attenuated CTL response could be related to the temporal "licensing" of APC as a result of damage induced by tumor cell injection or to the induction of tolerizing processes. Similarly, others have reported tolerizing events occurring within the CD8 population in tumor models which, in some cases, could be reversed by manipulation of APC function (40, 41). In contrast and in contradiction to our expectations, use of in vitro and in vivo CTL assays showed that our nonimmunogenic tumor induces tumor Ag-specific CTL which are very active in vivo on Ag-bearing cells and maintained throughout tumor growth. Clearly, in this model no tolerance was occurring.

These CTL were detectable at day 7, a time when cross-presentation was not detectable in any lymphoid site. This suggests that the in vivo cross-presentation assay does not detect low levels of presentation and that tumor-draining APC are presenting tumor Ags, even when the tumor burden is minimal, at levels that are sufficient to induce CTL precursors. The APC trafficking through our tumor appeared to be mature or "licensed" (42) and generated, or maintained, specific CTL in association with continuous Ag presentation. Additionally, specific CTL were induced early in tumor progression, indeed before the tumor was even palpable and well before macroscopic necrosis occurred. Therefore, tissue damage or danger (43) is not likely to be the explanation for our findings. It is possible that endogenous CD4 help may have been generated (37), but there is also some evidence that active CD8 cells may help in the licensing of APC (44). Thus, while the failure of the host immune response to eradicate some immunogenic tumors may relate to the down-regulation of T cell function or the induction of regulatory CD4 cells, this does not apply to our nonimmunogenic tumor.

The presence of a potent CTL response that is restricted to the dominant epitope does not induce alternative means of escape

It is possible that the generation of a persisting CTL response to a single dominant epitope may provide other escape mechanisms for the tumor. A number of studies have described the progressive selection of tumor Ag-loss variants as a means of escaping immune detection (19–21). We did not see any evidence of Ag loss during tumor growth, despite the potential selection pressure exerted by active CTL specific to a single epitope throughout the period of tumor growth.

Alternatively, a response limited to a single epitope may not be enough to destroy an established solid tumor. Tumors may focus the CTL response on this single epitope and block spreading to other epitopes escaping eradication. We found no evidence for a

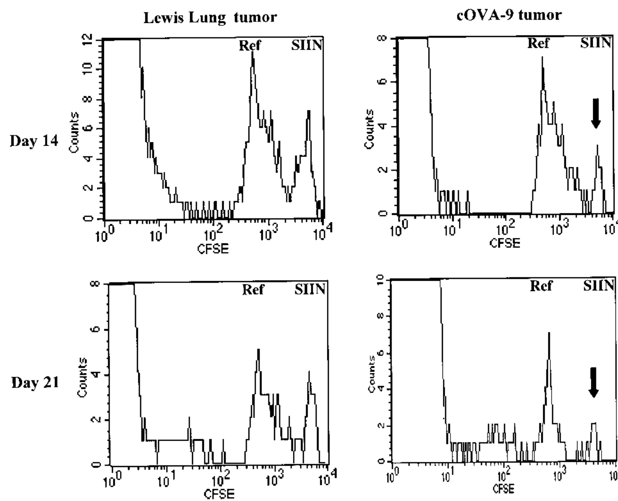

FIGURE 10. Lymphocytes can traverse tumors at any time during tumor growth and those CTL within the tumor are functional. Target cells were prepared as described for Fig. 6. SIINFEKL-pulsed (SIIN) and control (Ref) target cells were labeled with high and low concentrations of CFSE, respectively. Cells (1×10^7) of each population were mixed before i.v. injection into tumor-bearing recipient mice and tumors were analyzed by flow cytometry 18–24 h later. Representative FACS histograms are from the tumors of individual mice bearing either cOVA-9 or the LL at days 14 and 21 of tumor growth. These experiments were repeated at least three times, all showing the same pattern, with varying levels of specific lysis; the data shown are from one experiment.

broadening or shift of the immune response to other CTL epitopes, as vaccination with the transfected tumor did not protect against challenge with the parental line. We are currently examining ways of spreading CTL responses to multiple tumor Ags to determine whether these in vivo-induced CTL can exert any influence on tumor progression.

Finally, another escape mechanism that tumors could employ is the destruction of recently emigrated tumor Ag-bearing APC upon their entry into the DLN (45, 46) or direct CTL-to-CTL killing within the DLN (47). Our data show that although dominant-epitope-specific effector CTL readily lyse tumor Ag-bearing targets in the DLN, neither tumor Ag presentation nor CTL activity ceased during tumor progression. These data imply that little or no significant CTL-to-CTL killing was occurring and that either 1) tumor-draining APC were not lysed while in the DLN, or 2) the turnover of APC from tumor to DLN was sufficiently rapid to negate any effect lysis of APC may have, or 3) the rate of lysis was slower than the rate of T cell activation, or 4) other DC within the LN picked up and presented tumor Ag from lysed APC.

Immunogenicity and tumor Ags

Nonimmunogenicty was assumed to relate to the absence of a strong tumor Ag and even though the level of immunogenicity of human tumors remains uncertain, they are widely assumed to be nonimmunogenic. We transfected the LL tumor cell line with a strong Ag, confirmed that it remained nonimmunogenic and could be a CTL target in vitro and in vivo when large numbers of specific CD8 cells are already present early in the period of tumor growth. This observation is consistent with studies that have demonstrated that transfection of cytokine genes and costimulatory molecules, such as B7-1, can unmask reactivities to tumor Ags thought to be nonimmunogenic (48–50). The explanation that nonimmunogenicity is due to a failure of tumor Ag cross-presentation to the host immune system (i.e., ignorance of the presence of tumor Ags) may not be generally correct. Indeed, our data suggest that as a tumor grows, it is likely that many proteins which are initially expressed at low levels progressively reach the threshold levels required for cross-presentation. Therefore, a larger proportion of proteins present in tumor cells may, in fact, be cross-presented than would be the case for normal tissues.

Continuing tumor growth in the presence of effective CTL relates to events occurring within the local tumor microenvironment

The data presented here imply that simply providing tumor Ags in a way that causes them to be successfully presented to the host immune system, even if they induce significant effector CTL responses, will not necessarily be successful in causing tumor eradication. In fact, we only saw protection when very high numbers of tumor-specific CTL were already present 24 h before tumor injection, at a time point when the tumor matrix/microenvironment was not established. Hence, even providing protection against tumor development appears to be tightly regulated and dependent upon the numbers of tumor-specific T cells already present in the host. Nevertheless, tumor cells could activate adoptively transferred specific (OT-I) effector CTL, resulting in tumor killing in vivo. These data, in conjunction with an absence of Ag-loss variants, imply that, in our model, T cells activated within the LN by tumor-transiting APC do have the capacity to be reactivated and lyse tumor cells in vivo early in the course of tumor growth. Once tumors are established however CTL may fail to enter the tumor or they enter the tumor but are immediately disabled. Immunohistochemistry revealed low levels of tumor-infiltrating lymphocytes in both the OVA transfectant and the parental tumor, and nonspecific syngeneic lymphocytes were seen trafficking through the tumors at each experimental time point, suggesting that lymphocytes were able to penetrate or traverse through the tumor. Furthermore, CTL that were in the tumor retained their lytic function.

The fact that these CTL do not prevent tumor growth implies a failure within the tumor microenvironment. Lymphocytes including tumor-specific CTL may be simply trafficking through the tumor vasculature and do not enter the tumor matrix. Our data imply that larger tumors are more resistant to CTL-mediated destruction, and this is supported by work in our HA-expressing immunogenic tumor model (51) and by others (1). Hence, the failure of CTL to mediate tumor regression may, in part, be due to the overwhelming ratio of Ag-bearing tumor cells relative to tumor-specific CTL (1). Also, tumor Ag-loaded APC, e.g., macrophages, which are abundant within most tumors, could theoretically act as preferential or competing targets for limiting numbers of tumor-infiltrating CTL. We now have evidence for both of our tumor models that the window in time during tumor growth for successful immunologically based therapies is narrow, and once a tumor is well established transferred tumor-specific CD4 and CD8 cannot induce regression (our unpublished data). We are currently investigating the trafficking and fate of the tumor-specific CTL.

In conclusion, after transfection with a strong Ag, the LL tumor cell line retained its nonimmunogenic status, as well as its potential to be a CTL target, in vivo. Tumor Ags were continuously cross-presented during tumor growth, inducing a potent and persisting in vivo CTL response. Yet the tumor continued to grow, either by preventing entry of T cell subpopulations into the tumor milieu or by outnumbering effector CTL, a process that appears to start at a very early phase of tumor growth. Overall, these data imply that tumor-draining APC are not, as has been suggested, incapable of spontaneously arming CD8 cells for lytic function. Therefore, anticancer immunotherapeutic approaches that focus solely on inducing CD8 activation and neglect the development of methods that facilitate the

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entry of large numbers of active tumor-specific T cells into tumor sites, in the early phases of tumor growth, are not likely to succeed.

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