IL-2 Intratumoral Immunotherapy Enhances CD8+ T Cells That Mediate Destruction of Tumor Cells and Tumor-Associated Vasculature: A Novel Mechanism for IL-2

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IL-2 Intratumoral Immunotherapy Enhances CD8+ T Cells That Mediate Destruction of Tumor Cells and Tumor-Associated Vasculature: A Novel Mechanism for IL-2

Connie Jackaman,* Christine S. Bundell,* Beverley F. Kinnear,§ Alison M. Smith,* Pierre Filion,† Deborah van Hagen,* Bruce W. S. Robinson,*‡ and Delia J. Nelson2*‡¶

Therapeutic use of IL-2 can generate antitumor immunity; however, a variety of different mechanisms have been reported. We injected IL-2 intratumorally (i.t.) at different stages of growth, using our unique murine model of mesothelioma (AE17; and AE17 transfected with secretory OVA (AE17-sOVA)), and systematically analyzed real-time events as they occurred in vivo. The majority of mice with small tumors when treatment commenced displayed complete tumor regression, remained tumor free for >2 mo, and survived rechallenge with AE17 tumor cells. However, mice with large tumors at the start of treatment failed to respond. Timing experiments showed that IL-2-mediated responses were dependent upon tumor size, not on the duration of disease. Although i.t. IL-2 did not alter tumor Ag presentation in draining lymph nodes, it did enhance a previously primed, endogenous, tumor-specific in vivo CTL response that coincided with regressing tumors. Both CD4+ and CD8+ cells were required for IL-2-mediated tumor eradication, because IL-2 therapy failed in CD4+-depleted, CD8+-depleted, and both CD4+ and CD8+-depleted C57BL/6J animals. Tumor-infiltrating CD8+ T cells, but not CD4+ T cells, increased in association with a marked reduction in tumor-associated vascularity. Destruction of blood vessels required CD8+ T cells, because this did not occur in nude mice or in CD8+-depleted C57BL/6J mice. These results show that repeated doses of i.t. (but not systemic) IL-2 mediates tumor regression via an enhanced endogenous tumor-specific CTL response concomitant with reduced vascularity, thereby demonstrating a novel mechanism for IL-2 activity. The Journal of Immunology, 2003, 171: 5051–5063.

Interleukin-2 has been used extensively in murine cancer models and human clinical trials, mainly due to its effects on T cells, particularly CTLs. Not all studies have shown a significant clinical outcome; however, those that have observed antitumor immunity in response to IL-2 therapy (in both human and mice) have reported conflicting mechanisms, ranging from immune- to nonimmune-mediated mechanisms (1). In early studies, IL-2 was administered systemically into patients, but beside effects and low response rates made this approach unacceptable. More recently, IL-2 has been applied directly into, or around, accessible solid tumors with promising results and minimal side effects (2, 3). Central to this intratumoral (i.t.)1 approach are observations that a range of different immune-associated cell types can be found infiltrating progressing tumors at various time points during disease. These cells include those that exert antitumor cytolytic activity such as CD8+ CTLs and NK cells, as well as cells that activate CTL and NK cells such as dendritic cells (DC). These i.t. cell populations represent ideal targets for local immunotherapy. In particular, tumor-infiltrating DC and CD8+ T cells are placed perfectly for their respective roles as tumor Ag-bearing APC and effector, tumor Ag-specific CTLs. Intratumoral IL-2 may boost antitumor T cell responses, either by acting as a second costimulatory signal in the activation of CTL or by further activating DCs expressing IL-2Rs. Furthermore, local exposure to IL-2 may activate a wider range of effector cell types, including NK cells, macrophages, B cells, and CD4+ T cells in vivo. It is possible that a combination of activated innate and adaptive immune responses, as well as nonimmune responses, may be required to induce an effective antitumor response. However, the effects of serial i.t. IL-2 injections on tumor and systemic cellular reactivity had not been methodically and systematically examined until now. Before IL-2 can be used effectively in the clinical situation, it is essential to fully understand how this cytokine acts in vivo in tumor-bearing hosts.

Vital questions include the following: 1) what regimen gives the optimal antitumor response (dosage, route, and timing of injections); 2) does tumor burden at the beginning of treatment impact upon the ultimate outcome of IL-2-based therapy; 3) what mechanisms, immune or otherwise, are critical for antitumor efficacy; 4) if an immune response is generated/enhanced, does IL-2 affect the induction arm and/or the effector arm of immunity; and 5) is an IL-2-induced antitumor response mediated by a single or multiple mechanism(s)?

In this study, we examined whether i.t. IL-2 could induce an antitumor response at different stages of tumor growth, using our unique murine malignant mesothelioma (MM) model (AE17). We
chose this model because there is currently no standard treatment for MM patients, as MM is generally resistant to conventional therapies (chemotherapy, radiation, and surgery). However, it has been suggested that MM may be susceptible to immunotherapy (4, 5). Given this, MM represents an ideal model of a solid, incurable tumor.

A MM murine model was established in our laboratory by inoculating mice with asbestos fibers, excising the consequent tumors, and cloning tumor cells in vitro. Reinoacultation of cloned tumor cells into syngeneic mice results in progressing tumors confirmed by histopathology to be representative of human MM. The cell line used in this study (AE17) grows slower than other well-studied tumor cell lines (including the melanoma B16 cell line and the Lewis lung carcinoma), allowing us to study treatment at late time points, which is more relevant to the clinical situation. Until recently, in vivo analysis of real-time immune responses was difficult. To overcome this problem, we transfected our AE17 MM cell line with cDNA coding for OVA (AE17-secretory OVA (sOVA)), so that in AE17-sOVA tumor-bearing C57BL/6J mice, OVA becomes a neo tumor Ag. The response to the class I-restricted dominant peptide of OVA, SIINFEKL, can be measured using an in vivo Ag presentation assay and an in vivo CTL assay (6, 7).

In this study, we report that high doses of i.t. IL-2 (administered three times per week) enhanced tumor-specific CTL activity and inhibited tumor-associated vascularity leading to tumor rejection. This response was dependent upon tumor size when treatment commenced and not on the duration of tumor burden, i.e., tumor rejection was induced when the tumors were established but relatively small when i.t. IL-2 treatment began. Reduction of blood vessels, leading to and within the tumor, required functional lymphocytes, in particular CD8 T cells, because it could not be induced in nude mice deficient in T cells, or in CD8-depleted mice. However, CD4 cells may also play a role, because the efficiency of IL-2 therapy was diminished in CD4-depleted animals. Our findings suggest that the failure of the endogenous immune response in controlling tumor progression is initially restricted to the local tumor microenvironment, and can be overcome by directly offering help via IL-2. However, as tumors progress, this failure is amplified and cannot be rescued by IL-2 alone. Thus, if i.t. IL-2 therapy coincides with early diagnosis, it can enhance a previously primed, endogenous, tumor-specific CTL response concomitant with destruction of local blood vessels, thereby mediating complete and permanent tumor rejection.

Materials and Methods

Mice

Female C57BL/6J (H-2Kb) and BALB/c nu/nu (nude) mice between 6 and 8 wk of age were obtained from the Animal Resources Centre (Mordoc, Western Australia, Australia) and maintained under standard housing conditions. The OT-I (H-2Kb) TCR-transgenic mouse line, expressing a TCR recognizing the dominant H-2Kb-restricted OVA epitope (SIINFEKL) (8) was kindly supplied by Dr. F. Carbone (University of Melbourne, Melbourne, Australia) and Dr. W. Heath (Walter Eliza Hall Institute, Melbourne, Australia).

Murine tumor cell lines

AE17 is a MM cell line derived from the peritoneal cavity of C57BL/6J mice injected with asbestos (crocidolite) fibers. Ascites, solid tumor, or peritoneal flushings were collected from tumor-bearing mice and cultured in vitro. Tumor cell lines were then passaged in vitro for 2–20 passages and reinoacultated into syngeneic mice. Ascites, solid tumor, or peritoneal flushings were once again removed and cultured. This form of passaging through mice occurred at least three times.

The thymoma cell lines EL4 (TIB-39) and EG7 (CRL-2113) were obtained from American Type Culture Collection (Manassas, VA). EL4 and EG7 are H-2Kb-restricted tumors derived from C57BL mice and have been described previously (9).

Cell culture and maintenance

Murine tumor cell lines were maintained in RPMI 1640 (Invitrogen, Victoria, Australia) supplemented with 10% FCS (Invitrogen, Victoria, Australia), 48 mg/L gentamicin (Pharmacia & Upjohn, Perth, Western Australia, Australia), 60 mg/L benzylpenicillin (David Bull’s Laboratory, Victoria, Australia), and 0.05 mM 2-ME (Metec, West Point, PA). Transfected tumor cell lines were maintained in conditioned medium supplemented with 400 μg/mL neomycin analog G418 (geneticin; Invitrogen). Cells were cultured at 37°C in a 5% CO2 atmosphere and passed when 70% confluent.

Plasmids and transfections

The naturally occurring secreted form of OVA (sOVA) was constructed under the control of the human β actin promoter in the mammalian expression plasmid pAC.Neo.OVA (kindly donated by Dr. F. Carbone (University of Melbourne, Melbourne, Australia)) and has been described previously (9).

AE17 transfectants were developed by stably transfecting the AE17 parental cell line with cDNA encoding for sOVA and the neomycin selection marker, using DOTAP (N,N,N',N'-trimethylammonium methyl sulfate according to manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Positive clones were isolated via growth in culture medium containing G418. Single adherent colonies were picked and recloned by limiting dilution assay.

In vivo tumor growth

Mice were injected s.c. in the left flank at day 0 with 5 × 105 or 105 tumor cells per mouse in 100 μL of PBS, and tumor growth was monitored using microcallipers. Mice were regularly checked and sacrificed when tumor dimension reached 100 mm3 as per University of Western Australia Animal Ethics Committee ethic approval.

Transmission electron microscopy (TEM)

Solid-tumor samples were thinly sliced (2-mm thick), immersed in 2.5% glutaraldehyde fixative in phosphate buffer at pH 7.4, and fixed for 48 h. In vitro cell monolayers were covered in the above fixative for 24 h. scraped off, pelleted, and rinsed three times in phosphate buffer before gelling with BSA. The resulting pellet was fixed for a further 24 h with 2.5% glutaraldehyde.

The solid tumor and cell culture pellet were cut to 1-mm3 pieces and processed in a LYNX automated tissue processor by postfixation in 1% osmium tetroxide, dehydration in gradedethanols and in propylene oxide, and infiltration in araldite. The tissue blocks were embedded in fresh araldite and hardened for 48 h. Sections (1-μm thick) were stained with toluidine blue for light-microscopic assessment. Ultrathin sections were then cut at 100-nm thickness on 200 mesh copper grids and counterstained with saturated solutions of uranyl acetate and lead citrate. The grids were viewed on a Philips (Eindhoven, The Netherlands) CM10 transmission electron microscope.

CD4+ and CD8+ depletions

YTS-191 (anti-CD4+ depletion Ab) and YTS-169 (anti-CD8+ depletion Ab) were maintained in RPMI 1640 supplemented with 5% FCS, 48 mg/L gentamicin, 60 mg/L benzylpenicillin, and 0.05 mM 2-ME. Supernatants collected from each cell line were purified using a HiTrap protein G column (Amersham Biosciences, Uppsala, Sweden), dialyzed against PBS overnight, and then assayed for protein levels using the Bradford assay (Bio-Rad, Hercules, CA).

For depletion of CD4+ and CD8+ cells, two doses (100 μg/dose) of either YTS-191 or YTS-169 were injected i.p. before treatment, and then continued (three doses per week) until the end of the experiment. Mice were sacrificed and tested before the start of treatment and at the end of the experiment for levels of CD4+ and CD8+ cells. This was determined by FACs staining as >90% depleted for CD4+ cells and >95% depleted for CD8+ cells (data not shown).

Use of rIL-2

The bioactivity of IL-2 was routinely assayed using a murine IL-2-dependent cytotoxic T cell line (CTLL), as previously described (10). Briefly, 1 × 106 CTLL cells were plated with equal volumes of various concentrations of recombinant human IL-2 (Cetus, Emeryville, CA). CTLL proliferation was determined 24 h later by [3H]thymidine incorporation and
measured by liquid scintillation counting (Hewlett-Packard, Palo Alto, CA). Activity was calculated as the median effective dose (ED$_{50}$; units per milliliter) where 1 U is the amount required for 50% maximum growth from 1 × 10$^5$ CTL cells in 0.1-ml culture volumes during a 24-h culture period. Using this assay, 0.2 μg of IL-2 is equivalent to 400 U of activity (i.e., 2 μg of IL-2 equals 4,000 U and 20 μg equals 40,000 U).

IL-2 injection

Concentrated stocks of IL-2 were stored in aliquots at −80°C and thawed before use. Stocks were diluted in PBS to the required concentration, and 100 μl was injected via a 26-gauge needle directly into tumors (i.t. experiments) or i.p. (i.p. experiments). This was performed three times per week.

Peptides

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

**OVA ELISA**

OVA levels were determined via ELISA. Microtiter plates (96-well) were coated overnight with 2 μg/ml anti-OVA Ab (Cappel-Organon Technica, Durham, NC) at 4°C. Plates were blocked with PBS plus 1% BSA (Sigma-Aldrich) for 1 h at 37°C, and washed thoroughly with PBS plus 0.05% Tween (Koch-Light Laboratories, Colnbrook Bucks, U.K.). Samples were serially diluted 1/2, and OVA (chicken OVA fraction V; Sigma-Aldrich) was used as a standard curve. The ELISA plate was incubated overnight at 37°C, followed by sequential incubations at 37°C for 1 h each, using rabbit anti-OVA (2 μg/ml; Harlan Sera-Lab, Loughborough, England), anti-rabbit HRP (Dako, Glostrup, Denmark), and the HRP substrate 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich), washing thoroughly between incubations with PBS plus 0.05% Tween. Quantification of OVA was determined by a Spectramax 250 spectrophotometer, using Softmax Pro, version 2.2.1, software (both Molecular Devices, Sunnyvale, CA).

**In vitro 51Cr release CTL assay**

Effector cells were prepared from the spleens of OT-I TCR mice and used to screen the OVA-transfected clones. These effector cells were expanded in vitro with a 1:1 ratio of whole splenic cells taken from naive C57BL/6J mice that were used as a source of normal APC after pulsing for 90 min with 1 μM SIINFEKL peptide. The APC were washed before addition to the effector cells and incubated at 37°C for 5 days in conditioned medium, in a humid environment. Target cells, which included our OVA-transfected AE17 tumor clones (AE17-oOVA), as well AE17 cells alone (negative control) and AE17 cells pulsed with 10 μM SIINFEKL (positive control), were labeled with 100 μCi of 51Cr for 90 min and washed four times before use. Effector cells were added to corresponding targets at various E/T cell ratios and incubated at 37°C for 4 h; 50 μl of each supernatant was harvested, and 51Cr release was determined (gamma counter; TopCount; Packard, Zurich, Switzerland). The mean of duplicate samples was calculated, and the percentage of specific 51Cr release was determined as follows: percentage of specific lysis = (experimental 51Cr release – control 51Cr release)/(maximum 51Cr release – control 51Cr release)) × 100. Experimental 51Cr release represented counts from target cells mixed with effector cells, control 51Cr release represented counts from targets incubated with medium alone (spontaneous release), and maximum 51Cr release represented 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 (6). Lymph node (LN) cells from TCR-transgenic OT-I mice were resuspended in 20 ml of RPMI 1640 at 10$^7$ cells/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 twice, followed by PBS twice, and 1 × 10$^7$ cells were injected i.t. into each recipient mouse. CFSE-labeled cells were recovered from secondary lymphoid organs 3 days post adoptive transfer and analyzed by FACS analysis.

**FACS analysis**

LN or spleen cells were stained for two-color FACS analysis using the PE-conjugated mAb anti-CD8 (clone 53-6.7; BD Pharmingen, San Diego, CA). Analysis was performed on a FACSscan (BD Biosciences, Mountain View, CA) using CellQuest software. To determine the percentage of CD8$^+$ OT-I cells proliferating, those cells no longer within the parental peak were divided by the total number of cells and multiplied by 100.

**In vivo analysis of CTL function (in vivo CTL)**

Target cells for in vivo evaluation of cytotoxic activity were prepared as described elsewhere (7). Briefly, C57BL/6J LN and spleen cell suspensions were RBC-lysed, and the cells were washed and divided into two populations. One population was pulsed with 5 μM 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). A total of 10^5 cells of each population was mixed in 200 μl of PBS and injected i.t. into each recipient mouse. Specific in vivo cytotoxicity was determined by collecting the draining LN (dLN), non-draining LN (non-dLN), and spleens from recipient mice 18 h postinjection, 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$^{hi}$/CFSE$^{lo}$) 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.

**Immunohistochemistry**

Frozen sections (10 μm) of OCT-embedded tumors were fixed in cold ethanol and washed, and endogenous peroxidase was blocked using 1% hydrogen peroxide. Endogenous biotin was blocked using Avidin blocking kit (Vector Laboratories). Primary Abs directed against murine CD4 and CD8 T cells (clones MT310 and MT25, respectively; BD Pharmingen), MHC class II molecules (TIB-120 from Prof. P. Holt (Institute of Child Health Research, Perth, Australia)), macrophages (F4/80), and CD11c (N418 expressed on DCs; both Abs supplied by Dr. A. McWilliam (University of Western Australia)), B cells (B220; clone RA3-6B2; BD Pharmingen), CD80 (B7.1; clone 1G10; BD Pharmingen), CD86 (B7.2; clone GL1; BD Pharmingen), CD43 (clone 1B11; BD Pharmingen), CD54 (ICAM-1; clone 3E2; BD Pharmingen), and CD31 (platelet endothelial cell adhesion molecule; clone 390; BD Pharmingen) were applied for 45 min at room temperature. This was followed by sequential incubations with secondary biotinylated Abs (rat anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) or mouse anti-hamster IgG (BD PharMingen), and streptavidin within HRP (DAKO), washing with PBS between steps. Staining was visualized by precipitating 3,3′-diaminobenzidine (Sigma-Aldrich) and counterstaining with hematoxylin. Sections were rinsed in ethanol, cleared in xylene, and mounted using Depex (Scot Scientific, Perth, Australia).

**Measurement of CD31 staining**

Photos were taken using a Zeiss Axiostkop 2 plus microscope with attached Zeiss AxiosCam digital camera (Carl Zeiss, Oberkochen, Germany). Axiosvision 3.1 software was used to measure the number and length of CD31 adhesion molecule; clone 390; BD Pharmingen) in vivo staining within tumors, thereby giving an indication of blood vessel supply from each experimental group. For each group, three to five tumors were analyzed. Photos were taken of three to four random fields per section, and two sections were examined per tumor.

**H&E staining**

Frozen sections were fixed in Carnoy’s fixative (10% acetate, 60% absolute alcohol, 30% chloroform) for 30 s, rinsed in 70% alcohol, washed in tap water before staining with Gill’s hematoxylin (BDH, Victoria, Australia) for 1 min, and blued in Scott’s water (7 g of NaHCO$_3$ and 40 g of MgSO$_4$ in 2 liters of water) for 30 s. To counterstain, slides were rinsed in 70% alcohol, alcohol and 95% alcohol for 30 s each, and placed in 1% alcoholic eosin (BDH) solution for 20 s. Slides were then rinsed in absolute ethyl alcohol (BDH), cleared in xylene (BDH), and mounted using Depex (Scot Scientific).

**Statistical analysis**

Statistical significance was calculated using GraphPad (San Diego, CA) PRISM. Student’s t test was used to determine differences between two populations. One-way ANOVA was used to determine differences between more than two populations.

**Results**

**AE17 is a murine mesothelioma cell line**

TEM was used to confirm that the parental cell line, AE17, is a murine MM. Samples were taken from in vitro cell culture and in...
vivo tumor growth for analysis. Ultrastructural features of mesothelial differentiation such as abundant long, thin microvilli, occasional tight cell junctions and intermediate filaments were observed in AE17 in vitro cell culture and AE17 tumor samples (64 mm²; day 18) collected (Fig. 1, A and B, respectively). AE17-sOVA was also similarly examined and verified as a murine mesothelioma by TEM (data not shown).

**When expressed as a tumor Ag, OVA loads MHC class I molecules with the dominant peptide (SIINFEKL) and exhibits unaltered growth kinetics in syngeneic mice**

The murine MM cell line AE17 was transfected with cDNA coding for OVA; thus, in AE17 tumor-bearing C57BL/6J mice, transfected OVA becomes a neo tumor Ag. Screening by an in vitro CTL assay, using effector T cells from OT-I mice that recognize SIINFEKL revealed a number of clones expressing OVA that had been loaded into MHC class I molecules at differing levels on the AE17 tumor cell surface. These clones were then screened by ELISA for the levels of OVA they secreted. One clone, referred to as AE17-sOVA, secreted 26 μg/ml OVA per 10⁵ cells in 24 h, and was effectively lysed by OT-I T cells (Fig. 1C), demonstrating efficient production and MHC loading of SIINFEKL onto class I molecules, as well as susceptibility to lysis by OVA-specific effector CTL.

Inoculation of various doses (1 × 10⁶ and 5 × 10⁵) of the parental AE17 tumor cell line and its OVA transfectant (AE17-sOVA) into normal, syngeneic mice revealed similar in vivo growth rates (Fig. 1, D and E) and tumor frequencies (>95% of each cell line grew in vivo; data not shown). Seven days after inoculation, palpable pins developed and were considered to be established tumors. Tumors developed rapidly, and the growth rates between the different concentrations were consistent. There was no significant difference in tumor incidence, or growth rates, between groups inoculated with 5 × 10⁵ or 1 × 10⁶ of either cell line.

The in vitro growth rates of the AE17 vs AE17-sOVA cell lines (determined by [³H]thymidine incorporation) were also similar

![FIGURE 1](http://www.jimmunol.org/). Establishing the tumor model: AE17-sOVA, a murine MM cell line, readily progresses in C57BL/6J mice and is lysed by specific CTL. A and B, TEM photos of AE17 in vitro cell culture (A) and AE17 tumor samples (64 mm²; day 18) (B) show features of mesothelial differentiation such as microvilli (mv) and tight junctions (arrows; see inset). Magnification, ×5000. C, Effector cells prepared from the LN and spleens of OT-I TCR mice were added to corresponding target cells (AE17, AE17 pulsed with SIINFEKL, and AE17-sOVA) at various E:T cell ratios, and the percentage of specific lysis was determined. AE17-sOVA tumor cells were lysed to similar levels to the positive control (AE17 pulsed with SIINFEKL); in contrast, the parental cell line AE17 was not killed by OT-I CTL (C). Results displayed are means of duplicate cultures. D–E, Tumor growth experiments in C57BL/6J mice showed that there was no statistical significant difference (p > 0.05) between the growth rates of AE17 and AE17-sOVA after s.c. injection with either 10⁶ (D) or 5 × 10⁵ (E) tumor cells. Data are represented from three experiments (15 mice per group) as mean ± SE.
Intratumoral IL-2 therapy prolongs survival and retards tumor growth

AE17 or AE17-sOVA cells were injected s.c. into C57BL/6J mice, and tumors were allowed to develop for 18–20 days. At this time point, mice were separated into two groups: those with smaller (13 ± 2 mm² for AE17; 10 ± 1 mm² for AE17-sOVA) or larger (32 ± 3 mm² for AE17; 29 ± 3 mm² for AE17-sOVA) tumors; and IL-2 treatment commenced. Mice given 0.2 μg of IL-2 per dose exhibited a lower survival rate (37.5% remaining alive 40 days after the commencement of therapy) relative to mice treated with the two higher doses. Interest-

ingly, mice treated with either 2 or 20 μg/dose IL-2 showed similar survival rates; 62.5 and 75.0% of mice were alive 40 days after commencing IL-2. If tumors were large at the start of IL-2 therapy, as indicated by continued tumor growth (Fig. 3D) and no survival advantage (B). The similar trends in survival and tumor growth rates in response to i.t. IL-2 therapy for both AE17 and AE17-sOVA suggest that transfecting AE17 with OVA did not significantly alter any immune responses to the tumor.

To confirm that this response relied on tumor size, and not on the duration of tumor burden, similar experiments were performed on small tumors at earlier time points (days 7 and 12) post-tumor cell inoculation. An identical dose-dependent response was seen (data not shown). Mice were inoculated with a higher dose (10⁶ cells) of AE17-sOVA cells, and treatment commenced on day 12 when tumors were small (9 ± 1 mm²). Twenty percent of mice in the 0.2 μg/dose, 50% in the 2 μg/dose, and 70% in the 20 μg/dose IL-2 treatment groups survived to day 40 (data not shown). Tumor

FIGURE 2. Intratumoral IL-2 inhibits tumor growth and enhances survival in small but not large AE17 tumors. AE17 cells (5 × 10⁶) were injected s.c. into C57BL/6J mice and allowed to develop. On day 20, mice were separated into those bearing small (13 ± 2 mm²) or large (32 ± 3 mm²) tumors. Treatment consisting of three i.t. doses per week of PBS, or 0.2, 2, or 20 μg of IL-2 per dose, was commenced (indicated by arrows), and was continued until the end of the experiment. A–D, The survival and tumor growth rates of mice bearing small (A and C, respectively) vs large AE17 (B and D, respectively) tumors are shown. Data are represented from three experiments (15 mice per group) as mean ± SE. IL-2-treated mice were compared with PBS-treated control mice. **p < 0.01. E, Shown are pooled survival data with the lines indicating the mean tumor size for each group; survival <40 days (31 mm²) was significantly different from survival >40 days (12 mm²) (p < 0.001).
growth was slower with all three concentrations relative to the PBS control mice (data not shown). A similar experiment was performed using 10⁶ AE17 cells, starting IL-2 treatment (20 μg/dose) on day 7 when the tumors were palpable (4 mm²). Comparable results were observed (data not shown).

IL-2 administered i.p. does not affect survival or tumor growth

The results described above show that IL-2 therapy could induce tumor regression if given i.t. We next wanted to determine whether IL-2 therapy could be effective if given systemically. Mice were inoculated with 5×10⁵ AE17 or AE17-sOVA cells, and when the tumors were small, treatment was commenced. Groups of 5–10 mice received treatment consisting of 0.2 μg/dose, 2 μg/dose, or 20 μg/dose IL-2 i.p. three times per week. In all treatment groups, for both cell lines, there was no effect on survival or tumor growth compared with PBS controls (Table I and data not shown). These results indicate that the route of administration is critical in determining the outcome of IL-2 therapy.

Long-term survivors are protected from tumor challenge

Responder mice with AE17 or AE17-sOVA tumors that had completely regressed were left for various periods of time ranging from 2 to 6 mo and were rechallenged with 5×10⁵ AE17 cells at the original site of tumor. Seven of 10 mice were completely protected from tumor regrowth. These data imply that either immune responses had been generated to tumor Ags other than SIINFEKL, or that the local tissue site was permanently altered in such a way that tumors could not become established.

Intratumoral IL-2 therapy mediates local destruction of tumor-associated blood vessels, necrosis, and infiltration of CD8⁺ T cells and CD86⁺ cells

Many different mechanisms have been reported to be involved in IL-2-mediated tumor rejection (11). IL-2 has been shown to inhibit (12, 13) or induce (14, 15) vascularity in different systems. IL-2 has also been reported to cause necrosis or induce an antitumor response that may involve lymphocytes (16, 17), neutrophils, macrophages (18, 19), NK cells (20, 21), and/or T cells (22, 23). Therefore, the next series of experiments were designed to determine which mechanism(s) mediated the IL-2 response seen in the data shown above.

C57BL/6J mice were inoculated with 10⁶ AE17-sOVA cells, and tumors were allowed to develop. At day 14, when tumors were 10 mm² (small) to 30 mm² (large), IL-2 treatment was commenced. Mice received three i.t. doses per week of either 20 μg of

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**Table I. Intratumoral but not i.p. IL-2 treatment induces tumor regression**

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>AE17-sOVA</th>
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<td>Intratumoral PBS</td>
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<td>0/20</td>
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<td>6/16</td>
<td>1/20</td>
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<tr>
<td>2 μg/dose</td>
<td>10/16</td>
<td>9/20</td>
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<td>Intraperitoneal PBS</td>
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</tbody>
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* Tumor cells (5 x 10⁵ or 10⁶) were injected s.c. into C57BL/6J mice and allowed to develop. When tumors were small, treatment was commenced, consisting of three doses per week of PBS, 0.2, 2, or 20 μg of IL-2 per dose (i.t. or i.p.). Shown are pooled survival data (survival >40 days after the start of treatment) from mice receiving either i.t. or i.p. IL-2 treatment.
IL-2 per dose or PBS. Eight to 10 days later, mice were sacrificed and organs were taken.

Necrosis was often observed macroscopically in IL-2-treated tumors. This was confirmed by H&E staining, which showed areas of necrosis in 3 of 10 tumors taken from mice with small tumors responding to IL-2 treatment (data not shown). In contrast, no necrosis was seen in PBS control mice, or mice with large tumors at the start of IL-2 therapy (data not shown).

Immunohistochemistry revealed increased numbers of CD8$^+$ and CD86$^+$ cell numbers in mice responding to IL-2 treatment. Mice were inoculated with $10^6$ AE17-sOVA tumor cells and i.t. IL-2 (20 μg/dose, three times per week), or PBS treatment was commenced on day 14 when tumor size reached 10 mm$^2$ (small) to 30 mm$^2$ (large). Eight to 10 days later, tumors were harvested from each experimental group for immunohistochemistry. Frozen sections (8 μm) were prepared and stained using mAbs directed against CD4, CD8, and CD86 (B7.2). Magnification, ×400. These experiments were performed twice (six mice per group total), and representative photos are shown. Sections from PBS-treated mice revealed similar infiltrates to mice with large tumors treated with IL-2 (data not shown). Similar results were seen in mice bearing AE17 tumors (data not shown).

Macroscopic examination showed a reduction in the tumor blood supply in mice bearing small tumors responding to IL-2, relative to controls (Fig. 5, A and D). No vascular reduction was seen in PBS-injected control mice and in mice with large tumors treated with IL-2 therapy (Fig. 5, B and C). Similarly, there was no reduction of blood vessels in normal non-tumor-bearing animals receiving s.c. IL-2 (data not shown). Microscopic examination using anti-CD31 staining also revealed decreased i.t. vasculature in small tumors responding to IL-2 (Fig. 5, E–H, and Table II). This suggests that destruction of tumor-associated blood vessels by IL-2...
was also dependent upon tumor size at the start of treatment. Interestingly, it. IL-2 treatment resulted in a significant reduction in the size but not the number of blood vessels in mice responding to therapy compared with controls (Table II).

Intratumoral IL-2 therapy does not alter the levels of tumor Ag presentation seen in vivo

The results described above suggest that tumor rejection may, at least partially, be mediated by a specific immune response involving CD8+ T cells in responder mice. However, tumors may evade immune-mediated destruction by disabling APCs (25, 26). Tumor Ag presentation by professional APCs is essential for the induction of a specific antitumor T cell response. For this to happen, APC must transport tumor Ags from the tumor into LN for presentation to T cells. Therefore, the next series of experiments assessed whether or not, and at what levels, tumor Ags are presented in vivo by small vs large tumors treated with IL-2. To do this, the AE17-sOVA cell line was used, in which transfected OVA becomes a tumor-specific Ag OVA to which immune responses can be followed.

Intravenously injected CFSE-labeled cells taken from TCR-transgenic (OT-I) mice with specificity for the class I-restricted epitope of OVA (SIINFEKL) were used to determine whether the tumor Ag (OVA) was presented in vivo within secondary lymphoid organs during tumor growth, with or without IL-2 therapy. If SIINFEKL is presented by APC to these T cells they will become activated and proliferate. This can be detected by FACS analysis and is seen as the sequential halving of CFSE fluorescent intensity on proliferating OT-I cells.

AE17-sOVA tumor-bearing mice were divided into two groups at day 14 of tumor growth, mice with larger tumors (25–30 mm²) and those with smaller tumors (9–20 mm²), and treated with either PBS or 20 μg of IL-2 per dose for 1 wk. Within this time frame, IL-2 therapy prevented tumor growth in mice with smaller tumors. In contrast, tumor progression continued in mice with larger tumors, as well as in mice treated with PBS.

In mice given the parental AE17 tumor cell line (i.e., the negative control), no OT-I proliferation was seen in any lymphoid organ examined (seen in a representative mouse in Fig. 6A). Mice inoculated with 200 μg of OVA/IFA demonstrated strong Ag presentation in both the dLN (Fig. 6B) and spleen (data not shown). Each peak represents a single cell division, with staining intensity decreasing with increasing divisions. In the OVA/IFA positive control mice, more cells are present in the latter divisions than in the parental peak (Fig. 6, B and F). Weak Ag presentation was observed in the dLNs of mice given AE17-sOVA with, or without, IL-2 (Fig. 6, C–E, shows representative mice; F shows pooled data). This was observed only in the dLNs and was not evident in non-dLN or in the spleen (data not shown). IL-2 treatment did not up-regulate Ag presentation. In fact, Ag presentation may be decreased in mice responding to therapy, because these mice had considerably smaller tumors than the PBS-treated control mice. Thus, any differences seen in Ag presentation may be a function of size and therefore tumor Ag levels and not of APC capabilities.

Intratumoral IL-2 therapy enhances effector CTL activity in vivo in small tumor-bearing but not in large tumor-bearing mice

The data shown in Fig. 6 clearly show that the tumor Ag is effectively presented to T cells in such a manner that tumor-specific CD8+ T cells (prepared from OT-I mice) are activated and proliferate. This implies that tumor Ags are constitutively presented in vivo to endogenous T cells during tumor growth. However, these

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**Table II. IL-2 leads to destruction of tumor-associated blood vessels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of CD31+ Cells per Field (×200)</th>
<th>Average Length of CD31+ Staining (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small tumor pretreatment</td>
<td>33.1 ± 2.3</td>
<td>33.2 ± 2.5</td>
</tr>
<tr>
<td>PBS treated</td>
<td>31.2 ± 1.5</td>
<td>31.5 ± 2.9</td>
</tr>
<tr>
<td>Large tumor + IL-2</td>
<td>34.6 ± 1.8</td>
<td>28.9 ± 3.5</td>
</tr>
<tr>
<td>Small tumor + IL-2</td>
<td>35.1 ± 3.5</td>
<td>17.3 ± 1.1*</td>
</tr>
</tbody>
</table>

*Sections stained for CD31 blood vessels were analysed for number and average length of CD31 staining as described in Materials and Methods. Data are from three to five tumors per treatment group. Results presented are for AE17-sOVA, and results for AE17 were similar (data not shown).

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**FIGURE 6. Intratumoral IL-2 does not up-regulate Ag presentation in the dLN. C–E, C57BL/6J mice were inoculated with 10⁶ AE17-sOVA tumor cells and, 14 days later, separated into those bearing small (9–20 mm²) or large tumors (25–30 mm²) and given three i.t. doses of PBS (C) or 20 μg of IL-2 (D and E) for 1 wk. Three days before analysis, 10⁷ CFSE-labeled OT-I T cells were adoptively transferred into recipient mice. The dLN were harvested from recipient mice, prepared as a single suspension, and stained for CD8. Flowcytometric analysis was performed by gating on CD8+ CFSE+ cells. A and B, C57BL/6J mice inoculated with AE17 tumor cells were used as negative controls (A), and mice inoculated with 200 μg of OVA/IFA 4 days earlier were used as positive controls (B). PBS-treated small and large tumors gave similar results. F, Shown are pooled data of the percentage of proliferating CD8+ OT-I cells from two experiments (six mice per group total), represented as mean ± SE. Values above columns are the average tumor size ± SE.**

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**NOTES:**

*Sections stained for CD31 blood vessels were analysed for number and average length of CD31 staining as described in Materials and Methods. Data are from three to five tumors per treatment group. Results presented are for AE17-sOVA, and results for AE17 were similar (data not shown).

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**REFERENCES:**

1. Tumor Ag presentation by professional APCs is essential for the induction of a specific antitumor T cell response. For this to happen, APC must transport tumor Ags from the tumor into LN for presentation to T cells. Therefore, the next series of experiments assessed whether or not, and at what levels, tumor Ags are presented in vivo by small vs large tumors treated with IL-2. To do this, the AE17-sOVA cell line was used, in which transfected OVA becomes a tumor-specific Ag OVA to which immune responses can be followed.

2. Intravenously injected CFSE-labeled cells taken from TCR-transgenic (OT-I) mice with specificity for the class I-restricted epitope of OVA (SIINFEKL) were used to determine whether the tumor Ag (OVA) was presented in vivo within secondary lymphoid organs during tumor growth, with or without IL-2 therapy. If SIINFEKL is presented by APC to these T cells they will become activated and proliferate. This can be detected by FACS analysis and is seen as the sequential halving of CFSE fluorescent intensity on proliferating OT-I cells.

3. AE17-sOVA tumor-bearing mice were divided into two groups at day 14 of tumor growth, mice with larger tumors (25–30 mm²) and those with smaller tumors (9–20 mm²), and treated with either PBS or 20 μg of IL-2 per dose for 1 wk. Within this time frame, IL-2 therapy prevented tumor growth in mice with smaller tumors. In contrast, tumor progression continued in mice with larger tumors, as well as in mice treated with PBS.

4. In mice given the parental AE17 tumor cell line (i.e., the negative control), no OT-I proliferation was seen in any lymphoid organ examined (seen in a representative mouse in Fig. 6A). Mice inoculated with 200 μg of OVA/IFA demonstrated strong Ag presentation in both the dLN (Fig. 6B) and spleen (data not shown). Each peak represents a single cell division, with staining intensity decreasing with increasing divisions. In the OVA/IFA positive control mice, more cells are present in the latter divisions than in the parental peak (Fig. 6, B and F). Weak Ag presentation was observed in the dLNs of mice given AE17-sOVA with, or without, IL-2 (Fig. 6, C–E, shows representative mice; F shows pooled data). This was observed only in the dLNs and was not evident in non-dLN or in the spleen (data not shown). IL-2 treatment did not up-regulate Ag presentation. In fact, Ag presentation may be decreased in mice responding to therapy, because these mice had considerably smaller tumors than the PBS-treated control mice. Thus, any differences seen in Ag presentation may be a function of size and therefore tumor Ag levels and not of APC capabilities.
data do not show whether or not OT-I T cells, or more importantly, endogenous T cells become effector CTLs in vivo. Therefore, to determine whether an endogenous tumor Ag (SIINFEKL)-specific CTL was induced in mice treated with IL-2, a recently described in vivo CTL assay was used (7). In this assay, CFSE-labeled target cells were i.v. injected into the following groups of tumor-bearing mice: 1) mice with large tumors (average size, $32 \pm 3$ mm², on day 18 of tumor growth when IL-2 treatment was commenced) vs 2) mice with smaller tumors (average size, $7 \pm 2$ mm², on day 12 of tumor growth when IL-2 treatment was commenced). Controls were also divided into mice with small and large tumors at days 12 and 18, respectively, of tumor growth.

Target cells were harvested from the LN and spleens of syngeneic naive C57BL/6J mice, and differentially labeled with CFSE. CFSEhigh-labeled cells represent peptide (SIINFEKL)-pulsed target cells, whereas CFSELow-labeled cells represent the reference (or control) targets. The presence of functional Ag-specific CTL results in the killing of SIINFEKL targets, leading to a reduction in the height of the SIINFEKL-pulsed target cell peak relative to that seen in control mice, and to the reference peak in experimental mice.

No SIINFEKL-specific lytic activity was seen in mice bearing the (nontransfected) AE17 parental tumor in the dLN (Fig. 7A shows a representative mouse) or in the non-dLN or spleen (data not shown). Mice bearing large and small AE17-sOVA tumors displayed some lytic activity in the dLN before treatment with IL-2, shown by a reduction of the SIINFEKL peak relative to the reference peak (Fig. 7, B and E). PBS-treated control mice with large or small tumors (representative mice shown in Fig. 7, C and F, and pooled data shown in H) also exhibited some specific CTL activity in the dLN. These data indicate that constitutive tumor Ag presentation results in the generation of effector CTLs, and that this occurs in the absence of IL-2 therapy. Mice with smaller tumors when treatment commenced (Fig. 7, G and H) exhibited an enhanced tumor-specific CTL response. This strong response was also evident in the spleen and non-dLN (Fig. 7H). This was not observed in mice with larger tumors when treatment was commenced (Fig. 7, D and H). Therefore, mice bearing small tumors treated with IL-2 generated a stronger CTL response relative to untreated mice carrying the same tumor burden and to mice with larger tumors when treatment commenced.

**T cells are important for IL-2-mediated regression**

The data shown in Fig. 7 suggest that CD8+ cells, particularly activated effector CTLs, may be required for IL-2-induced tumor regression. The next series of experiments was performed to determine whether T cells, particularly CD8+ cells, were essential for IL-2-induced activity against small tumors.

BALB/c nu/nu mice were inoculated with $5 \times 10^4$ AE17 tumor cells. When tumors reached a small size ($12 \pm 2$ mm²; day 12), mice were treated with either 20 $\mu$g of IL-2 or PBS i.t. three times per week. There was no significant delay in tumor growth in IL-2-treated mice compared with PBS control mice indicating that T cells were required for IL-2-dependent tumor regression (Fig. 8A). C57BL/6J mice were inoculated with $5 \times 10^5$ AE17 tumor cells and, before IL-2 treatment, were depleted of CD4+ or CD8+ cells, or both CD4+ and CD8+ (see *Materials and Methods*). Small tumors ($14 \pm 2$ mm²; day 13) were injected with either PBS or 20 $\mu$g of IL-2 three times per week and monitored for tumor growth. Mice depleted of CD4+ or CD8+ cells demonstrated only a marginal reduction in tumor growth; however, this was not significantly different from PBS-treated controls (Fig. 8, B and C).
Mice depleted of both CD4\(^+\) and CD8\(^+\) cells did not respond to IL-2 therapy, because tumors continued to grow at the same rate as control PBS-injected tumors (Fig. 8D). These results confirm that both CD4\(^+\) and CD8\(^+\) cells are critical for IL-2-induced antitumor immunity.

**Destruction of tumor-associated vasculature is dependent on CD8\(^+\) but not CD4\(^+\) cells**

Our experiments revealed two main mechanisms that mediated IL-2 activity against tumors: 1) destruction of tumor-associated blood vessels and 2) enhanced CD8\(^+\) effector CTL activity. Blood vessels leading to the tumor were not destroyed in nude mice or in CD8\(^+\)-depleted animals as they were in C57BL/6J mice bearing small tumors treated with IL-2. However, in a few CD4\(^+\)-depleted mice treated with IL-2, blood vessels were diminished compared with PBS-treated controls (Table III and data not shown). This suggests that, for blood vessels to be destroyed via IL-2, activated fully functional effector CD8\(^+\) cells, but not necessarily CD4\(^+\) cells, are critically required.

### Table III. CD8\(^+\) cells are required for tumor-associated blood vessel destruction in mice responding to IL-2 therapy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor-Bearing Animals</th>
<th>Inhibited Tumor Growth</th>
<th>Number of CD31(^+) Cells per Field ((\times)200)</th>
<th>Average Length of CD31(^+) Staining ((\mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>C57BL/6</td>
<td>0/10</td>
<td>30.1 (\pm) 1.4</td>
<td>29.2 (\pm) 2.8</td>
</tr>
<tr>
<td></td>
<td>BALB/c nu/nu mice</td>
<td>0/10</td>
<td>29.1 (\pm) 3.6</td>
<td>30.2 (\pm) 2.2</td>
</tr>
<tr>
<td></td>
<td>CD4-depleted C57BL/6</td>
<td>0/10</td>
<td>28.8 (\pm) 1.7</td>
<td>32.7 (\pm) 2.3</td>
</tr>
<tr>
<td></td>
<td>CD8-depleted C57BL/6</td>
<td>0/10</td>
<td>33.1 (\pm) 1.6</td>
<td>32.6 (\pm) 2.4</td>
</tr>
<tr>
<td>IL-2</td>
<td>C57BL/6</td>
<td>8/10(^a)</td>
<td>33.0 (\pm) 1.1</td>
<td>16.5 (\pm) 1.5*</td>
</tr>
<tr>
<td></td>
<td>BALB/c nu/nu mice</td>
<td>0/10</td>
<td>33.6 (\pm) 2.3</td>
<td>29.7 (\pm) 2.0</td>
</tr>
<tr>
<td></td>
<td>CD4-depleted C57BL/6</td>
<td>4/10(^a)</td>
<td>28.3 (\pm) 1.5</td>
<td>19.2 (\pm) 1.1*</td>
</tr>
<tr>
<td></td>
<td>CD8-depleted C57BL/6</td>
<td>3/10(^a)</td>
<td>35.6 (\pm) 1.4</td>
<td>27.6 (\pm) 1.2</td>
</tr>
</tbody>
</table>

\(^a\) Two weeks after treatment (PBS or IL-2), AE17 tumors were collected from BALB/c nu/nu mice, C57BL/6 normal mice, and mice depleted of CD4\(^+\) or CD8\(^+\) (shown in Fig. 8); tumor growth and tumor-associated blood vasculature were assessed. Note: all mice had small tumors when treatment began.

\(^b\) Mice that responded to IL-2 treatment; 7/10 C57BL/6J mice treated with IL-2 exhibited complete tumor regression, and 1 mouse showed a delay in tumor growth.

\(^c\) Partial response; 4/10 mice depleted of CD4\(^+\) cells and 3/10 mice depleted of CD8\(^+\) cells displayed delayed tumor growth.

Tumor-associated blood vasculature was determined in PBS-treated mice or mice responding to IL-2 therapy by CD31 staining within the tumor (as per Fig. 5 and Table II) and compared to PBS controls.

\(*\, p < 0.001\) comparing IL-2-treated to PBS-treated controls.
Discussion

Intratumoral but not i.p. administration of IL-2 induces tumor regression

We show in this study that serial i.t. injections of IL-2 can be highly effective in eradicating established tumors; however, systemic IL-2 (via i.p. injection) failed to induce tumor eradication. The most likely reason for this failure could be that there are insufficient levels of IL-2 at the tumor site with i.p. administration, and that this is overcome by direct i.t. injection. If a solid tumor is readily accessible, i.t. administration of antitumor immunotherapeutic agent(s) offers a straightforward procedure that specifically targets tumor-associated cell populations and reduces unwanted side effects.

Effect of tumor burden on development of an effective antitumor immune response

In this study, we addressed the importance of tumor burden and immune effector cells on the efficacy of i.t. IL-2 treatment in a unique murine model of MM, AE17. Local IL-2 therapy was effective when tumors were detectable (i.e., well established and growing), but the tumor burden was relatively small. The locally administered IL-2 enhanced a previously primed endogenous, tumor-specific CTL response concomitant with immune-related destruction of blood vessels. Tumor progression inevitably occurred in mice with large tumors when IL-2 treatment was commenced. Timing experiments confirmed that tumor burden, and not duration of disease, was the critical factor that determined the outcome of i.t. IL-2 therapy.

The failure seen in large tumors may be because tumor-specific CTL numbers cannot catch up with and control the large (and exponentially increasing) numbers of tumor cells as has been suggested (27, 28). Another mechanism may be that tumor-related mechanisms mediate partial tolerance, ensuring that their lytic function remains weak (29). It is also possible that the cytokine milieu in larger tumors may irreversibly reduce the survival of tumor-infiltrating lymphocytes (30).

Tumors, including MM, secrete a number of suppressor molecules including TGF-β (31, 32) and vascular endothelial growth factor (VEGF) (33, 34). By delaying treatment, the concentration of these molecules, both locally and systemically, may be increased, negating the effects IL-2 has on the tumor-specific CTL response. TGF-β inhibits T cell cytotoxic activity (35, 36), while abnormally high blood levels of VEGF has been associated with resistance to IL-2 immunotherapy (37, 38), and VEGF can inhibit DC function (39, 40). We can detect VEGF using immunohistochemistry throughout MM growth, suggesting increasing concentrations associated with increasing tumor burden (data not shown). However, there was no change in VEGF levels in mice responding to IL-2 treatment (data not shown). Although i.t. IL-2 treatment did not alter the levels of VEGF, escalating VEGF concentrations may explain why i.t. IL-2 reduced blood vessels in small, but not in large, tumors.

Intratumoral IL-2 does not exert obvious effects on APC function, but does enhance CTL activity

Cross-presentation is thought to be the predominant pathway for activation of tumor-specific CD8+ T cells (41, 42), because tumor cells generally lack the ability to directly activate immune responses. In this tumor model, low numbers of tumor-infiltrating DCs were seen in normal, PBS-treated, and IL-2-treated tumors (data not shown). Although there is evidence that tumor-traversing DC are dysfunctional in their APC capacity and/or in their migratory responses (25, 40), we have shown that tumor-associated APC can present tumor Ags to naive T cells in dLN (43, 44). These naive T cells may then differentiate into fully functional effector CTL (44). Similarly, we show in this study that MM-associated tumor Ags are presented in dLN, resulting in the generation of functional, effector CTL. However, in this case, the lytic levels were relatively weak, suggesting that while priming was occurring; either 1) low numbers of precursor CTL were generated or 2) some level of incomplete tolerance was operating. Importantly, this immune response can be enhanced via local IL-2 therapy to levels that mediate tumor regression.

In vivo experiments were undertaken to examine the effects of IL-2 treatment on tumor Ag (OVA) presentation within the secondary lymphoid system. Our data clearly show that the tumor Ag is presented within tumor-draining LN during tumor growth in the absence of any therapy. However, IL-2 did not up-regulate Ag presentation levels above those seen in untreated animals. It may be that the Ag presentation levels seen were a function of tumor size, because mice responding to IL-2 therapy had much smaller tumors and therefore may have lower levels of Ag. It is also possible that the time points we chose to examine were too late, and that tumor Ag presentation may well have been transiently enhanced within hours, or a few days, after the first IL-2 injection.

To determine whether a tumor-specific CTL could be generated in mice treated with IL-2, an in vivo CTL assay was performed. This assay indicates the physiological strength of CTL activity occurring within specific anatomical locations. Our data show that, during tumor progression, a low-level specific CTL response is induced that cannot mediate tumor regression. In mice bearing small tumors when treatment with IL-2 was commenced, strong CTL activity was demonstrated within all compartments of the secondary lymphoid system. A weaker tumor-specific CTL was observed in IL-2-treated mice with large tumors when treatment was commenced, which was not significantly different to the PBS-treated control mice. These results strongly suggest that i.t. IL-2 therapy can, under specific circumstances, enhance CTL numbers and/or their lytic capacity to levels that mediate tumor regression.

Multiple mechanisms are responsible for the antitumor response seen after i.t. treatment with IL-2

IL-2 has been shown to induce a variety of antitumor effects. IL-2 may induce necrosis, leading to an effective immune response with (16) or without lymphocytes (17). We observed increased necrosis in some of our IL-2-treated mice (data not shown). A few studies have shown that IL-2 can affect blood vessels by increasing (14, 15) or decreasing vasculature (12, 13). Similarly, IL-2 has been shown to trigger the vascular leakage system, which results in endothelial cell damage (45). In our study, mice responding to IL-2 therapy demonstrated decreased vascularity around and within the tumor microenvironment. Hence, increased cell death via necrosis and, more importantly, reduced tumor-associated vasculature appear to have been induced by direct, serial exposure to i.t. concentrations of IL-2. Interestingly, studies have shown that both CD4+ (46) and CD8+ cells (47, 48) can target and destroy endothelial cells directly. Also, work involving the IL-2-induced vascular leakage system showed that Fas ligand and perforin were required for endothelial cell damage (45). Our experiments clearly identified a role for CD8+ T cells as tumor-associated blood vessels were not destroyed in CD8−-depleted mice. These T cells may be autoimmune CTL cells (as has been suggested by others (48)) whose lytic activity was enhanced by IL-2 treatment in the same way as the tumor-specific CTL. However, blood vessels remained intact in normal, non-tumor-bearing mice that received s.c. IL-2 (data not shown), suggesting tumor-associated CD8+ T cells were required. Alternatively, activated CD8+ cells secrete molecules...
such as IFN-γ, which can result in blood vessel destruction (49). In mice responding to IL-2, we observed enhanced CTL activity, which could lead to increased IFN-γ production. Importantly, this study demonstrates a novel mechanism for IL-2 in antitumor immunity via immune-related destruction of tumor-associated vasculature.

IL-2 can affect many different cell types resulting in antitumor immunity, including nonspecific cells, such as NK cells (12, 50, 51), neutrophils, or macrophages (18, 19). IL-2 therapy failed to work in nude mice that lack T cells but still have functional NK cells, suggesting that NK cells on their own are not sufficient for IL-2 induced regression. Macrophage and neutrophil numbers were not altered in these studies (data not shown); however, IL-2 may activate these cells, reversing their phenotype and resulting in tumor destruction. Both neutrophils and macrophages are able to respond to IL-2 to induce tumoricidal activity (52, 53).

Our depletion studies clearly showed that T cells, both CD8+ and CD4+ T cells, were critical for IL-2-induced tumor regression. However, higher numbers of CD8+, and not CD4+, T cells were seen infiltrating tumors responding to IL-2 therapy. Interestingly, evenly dispersed CD25+ cells could be detected throughout tumors, and their numbers did not change in responder mice (data not shown). We are currently determining whether these cells represent regulatory CD4+ T cells. If so, although this population does not secrete IL-2, it may respond to IL-2, thereby losing its suppressive capacity (54, 55), i.e., another potential mechanism offered by i.t. IL-2 is bypassing the effects of local regulatory cells.

These studies demonstrated a direct response dose, with prolonged i.t. doses generating an improved survival outcome. In contrast, recent reports have shown that the optimal anticancer IL-2 regimen was a short time course (2 days) with low doses of IL-2 that generated a prolonged and expanded tumor-specific CTL response (56). When IL-2 treatment was more prolonged, the therapy was less effective due to elimination of tumor-specific CTL (56). In our system, ceasing IL-2 treatment reversed tumor regression, i.e., continued treatment was required for complete tumor regression. This suggests that, in our model, IL-2 does not induce the elimination of tumor-specific CTL. Furthermore, the addition of IL-2 may potentially override an activation-induced nonresponive state (57) of the endogenously generated tumor-specific CTL. The use of a different tumor model (i.p. EG7) in association with the adoptive transfer of tumor-specific CD8 T cells and route of IL-2 administration (i.p.) may explain the contrasting results. In particular, i.p. administration of IL-2 may lead to more widespread effects due to systemic leakage of IL-2.

In conclusion, we report that a simple approach using i.t. IL-2 can reliably induce tumor regression and provide long-term protection under specific conditions. Tumor size, and not duration, ultimately determines a successful outcome; small tumor burdens are associated with an effective response. Furthermore, reduced vascularity in association with infiltrating functionally efficient, effector CD8+ T cells is critical for a successful outcome. Importantly, CD8+ T cells were required for the reduction in tumor-associated vascularity, demonstrating a novel mechanism for IL-2-mediated antitumor immunity. These results give new insights into the antitumor mechanisms mediated by IL-2, which are useful if IL-2 is to be successfully used in the clinical setting. This work highlights reasons for the failures seen in numerous IL-2-based clinical trials and clearly demonstrates that IL-2 therapy can be highly effective if the timing (low tumor burden), dose, and route (i.t.) are correct.

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References


