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# Complete Regression of Established Spontaneous Mammary Carcinoma and the Therapeutic Prevention of Genetically Programmed Neoplastic Transition by IL-12/Pulse IL-2: Induction of Local T Cell Infiltration, Fas/Fas Ligand Gene Expression, and Mammary Epithelial Apoptosis<sup>1,2</sup>

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Using a novel transgenic mouse model of spontaneous mammary carcinoma, we show here that the IL-12/pulse IL-2 combination can induce rapid and complete regression of well-established autochthonous tumor in a setting where the host immune system has been conditioned by the full dynamic process of neoplastic progression and tumorigenesis. Further, this regimen inhibits neo-vascularization of established mammary tumors, and does so in conjunction with potent local induction of genes encoding the IFN- $\gamma$ - and TNF- $\alpha$ -inducible antiangiogenic chemokines IFN-inducible protein 10 and monokine induced by IFN- $\gamma$ . In contrast to untreated juvenile C3(1)TAg mice in which histologically normal mammary epithelium predictably undergoes progressive hyperplasia, atypical changes, and ultimately transition to overt carcinoma, the current studies also demonstrate a unique preventative therapeutic role for IL-12/pulse IL-2. In juvenile mice, early administration of IL-12/pulse IL-2 markedly limits the expected genetically programmed neoplastic transition within the mammary epithelium and does so in conjunction with enhancement of constitutive Fas and pronounced induction of local Fas ligand gene expression, T cell infiltration, and induction of apoptosis within the mammary epithelium. These events occur in the absence of a durable Ag-specific memory response. Thus, this novel model system demonstrates that the potent therapeutic activity of the IL-12/pulse IL-2 combination rapidly engages potent apoptotic and antiangiogenic mechanisms that remain active during the delivery of IL-12/pulse IL-2. The results also demonstrate that these mechanisms are active against established tumor as well as developing preneoplastic lesions. *The Journal of Immunology*, 2001, 166: 1156–1168.

Interleukin 12 is a key immunoregulatory cytokine that can potentially activate T lymphocyte and/or NK cell function and appears to play a central role in linking nonspecific immune surveillance mechanisms with the engagement and expansion of specific T cell-mediated immune responses (1–3). As a single

agent, IL-12 has demonstrated substantial antitumor activity in a wide range of murine transplantable tumor models (4–6), including various models of primary and/or metastatic disease. Several studies have shown that IL-12 and IL-2, another potent immunoregulatory cytokine with significant antitumor activity, may interact favorably in the enhancement of various measures of immune function. More specifically, IL-12 and IL-2 use parallel intracellular signaling pathways (7), reciprocally up-regulate the expression of their respective receptors (8, 9), and may additively or even synergistically enhance proliferation, cytokine production, and cytolytic function by T and/or NK cells (10–14) and the production of NO by murine peritoneal macrophages (15).

We have reported that repeated systemic administration of IL-12 in combination with intermittent, weekly doses of IL-2 (pulse IL-2) is not only well tolerated, but can induce rapid and complete regression of well-established transplantable primary and/or metastatic murine renal carcinoma in up to 100% of treated mice (16). Given its striking antitumor activity, the IL-12/pulse IL-2 regimen may provide a unique model for the elucidation of key mechanisms in successful cytokine-induced eradication of established tumor. To investigate the antitumor activity in a setting where the host, as occurs in human patients with malignancy, has been physiologically conditioned by the entire dynamic process of genetically programmed neoplastic progression and tumorigenesis, we have studied this combination in a transgenic mouse model of spontaneous mammary carcinoma. In this unique model, tissue-specific targeted expression of the early region of the SV40 large

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tumor Ag (TAg)<sup>4</sup> gene was achieved by introduction of a fusion gene consisting of TAg and the 5'-flanking region of the rat prostatic steroid binding protein gene (C3(l)) (17). As a consequence, male mice of this transgenic strain experience progressive prostatic hyperplasia leading to the development of overt carcinoma by ~7 mo of age. Female mice uniformly develop mammary hyperplasia with progressive atypia and ultimately transition to overt carcinoma with multifocal mammary tumors by ~6 mo of age. Such an autochthonous model may afford the advantage of more closely reflecting the physiologic interplay of tumor-promoting genetic lesions and conditioning of the host immune system during the processes of neoplastic progression and tumorigenesis, in contrast to the rapid exposure of normal mice to large numbers of tumor cells as occurs in transplantable models. The present studies were undertaken to investigate the therapeutic activity of IL-12/pulse IL-2 against established autochthonous murine mammary tumors, to assess the ability of this regimen to delay and/or prevent the neoplastic transition observed in this model, and to gain insight into antineoplastic mechanisms engaged by this combination.

## Materials and Methods

### Mice

Female FVB/N-transgenic mice bearing the C3(l)-TAg fusion transgene produced as previously described (17) were used in all experiments. They were maintained in a dedicated pathogen-free environment and used at various ages as specified below. Animal care was provided in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, 1985).

### Reagents

Recombinant murine IL-12 (sp. act.,  $7 \times 10^6$  U/mg) was provided by Hoffmann-La Roche (Nutley, NJ) and Genetics Institute (Cambridge, MA). Stock solutions prepared in Dulbecco's PBS were stored at  $-70^\circ\text{C}$  until use. For *in vivo* administration, the stock solutions were diluted as necessary with PBS containing 0.1% (v/v) sterile-filtered homologous mouse serum. Highly purified, recombinant human IL-2 (from *Escherichia coli*) was provided by Chiron (Emeryville, CA) (18, 19). After reconstitution with sterile water, IL-2 was diluted with HBSS containing 0.1% sterile-filtered homologous mouse serum for *in vivo* administration.

### Treatment regimens

Based on our previous experience using the Renca murine renal carcinoma model, we initially evaluated the influence of IL-12/pulse IL-2 on the growth of established mammary carcinoma. Mice were ear-tagged to facilitate monitoring of the kinetics of tumor growth at each site in each mouse. Using a group of 10 mice, we monitored for the initial emergence of tumor. When the estimated cumulative tumor volume in a given mouse reached 500 mm<sup>3</sup> (range, 512–1100 mm<sup>3</sup>), therapy was initiated with IL-12/pulse IL-2. Based on the timing of initiation of therapy, mice were treated in one of four cohorts. Cohort 1 included two mice that received IL-2 (300,000 IU/dose) administered *i.p.* twice daily on days 0, 8, 14, 21, 28, 35, and 42. IL-12 (0.5  $\mu\text{g}$ /dose) was given *i.p.* on days 0–4, 8–11, 14–18, 21–25, 28–32, 35–39, and 42–46. Cohort 2 included four mice that received IL-2 on days 0, 6, 13, 20, 27, and 34 and IL-12 on days 0–3, 6–10, 13–17, 20–24, 27–31, and 34–38. Cohort 3 included two mice that received IL-2 as described above on days 0, 7, 14, and 21 and IL-12 on days 0–4, 7–11, 14–18, and 21–25. Cohort 4 included two mice that received IL-2 on days 0, 6, 13, and 20 and IL-12 on days 0–3, 6–10, 13–17, and 20–24 of therapy. Mice were monitored daily for evidence of toxicity, tumor dimensions at each mammary site were measured one or two times per week, and the estimated cumulative tumor volume for each mouse was calculated as outlined below.

Serum IFN- $\gamma$  and TNF- $\alpha$  concentrations were measured in tumor-bearing mice that had been treated with IL-2 or vehicle alone *i.p.* on days 0 and 7 and IL-12 or vehicle alone on days 0–4 and 7–9. On day 10 mice were euthanized via CO<sub>2</sub> asphyxiation and/or cervical dislocation, and serum samples were obtained as outlined below.

To evaluate the influence of IL-12/pulse IL-2 administration on tumor neovascularization and local expression of the genes encoding various modulators of angiogenesis within established mammary carcinoma, mice bearing established tumors were assigned to treatment with IL-12/pulse IL-2 or vehicle alone. An attempt was made to match the distribution of ages and/or baseline tumor volumes in the respective groups. Mice received IL-2 or vehicle alone twice daily on days 1 and 7, and IL-12 or vehicle alone daily on days 1–4 and 7–9. On day 10, mice were euthanized, and tumors were resected cleanly, snap-frozen, and processed for analysis of gene expression as outlined below. Tumors from mice treated with IL-12/pulse IL-2 or vehicle alone also were resected, fixed in formalin, and assessed for vascularity after staining with hematoxylin and eosin as noted below. To investigate the impact of IL-12/pulse IL-2 administration on local expression of the genes encoding Fas and Fas ligand (FasL) within established mammary carcinoma, we randomly assigned mice bearing established tumors to receive treatment with either IL-12/pulse IL-2 or vehicle alone. Mice received IL-2 or vehicle alone daily on days 0–4, 7–11, and 14–16. On day 17 mice were euthanized, and all involved tumor sites were cleanly resected, snap-frozen, and processed as outlined below.

We also assessed the therapeutic activity of IL-12/pulse IL-2 in mice bearing advanced disease characterized by multiple tumors at baseline. In a study to compare the antitumor activities of IL-12/pulse IL-2 vs IL-12 or pulse IL-2 alone in the setting of advanced multifocal disease, five mice per group were assigned to therapy with IL-12 with or without IL-2 or vehicles alone. Mice received IL-2 or vehicle alone twice daily on days 1, 7, 14, and 21 and IL-12 or vehicle alone daily on days 1–4, 7–11, and 14–18. In a subsequent study using a cohort of 11 mice, mice were randomly assigned to receive therapy with IL-12/pulse IL-2 (six mice) or vehicle alone (five mice). Mice received IL-2 or vehicle alone twice daily on days 0, 7, 14, and 21 and IL-12 or vehicle alone daily on days 0–4, 7–11, 14–17, 19, and 21–25. The number of mammary sites with measurable tumor and the respective dimensions were monitored at baseline and throughout the course of therapy, and cumulative estimated tumor volumes were calculated as outlined below.

To assess the impact of IL-12/pulse IL-2 on the progressive hyperplasia, atypia, and emergence of overt carcinoma that occur in this model, we used early chronic administration of this regimen to juvenile C3(l)-TAg mice. A cohort of 11 age-matched 2-mo-old mice was randomly assigned to therapy with IL-12/pulse IL-2 or vehicle alone. Five mice were assigned to treatment with IL-12/pulse IL-2, and six were given vehicles alone. The mice received IL-2 or vehicle alone twice daily on days 0, 7, 14, 21, 28, 35, 42, and 49, and IL-12 or vehicle alone on days 0–4, 7–11, 14–18, 21–25, 28–32, 35–39, 42–46, and 49–51 and were then monitored for emergence of tumor. To further characterize the influence of IL-12/pulse IL-2 on the neoplastic progression observed in this model, three mice per group were euthanized on day 53 (the completion of therapy), and all mammary sites were resected. Individual sites were either snap-frozen for subsequent evaluation of local gene expression by RT-PCR or placed in 10% neutral buffered formalin for fixation and subsequent histology studies as outlined below. In a subsequent study to help clarify the cell populations undergoing apoptosis, cohorts of age-matched mice were treated with IL-2 or vehicle alone twice daily on days 0, 7, and 14 and with IL-12 or vehicle alone on days 0–4, 7–11, and 14–17. On day 18, mice were euthanized, and individual mammary sites were resected and placed in 10% neutral buffered formalin for fixation and assessment of apoptosis as noted below.

To investigate the impact of IL-12/pulse IL-2 administration on the ultrastructural histology of the mammary epithelium and the occurrence of apoptosis in these cells, a cohort of eight juvenile mice was randomly assigned to treatment with IL-12/pulse IL-2 or vehicle alone. Mice were treated with IL-2 or vehicle alone twice daily on days 0 and 7, and with IL-12 or vehicle alone on days 0–4 and 7–8. On day 9 mice were euthanized, and individual mammary sites were resected and prepared as outlined below for subsequent examination using electron microscopy.

### Serum cytokine measurements

Whole blood was collected via cardiac puncture from individual mice treated with IL-12/pulse IL-2 or vehicle alone as described above and placed into glass red-top tubes to facilitate clot formation. After clot formation, the tubes were centrifuged at 3000 rpm, and serum was removed and stored at  $-20^\circ\text{C}$  until further use. Serum IFN- $\gamma$  and TNF- $\alpha$  concentrations were determined according to the manufacturer's instructions using the Quantikine-M immunoassay kits for murine TNF- $\alpha$  and IFN- $\gamma$  (R&D Systems, Minneapolis, MN).

### Tissue processing histology studies

Tumors or mammary sites, as indicated, were resected after mice were euthanized using CO<sub>2</sub> asphyxiation and/or cervical dislocation. Specimens

<sup>4</sup> Abbreviations used in this paper: TAg, large tumor Ag; FasL, Fas ligand; IP-10, IFN-inducible protein 10; MIG, monokine induced by IFN- $\gamma$ ; VEGF-B, vascular endothelial growth factor B.



for routine histologic examination were fixed in formalin and embedded in paraffin. Sections were then cut at 5- $\mu$ m thickness and stained with hematoxylin and eosin. Where indicated, tumor vascularity was assessed by identifying hypervascular zones in tumor sections scanned at  $\times 100$  magnification. Microvessel count within hypervascular areas, or so-called hot zones, has been widely reported as an index of tumor vascularity. Using such approaches, increased tumor vascularity has been found to reflect the presence of more aggressive disease in a wide variety of human tumors. Hypervascular zones were defined in the present studies as focal aggregates of vessel lumina with  $>10$  visible vessel lumina per field at  $\times 100$  magnification. Intact vessel lumina were defined by characteristic morphology with identifiable lining endothelial cells and the presence of intact RBC and/or leukocytes within the vessel lumen. Branching vascular structures were counted as one lumen. Vessel densities in hypervascular zones were assessed at  $\times 100$  with confirmation of morphology and vessel counts at  $\times 200$  as appropriate.

For immunohistochemical evaluation of local T cell infiltration, 5- $\mu$ m-thick tissue sections of formalin-fixed paraffin-embedded mammary sites were warmed in a 600°C oven for 10 min, deparaffinized, and hydrated with deionized water. Sections were then digested with 0.5% protease VIII for 3 min at 37°C. Slides were rinsed in deionized water, and endogenous peroxidase activity was quenched by incubation at room temperature in 3% hydrogen peroxide for 10 min, followed by rinsing with 0.5% Tween 20/PBS. Nonspecific binding of reagents was blocked by incubation of sections for 20 min in a solution consisting of 1% BSA and 1.5% normal goat serum. Sections were then incubated with rabbit anti-human CD3 (Dako, Carpinteria, CA) primary Ab or rabbit anti-human chloramphenicol acetyltransferase as an irrelevant isotype control Ab (5'3', Boulder, CO). Sections were subsequently incubated for 30 min with a biotinylated goat anti-rabbit IgG secondary Ab (Vector, Burlingame, CA) and then with ABC Elite reagent (Vector) for 30 min. Diaminobenzidine (Sigma, St. Louis, MO) was then applied for 4 min as a substrate for the peroxidase reaction. Slides were counterstained with hematoxylin, dehydrated, and coverslipped with Permount for light microscopic evaluation. Apoptotic cells in mammary sites were detected using the in situ end-labeling technique (ApopTag, Oncor, Gaithersburg, MD) performed on formalin-fixed paraffin-embedded 5- $\mu$ m-thick tissue sections.

### Electron microscopy

Tissue preparation for electron microscopic ultrastructural studies was described previously (27). Individual mammary sites were initially fixed in 4% paraformaldehyde and 2% glutaraldehyde in PBS (Tousimis, Rockville, MD). Fixed tissues were rinsed thoroughly in sodium cacodylate buffer (0.1 M, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA), trimmed into 3- to 4-mm<sup>3</sup> pieces with a single-edged razor blade, and postfixed in 1% osmium (Stevens Metallurgical Corp., New York, NY) in the same buffer overnight at 4°C. Dehydration, infiltration, and embedding of the tissues were conducted in a series of graded ethanol (e.g., 25, 50, 75, 95, and 100%), 100% propylene oxide (Electron Microscopy Sciences), epoxy resin LX-110 (Ladd Research, Burlington, VT), and finally in the pure epoxy resin. The epoxy resin was cured at 60°C for 48 h. The cured blocks were then sectioned to ~50–60 nm with an ultramicrotome (Leica, Deerfield, IL) using a diamond knife (Diatome U.S., Fort Washington, PA), and mounted on a copper mesh grid. The thin sections were stained in uranyl acetate and lead citrate solution to enhance the contrast. Thin sections were stabilized by carbon evaporation in a vacuum evaporator (Denton, Cherry Hill, NJ). Tissue sections were examined and photographed with an H7000 electron microscope (Hitachi, Tokyo, Japan).

### RT-PCR

Specimens to be used for analysis of local gene expression were snap-frozen immediately after resection and stored at  $-70^{\circ}\text{C}$  until further use. For the analysis of gene expression in mammary sites and/or established tumors, total cellular RNA was isolated from snap-frozen tissue specimens by the TRIzol method. Reverse transcription was performed in a 50- $\mu$ l reaction mixture containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 250  $\mu$ M dATP, 250  $\mu$ M dCTP, 250  $\mu$ M dTTP, 250  $\mu$ M dGTP, RNase inhibitor (0.75 U/ $\mu$ l), Moloney murine leukemia virus reverse transcriptase (5 U/ $\mu$ l), 2.5  $\mu$ M oligo(dT) primer, and 10  $\mu$ g denatured RNA. The reverse transcription reaction mixture was incubated at 42°C for 60 min and at 95°C for 5 min, then stored at  $-20^{\circ}\text{C}$  until further use.

The PCR was performed in a 25- $\mu$ l reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dATP, 200  $\mu$ M dTTP, 200  $\mu$ M dCTP, 200  $\mu$ M dTTP, 0.6 U of Taq DNA polymerase, 0.5  $\mu$ M sense primer, 0.5  $\mu$ M antisense primer, and 2.5  $\mu$ l of the products of the reverse transcription reaction. Amplification was performed in a thermocycler

(Gene Amp PCR System 2400, Perkin-Elmer/Cetus) as follows: 94°C for 3 min (one cycle), 94°C for 30 s/56–57°C for 1 min/72°C for 45 s (optimized at between 21–32 cycles depending on the primer pair and tissue source used), and 72°C for 5 min (one cycle). PCR products were then separated on 1% agarose gels impregnated with 0.5  $\mu$ g/ml ethidium bromide or stored at  $-20^{\circ}\text{C}$  until further use.

The PCR primer sequences and predicted product sizes for the genes evaluated in the present studies were as follows: G3PDH: sense, GCCAC CCAGAAGACTGTGGATGGC; antisense, CATGTAGGCCATGAGGTCCACCAC (product = 446 bp); Fas: sense, CTTCTACTGCGATTCTCTGGCT; antisense, TCTCTCTCTTCATGGCTGGAAC (product = 635 bp); FasL: sense, CAGCTCTTCCACCTGCAGAAGG; antisense, AGATTC CTCAAATTTGATCAGAGAGAG (product = 510 bp); IFN-inducible protein 10 (IP-10; CRG-2): sense, ACCATGAACCAAGTGCTGCCGTC; antisense, GCTTCACTCCAGTTAAGGAGCCCT (product = 312 bp); monokine induced by IFN- $\gamma$  (MIG): sense, GATCAAACCTGCCTAGATCC; antisense, GGCTGTGTAGAACACAGAGT (product = 399 bp); vascular endothelial growth factor-B (VEGFB): sense, AGTGGTGCCATGGATAGACG; antisense, TGGCTTCACAGCACTCTCTCT (product = 304 bp).

### Statistical analysis calculations

Tumor number data were analyzed untransformed using parametric methods. The mean number of tumors per mouse in control vs IL-12/pulse IL-2-treated groups were compared in pairwise fashion at day 0 (baseline) and day 27 (end of therapy) time points using Student's *t* test. The rate of change in tumor number in the respective groups was compared by calculating the result (number of tumors at day 27 – number of tumors at day 0) for each mouse. Mean values for the respective control and IL-12/pulse IL-2-treated groups were then calculated and compared using Student's *t* test.

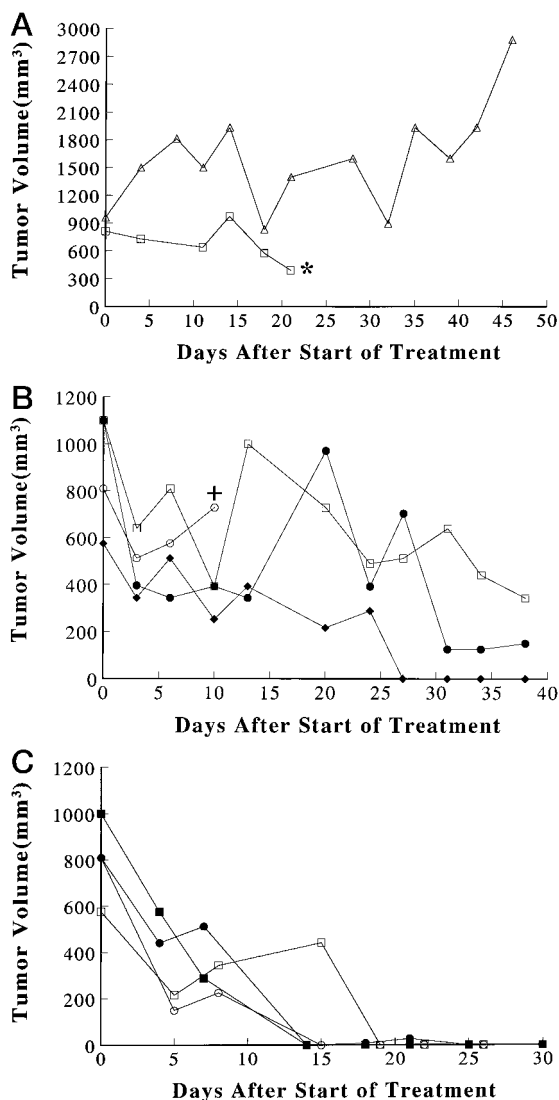
Tumor volumes were estimated by measuring the smallest and largest dimensions of each tumor and calculating the product of the square of the smallest dimension multiplied by the largest dimension. Cumulative tumor volumes for each mouse were determined by summing the estimated values calculated for each site of tumor involvement. Tumor volumes were transformed to square roots or cube roots before analysis to achieve variance stability and were analyzed using parametric methods. The mean estimated cumulative tumor volume in control vs IL-12/pulse IL-2-treated groups was compared in pairwise fashion at the indicated time points using Student's *t* test. Where indicated, the rate of change in tumor volume in the respective groups was compared by calculating the result (cumulative tumor volume at day 27 – cumulative tumor volume at day 0) for each mouse. Mean values for the respective control and IL-12/pulse IL-2-treated groups were then calculated and compared using Student's *t* test. The presence of tumor hypervascular zones in the respective treatment groups were compared using Fisher's exact test.

Comparisons of tumor number and volume data using the nonparametric Jonckheere test for trend and pairwise Wilcoxon rank-sum tests yielded generally the same conclusions. All *p* values were obtained from two-tailed tests of statistical significance and were considered significant when *p* < 0.05.

## Results

### Treatment of established tumor

Several previous studies in transplantable tumor models have documented the therapeutic advantages of IL-12 plus IL-2 over IL-2 or IL-12 alone (15, 16). To assess the antitumor effects of the IL-12/pulse IL-2 regimen in a setting where the host has been subjected to the full spectrum of physiologic pressures associated with genetically programmed neoplastic progression and tumorigenesis, we investigated its activity in the C3(l)Tag-transgenic mouse model of spontaneous mammary carcinoma. Using a cohort of 10 mice, all presenting with single established mammary tumors, complete responses were induced in 5 of 10 (50%) treated mice (Fig. 1, A–C). An additional 2 of 10 (20%) treated mice experienced a partial response (defined here as less than complete response, but  $>50\%$  reduction in estimated tumor volume), while 1 of 10 (10%) mice had progressive disease (defined here as  $>25\%$  increase in established tumor volume) despite therapy. One of 10 (10%) mice died due to apparent mechanical trauma from i.p. drug administration after having undergone a partial response 21 days into therapy, and 1 of 10 (10%) mice died for unknown reasons



**FIGURE 1.** Impact of administration of IL-12/pulse IL-2 on growth of established autochthonous mammary carcinoma in C3(l)TAg mice. A cohort of 10 mice was observed for the development of tumor. Therapy with IL-12/pulse IL-2 was initiated after tumor growth reached an estimated cumulative volume of 500 mm<sup>3</sup> or greater. Mice were treated in four distinct cohorts as outlined in *Materials and Methods*. Each line represents the estimated cumulative tumor volume over the course of therapy for an individual mouse. Cohort 1 is shown in A, cohort 2 in B, and cohorts 3 and 4 in C. Administration of IL-12/pulse IL-2 induced complete tumor regression in 50% of treated mice. The death of a mouse from traumatic injection is indicated by an asterisk, and death from apparent drug-related toxicity is indicated by a plus sign.

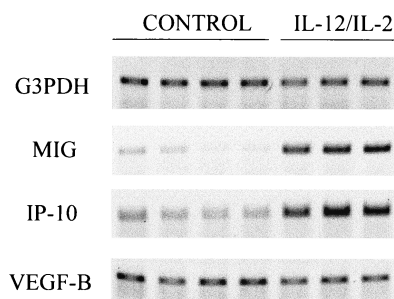
(presumed to be drug-related toxicity) with stable disease (defined as <50% reduction and no greater than 25% increase in tumor volume) 11 days into therapy. We also found here that IL-12/pulse IL-2 induced very rapid tumor regression, with three of five mice (60%) that would ultimately undergo a complete response having done so after only two cycles of therapy, and four of five (80%) mice undergoing a complete response after three cycles of therapy. Thus, overall, 7 of 10 mice (70%) bearing established tumors underwent complete or partial responses, which is in stark contrast to the inevitable tumor development progressive growth and death that occur in 100% of untreated C3(l)TAg mice (17). Follow-up of treated mice demonstrated recurrence of tumors beginning several weeks after cessation of therapy. Interestingly, new tumors gener-

ally did not recur at sites of complete response, but, rather, occurred at different mammary sites. These results show that although potent antitumor mechanisms were engaged by IL-12/pulse IL-2, an adaptive memory immune response capable of overcoming the chronic oncogenic stimulus provided by the TAg was not achieved.

*Administration of IL-12/IL-2 induces production of IFN- $\gamma$  plus TNF- $\alpha$ , up-regulates IFN- $\gamma$ -inducible antiangiogenic chemokines, and inhibits neovascularization*

To investigate mechanisms accounting for the rapidly induced antitumor activity of IL-12/pulse IL-2, we have evaluated its immunomodulatory and antiangiogenic activity in C3(l)-TAg mice bearing established tumors. Administration of IL-12/pulse IL-2 induced substantial increases in circulating serum concentrations of both IFN- $\gamma$  and TNF- $\alpha$  compared with control mice treated with vehicle alone. Serum IFN- $\gamma$  levels were greater than 1200 pg/ml in mice treated with IL-12/pulse IL-2 vs 4 pg/ml in control mice treated with vehicle alone (not shown). Serum TNF- $\alpha$  levels were 116 pg/ml in mice treated with IL-12/pulse IL-2 compared with <1 pg/ml in control mice treated with vehicle alone (not shown).

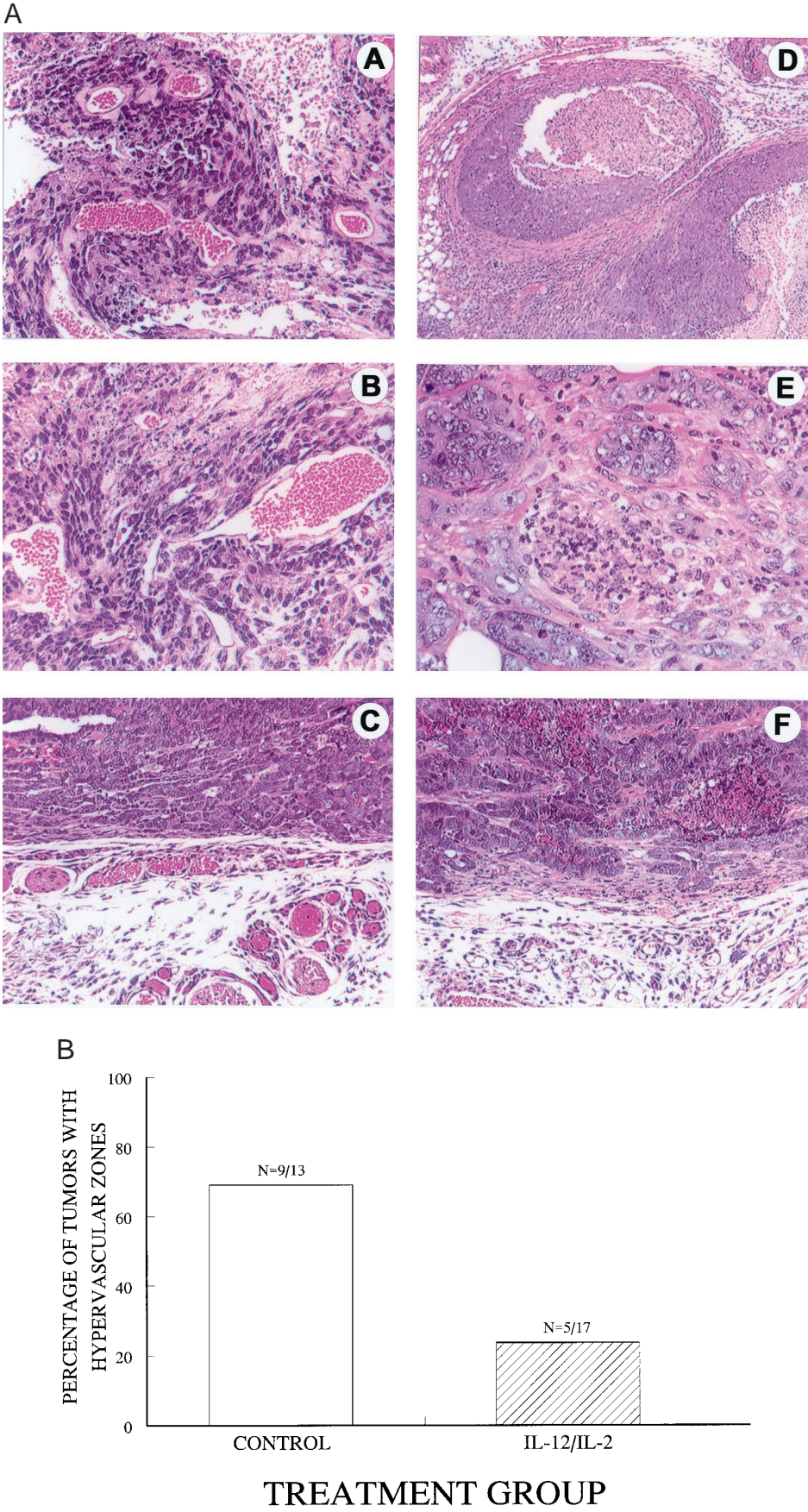
Because several IFN- $\gamma$ -inducible genes have been previously associated with anti-neovascular effects, we investigated the expression of several of these genes. As assessed by RT-PCR, the IL-12/pulse IL-2 regimen potentially up-regulated expression of genes encoding the IFN- $\gamma$  and TNF- $\alpha$ -inducible, antiangiogenic chemokines IP-10 and MIG within the local tumor site, while expression of the gene encoding the proangiogenic mediator, VEGF-B, was unaltered by treatment of mice with IL-12/pulse IL-2 (Fig. 2). Further, hematoxylin- and eosin-stained sections of established tumors from mice treated with IL-12/pulse IL-2 or vehicles alone revealed substantial differences in vascularity (Fig. 3A). Although tumors from control mice were highly vascularized, those from mice treated with IL-12/pulse IL-2 were poorly vascularized and revealed large areas of focal apoptosis/necrosis. The number of tumors with associated hypervascular zones was significantly less in mice treated with IL-12/pulse IL-2 vs control mice treated with vehicle alone ( $p = 0.0247$ ; Fig. 3B). Nine of 13 (69%) tumors from control mice had hypervascular zones compared with only 4 of 17 (23.5%) tumors from mice treated with IL-12/pulse IL-2.



**FIGURE 2.** Local expression of genes encoding modulators of angiogenesis within established mammary tumors. Mice were treated with IL-12/pulse IL-2 or vehicles alone as outlined in *Materials and Methods*, tumors were resected, and RNA was isolated from individual tumors using the TRIzol method. Expression of the genes encoding IP-10, MIG, and VEGF-B within established tumors was assessed via RT-PCR. Each lane represents analysis of material derived from an individual tumor site. Administration of IL-12/pulse IL-2 enhanced expression of the genes encoding IP-10 and MIG, while VEGF-B gene expression was unaltered by IL-12/pulse IL-2.

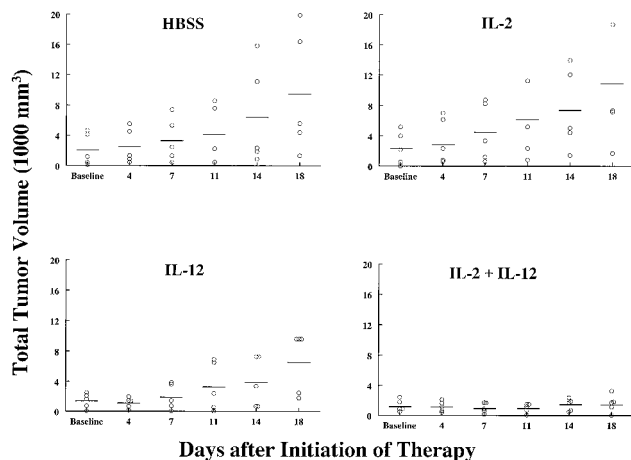


**FIGURE 3.** Antineovascular effects of IL-12/pulse IL-2 treatment of established mammary tumors in C3(l) TAg mice. Mice bearing established mammary cancers were used to evaluate the impact of IL-12/pulse IL-2 on tumor neovascularization. A, A–C are hematoxylin- and eosin-stained sections from vehicle-treated control mice as follows: A ( $\times 300$ ) and B ( $\times 300$ ) show areas of tumor with large numbers of vessel lumina, while C ( $\times 300$ ) shows cross-sections of tumor neovasculation coursing over the outside edges of the tumor. D ( $\times 150$ ), E ( $\times 750$ ), and F ( $\times 300$ ) were obtained from mice treated with IL-12/pulse IL-2 (A). These sections reveal limited vascularization either within or coursing over the surface of the tumors and several areas of evolving coagulative necrosis. The number of tumor with associated hypervascular zones was significantly less in mice treated with IL-12 compared with control mice treated with vehicle alone (B). Nine of 13 tumors (69%) from control mice had hypervascular zones, whereas only 5–17 tumors (23.5%) from mice treated with IL-12/pulse IL-2 had such zones.

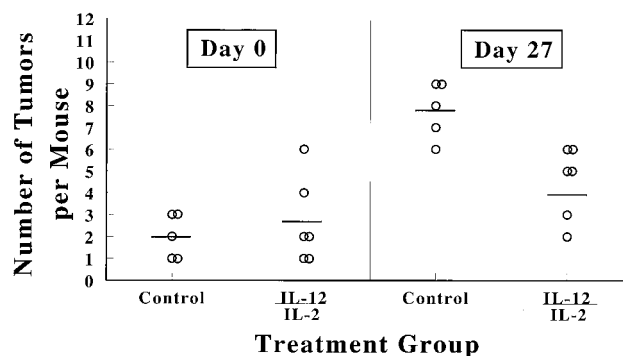


### Treatment of advanced established tumor

The ability of IL-12/pulse IL-2 to treat advanced disease as defined by the existence of multiple mammary tumors at the time of initiation of therapy was tested in two separate experiments. In the first experiment we compared the antitumor abilities of IL-2 and IL-12 as single agents with the combination of IL-12 plus IL-2 in a setting of very advanced disease (Fig. 4). In this study mice were randomized to therapy at about 20 wk of age when they were all presenting with multiple tumors and had a mean total tumor burden of  $>1200 \text{ mm}^3$ . These results showed that the combination of IL-12/pulse IL-2 blocked the progression of tumors as determined by total tumor burden compared with groups treated with IL-2, IL-12, or vehicle, such that by day 18 of therapy the mean total tumor volume in all these groups was significantly greater than that in mice treated with IL-12/pulse IL-2 ( $p < 0.05$ ). In this transgenic model of spontaneous tumor, these results confirm previous observations in transplantable tumor models (15, 16), which demonstrate enhanced therapeutic efficacy by IL-12/pulse IL-2 vs IL-2 or IL-12 alone. Based on these results we performed a second experiment with the goal of confirming the antitumor effects of IL-12/pulse IL-2 in the setting of advanced, multifocal disease (Fig. 5) and to investigate the impact of therapy on neoplastic progression and the development of new tumors. In this study although IL-12/pulse IL-2 was unable to induce complete tumor regression, as expected it did effectively inhibit the growth of tumors compared with control mice treated with vehicle alone (not shown). At baseline, the mean cumulative tumor volume was  $192 \text{ mm}^3$  for mice assigned to the control group and  $393 \text{ mm}^3$  for mice assigned to treatment with IL-12/pulse IL-2 ( $p = 0.32$ , not significant). At the end of therapy (day 27), the mean cumulative tumor volume in control mice was  $4,658 \text{ mm}^3$  (a 24-fold increase from baseline) compared with  $1659 \text{ mm}^3$  (a 4-fold increase from baseline) in mice treated with IL-12/pulse IL-2. The rate of increase in tumor volume was significantly slower in mice treated with IL-12/pulse IL-2 than in control mice treated with vehicle alone ( $p = 0.0178$ ), and the mean cumulative tumor volume on day 27 was significantly smaller in mice treated with IL-12/pulse IL-2 than in control mice ( $p = 0.034$ ). Of most interest in this study, administration of IL-12/pulse IL-2 also suppressed the emergence of new sites of tumor



**FIGURE 4.** Impact of IL-12/pulse IL-2 administration on total tumor burden in C3(1)TAG mice. Twenty mice were randomly assigned to treatment with IL-12/pulse IL-2, IL-12 or IL-2 alone, or HBSS vehicle as outlined in *Materials and Methods*. Therapy was initiated at a time when the mean total multifocal tumor volume was  $>1200 \text{ mm}^3$ . Administration of IL-12/pulse IL-2 delayed tumor growth more effectively than either IL-12 or IL-2 alone in mice bearing advanced multifocal mammary carcinoma.



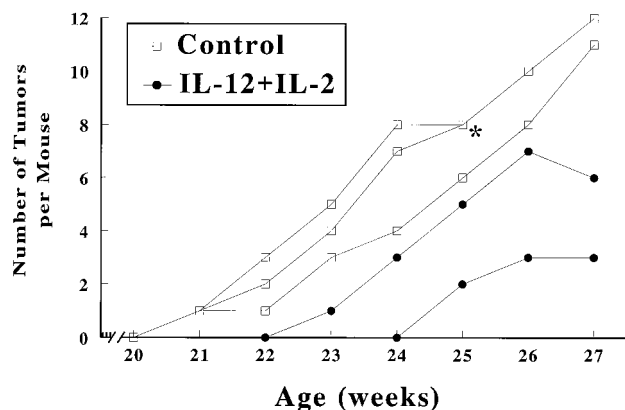
**FIGURE 5.** Impact of IL-12/pulse IL-2 administration on emergence of new tumors in C3(1)TAG mice. Eleven mice were randomly assigned to treatment with IL-12/pulse IL-2 (five mice) or vehicles alone (six mice) as outlined in *Materials and Methods*. The number of mammary sites with palpable tumor are indicated by open circles for each mouse, and mean values for the respective groups are represented by solid bars. Values on day 0 represent the pretreatment baseline, and day 27 values were obtained at the completion of therapy with IL-12/pulse IL-2. Administration of IL-12/pulse IL-2 inhibited the emergence of new sites of tumor involvement compared with those observed in control mice treated with vehicle alone.

involvement, in contrast to the progressive multifocal mammary carcinoma that developed in control mice (Fig. 5). At baseline, control mice had a mean of 2.0 tumors/mouse, compared with a mean of 2.7 tumors/mouse in mice treated with IL-12/IL-2 ( $p = 0.51$ , NS). On day 27, the mean number of tumors per mouse was 7.8 in control mice (a 3.9-fold (390%) increase from baseline) compared with 4.5 tumors/mouse in mice treated with IL-12/pulse IL-2 (an 1.7-fold (70%) increase from baseline). The rate of emergence of new tumors was significantly slower in mice treated with IL-12/pulse IL-2 than in control mice treated with vehicle alone ( $p = 0.00077$ ), and the mean number of tumors per mouse was significantly less in mice treated with IL-12/pulse IL-2 than in control mice on day 27 ( $p = 0.0055$ ). Although the administration of IL-12/pulse IL-2 effectively limited tumor progression over a period of 4 wk, tumor growth does accelerate within several weeks of cessation of therapy. This result suggests that the operative tumor mechanisms induced by IL-12/pulse IL-2 do not result in effective immunologic memory.

### Delay of neoplastic progression in juvenile mice

The ability of IL-12/pulse IL-2 to prevent the emergence of new tumor in this model suggested that this regimen might delay or prevent the genetically programmed neoplastic transition and progressive hyperplasia, atypia, and overt multi focal carcinoma that develop in the mammary epithelium of female C3(1)-TAG mice (17), a process associated with loss of p53 function, dysregulation of endogenous apoptosis, and disruption of normal epithelial cell turnover (20). Specifically, TAG expression becomes evident in the mammary gland between 14 and 21 days of age (20, 67). Early administration of IL-12/pulse IL-2 to juvenile C3(1)-TAG mice, initiated before the expected development of severe hyperplasia, atypia, and/or carcinoma, delayed the initial emergence of tumor by up to 4 wk compared with that in age-matched control mice treated with vehicle alone (Fig. 6). Further, hematoxylin- and eosin-stained sections of mammary sites obtained from mice after the completion of therapy with IL-12/pulse IL-2 revealed marked attenuation of the expected hyperplasia, atypia, and overt carcinoma noted in sections from mammary sites of age-matched control mice treated with vehicle alone (Fig. 7). Further, the mammary stroma of mice treated with IL-12/pulse IL-2 was extensively infiltrated with mononuclear cells (Fig. 7, C and D), in contrast to the



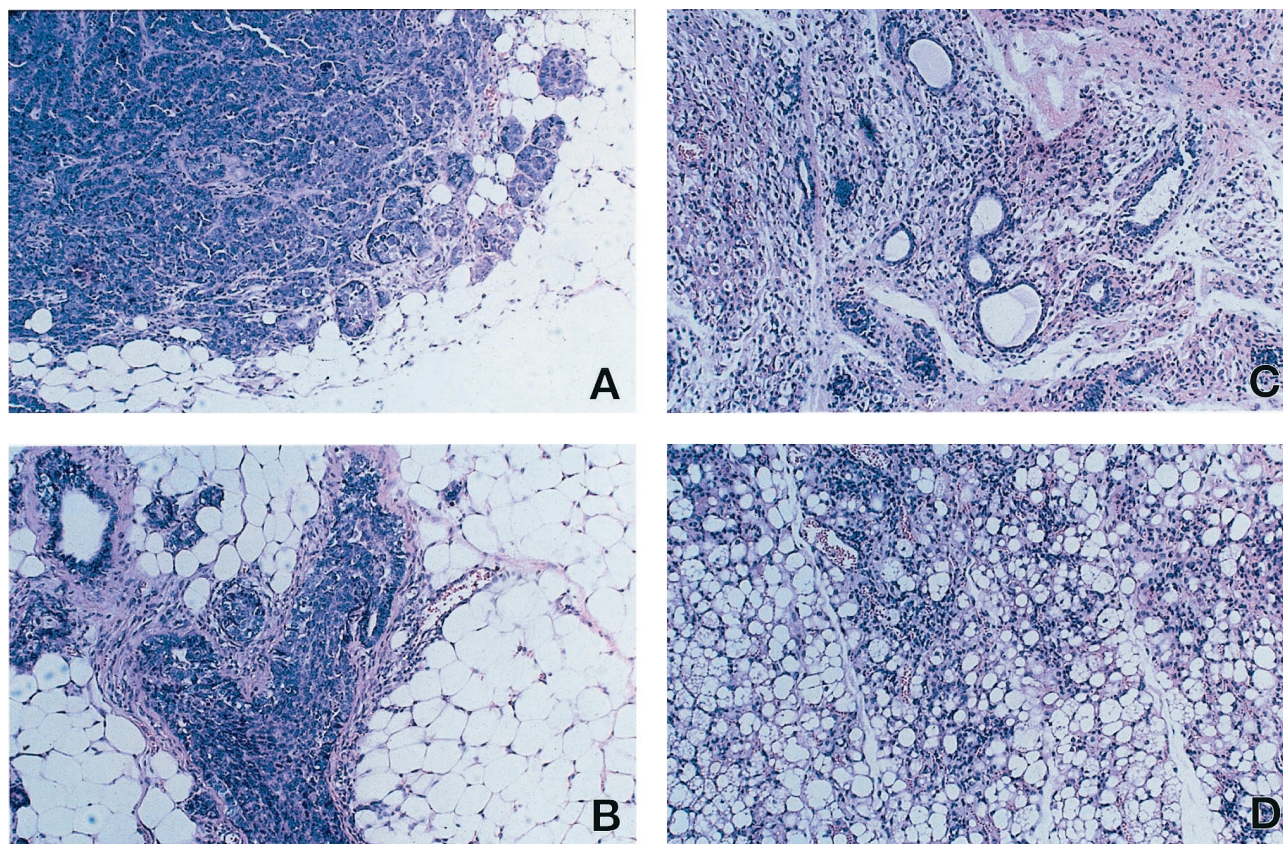


**FIGURE 6.** Impact of IL-12/pulse IL-2 on neoplastic progression in juvenile C3(l)TAg mice. A cohort of 11 age-matched 2-mo-old C3(l)TAg mice was randomly assigned to treatment with IL-12/pulse IL-2 (six mice) or vehicle alone (five mice) as outlined in *Materials and Methods*. At the completion of therapy, three randomly selected mice per group were euthanized for use in histology studies and analysis of gene expression (see also Figs. 7–9). The timing of emergence of tumor(s) at the respective mammary sites was monitored in the remaining mice. Each line represents the number of mammary sites with tumor in individual mice over time. Administration of IL-12/pulse IL-2 delayed the initial emergence of tumor by 3–4 wk compared with that observed in control mice treated with vehicle alone.

characteristic fatty stroma with infrequent leukocytes observed in sections from control mice treated with vehicle alone (Fig. 7, A and B). Administration of IL-12/pulse IL-2 also enhanced the constitutive expression of Fas and potentially induced detectable FasL gene expression in the mammary sites of treated mice compared with that observed in control mice treated with vehicle alone (Fig. 8), a finding later also confirmed by RNase protection assay (data not shown). Of note, similar enhancement of Fas and FasL gene expression has been observed within established tumors of mice that have been treated with IL-12/pulse IL-2 vs vehicle alone (not shown).

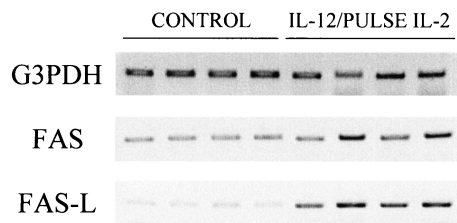
#### Local T lymphocyte infiltration and induction of apoptosis

Immunohistochemical staining of mammary sites resected from control transgenic mice revealed a paucity of CD3<sup>+</sup> T cells with no clear preference for localization to ductal epithelium (Fig. 9, A–C), while in mice treated with IL-12/pulse IL-2 there were large numbers of infiltrating CD3-positive T lymphocytes compared with those in control mice, with a substantial portion of the infiltrating T lymphocyte population localized at the basement membrane of the mammary epithelium and/or infiltrating the epithelium itself (Fig. 9, D–F). Large numbers of T lymphocytes also were observed throughout the stroma of mice treated with IL-12/pulse IL-2. Further, using a peroxidase-based end-labeling in situ detection technique, tissue sections of mammary sites resected from mice immediately after the completion of chronic therapy with



**FIGURE 7.** Histologic evaluation of impact of IL-12/pulse IL-2 administration on neoplastic progression in juvenile C3(l)TAg mice. A cohort of 11 age-matched 2-mo-old C3(l)TAg mice was randomly assigned to treatment with IL-12/pulse IL-2 (five mice) or vehicle alone (six mice) as outlined in *Materials and Methods*. As noted in Fig. 6, three randomly selected mice per group were euthanized at the completion of therapy, and mammary sites were resected for use in histology studies or analysis of local gene expression (see also Figs. 8 and 9). Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, and representative sections were photographed at  $\times 100$  magnification using an Olympus BHTU microscope fitted with an Olympus PM-10A DSP 35-mm camera. A and B, Sections from control mice; C and D, sections from mice treated with IL-12/pulse IL-2. Administration of IL-12/pulse IL-2 attenuated the progressive hyperplasia, atypia, and overt carcinoma that developed in control mice treated with vehicle alone (see A–C) and induced a marked increase in the cellularity of the mammary stroma (see B vs D).





**FIGURE 8.** Impact of IL-12/pulse IL-2 administration on local expression of Fas and FasL genes in the mammary sites of C3(l)TAg mice. A cohort of 11 age-matched 2-mo-old C3(l)TAg mice was randomly assigned to treatment with IL-12/pulse IL-2 or vehicle alone as outlined in *Materials and Methods*. As noted in Fig. 6, three randomly selected mice per group were euthanized at the completion of therapy, and mammary sites were resected for use in histology studies or analysis of local gene expression (see also Figs. 7 and 9). Fas and FasL gene expression was assessed using RT-PCR, as outlined in *Materials and Methods*. Each lane represents analysis of material derived from individual mammary sites. Administration of IL-12/pulse IL-2 enhanced expression of the genes encoding Fas, and in particular FasL, within the local mammary site compared with that observed in control mice treated with vehicle alone.

IL-12/pulse IL-2 revealed marked increases in the proportion of apoptotic cells compared with sites resected from control mice treated with vehicle alone (not shown). To further delineate the cell populations undergoing apoptosis within the local mammary site, sections were obtained from juvenile mice treated with a shorter course of IL-12/pulse IL-2 (i.e., 3 wk). These sections demonstrate that there is a substantial increase in cells undergoing apoptosis within the mammary epithelium itself in mice treated with IL-12/pulse IL-12 (Fig. 10, *D–F*) vs control mice (Fig. 10, *A–C*). To clearly document the nature of the cells undergoing apoptosis, a detailed electron micrographic analysis of mammary tumor site resected from control vs IL-12/pulse IL-2-treated mice was performed. In sections from control mice, characteristic hyperplastic epithelium is noted in a mammary duct (Fig. 11*A*), with normal ultrastructural histology apparent on higher magnification images (Fig. 11*B*). In sections from mice treated with IL-12/pulse IL-2, attenuation of the expected hyperplasia is again noted (Fig. 11*C*). Loss of cell-cell contact, extensive cytoplasmic vacuolization, as well as cellular degeneration and separation of the epithelium from underlying basement membrane are readily observed (Fig. 11, *C* and *D*). In other sections, ultrastructural changes consistent with apoptosis, including nuclear condensation and fragmentation, cytoplasmic vacuolization, and overt cellular degeneration with apoptotic bodies and cellular ghosts, are noted (Fig. 11, *E* and *F*).

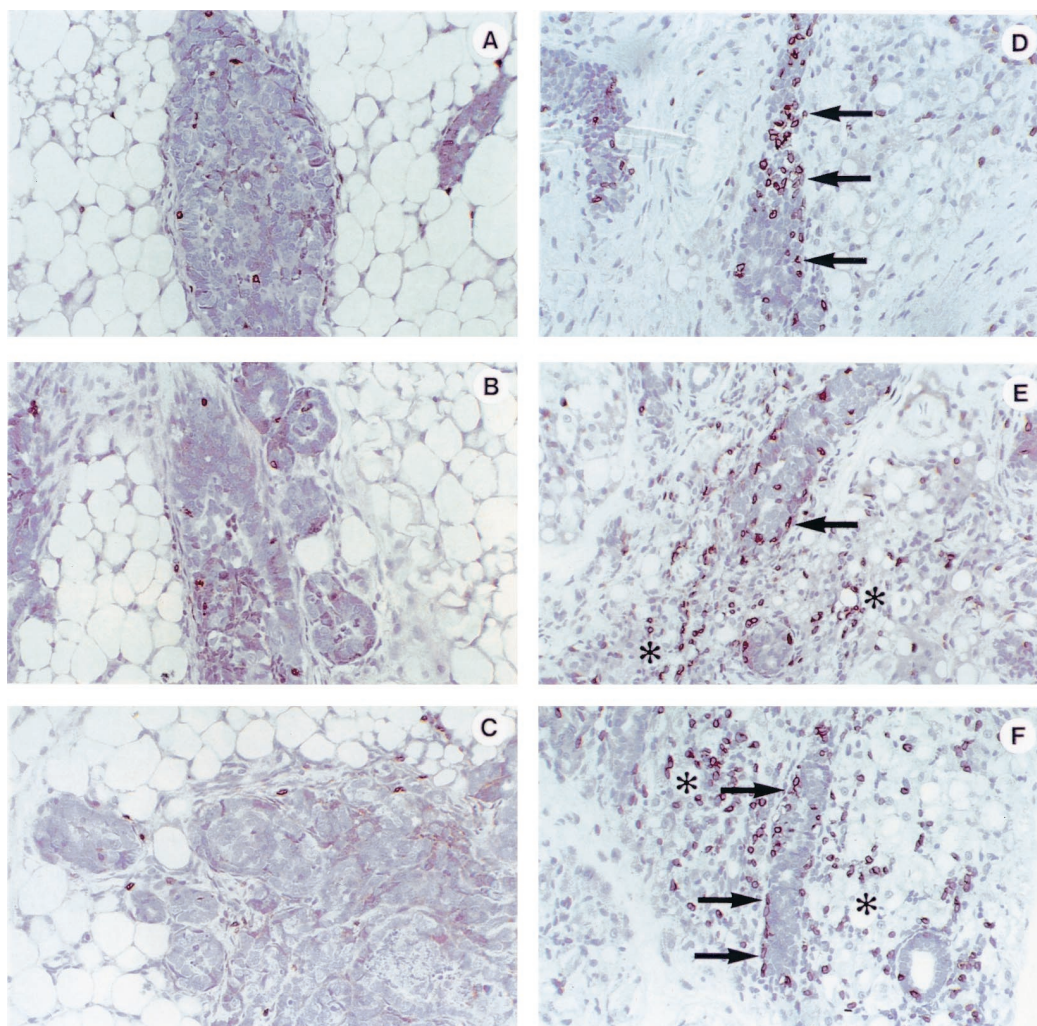
## Discussion

Using a novel transgenic mouse model of spontaneous mammary carcinoma (17), the present studies provide new evidence that systemically administered IL-12/pulse IL-2 possesses potent antitumor activity even in a setting where the host, as occurs in humans with malignancy, has been subjected previously to the complete genetically programmed process of neoplastic progression and tumorigenesis. We found that administration of IL-12/pulse IL-2 can induce complete regression of established autochthonous mammary carcinoma in a substantial portion of treated mice. Further, this regimen exerted its antitumor effects very rapidly, with 60% of the mice that would ultimately undergo a complete response having done so after only two 5-day cycles of therapy. Several studies in vitro and in vivo now have shown that in combination, IL-12 and IL-2 may possess more potent antitumor activity than that observed with either agent alone. IL-12 and IL-2 synergistically enhance cytolytic activity by human PBMC against various tumor

cell lines and/or autologous tumor (21, 22), while administration of low dose systemic IL-2 may enhance the ability of IL-12-secreting fibroblasts to limit the growth of established MC-38 colon carcinoma pulmonary metastases in vivo (23). Further, systemic IL-12 administration has been reported to potentiate the antitumor effects of vaccination with IL-2-producing colon carcinoma (24) or glioma (25) cells, and systemic IL-12/pulse IL-2 has been shown to synergistically enhance the survival of mice bearing established M5076 reticulum cell sarcoma (26).

We are actively investigating mechanisms that may account for the antineoplastic activity of this combination in the autochthonous mammary carcinoma model used for these studies as well as a transplantable model of primary and/or metastatic murine renal carcinoma. These studies support a role for IFN- $\gamma$  and/or TNF- $\alpha$  in the antineoplastic activity of IL-12/pulse IL-2. We have shown here that this regimen induces high circulating serum levels of both IFN- $\gamma$  and TNF- $\alpha$ . IL-12 and IL-2 also potentially enhance serum IFN- $\gamma$  and TNF- $\alpha$  levels and ex vivo IFN- $\gamma$  production by activated splenocytes from mice bearing established renal carcinoma (J. M. Wigginton and R. H. Wiltout, unpublished observations). Further, the antitumor activity of this combination against murine renal carcinoma is abrogated in mice with targeted disruption of the IFN- $\gamma$  gene (J. M. Wigginton et al., manuscript in preparation). We have shown here that administration of IL-12/pulse IL-2 inhibits tumor neovascularization and potentially enhances the expression of the genes encoding the IFN- $\gamma$ - and TNF- $\alpha$ -inducible, antiangiogenic chemokines IP-10 and MIG within established mammary tumors. Production of IP-10 and/or MIG by various cell types is very potently enhanced by IFN- $\gamma$  (27, 28) or TNF- $\alpha$  alone (29, 30), and the combination of IFN- $\gamma$  and TNF- $\alpha$  can synergistically enhance the production of IP-10 and/or MIG by various cell types (31–33). These studies suggest that the ability of IL-12/pulse IL-2 to enhance IP-10 and MIG gene expression may be accounted for at least partially by the potent induction of IFN- $\gamma$  and/or TNF- $\alpha$  production by this combination. IP-10 and MIG both possess antiangiogenic activity (34–36), as does IL-12 (37). IL-12 administration induces IP-10 gene expression within established murine (38) and human (39) renal carcinoma, and in a human tumor xenograft immunodeficient host model, the antiangiogenic activity of IL-12 appears to be accounted for largely by the induction of IP-10 production by IL-12 (40). In the Renca murine renal carcinoma model, we have found that administration of IL-12 and pulse IL-2 can additively enhance ex vivo production of IP-10 protein by activated splenocytes and induce local expression of the genes encoding IP-10 and MIG within s.c. tumor implants via an IFN- $\gamma$ -dependent mechanism (J. M. Wigginton, D. Taub, J. Farber, P. Strieter, and R. H. Wiltout, unpublished observations). Further, concurrent administration of neutralizing polyclonal antisera directed against IP-10 may partially attenuate the antitumor activity of IL-12/pulse IL-2 in Renca-bearing mice. These studies suggest a role for IFN- $\gamma$  with or without TNF- $\alpha$  and the chemokines IP-10 and/or MIG in the antitumor activity of IL-12/pulse IL-2. We propose a model in which administration of IL-12/pulse IL-2 markedly enhances IFN- $\gamma$  and TNF- $\alpha$  production, leading indirectly to induction of IP-10 and/or MIG gene expression, inhibition of tumor neovascularization, and delay of tumor growth as a component of the overall antitumor activity of this regimen.

In mice bearing advanced mammary carcinoma with multiple tumors at baseline, we found that IL-12/pulse IL-2 yielded enhanced antitumor effects compared with IL-12 or IL-2 alone, and that treatment with IL-12/pulse IL-2 not only delayed the growth of established tumor, but also prevented the emergence of new tumors at other mammary sites. These findings led us to hypothesize that early administration of this regimen to juvenile



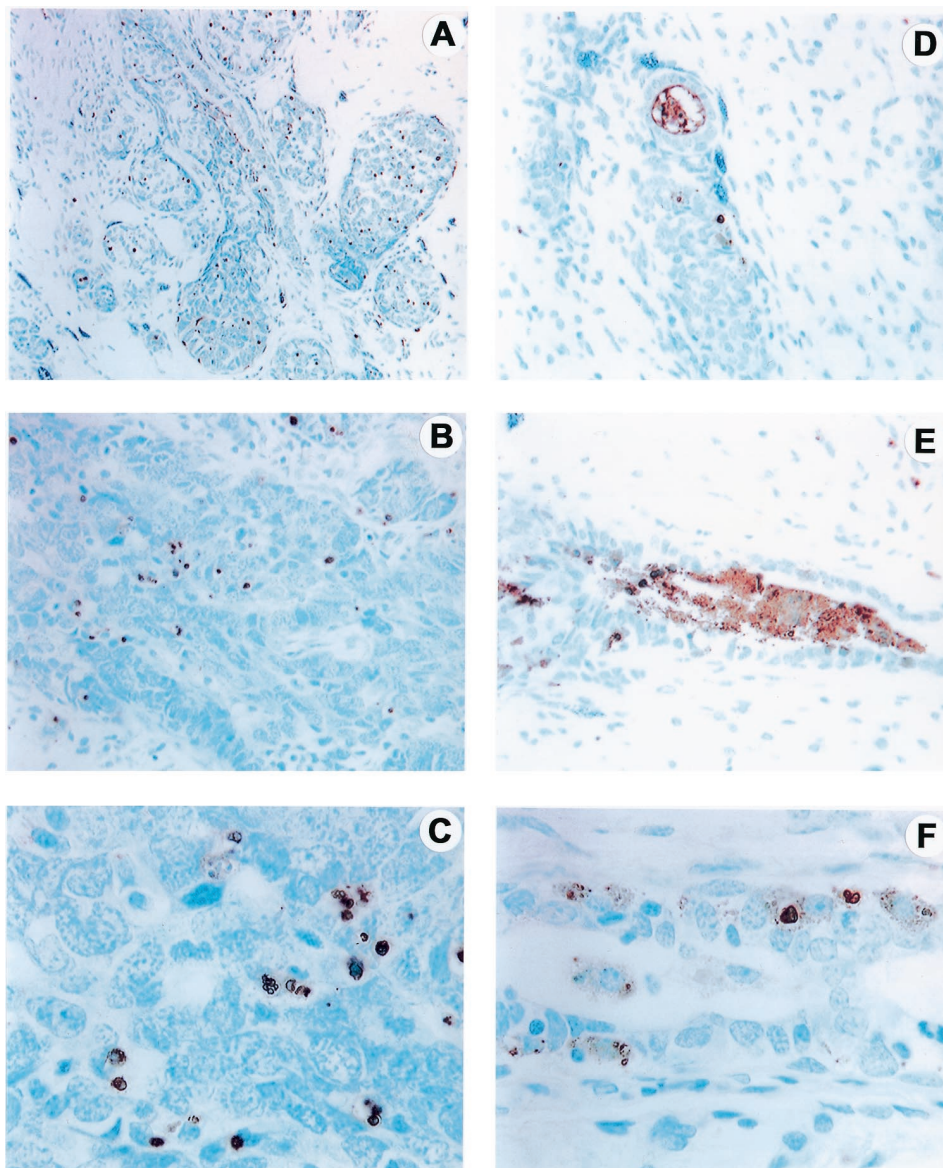
**FIGURE 9.** Infiltration of T lymphocytes into mammary sites of juvenile mice treated with IL-12/pulse IL-2. A cohort of 11 age-matched 2-mo-old C3(l)TAg mice was randomly assigned to treatment with IL-12/pulse IL-2 or vehicle alone as outlined in *Materials and Methods*. As noted in Fig. 6, three randomly selected mice per group were euthanized at the completion of therapy, and mammary sites were resected for evaluation in histology studies or analysis of local gene expression (see also Figs. 7 and 8). Formalin-fixed paraffin-embedded tissue sections were stained with Ab directed against CD3, the pan T lymphocyte marker, as described in *Materials and Methods*. Representative sections were photographed at  $\times 100$  magnification as described in Fig. 7. A–C, Sections of mammary sites from control mice; D–F, sections from mice treated with IL-12/pulse IL-2. Administration of IL-12/pulse IL-2 induced infiltration of large numbers of CD3-positive T lymphocytes that are found not only within the mammary stroma (starred), but also localized in close proximity to and/or invading the mammary epithelium (arrows; see D–F).

C3(l)TAg mice might delay or prevent the progressive hyperplasia, atypia, and carcinoma that occur in this model (17). IL-12 alone has been shown to delay the emergence of tumors induced by exogenous administration of the tumorigenic agent, 3-methylcholanthrene (41). We found that administration of IL-12/pulse IL-2 delayed the emergence of tumor by up to 4 wk compared with age-matched control mice. Further, histologic evaluation of mammary sites resected from mice treated with IL-12/pulse IL-2 revealed marked attenuation of the genetically programmed hyperplasia, atypia, and overt carcinoma noted in sections obtained from age-matched control mice and extensive infiltration of the mammary stroma with mononuclear cells.

Previous studies in this model have demonstrated that a loss of p53 function and endogenous apoptosis occur in conjunction with neoplastic progression (20) in the mammary epithelium. In light of these findings, and the ability of IL-12/pulse IL-2 administration to delay neoplastic transition in juvenile mice, we investigated the impact of this regimen on local expression of genes encoding po-

tential mediators of apoptosis, in particular Fas and FasL. Fas expression has been observed in a range of cell types (42, 43), including nontransformed (44) and preneoplastic (45) mammary epithelial cell lines, and a wide variety of tumors or tumor cell lines (42, 46, 47). FasL expression has been demonstrated in the eye and testis (48, 49) as well as the small intestine, lung, and various lymphoid organs (43). More specifically, FasL gene expression is up-regulated in activated CD8<sup>+</sup>, but not CD4<sup>+</sup> murine splenocytes, and is detected in cytotoxic T cell clones (43) and NK cells (50). The Fas/FasL apoptosis pathway has been implicated in modulation of T cell homeostasis (51, 52) and maintenance of local immune privilege (48, 49), and is a key effector mechanism in the cytolytic activity of T and/or NK cells (50, 53, 54). More recently, increasing attention has focused on the role of this pathway in the interaction between tumors and the host immune system. A loss or reduction in Fas expression is associated with transition of some cells from a normal to a malignant phenotype (44, 55, 56). Further, some tumors express high levels of FasL, and



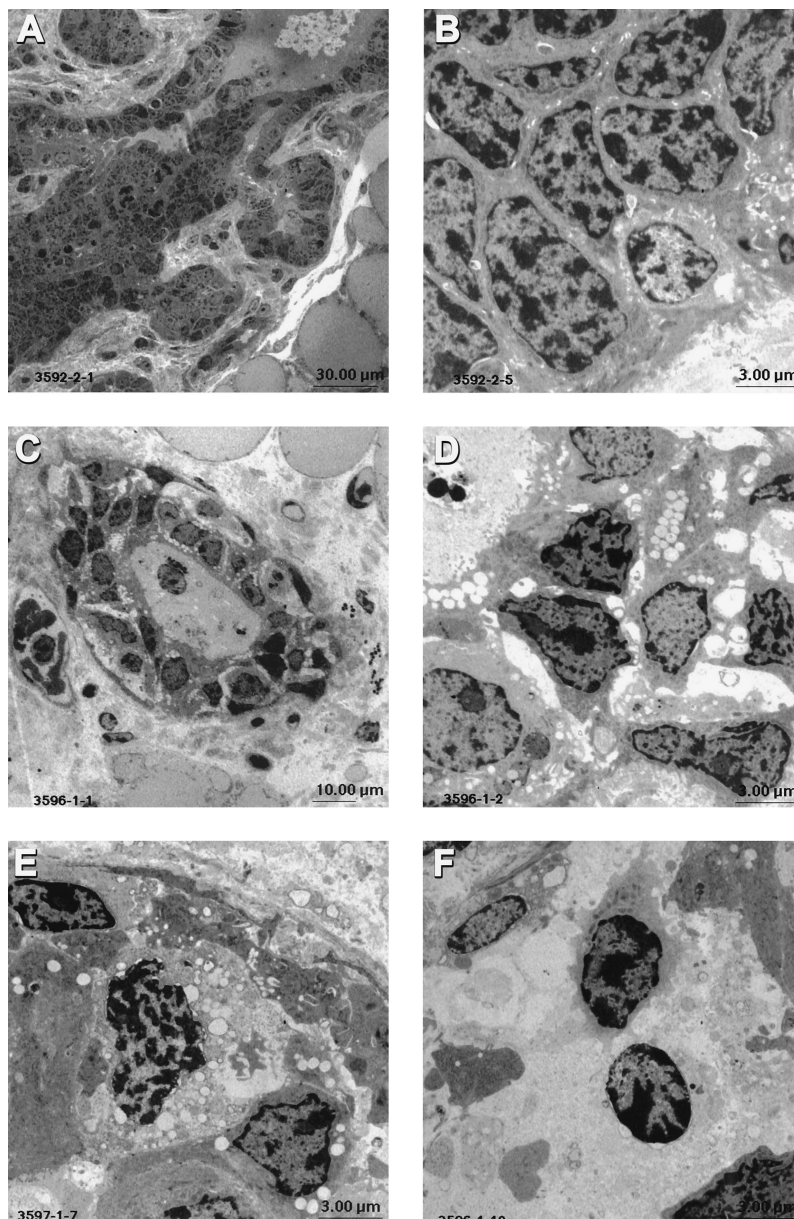


**FIGURE 10.** Rapid induction of apoptosis in evolving preneoplastic lesions in C3(l)Tag mice treated with IL-12/pulse IL-2. A cohort of seven age-matched C3(l)Tag mice was randomly assigned to treatment with IL-12/pulse IL-2 or vehicle alone as outlined in *Materials and Methods*. After three cycles of therapy, all mice were euthanized, and formalin-fixed paraffin-embedded mammary gland tissue sections were analyzed for apoptosis in situ. *A–C*, Sections from vehicle-treated control mice as follows: *A* ( $\times 150$ ) shows characteristic intraductal hyperplastic/neoplastic proliferative lesions and the low background level of apoptosis associated with this model; *B* ( $\times 300$ ) shows intraductal carcinoma, again with low background levels of apoptosis; *C* ( $\times 750$ ) shows intraductal neoplasia with a low apoptosis index. In contrast to *A–C*, *D* ( $\times 300$ ) shows little intraductal neoplastic proliferation with apoptotic cells and intraluminal debris. *E* ( $\times 300$ ) shows a large amount of intraductal debris and apoptotic bodies in the epithelium, while *F* ( $\times 750$ ) shows nonneoplastic ductal epithelium in a regressing lesion with apoptotic cells within the epithelium.

evidence suggests that these tumors may defend themselves via the induction of apoptosis of tumor-infiltrating Fas-positive effector cells (56–58). In contrast, a number of other studies have shown that cross-linkage of Fas on tumor and/or preneoplastic cell lines with anti-Fas Abs or ligation with soluble FasL may induce apoptosis in vitro (44–46, 59–61) and in vivo (60–62), suggesting that activation of the Fas/FasL pathway and induction of tumor apoptosis could serve a useful role in cancer treatment.

Existing literature suggests that administration of IL-12/pulse IL-2 and secondary induction of IFN- $\gamma$  and TNF- $\alpha$  production may provide a unique array of signals leading to activation of the Fas/FasL apoptosis pathway. As noted above, administration of IL-12/pulse IL-2 potentially enhances circulating serum levels of both IFN- $\gamma$  and TNF- $\alpha$ . We also have recently found that IFN- $\gamma$

and TNF- $\alpha$  rapidly up-regulated the expression of Fas on the transplantable M6 cell line derived from a spontaneous mammary tumor from C3(l)Tag-transgenic mice (J. M. Wigginton et al., unpublished observation). Several previous reports have shown that IFN- $\gamma$  and/or TNF- $\alpha$  enhance tumor cell expression of Fas- and Fas/FasL-mediated tumor cell apoptosis (42, 44, 46). Although neither IFN- $\gamma$  nor TNF- $\alpha$  directly modulates the expression of FasL (43), IL-2 induces FasL expression by murine splenocytes (43) and human T cells (63), and enhances FasL-mediated cytotoxicity by human CD8 $^{+}$  T cells (63). In fact, we also have recently shown that CD8 $^{+}$  T cells from BALB/c mice up-regulate FasL protein in response to IL-12 and IL-2 (J. M. Wigginton et al., unpublished observation). Further, IL-12 itself enhances FasL-mediated cytotoxicity by murine Th1 clones in vitro (64).



**FIGURE 11.** Ultrastructural histology and the induction of apoptosis and destruction of mammary ductal epithelium in mice treated with IL-12/pulse IL-2. A cohort of eight juvenile C3(l)TAg mice was randomly assigned to treatment with IL-12/pulse IL-2 or vehicle alone as outlined in *Materials and Methods*. At the completion of therapy, all mice were euthanized, and individual mammary sites were resected, fixed in 4% paraformaldehyde/2% glutaraldehyde in PBS, and processed for evaluation via electron microscopy as described in *Materials and Methods*. Sections in A ( $\times 450$  magnification) and B ( $\times 4500$ ) are from control mice, while C ( $\times 900$ ) and D–F ( $\times 4500$ ) are sections from mice treated with IL-12/pulse IL-2.

We have found that administration of IL-12/pulse IL-2 not only delayed neoplastic progression in the mammary epithelium of treated mice, but also potently up-regulated the production of IFN- $\gamma$  and TNF- $\alpha$  and the expression of the genes encoding Fas and FasL within the local mammary site. Further, a large portion of the mononuclear cells observed infiltrating the local mammary site in mice treated with IL-12/pulse IL-2 are CD3 $^{+}$  T cells, and IL-12/pulse IL-2 treatment induces apoptosis of mammary epithelial cells as evidenced by in situ end-labeling techniques (ApopTag) as well as direct examination of the ultrastructural histology of the mammary epithelium using electron microscopy. More recently, in our transplantable murine renal carcinoma model (Renca), we have shown that administration of IL-12/pulse IL-2 enhances local Fas/FasL gene expression in an IFN- $\gamma$ -dependent manner within established tumors, and using FasL mutant GLD mice, that the overall antitumor activity of this regimen is critically dependent on FasL (J. M. Wigginton and R. H. Wiltrott, unpublished observations). We propose a model in which the combination of IL-12 and IL-2 directly and/or indirectly, via induction of

IFN- $\gamma$  and TNF- $\alpha$  production, enhance local constitutive expression of Fas on mammary epithelial cells and FasL expression on infiltrating activated T cells. In this setting we propose that infiltrating FasL-positive effector cells induce apoptosis of Fas-bearing target cells in the mammary epithelium and in so doing reverse the dysregulation of endogenous apoptosis and cell turnover that occurs in this model and limit neoplastic progression. Administration of IL-12/pulse IL-2 also up-regulates the expression of both Fas and FasL within established mammary tumors, although its potential role in mediating tumor regression in that setting remains to be defined in future studies. Previous attempts to activate Fas-mediated apoptosis in vivo using systemic administration of anti-Fas Abs or soluble FasL have been limited by the occurrence of severe toxicity (60, 65). In the present studies we have demonstrated the induction of local Fas/FasL gene expression within preneoplastic lesions and/or established tumors in vivo, using an approach that is not only well tolerated, but delays transition of preneoplastic lesions and induces complete tumor regression in a large percentage of treated mice. Further, the current studies represent the first report of successful application of IL-12/pulse IL-2 as a preventative strategy to limit spontaneous, genetically programmed neoplastic



progression and the use of immunotherapy to induce Fas/FasL gene expression and enhance apoptosis within preneoplastic tissue.

The potential role of SV40 TAG in these observations remains to be elucidated. Although the kinetics of TAG expression have been established in this transgenic model (17), it is not completely clear whether C3(1)-TAG mice are completely or only partially tolerant to TAG. It does appear that the antitumor effects of IL-12/pulse IL-2 in this model are not dependent on breaking tolerance to SV40 TAG. Our results demonstrate the engagement of nonadaptive immunophysiologic mechanisms that then mediate rapid antitumor effects that occur in conjunction with T cell infiltration, inhibition of neovascularization, and induction of epithelial apoptosis. Previous studies have shown that although specific immunization of mice that carry the SV40 TAG transgene can induce CTL responses against T Ag and delay tumor growth, the ability to do so is highly sensitive to the timing of onset for the expression of the transgene in developing mice (66). More specifically, immunization of mice after the onset of T Ag expression was ineffective in inducing such responses. We have performed several experiments to investigate whether TAG-specific T cells can be detected in mice after treatment with IL-12/pulse IL-2. Specifically, we have challenged spleen and lymph node T cells from control and IL-12/pulse IL-2-treated mice with syngeneic TAG-pulsed dendritic cells or TAG-expressing M6 syngeneic tumor cells in vitro and have been unable to detect TAG-specific proliferative or cytotoxic responses. These results suggest the initial potent tumor regression induced by IL-12/pulse IL-2 is independent of a T cell memory response, but that this inability to generate a TAG-specific adaptive response renders the mice susceptible to the appearance of new tumors because of the constant oncogenic stimulus provided by the continued expression of TAG.

The current investigations provide further evidence of the potent antitumor activity of the IL-12/pulse IL-2 combination and demonstrate successful treatment of established tumor in a setting where the host immune system has been conditioned by the complete process of neoplastic progression and tumorigenesis. The present studies also provide conceptual support for a novel application of immunotherapy, using IL-12/pulse IL-2 to limit neoplastic progression, a process shown here to occur in conjunction with potent local activation of Fas/FasL gene expression, T cell infiltration, and the induction of apoptosis within preneoplastic mammary lesions. These studies suggest that IL-12/pulse IL-2 might be used to therapeutically induce apoptosis in preneoplastic mammary lesions and/or overt carcinoma, and in so doing not only induce regression of established tumor, but also limit or prevent progression of preneoplastic lesions to overt carcinoma. In that autochthonous tumor models may more closely reflect the spontaneous occurrence of malignancies in humans and the complexity of interactions between an evolving tumor and the host immune system, the efficacy of IL-12/pulse IL-2 in this model provides further supportive rationale for investigation of its potential clinical utility in the treatment of human malignancy, studies that are now being initiated.

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