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Intralesional Injection of Adenovirus Encoding CC Chemokine Ligand 16 Inhibits Mammary Tumor Growth and Prevents Metastatic-Induced Death after Surgical Removal of the Treated Primary Tumor¹

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The CC chemokine ligand (CCL)16 exerts chemotactic activity on human monocytes and lymphocytes. Although no murine homologous has been defined, the TSA mouse adenocarcinoma cells engineered to express human CCL16 are rapidly rejected by syngenic mice. An adenovirus encoding CCL16 (AdCCL16) was generated using a Cre-Lox-based system and was used to determine whether this chemokine might also block pre-existing tumors. Both recombinant and viral CCL16 showed in vitro chemotactic activity for murine CD4⁺ and CD8⁺ lymphocytes and dendritic cells (DC). AdCCL16, but not the control empty vector, when injected in established nodules significantly delayed tumor growth. Immunohistochemistry revealed accumulation of CD4⁺ and CD8⁺ T cells and DC in the treated tumors as well as in draining lymph nodes. DC from such lymph nodes stimulated IFN- γ by a T cell clone specific for the known TSA tumor-associated Ag (TAA), suggesting the tumor origin of these cells. Lymphocytes from the same nodes showed specific CTL activity against TSA tumor cells and their immunodominant TAA peptide. Antitumor activity required CD4, CD8, and IFN- γ production, as shown using subset-depleted and knockout mice. Despite the robust and rapid immune response triggered by intratumoral injection of AdCCL16, the lesions were not completely rejected; however, the same treatment given before surgical excision of primary lesions prevented metastatic spread and cured 63% of mice bearing the 4T1 mammary adenocarcinoma, which is perhaps the most compelling model of spontaneous metastasis. *The Journal of Immunology*, 2004, 172: 4026–4036.

Immunotherapy strategies that target single tumor-associated Ags (TAAs)³ identified by CTL have shown little success, suggesting the need for alternative approaches, including the use of whole tumor cells as a source of the entire antigenic repertoire of a particular tumor. In this context, attempts have been made to derive vaccines based on tumor cells transduced with cytokines and costimulatory molecules (1, 2). Such molecules can be expressed directly in primary tumors by means of adenoviral vectors, an approach that induced tumor rejection and systemic immunity in some tumor models (3).

Chemokines belong to a family of 8- to 10-kDa proteins that orchestrate the recruitment of leukocytes from the vasculature (4–6). Given their immunomodulatory properties, chemokines have been used extensively in the immunotherapeutic setting. The outcome of these studies largely depended on the tumor model and the chemokine used (7, 8). When used to transduce mouse tumor cell lines, single chemokines, such as CC chemokine ligand (CCL)2, CCL3, CCL5, CCL19, CCL21, and CXC chemokine ligand (CXCL)10, induce rejection of transduced cells and induction of immunity, as indicated by the ability of immune mice to reject a second challenge with parental cells (9–15).

When delivered by adenovectors to cure established tumors, CCL20 and CCL22 induced rejection in a significant number of treated animals (40–80%) (16, 17). However, several studies have pointed to the limited curative potential of chemokines used as single agents (7). Indeed, combinations of chemokines and cytokines have proven superior to single agents, as exemplified in studies of intratumoral coinjection of two adenoviruses, one encoding CXCL10 or CXCL9, and the other encoding IL-12 (18, 19). Lymphotactin, which is ineffective alone, induced rejection of established myeloma tumors or breast carcinoma when combined with adoptive transfer of T cells (20) or IL-12 (21).

In vaccine research, chemokines are most exploited for their ability to induce migration of dendritic cells (DC) (22). Immature DC capture and process Ags in peripheral tissue and, upon maturation, migrate to secondary lymphoid organs to prime naive T cells (23). Immunotherapy strategies that focus on DC are all based on the premise that the quality of the T cell response depends largely on the ability of DC to process and present tumor Ags to T cells; in the final phase of this process, DC provide T cells with the

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³ Abbreviations used in this paper: TAA, tumor-associated Ag; BM-DC bone marrow-derived dendritic cell; DC, dendritic cell; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; GKO, IFN- γ knockout mice; TIDC, tumor-infiltrating dendritic cell.

costimulatory molecules required for the development of T cell effector function (22, 23).

Administration of chemokines that recruit DC directly to the tumor site obviates the need for DC purification and *in vitro* manipulation and ensures the continuous availability of DC precursors, which, upon Ag uptake, undergo migration and encounter with T cells in a more physiological manner.

DC and T lymphocyte recruitment has been described in pre-established B16 melanoma tumors treated with recombinant CCL19, although tumor rejection was incomplete (24, 25). Two other studies also reported DC recruitment to the tumor site in mice injected intranodally with adenovirus carrying the human CCL20, a gene that induced rejection in 60–80% of mice in two of three tumor models tested, and in mice treated with adenovirus encoding CCL22/MDC, which was effective in 60% of mice (17).

However, most investigations have not directly addressed the actual role of DC in tumor rejection, and no compelling animal model for the prevention and cure of distant metastases, the critical issue in cancer therapy, has been tested. We addressed these points using human CCL16, a chemokine poorly characterized at the functional level (26, 27). Human CCL16 is a member of the CC family, and its gene maps to human chromosome 17q. In the mouse, only a pseudogene has been identified to date (27, 28). CCL16 is a functional ligand for CCR1, CCR2, CCR5 (29), and CCR8 (30). Recombinant CCL16 demonstrated chemotactic activity on human monocytes and lymphocytes (27). Based on the ability of human chemokines to exert activity on and bind to murine receptors, the TSA mouse adenocarcinoma cell line was transfected with human CCL16 cDNA and, in comparison with other cytokines (31), was shown to be the faster inducer of systemic immune response due to massive, prompt infiltration of leukocytes (32). No other studies have addressed CCL16 activity in the mouse, and no information is available on its true biological function in mice and humans.

We have cloned and inserted the coding region of CCL16 into an adenoviral vector, as these vectors efficiently infect tumor cells *in vivo*, induce high levels of transgene expression, and provide a certain adjuvant activity in mounting an immune response (33, 34). Injection of our construct AdCCL16 into pre-established TSA mammary carcinomas in mice induced a strong antitumor response, leading to accumulation of T cells and DC at the tumor site and rapid swelling of draining lymph nodes with accumulation of DC in the T cell area. However, despite the robust systemic immune response, large primary tumors were not completely rejected. The use of an approach involving surgical removal of the primary tumor, as usually required in clinical practice, showed that AdCCL16 significantly inhibited metastatic spread and cured up to 63% of treated mice.

Materials and Methods

Tumors and mice

TSA tumor is a murine mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse (35). 4T1 tumor is a 6-thioguanine-resistant cell line derived from a spontaneous mammary carcinoma (ATCC-LGC Promochem; American Type Culture Collection, Teddington, U.K.) (36). Tumor cells were cultured in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (BioWhittaker, Walkersville, MD).

Eight- to 10-wk-old female BALB/c mice BALB/c *nu/nu* mice were purchased from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. IFN- γ knockout mice (GKO) mice on the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME).

Tumor injection and treatment

BALB/c mice were inoculated s.c. into the right flank, equidistant from the inguinal and axillary lymph nodes, with 1×10^5 TSA or 7×10^3 4T1 cells.

After 7–10 days, when tumors reach 3–4 mm in diameter, nodules were inoculated with 1×10^9 PFU of AdCCL16 or control virus Add170-3, and the treatment was repeated 4 days later or was not repeated, as indicated.

In some experiments mice were depleted of CD4 and CD8 cells by *i.p.* injection of 200 μ g/mice of anti-CD4 (GK1.5 hybridoma, Lyt2; American Type Culture Collection) or anti-CD8 (clone 2.43; American Type Culture Collection) mAbs. Depletion was started 1 wk before tumor challenge and twice per week thereafter until the end of the experiment. FACS analysis of peripheral blood confirmed depletion of no less than 95%. Tumor size was monitored twice per week and was recorded as the longest diameter \times the shortest diameter² (in cubic meters). Mice were euthanized when tumors reached \sim 10 mm in diameter.

For surgical excision of primary 4T1 tumors, mice were anesthetized before surgery, and tumors were resected with sterilized instruments. Wounds were closed with metallic clips. All mice survived surgery. Mice in which primary tumors recurred at the site of the surgical excision (four of 60) were eliminated from the experiment. Metastases were evaluated as previously described (37, 38).

Briefly lungs were collected and dissociated in HBSS containing 1 mg/ml collagenase type 4 and 6 U/ml elastase for 1 h at 4°C; organs were then plated at various dilution in a medium supplemented with 6-thioguanine. Individual colonies representing micrometastasis were counted after 10–15 days.

Construction of recombinant adenoviral vector

Recombinant adenovirus carrying the human CCL16 gene under the control of the mouse CMV promoter was constructed using the Cre-Lox recombination system (Microbix, Toronto, Canada) (39). The cDNA for CCL16 was amplified from the cDNA of HepG2, a human hepatocarcinoma cell line that constitutively expresses CCL16 (29). A standard PCR using Ampli-Taq polymerase and GeneAmp PCR system 9700 (both from PerkinElmer, Foster City, CA) was used to amplify CCL16 cDNA with the following pairs: (5'-tgagaggatgaaggtctccc-3') and (5'-agcctgtcatcactggagg-3'). The empty adenoviral vector Add170-3 was provided by Dr. F. Graham (McMaster University, Hamilton, Canada).

Recombinant adenovirus was isolated from a single plaque amplified on 293 cell monolayers and purified by double-cesium chloride gradient centrifugation as previously described (40). Experiments were conducted with at least five independently prepared adenovirus stocks. Virus titer was determined by plaque assay in 293 cells and is expressed as PFU. Expression of CCL16 by AdCCL16 was confirmed by ELISA using specific mAbs (PeproTech, Rocky Hill, NJ).

Chemotaxis assay

CD4 and CD8 lymphocytes were obtained from naive mouse spleen; the two populations were enriched >95% by positive selection using Mini-Macs separation columns (Miltenyi Biotec). DC were obtained as follows: bone marrow cells from femurs and tibias were plated at 2×10^6 cells/ml in RPMI/5% FCS supplemented with recombinant murine GM-CSF (5 ng/ml) and recombinant murine IL-4 (10 ng/ml) of (PeproTech). On day 2, one-half of supplemented medium was replaced with fresh medium. On day 4, floating cells were centrifuged and plated in fresh medium at 1×10^6 /ml. On day 5 nonadherent cells were harvested. Cells were further using anti-CD11c magnetic beads to 95% (Miltenyi Biotec, Bergisch Gladbach, Germany). DC collected on day 5 of culture displayed the following characteristic phenotype: CD11c⁺, MHC class II^{int}, CD80^{low}, CD40^{low}, CD86^{low}.

Chemotactic activity was tested using the Boyden chamber method as previously described (41). Briefly, triplicate assays were conducted in 96-well microchemotaxis chambers (NeuroProbe, Cabin John, MD) with a 5- μ m pore size polycarbonate membrane (Costar, Cambridge, MA) separating cells from the medium containing the chemoattractant. T cells (4×10^6 /ml) or DC (2×10^6 /ml) were allowed to migrate for 3 or 2 h, respectively, in DMEM containing 0.1% of BSA. When necessary, cells were preincubated with 200 ng/ml pertussin toxin (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C. The membrane was removed and stained with Diff Quick kit. Migrated cells were counted with a Nikon microscope ($\times 40$ objective; Melville, NY).

In some experiments viral supernatants were used as the source of CCL16. To obtain these supernatants, TSA cells were plated at a concentration of 2×10^5 /ml and were infected with 100 multiplicity of infection of AdCCL16 or Add170-3 1 day later, and supernatants were harvested 48 h later. CCL16 in the medium was quantified by ELISA.

The migration index was calculated as: number of cells migrated in the presence of recombinant or viral CCL16/number of cells migrating in medium alone or in supernatant of Add170-3-infected TSA cells, respectively.

Histological and immunohistochemical analysis

For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm , and stained with H&E.

For immunohistochemistry acetone-fixed cryostat sections were immunostained with Abs against anti-CD11c (clone N418; Chemicon International, Temecula CA); anti-CD11b/CD18 (clone M1/70.5), anti-CD8 (Ly/T2, clone YT5 169.4), and anti-CD4 (LT34, clone YT5.191.1.2; all from Sera-lab, Crawley Down, U.K.); anti-GR1 (clone RB6-8C5; American Type Culture Collection); anti-NK (asialo GM1; Wako Chemicals, Dusseldorf, Germany); and anti-IFN- γ (clone XMG1.2; provided by Dr. S. Landolfo, University of Turin, Turin, Italy). After washing, sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit and horse anti-goat Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ig was removed by washing, and slides were incubated with ABC complex/AP (DAKO, Glostrup, Denmark). Tetramethyl rhodamine isothiocyanate-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary Ab for CD8⁺ cell immunofluorescence, and FITC-conjugated chicken anti-goat IgG (Rockland, Gilbertsville, PA) was used as secondary Ab for IFN- γ immunofluorescence.

Quantitative studies of stained sections were performed independently by three pathologists in a blind fashion. Cell counts were obtained in 10 randomly chosen fields under a light microscope (Leica DMLB; Leica, Deerfield, IL; $\times 400$ field, 0.180 mm²/field). Confocal microscopy was performed with an LSM 510 META (Zeiss, New York, NY).

Lymph node analysis

At the indicated time points, inguinal and axillary lymph nodes draining the treated tumors were harvested, mechanically disrupted, and digested for 45 min at 37°C with a solution of 1 mg/ml collagenase D (Hoffmann-La Roche, Nutley, NJ) in HBSS (BioWhittaker, Walkersville, MD). Lymph nodes were passed over a 70- μm pore size nylon filter, and cells were counted and used for FACS analysis. All Abs used were purchased from BD PharMingen (San Diego, CA). For analysis of T cell subsets and DC populations, cells were stained with PE-conjugated anti-CD11c, anti-CD4, and anti-CD8. Analyses were performed on a FACScan (BD Biosciences, Franklin Lakes, NJ). When indicated, lymph node cells were suspended at 1×10^6 cells/ml and stimulated for 10 h with anti-CD3 Ab (1 $\mu\text{g}/\text{ml}$; BD PharMingen). Supernatants were tested for IFN- γ production by ELISA using specific Abs (BD PharMingen). Cells were analyzed for the presence of IFN- γ production in the CD4 and CD8 subsets using mouse IFN- γ secretion assay kit (Miltenyi Biotec). Stained cells were analyzed by FACS analysis.

IFN- γ production by env-specific CTL clones and cell-mediated cytotoxicity assay

Six mice bearing TSA tumors were treated with AdCCL16 on days 0 and 4. Two days after the last treatment, mice were divided into two groups. Draining lymph nodes from each group were pooled and treated independently. DC were enriched from the lymph node populations using anti-CD11c magnetic beads (Miltenyi Biotec) and were used to stimulate a specific CTL clone. The negative fraction of DC purification containing lymphocytes was used to test CTL activity (see below). The generation and maintenance of the CTL line E-88 specific for murine leukemia virus env-derived peptide 423–431 SPSYVYHQF (AH1) has been previously described (42). E-88 cells (10^5 wells of 96-well plates) were incubated with stimulating cells (2×10^5 DC or 10^5 TSA cells) for 18 h at 37°C in 200 μl of complete medium (RPMI supplemented with 10% FCS). Supernatants were tested for IFN- γ content by specific ELISA (BD PharMingen).

Lymphocytes from the CD11c-negative fraction were restimulated in a mixed lymphocyte tumor culture with the irradiated parental tumor cells (1:10 ratio) or the specific peptide AH1 (1 $\mu\text{g}/\text{ml}$) medium. After 5 days, cytotoxic activity was tested in a standard 4-h ⁵¹Cr release assay. TSA and C26 (sharing the env Ag with TSA) were the specific target, and blast cells, pulsed or not with AH1 peptide, were the control for TSA tumor-specific lysis.

Statistical analysis

Data were analyzed using a two-sided Student's *t* test, except for metastasis formation data, which were analyzed using the Mann-Whitney test. All analysis were performed using PRISM software (GraphPad, San Diego, CA). Differences were considered significant at $p < 0.05$.

Results

CCL16 is chemotactic for DC, and CD4 and CD8 lymphocytes

Analysis of CCL16 *in vitro* chemotactic activity on different murine leukocyte subpopulations using both recombinant and virus-produced CCL16 showed that CCL16 induces migration of CD4 and CD8 cells and bone marrow-derived DC in a dose-dependent manner and that virus-produced chemokine is biologically active (Fig. 1). The chemotactic response was abrogated by preincubation of cells with pertussin toxin, suggesting that CCL16 mediates its chemotactic activity by G α_i -coupled receptors.

AdCCL16 inhibits tumor growth *in vivo*

TSA tumor nodules were injected with 1×10^9 PFU of AdCCL16 or the empty vector Addl70-3 and collected after 48 h to assay the amount of CCL16 produced *in vivo*. Supernatants from four different tumors were analyzed separately by ELISA. CCL16 production ranged from 100 to 120 ng/ml $\times 10^5$ cells/ml and is detectable for at least 10 days to decline thereafter. No CCL16 production was detected in tumors transduced with the empty vector (data not shown).

Growth of pre-established tumors (3–4 mm) injected once or twice with AdCCL16 was significantly inhibited, especially after two injections, whereas no inhibition was observed in the group treated with Addl70-3 virus and in the untreated group (Fig. 2A).

Fifteen of 49 (30%) mice were cured when treated with two injections of AdCCL16 vs none of 49 mice treated with the control empty vector (pooled data from seven independent experiments; $p = 0.001$). Mice that rejected the tumor after AdCCL16 treatment acquired long-term immunity, as indicated by their ability to reject a challenge with 2×10^5 live TSA cells 90 days later.

This finding was confirmed with the 4T1 (Fig. 2B) mammary carcinoma, which is the metastatic model used in the experiments described thereafter. This tumor produces an amount of CCL16 upon AdCCL16 infection (200–240 ng/ml $\times 10^5$ cells/ml) similar to that produced by TSA tumor.

Morphologic analysis of TSA tumors after AdCCL16 or Addl70-3 treatment

Histological analysis of TSA tumors collected 1 day after the last AdCCL16 injection revealed marked reactive cell infiltration well inside the tumor mass where small and multiple necrotic foci were also found. Addl70-3-injected TSA tumors evidenced much less infiltration and a barely detectable necrotic area (Fig. 3, A and B). Consistent with the intrinsic immunogenicity of adenoviruses (33), in tumors injected with the empty vector, leukocyte infiltration was greater than that in PBS-treated tumors; however, the infiltration induced by AdCCL16 was significantly greater than that of Addl70-3-treated tumors (Table I). All leukocyte subsets examined were more abundant in AdCCL16-treated tumors with a predominance of CD11c⁺, CD4⁺, and especially CD8⁺ T cells (Table I and Fig. 3, C and D). The latter cells were the main source of tumor-associated IFN- γ (Fig. 3, E and F), as confirmed by laser scanning confocal microscopy (Fig. 3, G and I).

Depletion of either CD4⁺ or CD8⁺ cells by means of specific Abs abrogated the effect of AdCCL16 (Fig. 4, A and B), indicating the functional role of both subsets in tumor rejection. Similarly, the role of IFN- γ , was confirmed in TSA tumor-bearing GKO mice in which AdCCL16 treatment of tumors was no longer inhibitory (Fig. 4C).

AdCCL16 induces inflammation of draining lymph nodes

One of the most evident consequences of AdCCL16, but not Addl70-3, treatment was the swelling of lymph nodes draining the

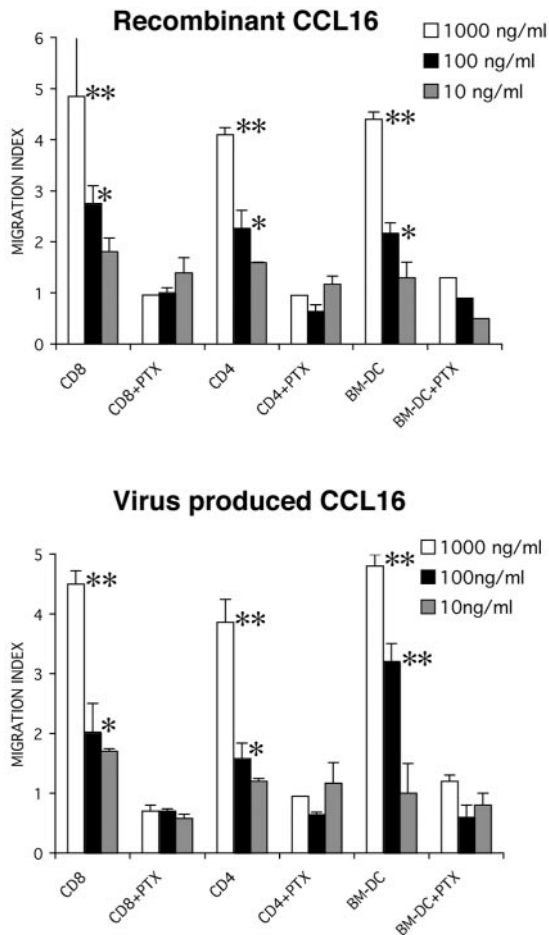


FIGURE 1. Recombinant and virus-produced CCL16 is chemotactic for CD4 and CD8 lymphocytes and DC. CD8⁺ and CD4⁺ cells purified from total splenocytes using magnetic beads as well as bone marrow DC were tested for migration in response to supernatants containing different amounts of recombinant or virus-produced protein. After 3 h for CD4⁺ and CD8⁺ and 2 h for DC, cells attached to the bottom of the filter, separating the top and the bottom of the Boyden chamber, were fixed and counted under a light microscope (×40 objective). Preincubation with pertussis toxin (PTX) inhibited CCL16-induced migration. Assays were performed in triplicate (means of two independent experiments; bars indicate ±SE). Specific migration (migration index) is expressed as the number of cells that migrated in the presence of recombinant or viral CCL16/the number of cells migrating in medium alone or in supernatant of Adl70-3-infected TSA cells, respectively. The migration index in the presence or the absence of PTX was compared for statistical analysis (*, $p < 0.05$; **, $p < 0.01$).

tumors. FACS analysis of cells from inguinal and axillary lymph nodes collected from the site of tumor injection at 2, 5, 8, 12, and 18 days after the first treatment, but not lymph node from the contralateral side, revealed a dramatic increase in total cells number as well as in the number of CD4⁺, CD8⁺ lymphocytes and CD11c⁺ DC in lymph nodes draining AdCCL16-treated tumor compared with Adl70-3-treated tumors at all points except day 18, when cell count returned to normal (Fig. 5 and data not shown). No significant differences between Adl70-3-treated and untreated mice were observed (not shown), suggesting that the inflammation induced by the empty adenovector is mainly local.

In addition, immunohistochemical analysis of inguinal lymph nodes collected on day 5 showed that CD11c⁺ cells were more represented in the paracortical T cell areas of the lymph nodes draining AdCCL16-injected TSA tumors than in those draining Adl70-3-injected tumors (Fig. 6, A and B). In the paracortical

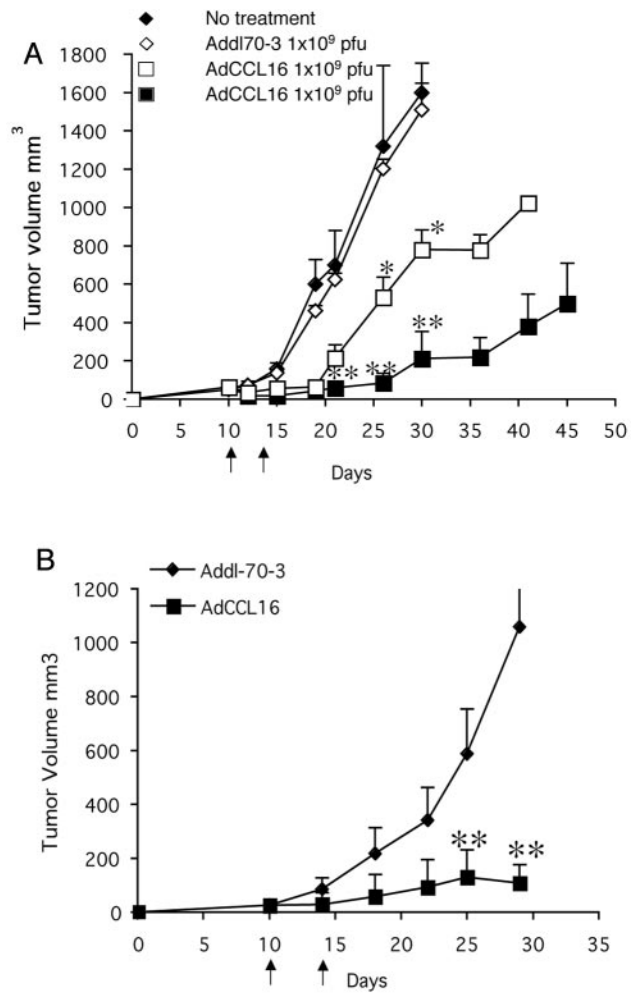


FIGURE 2. Antitumor effects of AdCCL16 on pre-existing tumors. A, BALB/c mice were injected s.c. on day 0 with 1×10^5 TSA cells. When tumors became palpable, mice were left untreated (◇) or were treated with a single (□) or a double injection (■) of 1×10^9 PFU of AdCCL16 or Adl70-3 (◆). Seven mice were used in each group. Results are from one experiment of three performed with similar results. Data are given as the tumor volume (mean ± SD). *, $p < 0.05$ for □ vs ◆ and ◇. **, $p < 0.01$ for ■ vs ◆ and ◇. B, BALB/c mice were injected s.c. on day 0 with 7000 4T1 cells, and 10 days later, 1×10^9 PFU of AdCCL16 or Adl70-3 was injected intratumorally. The treatment was repeated 4 days later. Twelve mice were used in each group. Data are given as the mean ± SD. **, $p < 0.01$ for ■ vs ◆. Arrows indicate the time of injection.

areas and sometimes in the medullary cords of lymph nodes draining AdCCL16-injected tumors, IL-12 expression was detected in cells with DC morphology (Fig. 6, C and D).

To test the locoregional activation of T lymphocytes from the draining lymph nodes, IFN- γ was measured by ELISA of total lymphocytes, whereas the percentage of IFN- γ -producing cells was evaluated by FACS analysis of CD4⁺ and CD8⁺ subsets. Cells from AdCCL16-treated mice produced significantly higher amounts of IFN- γ than cells from mice treated with control virus (Fig. 7A), and the majority of both CD4⁺ and CD8⁺ cells actively produced the cytokine (Fig. 7B). No IL-4 production was detected in the same populations (data not shown).

DC in lymph nodes draining AdCCL16-treated tumors are loaded with TAA and stimulate TAA-specific T lymphocytes

As DC proliferation in lymph nodes is unlikely (43), their accumulation in these nodes after AdCCL16 treatment suggests their

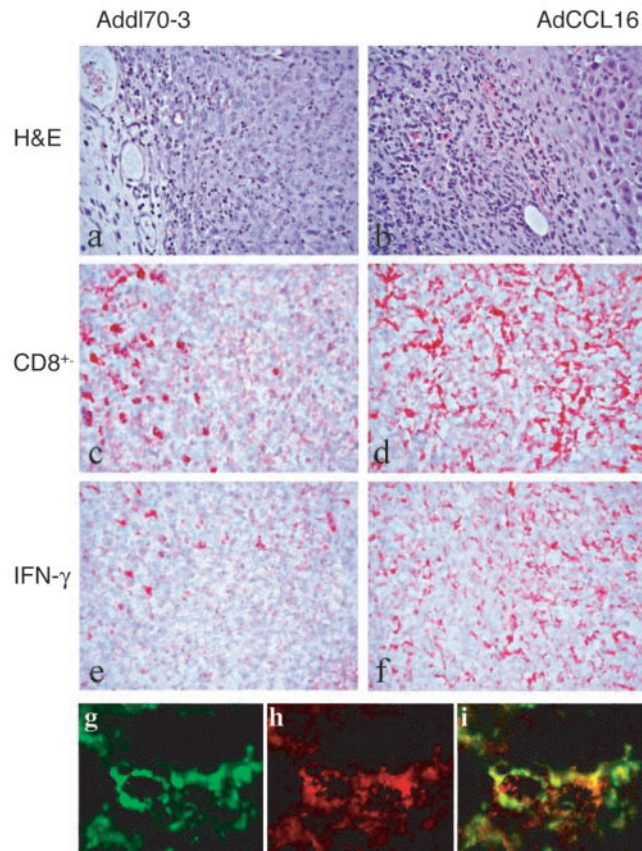


FIGURE 3. Histological and immunohistochemical analysis of TSA tumors after AdCCL16 or Add170-3 treatment. TSA tumors of 3–4 mm were injected on days 10 and 14 with AdCCL16 or Add170-3 and were processed for immunohistochemical analysis the following day. TSA tumors, formed by round to polygonal epithelial cells with frequent mitotic figures, when injected with AdCCL16 evidenced a marked inflammatory reaction, particularly in the peripheral areas (B), whereas Add170-3-injected TSA tumors showed much less inflammatory reaction than AdCCL16-treated tumors (A). CD8⁺ lymphocytes and cells expressing IFN- γ were the most represented leukocyte population in AdCCL16-treated tumors (D and F, respectively) compared with tumors injected with Add170-3 (C and E, respectively; $\times 400$). Laser scanning confocal microscopy revealed frequent colocalization of IFN- γ -producing cells (green staining in G) and CD8 cells (red staining in H), as shown in the yellow regions of the merged image (I; $\times 1000$).

migration from the tumor. To address this point, CD11c⁺ DC from lymph nodes draining AdCCL16-treated tumors were purified using magnetic beads and used to stimulate IFN- γ production by the T cell clone E/88 as an indication of TAA capture by DC. The *env* gene of the endogenous ecotropic murine leukemia virus, containing the L^A-restricted peptide AH1 (44), is expressed by TSA tumor as an immunodominant Ag (45), and clone E/88 specifically recognizes this epitope (42).

As shown in Fig. 8A, E/88 cells produced IFN- γ when cocultured with DC from lymph nodes of treated mice, whereas no IFN- γ production was observed by DC alone, E88 alone, or the CD11c⁻ fraction of the magnetic DC separation containing lymphoid cells. TSA tumor cells were used as positive controls. The low yield of DC from lymph nodes draining tumors treated with Add170-3 precluded their use as controls in this assay. The CD11c⁻ fraction of the magnetic separation, containing lymphoid cells, was restimulated for 5 days in the presence of irradiated tumor cells or the specific AH1 peptide and then used to test CTL activity. Lymphocytes from the CD11c⁻ fraction from AdCCL16-

Table I. Reactive cell infiltration induced in TSA tumors after injection with PBS, Add170-3, or AdCCL16

	PBS	Add170-3	AdCCL16
Cells ^a			
Macrophages	21 \pm 4	28 \pm 5 ^b	32 \pm 6 ^b
CD11c ⁺ cells	14 \pm 3	23 \pm 3 ^b	32 \pm 5 ^c
CD8 ⁺ cells	15 \pm 3	21 \pm 3 ^b	44 \pm 9 ^c
CD4 ⁺ cells	12 \pm 2	24 \pm 4 ^b	34 \pm 5 ^c
Granulocytes	3 \pm 1	7 \pm 1	8 \pm 2
NK cells	7 \pm 2	10 \pm 2	14 \pm 3

^a Cell counts performed at $\times 400$ in a 0.180-mm² field. At least three samples (one sample per tumor growth area) and 10 randomly chosen fields/sample were evaluated. Results are expressed as the mean \pm SD of positive cells per field evaluated on cryostat sections by immunohistochemistry.

^b Values significantly different ($p \leq 0.005$) from corresponding values of TSA tumors injected with PBS.

^c Values significantly different ($p \leq 0.005$) from corresponding values of TSA tumor injected with Add170-3.

treated, but not Add170-3-treated, tumors elicited specific CTLs against TSA cells, AH1-pulsed blasts cells, and C26 colon carcinoma cells, which share the same TAA (Fig. 8B). Together these results suggest that DC infiltrating the lymph nodes took up and processed the TAA for presentation to CTL clones as well as for priming of naive T cells in the draining lymph nodes.

AdCCL16 treatment of metastatic disease

Despite the strong proinflammatory activity and the initiation of an effective immune response, intralesional administration of AdCCL16 led to tumor rejection in only 30% of mice. Although addition of costimuli and/or cytokines might have increased the cure rate, we focused on the potential usefulness of AdCCL16 in preventing metastatic spread. Perhaps the most compelling model for studying spontaneous metastases, at least on a BALB/c background, is the 4T1 mammary carcinoma (38), because metastases spread immediately after tumor injection, and the process is not impaired by removal of primary tumor (46). The 4T1 tumor model has been used extensively by Ostrand-Rosenberg and colleagues (37, 47, 48) to study the therapeutic potential of cellular vaccines composed of various immunomodulatory molecules on metastatic spread.

As in TSA model, treatment of the 4T1 established tumors with AdCCL16 significantly inhibited primary tumor growth, but tumors eventually regrew, and no complete rejection was observed (Fig. 2B).

To determine whether AdCCL16 treatment of the primary 4T1 tumor might affect metastatic dissemination and whether surgical removal of the primary tumor after treatment might increase survival, tumor-bearing mice were inoculated with AdCCL16 or the control virus on days 10 and 14; in one group of mice, the primary tumor was surgically removed on day 19. At this time tumors size ranged between 3 and 6 mm in diameter, and it is well known that mice with tumors of 2 mm have already disseminated metastases (37). All mice were sacrificed on day 29, and the number of lung metastasis was scored by clonogenic assay. Fig. 9A schematizes the experimental protocol.

AdCCL16 reduced the number of lung metastases relative to the group that received the control virus (range, 0–40 vs 50–5000; $p < 0.001$), and when CCL16 treatment was combined with surgical removal of the primary tumor, 11 of 14 mice were metastases free, and the remaining three mice had < 10 metastases (range, 0–8 vs 90–4000 in mice receiving Add170-3 plus surgery; $p < 0.001$; Fig. 9B). The abrogation of lung metastases obtained by

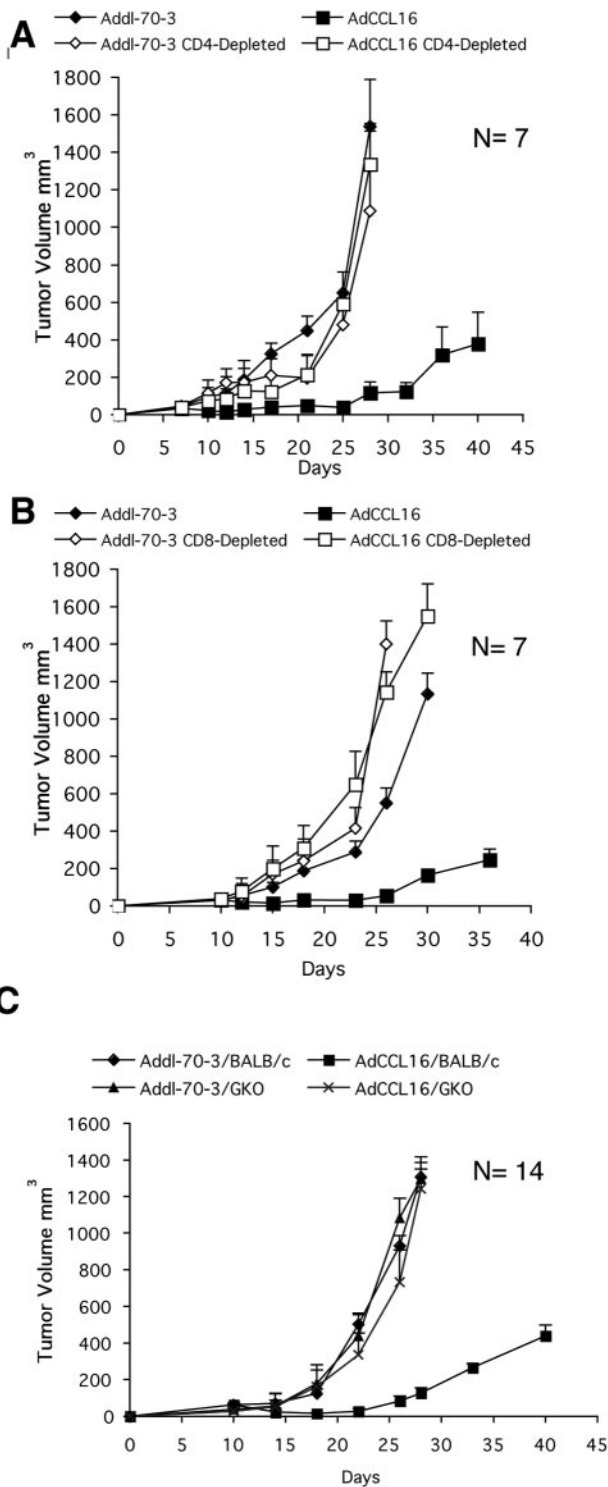


FIGURE 4. Contribution of CD4⁺ and CD8⁺ cells and IFN- γ to the AdCCL16 antitumor effect. Tumor growth was monitored in mice depleted of CD4⁺ cells (A) and CD8⁺ cells (B) and in GKO mice (C) treated with AdCCL16 or Addl70-3. The number of mice is indicated in each panel. Data are given as the mean \pm SD tumor volume.

combining CCL16 treatment with surgery was mainly T cell mediated, as the experiment performed in *nu/nu* mice showed a full metastatic capacity of 4T1 cells (Fig. 9C).

Moreover, of a total of 30 mice that received AdCCL16 and surgery in three independent experiments, 19 were cured (63%; $p < 0.001$), whereas all 24 mice treated with Addl70-3 and surgery

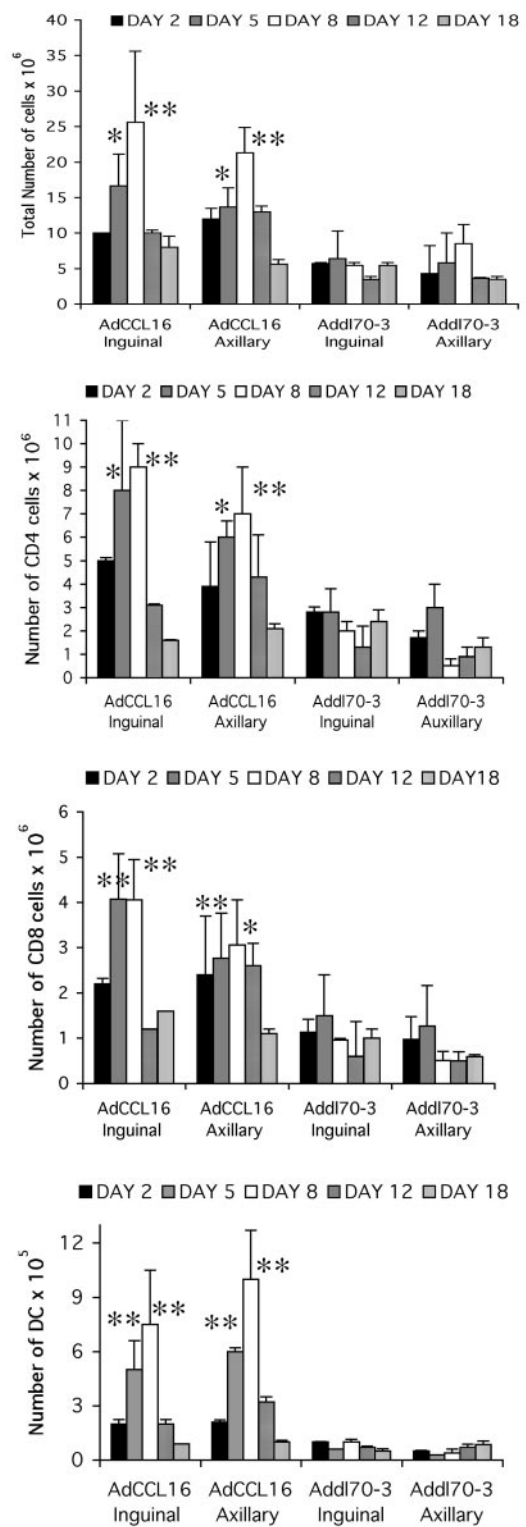


FIGURE 5. Enlargement of draining lymph nodes after AdCCL16 treatment. Inguinal and axillary draining lymph nodes were harvested 2, 5, 8, 12, and 18 days after the first injection of AdCCL16 or Addl70-3 and processed as described in *Materials and Methods*. Lymph node enlargement was due to the increased numbers of cells per lymph node. The numbers of CD4⁺ and CD8⁺ lymphocytes as well as of CD11c DC were greatly increased. Three mice were examined at each time point. Data are given as the mean \pm SD number of cells. Results are representative of two independent experiments with similar results. Statistical difference is given for each time point and compares the numbers of cells collected from lymph nodes draining AdCCL16- or Addl70-3-injected tumors: *, $p < 0.05$; **, $p < 0.01$.

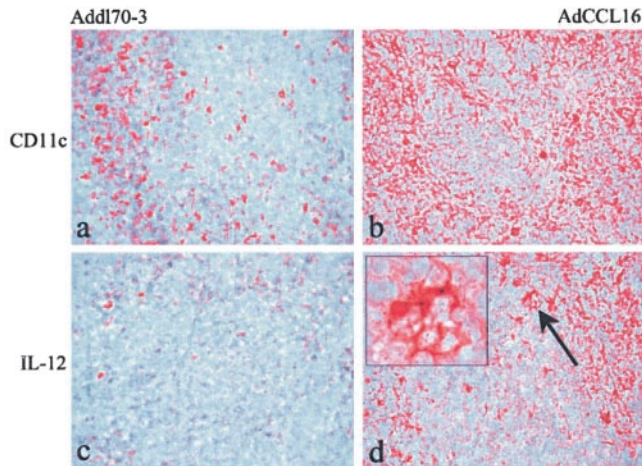


FIGURE 6. Immunohistochemical analysis of inguinal lymph nodes draining TSA tumors on day 5 after treatments. CD11c⁺ cells were more represented in the paracortical T cell areas of the lymph node draining AdCCL16-injected tumors (B) composed with those draining Addl70-3-injected tumors (A). In addition, lymph nodes draining AdCCL16-injected TSA tumors revealed distinct expression of IL-12 in cells with cytoplasmic projections identifiable as DC (the inset shows the detail indicated by the arrow at $\times 1000$ magnification) inside the paracortical areas and sometimes in the medullary cords (D). By contrast, IL-12 expression was barely detected in the lymph node draining Addl70-3-injected tumor (C; $\times 400$).

succumbed (Fig. 9D). Challenge of surviving mice with 10^4 live 4T1 cells 90 days after primary tumor injection revealed protection in 12 of 19 mice (63%), indicating a long term immunity (not shown).

Discussion

Currently, the most advanced vaccine formulations are thought to be those that involve presentation of TAAs directly by DC or indirectly through DC-mediated cross-priming (23). Most experimental data obtained with transplantable tumors show that the best vaccine formulations effectively preimmunize mice against even a poorly or apparently nonimmunogenic tumor challenge (49), but their efficacy declines if administered when the tumor overcomes a critical threshold and becomes clinically evident (2). Generally, only a minority of mice bearing established tumors have been cured, and only when the vaccine was administered in the first few days after tumor cell challenge (50). Similarly, few patients with established tumors display an objective response, which, in any event, are only temporary (51). Those results are not altogether surprising, considering that most of those patients have already been treated in various ways and no longer respond to conventional therapy. These considerations underline the need to investigate the use of molecules involved in the immune response in the context of existing tumors and after removal of primary tumor (2).

CCL16 is remarkable *in vivo* with respect to rapid induction of systemic immunity and tumor rejection. Both features are important when dealing with transplantable murine carcinomas, as tumors can double or triple in size during the time required for vaccination to “instruct” the immune system. When transfected into TSA cells, CCL16 rendered these cells unable to form tumors *in vivo* due to rapid rejection by locally recruited monocytes, DC, and CD8⁺ T cells. Moreover, mice injected with CCL16-transfected tumor cells were able to reject a contralateral challenge of the parental cells as early as 6 days later, indicating rapid development of systemic immunity (32).

These effects seem paradoxical in light of the weak chemotactic properties described for CCL16, *i.e.*, it requires 6–12 times the

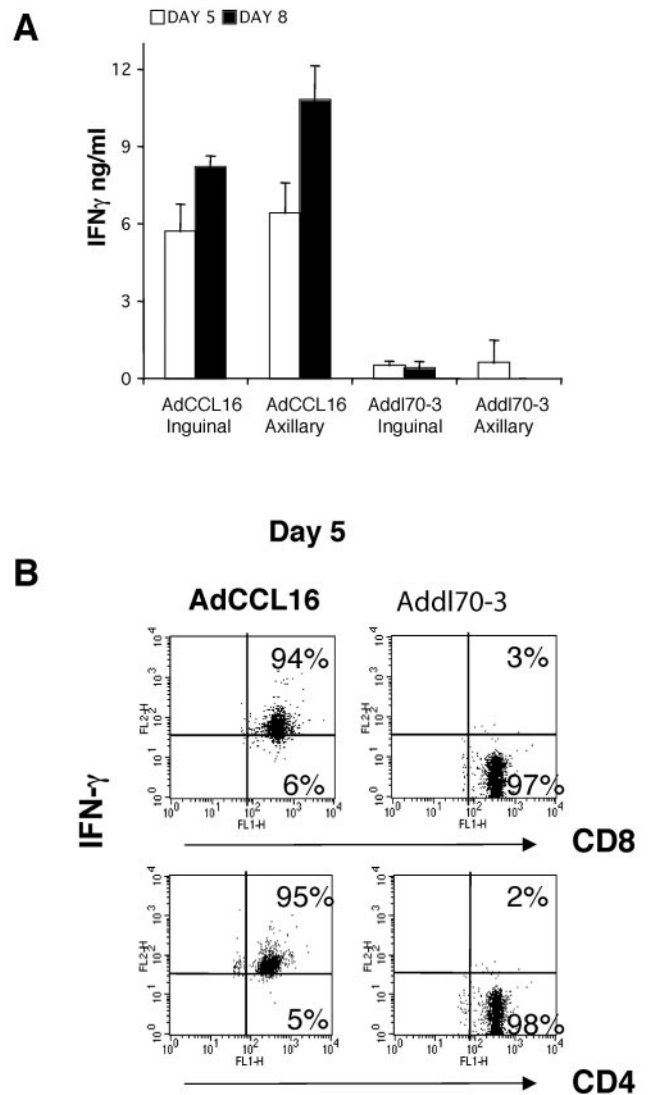
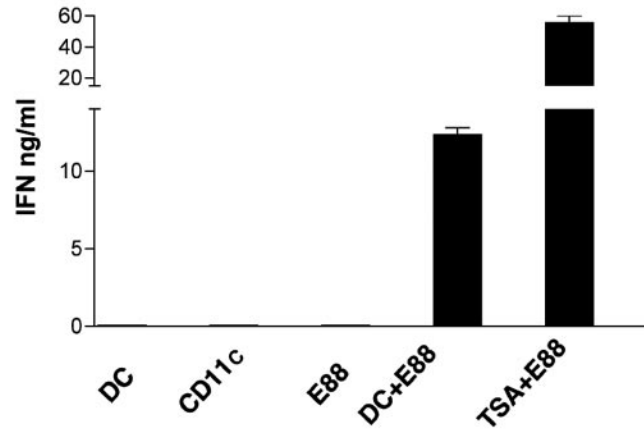


FIGURE 7. Production of IFN- γ by lymph nodes draining treated tumors. Lymph node cells were isolated on days 5 and 8 after the first adenoviral injection. IFN- γ production was evaluated by ELISA (A) and by mouse IFN- γ secretion assay (B) as described in *Materials and Methods*. Three mice were analyzed for each time point. The data in A are given as the mean \pm SD IFN- γ level ($p < 0.05$). Flow cytometry plots in B show the expression of IFN- γ among gated CD4⁺ or CD8⁺ cells; the percentage of positive cells is indicated in the top right corner of the plot. Results are representative of two independent experiments.

concentration of other CC chemokine for activity (30). Most likely, CCL16 induces chemokines and cytokines by nearby leukocytes that provide the appropriate signal for massive inflammatory infiltration. In this context, we found that 1) CCL16 induces direct migration *in vitro* of CD4⁺ and CD8⁺ lymphocytes other than DC at concentrations of 1000–10 ng/ml, and 2) CCL16 induces potent CCL2 production by macrophages (52), DC, and T cells, but not CCL3 or CCL20 production (C. Guiducci, unpublished observation).

The treatment of established 3- to 4-mm TSA tumors, instead of injection of CCL16-transfected cell suspension, necessitated delivery of CCL16 via an adenoviral vector. This size of the TSA tumors exceeded that of any previous therapeutic attempt using immunological strategies (53). *In vivo*, CCL16 production upon adenoviral delivery was quite similar to that of *in vitro* transduced TSA cells, and the extent of ensuing tumor leukocyte infiltration was also similar. In contrast, the use of *in vitro*-transfected cells

A CD11c⁺ fraction



B CD11c⁻ fraction

— AdCCL16
 Add170-3

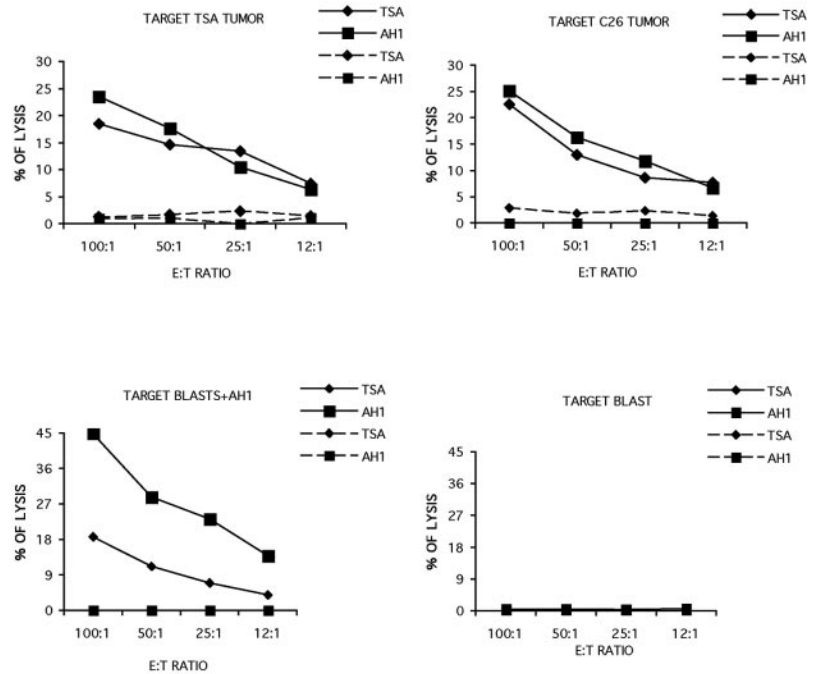


FIGURE 8. Functional characterization of DC from lymph nodes draining treated tumors. Tumors were treated with AdCCL16 or Add170-3 on days 10 and 14 after challenge. Two days after the last treatment, draining lymph nodes were isolated, and DC were separated by magnetic beads. Two lymph node pools, each from three mice, were processed independently. *A*, E88 (1×10^5) cells were incubated with stimulating cells at a ratio of 1:1 for TSA cells and 1:2 for purified DC for 18 h. DC-, E88-, or CD11c-negative fractions were incubated alone as negative controls. IFN- γ in the medium was tested by ELISA (mean \pm SD relative to the two independent groups). *B*, Lymphocytes from the CD11c-negative fraction were restimulated in vitro with TSA cells or AH1 peptide and tested 5 days later for CTL activity. Results are representative of two independent experiments

led to 85% tumor rejection vs 30% complete rejection when AdCCL16 was inoculated to established tumors. This observation is consistent with the difficulties for the immune response in eradicating large tumors, as underlined by the ability to reject normal tissue but not tumors expressing the same target Ag (54), and by the numerous mechanisms used by tumors to escape immune attack (55). We studied the mechanisms underlying the rapid systemic immune

response starting from the observed swelling of lymph nodes draining the AdCCL16-injected, but not control Add170-3-injected, tumors. Node enlargement reflected the increased numbers of CD4⁺, CD8⁺ T lymphocytes and DC. Among DC purified from these lymph nodes, some might come from the tumor, as no tumor cells were found by PCR analysis in the node at that time point (not shown). Such DC stimulated a T cell clone specific for the TSA TAA (45), but not a

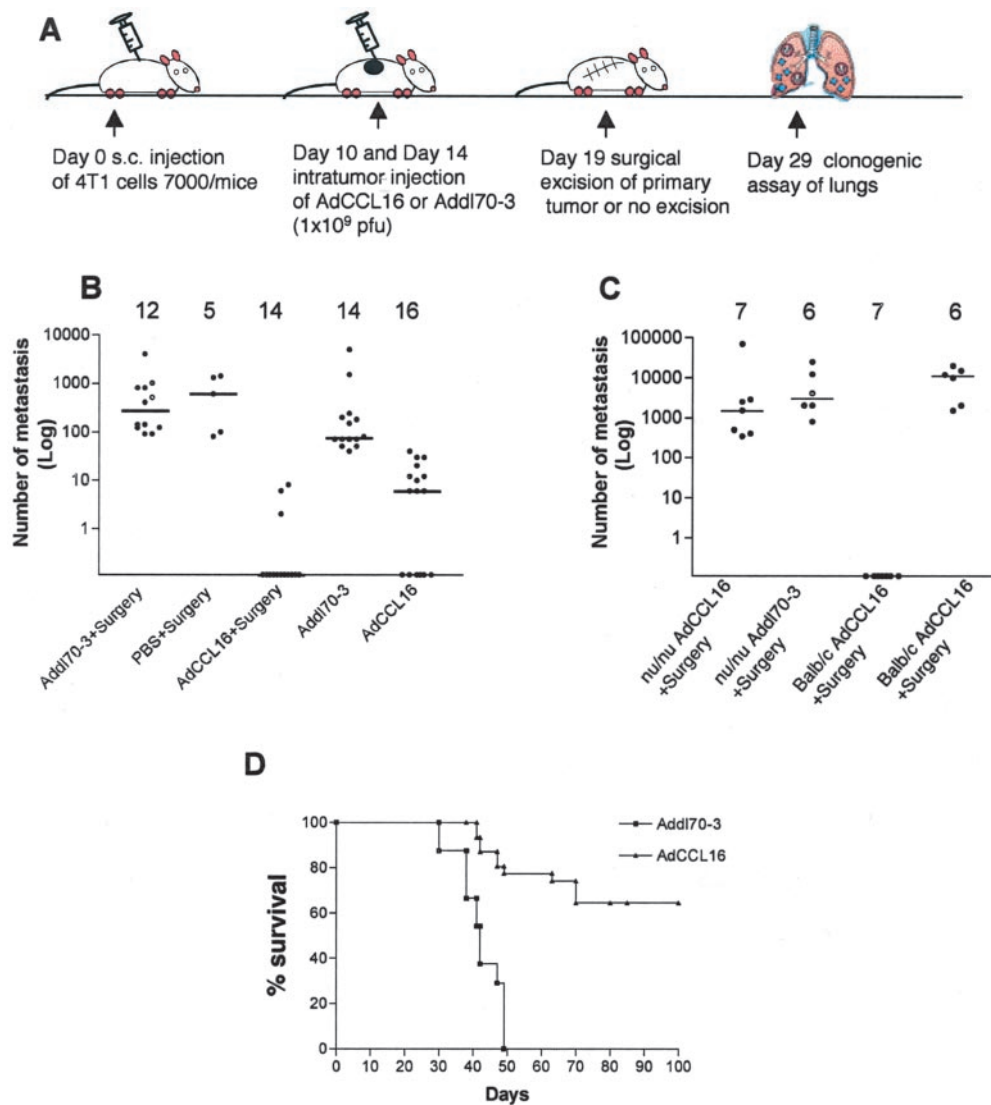


FIGURE 9. Effects of AdCCL16 treatment on metastatic spread of 4T1 tumors. *A*, Experimental scheme. *B*, Number of metastasis. The total number of treated mice is indicated at the top of the panel. Results are from two independent experiments. Horizontal lines represent the median. *C*, Number of metastasis in *nu/nu* mice compared with that in WT BALB/c mice. *D*, Survival curve after AdCCL16 treatment and surgical excision of the primary tumor. Cumulative results of three independent experiments (a total of 30 mice treated in the AdCCL16 treatment group and 24 in the Add170-3 control group).

clone recognizing the same Ag in the context of a different H-2 haplotype (Fig. 8*A* and data not shown) (56). Consistent with this finding, the lymphoid fraction remaining after CD11c magnetic beads sorting produced IFN- γ (data not shown) and induced CTL activity against TSA and its TAA immunodominant epitope AH1 (Fig. 8*B*). Induction of IFN- γ production by T lymphocytes was not a direct effect of CCL16 expression, as CD4⁺ and CD8⁺ purified T cells *in vitro* did not produce IFN- γ upon addition of recombinant CCL16 (data not shown).

The robust and specific immune response against the tumors was therapeutic for only ~30% of mice. Our effort to improve this rate disliked the idea to increase the number of AdCCL16 injections because of the vector immunogenicity; rather, it focused on application of AdCCL16 in a more clinically relevant context that generally requires surgical removal of primary tumor, because tumor lethality usually stems from the relatively small number of cells that remain after surgical excision, radiotherapy, and chemotherapy.

Although the TSA carcinoma has metastatic capacity, this model is cumbersome (53). Perhaps the most compelling model for studying spontaneous metastases, at least on a BALB/c back-

ground, is the 4T1 mammary carcinoma (38), as metastases spread immediately after tumor injection, and the process is not impaired by removal of primary tumor (46). The 4T1 tumor model has been used extensively by Ostrand-Rosenberg and colleagues (37, 47, 48) to study the therapeutic potential of cellular vaccines composed of various immunomodulatory molecules on metastatic spread.

Vaccination with tumor cells transfected with MHC class II and B7.1 without removal of the primary tumor reduced the number of metastases, but had no impact on survival (28). Also, treatment with an immunostimulator as mB7-2 Ig fusion protein combined with the angiogenesis inhibitor SU668 started as earlier as 3 days after tumor injection significantly reduced tumor growth and number of lung metastases, but did not achieve a cure (57).

A critical issue is the size of the tumor at the time of vaccination, as tumors >4 mm in diameter already have a large metastatic load, precluding successful treatment (28). Surgical removal of the primary tumor as the first line of treatment in the 4T1 model was not sufficient to arrest metastases-induced death, but did change the setting in which a vaccine is called to act against residual disease

(numerous small metastases). In 4T1 tumor-bearing mice, removal of primary tumors followed by repeated vaccination with MHC class II- and B7-1-cotransfected cells plus cells transfected with the staphylococcal enterotoxin B superantigen-encoding gene, significantly extended survival time, but did not cure the mice (48). A more effective strategy might involve the treatment of primary tumors before surgery, an approach that might reduce the time for priming the host against its tumor. In fact, AdCCL16 injected into tumor nodules twice on days 10 and 14, followed by surgical removal on day 19, cured 19 of 30 mice (63%) and reduced the number of lung metastases in mice sacrificed for clonogenic assay (three mice with <10 metastases and 11 free of metastases). A similar approach using a combination of adenoviruses encoding thymidine kinase, IL-2, and GM-CSF reduced the number of lung metastases and prolonged survival of six mice of 20 in a follow-up of only 35 days (58). A rough comparison of those data and ours suggests that CCL16 is more effective than such multiple combinations.

However, improved results can be obtained only through a more intensive treatment protocol such as surgery, followed by vaccination with syngeneic tumor cells mixed with bystander cells, and finally donor lymphocyte infusion of mice transplanted with allogeneic stem cells under nonmyeloablative conditions (59).

A curative rate of 60% was also obtained by surgical excision of primary 4T1 tumor injected into STAT6^{-/-} mice (60, 61). Although the lack of STAT6 signaling protects these mice from IL-13-mediated immunosuppression (61, 62), the recognition of tumor-associated STAT6 as a foreign Ag is a possibility (62).

It is unclear why the effectiveness of this AdCCL16 treatment on primary TSA and 4T1 tumor regression is limited to a small number of mice. In the 4T1 model, the lack of STAT6 impairs the development of metastases, but is not sufficient to induce primary tumor rejection (61). In addition to NKT cell-produced IL-13 immunosuppression of an unknown target cell (63), other immunosuppressive cytokines, such as IL-10, PGE₂, and TGF- β , produced by tumors or infiltrating leukocytes impair the function and survival of tumor-associated APC (64). In this context, strategies aimed at neutralizing IL-10 in the TSA model (65) or TGF- β in the 4T1 model have led to the functional restoration of tumor-infiltrating APC (66). The promising results obtained with CCL16 given alone suggest that the therapeutic potential of this approach might be augmented by strategies that further improve Ag-presenting functions of DC recruited at the tumor site.

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