

Redirecting *In vivo* Elicited Tumor Infiltrating Macrophages and Dendritic Cells towards Tumor Rejection

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Abstract

A hostile tumor microenvironment interferes with the development and function of the adaptive immune response. Here we report the mechanisms by which large numbers of tumor-infiltrating macrophages and dendritic cells (DC) can be redirected to become potent effectors and activators of the innate and adaptive immunity, respectively. We use adenoviral delivery of the CCL16 chemokine to promote accumulation of macrophages and DC at the site of preestablished tumor nodules, combined with the Toll-like receptor 9 ligand CpG and with anti-interleukin-10 receptor antibody. CpG plus anti-interleukin-10 receptor antibody promptly switched infiltrating macrophages infiltrate from M2 to M1 and triggered innate response debulking large tumors within 16 hours. Tumor-infiltrating DC matured and migrated in parallel with the onset of the innate response, allowing the triggering of adaptive immunity before the diffuse hemorrhagic necrosis halted the communication between tumor and draining lymph nodes. Treatment of B6>CXB6 chimeras implanted with BALB/c tumors with the above combination induced an efficient innate response but not CTL-mediated tumor lysis. In these mice, tumor rejection did not exceed 25%, similarly to that observed in CCR7-null mice that have DC unable to prime an adaptive response. The requirement of CD4 help was shown in CD40-KO, as well as in mice depleted of CD4 T cells, during the priming rather than the effector phase. Our data describe the critical requirements for the immunologic rejection of large tumors: a hemorrhagic necrosis initiated by activated M1 macrophages and a concomitant DC migration to draining lymph nodes for subsequent CTL priming and clearing of any tumor remnants. (Cancer Res 2005; 65(8): 3437-46)

Introduction

Although tumors are capable of autonomous growth, their progression is highly influenced by their stromal component (1). Thus, the tendency of novel therapeutic approaches is to target both the tumor cells and their embedding stroma. The extracellular matrix, blood vessel fibroblasts, and tumor-infiltrating leukocytes such as macrophage, in addition to their structural role, may exert either inhibiting or promoting effects on tumor growth (2, 3). Two distinct activation states of macrophages, which

are among the most abundant leukocytes infiltrating mouse and human tumors, have been described: conventionally activated M1 macrophages produce high levels of inducible nitric oxide synthase, interleukin (IL)-12, and tumor necrosis factor (TNF), whereas the alternatively activated M2 macrophages produce arginase, IL-10, transforming growth factor- β (TGF- β), and prostaglandin E2 (PGE2). M1 macrophages are extremely potent effector cells that kill tumor via nitric oxide and TNF (4), whereas M2 macrophages promote angiogenesis and tissue remodeling and limit T-helper 1 immune responses (2). Macrophages with M2 phenotype have been isolated from mouse and human tumors and shown to suppress T-cell activation and proliferation through IL-10 and TGF- β (5). Thus, although tumor-infiltrating macrophages are potentially able to induce an antitumor innate response, their activation state is modulated in the tumor microenvironment and they may be redirected to exert a tumor growth-promoting effect. Similarly to macrophages, tumor-infiltrating dendritic cells (DC) have been shown to be defective in their ability to produce proinflammatory cytokine such as IL-12 and to induce innate IFN- γ production or adaptive T-helper 1 responses.

The limited success of immunotherapy protocols targeting a single tumor-associated antigen (TAA) suggests that protocols attempting to elicit a response against the whole antigenic repertoire of TAAs expressed by the tumor cells could be more effective. Although the inadequate presentation of TAAs to T cells is one of the mechanisms of tumor escape (6), DC, the most effective antigen-presenting cells, could potentially trigger such a response on infiltration of tumors.

To improve antitumor immunity, tumor cells have been transduced with genes encoding molecules able to attract and to activate DC or DC recruitment at the tumor site has been induced by *in situ* delivery of chemokines (7). However, several studies underlined the limited efficacy of chemokines, used as single agents, in curing established (>3 mm) tumors (7, 8).

We have previously reported that the delivery via adenoviral vector of the chemokine CCL16 in established TSA mammary carcinomas induced a specific systemic antitumor response and a massive accumulation of leukocytes (T cells, macrophages, and DC) at the tumor site (8). However, a complete tumor rejection was not accomplished, possibly due to the lack of proper activation of the tumor-infiltrating DC. Indeed, to initiate and maintain an effective antitumor response after TAA uptake, DC should migrate to draining lymph nodes and prime T cells. This process is triggered by an activation-driven maturation process of DC characterized by up-regulation of costimulatory molecules (CD40, CD80, and CD86), a switch in the chemokine receptor repertoire, and production of immunomodulatory cytokines (IL-12 and INF- α) necessary for the generation of CTL (9). However, immunosuppressive cytokines such as IL-10, TGF- β , PGE2, and vascular endothelial growth factor interfere with DC maturation

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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and migration, hampering the antitumor response. DC paralysis is not always restricted to the tumor microenvironment but has also been found systemically in cancer patients as well as in tumor-bearing mice (6). Thus, vaccines based on DC-mediated activation of antitumor response might be ineffective due to tumor-induced inhibition of antigen presentation.

A possible immunotherapeutic approach would be to target the tumor escape mechanisms that paralyze the macrophage/DC tumor infiltrate and prevent both innate and adaptive antitumor responses. Indeed, repeated treatments with a combination of a microbial stimulus (a Toll-like receptor 9 ligand CpG oligonucleotide) and an antibody blocking the IL-10 receptor (IL-10R) were able to revert the functional paralysis of tumor-infiltrating DC and to reestablish their ability to produce IL-12 (10). Here, we report that the combination of local treatment with CCL16 and CpG together with the systemic administration of a monoclonal antibody to IL-10R cured the majority of mice bearing large tumors. This treatment induced within a few hours a shift in resident and recruited tumor-infiltrating macrophages from the M2 into the M1 type. In addition, tumor-infiltrating DC rapidly up-regulated costimulatory molecules and secreted inflammatory cytokines such as IL-12 and TNF. This potent inflammatory response not only rapidly induced massive hemorrhagic necrosis and tumor shrinkage but also induced DC migration to draining lymph nodes before tumor necrosis and the generation of a tumor-specific immune response able to clear any tumor remnant.

Materials and Methods

Tumors and mice. Eight- to ten-week-old female BALB/c and C57/BL6 mice were purchased from Charles River (Calco, Italy). IL-12p35-knockout (KO) mice (11) on BALB/c background and TNF-KO mice on C57/BL6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). CD40-KO mice, backcrossed for 10 generations to BALB/c, were kindly provided by L. Adorini (Roche, Milan, Italy; ref. 12). B6.SJL/J (CD45.1) were purchased from The Jackson Laboratory. CCR7-KO mice were kindly provided by M. Lipp (Max-Delbrück-Center, Berlin, Germany).

Mice were maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. TSA tumor (H-2^d) is a mouse mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse (13). MCA38 (H-2^b) is a colon carcinoma cell line established from primary cultures (14). 4T1 (H-2^d) tumor is a 6-thioguanine-resistant cell line derived from a spontaneous mammary carcinoma [American Tissue Culture Collection (ATCC)-LGC Promochem, Teddington, United Kingdom; ref. 15]. Tumor cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (BioWhittaker, Walkersville, MD).

In vivo procedures. Mice were inoculated s.c. into the right flank, equidistant from the inguinal and axillary lymph nodes, with 2×10^5 TSA, 1×10^5 MCA38, or 1×10^4 4T1 cells. After 10 to 14 days, when tumors reach 5 mm in diameter, nodules were inoculated with 1×10^9 plaque-forming units (pfu) of AdCCL16 or control virus Addl70-3 and 36 hours later with 5 μ g of CpG 1668 (MWG, Ebersberg, Germany) whereas 200 μ g of anti-IL-10R antibody (1B1.3a; IgG1) or of matching control rat IgG 1 antibody (clone GL113; ref. 10) were given i.p.

In some experiments, mice were depleted of CD4 and CD8 cells by i.p. injection of 250 μ g/mice of anti-CD4 (GK1.5 hybridoma, Lyt2; ATCC) or anti-CD8 (clone 2.43; ATCC) monoclonal antibody. Depletion was started 1 week before tumor challenge by injecting mice twice per week thereafter until the end of the experiment. In some experiments, depletion was started 5 days after tumor treatments. Fluorescence-activated cell sorting analysis (FACS) of peripheral blood confirmed that depletion was never inferior to 95%. Tumor size was monitored twice per

week and recorded as the longest diameter \times (shortest diameter)² (in cubic millimeters). Mice were sacrificed when tumors reached about 10 mm in diameter.

Lung metastases of 4T1 tumors were evaluated according to ref. 16. Briefly, lungs were collected and dissociated in a HBSS containing 1 mg/mL collagenase type 4 and 6 units/mL elastase for 1 hour at 4°C; organs were then plated at various dilutions in a medium supplemented with 6-thioguanine. Individual colonies representing micrometastasis were counted after 10 to 15 days.

In vitro and in vivo assay with tumor-infiltrating dendritic cells. Tumor-infiltrating DC were purified from TSA nodules as previously described (9). In brief, tumor masses were perfused with collagenase D solution (400 units/mL; Roche, Basel, Switzerland) and incubated in collagenase for 1 hour at 37°C. After gentle pipetting, the suspension was allowed to adhere for 4 hours at 37°C in complete RPMI medium, after which nonadherent cells were collected and purified using CD11c⁺ microbeads (Miltenyi Biotec, Bergischgladbach, Germany). Flow cytometry showed that the cells were more than 80% pure. Phenotypic characterization of tumor-infiltrating DC was done by FACS analysis using FITC-conjugated monoclonal antibodies: anti-CD40-FITC clone 3/23, anti-CD80 clone IG10, anti-CD86 clone GL1, anti-MHC-II clone B21.2, and isotype-matched monoclonal antibodies of unrelated specificity (BD Bioscience, San Diego, CA). Tumor-infiltrating DC were seeded in round-bottomed noncoated 96-well plates at a concentration of 1×10^6 /mL; after 24 hours, supernatants were collected and assayed by ELISA using 9A5 as capture antibody and C17-8 as detection antibody for IL-12, and using XT22 as capture antibody and XT3 as detection antibody for TNF (Endogen, Rockford, IL). For MLR assay, CD4⁺ T lymphocytes were purified from spleen of C57/BL6 mice using CD4 microbeads (Miltenyi Biotec). Triplicates of 2×10^5 CD4 T cells were seeded in round-bottomed noncoated 96-well plates with various ratios of irradiated (3,000 rad) tumor-infiltrating DC from TSA-treated tumors for 5 days. [³H]Thymidine (1 Ci/well, Amersham, Chalfont St. Giles, United Kingdom) was added for the last 10 hours of culture. For *in vivo* DC migration experiments, mice bearing TSA tumors were intratumorally injected with the indicated treatment plus 2×10^7 FITC-conjugated latex particles of 1 μ m in diameter (Polysciences, Warrington, PA). At the indicated time points, mice were sacrificed and draining lymph nodes were collected, teased, and incubated for 1 hour in collagenase D solution (400 units/mL).

Cells were stained for CD11c expression and 5×10^5 events per lymph node were acquired by cytofluorimetry. The total number of double-positive CD11c-phycoerythrin/FITC-bead events was calculated for each lymph node.

Histologic and immunohistochemical analysis. For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with Masson's trichrome.

For immunohistochemistry, acetone-fixed 5- μ m cryostat sections were incubated for 5 minutes in 3% methanol/H₂O₂ to block endogenous peroxidase. After washing, sections were incubated for 1 hour with the following antibodies: purified rat anti-mouse CD11c (clone N418, eBioscience, San Diego, CA), biotin-conjugated rat anti-mouse CD11b (clone M1/70.5, BD Bioscience), and FITC-conjugated rat anti-mouse F480 (C1:A31, Caltag, Burlingame, CA). After washing, sections were sequentially overlaid with biotinylated goat anti-rat IgG for 30 minutes (Vector, Burlingame, CA), and with avidin-peroxidase complex (Sigma-Aldrich, St. Louis, MO) for 30 minutes. Avidin-peroxidase complex or peroxidase-conjugated rabbit anti-FITC (DakoCytomation, Glostrup, Denmark) was used as secondary reagent for sections incubated with biotinylated or FITC-conjugated primary antibodies, respectively. Antigens were revealed with 3,3'-diaminobenzidine (Sigma-Aldrich) according to the instructions of the manufacturer. Sections were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol (70%, 95%, and 100% ethanol), and mounted in BDH medium (Merck Eurolabs, Lutterworth, United Kingdom).

For the intracellular staining of cytokines, 5- μ m cryostat sections were fixed with 2% paraformaldehyde for 20 minutes and washed. Sections were sequentially incubated for 30 minutes with the following solutions made in PBS: (a) 1% bovine serum albumin; (b) 0.1% saponin/1% FCS. After washing, sections were incubated with avidin-biotin blocking solution

(Vector) for 15 minutes. After washing, sections were incubated with the following monoclonal antibodies: rat anti-mouse IL-10 (clone JES5-2A5, eBioscience), rat anti-mouse TNF (clone XT22, BD Bioscience), and rat anti-mouse IL-12p70 (clone 9A5, Endogen). One hour later, sections were washed and then incubated with secondary antibodies and revealed as previously described.

All images were captured on a Nikon microscope equipped with a digital camera (DXM 1200, Nikon) and analyzed using ACT1 (Nikon) software. All immunolocalization experiments were repeated thrice with multiple serial sections including negative controls to determine the background staining. The number of immunostained cells was determined by light microscopy (magnification, $\times 400$) in 10 randomly chosen fields on a 1-mm² grid and is given as cells per square millimeter (mean \pm SD).

Nitric oxide evaluation. Total nitric oxide in tumor samples was determined as function of nitrate and nitrite concentrations using a specific kit (nitrate and nitrite colorimetric assay kit, Cayman, Ann Arbor, MI). In brief, 5-mm tumors were treated with the various combinations, collected 4 hours later, and perfused and minced in 300 μ L of PBS. The homogenate was centrifuged at 10,000 rpm for 20 minutes and then ultra filtered using a 30-kDa molecular weight cutoff (Millipore, Billerica, MA). Forty microliters of each filtrate were assayed for nitrate and nitrite concentration according to the instructions of the manufacturer.

Bone marrow chimera. CB6F1 mice were lethally irradiated with 900 cGy (given as a split dose of 450 + 450 cGy at 3-hour interval). Two hours later, mice were injected i.v. with 2×10^7 bone marrow nucleated cells obtained from BALB/c or C57L/B6 mice. Bone marrow cells were obtained by flushing the cavity of freshly dissected femurs with complete RPMI medium. To verify engraftment, peripheral blood mononuclear cells withdrawn from the retro-orbital sinus at 8 weeks after bone marrow transplantation were stained with FITC-conjugated anti-mouse H-2K^b and phycoerythrin-conjugated anti mouse H-2K^d, as well as matching isotype controls. B6.SJL mice were reconstituted with bone marrow from TNF-KO mice to obtain mice lacking TNF in the bone marrow compartment. Engraftment was verified using CD45.2 antibody (BD Bioscience). We used mice showing at least 95% of donor phenotype.

Cell-mediated cytotoxicity assay. Splenocytes from treated mice were restimulated *in vitro* with the TSA-specific peptide AH1 (1 μ g/mL) in complete medium. The *env* gene of the endogenous ecotropic MuLV, containing the L^d-restricted peptide AH1, is expressed by TSA tumor as an immunodominant antigen (8, 17). After 5 days, cytotoxic activity was tested in a standard 4-hour ⁵¹Cr release assay. Blast cells, pulsed or not with AH1 peptide, as well as TSA cells were used as specific targets 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, and for survival curves, which were analyzed using the log-rank test. All analyses were done using Prism software (GraphPad Software). Differences were considered significant at *P* < 0.05.

Results

CCL16 potently synergizes with CpG and anti-interleukin-10 receptor to reject large tumors. We tested whether adenoviral vector delivery of CCL16 into large established tumors in combination with a single injection of CpG and anti-IL-10R synergized to reject larger primary tumors. Tumors of 5 mm or larger were injected with AdCCL16 and 36 hours later with CpG whereas anti-IL-10R was given systemically. Three models of transplantable tumors were tested: TSA and 4T1, two mammary carcinomas of BALB/c origin, and MCA38, a C57BL/6 colon carcinoma. When mice were treated with CCL16 in combination with either anti-IL-10R or CpG, less than 20% of mice rejected the tumor. Similarly, control virus Addl70-3 combined with CpG and anti-IL-10R showed little therapeutic effect (20% to 30% tumor rejection). In contrast, when CCL16 was combined with CpG and anti-IL-10R, up to 90% of the mice rejected established TSA

tumors, 74% rejected the MCA38 tumors, and 60% rejected the 4T1 tumors (Fig. 1A-C; all *P* < 0.001 versus Addl70-3 + CpG + anti-IL-10R control groups). Mice rendered tumor free after a single treatment with CCL16 and CpG plus anti-IL-10R acquired long-term immunity and rejected a challenge with live tumor cells 100 days later (data not shown).

We already had confirmed (8) the metastatic potential of 4T1 cells that disseminate to several distant organs as soon as the primary tumor reaches 2 mm in diameter (18).

To determine whether the combination treatment affected the metastatic dissemination of 4T1 mammary carcinoma, 4T1 nodules were treated with AdCCL16 or Addl70-3 combined or not with CpG plus anti-IL-10R. Mice were sacrificed at day 32 after tumor inoculation and scored for lung metastases by a clonogenic assay. Whereas treatment with AdCCL16 significantly reduced the number of lung metastases (range of 12,000-160,000 versus 1,000-23,600 for Addl70-3 versus AdCCL16; *P* = 0.02), the additional treatment with CpG plus anti-IL-10R completely prevented metastases in 7 of 14 mice and reduced the number to less than 10 in the remaining 7 mice (Fig. 1D; range of 1,000-2,000 versus 0-12 for AdCCL16 or Addl70-3 versus combination with CpG + anti-IL-10R; *P* < 0.001).

The early antitumor effect of CCL16 and CpG plus anti-interleukin-10 receptor combination is the result of a rapid innate inflammatory response. Tumor-bearing mice treated with Addl70-3 or AdCCL16 combined or not with CpG and anti-IL-10R were sacrificed for macroscopic evaluation of the tumor area 6 and 16 hours posttreatment. Although CpG plus anti-IL-10R combined with control Addl70-3 showed partial necrosis, only the combination with AdCCL16 induced tumor shrinkage associated with a massive necrosis that was mostly colliquative as indicated by blood extravasation already present 6 hours posttreatment (Supplementary Fig. 1). Microscopically, tumors from the animals treated with Addl70-3 and CpG plus anti-IL-10R showed discrete areas of necrosis, whereas in the nodules treated with AdCCL16 and the same combination the necrotic area extended to almost the entire tumor mass (Fig. 2).

To gain insight on the mechanism of the synergistic effect of CCL16 and CpG plus anti-IL-10R, we analyzed the cellular infiltrate at the tumor site that was presumably recruited through the chemotactic properties of CCL16. TSA tumors treated with a single injection of AdCCL16 showed a significantly higher number of F480⁺ macrophages and CD11c⁺ DC than tumors treated with control adenovector or PBS (*P* < 0.001; Supplementary Fig. 2A). Macrophages infiltrating CCL16-treated tumors produced IL-10, as suggested by staining colocalization, but not detectable levels of TNF or IL-12, consistent with the M2 phenotype (ref. 2; Supplementary Fig. 2A and B and data not shown). In tumors treated with the control adenovector or PBS, macrophages, although less numerous, also colocalized with IL-10 expression (not shown), indicating that, although CCL16 is chemotactic for macrophages, it is unable to prevent their alternative activation into a M2 phenotype once they have been recruited into the tumor microenvironment.

The addition of CpG plus anti-IL-10R to the CCL16 treatment drastically changed the activity of tumor-associated macrophages that became able to produce TNF and IL-12 (Fig. 3A-D). Also, AdCCL16 induced nitric oxide thrice more than Addl70-3 when combined with CpG plus anti-IL-10R (Fig. 3E). Together these results indicated the role of CCL16 in recruiting macrophages and of CpG plus anti-IL-10R in restoring their ability to exert an antitumor activity through mechanisms of the innate response.

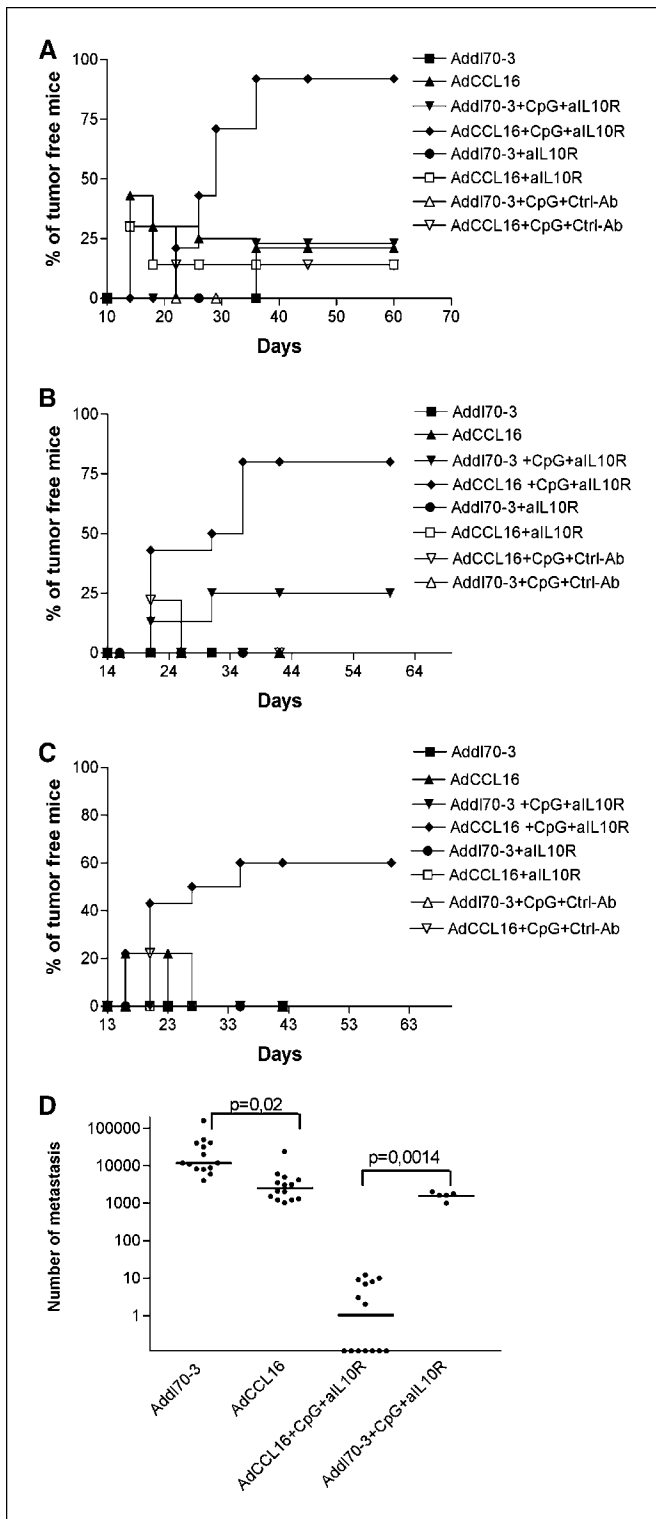


Figure 1. Combination of CCL16 and CpG plus anti-IL-10R induces rejection of preexisting tumors. Mice bearing TSA (A), MCA38 (B), or 4T1 (C) tumors were injected intratumorally with 1×10^9 pfu of AdCCL16 or control Addl70-3 and 36 hours later left untreated or treated with anti-IL-10R (200 μ g) i.p. and CpG intratumorally (5 μ g). D, effects of the combinatory treatment on the metastatic spread of 4T1 tumors. Mice bearing 4T1 tumors were intratumorally injected with 1×10^9 pfu of AdCCL16 or Addl70-3 and 36 hours later mice were left untreated or treated with CpG plus anti-IL-10R. Metastases were evaluated in the lungs 32 days after tumor inoculation and results were expressed per individual mice. Cumulative results of two independent experiments, each done with seven to eight mice.

Both innate and adaptive responses participate in the eradication of established large tumors. TSA tumor-bearing BALB/c mice treated with AdCCL16 and CpG plus anti-IL-10R developed tumor-specific systemic CTL activity as early as 7 days posttreatment (Fig. 4A). Thus, we tested whether innate resistance was sufficient or adaptive immunity was also required for complete tumor rejection. When TSA tumor-bearing Rag-KO mice, which are deficient of T and B cells, were treated with AdCCL16 and CpG plus anti-IL-10R, only 28% of them completely rejected the tumors ($P < 0.001$; Fig. 4B). Antibody-mediated depletion of either CD4 or CD8 T cells starting 1 week before tumor challenge also reduced to 50% the number of BALB/c mice rejecting TSA tumors ($P < 0.01$ versus nondepleted group; Fig. 4B). To better dissect the role of CD4 and CD8 T cells in the effector phase of the rejection process, they were depleted by antibody treatment of the mice 5 days after treatment with AdCCL16 and CpG plus anti-IL-10R. Late CD8 T-cell depletion still reduced the rejection rate to 37% compared with 85% in the nondepleted group ($P < 0.01$). Unlike CD8 T cells, late CD4 T-cell depletion did not affect the rejection rate, suggesting that CD4 T cells had a role during the priming rather than the effector phase whereas CD8 T cells were required in both phases (Fig. 4C).

Together, the above results unambiguously indicated that complete eradication of the tumors required the participation of T cells and suggested that both innate and adaptive responses were involved in tumor eradication. However, both conventional T cells as well as nonconventional T-cell subsets such as natural killer (NK) and $T\gamma/\delta$ cells can be recruited in a non-antigen-specific fashion in amplifying the innate response (e.g., by producing tumor-inhibiting cytokines such as IFN- γ). Thus, it was important to test whether mice in which T cells were present but were unable to mount a tumor-specific adaptive response were able to eradicate the tumors when treated with the combination protocol. This was accomplished by using bone marrow chimeric mice in which only

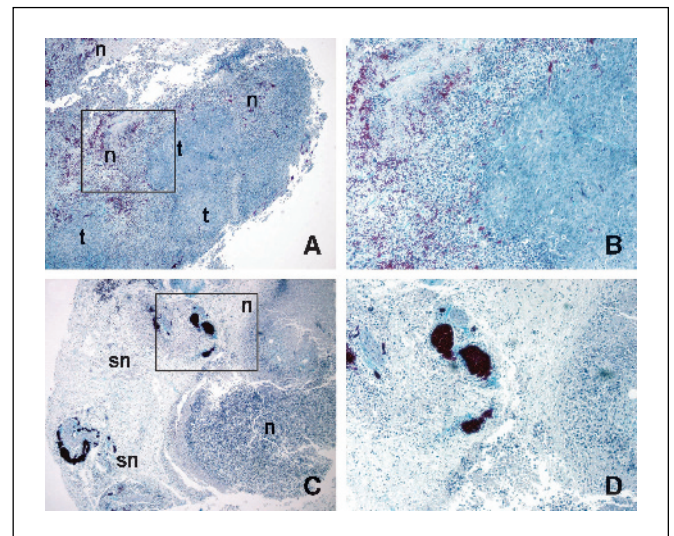


Figure 2. Histologic evaluation of treated TSA tumors. Representative sections of treated TSA tumors were stained with Masson's trichrome. Preestablished TSA tumors were treated with Addl70-3 (A and B) or AdCCL16 (C and D) together with CpG and anti-IL-10R. Mice were sacrificed 16 hours later. Tumors treated with AdCCL16 and CpG plus anti-IL-10R showed massive necrosis in both tumors (n) and stroma (sn), consistent with hemorrhagic necrosis. On the contrary, Addl70-3 combined with CpG and anti-IL-10R showed extended areas of necrosis alternated with large areas of live tumor tissue (t; $\times 40$). B and D, enlargement of boxed area ($\times 100$).

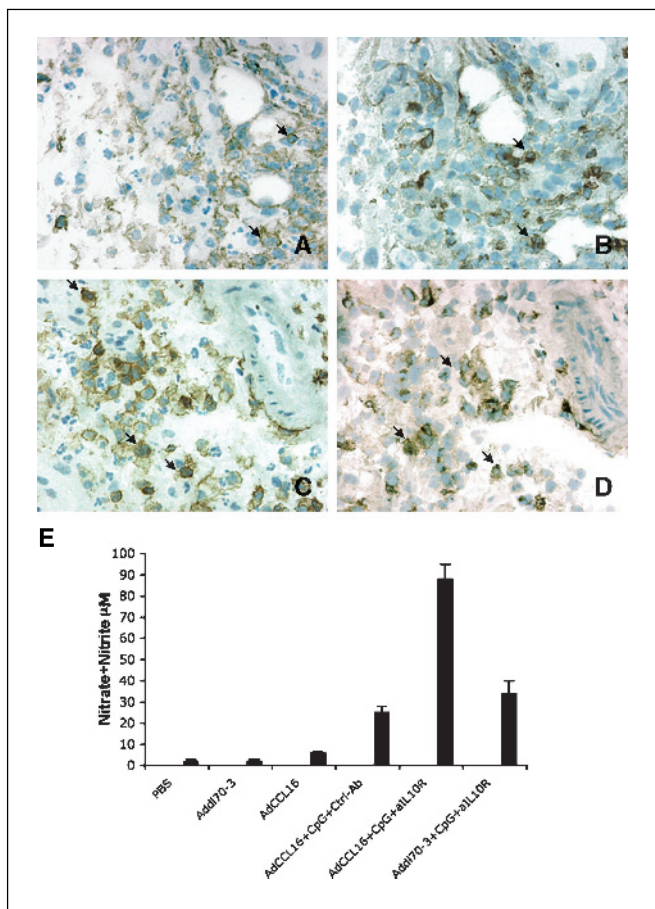


Figure 3. Macrophages infiltrating treated tumors express TNF, IL-12, and nitric oxide. TSA tumors were injected with AdCCL16 and CpG plus anti-IL-10R. Tumors were collected for immunohistochemical analysis 6 hours later. Macrophages (F4/80; A and C) colocalized in serial sections with TNF (B) and IL-12 (D; $\times 400$). Arrows, colocalization of stained cells in serial sections. E, production of nitric oxide in response to the indicated treatments. Tumors were collected for analysis 4 hours posttreatment (columns, mean of five tumors per group; bars, SD; one of two independent experiments is shown).

the adaptive response was impaired because of H-2 mismatch. Irradiated CB6F1 mice, reconstituted with bone marrow cells from either C57BL/6 or BALB/c, were injected with TSA tumor cells (H-2^d). CTL were induced to recognize H-2^d-presented TSA antigens in BALB/c>CB6 but not in B6>CB6 chimera. Whereas 92% of BALB/c>CB6 mice treated with AdCCL16 and CpG plus anti-IL-10R rejected the TSA tumors, only 26% of B6>CB6 did so (Fig. 4D). The innate response measured as nitric oxide production was comparable in the two groups of treated mice (Fig. 4E). These results strongly indicate that in mice in which the innate response failed to completely eradicate the primary tumor, adaptive T-cell responses, in particular CD8 T cells, were essential to control local recurrence.

The combination of CCL16 and CpG plus anti-interleukin-10 receptor restores tumor-infiltrating dendritic cell functions and bridges innate and adaptive immunity. In addition to macrophages, AdCCL16 treatment recruited DC in TSA tumors. The functions of these tumor-infiltrating DC, however, can be impaired by the tumor milieu. The generation of CD8 tumor antigen-specific T-cell response in the treated mice and their requirement for tumor eradication suggested that the combined treatment restored DC functions. To test the potential role of DC,

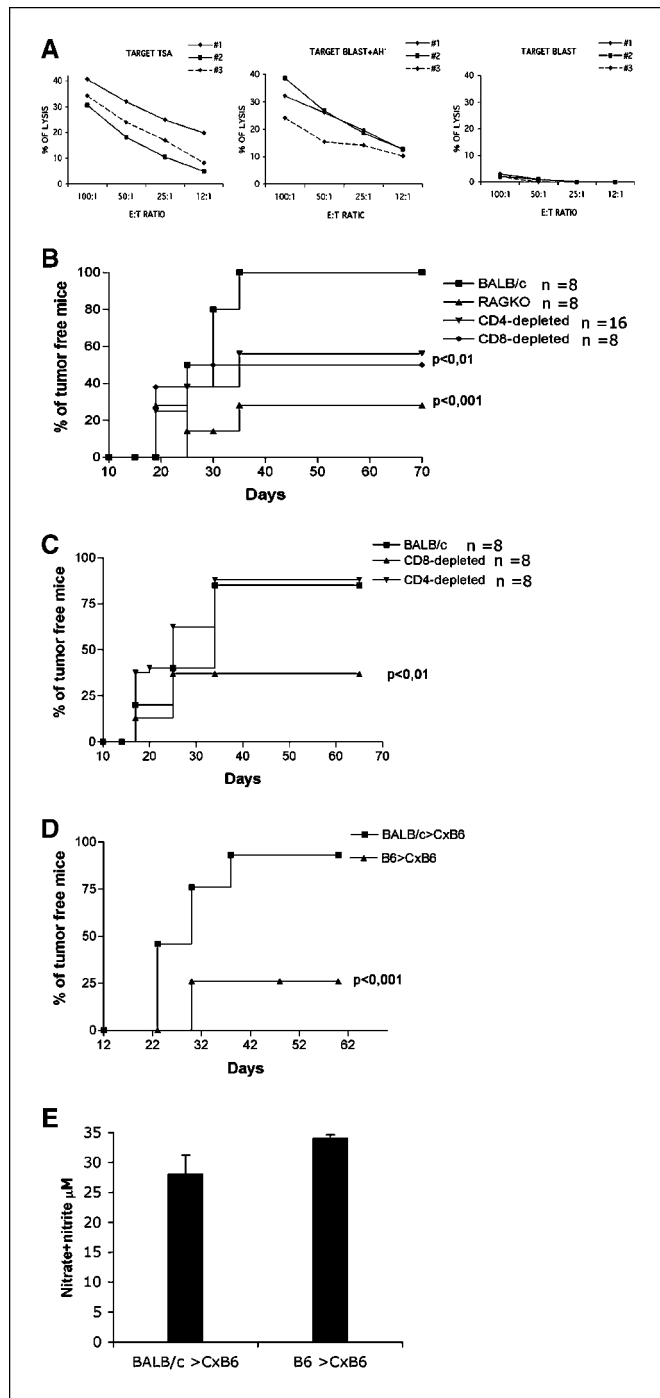


Figure 4. Adaptive response is required to cure mice. A-C, role of CD4 and CD8 T cells in tumor rejection induced by CCL16 and CpG plus anti-IL-10R. A, induction of CTL in treated mice. Splenocytes collected 7 days posttreatment were *in vitro* restimulated with the AH1 antigenic peptide of TSA and cytotoxicity measured against TSA tumor cells or blast cells loaded or not with the AH1 peptide. Results are representative of two independent experiments, each done with three mice. B, tumor rejection was monitored in mice depleted of CD4 or CD8 T cells before treatment as well as in RAG-KO mice. C, tumor rejection was monitored in mice in which CD4 and CD8 T cells were depleted starting 5 days posttreatment. D, BALB/c>CxB6 and B6>CxB6 chimeras were inoculated with 2×10^5 TSA tumors. Twelve days later, tumors were treated with AdCCL16 and CpG plus anti-IL-10R. Cumulative data from two independent experiments, each done with eight mice. E, in a separate experiment, AdCCL16-treated tumors ($n = 5$ /group) were collected 4 hours after treatment with CpG plus anti-IL-10R and processed to measure nitric oxide production (results expressed as mean \pm SD). For B and C, the number of mice is indicated in the figure.

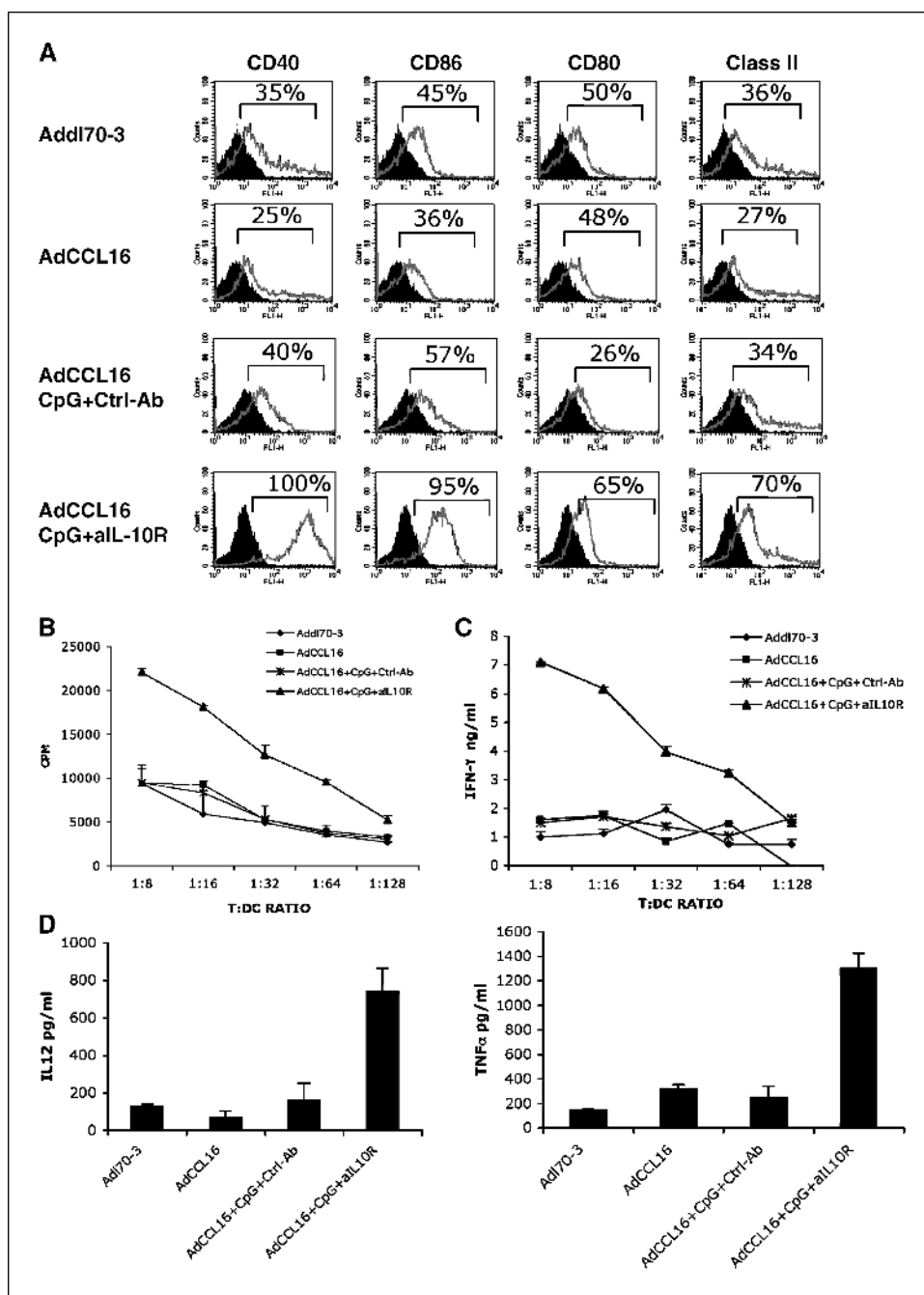


Figure 5. Phenotypic and functional characterization of tumor-infiltrating DC. Tumor-infiltrating DC were purified from TSA tumors treated with the indicated combinations and analyzed as follows: by FACS analysis for the activation markers CD40, CD80, CD86 and MHC-II (A); for their ability to stimulate proliferation (B) and IFN- γ production of allogeneic T cells in an MLR assay (C); and for IL-12 and TNF production by ELISA (D). Representative experiment of three with similar results (columns, mean; bars, SD).

we used mice genetically deficient for CD40, a key molecule for the interaction between DC and CD4 T cells during CTL induction. Because engagement of CD40 on DC induces IL-12, which is the primary cytokine bridging innate and adaptive immune responses, we also tested the efficacy of the combined treatment in IL-12p35-KO mice. Both CD40-KO and IL-12p35-KO mouse strains failed to reject TSA tumors in response to the combination protocol (Supplementary Fig. 3A). Moreover, both mouse strains failed to generate CTL (Supplementary Fig. 3B).

We characterized the phenotype and functions of tumor-infiltrating DC from treated versus nontreated animals. AdCCL16 combined with CpG and anti-IL-10R induced up-regulation of CD40, CD80, and CD86 in CD11c⁺ DC whereas the omission of anti-IL-10R abrogated this effect (Fig. 5A). Consistent with the

activated phenotype, tumor-infiltrating DC from the treated mice produced high levels of IL-12 and TNF (Fig. 5D) and vigorously stimulated allogeneic naive T cells to proliferate and produce IFN- γ (Fig. 5B and C).

To prime the antitumor CTL response observed in treated mice, either tumor-infiltrating DC migrated to the draining lymph nodes before the fast and massive necrosis of the tumor mass affected their viability or DC other than tumor-infiltrating DC were involved in the onset of the adaptive response (e.g., by uptaking tumor antigens released into lymphoid sites following tumor necrosis). To investigate these possibilities, draining lymph nodes were collected 6 and 16 hours posttreatment from mice in which FITC-labeled beads were injected as tracer in the tumor mass. CCL16 treatment combined or not with CpG plus

anti-IL-10R induced the highest number of FITC-labeled DC detected in draining lymph nodes collected 6 hours posttreatment, a number that did not significantly differ from that of DC collected after 16 hours (median, 15,000 versus 1,970 at 6 hours and 26,000 versus 5,000 at 16 hours for AdCCL16 or AdI70-3 combined with CpG + anti-IL-10R, respectively; Fig. 6A and B). This observation suggested that the majority of tumor-infiltrating DC migrated to the draining lymph nodes during the first 6 hours posttreatment. Indeed, mice in which the injection of FITC beads was delayed 6 hours posttreatment showed a significantly lower number of FITC-labeled DC in draining lymph nodes than the mice receiving the beads together with the treatment (median 868 versus 46,000; Fig. 6C and D). Finally, the functional requirement of DC migration for tumor eradication was tested using CCR7-KO mice, of which DC cannot respond to CCL19 and CCL21. No more than 25% of TSA tumor-bearing CCR7-KO mice eradicated the tumor in response to the combined treatment (Fig. 6E; $P < 0.001$ versus wild-type mice).

Tumor necrosis factor-null infiltrating leukocytes do not respond to treatment with AdCCL16 and CpG plus anti-interleukin-10 receptor. As a way to test for the key factor responsible for the innate response, tumor-associated necrosis, and conditioning adaptive response, we generated chimeras in which bone marrow from TNF-KO mice was transferred into B6.SJL mice. In this way we tested the role of TNF produced by infiltrating leukocytes and, because *TNF* gene deletion spans nearby MHC sequence, we tolerized donor cells to host MHC, avoiding tumor rejection because of subtle MHC disparity. Injection of MCA38 in these mice produced large tumors that, if treated with the combined treatment, were not rejected (Fig. 7C), lacking hemorrhagic necrosis (Fig. 7A and B), and had impaired tumor-

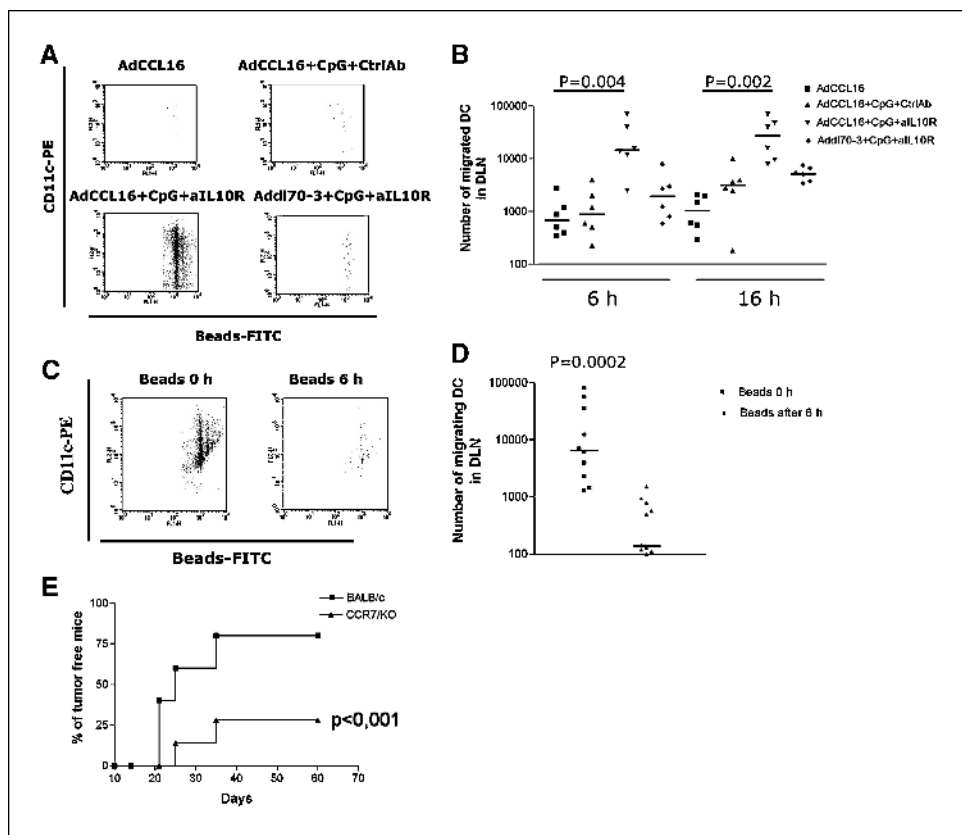
infiltrating DC migration (Fig. 7D). These results suggest that the early inflammatory response is instrumental for both tumor destruction and migration of DC to draining lymph nodes.

Discussion

Many studies have definitively shown that both in experimental animals and in humans, mechanisms of immune surveillance are able to control the appearance of new tumors or affect the progression of existing tumors (19).

Whereas locally injected chemokines can directly recruit DC at the tumor site, they are unable to revert the effect of suppressive tumor environment, and therefore showed limited curative potential when used as single agents (7). Inflammatory chemokines act on a heterogeneous population of circulating monocytes and DC that, after entering tumors, may differentiate not only into tumor-infiltrating DC but also into tumor-associated macrophages. Tumor-associated macrophages remain in the invaded tissue whereas DC maintain the ability to migrate into the draining lymph nodes (20). Tumor-infiltrating DC and tumor-associated macrophages are components of the tumor stroma and the functions of the cells recruited through chemokine manipulation are likely to be modulated by the tumor microenvironment (1). Tumor-associated macrophages are the main source of intratumor IL-10, a potent immunomodulating molecule able to turn down both innate and immune responses by affecting DC functions and acting in an autocrine fashion by maintaining the infiltrating macrophages in the M2 state (21). In such a hostile tumor environment and in the absence of proper inflammation, recruited DC may present antigen in an inappropriate T-cell context, generating tolerance to TAA instead of immunity (22). Strategies

Figure 6. CCL16 and CpG plus anti-IL-10R substantially increase the number of CD11c⁺ DC migrating to draining lymph nodes. *A*, TSA tumors treated as indicated received an intratumoral injection of fluorescent latex beads and 6 or 16 hours later, draining lymph nodes (DLN) were analyzed for the presence of CD11c⁺ cells carrying FITC⁺ beads (*left*, representative plots; *right*, total number of migrated DC). *B*, tumors were treated with AdCCL16 and fluorescent beads were injected together with CpG or 6 hours later. Draining lymph nodes were collected 16 hours after bead injection (*left*, representative plots; *right*, cumulative data of migrated DC). Each dot in cumulative graphs refers to a single draining lymph node collected from an individual treated mouse. *C*, reduced TSA tumor rejection in CCR7-KO mice treated with AdCCL16 and CpG plus anti-IL-10R ($n = 8$).



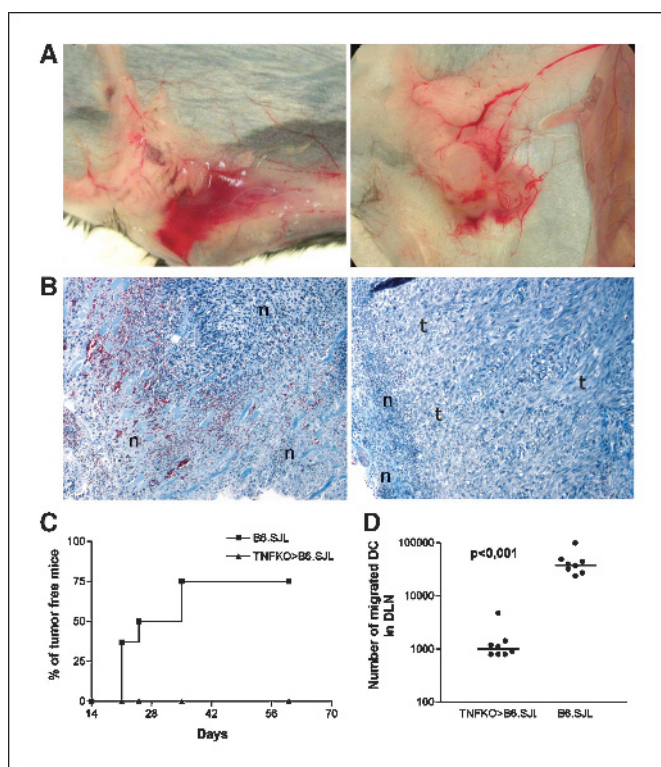


Figure 7. Role of TNF- α in hemorrhagic necrosis, tumor rejection, and tumor-infiltrating DC migration. TNF-KO>B6.SJL chimeras, differing in the *CD45* allele, were used to test the role of leukocyte-produced TNF α . *A* and *B*, B6.SJL (*left*) and chimeras (*right*) were injected with MCA38 carcinoma cells and were treated, when palpable, with AdCCL16 combined with CpG plus anti-IL-10R. Macroscopic evaluation (*A*) and trichomic staining (*B*) of tumor sections collected 16 hours posttreatment. In absence of TNF- α , tumor rejection did not occur (*C*) and DC migration was severely impaired (*D*).

able to overcome such tumor escape mechanisms are the key to effective immunotherapy. Although various soluble and cellular factors have been implicated in tumor-induced immune suppression, we have previously shown that IL-10 has a sufficient and essential role in determining the anergic state of tumor-infiltrating DC. Indeed, DC infiltrating a number of different mouse tumors were shown to be refractory to maturation stimuli, unable to produce IL-12, and to stimulate T lymphocytes, unless the inhibitory effect of IL-10 was blocked using repeated treatments with an anti-IL-10R antibody (10).

In the present work, we tested the hypothesis whether the treatment of tumor-bearing mice with a chemokine that is able to enhance the tumor infiltration by macrophages and DC would synergize with a treatment that simultaneously fights DC paralysis and subverts the macrophage phenotype from M2 to M1. We used the CCL16 chemokine that was previously shown to recruit macrophages and DC at the tumor site (8, 23). Here, we show that macrophages recruited by CCL16 produced IL-10, but not IL-12 or TNF, consistent with the M2 phenotype, which also characterizes the resident tumor-associated macrophages. This contrasts with *in vitro* results showing that recombinant CCL16 effectively induced peritoneal macrophages to produce proinflammatory cytokines, such as CCL2, IL-12, and TNF, and to kill TSA tumor cells (24). Our *in vivo* results indicate that CCL16-recruited macrophages became functionally inhibited by the tumor unless CpG and anti-IL-10R are added to the treatment. Mice treated with this combination protocol successfully rejected established TSA,

MCA38, and 4T1 tumor nodules. When combined with CpG plus anti-IL-10R, not only 60% of CCL16-treated animals rejected the 4T1 primary tumors but they were also cleared of distant metastases, whereas, as we previously reported, AdCCL16 given alone, although unable to eradicate the transplanted 4T1 tumors, was effective in curing their distant metastases when associated with the surgical excision of primary nodules, consistent with the hypothesis that the tumor is the source of both local and systemic immunosuppression (8).

The Toll-like receptor 9 agonist CpG has been widely used as an adjuvant of tumor-specific antigen vaccines or given intratumorally in a therapeutic setting (25). Peritumoral injection of CpG as a single agent induced tumor regression in some tumor models (26, 27) but has proven ineffective in others (28, 29). In all these reports, CpG was given repeatedly at a high dose (50-100 μ g/dose) to animals bearing small tumors. In combination with CCL16 and an antibody blocking IL-10R, a single CpG administration of 5 μ g is sufficient to eradicate large vascularized tumors. Considering that CpG is entering clinical trials for different applications, including cancer immunotherapy, and that repetitive high dose has been recently described to be toxic and to induce immunosuppression (25, 30), we feel that our findings provide proof of concept that combination protocol can reduce both the amount and frequency of CpG administrations, yet maintaining effectiveness. In comparison with our previous work with CpG and anti-IL-10R alone (10), the addition of CCL16 allowed us to successfully treat tumors at least twice in size than those previously tested using a single rather than three injections.

One of the most impressive observations in the present study was the rapid triggering of events leading to tumor necrosis and systemic immunity. The massive hemorrhagic tumor necrosis was comparable to a localized Shwartzman reaction (31), completed in 16 hours, and accompanied by the release of nitric oxide. Macrophages recruited by CCL16 produced IL-12 and TNF as soon as 6 hours after treatment with CpG plus anti-IL-10R and were likely the major inflammatory cell type responsible for the induction of tumor necrosis. Polymorphonuclear leukocytes, although frequently described to take part in necrosis and capable of tumor toxicity via production of the oxidant HOCl (32), were observed to be localized at the edge of tumors treated with the combinatory regimen (not shown); thus, they are unlikely to take part in the process of tumor destruction. The observed extensive hemorrhagic necrosis points to a central role of TNF and other proinflammatory cytokines and the participation of innate resistance mechanisms in tumor destruction. Indeed, in tumor-bearing chimeric mice reconstituted with bone marrow from TNF-KO mice, the combination treatment failed to induce hemorrhagic necrosis and to provoke tumor debulking. These results show that subversion of M2 phenotype requires that the inflammatory stimulus be combined with block of the IL-10 pathway. To our knowledge, this is the first report showing *in vivo* such a rapid switch from M2 to M1 phenotype.

Tumors treated with CCL16 were also enriched in tumor-infiltrating DC that showed the typical immature myeloid phenotype of the resident tumor-infiltrating DC in the absence of added chemokines (ref. 10 and data not shown) that is characterized by low lymphocyte stimulatory activity and low ability to produce inflammatory cytokines like TNF and IL-12. The refractory state of tumor-infiltrating DC, together with the observed M2 phenotype of infiltrating macrophages, correlates with the poor rejection of tumors in animals treated with AdCCL16

alone (8). Along with macrophages, DC also shifted phenotypically and functionally in response to the combinatory treatment. Tumor-infiltrating DC up-regulated costimulatory molecules, produced IL-12 and TNF, and stimulated T-lymphocyte proliferation and IFN- γ secretion. However, the rapid hemorrhagic necrosis induced by the treatment was so massive that it should have been expected to destroy infiltrating leukocytes along with tumor cells, questioning whether tumor-infiltrating DC could reach the draining lymph nodes before their viability was affected. The effect of the inflammatory response on DC mobilization from the tumor was confirmed in chimeric mice reconstituted with bone marrow from TNF-KO mice that showed both reduced inflammation and DC migration to the draining lymph nodes.

We hypothesize that the rapid migration from the tumor to the draining lymph nodes of mature DC, at least some of which were loaded with TAA, is instrumental in activating systemic immune responses and generating CTL before the progressive necrosis destroys intratumoral DC or prevents their ability to migrate to the draining lymph nodes. Accordingly, in B6>CXB6 chimera mice in which B6-induced CTL cannot recognize TSA (H-2^d) tumors, the observed low tumor rejection rate was due to the innate response. A more detailed analysis of the role of CD4 and CD8 T cells indicated that although depletion of CD8 T lymphocytes, either during the priming or the effector phase, reduces antitumor efficacy, CD4 T cells are needed for efficient priming. CD4 T-cell help for the induction of effector CD8 T cells is mediated by CD40/CD40L interaction with antigen-presenting cells (33). Accordingly, lack of tumor rejection and defective CTL induction were observed in mice lacking CD40, indicating the need for a correct cross-talk between antigen-presenting cells and T cells.

IL-12 bridges innate and adaptive immunity by promoting CTL induction, NK function, and T-helper 1 differentiation of CD4 T-helper cells (34). Impaired antitumor response in the absence of CD40/CD40L interaction might result from a defective IL-12 production (35). Indeed, abrogation of both tumor rejection and CTL generation was observed in mice lacking IL-12. Although alternative pathways of CTL induction independent from CD40 and IL-12 have been described, they were mainly effective against nontolerized antigens and/or in the

presence of a high number of specific T-cell precursors (36, 37), and thus unlikely to be effective in an antitumor response. Indeed, we showed that to induce adaptive immunity to TAA, the presence of high inflammatory stimuli cannot compensate for the lack of CD40/CD40L costimulation that is probably required for full maturation and activation of DC in the lymph nodes, including optimal production of IL-12 (38).

In the present studies in mice, we used human CCL16 to promote macrophage and DC recruitment within mouse tumors because its mouse homologue exists only as a pseudogene (39, 40), thus facilitating the possibility of translation of the therapeutic protocol into the clinical setting. However, the fact that different subsets of cells respond to CpG in humans and mice raises the question of whether CpG would induce a similar response in human tumors. In humans, plasmacytoid DC are the main cells triggered by CpG that, in the mouse, activates indistinctly myeloid DC, monocytes/macrophages, and plasmacytoid DC. Although the exact role of plasmacytoid DC infiltrating human tumors is yet to be determined, the observation of their immature phenotype and their inability to release IFN- α suggest that infiltrating plasmacytoid DC are negatively regulated by the tumor microenvironment. Indeed, *in vitro*, blocking IL-10R reestablished the ability of plasmacytoid DC extracted from tumors to stimulate T lymphocytes (41). Alternatively, other ligands of Toll-like receptors that can activate both tumor-associated macrophages and tumor-infiltrating DC in humans may replace CpG for the treatment of human cancer. Taken together, the present results strongly support that the manipulation of macrophages and DC recruitment within tumors together with their activation and relief of tumor immunosuppression may represent a potentially effective antitumor therapy.

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Redirecting *In vivo* Elicited Tumor Infiltrating Macrophages and Dendritic Cells towards Tumor Rejection

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