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Intratumoral Coinjection of Two Adenoviruses, One Encoding the Chemokine IFN-γ-Inducible Protein-10 and Another Encoding IL-12, Results in Marked Antitumoral Synergy[1]

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We have constructed a recombinant defective adenovirus that expresses functional murine IFN-γ-inducible protein-10 (IP-10) chemokine (AdCMVIP-10). Injection of AdCMVIP-10 into s.c. tumor nodules derived from the CT26 murine colorectal adenocarcinoma cell line displayed some antitumor activity but it was not curative in most cases. Previous studies have shown that injection of similar s.c. CT26 tumor nodules with adenovirus-encoding IL-12 (AdCMVIL-12) induces tumor regression in nearly 70% of cases in association with generation of antitumor CTL activity. AdCMVIP-10 synergizes with the antitumor effect of suboptimal doses of AdCMVIL-12, reaching 100% of tumor eradication not only against injected, but also against distant non-injected tumor nodules. Colocalization of both adenoviruses at the same tumor nodule was required for the local and distant therapeutic effects. Importantly, intratumoral gene transfer with IL-12 and IP-10 generated a powerful tumor-specific CTL response in a synergistic fashion, while both CD4 and CD8 T cells appeared in the infiltrate of regressing tumors. Moreover, the antitumor activity of IP-10 plus IL-12 combined gene therapy was greatly diminished by simultaneous in vivo depletion of CD4$^+$ and CD8$^+$ T cells but was largely unaffected by single depletion of each T cell subset. An important role for NK cells was also suggested by asialo GM1 depletion experiments. From a clinical point of view, the effects of IP-10 permit one to lower the required gene transfer level of IL-12, thus preventing dose-dependent IL-12-mediated toxicity while improving the therapeutic efficacy of the elicited antitumor response. ***The Journal of Immunology,*** **2000, 164: 3112–3122.**

Interferon-γ inducible protein-10 (IP-10)[5] (1, 2), also called Crg-2 (3, 4) in mice, is a chemokine that belongs to the CXC family known to stimulate the CXCR3 chemokine receptor (5–7). The pattern of CXCR3 expression explains that IP-10 attracts, at least in vitro, only activated but not resting T lymphocytes and NK cells (5, 8–10). Stably transfected tumor cell lines expressing IP-10 were rejected through an immune system-mediated mechanism (11). However, it has been recently shown that IP-10 also displays antitumoral properties related to its ability to impair tumoral angiogenesis (12–14). Such an effect seems not to be mediated by CXCR3 (15) and has been found to be important for the antitumoral effects of IL-12 in some models (16). Therefore, IP-10 has been involved on the interface of the immune (T and NK recruitment to the malignancies) and nonimmune antitumor mechanisms (angiostatic effect), making the IP-10 gene a good therapeutic candidate to be delivered into malignant cells.

Other chemokine genes such as the one encoding lymphotactin (17) and macrophage inflammatory protein-1α (18) have been transfected into tumor cells showing that although they attracted T lymphocytes to the malignant tissue, they failed to induce rejection (17, 18). However, combination of those chemokines with other cytokines or costimulatory molecules that ultimately result in lymphocyte activation such as IL-2, B7-1 (CD80), and IL-12 resulted in a marked antitumor effect (17, 19, 20).

IL-12 is a cytokine naturally produced by macrophages (21) and dendritic cells (22) and plays a key role in the induction of cellular immune responses (23). IL-12 has been found to mediate potent antitumor effects that are the result of a pleyade of actions involving not only the induction of CTL, Th1-mediated immune responses, and NK activation (21), but also impairment of tumor vascularization (24). Unfortunately, the first attempts to test IL-12 in the clinic underwent failure due to unacceptable dose-related toxicities leading to some fatalities (25, 26). Thus, viral transfer of IL-12 genes into tumors holds promise to be an efficacious alternative as proved in several animal models (27–30). It is noteworthy that IL-12 triggers important secretion levels of IFN-γ from T and NK cells, which in turn lead to induction of IP-10 in many cell types (21). Because IL-12 toxicity is reported to be largely related to IFN-γ hyperproduction, it is reasonable to assume that enrichment of the malignant environment directly with IP-10 could enhance the therapeutic effects of low doses of IL-12, thus permitting antitumor activity, while avoiding toxicity due to excessive IFN-γ production.

To study the effects of transducing tumor cells in vivo with the IP-10 gene, we have generated a recombinant defective adenovirus encoding for the chemokine (AdCMVIP-10). Intratumoral injection of high doses of AdCMVIP-10 had only mild antitumor

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[5] Abbreviations used in this paper: IP-10, IFN-γ-inducible protein-10; AdCMVIP-10, adenovirus encoding IP-10; AdCMVIL-12, adenovirus encoding IL-12; AdCMV-LacZ, adenovirus encoding LacZ; mIP-10, murine IP-10; MOI, multiplicity of infection.

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activity. Nonetheless, such treatment synergized with adoptive T cell therapy mediated by short-term cultured antitumor T lymphocytes and importantly with suboptimal levels of IL-12 gene transfer within the same tumor nodule. Gene transfer by coinjection into tumors of IP-10 and IL-12 recombinant adenoviruses resulted in a potent enhancement of tumor-specific CTL activity, which could account, at least in part, for the improved therapeutic effects.

Materials and Methods

Animals

Five- to 8 wk-old female BALB/c mice were purchased from Harlan (Barcelona, Spain). Six-week female BALB/c$^{nu/nu}$ mice were obtained from Harlan (Barcelona, Spain) and housed in pathogen-free conditions.

Cells and Abs

The 293 cell line (adenoviral E1 transformed human embryonic kidney cells) and the HepG2 cell line (human hepatoblastoma) were obtained through American Type Culture Collection (Manassas, VA). The CRE8 selective cell line was kindly provided by Dr. S. Hardy (University of California, San Francisco, CA). It has a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells (31). The BALB/c (H-2^d) mouse-derived CT26 tumor cell line is an undifferentiated murine colorectal adenocarcinoma (32) that was established from an *N*-nitroso-*N*-methylurethan-induced transplantable tumor, obtained from Dr. K. Brand (Max-Planck-Institut fur Biochemie, München, Germany). P815 and YAC-1 cells were obtained through American Type Culture Collection. The CRE8, 293, and HepG2 cell lines were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin (complete medium). CT26 cells were cultured in RPMI 1640 medium identically supplemented. All cell culture reagents were obtained from Life Technologies (Basel, Switzerland). Hybridomas GK1-5 and H35.17.2 (American Type Culture Collection) were used to obtain anti-CD4 and anti-CD8 Abs from ascitic fluid that was obtained from pristane-primed nude mice injected i.p. with 10^6 hybridoma cells. Asialo GM1 antiserum was obtained from Wako (Osaka, Japan). Anti IP-10 mAb and anti IP-10 polyclonal anti-serum were purchased from R&D Systems (Minneapolis, MN) and PeproTech (London, U.K.). FITC- and PE-tagged anti-CD4, anti-CD8, anti-CD3, and anti-Pan-NK (DX5) mAbs were obtained from PharMingen (San Diego, CA).

Construction of recombinant adenoviral vectors

Recombinant adenovirus carrying murine IP-10 under the control of CMV promoter was constructed using Cre-*lox* recombination system (31). Splenocytes were obtained from an 8-wk female BALB/c mouse, cultured with complete medium in 10-cm^2 culture dish (Techno Plastic Products, Trasadingen. Switzerland), and stimulated for 3 h with LPS (20 ng/ml) (Sigma, Madrid, Spain). Subsequently, total cellular RNA was isolated with Ultraspec (Biotecx Laboratories, Houston, TX), and amplified by RT-PCR using specific primers for murine IP-10 (mIP-10) (33). The 311-bp fragments corresponding to mIP-10 cDNA were cloned into pGEM-T vector system (Promega, Madison, WI) and sequenced (3, 4). The fragment containing the mIP-10 cDNA was *Sph*I/*Sal*I excised from pGEM-T/mIP-10, filled in by Klenow, and blunt-end ligated into *Bgl*II-digested alkaline phosphatase-treated pAdlox. CRE8 cells were CaPO$_4$ cotransfected with *Sfi*I-predigested pAdlox/mIP-10 and ψ5 DNA (31). After 10 days, lysate from the cotransfected cells was used to infect CRE8 cells to eliminate the ψ5 virus contamination (two similar passages were performed to assure purity). Lysate from the last passage was used to infect 293 cells to amplify AdCMVIP-10. Packaged viral DNA was prepared as described by Hardy et al. (31) and, when digested with *Bsa*BI, confirmed the expected pattern upon 1% agarose electrophoresis analysis (all samples were ψ5 DNA-free).

Recombinant adenovirus carrying IL-12 (AdCMVIL-12) has been previously described (27). Briefly, an expression cassette of IL-12 under the control of CMV promoter was constructed encompassing IL-12 p35 cDNA, an internal ribosomal entry site, IL-12 p40 cDNA, and a polyadenylation signal. Recombinant adenovirus encoding the IL-12 cassette of expression (AdCMVIL-12) was generated by cotransfection of 293 cells according to standard procedures (34). Adenovirus carrying LacZ reporter gene under the control of CMV promoter (AdCMVLacZ) was produced similarly (35). All recombinant adenoviruses were isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride gradient ultracentrifugation (35). Purified virus was extensively dialyzed against 10 mM Tris/1 mM MgCl$_2$ and stored in aliquots at 80°C, and it was carefully titred by plaque assay.

Western blot analysis

HepG2 cells cultured to 75% confluence in 10-cm diameter dishes were infected with AdCMVIP-10 (multiplicity of infection (MOI) = 65), transfected with Padlox/IP-10 (Fugene, Roche, Barcelona, Spain) or left untransfected and maintained in serum-free RPMI 1640 for 36 h. Supernatants were collected and concentrated using Centricom YM-3 (Amicon; Milipore, Madrid, Spain). Recombinant murine IP-10 (R&D Systems) was used as positive control. Proteins were resolved by 15% SDS-PAGE using Tris/Tricine buffer (36) and transfered to Hybond-P membranes (Amersham-Pharmacia Biotech, Madrid, Spain) that were blocked in 5% nonfat milk in PBS 0.1% Tween 20 (PBS-T) overnight at 4°C. After washing four times in PBS-T, the membranes were incubated for 1 h at room temperature in diluted (1/1000) anti-IP-10 goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed five times in PBS-T and incubated with diluted (1/5000) HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed by enhanced chemiluminescence detection reagents (ECL Plus; Amersham-Pharmacia Biotech).

Chemotaxis assay

Chemotactic activity was measured by migration assays across polycarbonate membranes (37). T cells from BALB/c spleens were enriched by plastic adherence and passage through nylon wool columns. T cells were activated in RPMI 1640, 10% FCS, IL-2 (20 IU/ml) (PeproTech) and Con A (2 μg/ml) (Sigma) for 2 days and then harvested. HepG2 cells at 80% of confluence in a 10-cm diameter dish were infected at a MOI of 65 with AdCMVLacZ, AdCMVmIP-10, or noninfected in RPMI 1640, 0.5% FCS. After 48 h, supernatants from AdCMVLacZ-infected, AdCMVmIP-10-infected, and noninfected cells were collected. Recombinant mIP-10 10 ng/ml protein, rIP-10 plus mAb anti-IP-10 (2 μg/ml), and uninfected cells were added to the lower chamber, and T cells (100 μl at 5×10^6/ml) were resuspended in RPMI 1640 plus 0.5% FCS added to the upper chamber. Migration assays were performed across polycarbonate membranes, 6.5 mm diameter, 10 μm thickness, 5 μm diameter pore size transwell cell culture chambers (Costar, Cambridge, MA). Migration was allowed at 37°C in 5% CO$_2$ atmosphere for 2 h. Filters were then fixed in 1% (v/v) glutaraldehyde in PBS for 1 h and stained in 0.5% (w/v) toluidine blue for 2–4 h. Cell migration was quantified by direct count of cells adhered to the bottom side of the polycabonate filters; 10 microscopic fields per point were counted. Four replicated wells were used for each condition.

In vivo gene therapy of established tumors

Tumors were established by s.c. or intrahepatic implantation of CT26 cells. A total of 5×10^5 cells were injected at the right hind flank of BALB/c syngenic mice. Ten days later, when tumors reached 5–7 mm (in diameter), different recombinant adenoviruses (AdCMVIP-10, AdCMVIL-12, and AdCMVLacZ) at indicated doses were injected intratumorally in a volume of 50 μl diluted in saline buffer (PBS). Tumor growth was monitored twice a week by measuring two perpendicular tumor diameters using a precision caliper. Animals showing severe distress or with tumors that exceeded 1.5 cm in two perpendicular diameters or 2 cm in one diameter were sacrificed for ethical reasons according to institutional guidelines. To induce bilateral tumors, 5×10^5 CT26 cells were injected s.c. into BALB/c mice at both right and left hind flanks.

T cell culture for adoptive therapy

Mice bearing bilateral 5–8 mm (diameter) s.c. CT26 tumors were treated with intratumor injections of 10^8 pfu of AdCMVIL-12. Draining lymph nodes were removed aseptically 5 days later, and single-cell suspensions were obtained by pressing them mechanically through mesh screens. Lymph node cells were cultured in 24-well plates (Greiner Labortechnik, Frickenhausen, Germany) for 7 days at 5×10^6 cells/well with 2×10^5 CT26 tumor cells/well pretreated for 1 h at 37°C with 150 μg/ml of mytomycin-C (Sigma) (reagent was extensively washed). Cells were cultured for 7 days in complete RPMI 1640 supplemented on day 5 with mIL-2 (8–10 IU/ml) (PeproTech).

For adoptive transfer of CD4$^+$ cells, splenocytes from mice who had rejected CT26 s.c. tumor nodules by treatment with AdCMVIP-10 plus AdCMVIL-12 15 days earlier were incubated with anti-CD4-coupled magnetic beads (MiniMACS, Miltenyi Biotech, Bergisch Gladbach, Germany), positively selected by magneting sorting according to manufacturer instructions and injected i.v. to BALB/c $^{nu/nu}$ mice.

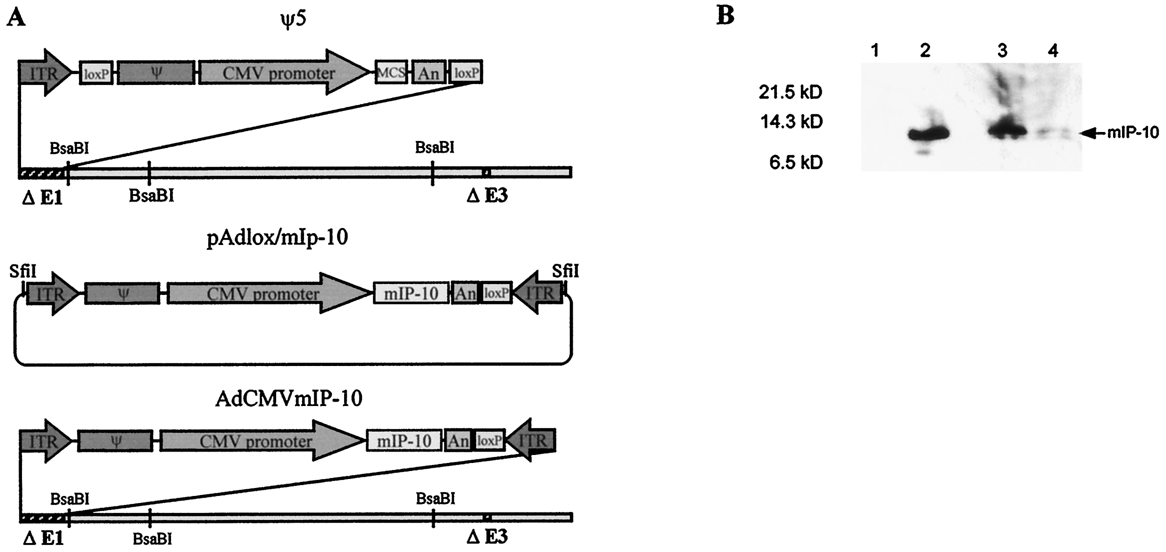


FIGURE 1. Construction of AdCMVIP-10. *A*, RT-PCR was used to clone mIP-10 cDNA from LPS-stimulated murine splenocytes. IP-10 cDNA was subcloned downstream the CMV promoter and upstream polyA signal (An) into pAdlox plasmid containing the adenoviral packaging signal (Ψ) and a *loxP* site, all flanked by the adenoviral inverted terminal repeats (ITRs). Recombinant adenovirus was created by cotransfection of pAdlox/IP-10 and Ψ5 (E1-E3 defective adenoviral viral DNA) in a 293 Cre-expressing cell line (CRE8). Cre recombinase catalyzed recombination at the *loxP* site directing the generation of AdCMVIP-10. *B*, Western Blot analysis with a polyclonal Ab against mIP-10 of cell culture supernatants from untransfected HepG2 cells (*lane 1*), recombinant mIP-10 protein as a positive control (*lane 2*), AdCMVIP-10 infected at a MOI of 65 (*lane 3*), and transfected pAdloxIP-10 (*lane 4*).

In vivo treatment of CT26 tumors by recombinant adenovirus and adoptive transfer of lymphocytes

BALB/c mice, in groups of five to six, received 5 × 10^5 CT26 cells in 25 μl of PBS injected surgically in the mid-lobe of the liver under general anesthesia. Ten days later, tumor diameters were assessed by surgical examination and injected with 5 × 10^8 pfu of recombinant adenoviruses or an equal volume (50 μl) of PBS. For cellular adoptive therapy, mice were injected i.v. with 5 × 10^6 cells from short-term antitumor T lymphocyte cultures 72 h after adenovirus administration. These mice received three i.p. injections of 2 × 10^4 IU of human rIL-2 (Chiron, Medfield, MA) in PBS on alternate days. Tumor size (mean diameter) was assessed by laparotomy using a precision caliper on day 19 after tumor challenge. Statistic significance of the differences among groups was evaluated by nonorthogonal contrasts.

Peptides

The H-2L^d-restricted peptides AH1 (SPSYVYHQF) (38) and P815AB (LPYLGWLVF) (39) were synthesized by F-moc chemistry as described (40), and purity was confirmed by HPLC.

^{51}Cr release assay

Cytotoxicity was analyzed in conventional 5-h ^{51}Cr release assays as described (41). Briefly, ^{51}Cr-loaded CT26, P815, and YAC-1 cells were incubated with effector cells at different E:T ratios in triplicate wells, and ^{51}Cr release (cpm) into the supernatants was measured in a gamma-counter to calculate percentage specific release as described (41). In some experiments, P815 cells were incubated during the assay with various concentrations of synthetic AH1 or P815AB peptides.

Depletion of lymphocytes and tumor growth

Tumor-bearing mice, four to five in each group, were depleted of CD4$^+$ or CD8$^+$ cells by i.p. injection of 100 μl of anti-CD4 or anti-CD8 ascitic fluid eight times, on days −3, −2, −1, 0, 5, 10, 14, and 21. Animals received treatment by intratumor injection of AdCMVIP-10 (5 × 10^8 pfu) and AdCMVIL-12 (7, 5 × 10^7 pfu) on day 0. Tumor growth was assessed twice a week. Experiments were repeated twice.

For NK depletion, anti-asialo GM1 (Wako) was given i.p. (100 μl/dose) when indicated. Gadolinium chloride (Sigma) was given i.v. (20 μg/dose). Depletions were monitored by FACS analysis of PBMC stained with fluorocrome-tagged anti-CD3, anti-CD4, anti-CD8, or anti-Pan-NK (DX5) (PharMingen) (41).

Histology and immunohistochemistry

For hemotoxylin-eosin staining, 4% formaldehyde-fixed tumor nodules were paraffin embedded, and sections of 4–6 μm thickness were stained according to standard procedures. For immunohistochemical staining, tumor tissues were embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen, and stored at −80°C. Tissues were sectioned on a cryostat at 4–6 μm, warmed at room temperature, air dried, and fixed in prechilled acetone for 10 min. After rinsing in PBS, endogenous peroxidase activity was neutralized using Dako peroxidase blocking reagent (Dako, Carpinteria, CA). Subsequently, sections were incubated with rat mAbs against mouse CD8 or CD4 (PharMingen) for 1 h at room temperature. Anti-rat IgG peroxidase conjugate (Sigma) was used as secondary Ab according to manufacturer's instructions. The chromogenic substrate diaminobenzidine (Dako) followed by Mayer's hematoxylin counterstaining was used to visualize positive reactions. Images are representative of multiple microscopic fields observed in at least three tumors equally treated.

Results

Construction of an IP-10-encoding recombinant defective adenovirus

IP-10 cDNA was amplified by RT-PCR from total purified BALB/c splenocytes stimulated with LPS (20 ng/ml) for 3 h. A 311-bp product was directly cloned into pGEM-T vector system and sequenced to rule out PCR errors and to confirm the identity of IP-10 cDNA.

To construct the recombinant adenovirus, a strategy based on Cre-*lox*-directed recombination was used (31). Thereby, IP-10 cDNA plus the encapsidation adenoviral signal was introduced into the defective adenoviral genome (ψ5) by cotransfection into Cre-expressing CRE8 cells, placing the IP-10 cDNA under the transcriptional control of CMV promoter as shown in Fig. 1*A*. Nonrecombinant helper adenovirus was purged by repeated infection of CRE8 cells, and, subsequently, recombinant adenovirus (AdCMVIP-10) was purified and produced by infection of 293 cells, as described in *Materials and Methods*.

Infection of HepG2 human hepatoblastoma cell line with AdCMVIP-10 (MOI = 65) resulted in the release to the cell culture

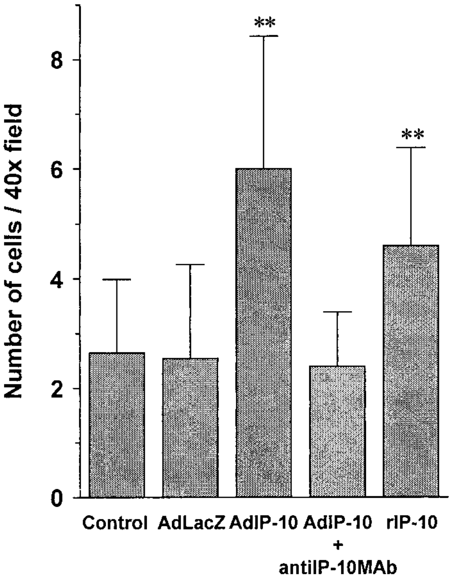


FIGURE 2. Chemotactic attraction of activated lymphocytes by AdCMVIP-10-encoded IP-10. Chemotactic activity of recombinant purified IP-10 10 ng/ml, 48 h tissue culture supernatant of HepG2 cells infected with AdCMVLacZ, AdCMVIP-10 (with or without neutralizing Ab), or uninfected cells, measured as the number per microscopic field of Con A T cell blasts migrating to the lower side of a 5-μm pore polycarbonate membrane separating two chambers in a 2-h transmigration assay. **, Significant differences at $p < 0.01$ according to Mann-Whitney *U* test with Bonferroni correction.

supernatant of a polypeptide recognized upon Western blot analysis by a specific anti-murine IP-10 polyclonal Ab (Fig. 1*B*). Slight immunoblot IP-10 reactivity was also observed in HepG2 cells transfected with liposome-coated IP-10-encoding plasmid (PAdlox/IP-10).

The culture supernatant of AdCMVIP-10-infected cells contains IP-10-dependent chemotactic activity for activated T cells

To verify that IP-10 expressed by AdCMVIP-10 was functional, HepG2 cells were infected at MOI = 65 and the culture supernatants were harvested 48 h later. Chemotactic activity for Con A T cells blasts was assessed by quantifying T cell migration across 5-μm pore polycarbonate membranes in a transwell cell culture chamber assay to measure the response to chemotactic stimuli placed into the lower chamber.

As shown in Fig. 2, supernatants from AdCMVIP-10-infected cells attracted Con A blasts well above control levels. Such an activity was abrogated by addition of a neutralizing anti-IP-10 mAb and it was not present in the supernatants of AdCMVLacZ-infected HepG2 cells. Our Con A-stimulated polyclonal T cell populations encompassed approximately equal numbers of CD4$^+$ and CD8$^+$ T cells. When using magnetic bead-purified CD4$^+$ and CD8$^+$ cells from such Con A blasts, we obtained data consistent with previous observations (8) that indicate a much stronger effect on the activated CD4$^+$ cell subset (data not shown).

Intratumoral injection of AdCMVIP-10-induced minor therapeutic effects

To explore whether AdCMVIP-10 had therapeutic effects against tumors, the CT26 colon adenocarcinoma cell line was s.c. injected into syngenic BALB/c mice. On day 9, nodules ranging from 4 to 8 mm in diameter were injected with 10^9 pfu of AdCMVIP-10, a control adenovirus (AdCMVLacZ), or saline buffer.

AdCMVIP-10 induced complete regressions of malignant tumors in 2 of 14 animals, whereas all tumors were lethal in the control groups. Fig. 3*A* shows the individual follow up of the diameter of the tumor nodules. In some animals treated with AdCMVIP-10, a tendency to delay tumor growth in comparison to control animals was noted.

Regardless of the mild effects on tumor diameter, microscopic examination of hemotoxylin and eosin-stained paraffin-embedded tumor sections disclosed gross areas of hemorragic necrosis in tumors treated with AdCMVIP-10 that were absent in tumor nodules receiving control adenovirus or saline buffer (data not shown).

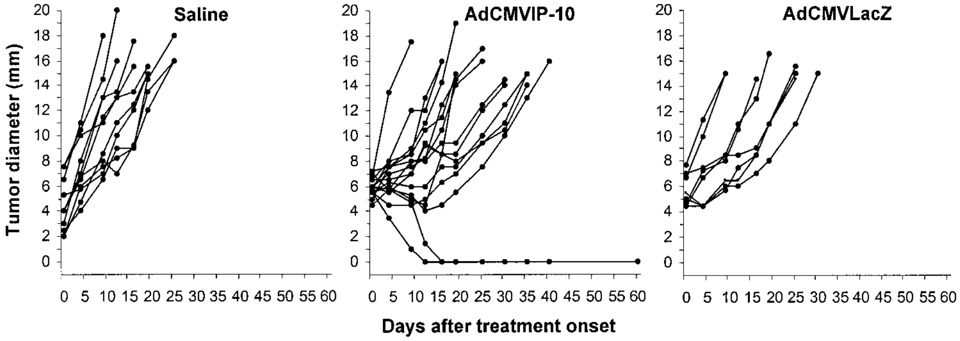
Intratumoral AdCMVIP-10 fosters the therapeutic efficacy of systemic T cell adoptive therapy

Because IP-10 expression can result in the attraction of activated T lymphocytes into the malignant tissue, we reasoned that AdCMVIP-10 infection of hepatic tumor nodules could potentiate the effect of adoptive transfer of antitumor T lymphocytes.

To study this issue, T cell cultures were obtained from the lymph nodes of mice who had rejected CT26 tumors upon intratumoral injection of an adenovirus-encoding murine IL-12 (AdCMVIL-12) by 7-day coculture with mitomycin-C-treated CT26 cells. Such short-term T cell cultures displayed potent CTL activity against CT26 and contained both activated CD4$^+$ and CD8$^+$ T cells, as we have previously reported (42).

Those T cell cultures were used for i.v. injection to treat tumor nodules derived from injection of CT26 cells into the mid-lobe of the liver in such a way that they gave rise to tumor nodules (4–8 mm in diameter) 8 days later. As shown in Fig. 4, adoptive transfer of 5×10^6 of such T cells did not show any effect against those CT26 liver tumor nodules. Under similar conditions, intratumor injection of 10^9 pfu of AdCMVIP-10 achieved only one tumor rejection of 11 mice treated. In contrast, 5 of 14 mice receiving both intratumor AdCMVIP-10 (10^9 pfu) on day 8 and antitumor T cells (5×10^6 i.v.) 36 h later showed complete regression of their tumors when surgically inspected 12 days later. Our results indicated a moderate synergistic effect of adoptive T cell therapy and

FIGURE 3. Effects of antitumor injection of AdCMVIP-10. Individual follow up of the diameter tumor nodules produced in BALB/c mice inoculated s.c. with 5×10^5 CT26 cells in the right flank and treated at day 9 by intratumoral injection with AdCMVIP-10 (10^9 pfu), AdCMVLacZ (10^9 pfu), or saline (PBS) as indicated.



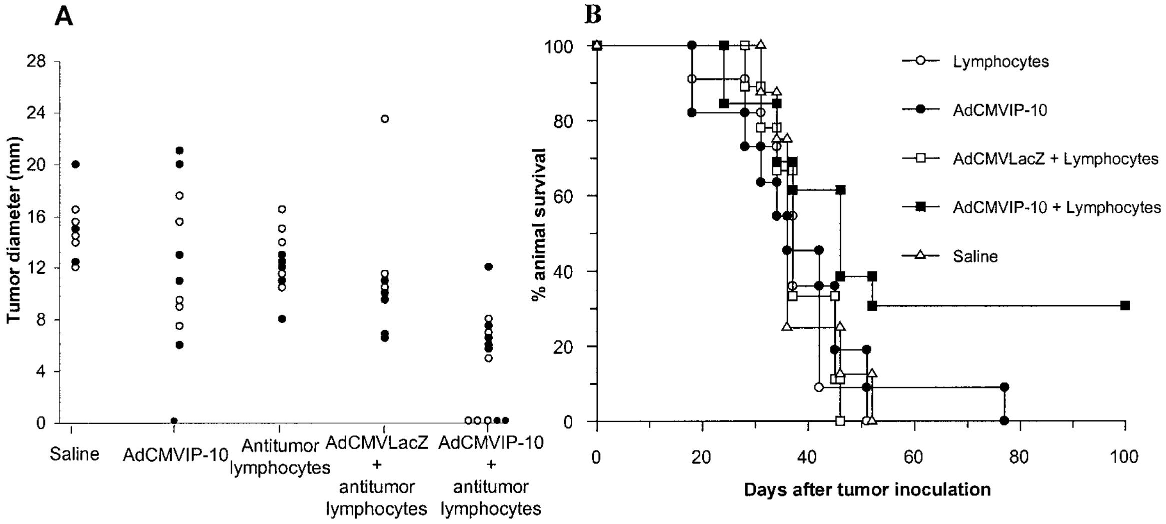


FIGURE 4. Combination of intratumoral AdCMVIP-10 and adoptive T cell therapy. Mice bearing tumors produced by CT26 cells injected into the hepatic mid-lobe were treated at day 9 after tumor cell inoculation with intratumoral injection of 10^9 pfu of the indicated recombinant adenoviruses or saline. In the indicated groups, mice were also i.v. injected on day 11 with 5×10^6 lymphocytes obtained from 7 day cultures of mytomicin-C-treated CT26 and lymph node cells from syngenic mice rejecting a CT26 s.c. tumor upon intralesional treatment with AdCMVIL-12. *A,* Individual liver tumor diameter at day 18 after tumor inoculation. *B,* Long-term survival follow up of these groups. Every experimental group in addition received low i.p. doses of IL-2 (2×10^4 IU) three times on alternate days from day 11. Statistical significance ($p < 0.01$) was found by nonorthogonal contrasts when comparing mice treated with AdCMVIP-10 plus antitumor lymphocytes with every other group. Two independent experiments represented by open or filled circles have been pooled.

gene transfer of IP-10 into established liver tumor nodules, which resulted in long-term survival in some cases (Fig. 4*B*). These data are reminiscent of our previous study with AdCMVIL-12 plus adoptive T cell therapy (42), although the synergy with adoptive T cell transfer displayed by IL-12 in the same system was more intense.

Intratumoral coinjection of AdCMVIP-10 and suboptimal doses of AdCMVIL-12 displays synergistic antitumor effects

We have described a potent antitumor therapeutic effect of AdCMVIL-12 against CT26-derived tumors when used at doses of 10^9 and 10^8 pfu (42). Under such conditions, AdCMVIL-12 directly injected into the tumor nodules led to eradication in 60–80% of the cases. When dealing with CT26 tumors, IL-12 gene transfer elicited an antitumor immune response that was $CD8^+$ T cell but not $CD4^+$ T cell dependent.

In Fig. 5, we show that lower doses of AdCMVIL-12 (7.5×10^7 pfu) given to s.c. tumor nodules that were allowed to grow for 9 days (4–8 mm diameter) only cured 4 of 11 cases with some delay of tumor growth in two additional mice. In striking contrast, combination of this suboptimal dose of AdCMVIL-12 (7.5×10^7 pfu) plus 5×10^8 pfu of AdCMVIP-10 consistently resulted in tumor regression of every treated tumor (10 of 10 cases). Such an outstanding result did not reflect transgene-unrelated effects caused by adenovirus combination because similar doses of AdCMVIP-10 and AdCMVLacZ did not show any significant change in tumor

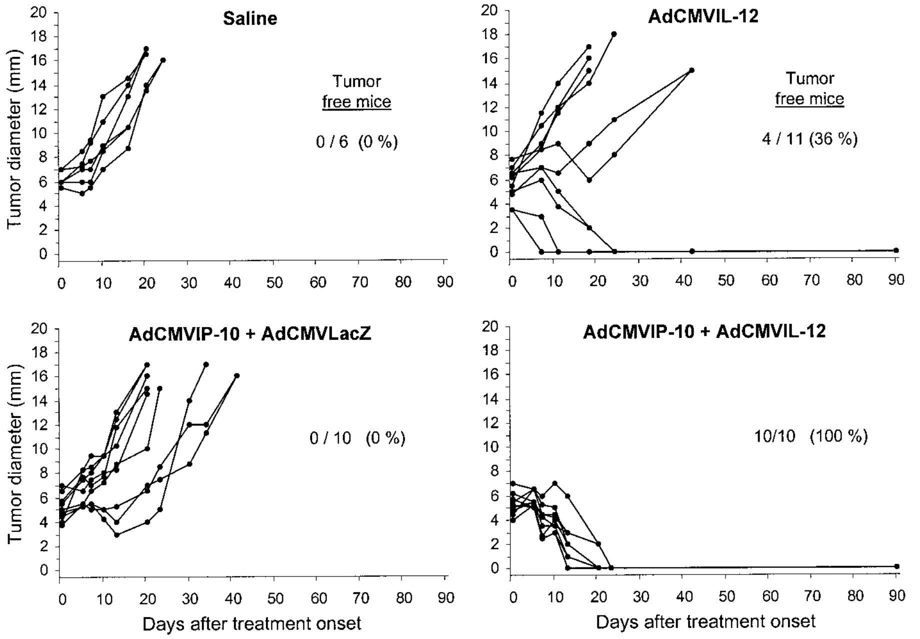


FIGURE 5. Combination of intratumoral AdCMVIP-10 and AdCMVIL12. Groups of mice bearing CT26 s.c. tumor nodules inoculated 9 days before treatment onset were intralesionally injected with the indicated adenoviruses or saline. Doses of 5×10^8 pfu were used with AdCMVIP-10 and doses of 7.5×10^7 pfu were used with AdCMVIL-12 and AdCMVLacZ. Individual evolution of the tumor diameter is shown, and the fraction of mice completely rejecting their tumors is indicated.

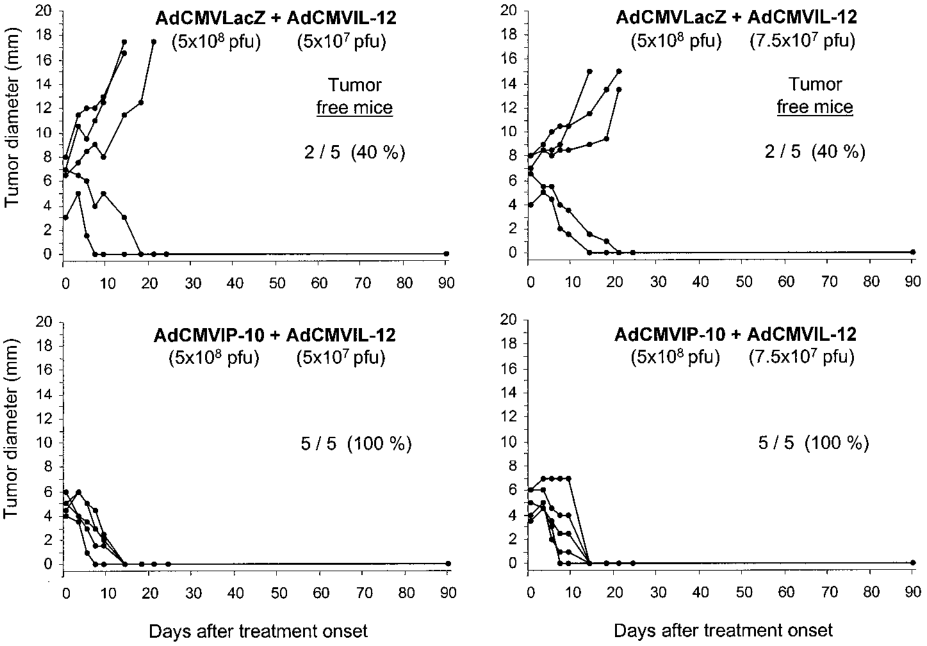


FIGURE 6. The therapeutic effects of combined intratumor injection of AdCMVIP-10 and AdCMVIL-12 are not transgene independent. Mice hosting s.c. CT26 tumors implanted 10 days earlier were injected intratumor with the indicated doses of adenovirus combinations, and the tumor size was monitored thereafter.

progression beyond a small delay in tumor growth in 3 of 10 cases if compared with intratumor injection of saline buffer. Moreover, in an additional set of experiments (Fig. 6) combination of AdCMVIL-12 (7.5 × 10^7 pfu or 5 × 10^7 pfu) with AdCMVLacZ (5 × 10^8 pfu) resulted in 40% tumor regression, while the same doses of AdCMVIL-12 combined with AdCMVIP-10 (5 × 10^8 pfu) caused 100% tumor disappearance. These results together with those of Fig. 5 indicate that the synergistic effect of AdCMVIP-10 on the antitumoral activity generated by AdCMVIL-12 is not transgene independent but mediated by IP-10 expression. In addition, repeated peritumoral injection of an antiserum anti-IP-10 delayed the rejection of tumors treated by AdCMVIP-10 plus AdCMVIL-12 for about 2 wk (data not shown).

AdCMVIP-10 plus AdCMVIL-12 synergy requires colocalization in the same tumor nodule

To ascertain as to whether adenoviral gene transfer of IP-10 and IL-12 required expression of the therapeutic genes on the same tumor nodule to display synergistic effects, we conducted experiments in which two tumor nodules were generated by CT26 s.c. inoculation into opposite flanks of the same mouse (Table I). Treatment with AdCMVIL-12 at suboptimal doses (7.5 × 10^7 pfu) led to tumor nodule regression of the treated site in only two of six cases, whereas the untreated site lethally progressed in every case. When AdCMVIP-10 (5 × 10^8 pfu) and AdCMVIL-12 (7.5 × 10^7 pfu) were injected in different nodules, two of six tumors regressed in the IL-12-transfected side and one of six regressed in the IP-10-transfected nodules. Interestingly, coinjection of the same doses of AdCMVIP-10 and AdCMVIL-12 led to tumor regression in every case (five of five cases) both at injected and at the untreated tumor location. Thus, synergy was greatly dependent on colocalization of both transgene products and thereby it could mediate a potent therapeutic effect against distant untreated tumors nodules. This is considered important to treat widespread metastatic disease.

Table I. *Tumor regression of bilateral s.c. CT26 tumors upon AdCMVIP-10 plus AdCMVIL-12 intratumoral treatment*[a]

Intratumoral Treatment		Tumor Regression After 90 Days[b]	
Right tumor	Left tumor	Right	Left
PBS	PBS	0/6	0/6
AdIL-12[c]	PBS	1/6	0/6
AdIP-10[d]	AdIL-12	1/6	2/6
AdIP-10+AdIL-12[e]	PBS	5/5	5/5

[a] Mice injected s.c. in opposite flanks with CT26 tumors received the indicated treatment in each side 10 days after tumor inoculation and were monitored for 90 days for complete tumor regression. A total of 5 × 10^5 CT26 cells s.c. in each side. Mean size, 5–6 mm.

[b] Fraction of mice with undetectable disease 90 days after treatment.

[c] AdCMVIL-12, 7.5 × 10^7 pfu.

[d] AdCMVIP-10, 5 × 10^8 pfu.

[e] AdCMVIP-10, 5 × 10^8 pfu plus AdCMVIL-12, 7.5 × 10^7 pfu.

CD4$^+$ and CD8$^+$ T cells and NK cells are involved in the antitumor efficacy of combined intratumor administration of AdCMVIP-10 and AdCMVIL-12

In the setting of CT26-derived s.c. tumor nodules treated with 5 × 10^8 pfu of AdCMVIP-10 and 7.5 × 10^7 pfu of AdCMVIL-12 given in a single bolus, we next studied the requirement of T cells for the observed therapeutic effect. Specific depletion of either CD4$^+$ or CD8$^+$ cells with specific mAb 3 days before intratumor treatment with the adenovirus combination resulted in a minor decrease of the antitumor activity (Fig. 7, *A* and *B*). However simultaneous depletion of CD4$^+$ and CD8$^+$ T cells resulted in lethal tumor progression in seven of nine mice, despite having received the AdCMVIP-10 plus AdCMVIL-12 combination (Fig. 7*C*). In BALB/c nude mice, intratumor treatment with AdCMVIP-10 plus AdCMVIL-12 also lacked antitumor activity (Fig. 7, *D* and *E*), confirming the T cell absolute requirement. These results are in contrast with depletion experiments in mice whose tumors had been treated with AdCMVIL-12 alone. In this case, only CD8$^+$ T cells are absolutely required for the antitumor effect, indicating a selective effect of IP-10 on activated CD4$^+$ T lymphocytes (27). Nonetheless, the nature of the effector cells under CD8$^+$ depletion was not clear, and experiments depleting NK cells with asialo GM1 and macrophages with gadolinium chloride were conducted (Fig. 8*A*). We found that depletion of asialo GM1$^+$ cells allowed the progression of two of four tumors treated with AdCMVIP-10

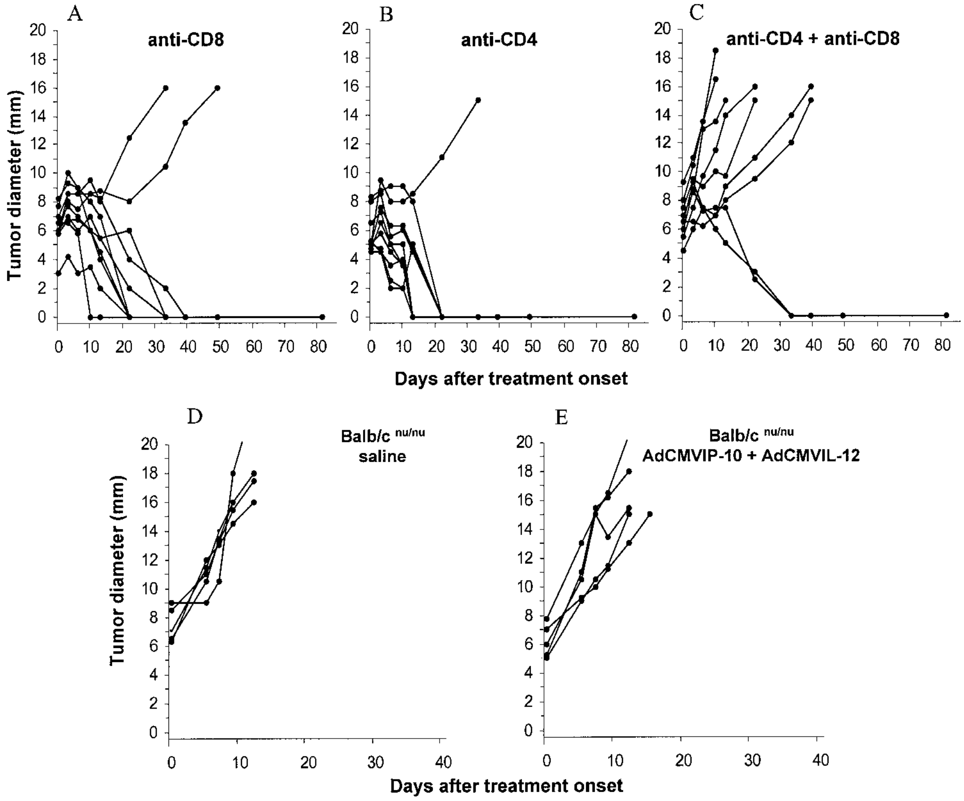


FIGURE 7. T cell requirement for tumor AdCMVIP-10 plus AdCMVIL-12 rejection. *A*, Individual tumor diameter evolution of BALB/c mice bearing 10 day s.c. CT26 tumors receiving a combination of intratumoral AdCMVIP-10 plus AdCMVIL-12, as in Fig. 5, that were in vivo depleted with injections of mAb with the indicated specificities. Depleting mAb i.p. injections started 3 days before adenovirus injections and were given daily for 3 days and weekly thereafter. Depletion was monitored by immunofluorescence and FACS analysis of PBL (not shown). A group of mice receiving polyclonal rat IgG rejected their tumors upon treatment with AdCMVIP-10 plus AdCMVIL-12 as fast as those in mice not receiving Ab (data not shown). *B*, Similar follow up of BALB/c$^{nu/nu}$ mice developing CT26 s.c. nodules for 10 days that were treated intratumorally with AdCMVIP-10 (5×10^8pfu) plus AdCMVIL-12 (7.5×10^7 pfu) or saline as indicated in the figure.

plus AdCMVIL-12. In contrast, gadolinium chloride did not impair the therapeutic effects (data not shown). Interestingly, under combined depletion of macrophages, NK, and CD8 cells, a residual antitumor activity was observed.

To specifically address if CD4$^+$ cells were mediating or orchestrating antitumor effects, such lymphocytes were immunomagnetically selected from spleens of mice who had rejected CT26 s.c. tumors upon treatment with AdCMVIP-10 plus AdCMVIL-12. Such T cells (1.7×10^7/mouse) were given i.v. to BALB/c$^{nu/nu}$ mice simultaneously challenged s.c. with 5×10^5 CT26 cells. The results (Fig. 8*B*) show that the adoptive transfer of CD4$^+$ cells clearly delayed the progression of the tumors nodules but did not cure them. Taken together, our data suggest that AdCMVIP-10 plus AdCMVIL-12 therapeutic effects are a result of combined functions of T cells (CD4$^+$ and CD8$^+$), NK cells, and nonimmune mechanisms possibly related to tumor vasculature.

In agreement with these experiments, specific immunostaining of frozen tumor sections treated 11 days earlier by a combination of AdCMVIP-10 plus AdCMVIL-12 showed a marked infiltration of CD4$^+$ lymphoid cells (Fig. 9*A*) with foci of CD8$^+$ infiltrate (Fig. 9*B*). Such results are reminiscent of the reported infiltrate induced by AdCMVIL-12 alone, which consists also of CD4$^+$ and CD8$^+$ T cells (Ref. 43 and our own results; data not shown).

Gene transfer to malignant cells of IP-10 and IL-12 synergizes to induce tumor-specific CTL activity

Splenocytes harvested from mice who have received intratumor treatment with AdCMVIP-10 plus suboptimal AdCMVIL-12 2–3 wk earlier displayed a potent lytic activity against CT26 after 6 days restimulation in vitro with mitomycin-C-treated CT26 cells. This activity was not detected in the spleen of mice who had been intratumorally treated with the same doses of either AdCMVIP-10 or AdCMVIL-12 (Fig. 10*A*). The CTL activity was directed, at least in part, to the described Moloney murine leukemia virus env gene-encoded tumor-associated Ag expressed by CT26 (38), because these lymphocyte cultures lysed P815 cells pulsed with the AH1 antigenic determinant presented by H-2L^d (Fig. 10*B*). Specificity of cytotoxicity was confirmed against nonpulsed P815, P815 pulsed with P815AB (control peptide), and the NK-sensitive target YAC-1 (Fig. 10*B*).

Our data strongly suggest that IP-10 enhance the CTL promoting activity of suboptimal gene transfer of IL-12 into malignant tissue.

Discussion

IP-10 (CRG-2) cDNA was chosen to construct recombinant adenovirus to transduce tumor nodules based on published data that demonstrate an enhanced immunogenicity of IP-10 stable transfectants (11). Genes encoding for several cytokines, including chemokines, have been transfected into tumor tissue with the help of adenovirus, and various degrees of interference with tumor progression have been reported (44). IP-10 might mediate antitumor activity through at least two possibly related mechanisms: 1) attraction into the malignant tissue of leukocytes and 2) inhibition of tumor angiogenesis.

Although our data demonstrate that functional murine IP-10 can be detected in the supernatant of cells infected in vitro with AdCMVIP-10, only a mild effect on established CT26-derived s.c. malignant nodules was noted after the injection of relatively large doses of the adenovirus. Despite the lack of measurable effects on the macroscopic tumor growth, histological examination of treated tumors disclosed areas of necrosis and vascular damage, consistent with the reported data for intratumoral injection of the recombinant protein (12). In our hands, supernatant from cells infected in vitro with AdCMVIP-10 attracted CD4$^+$ T cells with higher intensity than CD8$^+$ T cells, an effect that could be abrogated with anti

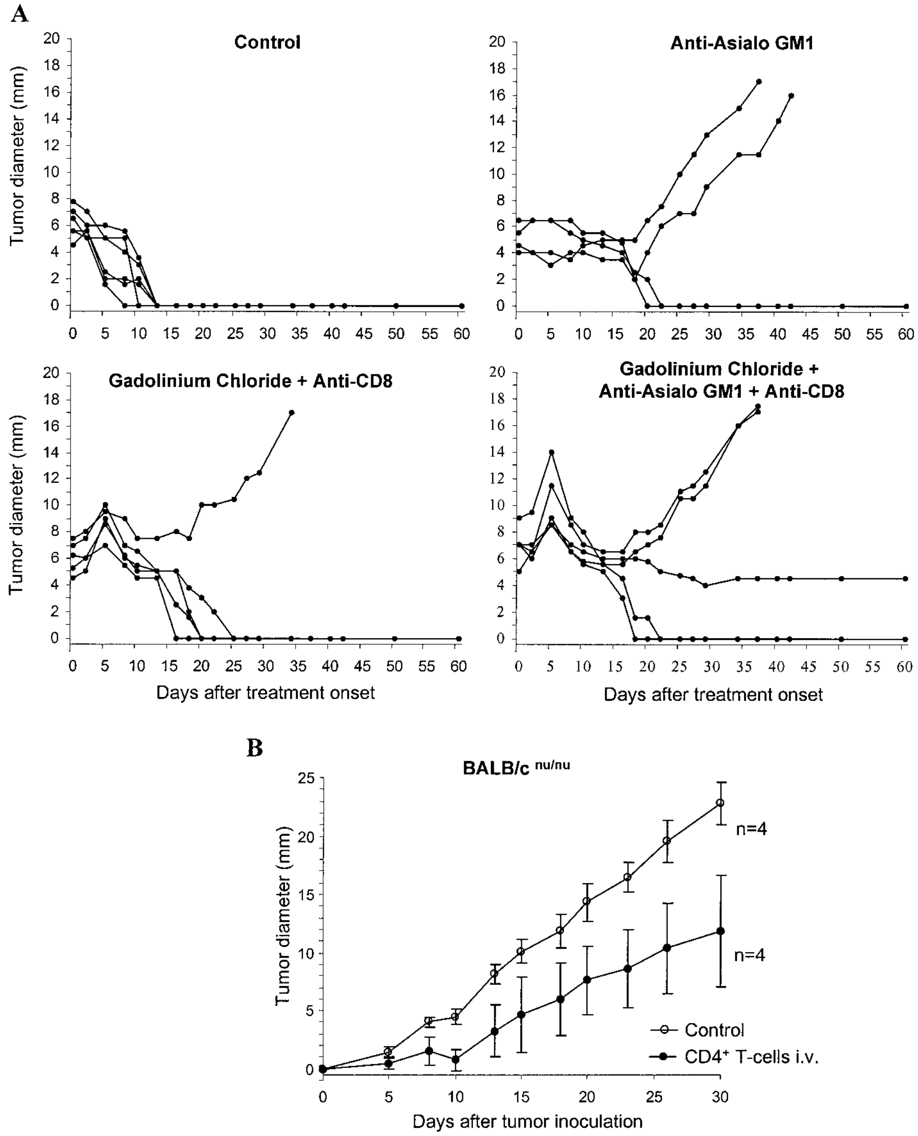


FIGURE 8. Complexity of the cellular mechanisms executing the antitumor effects of IP-10 plus IL-12 gene transfer. *A*, Mice hosting CT26 s.c. tumors were treated with AdCMVIP-10 (5×10^8pfu) plus AdCMVIL-12 (7.5×10^7 pfu) on day 10 after tumor cell injection (day 0). The different groups were treated with the indicated agents. Asialo GM1 antiserum (100 μl/dose) was injected i.p. on days −1, +2, +5, +10, +13, +15, and +17. The anti-CD8 mAb (100 μl of ascitic fluid/dose) was injected i.p. on days −3, −2, −1, +5, +10, +14, +21, and +28. Gadolinium chloride (20 μg/dose) was given i.v. on days −1, 0, +2, and every other day thereafter until day 23. Depletions were monitored by immunostaining of PBMC. *B*, Follow up of (mean diameter ± SD) s.c. tumors development in BALB/$c^{nu/nu}$ mice injected with 5×10^5 CT26 cells that were injected i.v. 3 h later with 1.7×10^7 $CD4^+$ cells purified by immunomagnetic sorting from the spleens of BALB/c mice who had rejected a CT26 s.c. tumor upon treatment with AdCMVIP-10 plus AdCMVIL-12. Purity of $CD4^+$ cells was >95% upon FACS analysis (data not shown).

IP-10 Abs. The preferential attraction of $CD4^+$ cells is in accordance with published data (8).

Intratumoral injection of AdCMVIL-12 (an adenovirus encoding for both chains of IL-12) at doses equal to those shown in Fig. 3 for AdCMVIP-10 induced tumor regression in ~70% of the cases when treating comparable tumor nodules associated with a marked increase in antitumor CTL activity (27). Because such lymphocyte cultures contained both activated $CD4^+$ and $CD8^+$ T cells, it was reasoned that IP-10 would probably help the homing of antitumor lymphocytes into the tumor tissue. Our data shows that IP-10 gene transfer into large well-established CT26 carcinomas growing in the liver had a synergistic effect with the adoptive transfer of short-term anti-tumor T lymphocyte cultures, an effect that was not observed with the control adenovirus-encoding β-galactosidase gene. It is conceivable that IP-10 expressed by the malignant tissue attracts $CD4^+$ cells and CTLs that in turn execute the antitumor effect.

These data are in agreement with previous reports in which intratumor injection of fibroblasts retrovirally modified with lymphotactin lack antitumor effects unless cotransfected with the immunostimulatory factor IL-2 (17). It was concluded that lymphotactin induced a tumor infiltrate but failed to expand and activate an antitumor immune response powerful enough to destroy the malignant inoculum. Similar pieces of information have been raised with the use of an adenovirus encoding for both IL-12 and lymphotactin, which were studied in comparison with adenovirus encoding separately each factor (20). In summary, in those studies lymphotactin increased the effects of IL-12 by attracting a more intense lymphoid infiltrate.

Because AdCMVIP-10 showed some synergy with adoptive transfer of lymphocytes derived from mice whose tumors were treated with AdCMVIL-12, the potential synergy of both adenoviruses when used together was explored. To this end, a suboptimal dose of AdCMVIL-12 was chosen. We show that simultaneous injection of AdCMVIP-10 along with AdCMVIL-12 induced tumor regressions in 100% of the cases using both optimal and suboptimal intralesional AdCMVIL-12 doses. This finding is important for two reasons: 1) in our CT26 model AdCMVIL-12 by itself failed to induce complete regressions in 20–30% of the cases, and 2) IL-12 can induce lethal shock (25), and reducing IL-12 doses, based in its synergistic effect with chemokines, might allow dose reduction with similar or even better effects in the absence of toxicity. Because we have not detected serious toxicity up to 10^{11} pfu of AdCMVIP-10 given intratumorally (data not shown), we believe that gene transfer of IP-10 will permit the use of safer doses of IL-12 without toxicity related to IP-10.

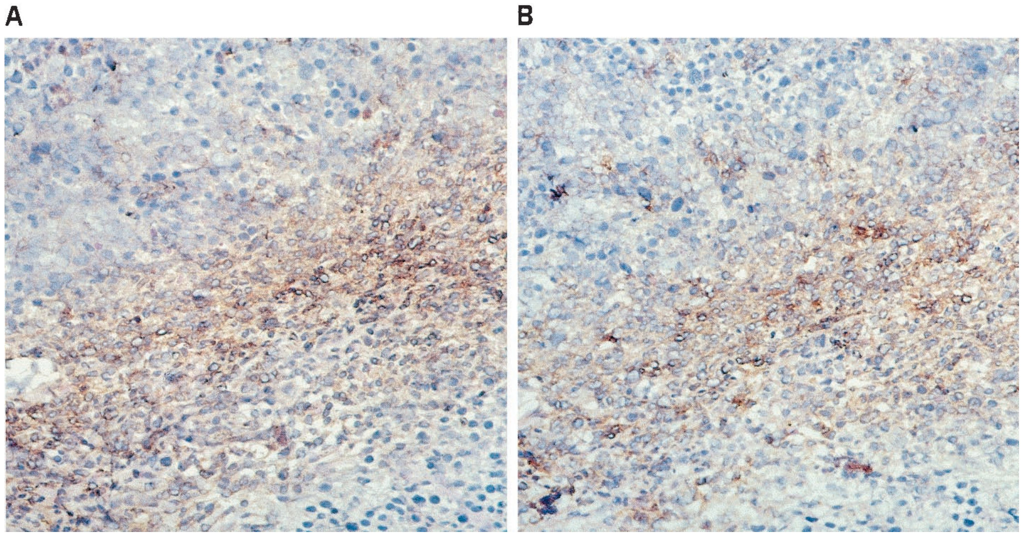


FIGURE 9. Lymphocyte infiltration of CT26 s.c. tumor nodules is secondary to intralesional injection of AdCMVIP-10 and AdCMVIL-12. Immunohistochemical staining with the indicated mAb of frozen sections of CT26 tumor nodules surgically removed 10 days after treatment with a combination of 5 × 10^8 pfu of AdCMVIP-10 and 7.5 × 10^7 pfu of AdCMVIL-12. Images are representative of multiple microscopic fields observed in at least five tumors equally treated. Lymphocyte infiltrates were absent in tumors injected with saline.

The combination of IL-12 and IP-10 transferred into the same tumor nodule displayed antitumor effects against a distant s.c. concomitant tumor. The relevance of these data is seen when we consider the natural evolution of metastasic colon cancer in which it will be often impossible to treat all the malignant sites at once. It is interesting to consider that synergy of IL-12 and IP-10 only took place when both recombinant adenoviruses were given to the same nodule, but not when identical doses were injected into distant tumor nodules. This is in agreement with the so-called "attraction and activation" hypothesis (45), which predicts the necessity of colocalization of immunostimulatory and chemoattractant factors as previously seen for lymphotactin (17, 20) and macrophage inflammatory protein-1α (18).

We have found an absolute requirement for T lymphocytes in the antitumor effect of IL-12 plus IP-10 local gene transfer. Total loss of the effect is only seen after double depletion of $CD4^+$ and $CD8^+$ cells indicating that both subsets can independently mediate the effects. This is in contrast with our own data obtained in the same CT26 model when using AdCMVIL-12 at optimal doses in which $CD8^+$ T cells were the only lymphocytes required for the effect (27). Our results pinpoint to a role for $CD4^+$ cells consistent with the observed mass infiltrate of $CD4^+$ and $CD8^+$ cells presumably attracted by IP-10. Results obtained after specific depletion with mAbs are not surprising, for $CD4^+$ T cells have been found to display antitumor properties despite the lack of MHC class II expression on malignant cells (46). The role of $CD4^+$ T cells could be to stimulate tumor cell killing by macrophages (46) and stimulation of dendritic cells to prime CTLs (47).

Cytokine secretion by CD4 T cells also can be activating NK cells to carry out their functions. In accordance to our depletion

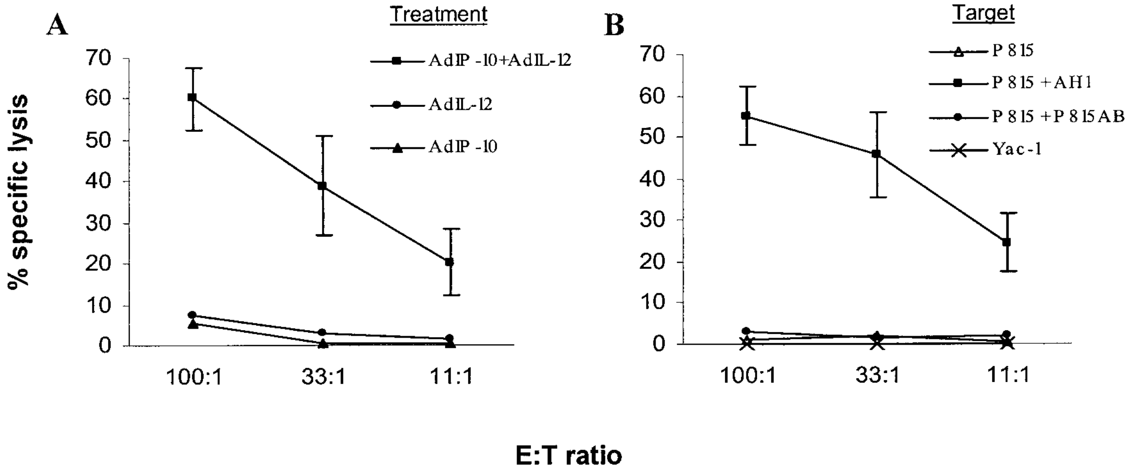


FIGURE 10. Intratumoral injection of AdCMVIP-10 and AdCMVIL-12 potently synergizes to raise tumor-specific CTL. *A*, Cytotoxic activity against CT26 measured in standard 5-h ^{51}Cr release assays displayed by splenocytes restimulated in vitro for 6 days with mytomicin-C-treated CT26 obtained from mice whose s.c. tumors were treated with the indicated adenovirus 3 wk before harvest of their spleens. Data are expressed by the mean specific lysis (%) ± SEM at different E:T ratios from three independent experiments. *B*, Cytotoxic activity displayed by lymphocytes of spleen cells from mice who had rejected a CT26 tumor upon treatment with AdCMVIP-10 and AdCMVIL-12 cocultured for 6 days with mytomicin-C treated CT26 against P815 and YAC-1 cell lines. In some experiments, P815 cells were incubated during the ^{51}Cr release assay with 10 ng/ml of the indicated 8-mer peptides AH1 or P815AB (control peptide). Data represent the specific lysis ± SEM in two experiments.

data with anti-asialo GM1, NK cells probably play a pivotal role in tumor rejection but their contribution is not sufficient, because combined therapy fails in nude mice who have normal NK activity but lack conventional T cells. The role for NK cells in the effects of IP-10 plus IL-12 combined therapy is in agreement with published observations from other groups (48, 49). We cannot rule out a contribution by NK-T cells as suggested by others (50) because it is noteworthy that asialo GM1 antiserum also depletes NK-T cells and possibly other T cell populations (51). In our experimental conditions, although macrophages could exert some antitumor activity, extensive gadolinium chloride treatment do not significantly alter the outcome, indicating that there is not important requirement for these cell populations. Partial efficacy of adoptive transfer of immune CD4 cells to nude mice further show a role for Th cells in the therapeutic activity. Despite the adoptive transfer of high numbers of such T cells, the antitumor effect was not complete, a result that can be interpreted in the sense that local conditioning of malignant tissue by the expression of the IL-12 and IP-10 transgenes is also required.

Our results show a dramatic increase in anti CT26 CTL activity upon intratumoral injection of AdCMVIL-12 plus AdCMVIP-10, which is probably related to an ongoing Th response favored by IP-10-mediated recruitment of activated CD4 cells. Nonetheless, other potential effects of IP-10 could favor CTL generation such as the observed necrosis of malignant tissue that could release tumor Ags in a fashion suitable for presentation by professional APCs (52). Increase in CTL activity has also been reported for the combination of IL-12 and lymphotactin by gene transfer (20).

Antitumor effects of IL-12 plus IP-10 most likely reflect the complexity of an interconnected network of immune and nonimmune mechanisms difficult to dissect and possibly redundant in achieving the outcome of tumor rejection. Regardless of the mechanisms involved, we are reporting a powerful tool to treat experimental murine malignancies by simultaneous adenoviral gene transfer of IP-10 and IL-12. Potency and safety profiles of the approach might make it find a place in immunotherapy of human malignancies.

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