Assessment of a Combined, Adenovirus-Mediated Oncolytic and Immunostimulatory Tumor Therapy

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Abstract
In this study, we identified murine breast cancer cell lines that support DNA replication of E1-deleted adenovirus vectors and which can be killed by an oncolytic adenovirus expressing adenovirus E1A and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) in a replication-dependent manner (Ad.IR-E1A/TRAIL). We showed that systemic or intratumoral (i.t.) injection of adenovirus vectors into mice increases plasma levels of proinflammatory cytokines and chemokines, including TNF-α, INF-γ, and MCP-1, which are potent inducers of dendritic cell maturation. Furthermore, we showed that in vivo expression of Flt3L from an adenovirus vector increases the number of CD11b+ and CD11c+ cells (populations that include dendritic cells) in the blood circulation. Based on these findings, we tested whether Ad.IR-E1A/TRAIL induced killing of tumor cells in combination with dendritic cell mobilization by Ad.Flt3L or, for comparison, Ad.GM-CSF would have an additive antitumor effect. As a model, we used immunocompetent C3H mice with syngeneic s.c. tumors derived from C3L5 cells. We found that vaccination of mice with C3L5 cells that underwent viral oncolysis in combination with Flt3L or granulocyte-macrophage colony-stimulating factor (GM-CSF) expression induces a systemic antitumor immune response. i.t. injection of the oncolytic and Flt3L expressing vectors into established tumors delayed tumor growth but did not cause efficient tumor elimination. This study shows the effectiveness of a combined oncolytic/immunostimulatory tumor therapy approach. (Cancer Res 2005; 65(10): 4343-52)

Introduction
Adenoviruses are known to elicit expression of proinflammatory cytokines as well as cell-mediated immune responses upon in vivo administration. Although adaptive immune responses are generally limited to the virally infected cells, there is some evidence that adenovirus infections can contribute to immune responses nonviral antigens. For example, adenovirus infections have been associated with transient autoimmune features (1) and graft versus host disease after allogeneic bone marrow transplantation (2, 3). These clinical observations suggest that adenovirus can break, or help break, tolerance, and for this reason, adenoviruses have been extensively studied as delivery vectors and adjuvant for vaccination (4). Notably, in the largest nonhuman primate study of HIV vaccines to date (using an SIV-HIV hybrid model), adenovirus vectors expressing the SIV gag protein were most efficient in providing true clinical protection when compared with various vaccine approaches (4).

It is thought that tumor cells dying in an environment containing proinflammatory cytokines are a potent source of antigens for presentation by and activation of dendritic cells. This implies that oncolytic adenoviruses might provide a means to stimulate an antitumor immune response. We have recently developed adenovirus vectors that achieve tumor-specific viral replication, tumor cell lysis, and viral spread. These vectors are deleted for the immediate early EIA and EIB regulatory genes (termed E1-deleted adenovirus vectors) and replicate their DNA in a variety of human tumor cell lines (5–7). In contrast, viral DNA replication of E1-deleted vectors is not detectable in primary human cells in vitro or in livers of mice and nonhuman primates after systemic application. We have modified the genome of these E1-deleted adenovirus vectors to construct new vectors (termed “Ad.IR” or inverse repeat adenovirus vectors) in which transgene expression is activated only upon replication-dependent homologous recombination between inverted homology regions (IR) flanking the transgene (ref. 8; Supplementary Fig. S1). This limits expression of transgenes to cells which support E1-deleted adenovirus vector DNA replication (i.e., tumor cells). To overcome low level viral replication and lysis of tumor cells (characteristic of most E1-deleted adenovirus vectors) the EIA gene was inserted into Ad.IR vectors (6). Ad.IR-E1A vectors showed efficient, tumor-specific transgene expression, replication, and spread in tumor cell lines in vitro and in vivo. We have also produced an Ad.IR-E1A vector expressing tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (9). i.v. administration of Ad.IR-E1A/TRAIL resulted in elimination of liver xenograft metastases derived from human tumor cell lines in mice (9) and caused no toxicity in nonhuman primates after i.v. injection (10).

In cancer patients, ineffective antitumor responses have been shown to be in part due to a functional impairment in tumor recognition and initiation of an immune response through antigen-presenting cells (APC), most notably dendritic cells. One approach to overcome these problems is to increase the number of tumor-infiltrating dendritic cells. The FMS-related tyrosine kinase 3 ligand (Flt3L) has been shown to mobilize and stimulate natural killer (NK) cells as well as dendritic cells and to induce antitumor activity in mouse models (11–13). The ability to increase the number of dendritic cells from myeloid and circulating precursors has also been reported for granulocyte-macrophage colony-stimulating factor (GM-CSF). A series of studies using adenovirus vectors for GM-CSF gene transfer showed stimulation of antitumor immune responses (14–18). Whereas Flt3L predominantly stimulates immature dendritic cells, GM-CSF can also promote dendritic cell maturation. In contrast to Flt3L, GM-CSF is not stimulatory for NK cells (19, 20).

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In this study, we tested a combination of adenovirus-based oncolytic and immunostimulatory anticancer therapies. An oncolytic virus (expressing E1A and TRAIL in a tumor-specific manner) was used to infect and induce cell death in tumor cells, which can subsequently serve as a source for tumor antigens. In parallel, adenovirus vectors expressing Flt3L or GM-CSF were used to mobilize APCs. Our goal was to investigate whether infection of tumor cells and induction of apoptosis by oncolytic vectors provides an antigenic stimulus that, in combination with expression of Flt3L or GM-CSF, results in control of tumor growth in a vaccination and therapeutic scheme.

Materials and Methods

Adenovirus vectors. AdBG (8) and Ad.CMV-GFP (21) are E1/E3-deleted adenovirus vectors that expresses *Escherichia coli* β-galactosidase and green fluorescent protein (GFP) under the control of the Rous sarcoma virus (RSV) promoter. AdIR-GFP (22) expresses GFP in a replication-dependent manner. AdIR-TRAIL (23) expresses the proapoptotic TRAIL in a replication-dependent manner. AdIR-E1A/AP (6) expresses Ad5 E1A and alkaline phosphatase (AP) in a replication-dependent manner. AdIR-E1A/TRAIL is an Ad5-based vector that has the same transgene cassette as Ad5/35E1A/IR1/TRAIL (9) and expresses Ad5 E1A and TRAIL in a replication-dependent manner. AdFt3L is an E1/E3-deleted adenovirus vectors that expresses human Flt3L under the control of the RSV promoter. The hFlt3-L cDNA was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cloned into pAd.BSV (8) to construct AdFt3L. Ad.GM-CSF was kindly provided by Richard Vile (Mayo Clinic, Rochester, MN). Ad.GM-CSF is a first-generation adenovirus vector containing a bicistronic HSVG/murine GM-CSF expression cassette (24, 25). Viral vectors were produced and propagated following standard procedures (26). Viruses were banded in CsCl gradients, dialyzed, and stored in aliquots as described previously. Titer were determined by plaque titration in 293 cells as described (26) and the contamination level of wild-type (wt) virus was examined by real-time PCR as described (6). Only vector preparations that contained less than one wt viral genome in 10^6 genomes were used in these studies. Lack of endotoxin contamination was shown using a *Limulus* amebocyte lysate endotoxin detection kit (Bio Whittaker, Walkerville, MD).

Cells. Murine breast cancer cell lines 4T1, JC, EMT-6, TM40D are derived from BALB/c mice. C3L5 is derived from C3H/Je mice. 4T1 and JC cells were obtained from the ATCC (CRL-2539). We are grateful to Dr. Rasey (Department of Radiation Oncology, University of Washington, Seattle, WA; ref. 27), Dr. Medina (Department of Cell Biology, Baylor College of Medicine, Houston, TX; ref. 28), and Dr. Lala (Department of Anatomy and Cell Biology, The University of Western Ontario, London, Ontario, Canada; ref. 29) for providing the EMT-6, TM40D, and C3L5 cell lines, respectively. 4T1 and JC cells were grown in RPMI with 2 mmol/L L-glutamine. Penicillin (100 units/mL) and streptomycin (100 μg/mL) were added to all media.

*In vitro replication assay.* A site-specific methylation strategy was used to monitor viral DNA replication in infected cells (30). Methylated adenovirus vectors were produced by propagation in 293 cells expressing PaeR7 methyltransferase (PMT). PMT protects XhoI sites from XhoI cleavage and loss of methylation through replication in PMT-negative tumor cells restores XhoI cleavage. Replicated genomic DNA can therefore be detected by Southern blotting of XhoI- and HindIII-digested genomic DNA from infected cells.

Flt3L/GM-CSF ELISA. C57Bl/6, BALB/c, and C3H/H3J mice received a single dose of 5 × 10^6 pfu of Ad.Ft3L or Ad.BG control virus via tail vein injection. A third group did not receive any virus. At 3, 7, and 14 days after tail vein injection, serial dilutions of plasma were subjected to ELISA analysis according to the manufacturer's instructions (Quantikine hFlt3L kit, R&D Systems, Minneapolis, MN). Ad.GM-CSF was injected into C3H mice and serum concentrations of murine GM-CSF were analyzed by ELISA (Quantikine mGM-CSF kit, R&D Systems).

Analysis of peripheral blood cells. C57Bl/6, BALB/c, and C3H/H3J mice received a single dose of 5 × 10^6 pfu of Ad.Ft3L or Ad.BG control virus via tail vein injection. At the indicated time points, blood was drawn for fluorescence-activated cell sorting analysis (FACS). Red cells were lysed using ammonium chloride (PharmLyse, BD Biosciences, Palo Alto, CA). Leukocytes were washed with PBS + 1% inactivated FBS and incubated with anti-CD4-PE (9145-2C11), anti-CD4-PRE (RM4-5), anti-CD8a-PE (53-6.7), anti-CD19-PE (1D3), anti-CD11b-PE (M1/70), anti-CD11c-PE (HL3), and appropriate isotype controls (all from BD Biosciences; 1 μL antibody per 10^6 cells). Cells were washed and the percentage of cells expressing each CD molecule was determined using flow cytometry (FACScan, BD Biosciences). Plasma samples from at least three mice were analyzed.

WBC and spleen weight was measured at day 10 after injection of 5 × 10^6 pfu of Ad.BG, Ad.Ft3L, or Ad.GM-CSF into C3H mice.

Plasma levels of cytokines/chemokines. To analyze serum levels of proinflammatory cytokines and chemokines, 30 minutes, 6, and 24 hours after i.v. administration of adenovirus vectors, blood samples were collected into heparin-treated Eppendorf tubes. Cells were pelleted for 5 minutes at 1,000 × g, and plasma was obtained and stored at −80°C in aliquots. To analyze plasma cytokine/chemokine levels, a “Mouse Inflammatory Cytometric Bead Array” (BD Biosciences) was used. Briefly, 10 μL of mouse plasma were diluted five times and mixed with cytometric beads capable of binding mouse TNFα, IL-6, MCP-1, and IFN-γ. Bound cytokines/chemokines were detected with corresponding secondary phycoerythrin-conjugated antibodies and analyzed by flow cytometry along with provided standard proteins. The collected data were processed using the manufacturer's software. Plasma samples obtained from at least three mice were analyzed in duplicate.

Crystal violet staining. Plated cells were infected and allowed to develop cytopathic effect (CPE). Before crystal violet staining, medium was removed and cells were fixed for 3 minutes in 3,7% paraformaldehyde at room temperature. Fixed cells were washed with PBS and incubated for 3 minutes in 1% crystal violet in 70% ethanol followed by three rinses with water. Air-dried cells were photographed.

Animal models. For s.c. tumors, 1 × 10^3 C3L5 cells (in a volume of 0.1 mL of DMEM) were injected into the right and left inguinal regions of C3H mice. S.c. tumors were measured with a caliper every other day, and the tumor volume was calculated using the formula largest diameter × (smallest diameter)^2. For vaccination studies (Fig. 7), C3L5 cells were infected with adenovirus vectors at a multiplicity of infection (MOI) of 100 plaque forming units (pfu) per cell overnight, trypsinized, and washed to remove noninternalized vector particles. Mice were sacrificed when the tumor volume exceeded 1,000 mm^3 or 10% of the body weight. Intratumoral (i.t.) adenoviral injections were done with 50 μL of adenovirus vectors in PBS at a dose of 1 × 10^9 pfu. i.v. injections of 5 × 10^9 pfu (in 200 μL PBS) were done via the tail vein.

Results

Identification of mouse breast cancer cell lines supporting efficient viral infection, DNA replication, and oncolysis. Several syngeneic mouse models of breast cancer have been described, including 4T1, EMT-6, TM40D, C3L5, and JC cells. We first selected cell lines that can be efficiently infected and killed by our oncolytic vector, AdIR-E1A/TRAiL. High sensitivity to AdIR-E1A/TRAiL implies that the cell line is readily infectible by adenovirus vectors, supports DNA replication of E1-deleted adenoviruses and formation of rearranged genomes, and is susceptible to TRAIL-mediated apoptosis. To compare infectibility, the cell lines were...
cells were infected at an MOI of 300 pfu per cell, C3L5 and TM40D * cells at DNA was extracted, digested with Hin
Ad5 virus to ensure similar uptake of viral DNA. At the indicated time points, 100 pfu per cell, and EMT-6 cells at 30 pfu per cell of a methylated, E1-deleted means of three individual experiments; Points, percentage of GFP expressing cells was determined using flow cytometry. Infectibility, viral DNA replication, and replication-activated gene expression from E1-deleted Ad.IR vectors.

Figure 1. Infectibility, viral DNA replication, and replication-activated gene expression from E1-deleted Ad.IR vectors. A, murine breast cancer cells, 4T1, JC, C3L5, EMT-6, and TM40D, were seeded at similar densities, left to adhere overnight, and exposed to varying MOIs of an E1/E3-deleted adenovirus vector expressing GFP from a CMV promoter. At 72 hours after infection, the percentage of GFP expressing cells was determined using flow cytometry. Points, means of three individual experiments; bars, SD. B, 4T1 and JC cells were infected at an MOI of 300 pfu per cell, C3L5 and TM40D * cells at 100 pfu per cell, and EMT-6 cells at 30 pfu per cell of a methylated, E1-deleted Ad5 virus to ensure similar uptake of viral DNA. At the indicated time points, DNA was extracted, digested with HindIII and XhoI, subjected to Southern blotting and probed for viral DNA. C, cells were infected with Ad.IR-GFP at MOIs ranging from 0.03 to 1,000 pfu per cell. At 72 hours after infection, cells were analyzed for GFP expression by flow cytometry. Points, means of three individual experiments; bars, SD. *TM40D cell could not be infected with MOIs above 300 pfu per cell due to toxicity.

infected with Ad.CMV-GFP (21), and the percentage of GFP-expressing cells was analyzed (Fig. 1A). Infectibility was best for EMT-6 cells followed by C3L5, TM40D, JC, and 4T1 cells.

Next, we studied whether the tumor cell lines support viral DNA replication and homologous recombination of an E1-deleted adenovirus vector. As outlined above, viral DNA replication is a prerequisite for homologous recombination in Ad.IR vectors and activation of transgene expression. Viral DNA replication was analyzed by a demethylation assay after infections at MOIs that allow for comparable uptake of adenovirus vectors (see Fig. 1B, ‘‘3 hour’’ lane). Efficient replication of E1-deleted vector genomes was observed in EMT-6 and C3L5 cells. Low-level DNA replication was found in the 4T1 cell line. To analyze replication-activated transgene expression through homologous recombination, a GFP-expressing Ad.IR vector was used in infections at increasing MOIs (Fig. 1C). Both EMT-6 and C3L5 cells efficiently supported GFP expression from the Ad.IR system, whereas 4T1 and JC cells allowed for replication-activated expression at higher MOIs. In contrast, TM40D cells did not show recombination-activated GFP expression, and increasing MOIs led to cell death from viral CPE before recombination could be observed.

We also tested how well our murine breast cancer cell lines support the replication of wild-type viral DNA and production of progeny viral particles (Supplementary Fig. S2). EMT-6, C3L5, and TM-40D cells supported wild-type adenovirus DNA replication at similar levels, in contrast with the data obtained for E1-deleted genomes, where TM40D cells were far more inefficient than the other two cell lines. EMT-6 and C3L5 cells also supported the production of progeny virions, as analyzed by plaque assay. However, the efficiency of progeny virus production was orders of magnitude lower than in human HeLa cells. No progeny virion production was detectable in 4T1, JC, and TM40D cells. Of all the cell lines analyzed, EMT-6 and C3L5 seemed the most suitable models to test oncolytic adenoviral vectors in syngeneic mouse models. However, when we tried to establish s.c. tumors with EMT-6 and C3L5 cells, in syngeneic immunocompetent C3H and BALB/c mice, respectively, only C3L5 cells engrafted. EMT-6 cells are highly immunogenic, which prevented their use in our tumor model. Subsequent studies were therefore done with C3L5 cells.

We tested whether Ad.IR vectors expressing TRAIL and E1A in a replication-activated manner, individually, or in combination, would efficiently kill C3L5 cells. Ad.IR-TRAIL, Ad.IR-E1A, Ad.IR.E1A/TRAIL, and an E1-deleted control vector (Ad.BG) were infected onto tumor cells at increasing MOIs. Six days after infection, viable cells were stained with crystal violet (Fig. 2A). Overall, the vector that expressed both E1A and TRAIL exerted greater cytotoxicity than vectors that express these genes individually. For example, an MOI of 10 pfu per cell of Ad.IR.E1A/TRAIL was sufficient to kill all cells. Figure 2B shows characteristic apoptotic features in C3L5 cells infected with Ad.IR-E1A/TRAIL, in comparison with Ad.BG-infected cells.

In vivo tumor model. We first studied the growth kinetics of s.c. tumors after injection of untransduced and transduced C3L5 cells. To ensure that Ad.IR-E1A/TRAIL exerts an antitumor effect in vivo, C3L5 cells were infected in vitro with Ad.IR-E1A/TRAIL, Ad.IR-E1A/AP, or an Ad.BG control vector. Infected cells were mixed at ratios of 1% and 10% with uninfected cells. Mice were inoculated s.c. with 10^7 uninfected cells or infected/uninfected cell mixtures, and tumor growth was monitored over time. All
mice developed tumors, however, Ad.IR-E1A/TRAIL more effectively delayed tumor growth than the two control vectors (P < 0.01 for 10% Ad.IR.E1A/TRAIL at day 21), and this effect was dose dependent (Fig. 3A).

Microdissected tumors showed high degrees of vascularization as shown by staining for the endothelial cell marker CD31 (Fig. 3B). Furthermore, infiltrating (F4/80-positive) monocytes/macrophages were detected in s.c. tumors (Fig. 3C). Tumor vascularization and infiltration by cells of the immune system is a prerequisite for immunotherapy.

Next we studied adenoviral transduction of tumor cells in s.c. tumors. Ad.BG was injected i.t. (1 × 10^9 pfu) and i.v. (5 × 10^9 pfu) and ß-galactosidase expression was analyzed in tumor sections 3 days later. Whereas i.t. adenoviral injection resulted in transduction of about 40% of tumor cells (Fig. 4A), i.v. Ad.BG injection conferred transgene expression in <1% of tumor cells in any given s.c. tumor (data not shown). Figure 4B shows that a significant fraction of vector genomes can be found in the blood circulation after adenoviral injection into s.c. tumors. This finding is in agreement with earlier observations by Lohr et al. (31). We also measured plasma levels of selected proinflammatory cytokines/chemokines (Fig. 4C). All analyzed cytokines and chemokines, including potent inducers of dendritic cell maturation, were elevated upon i.v. and i.t. adenoviral injection. In summary, s.c. C3L5 tumors grow aggressively and can be efficiently transduced by adenovirus vectors upon i.t. injection. Tumors are vascularized, which allows for leukocyte infiltration. Adenovirus injection results in release/expression of cytokines that potentially can activate maturation of dendritic cells.

Mobilization of CD11b/c^+ populations by Ad.Flt3L. Murine and human Flt3L cross-react, and numerous studies showed the effect of human Flt3L in healthy and tumor-bearing mice. We therefore decided to work with the human flt3-L cDNA. The target cell for Flt3L in this therapeutic approach (immature dendritic cells) does not reside within the tumor. In this study, we constructed an adenoviral vector that expresses Flt3L under the direct control of the strong RSV-promoter (Ad.Flt3L). In earlier studies, we have shown that i.v. injection of this type of vector results in high and sustained systemic expression levels of various transgenes for a period of at least 2 weeks. Groups of eight mice of different genetic backgrounds, C57Bl/6, BALB/c, C3H (known to mount different antivector immune responses; ref. 32), were injected with Ad.Flt3L. At the indicated time points, blood samples were drawn and analyzed for the presence of human Flt3L by ELISA and for leukocyte subsets by FACS analysis (Fig. 5). Three days after Ad.Flt3L injection, high systemic Flt3L plasma levels were measured in mice that had received Ad.Flt3L (data not shown). Plasma levels peaked at day 7 post injection and declined afterwards in BALB/c and C3H mice (Fig. 5A). These mouse strains have previously been reported to mount a strong antiadenovirus immune response (32) and the decline in Flt3L

Figure 2. Cytopathic effect of C3L5 cells. C3L5 cells were plated in 24-well dishes and left to adhere overnight. The following day, cells were infected with adenovirus vectors at the indicated MOIs. After 7 days, cells were fixed in formaldehyde, washed, and stained with crystal violet (A) or analyzed by light microscopy (B).
expression is most likely due to elimination of transduced hepatocytes. A small peak in plasma Flt3L levels was observed on day 7 after the injection of the Ad.BG control virus. This may also reflect an immune response caused by viral infection of a large number of hepatocytes. Analysis of leukocyte subsets in Ad.Flt3L-injected C57Bl/6 mice shows a substantial increase in the percentage of both myeloid CD11b+ and CD11c+ cells, a subpopulation which comprises dendritic cells (Fig. 5C). Similar results were obtained with BALB/c and C3H mice (Fig. 5B). The spleen weight and total number of leukocytes were also found to be substantially increased in Ad.Flt3L-injected mice (data not shown). A small increase in CD11b-positive but not CD11c-positive cells was noted in control virus–injected mice. In addition, the spleen size but not the number of peripheral circulating white cells was increased in these mice. In conclusion, the administration of a standard dose of Ad.Flt3L leads to high transient plasma levels of Flt3-L and mobilization of several leukocyte subsets including CD11c-positive cells.

For comparison, we included an adenovirus vector expressing murine GM-CSF (24, 25) into our studies. (In contrast to Flt3L, GM-CSF is species-specific; ref. 33); 5 × 10⁹ pfu of Ad.BG, Ad.Flt3L, or Ad.GM-CSF were i.v. injected into C3H mice and serum GM-CSF and Flt3L levels were measured by ELISA at days 3, 7, and 10. GM-CSF expression peaked at day 3 after vector injection; however, compared with Flt3L levels in Ad.Flt3L-injected mice, serum GM-CSF levels were 10-fold lower and declined much faster (data not shown). In addition to intrinsic differences in the ELISA, the lower GM-CSF levels might be due to the nature of the adenovirus construct that carries a bicistronic expression cassette with the GM-CSF cDNA as second cistron. To assess the functionality of GM-CSF expressed from the adenovirus vector, we compared WBC and spleen weight at day 10 after adenoviral injection. Whereas both Ad.Flt3L and Ad.GM-CSF stimulated leukocytosis and caused splenomegaly (Fig. 5D), these effects were less pronounced for the GM-CSF–expressing vector, which might be due to the lower serum concentration of this cytokine.

**Combined oncolytic and immunostimulatory therapy.** We first tested whether Ad.IR-E1A/TRAIL-mediated tumor cell lysis in combination with Ad.Flt3L or Ad.GM-CSF–mediated dendritic cell mobilization would induce an antitumor effect after injection of adenovirus vectors into s.c. C3L5 tumors. Preestablished tumors were injected twice with a total dose of 1 × 10⁹ pfu of adenovirus. This dose results in transduction of about 40% of tumor cells in any given s.c. tumor (see Fig. 4A). Tumors were mock-injected or injected with a combination of Ad.IR-E1A/TRAIL + Ad.Flt3L or Ad.IR-E1A/TRAIL + Ad.GM-CSF, with either vector alone, or with control vector (Ad.BG). The tumor sizes of all groups were measured before injection (day 0) and at days 4, 9, 13, and 18 after adenoviral injection. Figure 6A shows the tumor size for all groups.

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**Figure 4.** Plasma cytokine levels upon i.v. or i.t. adenovirus administration. A, mice bearing tumors with an average volume of 100 mm³ were i.t. injected twice (on two consecutive days) with 1 × 10⁹ pfu of Ad.BG. Three days after the last injection, tumor sections were analyzed for β-galactosidase expression. The tumor appears as a compact mass surrounded by lose connective tissue. Magnification, 4× (left) and 20× (right). B, systemic leakage of i.t. injected adenovirus. Ad.BG were injected into s.c. tumors (i.t.) at a total dose of 2 × 10⁹ pfu or into the tail vein (i.v.) at a dose of 5 × 10⁹ pfu. Adenovirus vector genomes in plasma 3, 15, and 120 minutes after adenovirus injection were quantitated by real-time PCR. C, plasma cytokine levels were measured 6 hours after Ad.BG injection. Previous studies showed that plasma cytokines reached peak levels 6 hours after i.v. injection of adenovirus vectors (41).
measured at day 13 after injection. (Mice with mock- and Ad.BG-injected tumor had to be sacrificed before day 18 because of the large tumor burden.) Both injection of the oncolytic vector Ad.IR-E1A/TRAIL and the Flt3L-expressing vector alone significantly slowed tumor growth compared with the mock- or Ad.BG-injected groups ($P < 0.00001$), whereas the Ad.GM-CSF vector alone had no significant antitumor effect. The combination of Ad.IR-E1A/TRAIL and Ad.Flt3L had an additive antitumor effect compared with either vector alone ($P < 0.00001$). The same is true for the combination of Ad.IE-E1A/TRAIL and Ad.GM-CSF. The combination of the oncolytic vector with Ad.Flt3L had a greater antitumor effect than the combination of the oncolytic and GM-CSF-expressing vectors ($P = 0.00038$). Importantly, tumor regression (one of five tumors) or absence of progression (three of five tumors) was seen in the Ad.IR-E1A/TRAIL + Ad.Flt3L–injected group (Fig. 6B). In the Ad.IR-E1A/TRAIL + Ad.GM-CSF–injected group, tumor growth stagnated in one of five tumors. All other tumors in this group and in all the other groups progressed. In conclusion, i.t. Ad.IR-E1A/TRAIL and Ad.Flt3L injection had an additive antitumor effect that lead to regression/attenuation of tumor growth in the majority of mice.

Systemic antitumor immune response after vaccination with adenovirus-transduced tumor cells. We assessed whether a combination of viral oncolysis and expression of Flt3L or GM-CSF can induce a protective antitumor immune response. Figure 7A shows the scheme of this experiment. We s.c. vaccinated C3H mice with mock-, Ad.BG-, or Ad.IR-E1A/TRAIL–infected C3L5 cells. C3L5 cells were infected 24 hours before transplantation at an MOI of 100 pfu per cell, which allows for efficient transduction and cell killing by Ad.IR-E1A/TRAIL. One day after vaccine tumor cell transplantation, mice received an i.v. administration of Ad.BG, Ad.Flt3L, or Ad.GM-CSF (labeled as Ad.GM) into C3H mice.
Three weeks after the vaccination, animals were challenged with nontreated tumor cells to assess whether mice had developed protective immunity against the tumor cells. Tumor sizes were measured 4 weeks later (Fig. 7B and C).

Ad.BG-infected C3L5 cells had a slight vaccination effect against subsequent challenge ($P = 0.023$ mock- versus Ad.BG-transduced cells), probably due to adenovirus-mediated cell death or expression of viral proteins. Induction of TRAIL-mediated apoptosis in C3L5 cells upon infection with Ad.IR-E1A/TRAIL had a significantly greater vaccination effect than Ad.BG infection alone ($P = 0.0002$ for mock/Ad.BG versus Ad.IR-E1A/TRAIL; $P = 0.024$ for Ad.BG/Ad.BG versus Ad.IR-E1A/TRAIL). Vaccination of mice with cells that underwent Ad.IR-E1A/TRAIL-mediated lysis in combination with Flt3 expression had a significantly greater antitumor effect than vaccination with mock- or Ad.BG-infected cells ($P = 0.0042$, $P = 0.034$, respectively). Notably, 7 of 10 mice vaccinated with Ad.IR-E1A/TRAIL infected cells and injected with Ad.Flt3L rejected tumors upon challenge, indicating strong antitumor immunity. Ad.GM-CSF injection alone and in combination with viral oncolysis had a similar vaccination effect ($P = 0.0004$ for mock/Ad.GM-CSF versus Ad.IR-E1A/TRAIL/Ad.GM-CSF), whereby the difference between the corresponding Ad.Flt3L- and Ad.GM-CSF–injected groups was not significant. In conclusion, vaccination with cells that underwent viral oncolysis in combination with Flt3L or GM-CSF expression induces a systemic antitumor immune response that is stronger than vaccination with mock- or control Ad.BG-infected cells.

**Discussion**

In this study, we showed that the combination of an oncolytic vector and an immunostimulatory vector expressing Flt3L or GM-CSF has an additive antitumor effect. The ability of cell death induced by an oncolytic adenovirus to assist the induction of tumor-specific immunity was particularly apparent in the vaccine scheme. I.t. injection of the oncolytic and Flt3L expressing vector into established tumors delayed tumor growth; however, it did not result in efficient tumor elimination. The following limitations of our model should be kept in mind when interpreting these results: (i) Productive replication of human adenoviruses is less efficient in murine than in human cells, even when adjusted for differences in receptor-mediated uptake and intracellular trafficking (34). The inefficient progeny virus production implies that viral spread in tumors in situ will likely...
be minimal with Ad.IR-E1A-based vectors. Whereas in the vaccination experiment cells were transduced in a monolayer under conditions that allow for infection of close to 100% of the cells, i.t. injection of adenovirus vectors resulted in transduction of <40% of tumor cells. It is likely that only cells directly expressing TRAIL and potentially immediately neighboring cells (due to a bystander effect of membrane localized TRAIL) were killed in this approach. (ii) C3L5 tumors grew relatively fast once they reached a size that made i.t. adenovirus vector injection physically possible. The timeframe from adenoviral injection to the point where mice had to be sacrificed was only 12 days. This is a short time for immunity to be established and an effective immune response to be carried out. Tumor models in which successful immunotherapy for established tumors was shown were usually slower growing (for example, see refs. 35, 36). (iii) The fast growth kinetics of C3L5 tumors also imply that the tumor burden was already relatively large before an effective antitumor immune response was elicited by our dual vector approach.

Whereas in the vaccination scheme both the Ad.Flt3L and Ad.GM-CSF vector displayed similar antitumor effects (Fig. 7), the Ad.GM-CSF vector was less efficient in slowing down tumor growth after i.t. injection (therapy scheme, Fig. 6). In contrast to GM-CSF, Flt3L is also stimulatory for NK cells (19, 20), and these cells might have been partly responsible for the early tumor regression/delayed growth seen within 13 days after injection of adenovirus vectors into preestablished tumors. However, as the vaccination study shows, both molecules are apparently comparable in their ability to induce a long-term protective antitumor immune response when expressed from adenovirus vectors.

A number of studies using tumor cells that express cytokines after retroviral gene transfer compared the ability of Flt3L and GM-CSF to induce an antitumor immune response. Whereas Alsheikhly et al. found no difference between GM-CSF, soluble or membrane-bound forms of Flt3L (14), Mach et al. reported that vaccination with irradiated tumor cells expressing GM-CSF stimulated a more potent antitumor immune responses than vaccination with Flt3L-expressing tumor cells (18). The latter studies argued that in contrast to Flt3L, GM-CSF can also promote dendritic cell maturation. We speculate that the inability of Flt3L to induce dendritic cell maturation might be compensated if adenovirus vectors are used for its expression. As outlined above, adenoviral transduction induces expression of potent stimulators of dendritic cell maturation. Other factors that might account for the

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**Figure 7.** Protection against challenge with C3L5 tumor cells. Mice were vaccinated with mock-, Ad.BG-, or Ad.IR-E1A/TRAIL–transduced C3L5 cells. Twenty-four hours after transplantation, mice received a tail vein injection of 5 × 10⁶ pfu of Ad.BG, Ad.Flt3L, or Ad.GM-CSF. Three weeks later, mice were challenged by s.c. injection of 1 × 10⁴ (untreated) C3L5 cells and tumor volumes were measured four weeks later. *Tumors developed in mice transplanted with mock- and Ad.BG-infected C3L5 vaccines but not in mice transplanted with Ad.IR-E1A/TRAIL–infected cells. Before challenge, these tumors were surgically removed. Notably, we did not observe that C3L5 tumor cells metastasize from the parental s.c. tumor. A, scheme of the experiment. B, volume of individual challenge tumors. Astersisks, absence of tumors. C, average (n = 10) tumor volumes (columns) and SD (bars).
discrepancy between Mach's study and our data include the use of different tumor cell lines, different transgene expression levels, and expression from tumor versus ectopic site (which in our case is hepatocytes, because i.v. injection of Ad5 vectors into mice leads to predominant transduction of liver; ref. 37). Although not compared directly, other studies showed that expression of GM-CSF (14–18) and Flt3L (14, 17, 18, 38) after adenovirus gene transfer induced strong stimulation of antitumor immune responses.

Our studies also support the hypothesis that AdLR-E1A/TRAIL-mediated oncolysis stimulates antitumor immune responses. This hypothesis is based on the assumption that TRAIL- and/or adenovirus-induced tumor cell apoptosis/lysis results in the release of cryptic tumorantigens in immunogenic form for presentation to dendritic cells and subsequent activation of antitumor T-cell responses. Based on our data, we conclude that the ability of Flt3L and GM-CSF to mobilize APCs together with the high local concentrations of proinflammatory cytokines and the release of tumor antigens upon adenovirus-mediated oncolysis is critical for the creation of protective immunity. Our data are in agreement with earlier studies showing that a combination of oncolytic and GM-CSF-expressing adenovirus vectors elicited greater antitumor efficacy in a xenograft (nude) mouse model compared with the oncolytic vector alone (15, 16).

It is unclear whether tumor-specific expression of Flt3L/GM-CSF is more effective in promoting antitumor immunity than systemic expression or administration, although one animal study has suggested that this may be the case (13). The target cells for Flt3L/GM-CSF in this therapeutic approach (progenitors for dendritic cells) do not reside within the tumor, and therefore, at least theoretically, local expression should not necessarily be advantageous.

Flt3 has been described as an important onco gene in acute myeloid leukemia (for a review, see ref. 39). In our model, due to the episcopal status of adenovirus genomes and the strong immunogenicity of adenovirus vectors, Flt3L expression was only transient and it is unlikely that this will result in neoplastic transformation of Flt3+ hematopoietic cells. Furthermore, in a recent clinical trial using HER-2/neu peptide vaccines in combination with Flt3L autoimmune phenomena were observed in some patients (40) which could suggest that Flt3L did mobilize antigen presenting cells, but these were unable to recognize there intended target. We did not systematically screen our mice for autoimmune responses, as this was beyond the scope of this study. However, we did not see any clinical signs of autoimmunity (such as hair depigmentation). It might be speculated that this is due to the use of adenovirus vectors for Flt3L expression as outlined above.

This study provides proof of principle for the feasibility of a combined adenoviral oncolytic and immunostimulatory therapy approach. Clearly, a more detailed characterization of tumor-specific T cells and NK cells is required to further validate this approach. Furthermore, the differences between the tumor cell lines in their ability to support DNA replication and homologous recombination of E1-deleted vectors are interesting and might be a basis to delineate cellular factors involved in regulating adenovirus DNA replication in future studies.

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References


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