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ORIGINAL ARTICLE

Adenovirus-mediated intratumoral expression of immunostimulatory proteins in combination with systemic Treg inactivation induces tumor-destructive immune responses in mouse models

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Tumor-associated antigens (TAAs) include overexpressed self-antigens (for example, *Her2/neu*) and tumor virus antigens (for example, HPV-16 E6/E7). Although in cancer patients, TAA-specific CD4+ and CD8+ cells are often present, they are not able to control tumor growth. In recent studies, it became apparent that tumor site-located immune evasion mechanisms contribute to this phenomenon and that regulatory T cells have a major role. We tested in *Her2/neu*+ breast cancer and HPV-16 E6/E7+ cervical cancer mouse models, whether intratumoral expression of immunostimulatory proteins (ISPs), for example, recombinant antibodies (αCTLA-4, αCD137, αCD3), cyto/chemokines (IL-15, LIGHT, mda-7) and costimulatory ligands (CD80), through adenovirus(Ad)-mediated gene transfer would overcome resistance. In both the breast and cervical cancer model, none of the Ad.ISP vectors displayed a significant therapeutic effect when compared with an Ad vector that lacked a transgene (Ad.zero). However, the combination of Ad.ISP vectors with systemic T regulatory (Treg) depletion, using anti-CD25 mAb (breast cancer model) or low-dose cyclophosphamide (cervical cancer model) resulted in a significant delay of tumor growth in mice treated with Ad.αCTLA4. In the cervical cancer model, we also demonstrated the induction of a systemic antitumor immune response that was able to delay the growth of distant tumors. Ad.αCTLA4-mediated tumor-destructive immune responses involved NKT and CD8+ T cells. In both models no autoimmune reactions were observed. This study shows that Ad.αCTLA4 in combination with systemic Treg depletion has potentials in the immunotherapy of cancer.
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Introduction

Many tumor-associated antigens (TAAs) are nonmutated self-antigens that are overexpressed in tumor cells. A standard example for this class of TAAs is *Her2/neu*, which is immunogenic and spontaneous; vaccine-induced generation of *Her2/neu*-specific CD8+ T cells in breast cancer patients is frequently reported.[1–3] However, boosting anti-*Her2/neu* immunity has mostly failed to show clinical benefits. This has been in part attributed to the presence of both central and peripheral tolerance to *Her2/neu*.[4–7] Another class of TAAs represents proteins encoded by tumor-associated viruses such as human papillomavirus type 16 (HPV-16), which is a major etiological factor in cervical cancer.[8,9] Following the demonstration that the E6 and E7 epitopes of HPV16 encode tumor rejection antigens,[10,11] many therapeutic vaccines, including E6/E7 peptides, fusion proteins and DNA vaccines, have been examined both in animal models and in phase-I or -II clinical trials in humans. However, as observed in breast cancer, intratumoral mechanisms that trigger anergy or tolerance against the HPV-derived TAAs appear to also limit the effect of therapeutic HPV vaccines (for a review, see ref. 12).

Several cytokines and antibodies have been identified or designed, which have the potential to counteract

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intratumoral immune tolerance and activate antitumor reactive lymphocytes against immunogenic tumors *in vivo*. Among cytokines and chemokines that are able to activate tumor-reactive immune cells are interleukin (IL)-15 (ref. 13), LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein-D for herpes simplex virus entry mediator, a receptor expressed by T lymphocytes; TNFSF14)[14] and human melanoma differentiation-associated gene-7 (mda-7)/IL-24 (ref. 15). Immunostimulatory antibodies include antibodies against CD137 (ref. 16; a costimulatory molecule, which is expressed on natural killer (NK) and T-effector cells), antibodies against cytotoxic T-lymphocyte-associated protein-4 (CTLA4, ref. 17; a main negative regulator of the immune system, which inhibits the costimulatory signaling for T cells) or antibodies against CD3 in combination with CD28 ligands (which can trigger direct activation of naive T cells).[18] However, the clinical usage of these immunostimulatory proteins (ISPs) is limited because their systemic administration or expression is associated with site effects, specifically with the induction of autoimmune responses. An approach to address this problem involves the tumor-localized expression of ISP by intratumoral injection or by transductional targeting of gene transfer vectors, thereby increasing the local concentration of ISPs in tumors and minimizing adverse effects associated with their systemic leakage. Another approach to achieve tumor-localized action of ISPs involves anchoring of otherwise secreted ISPs to the membrane of tumor cells.[19] Activation of tumor-destructive immune responses has been shown with membrane-tethered antibodies with specificity for CD3 in combination with CD28 ligation by ectopically expressed B7-2.[20] Likewise, tumor-located expression of a membrane-tethered antibody against CD137 caused extensive rejection of poorly immunogenic tumors in mice without induction of autoimmunity.[16]

In a recent study, we demonstrated that stable expression of an anti-CTLA4 antibody by tumor cells conferred tumor-destructive immune responses, which could be further enhanced by systemic T regulatory (Treg) depletion.[21] To achieve anti-CTLA4 expression, we transduced the tumor cells *ex vivo* with a lentivirus vector. This approach, however, is difficult to employ clinically. In this study, we therefore tested the antitumor effect of the expression of ISPs in the tumor environment by using adenovirus (Ad) vectors for gene transfer into tumors. Ad vectors have a number of advantages for *in vivo* gene transfer into tumors, including the ability to efficiently transduce tumor cells after systemic and intratumoral injection and the ability to induce Ad-associated innate and adaptive immune responses that create a proinflammatory environment inside the tumor and facilitate the activation of tumor-antigen-specific T cells, thereby triggering not only anti-virus but also antitumor immunity. Recently, we showed that injection of Ad vectors caused a strong CD4 and CD8 T-cell-mediated immune response against the vectors and vector-transduced tumor cells inside the tumor-draining lymph nodes and in the tumor microenvironment. Importantly, Ad-triggered T-effector cells delayed tumor growth, despite the presence of intratumoral Treg-mediated immune tolerance that suppressed tumor-specific CTLs.[22]

Here, we screened a series of *ISP* genes delivered by Ad vectors for their ability to delay tumor growth in two mouse models that are functionally relevant for breast and cervical cancer. The breast cancer model involves *Her2/neu* transgenic mice (*neu*-tg) that harbor nonmutated, nonactivated rat *Her2/neu* under control of the mouse mammary tumor virus promoter. It has significant biological and pathological similarity to human neu-associated estrogen receptor-negative breast cancer.[23] The mice spontaneously develop breast tumors, which are immunogenic due to the expression of a panel of TAAs (including *Her2/neu*) similar to human cancer. As most TAAs are nonmutated self-antigens that have triggered both central and peripheral tolerance, *neu*-tg mice mimic such tolerance to an endogenous tumor antigen. Overall, the tumor antigen repertoire in mouse mammary carcinoma (MMC)-tumor-bearing mice appears to be predictive for human breast cancer antigens.[23] Immune tolerance against the *Her2/neu*-positive MMC cells is primarily mediated by regulatory T cells. We have recently shown that although implantation of MMC tumors in *neu*-tg mice triggered *Neu*-specific T-effector cells inside the sentinel lymph nodes, these cells poorly infiltrated tumors and were functionally inactive in the tumor microenvironment. We also demonstrated that depletion of regulatory T cells enabled activation and clonal expansion of *neu*-specific $CD8^+$ T cells *in vivo*, which subsequently mediated MMC tumor regression.[22]

A second tumor model (for cervical cancer) is based on TC-1 cells, a mouse epithelial cancer cell line that expresses HPV-16 E6 and E7 proteins. It is propagated in C57Bl/6 mice and has been frequently used.[24–31] We have previously shown that TC-1 tumors are infiltrated with E7-specific cells but also contain large numbers of Tregs[21,32] and that immunotherapy was successful in the TC-1 model, if Tregs were systemically depleted by low-dose cyclophosphamide (CY) injection.[21,32]

Materials and methods

Cells

MMC cells were established from a spontaneous tumor in a *neu*-tg mouse. MMC cells have been shown to display high levels of *Neu* expression, an epithelial phenotype, expression of major histocompatibility complex class I and II and presentation of the immunodominant *Neu*-epitope H-2Dq/$RNEU_{420–429}$.[33] TC-1 cells were from the American Type Culture Collection (ATCC, Manassas, VA). TC-1 cells are immortalized murine epithelial cells that stably express HPV-16 E6 and E7 proteins. MMC and TC-1 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 1 mmol l^{-1} sodium pyruvate, 10 mmol l^{-1} HEPES, 2 mmol l^{-1} L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Antibodies for immune cell depletion

Monoclonal rat anti-mouse CD25 antibody (PC61.5 hybridoma, ATCC), rat anti-mouse CD4 immunoglobulin-G (IgG; GK1.5, ATTC) and rat anti-mouse CD8 IgG (169.4; ATTC) were produced by culturing hybridoma cell lines in CELLine 1000 culture flasks (BD Biosciences, San Diego, CA) using Dulbecco's modified Eagle's medium supplemented with 10% low IgG fetal bovine serum (Hyclone, Logan, UT), 4 mM L-glutamine, 100 U penicillin ml^{-1} and 100 μg ml^{-1} Pen/Strep. Antibodies were purified from hybridoma supernatant using protein-G purification (Amersham Biosciences, Piscataway, NJ) followed by dialysis against phosphate-buffered saline (PBS). NK cell depleting rabbit anti-mouse asalio GM1 IgG were from Cedarlane (Ontario, Canada). Respective IgG isotype controls (isotype1, rat IgG; isotype2, rabbit IgG) were from Jackson ImmunoResearch (West Grove, PA).

Ad vectors

All vectors were based on Ad serotype 5 and had the *E1A* and *E1B* genes and the *E3* genes deleted. The genomic structures of all vectors are summarized in Supplementary Figure 1. Ad.zero is a transgene-devoid vector. Ad.bGal, Ad.IL-15, Ad.LIGHT and Ad.mda7 expressed *Escherichia coli* β-galactosidase, rat Her/neu, mouse IL-15, mouse LIGHT or human mda7 under the control of the Rous Sarcoma Virus (RSV) promoter.[34] Ad.αCD3 and Ad.αCD137 expressed membrane-bound anti-mouse CD3scFv and anti-mouse CD137scFv under the control of the RSV promotor, respectively.[35,36] Ad.αCTLA4 was generated by cloning an *Eco*RV/*Xho*I fragment of p4F10-γ1 (ref. 21) into the *Eco*RV/*Sal*I sites of pAd.RSV.[37] Ad.CD80 was produced by cloning the coding region for murine CD80 (ref. 38) into pAd.RSV. Recombinant viruses were propagated in 293 cells, banded in CsCl gradients, dialyzed and stored in aliquots as described.[39] To assess contamination of $AdE1^-$ vector preparations with $E1^+$ replication-competent Ad (RCA), real-time PCR quantification for $AdE1^+$ genomes was performed.[40] Only virus preparations that contained less than one $E1^+$ (RCA) viral genome in 1×10^9 genomes were used. Ad-particle (viral particle) concentrations were determined spectrophotometrically by measuring the optical density at 260 nm (OD_{260}). Plaque titering (plaque forming units, p.f.u.) was performed using 293 cells as described elsewhere.[39] The p.f.u./viral particle ratios for all Ad preps was ~1:20. Multiplicities of infection in this study were stated as p.f.u. per cell for all assays. Endotoxin contamination was tested with an Endotoxin detection kit (BioWhittaker, Walkerville, MD). All vectors were free of endotoxin.

Validation of transgene expression from Ad vectors

MMC and TC-1 cells were transduced with Ad vectors (multiplicity of infection 50) and transgene expression was analyzed 48 h later. Expression of IL-15 and LIGHT proteins was quantified using an IL-15 ELISA Kit (eBioscience, San Diego, CA) and a LIGHT/TNFSF14 ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. For analysis of α*CD3*, *CD80* and α*CD137* expression, Ad-transduced cells were detached 48 h post-transduction using Versene (Gibco, Carlsbad, CA), and expression of membrane-bound proteins was detected by flow cytometry. For Ad.αCTLA4, serial dilutions of the culture medium were assayed for binding to recombinant CTLA-4 protein coated in microtiter plates by enzyme-linked immunosorbent assay as described earlier.[21] Mda-7 was detected by western blot of cell lysates using rabbit anti-mda7 antibodies (Santa Cruz Biotech., Santa Cruz, CA) as described previously.[41]

Animals

All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. All mice were housed in specific-pathogen-free facilities. C57Bl/6 mice were obtained from Charles River (Wilmington, MA). *Neu*-tg mice (strain name: FVB/N-Tg(MMTVneu)202Mul) were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice harbor nonmutated, nonactivated rat *neu* under control of the mouse mammary tumor virus promoter. The *neu* tg is expressed at low levels in normal mammary epithelium, salivary gland and lung. Until the age of 8 months, ~35% of female *neu*-tg mice spontaneously develop mammary carcinomas that display high Neu expression levels. Immunodeficient NOD.CB17-$Prkdc^{scid}$/J (CB17/SCID/beige) mice were obtained from the Jackson Laboratory.

Tumor cell transplantation

MMC and TC-1 cells were collected using Versene (Gibco) and washed in RPMI-1640 medium (without supplements) before injection. Mice received anesthesia and were injected subcutaneously. Tumors were measured every other day and tumor volume was calculated as the product of length × width × width. For survival studies, tumor sizes $\geqslant 500\,mm^3$ were considered the experimental end point. Animals with skin surface ulcerations were excluded from experiments and killed immediately.

Intratumoral Ad injection

When tumors reached a size of 3–4 mm diameter, mice were randomly assigned to treatment groups. Mice were given anesthesia (Avertin (Sigma, St Louis, MO) intraperitoneally (i.p.)) and then Ad (1×10^9 p.f.u. diluted in 50 μl PBS), or 50 μl PBS was intratumorally injected with constant and low pressure using an insulin syringe (BD Pharmingen).

MMC therapy model

A total of 5×10^5 MMC cells in 100 μl PBS were subcutaneously injected into *neu*-tg mice. When tumors reached a size of 4 × 3 mm diameter (usually at day 6 after cell injection), 250 μg of anti-CD25 (or control antibody) in 500 μl PBS was injected i.p. Four days later, Ad vectors were injected intratumorally. Mice then received anti-CD25 or control antibody i.p. once a week.

TC-1 therapy model

A total of 5×10^4 TC-1 cells in 100 μl of PBS cells were subcutaneously injected into C57Bl/6 mice. When tumors

reaches a diameter of 2×3 mm (usually at day 5 after cell injection), mice received an intraperitoneal injection of CY (2 mg per mouse in 500 µl PBS) as described previously.[21] Four days later, Ad vectors were injected intratumorally.

TC-1 vaccination model

A total of 1×10^4 TC-1 cells were injected subcutaneously into the right inguinal flank of C57Bl6 mice. When tumors reached a diameter of $\sim$2 mm, mice received an intraperitoneal injection of low-dose CY. Four days later, a total of 1×10^6 Ad-transduced TC-1 cells were transplanted into the left inguinal flank. TC-1 cells were infected *ex vivo* with Ad vectors at a multiplicity of infection of 100 p.f.u. per cell. Twenty-four hours after Ad infection, cells were trypsinized, washed and used for transplantation.

Immune cell depletion

$CD4^+/CD8^+$ T cells and NK cells were depleted using intraperitoneal injection of the following antibodies diluted in 500 µl PBS: 200 µg rat anti-mouse CD4 IgG (GK1.5, ATTC), 200 µg rat anti-mouse CD8 IgG (169.4; ATTC) or 20 µl rabbit anti-mouse asalio GM1 IgG (Cedarlane). A quantity of 200 µg of the respective IgG isotype control (Jackson ImmunoResearch) was used in control animals (isotype1, rat IgG; isotype2, rabbit IgG). For some experiments, injection of each antibody was repeated every 3 days to maintain the depletion.

Flow cytometry

Flow cytometry was performed using a BD FACSCalibur (BD Biosciences). Samples were pretreated with Fc-block (anti-CD16/CD32, BD Biosciences) for 15 min, stained for 30 min on ice in washing buffer (PBS-1% fetal bovine serum) and washed three times with washing buffer. Fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated isotype controls were included in all experiments. Anti-E-cadherin-phycoerythrin antibody (clone 114420, R&D Systems) was used to detect MMC cells. For flow cytometry analysis of immune cells, the following monoclonal antibodies were used: anti-FoxP3-phycoerythrin (clone FHK16s; cells were permeabilized according to the manufacturer's instruction; eBioscience), anti-CD4-FITC (clone RM4–5), anti-CD8-FITC (clone 53-6.7), anti-NK1.1-phycoerythrin (clone PK136). For CD25 flow cytomety, another monoclonal antibody isotype was used than for Treg depletion.

Immunofluorescence studies

Tumor slides were fixed with acetone/methanol (10 min) and washed twice with PBS. Slides were blocked for 20 min at room temperature using PBS-5% blotting grade milk (Bio-Rad, Hercules, CA) followed by incubation with primary antibodies in PBS for 1 h at room temperature. Then slides were washed twice with PBS and incubated with secondary antibodies for 1 h at room temperature followed by washing with PBS three times. Slides were washed twice with PBS, mounted with Mounting Medium for Fluorescence (Vector Laboratories, Burlingame, CA) and then analyzed using a fluorescence microscope. *Laminin* was detected using anti-laminin polyclonal (primary) antibody (1:200; #Z0097; Dako, Carpinteria, CA) and goat anti-rabbit IgG Alexa Fluor568 (secondary) antibody (1:200; Molecular Probes, Carlsbad, CA); the following cell surface markers were used: FITC-labeled anti-E-cadherin antibody (1:100; clone 36/E-Cadherin, BD Biosciences) or FITC-labeled anti-CD4, anti-FoxP3, and anti-CD8 antibodies (see 'Flow cytometry'). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma).

Immunohistochemistry of mouse tissue and organs

For histological assessment of autoimmune disease, mouse tissues and organs (heart, lung, brain, stomach, mesenterium, liver, kidney, muscle and skin) were fixed in 10% formalin and processed for hematoxylin and eosin staining. All samples were examined by two experienced pathologists for typical inflammation signs in a blinded manner. Immunohistochemistry for IgG on kidney sections was performed as described for tumor sections using a polyclonal, horseradish peroxidase-labeled, anti-mouse IgG antibody (eBiosciences).

Statistical analysis

Statistical significance of *in vivo* data was analyzed by Kaplan–Meier survival curves and log-rank test (GraphPad Prism Version 4, La Jolla, CA). Statistical significance of *in vitro* data was calculated by two-sided Student's *t*-test (Microsoft Excel). *P*-values >0.05 were considered not statistically significant.

Results

Studies in Her2-transgenic mice with MMC tumors

We tested seven E1/E3 deleted, Ad5-based Ad.ISP vectors expressing the following ISPs: anti-CD3, mouse CD80, anti-CD137, anti-CTLA4, mouse IL-15, mouse LIGHT or mda7. Transgenes were under the control of the RSV promoter (Supplementary Figure 1). This promoter provides efficient transgene expression in MMC and TC-1 cells *in vitro* and *in vivo*.[21,32] A control vector (Ad.zero) contained the RSV promoter without any transgene. Expression of ISPs after Ad infection of MMC and TC-1 cells was validated as described earlier[22] and in the Materials and methods section.

MMC tumors were established by subcutaneous injection of tumor cells into *neu*-tg mice. MMC tumors grew aggressively and reached a size of 500 mm^3 within 14 days after cell implantation. Tumors contained nests of epithelial tumor cells with intercellular tight and adherens junctions (Figure 1a, E-cadherin staining). Tumor nests were surrounded by extracellular matrix (Figure 1b, laminin staining). Tumors were infiltrated with CD4, CD8 and Tregs (Figures 1c and d). Overall, this histology is very similar to that observed in breast cancer patient biopsies.[21] Intratumoral injection of Ad.bGal resulted in efficient transgene expression predominantly localized around the needle track (Figure 1e). Flow cytometry

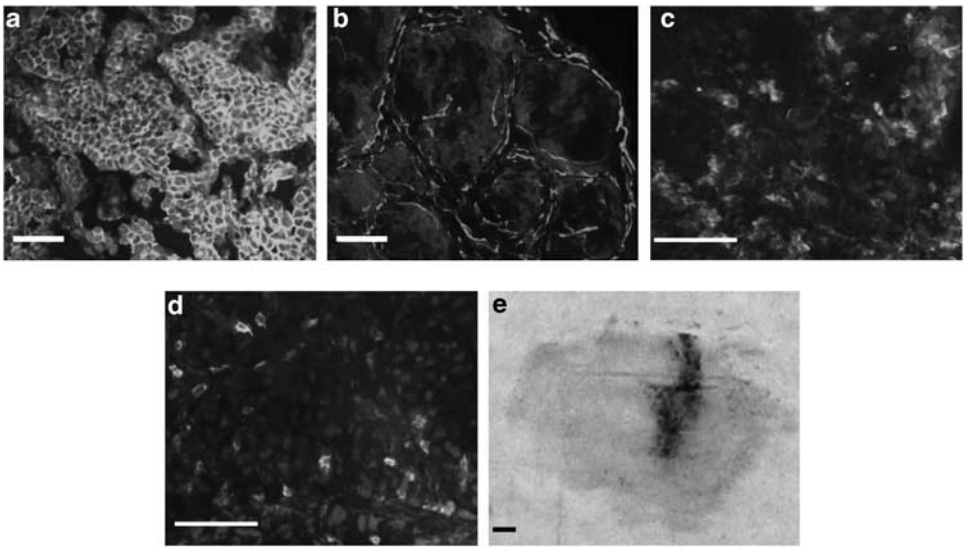

Figure 1 Histology of MMC tumors. MMC cells were subcutaneously transplanted into *neu*-tg mice. (**a**–**d**) Two weeks after transplantation, tumor sections were stained for E-cadherin (**a**), laminin (**b**), FoxP3 (red) and CD4 (green) (**c**), and CD8 (green) (**d**). Cell nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). (**e**) β-galactosidase expression 2 days after intratumoral injection of 1×10^9 p.f.u. of Ad.bGal.

analyses of tumor cell suspensions showed β-galactosidase expression in 18(±7)% of tumor cells (data not shown).

To assess tumor toxicity of Ad.ISP vectors by mechanisms other than immune-mediated tumor destruction, we studied the effect of Ad.ISP injection in MMC tumors established in immunodeficient CB17-SCID/beige mice, which are impaired for T-cell and NK-cell function. These tumors were similar to those in *neu*-tg mice with regard to epithelial/nodular phenotype and extracellular matrix. In the MMC/CB17-SCID/beige model, we did not find significant differences between PBS, Ad.zero and Ad.ISP-injected animals, indicating that the expression of the transgene product or the Ad injection procedure did not exert antitumor effects. Notably, T-cell stimulation through CD3 ligands requires costimulatory signals, such as activation of CD28. We therefore employed AdαCD3 together with Ad.CD80.

In a therapy study in *neu*-tg mice, 2×10^9 p.f.u. of Ad.ISP vectors were injected into MMC tumors (average size $\sim 50\,mm^3$). All vectors significantly increased the median survival in *neu*-tg mice for at least 5 days compared with PBS-injected mice ($P < 0.05$; Figure 2a, Supplementary Figure 2). However, there was no significant difference between the control vector, Ad.zero and the Ad.ISP vectors. This indicated that vector-triggered immune responses have a significant antitumor effect in this model, as we did not observe a tumor-growth delay in immunodeficient mice. However, expression of ISP did not have an adjuvant effect on this antitumor immune response. As Treg cells have been shown to mediate immune tolerance toward tumor antigens in this model,[22] we decided to test Ad.ISP vectors in combination with systemic depletion or inactivation of Tregs. An approach that we and others successfully applied in various tumor models (TC-1, B16 or PC61) utilizes intraperitoneal injections of low-dose ($100\,mg\,kg^{-1}$) CY.[21,42,43] To evaluate the effect of low-dose CY on MMC tumors, we injected MMC-tumor-bearing CB17-SCID/beige mice with $100\,mg\,kg^{-1}$ CY and measured tumor growth (Figure 2b, Supplementary Figure 3A). CY resulted in a marked inhibition of tumor growth, indicating a direct toxic effect on MMC cells. Decreasing the CY dose to $50\,mg\,kg^{-1}$ affected MMC tumor growth less, but did not result in efficient depletion of CD4/CD25 cells.[21] In contrast, an approach for Treg depletion that involved intraperitoneal injection of anti-CD25 antibodies (250 μg per mouse i.p.), which did not delay MMC tumor growth in CB17/SCID-beige mice (Figure 2c, Supplementary Figure 3B), significantly reduced Tregs in *neu*-tg mice, as CD4, CD25 and FoxP3 flow cytometry studies with splenocytes showed (Figure 2d). To test the therapeutic effect of the combined anti-CD25/Ad.ISP approach, MMC tumors with an average volume of $50\,mm^3$ were intratumorally injected with Ad.ISP or Ad.zero (1×10^9 p.f.u.) 4 days after the mice had been injected with anti-CD25 or isotype control Ig. Pre-injection of anti-CD25 did not significantly increase median survival of Ad.zero (Figure 2e), Ad.IL-15, Ad.LIGHT, Ad.*a*CD3/Ad.CD80 and Ad.αCD137-injected mice (not shown). However, the combination of anti-CD25 and Ad.αCTLA4 had a significant therapeutic effect reflected in prolonged survival compared with Co-Ig/Ad.αCTLA4 and anti-CD25/Ad.zero groups (Figure 2f). None of the anti-CD25/Ad.αCTLA4-treated mice displayed signs of autoimmune responses detectable as changes in fur color or organ inflammation (assessed on tissue sections).

Studies in C57Bl/6 mice with TC-1 tumors

Because the outcome of immunotherapy approaches greatly depends on the phenotype of the tumor and mouse strain, we tested our Ad.ISP vectors in a second

a

	median survival (days)
PBS	14 (+/-4)
Ad.zero	19 (+/-5)
Ad.IL15	17(+/-3)
Ad.LIGHT	17(+/-4)
Ad.mda7	17(+/-3
Ad.aCD3/ Ad.CD80	19 (+/-5)
Ad.aCD137	18(+/-4)
Ad.aCTLA4	17(+/-2

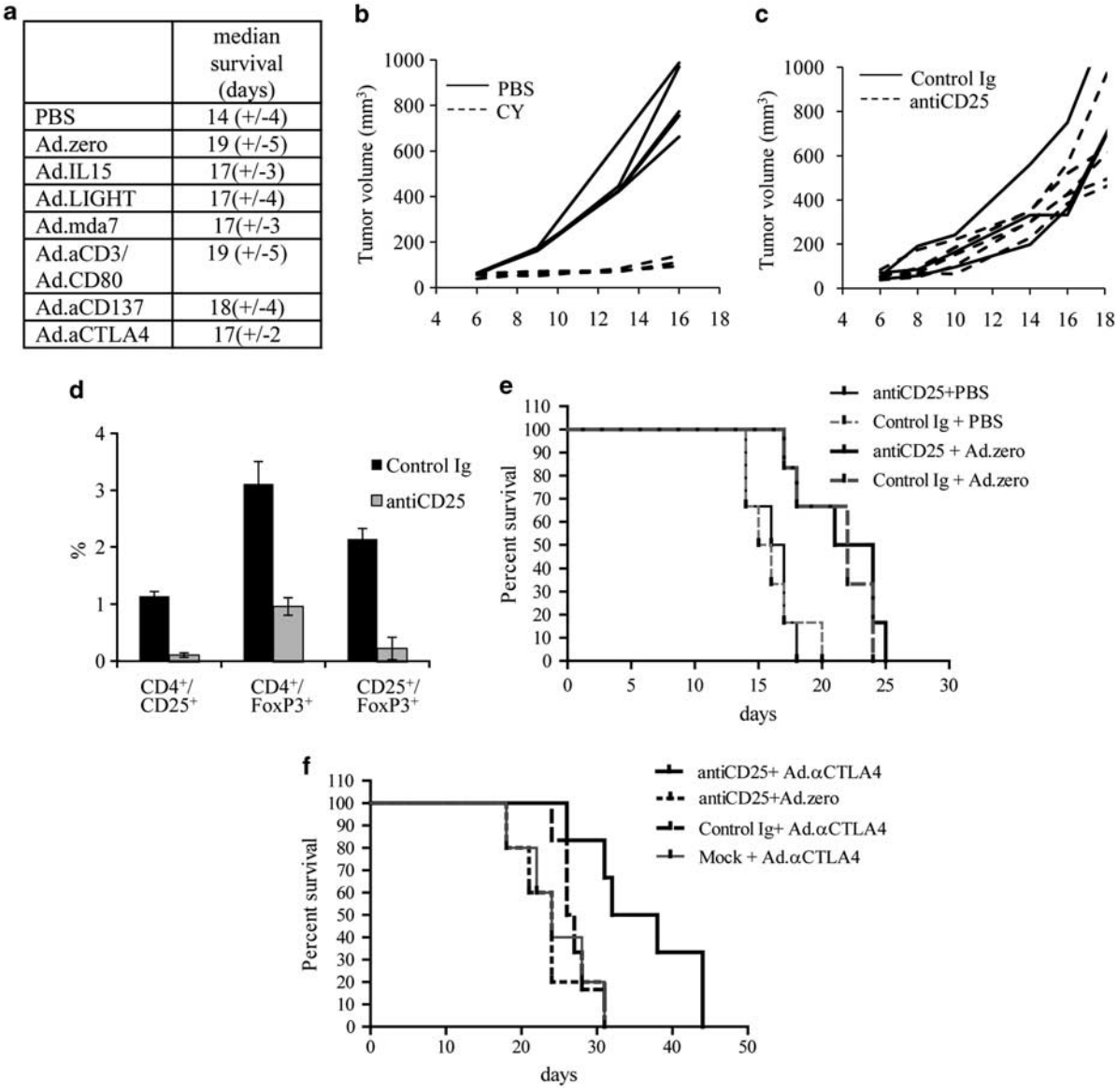

Figure 2 Therapy studies with MMC tumors. (**a**) Median survival of MMC-tumor-bearing *neu*/tg mice after intratumoral injection of PBS, Ad.zero and Ad.ISP vectors. Tumor size was measured every other day. The day tumors reached a volume of 400 mm^3 represented the end point in Kaplan–Meier survival studies. Median survival was calculated using GraphPad-Prizm4 software, $N=5$. (**b**, **c**) Studies in CB17-SCID/beige mice. (**b**) MMC tumor cells were implanted and low-dose CY (100 mg kg^{-1}) or PBS was injected intraperitoneally (i.p.) 6 days later. Tumor volumes at day 16 (end of the observation period) were significantly less in CY versus PBS-treated groups ($P<0.001$); see also Supplementary Figure 3A. (**c**) In another experiment, tumor-bearing CB17-SCID/beige mice received 250 μg of anti-CD25 or control antibody 6 days after MMC cell implantation. Shown is the tumor volume in individual mice. Tumor volumes at day 16 (end of the observation period) did not significantly differ between Control Ig and anti-CD25 treatment groups ($P=0.07$); see also Supplementary Figure 3B. (**d**) Treg depletion by anti-CD25 in *neu*/tg mice. Mice were i.p. injected with 250 μg anti-CD25 or control antibody and 4 days later, splenocytes were analyzed for CD4, CD25 and FoxP3 by flow cytometry ($N=3$). CD4 + /CD25 + Co Ig versus anti-CD25: $P=0.004$; CD4 + /FoxP3 + Co Ig versus anti-CD25: $P=0.019$; CD25 + /FoxP3 + : $P=0.009$. (**e**) Effect of anti-CD25 on tumor growth. MMC-tumor-bearing *neu*/tg mice were injected with 250 μg anti-CD25 or control antibody 4 days before intratumoral injection of PBS or 2×10^9 p.f.u. of Ad.zero. The day tumors reached a volume of 400 mm^3 represented the end point in Kaplan–Meier survival studies, $N=8$; anti-CD25 + PBS vs antiCD25 + Ad.zero: $P=0.0061$; control Ig + PBS versus control Ig + Ad.zero: $P=0.0058$; antiCD25 + PBS versus control Ig + PBS: $P=0.9677$; antiCD25 + Ad.zero versus control Ig + Ad.zero: $P=0.5722$. (**f**) Effect of anti-CD25/Ad.αCTLA4 injection on tumor growth. MMC-tumor-bearing *neu*/tg mice were injected with 250 μg anti-CD25 or control antibody 4 days before intratumoral injection of 2×10^9 p.f.u. of Ad.zero or Ad.αCTLA4. The day tumors reached a volume of 400 mm^3 represented the end point in Kaplan–Meier survival studies, $N=10$; anti-CD25 + Ad.αCTLA4 versus antiCD25 + Ad.zero: $P=0.0066$; anti-CD25 + Ad.αCTLA4 versus control Ig + Ad.αCTLA4: $P=0.0104$; anti-CD25 + Ad.αCTLA4 versus Mock + Ad.αCTLA4: $P=0.0105$.

tumor model that involved C57Bl/6 mice and TC-1 cells. The TAAs in this model represent HPV type 16 proteins E6 and E7. Notably, these proteins have been targeted in various immunotherapy approaches of cervical cancer.[44] Upon subcutaneous transplantation into syngeneic C57Bl/6 mice, TC-1 cells form aggressively growing,

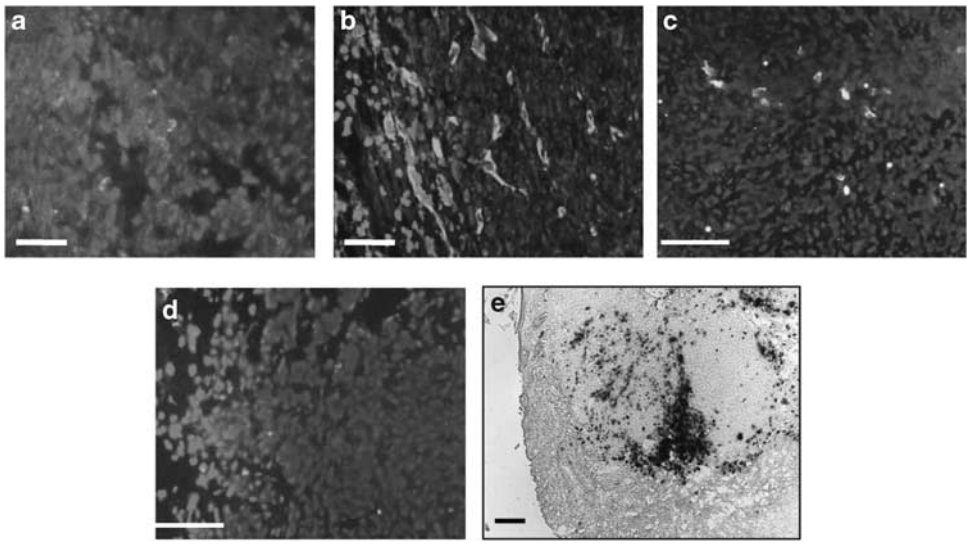

Figure 3 Histology of TC-1 tumors. TC-1 cells were subcutaneously transplanted into C57Bl/6. Two weeks after transplantation, tumor sections were stained for E-cadherin (**a**), laminin (**b**), FoxP3 (red) and CD4 (green) (**c**) and CD8 (green) (**d**). Cell nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). (**e**) β-Galactosidase expression 2 days after intratumoral injection of 1×10^9 p.f.u. of Ad.bGal. The scale bar represents 40 μm.

vascularized tumors (Figure 3). Histology studies of TC-1 tumor sections revealed intratumoral Tregs and CD8 cells (Figures 3c and d). Intratumoral injection of Ad.bGal into TC-1 tumors results in efficient tumor cell transduction (Figure 3e).[32]

As seen in the MMC/*neu*-tg model, intratumoral injection of Ad.ISP vectors into TC-1 tumors as a single agent did not result in significant delay of tumor growth (Figure 4a). While in the MMC model, Ad injection (including Ad.zero injection) delayed tumor growth compared with PBS-injected mice, this effect was not observed in the TC-1 model, which might be due to less immunogenicity of Ad in this mouse strain.

Previous studies showed that low-dose CY injection in the TC-1 model resulted in efficient Treg depletion without direct killing of TC-1 tumor cells.[21] We therefore tested our Ad.ISP vectors in combination with Treg depletion by low-dose CY. This approach resulted in significant prolongation of survival when mice received intratumoral Ad.αCTLA4 and Ad.αCD3/Ad.CD80 in combination with systemic Treg depletion (Figure 3b). Intratumoral injection of Ad.zero and the other Ad.ISP vectors (Ad.IL-15, Ad.αCD137, Ad.LIGHT and Ad.mda7) combination with CY did not result in a significant increase of survival compared with PBS-injected mice that received CY (Supplementary Figure 4). Separate injection of Ad.αCD3 and Ad.CD80 plus CY also failed to exert therapeutic effects.

Next we employed a vaccination regimen to study whether this approach mediates the induction of a systemic antitumor response that is able to control growth of tumors at distant sites that were not transduced by Ad vectors.

To avoid Ad dissemination *in vivo* and minimize growth of the 'vaccination' tumor, we infected TC-1 cells *ex vivo* with Ad.zero, Ad.αCTLA4 or Ad.αCD3/Ad.CD80 vectors and, after incubation for 24 h, injected them into the right inguinal site of mice with pre-established TC-1 tumor (established at the left inguinal site). The size of pre-established TC-1 tumors was measured. Figures 4c and d show the outcome of these studies. Vaccination with Ad.αCTLA4-infected TC-1 cell significantly inhibited the growth of pre-established TC-1 tumors (PBS/CY vs Ad.αCTLA4/CY: $P = 0.005$; Ad.zero/CY vs Ad.αCTLA4/CY: $P = 0.033$). The antitumor effect of Ad.αCD3/CD80-infected cells in combination with CY injection was borderline significant (PBS/CY vs Ad.αCD3/Ad.CD80/CY: $P = 0.05$; Ad.zero/CY vs Ad.αCD3/CY: $P = 0.05$). This study also demonstrates that both Ad.ISP vectors trigger TC-1-specific immune responses, excluding a major role of anti-Ad responses in tumor destruction in the therapy scheme shown in Figure 4c.

We used the TC-1 model to study which arm of the immune system conferred Ad.αCTLA4-mediated antitumor immune responses. Three weeks after TC-1 cell implantation, primary (pre-established) tumors, tumor-draining lymph nodes and spleens from treated mice were analyzed by flow cytometry for CD4, CD8, FoxP3 (Tregs) and CD3/NK1.1 (NKT cells; Figure 5a). At all three sites, we found more CD3+/NK1.1+ NKT cells in mice that were vaccinated with Ad.αCTLA4-infected TC-1 cells compared with mice that received Ad.zero-transduced TC-1 cells. Furthermore, in tumors the number of CD8+ T cells was significantly increased in the Ad.αCTLA4 group (2.4-fold). Because CY was given at day 5 after TC-1 cell transplantation, it is not expected that, at the time of T-cell analysis (day 22), the Treg cell numbers are still affected by CY.

To corroborate our findings, we depleted $CD4^+$, $CD8^+$ T cells and NK cells over the entire period of

a

	median survival (days)
PBS	19(+/-4)
Ad.zero	18(+/-3)
Ad.IL15	17(+/-2)
Ad.LIGHT	18(+/-3)
Ad.mda7	19(+/-3)
Ad.αCD3/ Ad.CD80	18(+/-2)
Ad.αCD137	18(+/-3)
Ad.αCTLA4	19(+/-3)

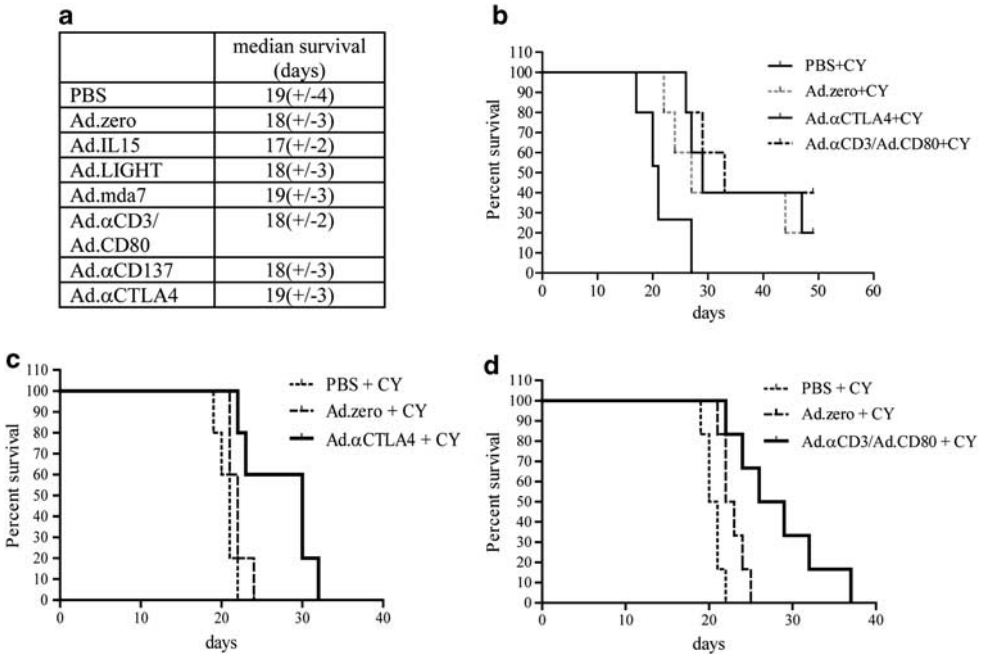

Figure 4 Therapy studies with TC-1 tumors. (**a**) Median survival of TC-1-tumor-bearing C57Bl/6 mice after intratumoral injection of PBS, Ad.zero and Ad.ISP vectors. Tumor size was measured every other day. The day tumors reached a volume of 400 mm^3 represented the end point in Kaplan–Meier survival studies. (**b**) Survival of TC-1-tumor-bearing mice after treatment with CY (100 mg ml^{-1}) 4 days before PBS or Ad injection. The day tumors reached a volume of 400 mm^3 represented the end point in Kaplan–Meier survival studies, $N=8$. PBS + CY versus Ad.zero + CY: $P=0.076$; PBS + CY versus Ad.αCTLA4 + CY: $P=0.028$; PBS + CY versus Ad.αCD3/Ad.CD80 + CY: $P=0.013$; Ad.zero + CY versus Ad.αCTLA4 + CY: $P=0.664$; Ad.zero + CY versus Ad.αCD3/Ad.CD80 + CY: $P=0.433$; Ad.αCTLA4 + CY versus Ad.αCD3/Ad.-CD80 + CY: $P=0.556$. (**c**, **d**) Effect of vaccination with Ad.αCTLA4 or Ad.αCD3/Ad.CD80-infected TC-1 cells on growth of pre-established TC-1 tumors. A total of 5×10^4 TC-1 cells were injected subcutaneously into the right inguinal flank of C57Bl6 mice. When tumors reached a diameter of ~2 mm, mice received an intraperitoneal injection of low-dose CY. Four days later, a total of 1×10^6 Ad-transduced TC-1 cells were transplanted into the left inguinal flank. TC-1 cells were infected *ex vivo* with Ad vectors at a total multiplicity of infection of 100 p.f.u. per cell. (This multiplicity of infection efficiently inhibits *in vivo* growth of infected cells). Twenty-four hours after Ad infection, cells were trypsinized, washed and used for transplantation. The day tumors reached a volume of 200 mm^3 represented the end point in Kaplan–Meier survival studies. $N=8$. (**c**) PBS + CY versus Ad.zero + CY: $P=0.059$; PBS + CY versus Ad.αCTLA4 + CY: $P=0.0004$; Ad.zero + CY versus Ad.αCTLA4 + CY: $P=0.0021$. (**d**) PBS + CY versus Ad.zero + CY: $P=0.0102$; PBS + CY versus Ad.αCTLA4 + CY: $P=0.0014$; Ad.zero + CY versus Ad.αCTLA4 + CY: $P=0.0174$.

tumor growth and treatment. Efficacy of lymphocyte-depleting antibodies was confirmed via flow cytometry. Reduction of splenic NK, CD4 +, CD8 + cells was 54, 83 and 87.5%, respectively, at day 4 after injection of corresponding depleting antibodies. In short, depletion of $CD8^+$ and NK cells inhibited the Ad.αCTLA4-mediated antitumor efficacy, as indicated by significantly faster tumor growth (Figure 5b, Supplementary Figure 5A). This indicates that NK cell and $CD8^+$ T-cell-mediated immune responses were an essential component of Ad.αCTLA4-mediated antitumor efficacy. Notably, similar mechanisms appear the act in Ad.zero-triggered antitumor immune responses (Figure 5c, Supplementary Figure 5B).

In both the MMC and TC-1 tumor models, none of the anti-CD25/Ad.αCTLA4-treated mice displayed signs of autoimmune responses detectable as changes in fur color. To further assess potential autoimmune reactions, organs of TC-1/Ad.αCTLA4 vaccinated, CY-treated mice were collected, paraffin embedded, and sectioned for hematoxylin and eosin staining. No evidence of inflammatory processes (for example, mononuclear infiltrations) was detectable in these mice (Figure 6). Furthermore, immunohistochemistry staining for IgG complexes on kidney sections did not reveal abnormalities.

Discussion

We tested a series of Ad.ISP vectors in two aggressively growing syngeneic tumor models. Similar to what is seen in humans, in both models intratumoral Tregs were involved in the protection of tumors against immune-mediated destruction. In our studies, we found that these mechanisms also blocked potential therapeutic effects of Ad.ISP vectors. Only when Tregs were depleted or functionally inactivated (either by low-dose CY or anti-CD25 injection), Ad.αCTLA4 and Ad.αCD3 + Ad.CD80 triggered significant, immune-mediated delay of tumor growth. The first vector expressed an antibody against CTLA4. CTLA4 is a main negative regulator of the

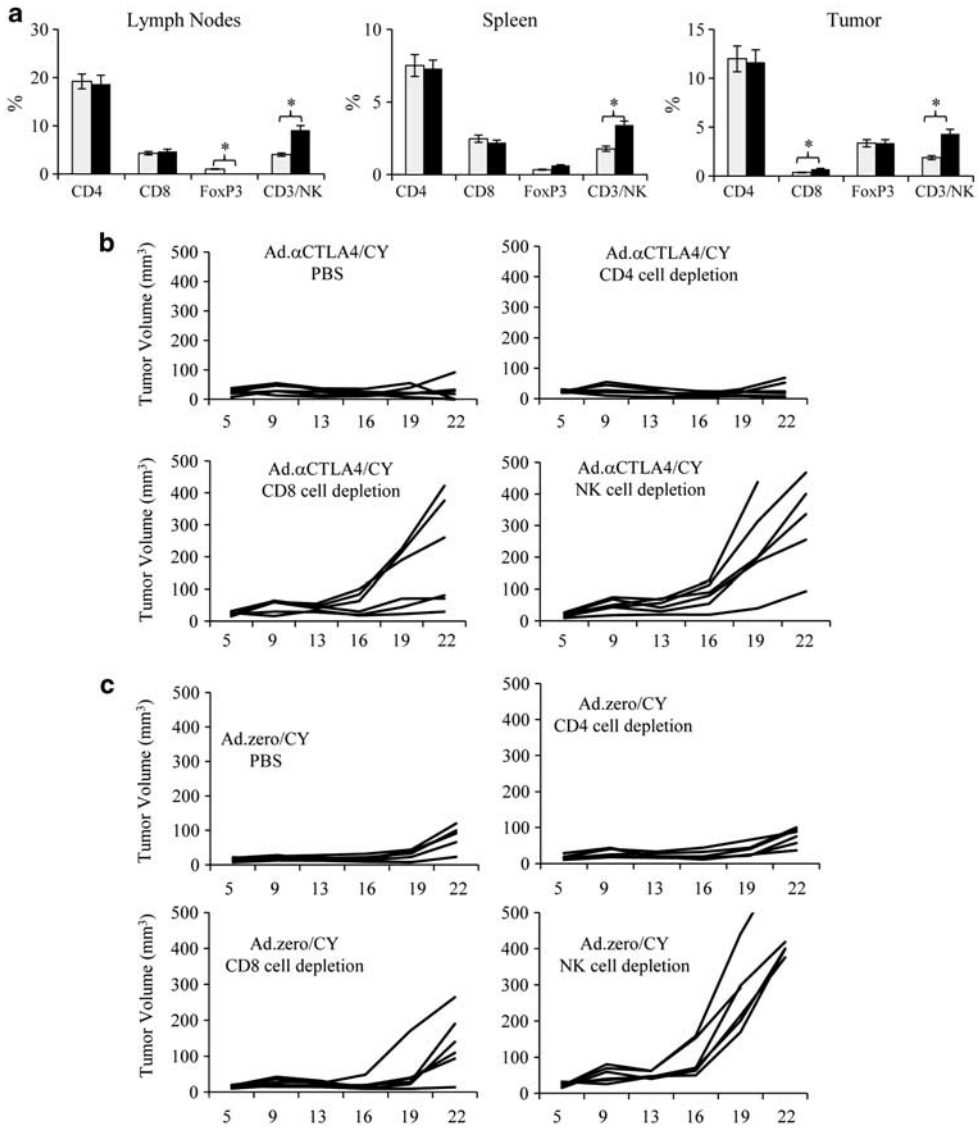

Figure 5 Mechanisms of Ad.αCTLA4 stimulated antitumor immune response in TC-1 model. A total of 1×10^4 TC-1 cells were subcutaneously injected into the left inguinal flank of C57Bl/6 mice at day 0. At day 5, mice received an intraperitoneal injection of CY. At day 9, 1×10^6 Ad.zero or Ad.αCTLA4-transduced TC-1 cells were injected into the right inguinal flank. (**a**) At day 22, tumors, tumor-draining lymph nodes and spleen were harvested and analyzed for CD4, CD8, FoxP3, CD3 and NK1.1 by flow cytometry. White bars: Ad.zero-transduced TC-1 cells, back bars: Ad.αCTLA4-transduced cells. $N=4$, $^*P<0.05$. (**b**, **c**) Four days before vaccination with Ad.αCTLA4 (**b**) or Ad.zero (**c**)-transduced cells, mice received intraperitoneal injection of CD4, CD8 or NK cell depleting antibodies. Injections were repeated every 3 days for the time of monitoring. Shown is the tumor volume of individual mice. Statistical analysis was performed using tumor volumes measured at the end of the observation period (day 22); see also Supplementary Figure 5. Ad.aCTLA4/CY: PBS versus CD4 depletion: $P=0.964$; PBS versus CD8 depletion: $P=0.029$; PBS versus NK depletion: $P=0.001$. Ad.zero/CY: PBS versus CD4 depletion: $P=0.678$; PBS versus CD8 depletion: $P=0.191$; PBS versus NK depletion: $P=0.00003$. Ad.aCTLA4/CY-PBS versus Ad.zero/CY-PBS: $P=0.020$.

immune system, which inhibits the costimulatory signaling for T cells. Anti-CTLA4 antibodies stimulated antitumor immune responses in preclinical and clinical studies. Several mechanisms are proposed for the action of anti-CTLA4 antibodies,[45] including (i) activation of antitumor T cells, (ii) inhibition of the functional activity of Tregs or plasmacytoid dendritic cells, (iii) longer interaction between activated T cells and cancer cells, lowering the threshold of TCR signaling and inducing cytotoxic effects on cancer cells and (iv) induction of apoptosis in CTLA4-positive tumor cells. Our data indicate that αCTLA4 expression triggers antitumor responses through NK and CD8 cells. αCTLA4 expression appeared not to affect the number of Tregs in the tumor and spleen; however, an effect on the function of Tregs cannot be excluded.

A therapeutic effect in the TC-1 model was also observed for Ad.αCD3/Ad.CD80. This vector combination provides T-cell stimulation by binding of αCD3 to the CD3 complex on T cells and ligation of CD28 by

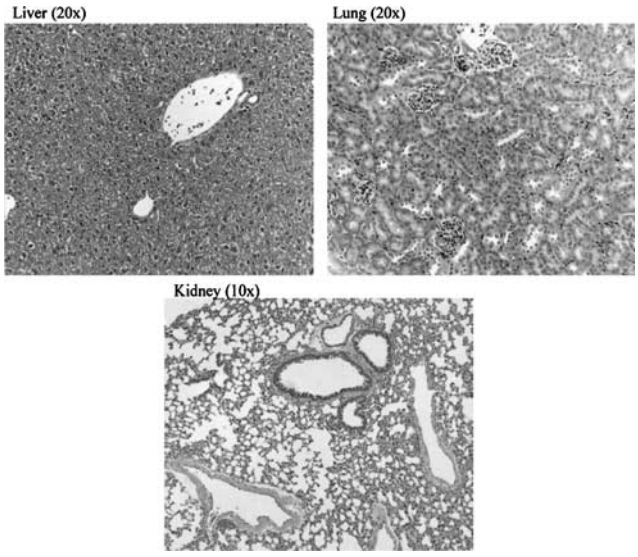

Figure 6 Organ histology. Tissues were collected from anti-CD25 + Ad.αCTLA4-treated mice with MMC tumors at day 40 (see Figure 2f) and from Ad.aCTLA4 + Cy-treated mice with TC1 tumors at day 30 (see Figure 4c). Representative hematoxylin and eosin-stained sections are shown.

CD80. It is thought that expression of both αCD3 and CD80 reduces the dependency of T-cell activation on major histocompatibility complex expression, which is often absent or low on tumor cells. Previous studies in the B16 melanoma model have shown that Ad-mediated overexpression of αCD3 and the CD28 ligand CD86 in combination with Treg depletion induced potent anti-tumor immune responses.[42]

A problem associated with systemic application of immunostimulatory antibodies in preclinical and clinical studies are toxic side effects. In humans who received anti-CTLA4 antibodies, these included colitis and skin rash as well as a variety of autoimmune and inflammatory processes against multiple organs.[46,47] There is a prevalent thought that toxicity and response are correlated after therapy with anti-CTLA4 blocking monoclonal antibodies. Our studies with tumor-localized expression of immunostimulatory antibodies, however, indicate that this is not necessarily correct as we did not observed signs of autoimmunity in our models. In this study, preferentially tumor-localized transgene expression is achieved through local application of Ad vectors into subcutaneous tumors. Our studies in the TC-1 model indicate that the Ad.αCTLA4-based approach has the potential to induce a systemic immune response that can also control distant tumor sites that were not injected with the Ad.ISP vector.

While Ad vectors have clear advantages for tumor cell immunotherapy (high efficiency *in vivo* tumor transduction, induction of a proinflammatory milieu inside the tumor and so on), they also trigger strong anti-Ad immune responses, which potentially can compete with immune responses against the relatively low immunogenic TAAs. In a recent study, we found, however, that anti-Ad T-cell immune responses after intratumoral Ad injection can cause collateral damage and kill tumor cells and that Ad-mediated antitumor efficacy is further enhanced by pre-existing anti-Ad immunity.[22]

Overall, our study provides a basis for Ad-based immunotherapy, particularly for the combination of Ad.αCTLA4 and systemic Treg depletion.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)