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

Liu Ying, M.D.[1,*], **Sebastian Tuve, M.D.**[1,*,$], **Jonas Persson, Ph.D.**[1], **Ines Beyer, M.D.**[1], **Roma Yumul**[1], **ZongYi Li, M.D.**[1], **Kajornsak Tragoolpua**[4], **Karl-Erik Hellström, M.D., Ph.D.**[2], **Steve Roffler, Ph.D.**[4], and **André Lieber, M.D., Ph.D.**[1,2]

[1] University of Washington, Department of Medicine, Division of Medical Genetics [2] University of Washington, Department of Pathology [3] Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand [4] Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Abstract

Tumor-associated antigens (TAAs) include overexpressed self-antigens (e.g. *Her2/neu*) and tumor-virus antigens (e.g. 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 play 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), e.g. recombinant antibodies (αCTLA-4, αCD137, αCD3), cyto/chemokines (IL-15, LIGHT, mda-7), and costimulatory ligands (CD80), via 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 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 anti-tumor 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 auto-immune reactions were observed. The study shows that Ad.αCTLA4 in combination with systemic Treg depletion has potentials in the immunotherapy of cancer.

Corresponding author: Andre Lieber, University of Washington, Box 357720, 1705 NE Pacific St., Seattle, WA 98195, phone: (206) 221-3973, fax: (206) 685-8675, lieber00@u.washington.edu.

*These authors contributed equally.

$present address: Medical Clinic and Policlinic I, University Hospital, Dresden, Germany

Conflict of Interest

There is no competing financial interest in relation to the work described.

Introduction

Many tumor-associated antigens (TAA) are non-mutated self-antigens that are overexpressed in tumor cells. A standard example for this class of TAAs is *Her2/neu. Her2/neu* is immunogenic and spontaneous or vaccine-induced generation of *Her2/neu*-specific CD8+ T-cells in breast cancer patients is frequently reported [1], [2], [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 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 [12]).

Several cytokines and antibodies have been identified or designed, which have the potential to counteract intratumoral immune tolerance and activate anti-tumor reactive lymphocytes against immunogenic tumors in vivo. Among cytokines and chemokines that are able to activate tumor-reactive immune cells are IL-15 [13], LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus [HSV] glycoprotein D [gD] for HSV entry mediator, a receptor expressed by T lymphocytes; TNFSF14) [14], and human melanoma differentiation-associated gene-7 (mda-7)/interleukin-24 (IL-24[15]). Immunostimulatory antibodies include antibodies against CD137[16] (a co-stimulatory molecule, which is expressed on NK and T effector cells), antibodies against cytotoxic T lymphocyte-associated protein 4 -CTLA4[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 naïve T cells) [18]. The clinical usage of these immunostimulatory proteins (ISP) is however, 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 Treg depletion [21]. To achieve antiCTLA4 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 pro-inflammatory environment inside the tumor and facilitate the activation of tumor-antigen-specific T cells, thereby triggering not only anti-virus but also anti-tumor 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 (MMTV) promoter. It has significant biologic and pathologic similarity to human neu-assoviated estrogen receptor-negative breast cancer[23]. The mice spontaneously develop breast tumors, which are immunogenic due to expression of a panel of TAAs (including *Her2/neu*) similar to human cancer. As most tumor-associated antigens are non-mutated self-antigens that have triggered both central and peripheral tolerance, *neu* transgenic mice mimic such tolerance to an endogenous tumor antigen. Overall, the tumor antigen repertoire in MMC-tumor bearing mice appears to be predictive for human breast cancer antigens[23]. Immune tolerance against the *Her2/neu* positive mouse mammary carcinoma (MMC) cells is primarily mediated by regulatory T cells. We have shown recently 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].

Material 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 MHC 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). 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% FCS,1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 2 mmol/L L-glutamine,100 units/mL penicillin, and 100 μg/mL streptomycin.

Antibodies for immune cell depletion

Monoclonal rat anti-mouse CD25 antibody (PC61.5 hybridoma, ATCC), rat anti-mouse CD4 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) using DMEM supplemented with 10% low IgG Fetal Bovine Serum (Hyclone, Logan, UT), 4 mM L-glutamine, 100 U penicillin/ml and 100 μg/ml Pen/Strep. Antibodies were purified from hybridoma supernatant using protein G purification (Amersham Biosciences) followed by dialysis against 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 theE3 genes deleted. The genomic structures of all vectors are summarized in Suppl. Fig. 1. Ad.zero is a transgene-devoid vector. Ad.bGal, Ad.IL-15, Ad.LIGHT, and Ad.mda7 expressed E.coli β-galactosidase, rat Her/neu, mouse IL-15, mouse LIGHT, or human Melanoma differentiation-associated gene-7 (mda7) under the control of the 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 EcoRV/XhoI fragment of p4F10-γ1 [21] into the EcoRV/SalI sites of pAd.RSV [37]. Ad.CD80 was produced by cloning the coding region for murine CD80 [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, VP) concentrations were determined spectrophotometrically by measuring the optical density at 260 nm (OD_{260}). Plaque titering (plaque forming units, pfu) was performed using 293 cells as described elsewhere [39]. The pfu:VP ratios for all Ad preps was ~1:20. Multiplicities of infection (MOIs) in this study were stated as pfu per cell (pfu/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 (MOI50) and transgene expression was analyzed 48 hours 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 *αCD3*, *CD80*, and *αCD137* expression, Ad-transduced cells were detached 48 h post transduction using Versene (Gibco) 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 ELISA 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*-transgenic (*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 (MMTV)promoter. The *neu* transgene is expressed at low levels in normal mammary epithelium, salivary gland, and lung. Until the age of 8 month ~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 harvested 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 ≥ 500mm^3 were considered the experimental endpoint. Animals with skin surface ulcerations were excluded from experiments and sacrificed 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 i.p.) and then Ad (1×10^9 pfu 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 100μl PBS were subcutaneously injected into *neu*-tg mice. When tumors reached a size of 4×3mm diameter (usually at day 6 after cell injection), 250μg of antiCD25 (or control antibody) in 500μl PBS was injected intraperitoneally. Four days later Ad vectors were injected intratumorally. Mice then received antiCD25 or control antibody i.p. once a week.

TC-1 therapy model—A total of 5×10^4 TC-1 cells in 100μl of PBS cells were subcutaneously injected into C57Bl/6 mice. When tumors reaches a diameter of 2×3mm (usually at day 5 after cell injection), mice received an intraperitoneal injection of CY (2mg/mouse in 500μl 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 was injected subcutaneously into the right inguinal flank of C57Bl6 mice. When tumors reached a diameter of ~2mm, 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 an MOI of 100pfu/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 i.p. 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, Ontario, Canada). 200 μg of the respective IgG isotype control (Jackson ImmunoResearch, West Grove, PA) 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 was performed using a BD FACSCalibur (BD Biosciences, San Diego, CA). Samples were pre-treated with Fc-block (anti–CD16/CD32, BD Biosciences) for 15 min, stained for 30 min on ice in washing buffer (WB; PBS-1%FBS) and washed three times with WB. FITC- or PE-conjugated isotype-controls were included in all experiments. Anti-E-cadherin-PE antibody (clone 114420, R&D Systems, Minneapolis, MN) was used to detect MMC cells. For flow cytometry analysis of immune cells the following monoclonal antibodies (mAbs) were used:anti-FoxP3-PE (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-PE (clone PK136). For CD25 flow cytomety another mAb 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 RT using PBS-5% blotting grade milk (BIO-RAD, Hercules, CA) followed by incubation with primary antibodies in PBS for 1h at RT. Then slides were washed twice with PBS and incubated with secondary antibodies for 1h at RT followed by washing with PBS three times. Slides were washed twice with PBS, mounted with Mounting Medium for Fluorescence (Vector Laboratories) and then analyzed using a fluorescence microscope. *Laminin* was detected usinganti-laminin polyclonal (primary) antibody (1:200; #Z0097; Dako, Carpinteria, CA) and goat anti-rabbit-IgG AlexaFluor568 (secondary) antibody (1:200; Molecular Probes, Carlsbad, CA); *Cell surface markers*: FITC-labeled anti-E-cadherin antibody (1:100; clone 36/E-Cadherin, BD Biosciences) or FITC-labeled anti-CD4, antiFoxP3, and antiCD8 antibodies (see "Flow cytometry"). Nuclei were stained with 4′,6-diamidino-2-phenylindole(DAPI; 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, 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 fashion.

Immunohistochemistry for IgG on kidney sections was performed as described for tumor sections using a polyclonal, HRP-labeled, anti mouse IgG antibody (eBiosciences).

Statistical analysis

Statistical significance of *in vivo* data was analyzed by Kaplan-Meier survival curves and logrank test (GraphPad Prism Version 4). 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: antiCD3, mouse CD80, antiCD137, antiCTLA4, mouse IL-15, mouse LIGHT, or mda7. Transgenes were under the control of the Rous Sarcoma Virus (RSV) promoter (Suppl. Fig. 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 Material 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 (Fig. 1A, E-cadherin staining). Tumor nests were surrounded by extracellular matrix (Fig. 1B, laminin staining). Tumors were infiltrated with CD4, CD8 and Tregs (Figs.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 (Fig. 1E). Flow cytometry 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 regards 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 anti-tumor 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 pfu of Ad.ISP vectors were injected into MMC tumors (average size ~50mm^3). All vectors significantly increased the median survival in *neu*-tg mice for at least 5 days compared to PBS injected mice ($p<0.05$) (Fig. 2A, Suppl. Fig. 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 anti-tumor effect in this model, since we did not observe a tumor-growth delay in immunodeficient mice. However, expression of ISP did not have an adjuvant effect on this anti-tumor immune response. Since regulatory T cells have been shown to mediate immune-tolerance towards 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 i.p. injections of low-dose (100mg/kg) cyclophosphamide (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 100mg/kg CY and measured tumor growth (Fig. 2B, Suppl. Fig. 3A). CY resulted in a marked inhibition of tumor growth, indicating a direct toxic effect on MMC cells. Decreasing the CY dose to 50mg/kg 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 antiCD25 antibodies (250μg/mouse i.p.), which did not delay MMC tumor growth in CB17/SCID-beige mice (Fig. 2C, Suppl. Fig. 3B), significantly reduced Tregs in *neu*-tg mice as CD4, CD25, and FoxP3 flow cytometry studies with splenocytes showed (Fig. 2D). To test the therapeutic effect of the combined antiCD25/Ad.ISP approach, MMC tumors with an average volume of $50mm^3$, were intratumorally injected with Ad.ISP or Ad.zero (1×10^9pfu) four days after the mice had been injected with antiCD25 or isotype control Ig. Pre-injection of antiCD25 did not significantly increase median survival of Ad.zero (Fig. 2E), Ad.IL-15, Ad.LIGHT, Ad.*a*CD3/Ad.CD80, and Ad.αCD137 injected mice (not shown). However the combination of antiCD25 and Ad.αCTLA4 had a significant therapeutic effect reflected in prolonged survival compared to Co-Ig/Ad.αCTLA4 and antiCD25/Ad.zero groups (Fig. 2F). None of the antiCD25/Ad.αCTLA4 treated mice displayed signs of auto-immune 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 tumor model that involved C57Bl/6 mice and TC-1 cells. The tumor-associated antigens 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 s.c. transplantation into syngeneic C57Bl/6 mice, TC-1 cells form aggressively growing, vascularized tumors (Fig. 3). Histology studies of TC-1 tumor sections revealed intratumoral Tregs and CD8 cells (Figs. 3C and D). Intratumoral injection of Ad.bGal into TC-1 tumors results in efficient tumor cell transduction (Fig. 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 (Fig. 4A). While in the MMC model, Ad injection (including Ad.zero injection) delayed tumor growth compared to PBS-injected mice, this effect was not observed in the TC-1 model, which might be due to less immunogenicity of Adin 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 (Fig. 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 to PBS-injected mice that received CY (Suppl. Fig. 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 anti-tumor 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 hours, 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 anti-tumor 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 Fig. 4C. We used the TC-1 model to study which arm of the immune system conferred Ad.αCTLA4-mediated anti-tumor 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). (Fig. 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 to 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 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 anti-tumor efficacy, as indicated by significantly faster tumor growth (Fig. 5B, Suppl. Fig. 5A). This indicates that NK-cell and $CD8^+$ T cell-mediated immune responses were an essential component of Ad.αCTLA4-mediated anti-tumor efficacy. Notably, similar mechanisms appear the act in Ad.zero-triggered anti-tumor immune responses (Fig. 5C, Suppl. Fig. 5B).

In both the MMC and TC-1 tumor models, none of the antiCD25/Ad.αCTLA4 treated mice displayed signs of auto-immune 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 H&E staining. No evidence of inflammatory processes (e.g., mononuclear infiltrations) was detectable in these mice (Fig. 6). Furthermore, immunohistochemistry staining for IgG complexes on kidney sections did not reveal abnormalities.

Discussion

We tested a series if 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 immune system, which inhibits the costimulatory signaling for T cells. Anti-CTLA4 antibodies stimulated antitumor immune responses in preclinical and clinical studies. Several mechanism are proposed for the action of anti-CTLA4 antibodies[45], including i) activation of anti-tumor T-cells, ii) inhibition of the functional activity of Tregs or plasmacytoid DCs (pDCs), 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 anti-tumor 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 CD80. It is thought that expression of both αCD3 and CD80 reduces the dependency of T-cell activation on MHC expression, which is often absent or low on tumor cells. Previous studies in the B16 melanoma model have shown that Ad mediated overexpression ofαCD3 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 that received antiCTLA4 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 necessary 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 pro-inflammatory milieu inside the tumor etc), they also trigger strong anti-Ad immune responses, which potentially can compete with immune responses against the relatively low immunogenic tumor-associated antigens. 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 anti-tumor 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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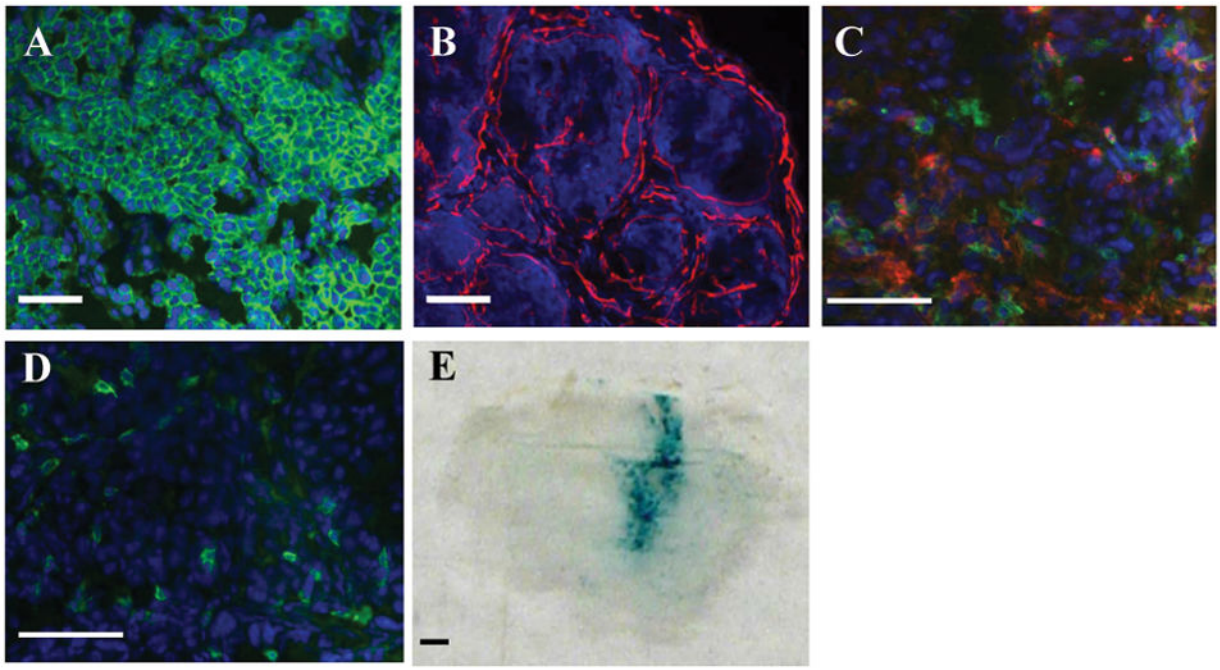


Fig. 1. Histology of MMC tumors

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 DAPI (blue). **E)**β-galactosidase expression 2 days after intratumoral injection of 1×10^9 pfu of Ad.bGal.

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

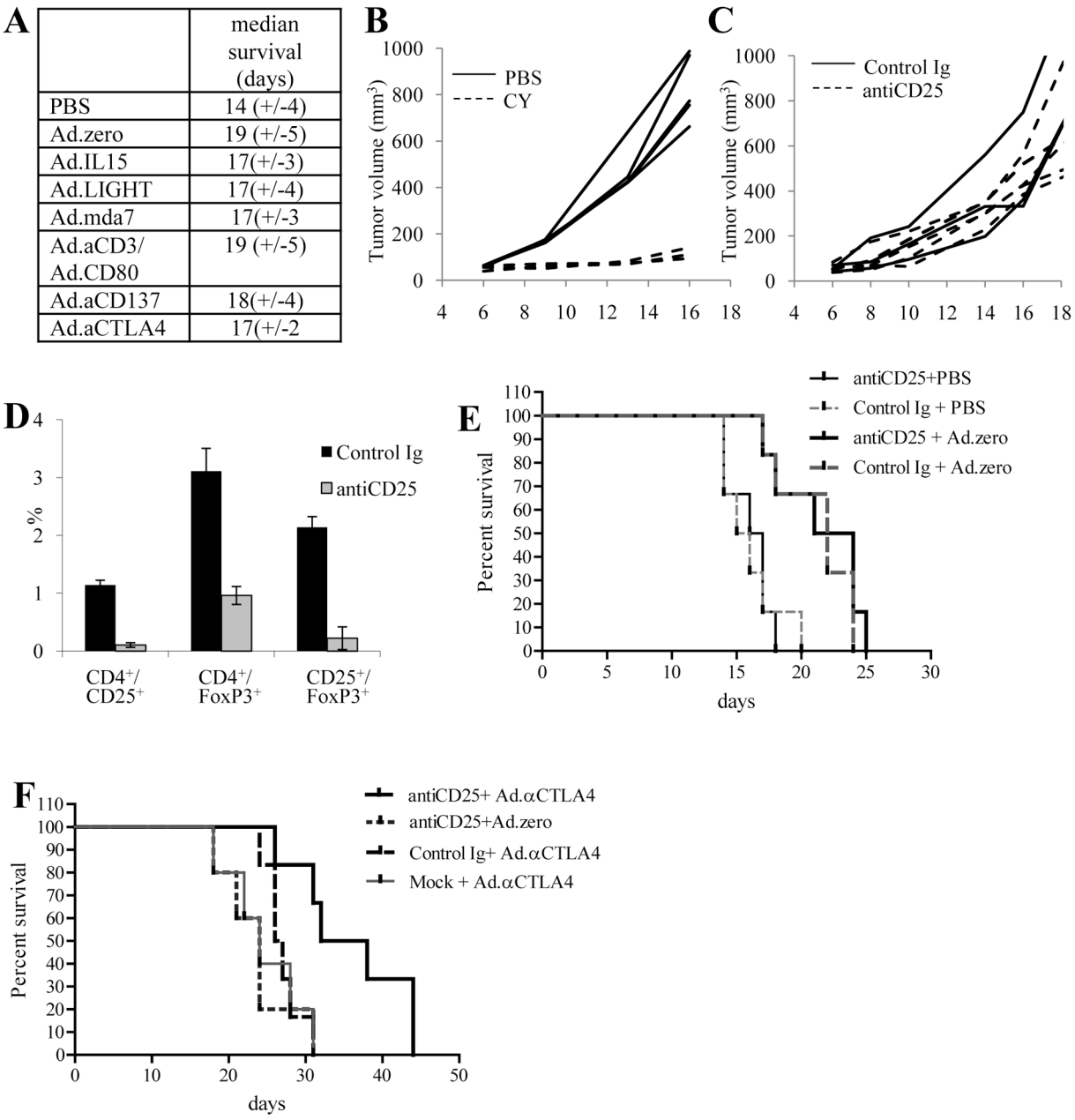


Fig. 2. Therapy studies with MMC tumors

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 400mm^3 represented the endpoint in Kaplan-Meier survival studies. Median survival was calculated using GraphPad-Prizm4 software. N=5.

B) and **C)** studies in CB17-SCID/beige mice. **B)** MMC tumor cells were implanted and low-dose CY (100mg/kg) or PBS was injected i.p. six days later. Tumor volumes at day 16 (end of the observation period) were significantly lessin CY vs PBS treated groups ($p<0.001$);see also Suppl. Fig. 3A. **C)**. In another experiment, tumor-bearing CB17-SCID/beige mice received 250μg of antiCD25 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 antiCD25 treatment groups (p=0.07); see also Suppl. Fig. 3B. **D)** Treg depletion by antiCD25 in *neu*/tg mice. Mice were intraperitoneally injected with 250μg antiCD25 or control antibody and 4 days later, splenocytes were analyzed for CD4, CD25, and FoxP3 by flow cytometry. N=3. CD4+/CD25+ Co Ig vs. antiCD25: p=0.004; CD4+/FoxP3+ Co Ig vs antiCD25: p=0.019; CD25+/FoxP3+: p=0.009. **E)** Effect of antiCD25 on tumor growth. MMC tumor bearing *neu*/tg mice were injected with 250μg antiCD25 or control antibody 4 days before intratumoral injection of PBS or 2×10^9 pfu of Ad.zero. The day tumors reached a volume of $400mm^3$ represented the endpoint in Kaplan-Meier survival studies. N=8. antiCD25+PBS vs antiCD25+Ad.zero: p=0.0061; control Ig+PBS vs. control Ig+Ad.zero: p=0.0058; antiCD25+PBS vs control Ig +PBS: p=0.9677; antiCD25+Ad.zero vs control Ig+Ad.zero: p=0.5722

F) Effect of antiCD25/Ad.αCTLA4 injection on tumor growth. MMC tumor bearing *neu*/tg mice were injected with 250μg antiCD25 or control antibody four days before intratumoral injection of 2×10^9 pfu of Ad.zero or Ad.αCTLA4. The day tumors reached a volume of $400mm^3$ represented the endpoint in Kaplan-Meier survival studies. N=10. antiCD25+Ad.αCTLA4 vs antiCD25+Ad.zero: p=0.0066; antiCD25+Ad.αCTLA4 vs control Ig+Ad.αCTLA4: p=0.0104; antiCD25+Ad.αCTLA4 vs Mock+Ad.αCTLA4: p=0.0105.

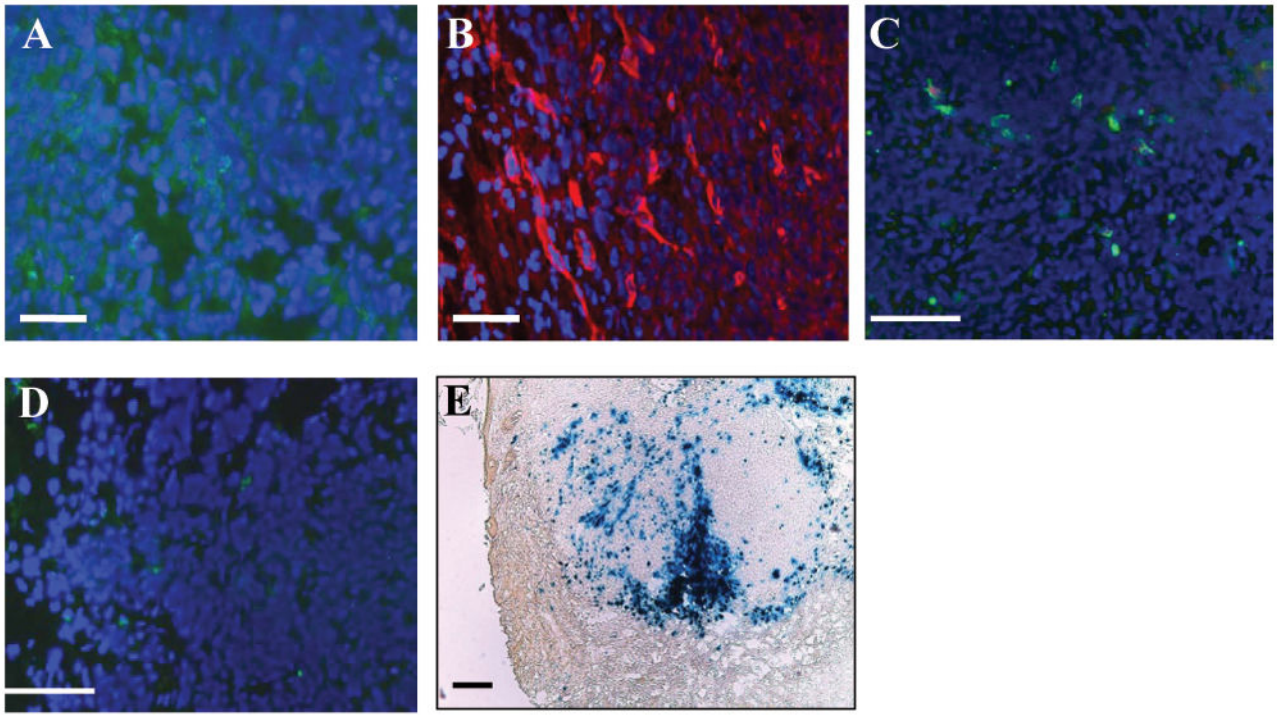
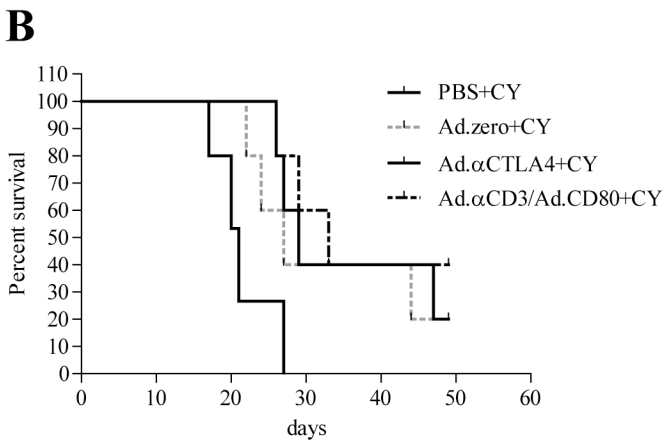


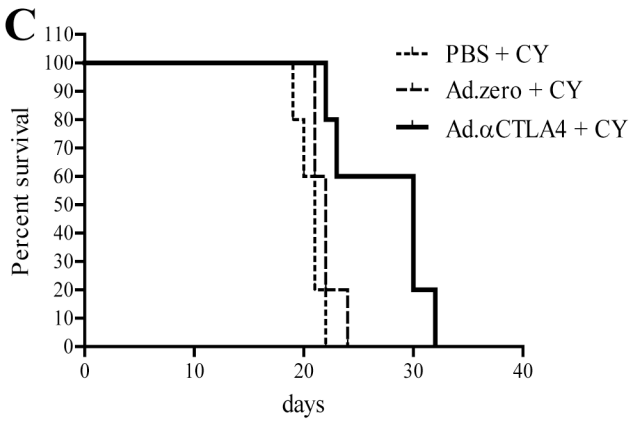
Fig. 3. Histology of TC-1 tumors

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 DAPI (blue). **E)**β-galactosidase expression 2 days after intratumoral injection of 1×10^9 pfu of Ad.bGal. The scale bar represents 40μm.

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)





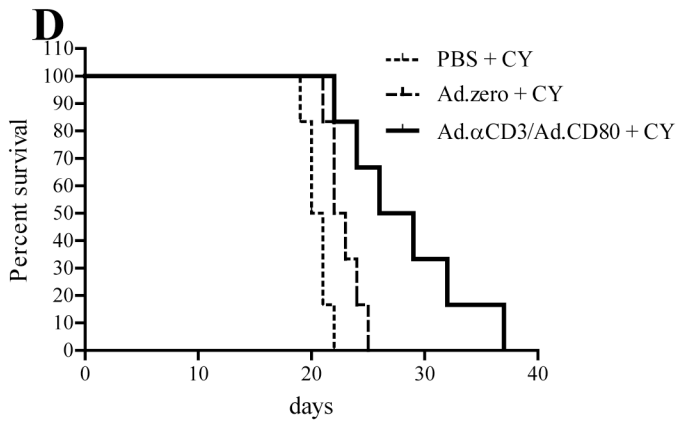


Fig. 4. Therapy studies with TC-1 tumors

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 400mm^3 represented the endpoint in Kaplan-Meier survival studies.

B) Survival of TC-1 tumor bearing mice after treatment with CY (100mg/ml) 4 days before PBS or Ad injection. The day tumors reached a volume of 400mm^3 represented the endpoint in Kaplan-Meier survival studies. N=8. PBS+CY vs Ad.zero+CY: p=0.076; PBS+CY vs Ad.αCTLA4+CY: p=0.028; PBS+CY vs Ad.αCD3/Ad.CD80+CY: p=0.013; Ad.zero+CY vs Ad.αCTLA4+CY: p=0.664; Ad.zero+CY vs Ad.αCD3/Ad.CD80+CY: p=0.433; Ad.αCTLA4+CY vs Ad.αCD3/Ad.CD80+CY: p=0.556.

C) and **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 was injected subcutaneously into the right inguinal flank of C57Bl6 mice. When tumors reached a diameter of ~2mm, 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 MOI of 100pfu/cell. (ThisMOI 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 200mm^3 represented the endpoint in Kaplan-Meier survival studies. N=8

C) PBS+CY vs Ad.zero+CY: p=0.059; PBS+CY vs Ad.αCTLA4+CY: p=0.0004; Ad.zero +CY vs Ad.αCTLA4+CY: p=0.0021.
D) PBS+CY vs Ad.zero+CY: p=0.0102; PBS+CY vs Ad.αCTLA4+CY: p=0.0014; Ad.zero +CY vs Ad.αCTLA4+CY: p=0.0174.

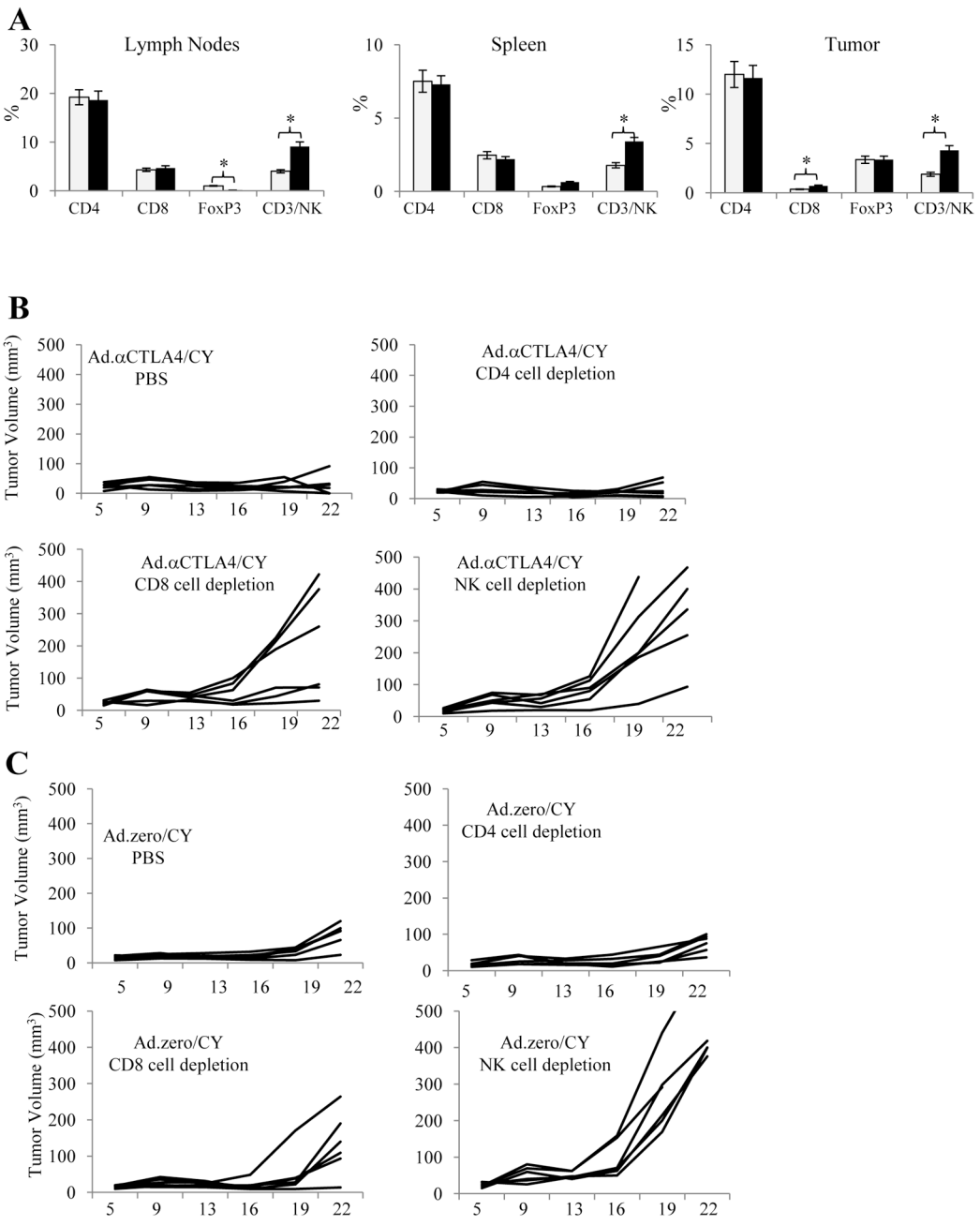


Fig. 5. Mechanisms of Ad. αCTLA4 stimulated anti-tumor immune response in TC-1 model

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 and 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 Suppl.Fig. 5.
Ad.aCTLA4/CY:
PBS vs CD4 depletion: p=0.964
PBS vs CD8 depletion: p=0.029
PBS vs NK depletion: p=0.001
Ad.zero/CY:
PBS vs CD4 depletion: p=0.678
PBS vs CD8 depletion: p=0.191
PBS vs NK depletion: p=0.00003
Ad.aCTLA4/CY-PBS vs. Ad.zero/CY-PBS: p=0.020

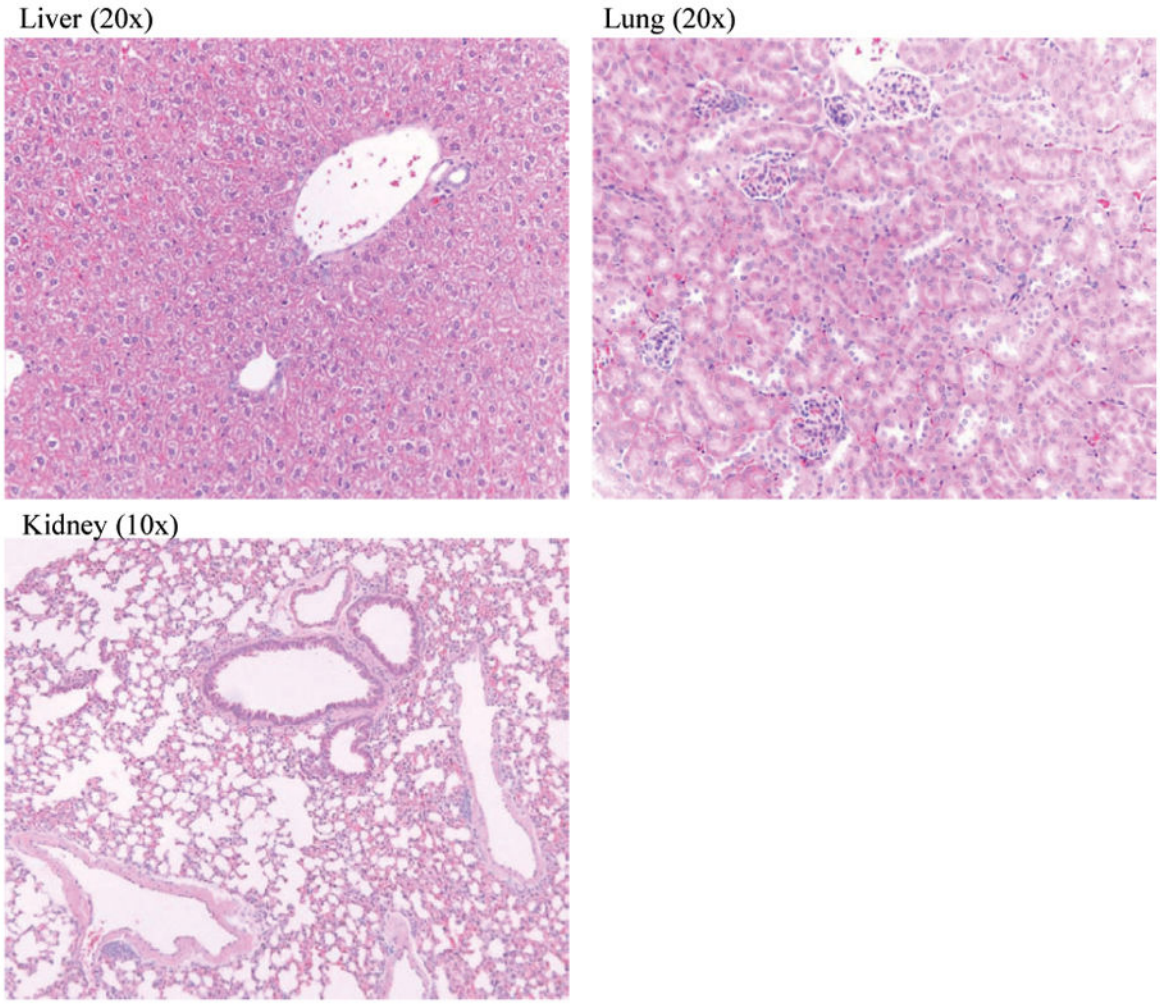


Fig. 6. Organ histology

Tissues were collected from antiCD25+Ad.αCTLA4 treated mice with MMC tumors at day 40 (see Fig. 2F) and from Ad.aCTLA4+Cy treated mice with TC1 tumors at day 30 (see Fig. 4C). Representative H&E-stained sections are shown.