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$CD8^+$ T cell response mediates the therapeutic effects of oncolytic adenovirus in an immunocompetent mouse model

YANG YaJun[1†], LI XiaoZhu[1†], WANG YaoHe[2,3] & WANG ShengDian[1*]

[1] *Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China;*
[2] *Sino-British Research Center for Molecular Oncology, Zhengzhou University, Zhengzhou 450001, China;*
[3] *Center for Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, London EC1M6BQ, UK*

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The role of anti-tumor immune responses in oncolytic adenoviral therapy has not been well studied due to lack of efficacious tumor model in immunocompetent mice. Here, we evaluated the contributions of immune components to the therapeutic effects of oncolytic adenoviruse in an immunocompetent murine tumor model permissive for infection and replication of adenovirus. We found that $CD8^+$ T cells were critical mediator for antitumor efficacy by oncolytic adenovirus. Intratumoral viral therapy induced intensive infiltration of $CD8^+$ T cells in tumor, increased tumor-specific IFN-γ (interferon-γ) production and CTL (cytotoxic T lymphocyte) activity of lymphocytes, and generated a long-term tumor-specific immune memory. Boosting $CD8^+$ T cell responses by agonistic anti-4-1BB (cluster differentiation 137, CD137) antibody showed synergistic anticancer effects with oncolytic virotherapy. Our results provide insight into antitumor mechanisms of oncolytic adenovirus in addition to their direct oncolytic effect.

oncolytic adenoviral therapy, $CD8^+$ T cells, immune responses, anti-4-1BB antibody

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Cancer is a leading cause of death worldwide and there is a critical need for novel cancer therapies. Primary tumors are currently treated by a combination of therapies including surgery, local radiotherapy, chemotherapy and antibody targeted therapies, which have been traditionally known to directly destroy or eliminate tumor cells. However, various studies recently showed that the host anti-tumor immune responses also play a critical role in the therapeutic effects of these conventional antitumor regimens [1,2].

Replication-selective oncolytic viruses are a rapidly expanding therapeutic platform for cancer. Adenovirus (Ad) has been the most commonly described oncolytic virus in the recent decade due to their efficacy, safety and ease of manipulation [3]. Oncolytic Ad vectors infect and kill cancer cells as a result of the normal Ad life cycle by replicating in cells and releasing progeny viruses. These vectors rely on replication and spread through the tumor to achieve efficacy. However, the replication of oncolytic virus is quite immunogenic and the influence of immune responses on the oncolytic therapy is complex. Even though several studies showed that suppressing the immune system enhances the efficacy of oncolytic vectors, recent preclinical and clinical evidence suggests that antitumor efficacy is partially mediated by the immune response [4–6].

Adenoviral replication is generally species specific, and it was assumed by many that viral replication would not occur in mouse tumors [7]. Consequently, oncolytic adenoviral vectors are commonly evaluated in immunodeficient mouse-human tumor xenograft models. However, this model does not accurately reflect how oncolytic adenovirus might behave in human because these mice lack an intact immune system [6,8]. We previously screened a large panel of mouse carcinoma cell lines for their ability to support adenovirus uptake, earl gene expression, late gene expression, replication and cytopathic effects (CPE) and identified

†These authors contributed equally to this work.
*Corresponding author (email: sdwang@moon.ibp.ac.cn)

four carcinoma lines with variable permissivity for adenoviral gene expression, cytopathic effects and/or replication. The antitumor efficacy of adenovirus serotype 5 (Ad5) was significantly higher in CMT-93 model than that in other three tumor models. Moreover, the efficacy of Ad5 against CMT-93 tumors was markedly less in athymic mice than in immunocompetent mice [9,10]. Here, we studied the roles of T cell responses in the tumor therapy of oncolytic adenoviruse by tansplanting murine rectal tumor CMT93 into immunocompetent C57B/6 mice as a tumor model. We found that antitumor efficacy of oncolytic adenovirus is dependent on $CD8^+$ T cell response, which can be enhanced by anti-4-1BB Ab treatment, a potent promoter of antitumor $CD8^+$ T cell responses.

1 Materials and methods

1.1 Animals

Female C57BL/6 mice aged 6–8 weeks were purchased from Wei Tong Li Hua experimental animal center (Beijing, China). All studies involving animals were approved by the Institutional Laboratory Animal Care and Use Committee.

1.2 Cell lines, adenoviruses and antibodies

Murine rectal cell line (CMT93), murine non-small-cell lung cancer cell line (CMT64) were maintained in RPMI 1640 (Hyclone, Laboratory Inc, Logan, UT, USA) supplemented with fetal bovine serum (10%, Hyclone), *L*-glutamine (2 mmol/L, Hyclone), penicillin (100 IU/mL), and streptomycin (50 μg/mL, Hyclone,). Adenovirus serotype 5 (Ad5) was grown on HEK-293 cells as previously described [11].

Anti-mouse CD137 (cluster differentiation 137) agonist mAb (clone 2A, rat IgG2a) was described previously [12]. Rat anti-KLH mAb (rat IgG2a) or rat IgG (Sigma-Aldrich, St. Louis, MO, USA) was used as control. All mAbs, including anti-CD8 (TIB210, rat IgG2a), anti-NK1.1 (PK136, rat IgG2a), anti-CD4 (GK1.5, rat IgG2b), and Rat anti-KLH mAb (rat IgG2a), used as control Ig, were purified from ascites of nude mice and treated with Triton-X 114 for LPS removal.

1.3 *In vivo* antitumor efficacy by oncolytic adenovirus and lymphocyte depletion

1×10^6 CMT93 tumor cells in 100 μL PBS were injected subcutaneously into the right flank of mice. 1×10^9 plaque-forming units (pfu) virus were injected intratumorally on days 8, 11, and 14 after tumor establishment. The injections were introduced through a single central tumor puncture site and 3–4 needles tracts were made radially from the center while virus was injected as the needle was withdrawn. CD4, CD8 or NK (natural killer) cells were depleted by intraperitoneal injection of anti-CD4 (GK1.5), anti-CD8 (TIB210), or anti-NK1.1 (PK136) mAbs at 200 μg respectively twice a week from the day before the viral therapy. For tumor re-challenge experiments, tumor free mice from virotherpay were kept for three months and then rechallenged with 2×10^6 CMT93 in the left flank (contrary side to the first inoculation). For combination therapy of Ad5 and anti-4-1BB mAb, CMT93 tumor-bearing mice were intratumorally injected with 1×10^9 pfu of Ad5 or PBS on days 12, 15 and 18 after tumor inoculation, and intraperitoneally with anti-4-1BB mAb or control Ig on days 14, 19 and 24. Tumor dimensions were measured every 3 d using a digital caliper (111-103-20G, Guilin Guanglu Measuring Instrument Co., Ltd., Guilin, China), and the volume of the tumors was calculated using the formula: volume = 0.52 × length × width × width.

1.4 CTL activity assays

Splenocytes were isolated from the mice 7 d after the last Ad5 administration and co-cultured with mitomycin C (MMC, 100 μg/mL)-treated CMT93 cells for 4 d as CTL effector cells. CMT93 cells or CMT64 tumor cells were used as target cells. The CTL activity were determined by lactate dehydrogenase (LDH) release assay with CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. In brief, target cells were washed three times with RPMI 1640, and mixed with effector cells at effector-to-target (E/T) ratios of 6∶1, 13∶1, or 40∶1 in a 96-well round-bottomed plate. The wells containing exclusively effector cells, target cells, or culture medium, respectively, were served as controls to estimate the LDH background. After 4 h incubation, 50 μL supernatant from each well was transferred to the corresponding well of enzymatic assay plate. 50 μL reconstituted substrate mix was added to each well. After 30 min incubation, the reactions were stopped by adding 50 μL stop solution and measured with an ELISA reader (Plate CHAMELEON, Hidex, Finland) at 490 nm. The percentage of specific lysate was calculated as 100 × (experimental release−spontaneous release)/(target maximum release−target spontaneous release).

1.5 Histopathology staining

The harvested tumor tissues were fixed with 10% formaldehyde and embedded in paraffin. The cross section was deparaffinized and stained with hematoxylin and eosin solution.

Frozen tumor tissues were cut into 5-μm sections and placed on polylysine-coated slides. After blocking with 3% hydrogen peroxide (H_2O_2) in methanol and goat serum, the slides were stained with rat anti-mouse CD8 (Clone 53-6.7, R&D) mAb overnight at 4°C. The slides were incubated with goat anti-rat IgG conjugated with horseradish peroxidase. 3-amino-9-ethy-carbazole solution was added as the substrate for peroxidase activity (Zhongshan Goldenbridge

Biotech, Beijing, China). For statistic analysis of $CD8^+$ T cell number in tumor tissues stained with anti-CD8 Ab, five fields (200×) were randomly selected in each tumor section and photographed with a camera (DFC300 FX Digital Color Camera, Leica Microsystems AG, Schmidt Marketing & Trading Ltd., Guangzhou, China). The pictures were analyzed by Image J image processing soft (NIH, USA) for $CD8^+$ T cell number in each sample. The results were expressed as the median of $CD8^+$ cells per mm^2, n=5.

1.6 Detection of IFN-γ production by ELISA

Splenocytes and tumor draining lymph node cells were stimulated with MMC-treated tumor cells in complete RPMI 1640 medium for 3 d. Supernatants were collected for the detection of IFN-γ (interferon-γ) by using IFN-γ ELISA kit (BD PharMingen, San Diego, CA, USA).

1.7 Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Error bars represent standard error of the mean. Two-tailed unpaired Student's t test was used. P value < 0.05 was considered to be significant.

2 Results

2.1 Therapeutic efficacy of intratumoral Ad5 treatment is dependent on $CD8^+$ T cell responses in immunocompetent mice

We previously found that adenovirus (Ad5) therapy could eliminate CMT-93 rectal tumors subcutaneously transplanted in immunocompetent syngeneic mice, but the efficacy of Ad5 against CMT-93 tumors was significantly less in athymic mice [9]. This result suggested that adaptive immunity play an important role in oncolytic adenoviral therapy.

To determine the components of adaptive immune response which are involved in the antitumor effect elicited by Ad5 therapy, the mice bearing subcutaneous CMT93 tumor were intratumorally injected with Ad5 at days 8, 11 and 14 after tumor inoculation and intraperitoneally injected with anti-CD8, anti-CD4 or anti-NK1.1 deletion mAbs respectively every 5 d from the day before the viral therapy. Adequate depletion of immune cells was evaluated by flow cytometry of peripheral blood from the mice (data not shown). As shown in Figure 1(a), intratumoral treatment of Ad5 resulted in the tumor regression in control antibody-treated mice, and depletion of NK or $CD4^+$ T cells did not affect the anti-tumor effect of Ad5 therapy. However, depletion of $CD8^+$ T cell almost abrogated the antitumor activity of Ad5 therapy. Histological examination of the treated tumor tissues also showed extensive leukocytic infiltration in Ad5-treated tumors compared with PBS-treated tumor. Immunohistochemical analysis with anti-CD8 antibody showed that Ad5 treatment led to intensive infiltration of $CD8^+$ cells in the tumors (Figure 1(b), (c)).

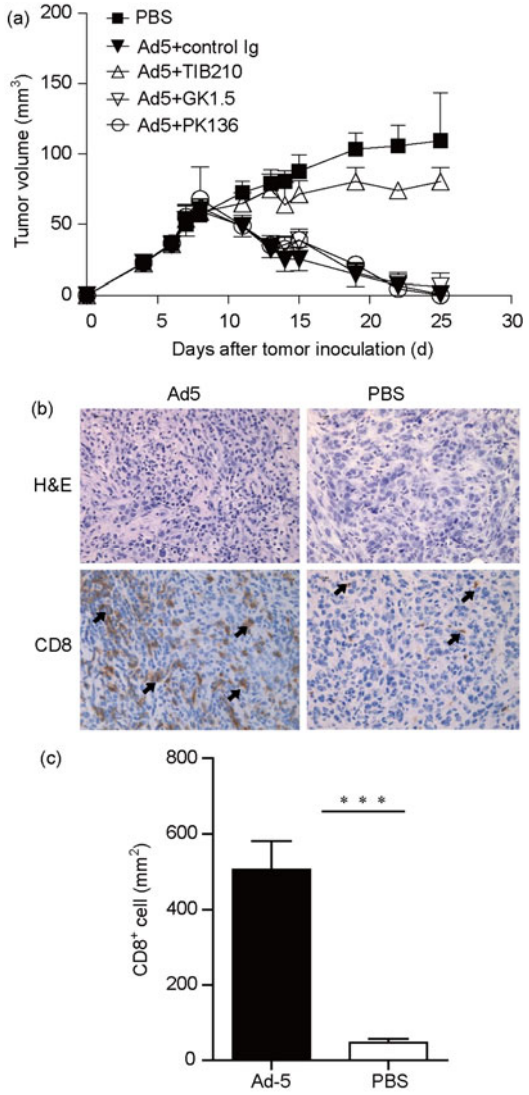

Figure 1 Intratumoral adenovirus therapy is mediated by $CD8^+$ T cells in an immunocompetent mouse model. C57B/6 mice were inoculated subcutaneously with 1×10^6 CMT93 cells. The established tumors were injected directly with 1×10^9 plaque-forming units (pfu) of Ad5 or PBS (control) on days 8, 11, and 14. (a) The deletion antibodies of anti-CD4 (NK1.5), anti-CD8 (TIB210) and anti-NK1.1 (PK136) or control antibody (control Ig) were intraperitoneally administrated twice a week, starting on day 7. Tumor sizes of individual mice were monitored every other day. (b) The tumors treated with Ad5 (left panel) or PBS (right panel) were harvested 7 d after treatment for H&E staining (above) and anti-CD8 mAb staining (down). Arrows indicate the typical $CD8^+$ cells. Original magnification ×100. (c) Statistical analysis of $CD8^+$ T cell number in Ad5 and PBS-treated tumor tissue samples. Ad-5, Ad-5 treatment group; PBS, PBS control group. *P<0.005. One of two experiments is shown.

2.2 Oncolytic Ad5 therapy induced tumor-specific $CD8^+$ T cell responses

To evaluate weather the intratumoral treatment of Ad5 induced specific anti-tumor $CD8^+$ T cells responses, we first

analyzed IFN-γ production of lymphocytes in tumor draining lymph node (DLN) and spleen in different time points after Ad5 therapy by re-stimulated the lymphocytes with CMT93 tumor cells or an irrelevant control lung tumor cells CMT64. As shown in Figure 2, intratumoral Ad5 treatment induced strong T cell responses which produced high level of IFN-γ than the PBS control group. On day 3 after Ad5 therapy, DLN cells from Ad5-treated mice produced almost two times higher IFN-γ than the cells from PBS control group. Even though the splenocytes produced low level of IFN-γ at this time, the splenocytes of Ad5-treated mice produced much higher level of IFN-γ than the cells of PBS control mice. On day 7 after Ad5 therapy, IFN-γ production of the splenocytes dramatically increased to two times higher level than that of DLN cells. Moreover, the splenocytes of Ad5-treated mice produced more than two times higher IFN-γ than the cells of PBS control group. The increase of IFN-γ production in lymphocytes induced by Ad5 treatment was tumor specific, because *in vitro* restimulation of lymphocytes with control CMT64 lung tumor cells did not notably increase the IFN-γ production in Ad5-treated mice compared with PBS control mice.

Next, we detected the tumor-specific CTL activities of lymphocytes induced by Ad5 treatment. The splenocytes isolated from the mice 7 d after treatment were re-stimulated with CMT93 tumor cells, and their cytotoxicity against CMT93 tumor and control CMT64 tumor were detected by lactate dehydrogenase (LDH) release assay. As shown in Figure 3, the CTL activity of Ad5-treated mice was increased more than three times at an effector-to-target ratio of 40:1 compared with that of PBS-treated animals. The CTL activity appeared to be specific for the transplanted CMT93 tumor because the syngeneic CMT64 lung cancer cells were not lysed.

The above results demonstrated that intratumoral oncolytic adenovirus therapy could induce tumor-specific $CD8^+$ T cell responses, which were required for therapeutic efficacy of adenovirus.

2.3 Ad5 therapy induced tumor-specific memory immune response

To determine whether the anti-tumor $CD8^+$ T cell responses initiated by Ad5 therapy resulted in memory, the hallmark of adaptive immune response, we evaluated the cured mice for long-term protection by tumor rechallenge. The mice that underwent complete tumor regression following Ad5 therapy were rechallenged with CMT93 tumor in the left

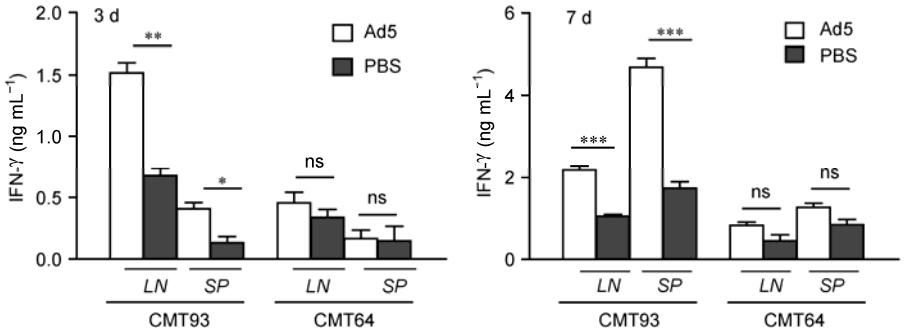

Figure 2 Oncolytic Ad5 therapy induced tumor-specific IFN-γ production of lymphocytes. Tumors were established and treated with Ad5 or PBS as in Figure 1. Draining lymph nodes and spleens were harvested on days 3 or 7 after final viral treatment and stimulated with CWT93 tumors or control CWT64 tumors for 3 d *in vitro*. The IFN-γ in the supernatants was detected by ELISA assay. CMT64 tumor cells were selected as irrelevant tumor control. **P*<0.05; ***P*<0.01; ****P*<0.005; ns, no significant difference. Similar results were obtained in three independent experiments.

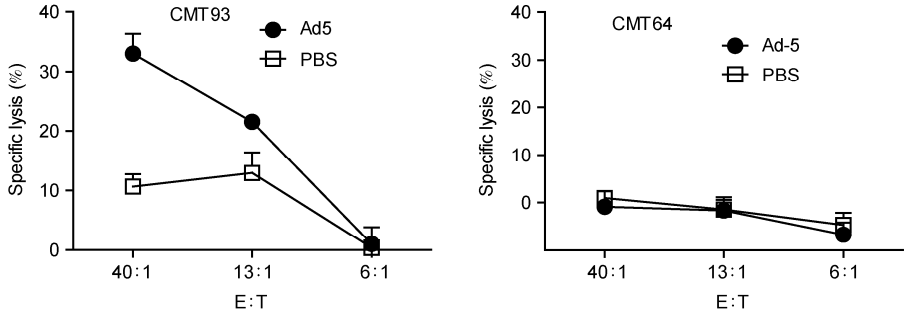

Figure 3 Increased activity of antitumor CTLs in tumor-bearing mice treated with Ad5. Tumors were established and treated with Ad5 or PBS as in Figure 1. Splenocytes were isolated 7 d after Ad5 or PBS treatment and re-stimulated with MMC (100 μg/mL) treated CMT93 tumor cells *in vitro* for 4 d. Cytotoxicity against CMT93 or CMT64 target tumor cells were examined in LDH release assay with different effector-to-target ratios (E:T). Results are representative of two independent experiments.

flank (contrary side to the first inoculation) and with control CMT64 tumor in the right flank after primary CMT93 tumor had not been detected for more than 3 months. Impressively, all mice rejected the rechallenged CMT93 tumors, but the control CMT64 tumors grew progressively. Both tumors grew progressively in naive mice (Figure 4). This result strongly supports that Ad5 therapy-induced tumor regression generated long-term tumor-specific immune memory capable of protecting the host from rechallenge, and presumably against relapse.

2.4 Anti-4-1BB mAb treatment enhance anti-tumor efficacy of Ad5 therapy

Although Ad5 therapy induces specific $CD8^+$ T cell responses against tumor, sustained immunity could be transient or diminished with the growth of tumor, or inhibited by immunosuppressive tumor environment. We reasoned that amplification and maintenance of tumor-specific $CD8^+$ T cell responses initiated by Ad5 therapy could enhance the therapeutic effects of oncolytic adenovirus. Agonistic mAb to mouse 4-1BB/CD137 has been shown to be a potent promoter of specific $CD8^+$ CTL in established tumor models [12,13]. To evaluate the combination therapy of Ad5 and anti-4-1BB mAb, mice bearing larger established CMT93 tumor were treated with intratumoral injection of Ad5 and intraperitoneal injection anti-4-1BB mAb. Neither Ad5 therapy, nor anti-4-1BB mAb treatment alone was sufficient to control tumor growth. The tumor growth in the Ad5-treated group was only delayed by about two weeks compared with that in PBS control group. In contrast, the combination treatment resulted in rejection of all transplanted tumors (Figure 5). Thus, promotion of T cell responses against tumor could enhance the therapeutic effects of oncolytic adenovirus.

3 Discussion

The lack of an immunocompetent tumor efficacy model has been a critical limitation for the field of oncolytic adenovirus therapy because the use of immunodeficient mice bearing human xenograft tumors fails to fully assess the safety of replicating vectors as well as the effect of the immune system on the vector or the tumor [7,8,11]. Despite adenovirus was thought unable to complete replicate in murine tumors, we previously identified four mouse tumor cell lines with variable permissivity for adenoviral gene expression, cytopathic effects and/or replication. Ad5 therapy lead to the tumor regression with acute intratumoral inflammatory responses and $CD8^+$ T cell infiltrations in immunocompetent mice, which is much less in athymic mice [9,10]. Here, we subcutaneously inoculated one of the permissive tumor cell lines, CMT 93, a murine colorectal carcinoma, into immunocompetent syngeneic C57B/6 mice to evaluate the roles of immune components in the tumor therapy of adenovirus. Intratumoral injection of Ad5 induced strong tumor-specific $CD8^+$ T cell responses, which were required for antitumor efficacy of adenovirus. Deletion of $CD4^+$ and NK cells did not affect adenoviral therapy. Anti-4-1BB mAb, a potent enhancer of specific $CD8^+$ CTL, has a synergistic anti-tumor effect with Ad5 therapy.

Adenoviruses have numerous interactions with immune response effectors. In a fully immunocompetent host, local intratumoral virotherapy not only primes antivirus immune response to control viral spread both within and outside of the tumor, but also induces anti-tumor immune response to clear tumor, both locally and distant sites of tumor growth. In this study, we just focused on the specific immune response against tumor and showed that deletion of $CD8^+$ T cells almost abrogated the therapeutic effects of Ad5. Although Ad5 gene expression, and cytopathic effects in CMT93 tumor are comparable to that in human cells *in vitro*, the oncolytic effects of Ad5 and production of new virons in mouse CMT93 tumor model might be inferior to that in human tumor therapy based on the data from adenovirus in normal mouse tissues. The direct oncolytic efficacy of Ad5 might be underestimated in this mouse tumor model. However, our results at least demonstrated that treatment of oncolytic adenovirus could induce tumor-specific T cell

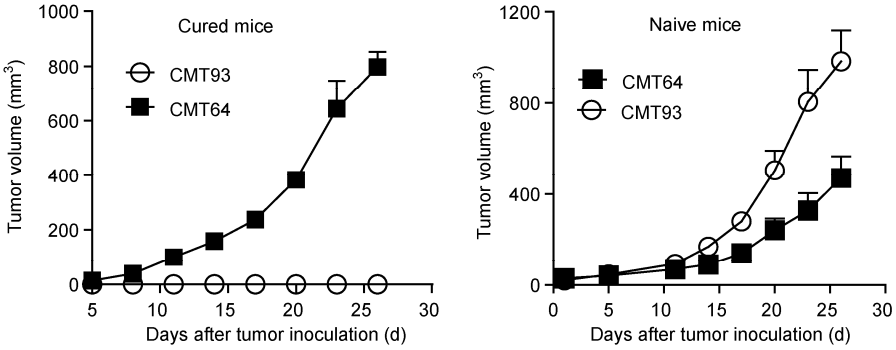

Figure 4 Mice cured of tumors by Ad5 therapy acquired long-lasting, specific immune memory. The Ad5-treated, tumor-free mice were kept for above 3 months and rechallenged with 2×10^6 CMT93 tumor cells in the left flank (contrary side to the first inoculation) and 2×10^5 CMT64 tumor cells in the right flank (n=5). Five naive age-matched mice were also challenged as controls. Tumor sizes were monitored every 3 d by a digital caliper. Similar results were obtained in two independent experiments.

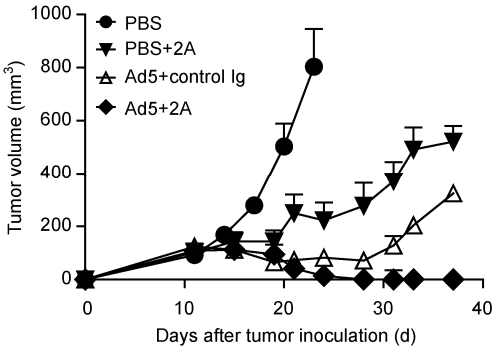

Figure 5 The combination of Ad5 and anti-4-1BB mAb immunotherapy eradicates established large tumors. CMT93 tumor-bearing mice (n=5) were intratumorally injected with 1×10^9 pfu/injection of Ad5 or PBS on days 12, 15 and 18 after tumor inoculation, and intraperitoneally with anti-4-1BB mAb or control Ig on days 14, 19 and 24. Tumor sizes were monitored every other day using a digital caliper. Results are representative of three independent experiments.

responses, which contribute to the therapeutic effects of oncolytic adenovirus.

In a fully immunocompetent host, viral oncolysis promote direct tumor destruction and tumor antigen release. Meantime, inflammatory milieu produced by viral replication provide 'dangerous signal' for host immune response, with the release of immunogenic intracellular contents and cytokine at the site of cell death. These factors favor to recruit and activate APC (antigen presenting cell) and promote presentation of tumor antigens. These series of events provide an opportunity to activate T cells. However, tumor microenvironment was stressed in patients' body and anti-tumor immune responses could be tightly suppressed by tumor immunosuppressive environment. Furthermore, large tumors are often refractory to single oncolytic virus administration. The potential synergistic therapy with chemotherapy has been described in head and neck cancer patients [14]. The curative effect could also be improved by adding immunostimulatory genes in viral vectors to enhance clearance of infected tumors [15,16]. Given that the efficacy of adenovirus therapy is dependent on $CD8^+$ T cells, we investigated combination therapeutic strategy of Ad5 and anti-4-1BB mAb, a potent stimulator of specific $CD8^+$ T cell responses. In order to enhance immune-based anti-tumor response while not clear virus too early to reduce oncolytic efficacy, we intratumorally injected Ad5 in first step which harnessed the property of virus replication within tumor and promoted tumor antigen release and presentation, then intraperitoneally administrated anti-4-1BB mAb, boosting the anti-tumor T cell response initiated by viral therapy. The delayed injection of anti-4-1BB mAb could avoid accelerating immune-mediated clearance of the virus to more effectively enhance the therapeutic effects of oncolytic virus.

In summary, in an immunocompetent murine tumor model which was permissive for infection and replication of adenovirus, we demonstrated that oncolytic adenoviral therapy could induce potent tumor-specific $CD8^+$ T cell responses, which were critical for the therapeutic effects of the virus. The strategies for boosting T cell responses could improve the tumor therapy of oncolytic virus. This study proposes a model and a new and more effective therapeutic regime for cancer treatment by combination therapies of oncolytic adenoviruse and targeted antibody immunotherapy.

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