Title

The efficacy of oncolytic adenovirus is mediated by T cell responses against virus and tumor in Syrian hamster model

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**Translational Relevance**

Oncolytic viruses are able to directly lyse cancer cells and represent an innovative approach to cancer therapy. Viruses additionally represent a potent immunologic “danger signal”, which facilitate the generation of antiviral and antitumor immune responses. The roles and the relative therapeutic importance of the direct tumor lysis, antiviral and antitumor immunity in antitumor efficacy of oncolytic virotherapy have remain uncertain. We explored the effects of T cells in oncolytic adenoviral therapy by using a deleting anti-Syrian hamster CD3 monoclonal antibody. We identified the key roles of T cells in oncolytic Ad therapy, in which viral-specific T cell response may act as a bridge linking Ad infection and oncolysis to the antitumor immune responses. These results shed light on developing new therapeutic regime of oncolytic Ad by enhancing antigen-specific T cell responses.
Abstract

Purpose: Oncolytic adenoviruses (Ad) represent an innovative approach to cancer therapy. Its efficacy depends on multiple actions including direct tumor lysis, stimulation of antiviral and antitumor immune responses. In this study, we investigated the roles of T cell responses in oncolytic adenoviral therapy.

Experimental Design: An immunocompetent and viral replication-permissive Syrian hamster tumor model was used. The therapeutic mechanisms of oncolytic Ad were investigated by T-cell deletion, immunohistochemistry staining, and CTL assay.

Results: Deletion of T cells with an anti-CD3 antibody completely demolished the antitumor efficacy of oncolytic Ad. Intratumoral injection of Ad induced strong virus- and tumor-specific T cell responses, as well as antiviral antibody response. Both antiviral and anti-tumor T cell responses contributed to the efficacy of oncolytic Ad. Deletion of T cells increased viral replication and extended the persistence of infectious virus within tumors, but almost abrogated the antitumor efficacy. Preexisting antiviral immunity promoted the clearance of injected oncolytic Ad from tumors, but had no effect on antitumor efficacy. Strikingly, the repeated treatment with oncolytic Ad has strong therapeutic effect on relapsed tumors or tumors insensitive to the primary viral therapy.

Conclusion: These results demonstrate that T cell-mediated immune responses outweigh the direct oncolysis in mediating antitumor efficacy of oncolytic Ad. Our data has a high impact on redesigning the regimen of oncolytic adenovirus for cancer treatment.
Introduction

Oncolytic viruses (OVs) are self-replicating, theoretically tumor selective, and possess an ability to directly lyse cancer cells. OVs are being developed as an attractive therapeutics for cancers(1). Viral replication is quite immunogenic. An active host immune response against the viruses which rapidly eliminates the virus had been considered as an important barrier to the successful cancer virotherapy. Indeed, several studies showed that suppressing the immune system enhanced the efficacy of oncolytic adenovirus (Ad) (2, 3). However, there have been accumulating evidence that the virus-induced immune activation can generate innate and adaptive immune responses that are critical to mediating the antitumor responses. Recently, preclinical and clinical data suggested that the virotherapy might in fact act as cancer immunotherapy (4, 5). The interactions between oncolytic viruses, tumor cells and the immune system are critical to the outcome of antitumor efficacy of OVs.

Like many OVs, Ad replication is species-specific and human Ads replicate poorly in cells from most other species like mice and rats. Consequently, most published efficacy data have come from immune-deficient mice bearing human tumor xenografts. These models cannot adequately address the effect of the host immune system on the vector, tumor, as well as the toxicity and biodistribution of the vector in normal tissues. The Syrian hamster has been characterized as a suitable immunocompetent, replication-permissive animal model for the assessment of human oncolytic Ads (6, 7). Intratumoral (i.t.) injection of oncolytic Ad resulted in viral replication and necrosis of tumors, suppression of primary and metastatic lesions, viral replication in the liver and lungs, and anti-Ad antibody induction. Wold W’s group used this model to study the effects of preexisting anti-Ad immunity and immunosuppression on replication and antitumor efficacy of oncolytic Ad vectors. They found that immunosuppression induced by elimination of all white blood cells with cyclophosphamide (CP) abrogated anti-Ad immune response, allowed sustained Ad replication, and significantly improved tumor suppression (2). However, the pre-immunization, which induced a persistent high-level of neutralizing anti-Ad antibody and disabled the detection of the infectious virus injected in tumors, had no effect on the antitumor efficacy of oncolytic Ad in immunocompetent animals. In the pre-immunized tumor-bearing animals, CP treatment had no effect on tumor growth, although the titer of neutralizing anti-Ad antibody stayed steady at all time points, and tumors had moderate amount of virus (8). These results suggest that curative viral oncolysis on its own probably only occurs in the instance when tumors are completely devoid of any virus defense system. On the other hand, promotion of immune
responses by arming oncolytic Ad with immune-stimulatory molecules in the immunocompetent hosts significantly enhanced antitumor effects (9-11). Thus, the host immune responses will probably be critical to the efficacy of oncolytic virotherapy, although it also mediate the rapid viral elimination.

In recent years, a lot of clinical data in cancer immunotherapy clearly demonstrated that T cell response is the dominant fighter against cancers. The cellular antiviral immune response may limit efficacy of virotherapy by eliminating tumor infection via clearance of infected tumor cells. Alternatively, clearance of infected tumor cells may play a key role in tumor regression. Some studies showed that T cell response triggered by oncolytic virotherapy included virus-specific cytotoxic activity, which might play a role in tumor therapy (12-14). Although T cells are major components for both anti-virus and anti-tumor immunity, they have been rarely studied in oncolytic Ad therapy in immunocompetent, replication-permissive hosts. In this study, we evaluated the effects of T cells in oncolytic Ad therapy by using an anti-Syrian hamster CD3 monoclonal antibody that can delete T cells in hamsters. We found that oncolytic Ad therapy induced strong virus-specific and tumor-specific T cell responses. Deletion of T cells extended the persistence of infectious virus in tumors, but almost abrogated the antitumor efficacy. These results suggest that T cell-mediated immune responses outweigh direct oncolysis of Ad in mediating antitumor efficacy in immunocompetent hosts.
Materials and methods

Cell lines, Viruses and reagents

The Syrian hamster pancreatic carcinoma cell line HPD-1NR was purchased from German Collection of Microorganisms and Cell Culture, and kidney tumor cell line HAK was originally purchased from American Type Culture Collection (ATCC). JH293, the human kidney epithelial cell line transformed with Ad5 DNA, was obtained from Cancer Research UK Central Cell Services, London, United Kingdom. All cell lines were cultured in basal medium (HPD-NR and HAK, RPMI 1640; JH293, DMEM) supplemented with 10% fetal bovine serum (Hyclone), 2 mmol/L L-glutamine (Hyclone), 100 IU/mL penicillin, and 50 μg/mL streptomycin. Cells were tested negative for mycoplasma. Cells were not further authenticated.

Wild-type adenovirus type 5 (Ad5) and E1A-deleted Ad5 (dl312) were from Sino-British Research Centre, Zhengzhou University, and grown on HEK-293 cells as previously described (15).

The mouse monoclonal antibody (mAb) against Syrian hamster CD3e (clone 4F11, IgG1 isotype) and anti-KLH mAb were prepared as previously described (16).

In vivo treatments

Four- to five-week-old female Syrian (Golden) hamsters (Mesocricetus auratus) were obtained from Vital River Corp (Beijing, China), and inoculated subcutaneously (s.c.) with 1 x 10⁶ HPD-1NR cells. The tumor-bearing animals were treated by intratumoral (i.t.) injection of Ad5 or PBS every other day for six times from day 11 after tumor inoculation. The injections were introduced through a single central tumor puncture site and 3–4 needles tracts were made radially from the center while the virus was injected as the needle was withdrawn. To delete T cells, an anti-hamster CD3 mAb (4F11) was injected intraperitoneally (i.p.) at doses of 500μg/injection every 5 days from the day before the viral therapy, and anti-KLH mAb was used as control. Tumor size was measured twice weekly using digital calipers, and tumor volume was calculated using the formula: volume = (length x width² x π /6). All animal experiments were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China) and were conducted in accordance with institutional and national regulations.

CTL assays

CTL assays were done as previously described with a little modification (17). In briefly, splenocytes were stimulated in vitro with ⁶⁰Co-irradiated HPD-1NR tumor cells infected with dl312 at a ratio of 5:1 in RPMI-1640 medium for 4 days. The
Lymphocytes were isolated by Lymphocyte Separation Media (LTS1077, TBD Corp., Beijing, China) as effector cells. Both HAK cells infected with d/h312 for 24 h and HPD-1NR cells without infection were stained with 10 mM CFSE (CFSE\textsuperscript{high}) as specific target cells. HAK cells without viral infection were stained with 0.5 mM CFSE (CFSE\textsuperscript{low}) as control target cells. A mixture of specific and control target cells at a 1:1 ratio was incubated with effector cells at different E/T ratios in a round-bottom 96-well plate for 16 h. Then the cells were harvested and stained with 7-aminoactinomycin D (7-ADD). The CFSE profiles were analyzed using 7-AAD\textsuperscript{+} CFSE\textsuperscript{−} gate. Cytotoxicity was determined by the following formula: Cytotoxicity(%)=100%×[1-Ratio (target/control)\textsubscript{effector}/Ratio (target/control)\textsubscript{effector}]⁻¹.

**Assessment of virus in tumors**

Fresh tumor tissues were homogenized and titrated on JH-293 cells to determine the 50% tissue culture infective dose (TCID\textsubscript{50}) as previously described (18). Viral DNA levels were evaluated as previously described (16). Results were expressed as genome copy number/gram based on the weight of tumor tissues.

**Immunohistochemistry (IHC)**

The harvested tumor tissues were processed and stained respectively with anti-hamster CD3 mAb (4F11), anti-Ad\textsubscript{5} E1A mAb (MA5-13643, Thermo scientific), and anti-Ad\textsubscript{5} Hexon mAb (ab8249, Abcam) followed by relevant peroxidase-conjugated secondary antibodies. The peroxidase activity was detected with DAB substrates (ZLI-9019, Zhongshan Goldenbridge Biotech, Beijing, China). Apoptosis was detected in situ using the Roche TUNEL kit (11684817910, In Situ Cell Death Detection Kit, POD, Roche,) according to manufacturer’s instructions. For assessment of the positive staining proportion of tumors, each sample was cut in at least three sections at different, equally spaced depths and five high-power fields (HPF, 200 x) were randomly selected for each section. A total of over 15 high-powered fields for each sample were counted and the exact number of positive cells was determined per HPF by an image analysis system (Image-Pro plus 6.0). The values for the different fields were then averaged to obtain the final values of each sample for statistical analysis.

**Detection of anti-Ad\textsubscript{5} antibodies**

Anti-Ad\textsubscript{5} antibodies were detected as previously described (16). The relative levels of total antibodies in all samples were shown as the value of OD\textsubscript{450nm}.

**Statistical analysis**

Statistical analysis was done with GraphPad Prism 5 software. The results are expressed as means with the standard deviations (±SD) when appropriate. Differences between groups were analyzed using the two-tailed Student’s t test or 2-way ANOVA. P < 0.05 (*)
was considered significant, and \( P < 0.01 \) (***) was considered highly significant.
Results

Antitumor efficacy of oncolytic Ad depends on T cells in hamster tumor model

To investigate the role of T cells in antitumor efficacy of oncolytic Ad therapy, Syrian hamsters were inoculated s.c. with HPD-1NR pancreatic carcinoma cells and treated with six i.t. injections of oncolytic adenovirus (Ad5) or PBS at one day interval from day 11 after tumor inoculation when the tumors were 6 to 7 mm in diameter. Meanwhile, anti-hamster CD3 mAb (4F11) or control Ig was injected i.p. at three days interval from the day before the start of viral therapy. Consistent with the previous studies(6), Ad therapy markedly inhibited tumor growth compared with i.t. injection of PBS. Moreover, the antitumor efficacy of Ad was dose-dependent (Fig.1A). The Ad treatment with a low dosage of $5 \times 10^8$ PFU/injection inhibited tumor growth and caused transient tumor regression, leading to significant delay of tumor growth in most animals (80%). One of five animals in the group was cured and remained tumor free more than 60 days (data not shown). The regressed tumors in other animals started rapid growth about two weeks after viral treatment. Ten times higher dosage ($5 \times 10^9$ PFU/injection) of Ad markedly suppressed tumor growth and caused tumor regression in all animals, in which tumors were completely disappeared in three of five animals. The recurrent tumors also started to grow about two weeks after Ad5 treatment. When T cells were deleted, Ad therapy still markedly inhibited tumor growth as well during the vector injections. However, it could not cause tumor regression even with high dosage of Ad, and all tumors started to grow about one week after Ad treatment, which was one week earlier than the recurrent tumors in immunocompetent animals. Importantly, the tumor growth after Ad treatment in T cell-deficient animals was faster than that in immunocompetent animals. These data showed that oncolytic Ad vector effectively inhibited tumor growth during viral treatment, but failed to cause tumor regression in the T cell-deficient animals. Deletion of T cells caused rapid re-growth of the suppressed tumors after viral therapies. To further assess the contributions of CD4$^+$ and CD8$^+$ T cells to the antitumor effect, we compared the effect of T cell deletion with that of CD4$^+$ T cell deletion in therapeutic efficacy of oncolytic Ad, by using an anti-mouse CD4 antibody that crossly react with T cells of hamsters(19). Compared with deletion of total T cells, deletion of CD4$^+$ T cells marginally attenuated the antitumor effect of oncolytic Ad, suggesting that the antitumor T cell response is majorly mediated by CD8$^+$ T cells (Fig. 1B). These results demonstrate that T cell responses, especially CD8$^+$ T cell-mediated response, play a critical role in the therapeutic effect of oncolytic Ad, especially in mediating tumor regression and preventing tumor recurrence after viral treatment.
To determine whether the T cell responses initiated by Ad vector results in immunological memory, the hallmark of adaptive immunity, we evaluated the cured hamsters for long-term protection by tumor rechallenge with or without T cell deletion. The hamsters that underwent complete tumor regression following Ad treatment were rechallenged with $2 \times 10^6$ HPD-1NR cells (two times of the primary tumor inoculation) after primary tumors had not been detected for one month. All animals rejected the rechallenged tumors. However, once the cured animals were i.p. injected with anti-CD3 mAb at the day before the rechallenge, all the rechallenged tumors grow progressively (Fig. 1C). This result strongly supports that oncolytic Ad therapy generates antitumor memory T cells capable of protecting the host from rechallenge, and presumably against tumor relapse.

**T cells are associated with elimination of infectious Ad and cytolysis of tumors**

T cell-deficiency has a contrast effect on antitumor efficacy of oncolytic Ad in Syrian hamster with the reported CP-induced immunosuppression, in which all immune cells were deleted.(2). Thus, it is interesting to see the effects of T cells on viral replication in tumor, and their contributions to antitumor efficacy. To this end, tumor-bearing hamsters were treated with six i.t. injections of low dosage of Ad5 with or without deletion of T cells, and the infectious viral yields, viral protein expression and viral DNA level in tumor tissues were determined at the indicated time points. The infectious virions of Ad5 detected by viral replication assay remained elevated at the same level ($\sim 10^6$ PFU/g or so) in both tumors of immunocompetent and T cell-depleted animals within three days after the first viral injection. Then, in immunocompetent animals, virus levels were decreased approximately tenfold ($10^5$ PFU/g) by day 7. At day 11, just one day after the last viral injection, only one of three animals showed a detectable virus in tumors, which is only slightly (about twofold) higher than the detection limit ($1 \times 10^3$ PFU/g). In contrast, the amount of infectious virus within tumors of T cell-deficient animals still maintained elevated ($\sim 10^7$ PFU/g or so) at day 7 after the first viral injection, and then started to gradually decrease and reached to $\sim 10^4$ PFU/g by day 17. All animals in both groups had no detectable infectious virus in tumors 21 days after the first viral injection (Fig. 2A). Consistent with the yields of infectious virus in tumors, viral protein E1A and Hexon detected by immunohistochemistry were highly expressed in tumors of T cell-deficient animals at day 7 after the first viral injection and dramatically decreased at day 17. While, the expression of viral proteins was significantly lower in tumors from the immunocompetent animals. The numbers of tumor cells expressing E1A and Hexon were 3 to 4 fold higher in tumor sections of T cell-deficient animals at both day 7 and 17 as compared with the tumor sections from the immunocompetent animals. At day 21, the
viral proteins were rarely detected in tumors of both animals (Fig. 2B). Thus, viral replication was initially similar in tumors injected with oncolytic Ad, regardless of immune status of animals. About one week later, the infectious virus and virus-infected tumor cells began to rapidly decline in immunocompetent animals compared with that in T cell-deficient hamsters. This timing suggested that the infectious virus and virus-infected tumor cells were eliminated by adaptive anti-viral immunity developed in immunocompetent animals. Although T cells deficiency allowed intratumoral virus levels to remain elevated for prolonged periods, the antitumor effects of oncolytic Ad were severely impaired in T cell-deficient hamsters (Fig. 1). These results further support the critical role of T cells in oncolytic Ad therapy.

There was no significant difference in virus DNA levels titrated by real-time PCR between the tumors of T cell-deficient and immunocompetent hamsters. Viral DNA level remained elevated in tumor until day 17 and could not be detected at day 21 after the first viral injection (Fig.2C). These data suggest that T cells play a major role in elimination of infectious virus, but have less effect on clearance of viral genome. The elimination of viral genome should be mediated by innate immune system.

To directly assess T cell responses and virus replication in tumors, as well as their contributions to tumor cytolysis, we histologically assessed T cell infiltration, virus replication and cell apoptosis in tumors on day 7 after the first viral injection when the level of intratumoral virus started to become different in immunocompetent and T cell-deficient animals. Compared to PBS treatment, oncolytic Ad therapy increased tumor infiltration of CD3+ T cells, and resulted in expression of viral protein E1A and marked apoptosis of tumor cells. Deletion of T cells dramatically eliminated tumor-infiltrating CD3+ T cells and increased expression of E1A protein, but significantly decreased apoptosis of tumor cells (Fig.2D). These data showed that the viral replication was not correlated with cytolysis in tumors even at day 7 after viral injection, while T cell response was associated with suppression of virus replication and increased apoptosis in tumors.

Overall, these results demonstrate that oncolytic Ad therapy rapidly induces T cell responses, which eliminate the virus-infected tumors cells and mediated antitumor efficacy although it also decrease the yield of infectious virus in tumors.

Oncolytic Ad therapy resulted in both tumor- and virus-specific CTL activities
CD8+ T cells are major effector cells for immune responses against tumors and virus infection. Next, we assessed the tumor- and virus-specific CTL activities induced by oncolytic Ad therapy. To exclude the influence of tumor size on the antitumor T cell
responses, splenocytes were harvested at day 4 and 7 after the first viral injection, and stimulated in vitro for four days with HPD-1NR tumor cells infected with replication-deficient dl312, an E1A-deleted Ad5. CTL activities were detected by an in vitro killing assay(17) using an irrelevant hamster renal cancer cell HAK as an internal reference. Tumor-specific cytotoxicity was detected against HPD-1NR tumor cells without virus infection, and virus-specific cytotoxicity was detected against dl312-infected control HAK tumor cells. Relative to cytolysis of HAK, the splenocytes from PBS-treated animals had no detectable cytolytic effects on any target cells. The splenocytes from Ad-treated animals displayed clear cytotoxicity against dl312-infected HAK tumor cells four days after the first viral injection, but no cytotoxicity against HPD-1NR tumor cells, suggesting that only anti-virus CTL response was induced at this moment. On day 7, Ad treatment-induced CTLs not only had increased cytolytic activity against dl312-infected HAK tumor cells, but also displayed strong cytotoxicity against HPD-1NR cells, indicating that a strong tumor-specific CTL activity also appeared at this time point (Fig. 3A). These results demonstrate that oncolytic Ad therapy induces both strong tumor- and virus-specific CTLs, and virus-specific CTL responses are first developed. The virus-specific CTLs have strong cytolytic effect on virus-infected tumor cells, suggesting that antiviral CTL response also contributes to oncolytic efficacy.

To further study the impact of anti-viral immunity on the therapeutic efficacy of oncolytic Ad5, 1 x 10⁶ HPD-1NR cells were inoculated respectively in right and left flanks of hamsters. The right tumors were treated with six i.t. injections of low dosage of Ad5 (5 x 10⁸ PFU/injection) or PBS. The viral treatment caused transient regression of the virus-injected tumors after the third viral injection (about 6 days after the first viral injection), and also markedly inhibited the growth of the untreated left tumors after completion of Ad treatment (about 14 days after the first viral injection) (Fig. 3B). Because the viral treatment just transiently inhibited tumor growth in T cell-deficient animals (Fig. 1A), the regression of the virus-injected right tumors suggested the therapeutic effect of anti-viral immunity. While the retarded growth of the untreated left tumors suggested the effect of anti-tumor immunity, which appeared later than that of anti-viral immunity. This result is consistent with the virus- and tumor-specific CTL activities.

**Preexisting anti-Ad immunity promotes viral clearance of tumors through T cells, but has no effect on therapeutic efficacy**
Preexistent anti-Ad antibodies are highly prevalent in the human population. The neutralizing anti-Ad antibody is likely to be the major factor of limiting the therapeutic efficacy of Ad by blocking viral infection and spreading (20, 21). So we next monitored anti-Ad antibody responses in immunocompetent and T cell-deficient hamsters treated with oncolytic Ad. Obvious anti-Ad antibody responses were detected in immunocompetent hamsters one week after the first viral injection, and continuously increased. But, anti-Ad antibody was hardly detected in T cell-deleted animals at all time points (Fig. 4).

To investigate the relative contributions of anti-viral antibody and T cells to viral clearance and their effects on antitumor efficacy, hamsters were pre-immunized with one dose of Ad5 (1 x 10^{10} PFU) by intramuscular injection, and s.c. inoculated with HPD1NR tumor cells one month later. Then, oncolytic Ad therapy was performed in the pre-immunized and naïve tumor-bearing hamsters with or without T cell deletion. The pre-immunization induced persistent high level of anti-Ad antibody in plasma and tumor tissues, which was not influenced by Ad therapy and T cell deletion (Fig. 5A). The infectious Ad could not be detected in tumors of pre-immunized immunocompetent animals even at the day after the first viral injection. However, a dramatic viral amplification was detected in tumors of pre-immunized animals with T cell deletion and showed a similar dynamic process with that in tumors of immunocompetent hamsters without pre-immunization (Fig. 5B, Left). These results demonstrated that preexisting anti-Ad immunity rapidly eliminated the infectious Ad from tumors once Ad was injected. Furthermore, infectious Ad was majorly eliminated by T cells and the preexisting anti-Ad antibodies could not effectively prevent viral infection of tumor cells. DNA detection showed the same kinetics of virus genome clearance from tumors in the pre-immunized hamsters with or without T cell deletion, as that in non-immunized hamsters (Fig. 5B, Right). These data suggest that the injected infectious virus could effectively infect tumor cells before it was cleared by the preexisting anti-viral immunity. Meanwhile, it further supports that Ad genome is cleared by innate rather than adaptive immunity. Consistent with the previous study (8), the preexisting anti-Ad immunity did not affect the antitumor efficacy of oncolytic Ad. However, T cell deletion also abrogated the antitumor effect of oncolytic Ad in pre-immunized animals (Fig. 5C). These results further prove that T cell responses play major roles in antitumor efficacy of oncolytic Ad, even under the condition of preexisting anti-Ad immunity that can rapidly eliminate the infectious virus from tumors.

**Repeated Oncolytic Ad therapy eradicates the primary therapy resistant tumors**
Next, we wonder if repeated oncolytic Ad therapy has therapeutic effects on the tumors that grow up from the primary oncolytic Ad therapy. To this end, tumor-bearing hamsters were treated with six i.t. injections of low dosage of Ad5 (5 × 10⁸ PFU/injection). Ad treatment caused complete regression of tumors in five of twenty hamsters. The other tumors just transiently shrank. 12 days after the last viral injection when the shrunk tumors started to grow, the tumor-bearing hamsters were randomly divided into two groups that were respectively treated with another six injections high dosage of Ad (2 × 10⁹ PFU/injection) or PBS as control. All tumors kept growing in the PBS-treated animals. Ad treatment caused complete regression of tumors in five of eight animals. The growth of tumors was also delayed in other three hamsters (Fig. 6). This result demonstrates that repeated oncolytic Ad therapy has strong therapeutic effect on relapsed tumors or tumors insensitive to the primary viral therapy. Most interestingly, the same treatment with high-dosage of Ad eliminated the relapsed tumors in more than 60% hamsters (Fig. 6), versus the primary tumors in 40% hamsters (Fig. 1A). Moreover, the relapsed tumors were at least two times bigger than the primary tumors when they were treated with Ad. This data suggests that the anti-viral recall immune response triggered by the repeated Ad therapy might have greater therapeutic effect on relapsed tumors.

Discussion

An intriguing aspect of oncolytic virotherapy is that by their very nature they potently stimulate multiple arms of the immune system. In return, the immune system exerts multiple effects on the outcome of tumor therapy: some positive, some negative. The most important things should be to determine the roles and the relative importance of each key components of immune system in antitumor efficacy of oncolytic virotherapy. To more fully explore the roles of T cells in oncolytic Ad therapy in the hamster model, we developed a monoclonal antibody against Syrian hamster CD3 (mAB4F11) that can efficiently delete T cells in vivo (16). Here, we show that deletion of T cells remarkably impairs the antitumor efficacy of oncolytic Ad, especially the long-term control of tumor recurrence. On the other hand, the deletion of T cells also abrogates production of anti-Ad antibodies, delays the clearance of infectious Ad in tumors and maintains elevated levels of intratumoral infectious virus for prolonged periods. The elevated infectious virus levels in tumors and the impaired antitumor efficacy of oncolytic Ad in T cell-deficient hosts fully demonstrate the
importance of T cell-mediated immune responses in antitumor effects of oncolytic Ad therapy. CP treatment was shown to cause deletion of total white blood cells and a prolonged high infectious Ad level in tumors in Syrian hamsters, which significantly enhanced the antitumor efficacy of oncolytic Ad (2, 3). The opposite effects of these two kinds of immune deficiency on the antitumor efficacy of oncolytic Ad further demonstrate that T cells are key components of immune system mediating antitumor effects of oncolytic Ad, whose antitumor effect is far outweigh its negative effects on the direct oncolytic efficacy of Ad. Moreover, this opposite effects suggest that the responses of other non-T cells immune components have negative effects on antitumor efficacy by limiting the oncolysis of Ad in T cell-deficient condition.

T cell-mediated immunity in oncolytic virotherapy includes virus-specific and tumor-specific responses. Their relationship and the contribution of virus-specific immune response to the therapeutic efficacy have been the hot topics of discussion in oncolytic virotherapy (22, 23). The adaptive immune response has been of serious concern for the delivery and spread of oncolytic virus in tumors. T cell response plays a dominant role in immune response against viral infection by clearance of viral infected cells. Our in vitro CTL assay shows that oncolytic Ad therapy induces strong antiviral and antitumor CTL activities. Deletion of T cells increases viral replication and markedly delays the clearance of virus, which were indicated by increased expression of viral protein E1A and elevated infectious virus levels in tumor tissues. These results demonstrate that antiviral T cell responses play important roles in eliminating the infectious virus from tumors, which might limit the direct oncolytic effect of Ad. On the other hand, antiviral T cell response itself should has a function in antitumor efficacy at the same time. The central problem in cancer immunotherapy is that most tumor-associated antigens are non-mutated self-antigens that have triggered both central and peripheral tolerance(24). Viral proteins expressed in the infected tumor cells are strong non-self antigens that can trigger robust virus-specific T cell response as being logically immunodominant over self-derived tumor epitopes on the tumor cells. The CTL assay shows that oncolytic Ad therapy induces strong virus-specific CTL response against the virus-infected tumor cells, which was produced earlier than the tumor-specific CTL response. Thus, the killing of virus-infected tumor cells by virus-specific CTLs should also contribute to oncolytic efficacy, especially at the early stage of the oncolytic therapy. Importantly, the oncolytic virus infect and debulk tumors, which leaves any residual tumors for the immune system and leads to the release of “danger signals” and tumor antigens that
could allow for antigen spreading and reduce local immunosuppression. So, the Ad-specific T cell response appears to be an important component of Ad-mediated antitumor responses.

A majority of the human population is seropositive for Ad5, which is acquired as a childhood infection. Elimination of the vector by preexisting immunity to Ad poses a possible concern with respect to achieving significant antitumor efficacy (25-27). The adaptive immune responses and, in particular, neutralizing antibodies were shown to be a common and powerful inhibitory end response to infection involving a variety of oncolytic viruses, including Ad (28-30). To study the effect of the preexisting anti-Ad immunity on the viral clearance and therapeutic efficacy of oncolytic virotherapy, we immunized the hamsters with one dose of intramuscular injection of Ad. Consistent with the previous study (8), the pre-immunization induced persistent high level of anti-Ad antibody in plasma and tumor tissues. Correspondingly, the infectious Ad could not be detected in tumors of the pre-immunized animals even at the very early time after the viral injection. However, once T cells were deleted in the pre-immunized hamsters, the infectious Ad was easily detected in the tumor tissues and showed a similar dynamic process with that in tumors of immunocompetent hamsters without pre-immunization, although T cell deletion had no effect on the titer of anti-Ad antibody in the pre-immunized hamsters. These results demonstrate that infectious Ad is majorly cleared from tumors by the antiviral T cells rather than antibodies, and the preexisting anti-Ad antibody could not effectively block viral infection of tumor cells. Anti-Ad antibodies might have important role in preventing the systemic toxicity of oncolytic Ad by inhibiting Ad spillover from the tumor and replication in normal tissues (8). The identical kinetics of viral DNA clearance from the tumor in T cell-deleted or immunocompetent hamsters with or without pre-immunization demonstrate that the adaptive immunity has no effect on the clearance of oncolytic Ad genome, and the innate immunity may be responsible for clearance of virus DNA from tumors following i.t. injection of oncolytic Ad. Most importantly, even though the pre-immunization induced a robust systemic immune response against Ad, the preexisting anti-viral immunity had no effect on the therapeutic efficacy of oncolytic Ad therapy, which is consistent with the previous study(8). The detection of viral DNA in tumors demonstrates that the injected infectious virus could effectively infect tumor cells before it was cleared by the preexisting antiviral immunity. This finding is unsurprising, as T cell response eliminates the virus by destruction of the viral-infected tumor cells only after the virus infected the tumor cells and express viral antigen during their replication. This offers
us an opportunity to improve oncolytic virotherapy by engineering the oncolytic virus to increase their rapid spreading and infection, and delay their replication within cells.

Due to the viral immunogenicity, it was generally accepted that the therapeutic efficacy of multiple injections of the same virus might be limited by the antiviral immune responses. Numerous strategies have been developed to circumvent this hurdle(31). For example, two or more antigenically distinct viruses were applied so that the specific immunity that arises subsequently to the first virus does not inhibit the therapeutic effects of the second therapeutic virus (16). Alternatively, the therapeutic viruses were masked from antibody neutralization with chemical conjugates (32, 33). However, the studies in the immunocompetent and viral replication-permissive hamster model, including ours result, demonstrated that preexisting anti-viral immunity did not affect the therapeutic efficacy of vector after i.t. injection of Ad (8). Most importantly, we, for the first time, have shown that, instead of attenuating the efficacy of oncolytic Ad, the repeated oncolytic Ad therapy significantly enhanced therapeutic effect on relapsed tumors compared with the first Ad treatments for the primary tumors. The repeated Ad treatment would trigger robust recall immune responses against the virus, which cause violent disruption to the virus-infected cells. The sudden death of large amounts of tumor cells will dramatically ameliorate local immunosuppression and release tumor antigens that trigger robust recall immune response against tumors eventually leading to the elimination of tumors. We can infer from our study that the viral-specific T cell response ought to play a critical role in efficacy of oncolytic Ad therapy by enhancing tumor oncolysis and triggering a long-term antitumor immune response.

In conclusion, we have identified the key roles of T cells in oncolytic Ad therapy in an immunocompetent, replication-permissive hamster tumor model. Our results suggest that viral-specific T cell response may play important role in oncolytic therapy as a bridge linking Ad infection and oncolysis to the antitumor immune responses. These results shed light on developing new therapeutic regime of oncolytic Ad by enhancing antigen-specific T cell responses.
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Author contributions

XL, HL, XD, ML, and QH conducted the experiments; XL, YW, and SW designed research studies; XL plotted and analyzed the data; XL and PW performed the histopathological staining and interpretation; PW and YW prepared the viruses; YW and SW conceived of the project; XL and SW wrote the manuscript.
Reference


Figure legends

Fig.1. Deletion of T cells by antibody severely impairs antitumor effects of oncolytic Ad. Syrian hamsters were inoculated s.c. with $1 \times 10^6$ HPD-1NR cells, $5 \times 10^6$ Pfu (Ad$_{\text{low}}$) or $5 \times 10^9$ Pfu (Ad$_{\text{high}}$) of Ad5, or PBS were injected i.t. on day 11, 13, 15, 17, 19 and 21 (indicated by dash arrow) after tumor inoculation. Anti-hamster CD3 (4F11) or control antibodies (ConIg) were injected i.p. at doses of 500μg/injection every 4th day from the day before the viral therapy (day 10 after tumor inoculation) to the end of the experiment (indicated by solid arrow). A, Tumor growth curves of individual animals were shown. One of three experiments was shown; n=5 animals per group. B, Anti-mouse CD4 (GK1.5), anti-hamster CD3 (4F11) or control antibodies (ConIg) were injected i.p. at doses of 500μg/injection every 4th day from the day before the viral therapy to the end of the experiment. n=7
animals per group. Mean tumor size & SEM are displayed. Statistical analysis was conducted using 2-way ANOVA test on time points after viral therapy. ***, $P<0.001$.

C, Ad5-treated, tumor-free hamsters (n= 5 animals per group pooled from Ad$_{low}$ and Ad$_{high}$-treated animals) were rechallenged with $2\times10^6$ HPD-1NR cells on different site from primary tumor at least 1 month after complete rejection of primary tumors. Anti-CD3e (4F11) or control antibodies were injected i.p. every 4th day from the day before the tumor rechallenge to the end of the experiment. Tumor sizes were measured twice weekly. Mean tumor size & SEM are displayed. Data are representative of three independent experiments.

Fig.2. T cell depletion extends the existence of infectious virus and decreases apoptosis in tumors treated with Ad5. (A-C) Tumor-bearing hamsters were treated with $5\times10^8$ Pfu Ad5 (indicated by arrow), and 4F11 or control antibody as they were treated in Fig.1. The hamsters were sacrificed at 1.5 hours, and 1, 3, 7, 11, 17 and 21 days after the first virus injection (n = 3 per time point), and tumors were harvested. A, Viral loads in tumor tissue homogenates were determined by TICD50 on JH-293 cells. B, The expression of viral protein E1A and Hexon were determined by immunohistochemistry. The representative of anti-E1A and Hexon staining of sections of tumors harvested at day 7, 17 and 21 were shown left (original magnification, 400x; Bars, 36 μm); Statistical analysis of positive staining cell number within tumors was shown right. C, Viral genome was determined by a quantitative PCR assay (copy number/g). Each point represents the yield from one animal. The sensitivity of the assay is illustrated by the dotted line. D, The tumor-bearing hamsters were injected i.t. with $5\times10^8$ Pfu of Ad5 or PBS on day 11, 13, 15, 17 after tumor inoculation. The virus-treated groups were injected i.p. with anti-CD3e (4F11) or control antibodies at doses of 500μg/injection at the day before the first viral injection and 4 days later. At the day after the last viral injection, Syrian hamsters were sacrificed and tumors were harvested for immunohistochemistry analysis. n=3 animals per group. Representatives of anti-CD3e, anti-E1A and TUNEL staining of sections of tumors derived from 3 different groups of Syrian hamsters (original magnification, 200x; Bars, 73 μm) (Up). Statistical analysis of positive staining cell number within tumors (Down). *, $P<0.05$. **, $P<0.01$. ***, $P<0.001$. ND: not detected. Data are representative of four independent experiments.

Fig.3. Ad5 therapy successively induced anti-viral and anti-tumor CTL responses
A, CTL assay. The Tumor-bearing hamsters were injected i.t. with 5 x 10^8 Pfu of Ad5 or PBS every other day for 4 times from day 11 post tumor inoculation, and sacrificed 4 and 7 days after the first injection of virus respectively. Splenocytes from 3 animals per group were pooled and stimulated with irradiated-HPD1NR tumor cells infected with replication-deficient dl312 (an E1A-deleted Ad5) for 24h in vitro for 4 days. HPD-1NR and dl312-infected HAK cells were respectively stained with 10 mM CFSE (CFSE<sup>high</sup>) as specific target cells. HAK cells without virus infection were stained with 0.5 mM CFSE (CFSE<sup>low</sup>) as control target cells. CTL activities were examined against HPD1NR cells and dl312-infected HAK cells with different effector-to-target ratios (E:T) by an in vitro killing assay using a mixture of CFSE<sup>high</sup>-labeled specific target cell (HPD1NR or dl312-infected HAK) and CFSE<sup>low</sup>-labeled control cell (HAK). Cytotoxicity was expressed as cytotoxicity(%) (100% × [1-Ratio (target/control)_{effector}/Ratio (target/control)_{effector}]). ND: not detected. Data are representative of two independent experiments. B, Syrian hamsters were inoculated s.c. with 1 x 10^6 HPD-1NR cells in right and left flanks. 5 x 10^8 pfu Ad5 or PBS were injected i.t. in right tumors on day 10, 12, 14, 16, 18 and 20 (indicated by dash arrow) after inoculation. n=7 animals per group. Mean tumor size & SEM are displayed. Statistical analysis was conducted using 2-way ANOVA test on time points after viral therapy. ***, P<0.001; ns, not significant.

**Fig.4. The effect of T cell deletion on anti-Ad antibody response induced by Ad5 therapy.** Tumor-bearing hamsters were treated with 5 x 10^8 Pfu of Ad5 (indicated by arrow), and 4F11 or control antibody as they were treated in Fig.1. Sera were harvested on indicated days and the levels of anti-Ad5 antibody were measured by ELISA assay. Each point represents the yield from one animal. Data are representative of three independent experiments.

**Fig.5. The contributions of anti-Ad antibody and T cell responses to viral clearance in tumor and antitumor efficacy of Ad5 therapy.** Hamsters were immunized intramuscularly with 1x10<sup>10</sup> pfu of Ad, and inoculated with HPD1NR cells one month later. Meanwhile another group of naïve animals were also inoculated with HPD1NR cells. The tumor-bearing hamsters were treated with 5 x 10^8 Pfu of Ad5 (indicated by arrow), and 4F11 or control antibody as they were treated in Fig.1. A, Sera and tumor tissue were harvested on the indicated days and the levels of anti-Ad5 antibody were measured by ELISA. B, Tumor tissues were harvested at the indicated
times and Viral loads (left) and genome (right) in tumor were determined by TICD50 on JH-293 cells and the quantitative PCR assay respectively. C, Tumor sizes were measured twice weekly. Error bars represent mean ± SD (standard deviations) from 7-8 animals per group. Statistical analysis was conducted using 2-way ANOVA test on time points after viral therapy. ***, P<0.001; ns, not significant. Data are representative of two independent experiments.

**Fig.6. The efficacy of repeated Ad5 therapy on progressive tumors.** Tumor-bearing hamsters were injected i.t. with 5 x 10⁸ Pfu of Ad5 on day 12, 14, 16, 18, 20, and 22 (indicated by dash arrow) after tumor inoculation. After 11 days of last injection, the hamsters with growing tumor were treated with six i.t. injections 2 x 10⁹ Pfu of Ad5 or PBS (indicated by solid arrow). Tumor sizes were measured twice weekly. Tumor growth curves of individual animals were shown. Data are representative of two independent experiments.
Figure 2

A

B

D7

D17

D21

Ad/ConIg     Ad/4F11     Ad/ConIg     Ad/4F11     Ad/ConIg    Ad/4F11

E1A

Hexon

C

Ad/ConIg     Ad/4F11

DNA level: copies/g  (Log_{10})

Days after Ad5 therapy

PBS

Ad/ConIg

Ad/4F11

TUENL

CD3

E1A

TUENL

PBSt

Ad/ConIg

Ad/4F11

CD3: cells per HPF

E1A: cells per HPF

TUENL: cells per HPF
Figure 3

A

B
Figure 4

[Graph showing OD450nm (Anti-Ad antibody) over days after Ad5 therapy, with points indicating measurements at different days and lines connecting them.]

Days after Ad5 therapy

OD450nm (Anti-Ad antibody)

- Conlg
- 4F11
Figure 5

A

Sera

Tumor

Days after Ad5 therapy

Days after Ad5 therapy

B

Viral load: pfu/g

DNA level: copies/g

Days after Ad5 therapy

Days after Ad5 therapy

C

Tumor volume (mm$^3$)

Days after tumor inoculation

Non-imm-PBS
Non-imm-Ad5
Imm-Ad5/ConIg
Imm-Ad5/4F11

***

ns
Figure 6
The efficacy of oncolytic adenovirus is mediated by T cell responses against virus and tumor in Syrian hamster model

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