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Tumor immunotherapy using adenovirus vaccines in combination with intratumoral doses of CpG ODN

S.M. Geary¹, C.D. Lemke¹, D.M. Lubaroff², and A.K. Salem¹

¹Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa, 52242, United States

²Departments of Urology & Microbiology, Holden Comprehensive Cancer Center, University of Iowa, Iowa City, Iowa, 52242, United States

Abstract

The combination of viral vaccination with intratumoral (IT) administration of CpG ODNs is yet to be investigated as an immunotherapeutic treatment for solid tumors. Here we show that such a treatment regime can benefit survival of tumor-challenged mice. C57BL/6 mice bearing ovalbumin (OVA)-expressing EG.7 thymoma tumors were therapeutically vaccinated with adenovirus type 5 encoding OVA (Ad5-OVA) and the tumors subsequently injected with the immunostimulatory TLR9 agonist, CpG-B ODN 1826 (CpG) 4, 7, 10 and 13 days later. This therapeutic combination resulted in enhanced mean survival times that were more than 3.5 x longer than naïve mice and greater than 40% of mice were cured and capable of resisting subsequent tumor challenge. This suggests an adaptive immune response was generated. Both Ad5-OVA and Ad5-OVA + CpG IT treatments led to significantly increased levels of H-2K^b-OVA-specific CD8⁺ lymphocytes in the peripheral blood and intratumorally. Lymphocyte depletion studies performed *in vivo* implicated both NK cells and CD8⁺ lymphocytes as co-contributors to the therapeutic effect. Analysis of tumor infiltrating lymphocytes (TILs) on day 12 post tumor challenge revealed that mice treated with Ad5-OVA + CpG IT possessed a significantly reduced percentage of regulatory T lymphocytes (Tregs) within the CD4⁺ lymphocyte population, compared to TILs isolated from mice treated with Ad5-OVA only. In addition, the proportion of CD8⁺ TILs that were OVA-specific was reproducibly higher in the mice treated with Ad5-OVA + CpG IT compared to other treatment groups. These findings highlight the therapeutic potential of combining intratumoral CpG and vaccination with virus encoding tumor antigen.

Keywords

Toll-like receptor; Adenovirus; Intratumoral therapy; Immunotherapy; Tumor Immunology; CpG ODN

*Corresponding author: Aliasger K. Salem, S228 PHAR, 115 S. Grand Avenue, Iowa City, IA 52242, (319) 335-8810 (telephone), (319) 335-9349 (fax), aliasger-salem@uiowa.edu.

Introduction

The search for alternative and supplementary cancer therapies is crucial since the current treatments of chemotherapy and radiotherapy have either undesirable side effects or are often inadequate [1]. Tumor immunotherapy has the attractive approach of specifically, and systemically, targeting tumors through their *de novo* expression of tumor-associated antigens (TAAs). The aim is to break the host's immunological tolerance to TAAs by inducing TAA-specific cytotoxic T lymphocyte (CTL) responses [2,3]. Such tolerance can be broken should the host be afforded the opportunity to recognize TAAs in an infectious-like setting [2]. A variety of strategies have been implemented that have yielded encouraging preclinical and clinical data, however, it is evident that further investigation of new and altered strategies is required [4,5].

Recombinant adenovirus type 5 vectors (Ad5) are strong candidates for the delivery of TAAs since they are efficient at transducing genes [6]. Apart from being efficient delivery systems to a range of cell types, Ad5 are also useful adjuvants for recombinant TAA genes due to their inherent ability to potently infect and stimulate dendritic cells (DCs) [7,8]. TAA-encoding viral vaccination alone has yielded promising but far from optimal results in limited clinical cancer trials performed thus far and therefore supplementary treatments are very likely to be necessary that will further improve the therapeutic potency of such vaccines [9]. Adjuvants capable of stimulating the innate arm of the immune response are now considered essential in maximizing adaptive immune responses [10]. CpG ODNs are adjuvants capable of stimulating T_H1-biased DC maturation resulting in efficient CTL activation. In recent years CpG ODNs have been used in clinical cancer trials, either as monotherapy or in combination with therapeutic antibodies or as an adjuvant to TAA peptides [11]. However, the therapeutic combination of CpG and TAA-encoding viral vectors is yet to be investigated in a clinical setting and has thus far only recently been reported in rare preclinical studies that are prophylactic rather than therapeutic [12,13].

EG.7 is an ovalbumin (OVA)-expressing thymoma cell line often used as a model for tumor immunotherapy where OVA is the model TAA [14]. Many studies have been performed where OVA has been delivered in various carrier systems to prophylactically protect against EG.7 tumor challenge in mice [15,16]. Such studies showed that treatment could significantly extend mouse survival times but ultimately most or all mice would succumb to the tumor challenge. Recent studies by our group have shown that the combination of Ad5-OVA and CpG-B ODN 1826 (CpG) could protect against subsequent challenge with EG.7 cells in approximately 50% of mice, and was significantly more protective than Ad5-OVA alone [13]. Therapeutic, as opposed to prophylactic, vaccinations however are less commonly described, yet are generally more apposite as it is from a therapeutic perspective that most cancers need to be managed [17,18,19]. When feasible, intratumoral (IT) therapy has the advantage of directly targeting the tumor and its environs and there have been studies showing the benefit of using such a route [20], [21]. In the studies presented here, C57BL/6 mice were challenged with syngeneic EG.7 cells and then, 3 days later, therapeutically vaccinated with Ad5-OVA. In order to enhance the anti-tumor immune response repeated IT doses of CpG were subsequently administered. Tumor volume, mouse

survival and the generation of Tregs and OVA-specific CD8⁺ T lymphocytes were assayed to determine the therapeutic benefit of this combinatorial approach.

Materials and Methods

Mice and cell lines

C57BL/6 (H2K^b) male mice between 6 - 8 weeks of age were obtained from Jackson Laboratories and maintained in filtered cages. EG.7 (thymoma cell line transfected with chick ovalbumin[14]), YAC-1 and P815 cell lines were obtained from American Type Culture Collection (ATCC) and grown in RPMI-1640 (GIBCO, Invitrogen, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA), 1 mM sodium pyruvate (GIBCO, Invitrogen, CA), 10 mM HEPES (GIBCO, Invitrogen, CA), 0.05 mM 2-mercaptoethanol, and 50 µg/ml gentamicin sulfate (Mediatech, Inc., VA). EG.7 cell culture was also maintained with 0.4 mg/mL G418 (GIBCO, Invitrogen, CA). All the experiments were performed in accordance with guidelines and regulations approved by the University of Iowa Institutional Animal Care and Use committee.

Adenovirus and CpG ODN

Replication-deficient adenovirus type 5 encoding chicken ovalbumin (Ad5-OVA) or beta-galactosidase (Ad5-LacZ) were obtained from the University of Iowa Gene Transfer Vector Core. Non-methylated CpG-B ODN 1826 (5'TCCATGACGTTTCCTGACGTT) was provided by Coley Pharmaceutical Group (Wellesley, MA) and had no detectable endotoxin. Non-methylated CpG-A ODN 2336 was kindly provided by Dr. Zuhair Ballas (Division of Immunology, Dept. of Internal Medicine, University of Iowa)

Tumor challenge and therapeutic protocol

For tumor challenge, 7 – 10 week old C57BL/6 mice (4 per group) were anesthetized by intraperitoneal injection of a ketamine/xylazine mix. Mice were injected subcutaneously (s.c.) with 10⁷ EG.7 cells (passages 9 – 13) in 100 µl of PBS into the dorsal right flank. Three days later, mice were immunized s.c. with 10⁸ pfu of Ad5-OVA or Ad5-LacZ. Beginning seven days after tumor challenge, single or multiple doses of intratumoral (IT) CpG ODN 1826 (CpG) were administered. Multiple injections of CpG were spaced three days apart. Tumor outgrowth, determined by tumor size as a function of time, was measured two – three times per week and tumor volume was calculated by the equation for determining the volume of an ellipsoid: [(Diameter 1 × Diameter 2 × Height) × (π/6)], as previously described [13].

Isolation of tumor infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL)

On day 12 post tumor challenge, variously treated mice were sacrificed and their tumors harvested and digested using 0.05 Wunsch units/ml Liberase TM Research Grade Enzyme Blend (Roche, Mannheim, Germany) for 21 minutes in a 37° C water bath, in the presence of 15 µg/ml DNase I (Sigma, St. Louis, MO) in HBSS. Single cell suspensions were collected by passing digested tissue through a 70 µm cell strainer and stained for Foxp3 or Tetramer (see below). PBLs were isolated by submandibular bleeds, red blood cell lysis

using ACK buffer (NH₄Cl/KHCO₃/EDTA solution) and stained for Foxp3 or tetramer (see below).

Tetramer and Foxp3 staining on peripheral blood or TILs

The frequency of OVA-specific CD3⁺ CD8⁺ T lymphocytes was determined by tetramer staining, as previously described [13]. The tetramer used was the H-2K^b SIINFEKL Class I iTagTM MHC Tetramer (K^b-OVA₂₅₇) labeled with PE (Beckman Coulter, Fullerton, CA). Surface CD8 and CD3 were stained with CD8-FITC and CD3-PE-Cy5 mAbs (eBioscience, San Diego, CA) respectively. The frequency of Foxp3⁺ CD3⁺ CD4⁺ Tregs was determined using a Foxp3 staining kit (eBioscience). The Foxp3 antibody was PE-labeled (eBioscience) and surface CD4 and CD3 were stained with CD4-FITC and CD3-PE-Cy5 mAbs (eBioscience). Samples were acquired using a FACScan flow cytometer (Becton Dickinson, NJ) and analyzed with FlowJo software (TreeStar, OR).

Lymphocyte depletion

Lymphocyte subset depletions were commenced on day 16 post tumor challenge (see results section) by I.P. administration of 150 µg of anti-CD4 (GK1.5), anti-CD8 (2.43) or anti-NK1-1 (PK136). Antibodies were re-administered on days 17 and 18 and then twice per week for the duration of the experiment. Effectiveness of depletions is shown in supplementary Figures S1 (A-C).

Statistics

Analysis of tetramer data (Fig. 5) was done using the student's unpaired two-tailed *t*-test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Data from pooled experiments (Figs. 5b, 5d, 5f and 5h) were normalized and the ratios generated were analyzed using the student's unpaired two-tailed *t*-test. Analysis of survival curves was performed using the log-rank (Mantel-Cox) test (GraphPad Prism)

Results

Combining therapeutic Ad5-OVA vaccination with multiple IT CpG enhances tumor protection and survival

We observed in preliminary tumor (EG.7) studies that the delivery of multiple IT injections of CpG following therapeutic adenovirus tumor vaccine (Ad5-OVA) significantly enhanced survival times, as compared to mice receiving vaccine only (data not shown). To further elucidate the level of protection conferred by this novel combination immunotherapy, we compared single versus multiple injections of IT CpG (50 µg/dose) administered following the tumor vaccine. The most dramatic tumor protection and survival was seen in mice that received both Ad5-OVA and multiple doses (three or four) of IT CpG after tumor challenge (Fig. 1 and Fig. 2a). This data combined with the repeated survival studies (summarized in Fig. 2b) make it apparent that multiple IT injections of CpG was of superior therapeutic benefit over Ad5-OVA alone or Ad5-OVA in combination with a single IT administration of CpG. Average overall survival from pooled experiments was > 3.5 x longer than the naïve mice and > 2 x longer than the Ad5-OVA alone group. The percentage of mice that became

tumor-free and remained so for greater than 70 days (i.e. until termination of experiment) was greatly increased in the mice that received the combination of Ad5-OVA plus multiple doses of IT CpG (Ad5-OVA/multiCpG), with > 40% of mice cured compared to 25% for the Ad5-OVA/CpG(x1) mice, and < 15% for all other groups (Fig. 2c). All mice that were cured by Ad5-OVA/multiCpG treatment were rechallenged with EG.7 cells (> 90 days post initial tumor challenge) and 100% (8/8) were capable of resisting the tumor.

Establishment of an optimal therapeutic intratumoral dose of CpG-B ODN 1826

Having observed a promising therapeutic effect of multiple administrations of IT CpG (50 µg), we next wanted to determine the optimal CpG dose for tumor protection. Mice were challenged with EG.7 tumor cells, followed by Ad5-OVA immunization and four IT CpG injections of 10, 20, 50, or 100 µg each, as described in the materials and methods. Regardless of dose, administration of IT CpG improved tumor protection and extended survival compared to the untreated mice (Fig. 3a), and to a greater extent than previously observed for Ad5-OVA alone (Fig. 2b). A dose response effect was seen and, although not statistically significant, doses of 50 and 100 µg CpG IT had enhanced mean survivals compared to 10 and 20 µg doses (Fig. 3b). Although in this experiment 100 µg doses of CpG resulted in 50% (2/4) tumor free mice versus 25% (1/4) for the 50 µg dose, we observed more consistently extended survival with the 50 µg IT dose group, suggesting that this was the optimal dose to use. Additionally, it was determined from pooled experiments, where 50 µg IT doses (x4) of CpG were used, that the overall cure rate was 44% (8/18) (Fig. 2c).

Lymphocyte-depletion assay

In order to determine which lymphocyte subsets were contributing to the therapeutic antitumor effect observed from combinatorial Ad5-OVA/CpG treatment, we performed lymphocyte depletion assays. Tumor-challenged mice, immunized with Ad5-OVA and given 4 IT doses of CpG (50 µg), were depleted independently of CD4⁺ and CD8⁺ lymphocytes as well as NK cells, as described in materials and methods. Tumor volumes (Fig. 4a) and survival times (Fig. 4b) indicated that both CD8⁺ lymphocytes and NK cells significantly contributed to the therapeutic response.

OVA-specific lymphocytes and Treg levels in the TME and peripheral blood: Comparison of Ad5-OVA versus Ad5-OVA/CpG treatments

It has been previously demonstrated that tumor protection conferred by adenoviral tumor vaccines is primarily mediated by CD8⁺ T cells [13,22]. Thus we wanted to determine if the combination of Ad5-OVA + IT CpG (Ad5-OVA/CpG), which we have shown here to be therapeutically more beneficial than Ad5-OVA alone, further enhanced the production of tumor Ag (OVA)-specific CD8⁺ T cells in PBLs or in the tumor microenvironment (TME). Thus on day 12 post-tumor challenge, tumors and peripheral blood were isolated, processed and stained for the presence of OVA-specific lymphocytes as described in the materials and methods. This time point was chosen as it represents a stage when the tumors, from treated mice, are in regression but are still large enough to yield sufficient TILs for analysis. Therefore, in this series of experiments, the Ad5-OVA/CpG treatment involved only two IT administrations of CpG (Ad5-OVA/CpG(x2) on days 7 and 10 post tumor challenge). In TILs, the percentage of CD3⁺CD8⁺ lymphocytes that were OVA-specific was observably,

though not significantly, higher for the Ad5-OVA/CpG(x2) group compared to the Ad5-OVA only group, in one representative experiment (Fig. 5a). This result was reproducible and when multiple experiments were pooled and analyzed it was found the statistical difference between these two treatment groups was marginally significant (Fig. 5b). In contrast, comparison of the percentage of CD3+CD8+ PBLs that were OVA-specific revealed no observed differences between the two treatment groups of Ad5-OVA/CpG(x2) and Ad5-OVA alone (Fig. 5e). When data from multiple experiments were pooled and statistically analyzed, the differences between these two treatment groups remained insignificant (Fig. 5f). These results suggest that the administration of CpG IT may enhance the levels of tumor-specific lymphocytes in the TME when used in combination with Ad5-OVA vaccination. CPG IT administration alone had no significant ability at generating tumor-specific lymphocytes in the TME or the peripheral blood.

In the same representative experiment as described above, it was shown that TILs isolated from tumors of Ad5-OVA/CpG(x2) treated mice possessed a lower percentage of CD3+CD4+ lymphocytes that were Foxp3+ Tregs compared to tumors from mice that received treatment with Ad5-OVA alone (Fig. 5c). This difference was highly statistically significant in each separately performed experiment and also when data from multiple experiments were pooled and analyzed (Fig. 5d). Although the Ad5-OVA/CpG treatment resulted in reduced Treg percentages it was noted that treatment with Ad5-OVA only or CpG IT only, significantly reduced the Treg percentage in the TME when compared to untreated (naïve) mice (Fig. 5c). In contrast, in PBLs, a marginal increase in the presence of Tregs was noted for the Ad5-OVA/CpG(x2) group compared to the Ad5-OVA only group. These differences were not significant when individual experiments were statistically analyzed (Fig. 5g) but proved to be significant upon analysis of pooled data from multiple experiments (Fig. 5h).

It would therefore appear that the TME of mice receiving the combinatorial treatment of Ad5-OVA/CpG possessed more favorable anti-tumor conditions with significantly more tumor-specific lymphocytes and significantly less Tregs being present in the tumor as a percentage of CD8+ lymphocytes and CD4+ lymphocytes respectively.

Discussion

The primary aim of most tumor immunotherapies is to break the tolerance of the tumor host to tumor associated antigens (TAAs) by inducing an effective tumor-specific CTL response that can eliminate the tumor, including micrometastases, and provide protection against tumor recurrence through immune memory [2]. EG.7 cells injected s.c. into immunocompetent syngeneic mice are generally not rejected, despite expressing a highly immunogenic xenogeneic protein, OVA. This is likely due to immunological tolerance or ignorance. Such tolerance is thought to be the result of immature DCs, which sample the tumor microenvironment (TME) and subsequently, in the draining lymph nodes, deliver a tolerogenic signal to T lymphocytes [23,24]. DCs require maturation signals, which can be provided by viral or bacterial components, to become effective promoters of cellular immune responses. In these studies we have therapeutically combined both viral delivery of a model tumor antigen (OVA) with subsequent administrations of a synthetic bacterial

pathogen-associated molecular pattern, CpG. When Ad5-OVA alone was used to vaccinate EG.7-challenged mice, although tumor regression was induced it was not long lasting and most mice (14/16) were required to be sacrificed due to tumor burden. However, the combination of Ad5-OVA vaccination with subsequent multiple IT doses (x3 or x4) of CpG (Ad5-OVA/multiCpG) resulted in significantly longer tumor regressions, improved survival times and dramatically increased cure rates (40%). Lymphocyte-depletion studies revealed that both NK cells and CD8+ lymphocytes were significant contributors to the therapeutic effect induced by Ad5-OVA/multiCpG. That CpG is capable of contributing to both improved NK-mediated and CTL-mediated functions has documented support [25] [26] [27]. We established that AdLacZ /multiCpG treatment was of only marginal therapeutic benefit. It is therefore likely that the tumor regression caused by effector CD8+ lymphocytes induced by AdOVA/multiCpG and Ad5-OVA only treatments was at least partially due to OVA-specific CD8+ T lymphocytes. This was further supported by: 1) the finding that tumor-free mice were protected from subsequent EG.7 tumor challenge and, 2) the induction of OVA-specific lymphocytes in the TME and peripheral blood of vaccinated mice during the tumor regression phase. Of particular importance was the finding that CpG IT treatment appeared to increase the percentage of OVA-specific lymphocytes in the TME compared to Ad5-OVA vaccination alone. What we also provide here is evidence that CpG when administered IT can substantially decrease the percentage of Tregs in the CD4+ lymphocyte population in the TME when compared to naïve mice. As far as we are aware this is the first report showing that CpG administered IT is capable of reducing Treg percentages in the TME. This phenomenon occurred with or without Ad5-OVA vaccination, and it was of particular interest that the reduction in Treg percentages was also significantly greater in tumors from Ad5-OVA/CpG treated mice compared to mice vaccinated with Ad5-OVA alone. Thus IT administration with CpG of Ad5-OVA vaccinated mice creates an environment within the tumor that favors anti-tumor immunity by increasing tumor-specific lymphocytes and reducing the proportion of Tregs.

The benefit provided by CpG may be due to the direct effect of inducing a T_H1-type DC maturation in the TME. This effect of CpG on DCs is well established [11]. The continued presence of CpG over a period of 6 – 9 days may have ensured that this T_H1 phenotype was maintained. These results highlight the advantage of directly targeting the TME through IT administration. It was demonstrated by others that IT administration of *ex vivo* expanded DCs, genetically engineered to produce IL-12, had the capacity to generate an effective CTL-mediated anti-tumor response against colon adenocarcinomas in mice [28]. Similar findings, by an independent group, were shown for a range of other weakly immunogenic tumors and both groups found that the anti-tumor immunity was also effective against coexisting non-injected tumors [29]. However, such treatments when applied clinically are time consuming and costly since the treatment must be tailored to each patient as they must be injected with their own DCs. Here we suggest a much simpler generally applicable treatment that has a potentially similar outcome. CpG in the TME is capable of promoting T_H1 cytokine (e.g. IL-12) production by infiltrating DCs which phagocytose and process tumor antigens for subsequent presentation to T lymphocytes in draining lymph nodes [30]. The amount of tumor antigen available to DCs would be substantial by days 10 - 13 post tumor challenge since the Ad5-OVA vaccination performed 7 – 10 days earlier would be

causing tumor cell death, as evidenced by the tumor volume measurements (see Fig. 1). It has been shown by others that the presence of apoptosing tumor cells within the TME enhances the capacity of infiltrating/injected DCs to affect a tumor antigen-specific CTL response [30]. It has also been demonstrated that direct IT injection of CpG into B cell lymphomas possessing dying tumor cells, results in the generation of anti-tumor CTL responses that eliminate the tumor and its metastases as effectively as the IT administration of DCs [31]. Another possible beneficial effect of CpG may be the direct or indirect abrogation of the function of myeloid derived suppressor cells (MDSCs). MDSCs are a recently discovered heterogeneous population of immature myeloid cells that are induced to become immunosuppressive and whose numbers and activity have been shown to increase in tumor-burdened individuals [32].

Skepticism as to the potential therapeutic benefit CpG may have in human cancers has been fueled by the fact that all murine DCs express TLR-9 whilst only plasmacytoid DCs express TLR-9 in humans. However, it has recently been reported that CpG is capable of inducing maturation of MDSCs and diminishing their immunosuppressive capacity in tumor-bearing mice through a mechanism suggested to be dependent on plasmacytoid dendritic cells producing α -IFN [33]. In addition CpG ODNs are a relatively newly discovered class of therapeutic molecules that still require further preclinical and clinical trials to establish their potential benefits and mechanisms of action. Most clinical cancer trials performed thus far have been phase I or phase II studies combining CpG treatment with monoclonal antibodies, chemotherapy or tumor antigen peptide vaccinations [34]. These studies have yielded mostly promising results showing that CpG is well tolerated by patients and even generating improved therapeutic outcome for certain cancers. Should CpG prove to be less than advantageous in future human trials, alternative TLR agonists that trigger immune signaling through the adapter protein, MyD88, could provide an alternative or supplement to CpG. Support for this comes from recent studies showing the immune-mediated antitumoral benefits of intralesional MyD88 overexpression [35].

To the best of our knowledge, this is the first report showing the therapeutic effect of combining a TAA (or a model TAA)-encoding virus with IT administration of CpG. Although CpG has started being used in clinical cancer trials there has only been one study to date where it has been applied intratumorally and that was a monotherapeutic study [36]. Current clinical studies that are focusing on treatments involving TAA-encoding viral vaccines are usually either using the virus alone or in combination with chemotherapy and/or one or more specific cytokines or surface-expressed costimulatory factors [37,38,39]. There have yet to be any trials that involve the combination of virally administered TAA and CpG. We have provided evidence that such a combination could be therapeutically beneficial in certain types of cancers. Specifically, it is possible that prostate cancer patients may be good candidates for such a treatment regime as we have previously shown in murine models of prostate cancer that the combination of Ad5-PSA and CpG (delivered subcutaneously) is capable of generating anti-tumor responses superior to vaccination with Ad5-PSA alone [40]. Whilst the Ad5-OVA vaccinations described here involved a xenogeneic protein as the immunogen, it has previously been shown that Ad5-OVA vaccinations are capable of breaking tolerance in an OVA-transgenic mouse model [22]. Additionally there is abundant evidence describing the breaking of tolerance through virally

administered TAA vaccinations and therefore supporting the anti-tumor potential of such a therapeutic approach [41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Comparison of the therapeutic effect of Ad5-OVA vaccination combined with no, single or multiple IT injections of CpG on tumor volumes. Individual tumor progression (volume) over time for indicated treatments. Tumor-challenged mice were vaccinated with either Ad5-LacZ or Ad5-OVA followed by IT injections of CpG (50 μ g) on days 7 (x1); days 7, 10 and 13 (x3); days 7, 10, 13 and 16 (x4) post tumor challenge, or no CpG treatment (i.e. Ad5-OVA alone).

Fig. 2.

Comparison of the therapeutic effect of Ad5-OVA vaccination combined with no, single or multiple IT injections of CpG. **a** Survival curve of one experiment (Log-rank (Mantel-Cox) test was used to determine statistical significance (see Table S1A in supplementary data)). **b** Survival curve summarizing pooled data from multiple experiments (Log-Rank (Mantel-Cox) test was used to determine statistical significance (see Table S1B in supplementary data)). **c** Percentage of mice tumor-free beyond 70 days from multiple experiments. Pooled experiments (**b** and **c**) were performed using indicated (below) number of mice (n) and indicated number of experiments (e). Naïve (n = 24, e = 6); AdLacZ (n = 12, e = 3); AdLacZ/CpG(x1) (n = 12, e = 3); AdLacZ/CpG(x3) (n = 8, e = 2); AdLacZ/CpG(x4) (n = 8, e = 2); AdOVA alone (n = 16, e = 4); AdOVA/CpG(x1) (n = 12, e = 3); AdOVA/CpG(x3) (n = 12, e = 3); AdOVA/CpG(x4) (n = 18, e = 5).

Fig. 3.

B> Comparative therapeutic responses to Ad5-OVA plus varied doses of IT CpG. Tumor challenged mice were subsequently vaccinated with Ad5-OVA on day 3 (except naïve group) and then 4x IT injections of 10, 20 50 or 100 μ g of CpG on days 7, 10, 13 and 16 post tumor challenge. **a** Survival curve representing one experiment (Log-Rank (Mantel-Cox) test determined no significant differences between treated groups). **b** Mean survival graph (Day 70 was censor time point). Error bars represent standard deviation.

Fig. 4.

Lymphocyte depletion study. Tumor challenged mice were subsequently vaccinated with Ad5-OVA on day 3 followed by 4x IT injections of CpG (50 µg) (days 7, 10, 13 & 16). On days 16, 17 and 18 mice were given IP 150 µg of depleting antibodies; anti-CD4, anti-CD8 or anti-NK1.1. Depletion was maintained twice a week until day 45. **a** Individual tumor progression (volume) over time for indicated treatments. **b** Survival curve representing one experiment (Log-Rank (Mantel-Cox) test was used to determine statistical significance (see Table S2 for supplementary data).

Fig. 5. OVA-specific CD8+ T lymphocyte responses and Treg levels in tumor microenvironment (TME) and peripheral blood. *a*: Percentage of CD8+ TILs that were OVA-specific. *b*: Pooled data comparing the relative proportion of CD8+ TILs that were OVA-specific ($n = 8$, $e = 2$). *c*: Percentage of CD4+ TILs that were Foxp3+. *d*: Pooled data comparing the relative proportion of CD4+ TILs that were Foxp3+ ($n = 8$, $e = 2$). *e*: Percentage of CD8+ PBLs that were OVA-specific. *f*: Pooled data comparing the relative proportion of CD8+ PBLs that were OVA-specific ($n = 11$, $e = 3$). *g*: Percentage of CD4+ PBLs that were Foxp3+. *h*: Pooled data comparing the relative proportion of CD4+ PBLs that were Foxp3+ ($n = 8$, $e = 2$). Statistical significances were determined using student *t* test as described in materials and methods (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars represent standard deviation.