Published in final edited form as:
Urol Oncol. 2014 February ; 32(2): 92–100. doi:10.1016/j.urolonc.2012.10.007.

Combined therapeutic effects of adenoviral vector-mediated GLIPR1 gene therapy and radiotherapy in prostate and bladder cancer models

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Abstract

Objectives—To explore the potential benefits of combining AdGlipr1 (or AdGLIPR1) gene therapy with radiotherapy using subcutaneous prostate and bladder cancer models.

Materials and methods—Combination adenoviral vector-mediated gene therapy and radiotherapy were applied to 178-2 BMA and TSU-Pr1 cells in vitro and colony formation and apoptosis were analyzed. Also, combination therapies were administered to mice bearing subcutaneous 178-2 BMA and TSU-Pr1 tumors and tumor growth suppression and survival extension were compared with the monotherapies (AdGlipr1/ AdGLIPR1, radiotherapy) or control vector Adv/CMV/βgal as well as comparing single cycle treatment with two cycle treatment.

Results—Combination treatment significantly suppressed colony formation and increased apoptosis in vitro. In vivo, combination therapy produced significant 178-2 BMA and TSU-Pr1 tumor growth suppression and survival extension compared with the monotherapies or the control. Further tumor growth suppression and survival extension were observed after two cycles of the combination treatment.

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Conflict of interest: T.C.T is a co-inventor on patents involving therapeutic applications of GLIPR1. These patents are held by Baylor College of Medicine, and licensed to Progression Therapeutics Inc., a private biotechnology start-up.

Conclusions—Combining AdGlipr1 (AdGLIPR1) with radiotherapy may achieve additive or synergistic tumor control in selected prostate and bladder tumors, and additional therapeutic effects may result with repeated treatment cycle.

Keywords

GLIPR1; gene therapy; radiation therapy; bladder cancer; prostate cancer

1. Introduction

Radiotherapy (XRT) plays an important role in cancer treatment and there has been significant development of combination therapies that include XRT. Relatively high radiation doses shown to improve both biochemical and clinical local tumor control of prostate cancer [1, 2] are limited by safety concerns. Further, some cancer cells such as prostate cancer (PCa) easily become radioresistant [3]. Radiation therapy for bladder cancer has been largely limited to aggressive, alternative treatments to radical surgery. However, there is currently a strong interest in partial-bladder radiation to compliment and limit toxicity of definitive therapy [4].

Our previous studies using herpes simplex virus thymidine kinase followed by ganciclovir gene therapy + XRT demonstrated improved tumor control, prolonged survival, and suppression of pre-existing metastases, vs. controls and monotherapy alone, in mouse PCa models [5]. And a phase I/II clinical trial combining in situ gene therapy + XRT showed minimal toxicity and excellent tumor control for PCa patients [6, 7].

Glioma pathogenesis–related protein 1 (human GLIPR1/mouse Glipr1) is a member of the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP) superfamily [8]. GLIPR1, a p53 target gene, is associated with myelomonocytic differentiation toward the macrophage phenotype [9], and is shown to be methylated and downregulated in PCa [10, 11]. Functional analysis of GLIPR1 revealed both growth suppression and proapoptotic activities in multiple cancer cell lines [10-13]. The proapoptotic activities depended on reactive oxygen species production and sustained c-Jun–NH2 kinase (JNK) signaling [12]. Additionally, GLIPR1/Glipr1 expression led to downregulated c-Myc at the transcriptional and posttranslational levels [13].

Significantly reduced tumor-associated angiogenesis and direct suppression of endothelial-cell sprouting were documented after adenoviral vector–mediated Glipr1 (AdGlipr1) therapy in immunocompetent orthotopic prostate mouse model [14]. Also, a novel Glipr1 gene–modified tumor cell vaccine had significant antitumor activity in recurrent PCa model [15]. These preclinical studies led to a limited clinical trial of an adenoviral GLIPR1 (AdGLIPR1) neoadjuvant injection preceding radical prostatectomy in men with high-risk PCa [16]. The results provided preliminary evidence of biologic antitumor activity and systemic immune response.

XRT increases GLIPR1/Glipr1 gene expression in human and mouse cancer cell lines in vitro [10]. Therefore, we reasoned that GLIPR1 may have synergistic therapeutic effects when combined with XRT. In this study, we explored the potential benefits of combining AdGlipr1 (AdGLIPR1) and XRT using a mouse PCa cell line, and a human bladder cancer cell line.

2. Materials and methods

2.1 Cells and adenoviral vectors

Mouse Pca cell 178-2 BMA and human bladder cancer cell TSU-Pr1 were grown as described previously [9, 10, 15, 17]. All chemicals for cell culturing were from GIBCO (Gaithersburg, MD, USA). AdGlipr1 (AdGLIPR1), and control Adv/CMV/βgal were prepared as described previously [9, 15]. Virus concentration was expressed as plaque-forming units (PFU).

2.2 In vitro studies

178-2 BMA and TSU-Pr1 cells were seeded in duplicate and infected with AdGlipr1 (178-2 BMA), AdGLIPR1 (TSU-Pr1), and Adv/CMV/βgal (hence control for both lines). Twenty-four hours after vector infection, cells were irradiated with single doses of 5, 10, 15, or 30 Gy (178-2 BMA) or 2, 4, 6, or 8 Gy (TSU-Pr1). Uninfected cells of both lines were exposed to the same radiation doses.

To assess clonogenic survival, 1% of the infected irradiated cells were reseeded and 12 days later colonies were stained and counted. Data given are from three or more independent groups of plates.

Apoptotic morphology was evaluated on fluorescence microscopy after 4′, 6-diamidino-2-phenylindole (DAPI; 0.2 μg/ml) staining. The percentage of apoptotic cells was determined as previously described [10, 11].

2.3. In vivo studies

Subcutaneous TSU-Pr1 tumors were established by injecting 5.0×10^6 cells in 100 μl of Hanks's balanced salt solution (HBSS): Matrigel (1:1) into the hind flanks of 10–11-week-old athymic male nude mice (Harlan Laboratories). Similarly, 5.0×10^4 178-2 BMA cells suspended in 50 μl of HBSS were injected to 4–6-month-old syngeneic 129/Sv male mice, as previously described [18]. Optimal adenovirus therapeutic dose was determined by intratumoral injection of escalating doses of AdGlipr1 (5×10^7, 1×10^8, 5×10^8, and 1×10^9 PFU/tumor), or Adv/CMV/βgal.

To determine a lowest safe dose for maximum cooperative effects for gene therapy and XRT [5, 19], tumors were irradiated with single doses of 5, 10, and 15 Gy by an orthovoltage x-ray generator.

For single-cycle combination-therapy studies, once the tumors reached 50–60 mm^3 (2 days post injection for TSU-Pr1 cells and 12-14 days for 178-2 BMA cells), AdGlipr1 (for 178-2 BMA), AdGLIPR1 (for TSU-Pr1), and the control vector were injected intratumorally. After 48 hours, selected group of tumor-bearing mice (control vector + XRT and AdGlipr1 (AdGLIPR1) + XRT) were irradiated with one 5-Gy dose. (Hereafter, XRT denotes Adv/CMV/βgal + XRT treatment). Five days after completion of single-cycle therapy, the tumors were re-injected with the same vectors and re-irradiated 48 hours later for two-cycle therapy.

Tumor size was measured every other day and calculated as described previously [18, 20]. 16 days after the initial adenoviral vector injection for178-2 BMA, and 72 days for TSU-Pr1, mice were euthanized.

For survival analysis, animals were evaluated at near death or when the tumors exceeded 25 mm in maximum diameter along any axis. At necropsy, we found no obvious cause of death other than extensive tumor load, splenic trauma, and/or abundant ascites.

Mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted according to the principles and procedures outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.4. Cytolysis assay

Fourteen days after the initial vector injection, the mice bearing TSU-Pr1 xenograft tumors were euthanized and the splenocytes were purified to determine natural killer (NK) activity as described previously [18, 21]. Athymic nude mice with no tumor and no prior treatment were included to control for immunogenic compromise.

2.5. Immunohistochemical analyses

Apoptotic and proliferative activities were evaluated on formalin-fixed, paraffin-embedded sections of the tumors from each treatment group by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) and immunostaining for proliferating cell nuclear antigen (PCNA) with a monoclonal antibody (PC10; Dako North America, Inc., Carpinteria, CA, USA), as previously described [22]. To determine AdGlipr1 gene transduction effects, c-Myc immunostaining was performed and quantified using procedure previously described [13]. Immunocyte infiltration in the tumors was also quantified by using antibodies to TNFα, CD4, CD8, MAC1, and MAC3 (BD Biosciences Pharmingen, San Diego, CA, USA) as previously described [21].

2.6 Statistical analysis

Analysis of variance followed by Fisher's least significant difference post hoc test or unpaired t test was used to make comparisons between the means. Kaplan-Meier method survival analysis was evaluated with log-rank Mantel-Cox test. Mann-Whitney rank test was used to analyze the differences in c-Myc immunostaining. All analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. In vitro: therapeutic effects of AdGlipr1/AdGLIPR1+ XRT

The growth of 178-2 BMA and TSU-Pr1 colonies was dose-dependently suppressed by XRT (Fig. 1a, 1c). AdGlipr1 + XRT statistically suppressed colony formation compared with the uninfected and the control groups, at a radiation dose of 5, 10 and 15 Gy ($P \leq 0.05$, $P \leq 0.05$ and $P \leq 0.01$ respectively). Similar dose-dependent effects were observed for AdGLIPR1-infected TSU-Pr1 cells relative to those in the control groups, with 2-, 4-, 6- and 8-Gy doses ($\underline{P} < 0.01$ for all doses) (Fig. 1c).

Corroborating the survival results, significantly greater apoptosis was observed in both cell lines after AdGlipr1 (AdGLIPR1) + XRT in radiation dose dependently manner (Figures 1b, 1d). However, statistical significance was observed only in 178-2 BMA at 10- and 30-Gy doses ($\underline{P} < 0.05$ and < 0.01, respectively). Our in vitro data thus indicate cooperative adenovirus + XRT effects on suppression of colony-formation and induction of apoptosis.

3.2 Selection of AdGlipr1/AdGLIPR1 gene therapy +XRT doses

Evaluating the effectiveness of the AdGlipr1 (AdGLIPR1) gene + XRT combinations required identification of effective but safe doses of each agent in vivo. No AdGlipr1 gene therapy dose was curative, but the 5×10^8 and 1×10^9 PFU/tumor doses delayed tumor growth (data not shown). We reasoned that the immunocompetent mouse model would also approximate the human xenograft model and that since titers of AdGLIPR1 and AdGlipr1

are obtained similarly, the dose responses for AdGlipr1 would be valid for both adenoviral vectors; thus, we did not repeat this testing with AdGLIPR1.

To choose a safe dose that provides the best optimal effects of combination therapy, we compared single-fraction doses of 5, 10, or 15Gy. Tumor growth delay was observed with all three doses, but the two higher doses produced statistically significantly greater delay ($P < 0.0001$ vs. 0 Gy; data not shown). On the basis of these findings, we selected a dose of 5×10^8 PFU for AdGlipr1 and AdGLIPR1 gene therapy and a radiation dose of 5 Gy for the combination studies. No apparent toxicity occurred in any treatment group.

3.3. In vivo: therapeutic effects of AdGlipr1/ AdGLIPR1 + XRT

Compared to the control vector treatment, XRT, AdGlipr1 and combined AdGlipr1 + XRT therapy significantly suppressed the growth of 178-2 BMA tumors (Fig. 2a). Single cycle XRT, AdGlipr1, and AdGlipr1 + XRT yielded 39%, 78% and 94% average reduction in tumor volumes respectively compared with the control treatment.

Two cycles of XRT, AdGlipr1, and AdGlipr1 + XRT combination therapy resulted in further tumor volume reduction and the reductions were significant when compared to the single cycle therapy (Fig. 2a). Nearly 98% average tumor volume reduction was found after the two-cycle AdGlipr1 + XRT combination therapy compared to the control.

As in 178-2 BMA tumors, XRT, AdGLIPR1 and AdGLIPR1 + XRT combination therapy significantly reduced TSU-Pr1 tumor volumes compared with the control group (Fig. 2b). Overall, the biggest tumor reduction (76% on average) was seen with combined AdGLIPR1 + XRT therapy compared to the control.

Two cycles of AdGlipr1 and AdGlipr1 + XRT combination therapy also resulted in c-Myc expression reduction as shown by quantitative immunostaining on the “hot spot” regions of positively labeled tumor cells. Compared to the control group, AdGlipr1 or AdGlipr1 + XRT combination therapy did not show statistically significant reduction in c-Myc expression ($P=0.066$ or $P=0.112$, respectively). However, when c-Myc expression in AdGlipr1 and AdGlipr1 + XRT groups were combined and then compared to the control group, we found statistically significant reduction in c-Myc expression ($p=0.043$, Mann-Whitney rank test) (Fig. 2c).

3.4. Survival extended by combined AdGlipr1/ AdGLIPR1 + XRT

A cumulative Kaplan-Meier survival plot for animals with 178-2 BMA tumors treated with the therapies were analyzed. The mice treated with XRT, AdGlipr1and AdGlipr1 + XRT had substantial extension of survival time compared to the control group mice ($P < 0.003$, $P < 0.0001$, and $P < 0.0001$ respectively) but the longest survival extension was found in AdGlipr1 + XRT group (Fig. 3a). Further extension of survival time resulted after two cycles of XRT, AdGlipr1, and AdGlipr1 + XRT therapy compared with the single-cycle therapy ($P = 0.027$, $P = 0.18$, and $P = 0.019$ respectively), and the two-cycle combination therapy resulted in 39 days of survival time compared to 17.6 days of the control.

Similar statistically significant extension of survival was found in the XRT, AdGLIPR1, and AdGLIPR1 + XRT combination therapy groups compared with the control vector group for mice bearing TSU-Pr1 tumors (Fig. 3b). Again, the combination therapy resulted in longest survival extension.

3.5. Immunostimulatory activity of AdGlipr1/ AdGLIPR1 + XRT

The immunostimulatory analysis of 178-2 BMA tumors show that treated groups have higher $TNF\alpha^+$ infiltration but the differences were not statistically significant compared to the control group or between the treatment groups. However, the densities of the infiltrates of $CD4^+$ and $CD8^+$ lymphocytes and $MAC1^+$ macrophages were significantly higher in the combination therapy group than in the control group ($P < 0.05$ for all three cell types) (Fig. 4). The densities of $CD4^+$, $CD8^+$ and $MAC1^+$ infiltrates in the AdGlipr1 group were also higher than the control or XRT groups but the statistical significance was found only in $MAC1^+$ macrophages ($P < 0.001$).

Quantitative image analyses of the TSU-Pr1 tumors showed that only combined AdGLIPR1 + XRT therapy produced significantly greater apoptosis compared with the control vector ($P = 0.029$) (Figure 5a,b). No significant differences were found in PCNA labeling in any of the treatment groups (Figure 5c).

3.6. NK activity

NK cell activities in splenocytes isolated from mice bearing TSU-Pr1 tumors in the AdGLIPR1 and the AdGLIPR1 + XRT group were significantly greater than that in the normal control nude mice at an E:T ratio of 100:1($P< 0.05$ for both) (Fig. 6). However, no difference in NK cell activity was found between AdGLIPR1 and AdGLIPR1 + XRT group or between XRT and the normal control group.

4. Discussion

Our study results illustrate the therapeutic potential of combining Glipr1/GLIPR1 gene therapy with XRT in subcutaneous cancer models. In vitro, survival of both mouse PCa and human bladder cancer cells was suppressed and apoptosis induced by the combined therapy. In subcutaneous cancer models, the combination therapy significantly suppressed tumor growth and prolonged survival compared with the monotherapies or the control vector therapy. Additional tumor growth suppression and extension of survival was observed with two cycles of treatment without untoward effect for 178-2BMA tumors. Reduction of c-Myc expression by immunohistochemical analysis of the tumor tissues from AdGlipr1 and AdGlipr1 + XRT treated mice suggests that downregulation of c-Myc was induced by increased Glipr1 expression after Glipr1 gene transduction, as previously reported for GLIPR1 [13]. The immunostimulatory effects of the combined therapy on MAC1-, CD4-, and CD8-positive cells were also significantly higher than with the monotherapies. The result of NK activity assay demonstrated that NK cells were predominantly responsible for the antitumor activity of the combination therapy for TSU-Pr1 tumors.

Numerous preclinical studies of gene therapy plus XRT have revealed synergistic effects of the combination [5, 19, 23-27] including a previous report which showed the p53 gene enhance the therapeutic potential of XRT in vivo. Our current investigation provides evidence that combining XRT with adenoviral GLIPR1/ Glipr1gene therapy synergize the therapeutic effects of XRT or gene therapy alone, and this effect is p53-independent since the 178-2 BMA and TSU-Pr1 cell lines are p53 null [17] and p53 mutant [27], respectively.

Ionizing radiation improves the efficiency of transfection and/or transduction and transgene integration [28]. Gene therapy and XRT may produce synergistic effects by targeting different parts of the cell cycle (e.g., the S phase for gene therapy and the G2-M phase for XRT [29], or by XRT enhancing the ‘bystander effect’ of cytotoxic gene therapy [30]. We will continue to study the mechanisms underlying this combined therapeutic approach.

In conclusion, combining Glipr1 gene therapy with XRT enhances the therapeutic effects of both gene therapy and XRT alone in subcutaneous prostate and bladder models, and the effects are greater after two treatment cycles. The result of this study suggests clinical benefit of combined GLIPR1 therapy + XRT therapy for cancers with low GLIPR1 expression.

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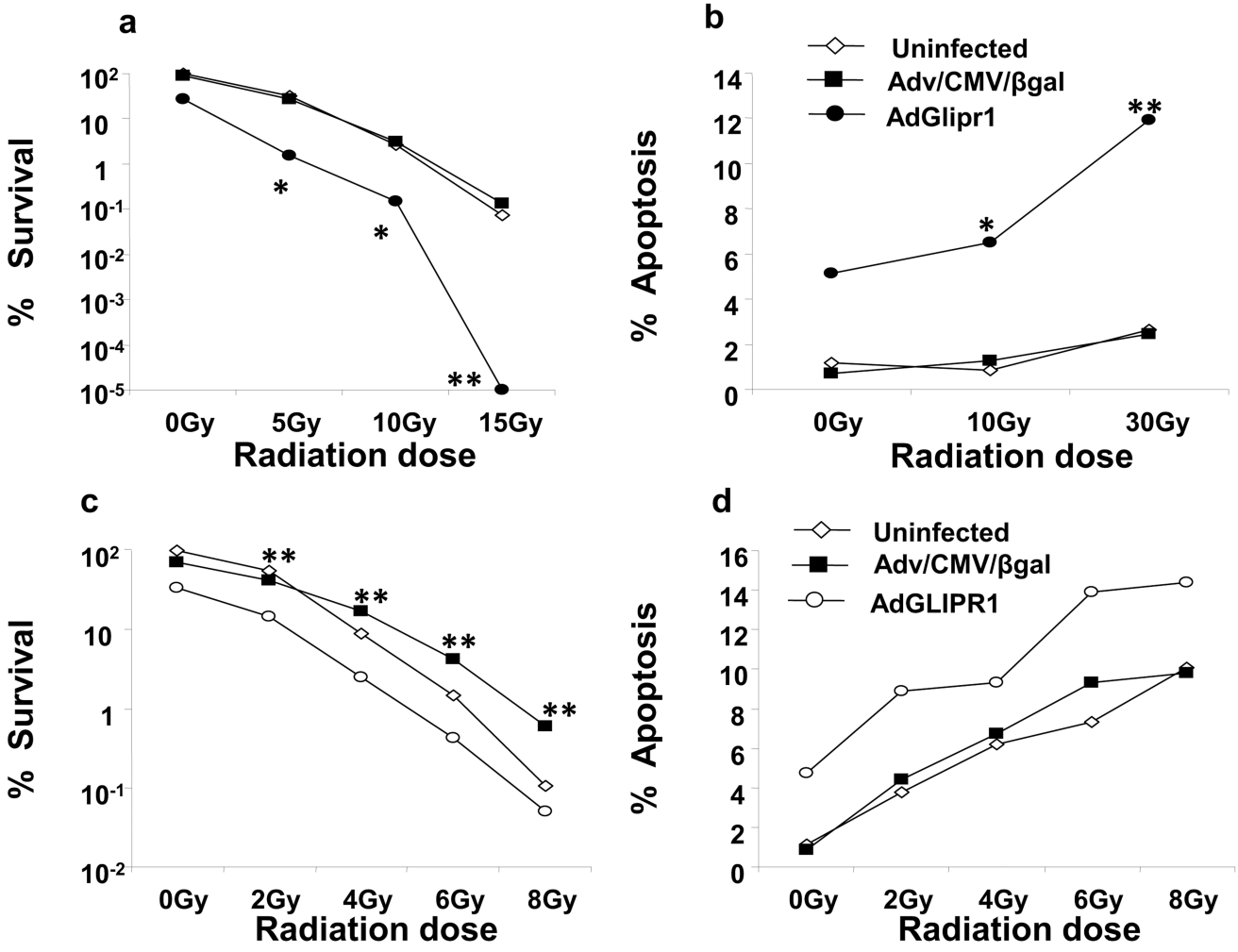
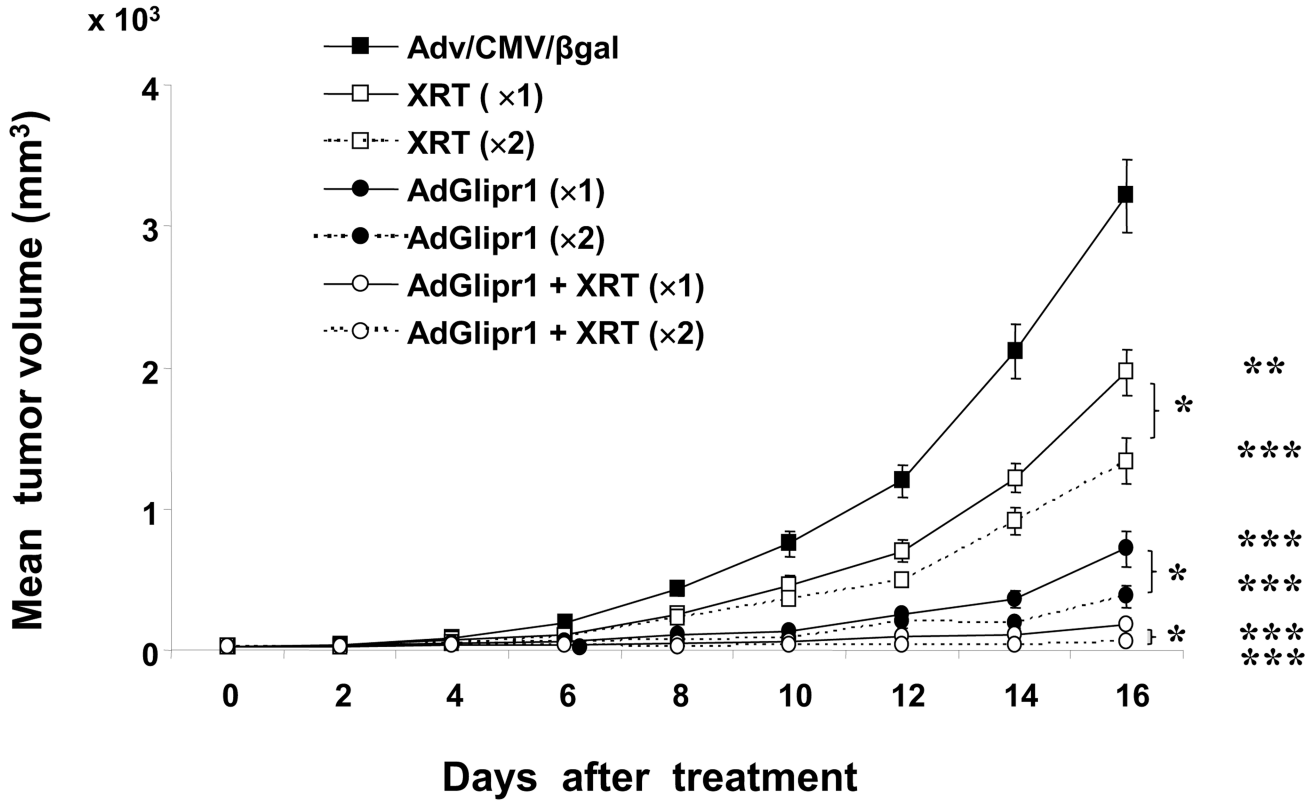


Figure 1. In vitro effects of combined AdGlipr1 (AdGLIPR1) + XRT.

Mouse 178-2 BMA PCa and human TSU-Pr1 cancer cells were seeded at 2×10^6 cells per 10-cm plate and infected the next day with AdGlipr1 (AdGLIPR1) or Adv/CMV/βgal at a MOI of 100. 24 hours after vector infection, cells were irradiated with escalating radiation doses and then reseeded. An uninfected group was added as a negative control. Colonies were counted 12 days after irradiation, 178-2 BMA (a), TSU-Pr1 (c) ; and apoptotic cells were counted 2 days after irradiation, 178-2 BMA (b); TSU-Pr1(d). As compared with the uninfected and Adv/CMV/βgal control groups, the combination treatment significantly suppressed colony formation and increased apoptosis. The data are represented as mean % survival from triplicate plates. *$\underline{P} \leq 0.05$, **$\underline{P} \leq 0.01$.

a



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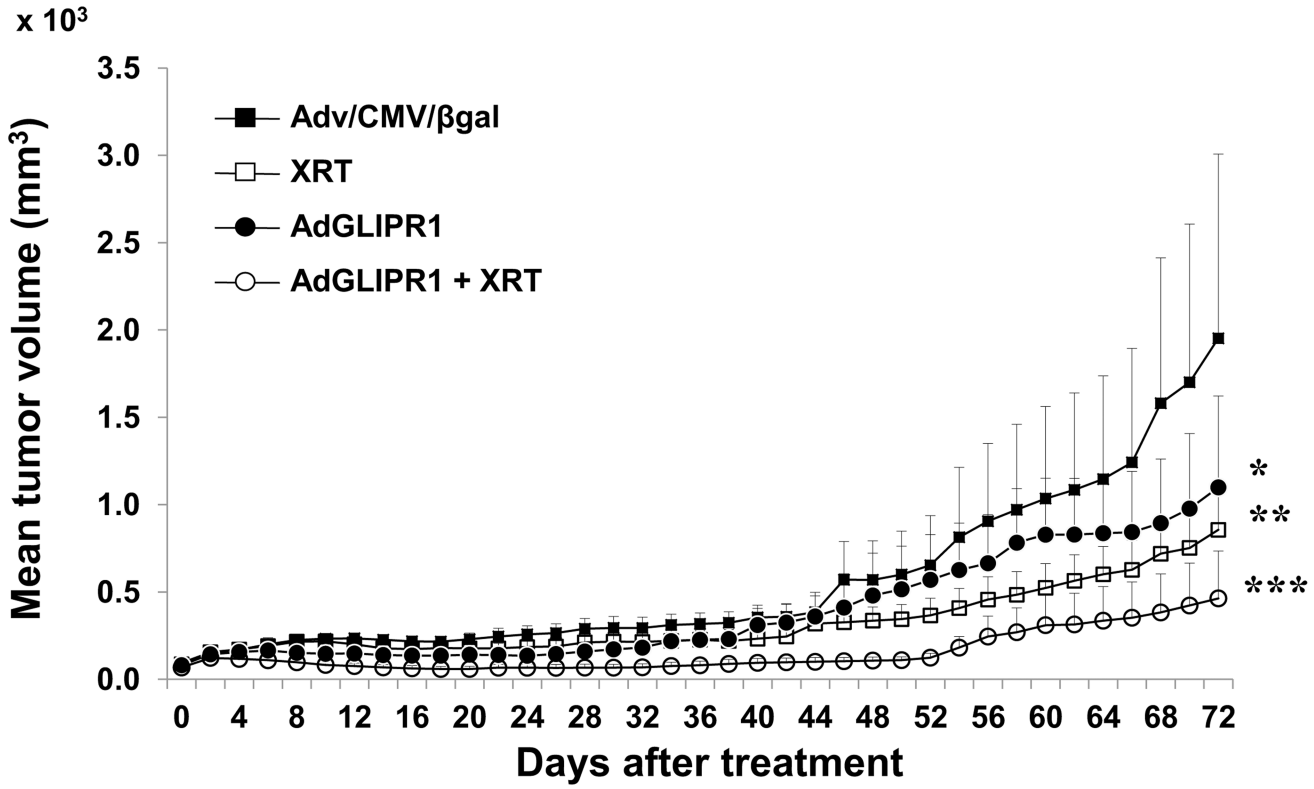


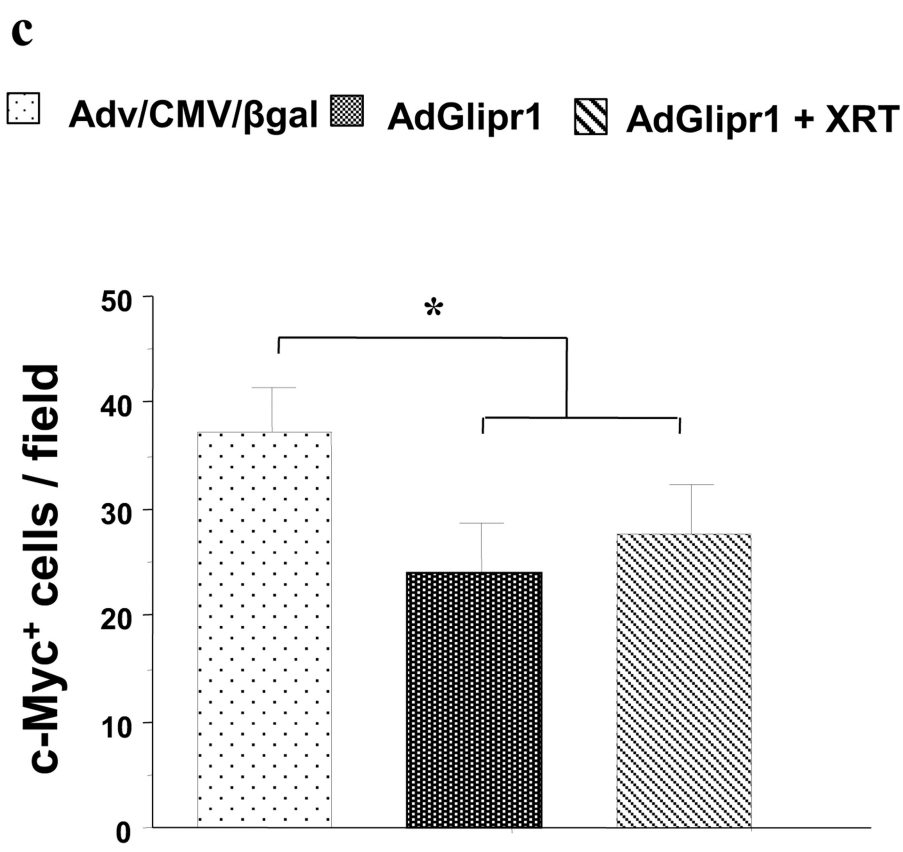
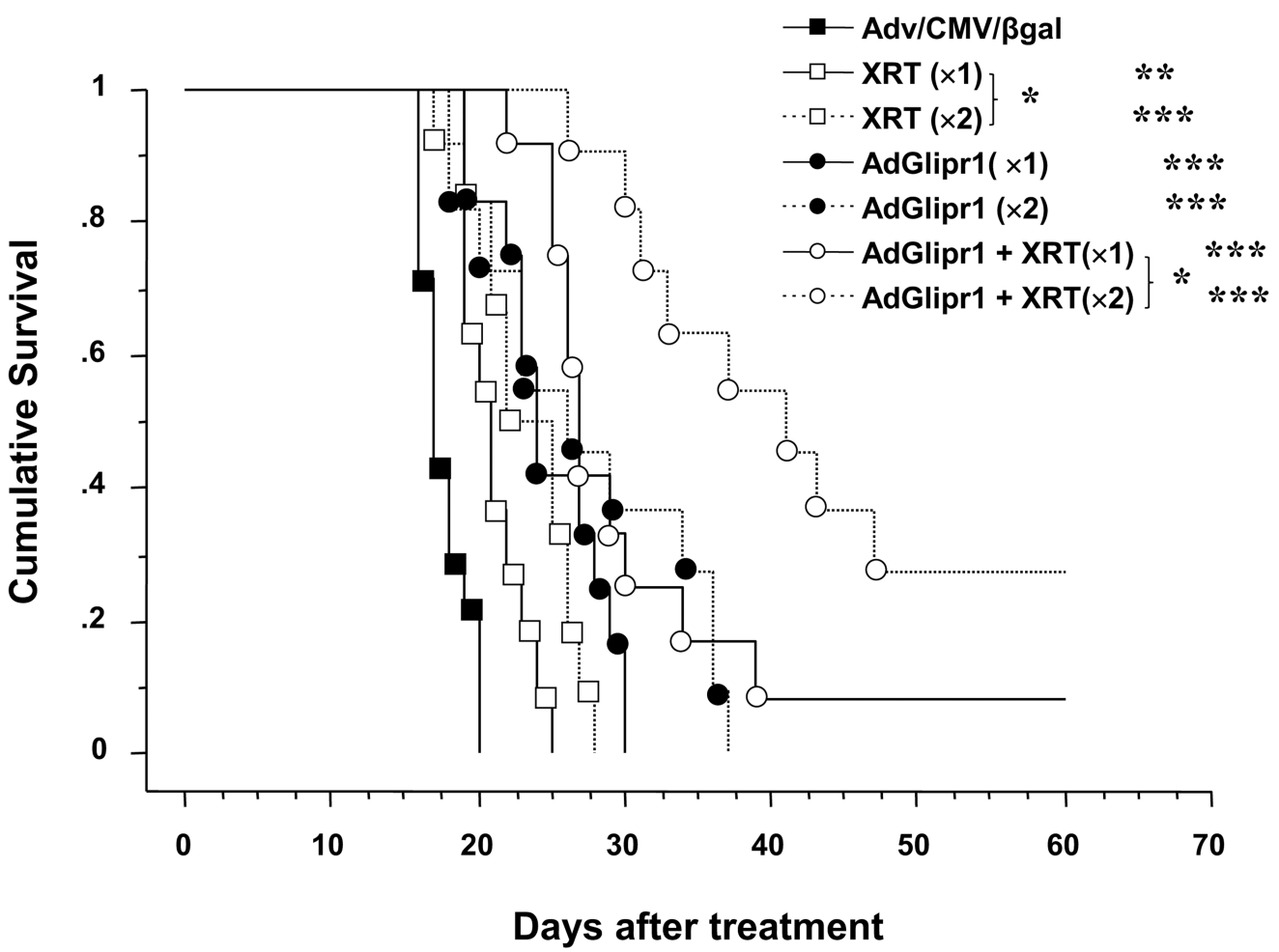


Figure 2. Suppression of 178-2 BMA and TSU-Pr1 subcutaneous tumor growth comparison after XRT, AdGlipr1(AdGLIPR1) and combined AdGlipr1(AdGLIPR1) + XRT therapy with the control Adv/CMV/βgal treatment

In comparison with the Adv/CMV/βgal (control) vector, XRT, AdGlipr1 monotherapies, and AdGlipr1 + XRT significantly suppressed 178-2 BMA tumor growth. Two cycles of combined treatment suppressed growth significantly more than single-cycle therapy did. Biggest tumor suppression was observed in the group of mice treated with two cycles of AdGlipr1 + XRT. The data are reported as means ± standard error. *P ≤ 0.05, ** P ≤ 0.005, ***P ≤ 0.0001. (Fig. 2a) In comparison with the control vector, the monotherapies significantly suppressed the TSU-Pr1 tumor growth but the biggest suppression was seen after combined AdGLIPR1 + XRT therapy. The data are reported as means ± standard error. *P ≤ 0.05, **P ≤ 0.002, ***P ≤ 0.0001. (Fig. 2b) Each treatment group contained 5–8 mice. Quantitative immunostaining analysis showed significant reduction of c-Myc-positive tumor cells in AdGlipr1 and AdGlipr1+ XRT treated mice when these two treatment groups were compared to Adv/CMV/βgal control group. *P=0.043, n=5 for each treatment group. (Fig. 2c)

a



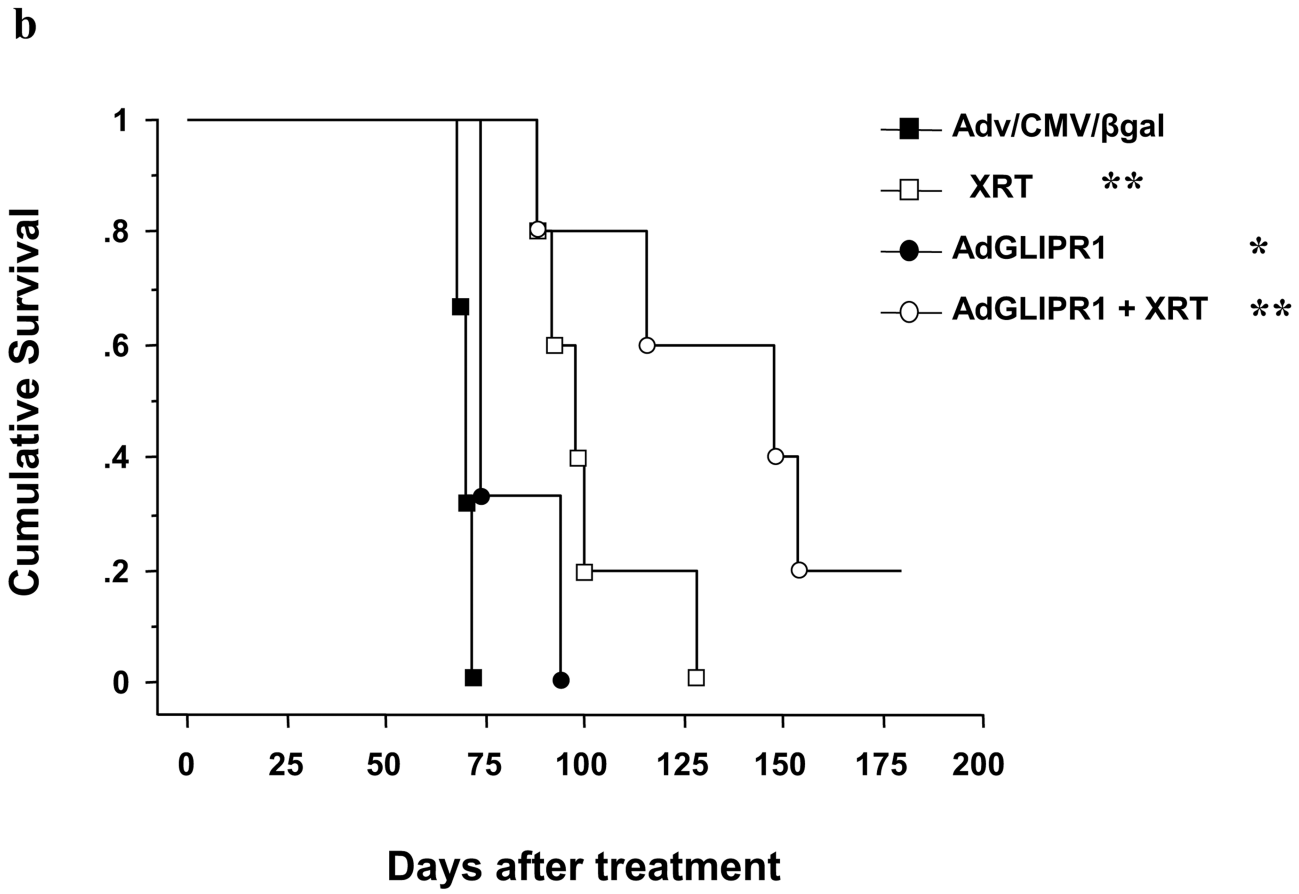


Figure 3. Kaplan-Meier survival plot shows the cumulative survival extension for mice with subcutaneous 178-2 BMA and TSU-Pr1 tumors

In comparison with control vector treatment, XRT, AdGlipr1monotherapies, and combined AdGlipr1 + XRT significantly extended survival of mice with 178-2 tumors after single cycle of treatment. Two cycle treatment significantly added to the survival time compared to the single cycle treatment. Longest survival time was observed in the group of mice treated with two cycles of AdGlipr1 + XRT *P ≤ 0.05, **P ≤ 0.002, ***P ≤ 0.0001. (Fig. 3a) Compared with the control Adv/CMV/βgal, AdGLIPR1 and XRT, combined AdGLIPR1 + XRT provided a significant survival advantage for mice with TSU-Pr1 tumor. Longest survival time was observed in the group of mice treated with AdGLIPR 1 + XRT. *P ≤ 0.05, **P ≤ 0.002, ***P ≤ 0.0001 (Fig. 3b).

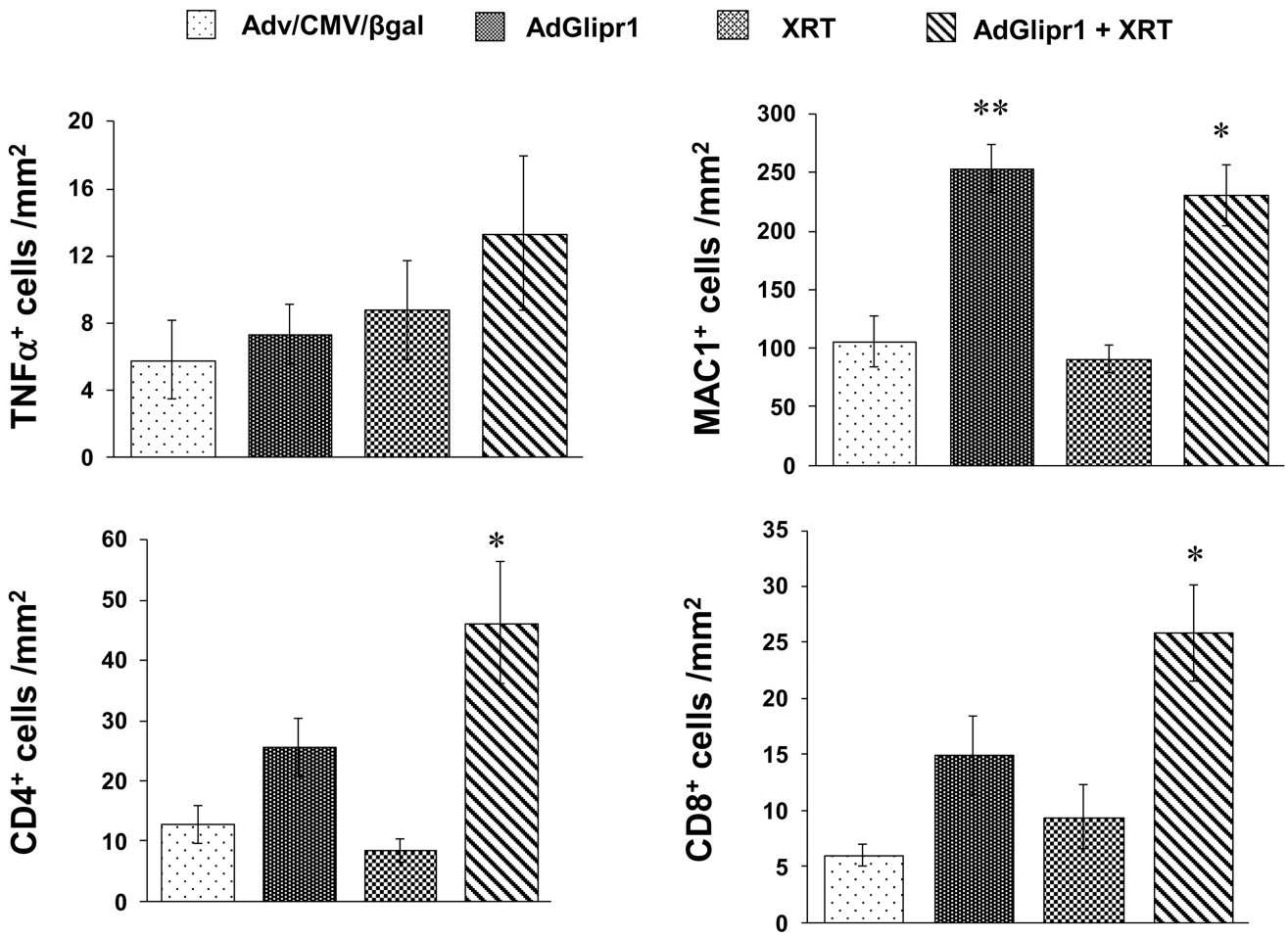


Figure 4. Immunocyte infiltration in subcutaneous 178-2 BMA tumors was enhanced by combined AdGLIPR1 + XRT treatment

Quantitative immunohistochemical analyses using antibodies to TNFα, MAC1, CD4, and CD8 were performed on the tumor sections obtained after two cycle treatment. The results were presented as mean± standard error of positively labeled cells per mm^2 tumor area. *P ≤ 0.05, **P ≤ 0.005.

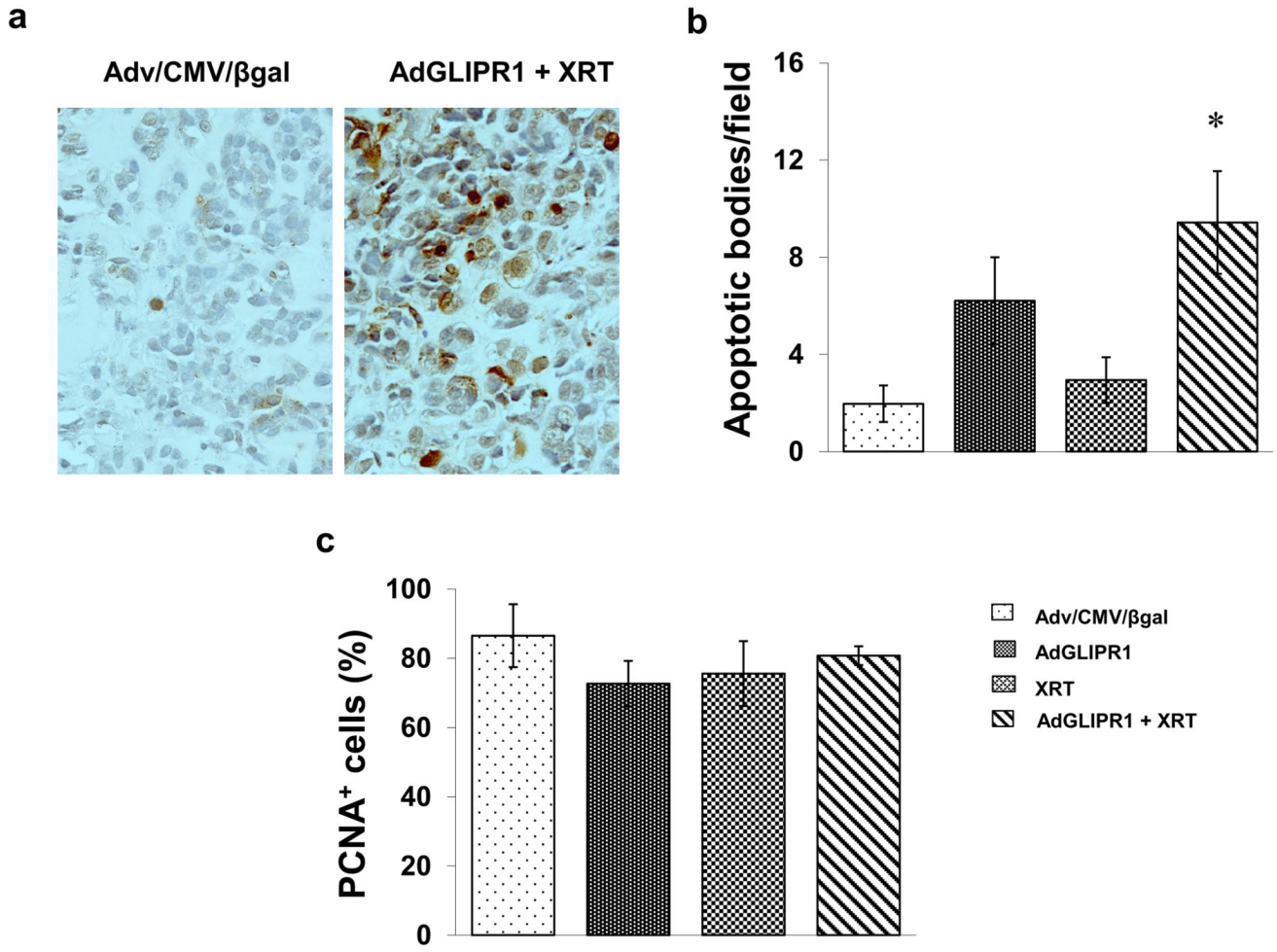


Figure 5. Quantitative immunohistochemical analysis of TSU-Pr1 tumor xenografts (n = 3 for each group) was performed 14 days after vector administration

A representative portion of each tumor was formalin fixed and paraffin embedded, cut into 5-μm sections, and stained with hematoxylin and eosin for histologic examination. Another portion was frozen in liquid nitrogen for frozen-section analysis. Apoptotic bodies and PCNA-positive cancer cells in each tumor were quantified in 20-30 microscopic fields at 200× using a Nikon Eclipse 90i image analysis system with NIS-element software (version 3.0). The results are expressed as the number of apoptotic bodies per microscopic field and the percentage of PCNA labeling. In comparison with the control Adv/CMV/βgal, AdGLIPR1 + XRT significantly increased apoptosis in the tumors as shown by TUNEL staining (a, 200×) or by quantitation (b), but caused no significant change in proliferation (PCNA) labeling rate (c). The data are reported as means ± standard error. *$P \leq 0.05$.

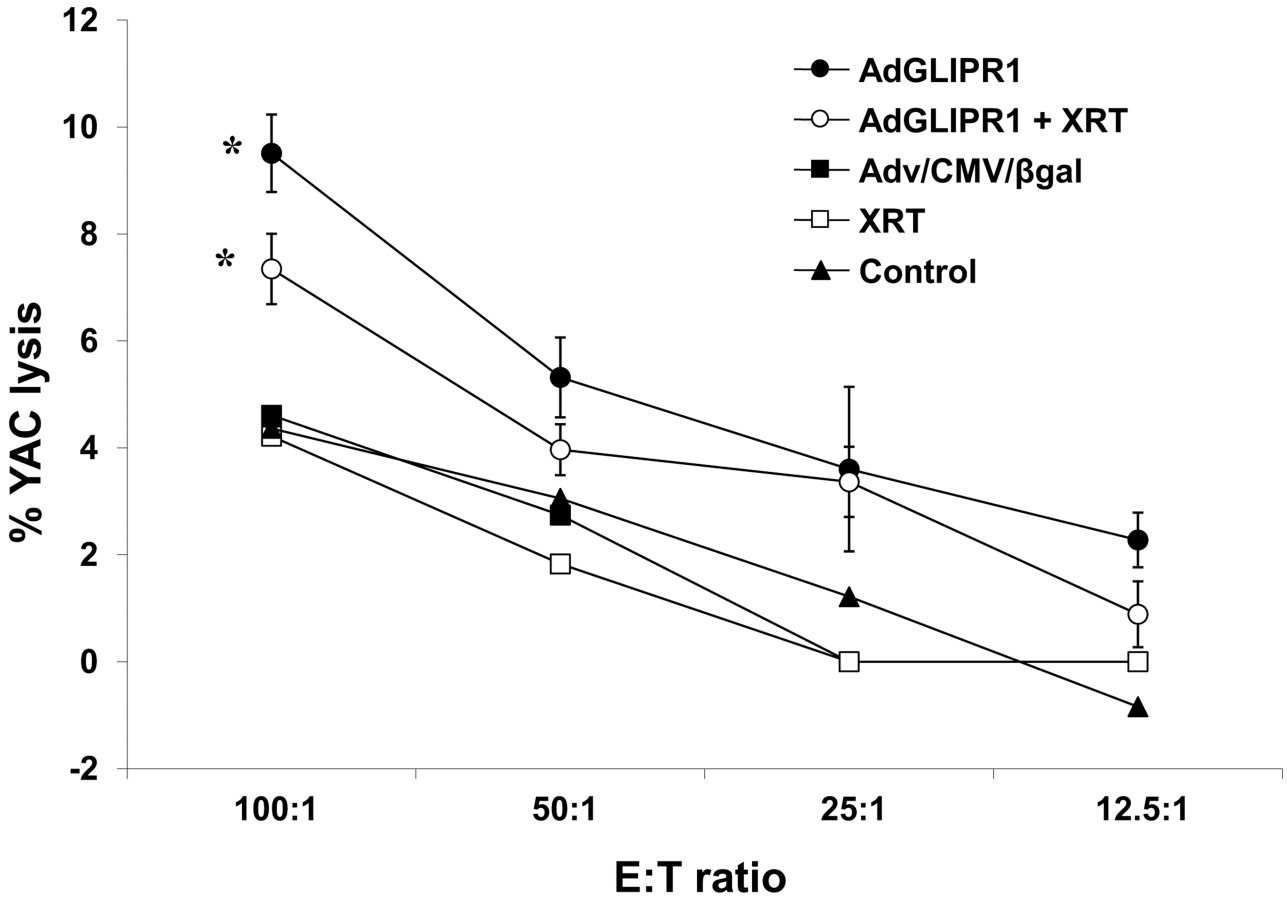


Figure 6. Systemic immune responses in TSU-Pr1 xenograft model

Natural killer (NK) cell activity in splenocytes from three animals per treatment group was determined 14 days after vector administration. Cells from the AdGLIPR1 and AdGLIPR1 + XRT groups had significantly greater NK activity than did those in the normal control group at the E: T ratio of 100:1. The data are reported as means ± standard error. *P < 0.05.