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Preclinical Evaluation of Sequential Combination of Oncolytic Adenovirus Delta-24-RGD and Phosphatidylserine-targeting Antibody in Pancreatic Ductal Adenocarcinoma

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Abstract

Delta-24-RGD (DNX-2401) is a conditional replication-competent oncolytic virus engineered to preferentially replicate in and lyse tumor cells with abnormality of p16/RB/E2F pathway. In a phase 1 clinical trial, Delta-24-RGD has shown favorable safety profile and promising clinical efficacy in brain tumor, which prompted us to evaluate its anticancer activity in pancreatic ductal adenocarcinoma (PDAC), which also has high frequency of homozygous deletion and promoter methylation of CDKN2A encoding the p16 protein. Our results demonstrate that Delta-24-RGD can induce dramatic cytotoxicity in a subset of PDAC cell lines with high Cyclin D1 expression. Induction of autophagy and apoptosis by Delta-24-RGD in sensitive PDAC cells was confirmed with LC3B-GFP autophagy reporter and Acridine Orange staining as well as Western blotting analysis of LC3B-II expression. Notably, we found that Delta-24-RGD induced phosphatidylserine (PS) exposure in infected cells independent of cells' sensitivity to Delta-24-RGD, which renders a rationale for combination of Delta-24-RGD viral therapy and PS targeting antibody for PDAC. In a mouse PDAC model derived from a liver metastatic pancreatic cancer cell line, Delta-24-RGD significantly inhibited tumor growth compared with control (p<0.001), and combination of PS targeting antibody 1N11 further enhanced its anticancer activity (p<0.01) possibly through inducing synergistic anticancer immune responses. Given that these two agents are currently in clinical evaluation, our study warrants further clinical evaluation of this novel combination strategy in pancreatic cancer therapy.

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Keywords

Delta-24-RGD; Oncolytic virus; Phosphatidylserine-targeting antibody; PDAC

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the leading cause of cancer-related death (1). Although advances have been made, the overall 5-year survival of PDAC patients remains less than 7 % mainly due to late diagnosis and limited number of available systemic agents. New effective therapeutic agents for this cancer are still urgently needed.

Oncolytic virus are engineered to take advantage of genetic mutations of specific tumor suppressor genes or oncogenes in cancer cells to allow preferential replication within and lysis of cancer cells while sparing normal cells (2, 3). Multiple types of oncolytic viruses including Ad5/3 adenovirus, herpes simplex viruses (HSV), vaccinia virus, Newcastle disease virus, and measles virus have been evaluated clinically (3). The first FDA-approved HSV1-based oncolytic virus T-VEC carrying a granulocyte-macrophage colony-stimulating factor (GM-CSF) gene significantly improved durable response rate of patients with unresectable melanoma compared with GM-CSF treatment alone (16.3 *vs* 2.1%, $p<0.0001$) (4). The approval of T-VEC for late stage melanoma brings the hope in oncolytic virus-mediated immunotherapy for cancer treatment. Delta-24-RGD is an adenovirus-based oncolytic virus with a deletion of 24 base pairs in the E1A region and a modification in virus fiber with a RGD-4C motif to enhance its infection of cancer cells independent of the expression of coxsackievirus and adenovirus receptor (CAR) (5, 6). Adenovirus E1A gene codes a 19 kDa protein that binds to RB protein thus releasing E2F factor from RB/E2F complex for cell cycle progression. The deletion of 24 base pairs in E1A region suppresses virus replication in normal cells but not in cancer cells with defect of p16/RB/E2F pathway. Delta-24-RGD has shown promising anticancer effect by stimulating anticancer immune response in brain tumor patients (7), and is currently in phase 2 clinical trial with combination of chemotherapy for brain tumor. Because p16/RB/E2F pathway is also frequently altered in pancreatic cancer due to the deletion, mutation or promoter methylation of CDKN2A gene which encodes the p16 protein, we hypothesize that Delta-24-RGD could be an active agent for pancreatic cancer therapy, especially for the tumors with abnormal p16/RB/E2F pathway.

Phosphatidylserine (PS), a membrane phospholipid, is localized in the inner leaflet of a plasma membrane in normal non-tumorigenic cells but is presented on the surface of apoptotic cells and cancer cells within the tumor microenvironment (8, 9). Although a signal for cell engulfment, PS is known to dampen the immune response. PS exposure on the outer membrane also occurs during viral cellular infection and replication. Monoclonal antibodies have been raised to target PS and investigated as anti-viral therapy (10). Recent data from an animal model of melanoma demonstrated that combining PS-targeting antibodies improved the effectiveness of immune checkpoint inhibitors, suggesting that antibodies to PS can reverse its immune dampening signals (11). PS targeting antibodies, Bavituximab, have also been raised to target PS-expressing tumor cells and investigated in phase I clinical trials of

several solid tumors systems including metastatic breast and lung cancers (12, 13). Together, these studies suggest that anti-PS antibodies could augment the anti-cancer effects of oncolytic virus therapy.

In this study we evaluated the anticancer activity of Delta-24-RGD in multiple pancreatic cancer cell lines and primary pancreatic cells established from patient-derived xenograt tumors (PDXs) and explored potential predictive biomarkers for sensitivity. We found that Delta-24-RGD induced dramatic cytotoxicity in a subset of pancreatic cancer cell lines with high expression of Cylin D1 and induced PS exposure in infected cells. In addition, combination with a PS targeting antibody further enhanced the anticancer effects of Delta-24-RGD *in vivo*, possibly through stimulating immune responses. Our study demonstrate that Delta-24-RGD could be a promising agent, alone or in combination, for pancreatic cancer therapy.

Materials and Methods

Reagents, cell lines and animals

Cell culture medium RPMI1640 was purchased from HyClone. Fetal bovine serum (FBS) and MTT cell viability reagent were purchased from Life Science Technologies (Grand Island, NY). Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture 100× was purchased from Sigma (Missouri, MO). Antibodies for PARP, Cleaved-PARP, Caspase-9, Cleaved-Caspase 9, Caspase-7, Cleaved-Caspase 7, Ki-67, and β-Actin were purchased from Cell Signaling Technology (Danvers, MA). E1A antibody and PS antibody [4b6] for in vitro immunofluorescence staining were purchased from Abcam (Cambridge, MA). CD68 and NKp46 antibodies were from Biolegend (San Diego, CA). PS antibody 1N11, also known as PGN635, a fully human anti-PS antibody was prepared by Dr. Rolf Brekken's lab (University of Texas (UT) Southwestern Medical Center) and used for in vivo study (14). Conventional pancreatic cancer cell lines were from ATCC, and primary pancreatic cancer cells were established in our laboratory as described previously (15). All primary pancreatic cancer cells were authenticated with unique fingerprinting. NOD/SCID and Nude mice (female, 6 weeks) were purchased from the National Cancer Institute and Jackson Laboratories.

Adenovirus infection

Delta-24-RGD and Ad-GFP-RGD virus were prepared as described previously (16). For cell infection with Ad-GFP-RGD or Delta-24-RGD, $1X10^4$ cells were seeded in 6-well plates, and after 24 hours, cells were infected with adenovirus at different multiplicity of infections (MOIs)..

Cytotoxicity Crystal violets staining

Cells ($1X10^4$) were seeded in 6-well plates and infected with Delta-24-RGD or Ad-GFP-RGD control virus at different MOIs, then 10 days post infection, cells were fixed with ice-cold methanol for 10 minutes. Cells were washed with PBS and stained with 0.5% Crystal violets solution under room temperature for 10 minutes. The plates were rinsed with water until no color coming off. The plates were dried at room temperature for overnight.

Cell viability MTT assay

Cells were seeded at the density of $1x10^3$ cells/well in 96 well plate, and 24 hours later, cells were infected with Dleta-24-RGD at MOIs of 0.1, 0.3, 1, 3, and 10. Cell viability was measured with MTT assay as described previously (15). The dose that causes 50% of cells death (IC_{50}) of infected cells compared with non-infected control was calculated with GraphpadPrism software (6.0).

Immunofluorescence staining and Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested with trypsinization and washed with PBS containing 10% FBS. About $2X10^6$ cells in 100 μl FACS buffer was added in polystyrene round-bottom tube, and incubated with another 100 μl Fc blocking buffer on ice for 20 minutes. Cells were centrifuged at 1500 rpm for 5 minutes at 4 C and resuspended with 100 μl FACS buffer. Primary antibodies were added in the buffer with a concentration of 1 μg/ml. Cells were incubated for 60 minutes at 4C. After incubation, cells were washed with FACS buffer 3 times by centrifugation at 1500 rpm and resuspended in 200 μl cold FACS buffer. Diluted Fluorochrome-labeled secondary antibody in FACS buffer was added to the cells and incubated them for 30 minutes at 4 C. Cells were washed 3 times with FACS buffer and resuspended in 200 μl FACS buffer. FACS analysis of fluorescence intensity was performed with FACSCalibur™ (BD) and the results were analyzed with FlowJo 10 software (FlowJo, Ashland, OR.).

Autophagy assay

Cells ($1X10^5$) seeded in the wells of chamber slide were treated with PBS or Delta-24-RGD virus at 1 MOI for 3–5 days, and 10 μl LC3B-GFP virus (BacMam 2.0, Thermo Fisher) was added into the well and incubated for 24 hours. Cells were checked under fluorescence microscope and LC3-GFP positive autophagasome were counted. For Acridine orange staining and flow cytometry analysis, cells ($1x10^6$) in 6-well plates were infected with Delta-24-RGD virus at MOIs of 0.1 1, and 10 for 5 days. Cells were harvested, washed with PBS and incubated with Acridine Orange (1μg/ml) for 15 minutes. Cells were washed with PBS twice and resuspended in 400 μl PBS for FACS analysis.

Analysis of virus replication

Cells ($1X10^5$) were seeded in 6-well plates and infected with Delta-24-RGD at 1 MOI. Cells were harvested and counted, and cell lysates were collected at day 1, 3 and 7 post infection. Virus DNA was isolated and purified with Adeno-X qPCR Titration Kit from Clontech Company (Mountain View, CA). The copy numbers of adenovirus in the cells were quantified with the kit following manufacturer's instruction and normalized by cell number.

Western blotting

Cells after treatment or infection were washed with cold PBS, and tissue lysates were extracted with RIPA buffer and quantified for protein concentration with BCA method. Thirty to 50 micrograms of protein was used for western blotting analysis. Detailed methods can be found in our previous publication (17).

Immunohistochemistry (IHC) and immunofluorescence (IF) staining of paraffin embedded tumor tissues

Tumor tissues harvested from mice were fixed for overnight. Paraffin embedded tumor tissues were cut into 5 μm sections. Immunohistochemistry or immunofluorescence staining was performed using the methods described previously (18). Images were captured with an Olympus DP72 camera and CellSens software (Center Valley, PA) on an Olympus BX51 microscope.

Animal experiment *in vivo*

Animal experiment protocol (00001089-RN00) was reviewed and approved by The University of Texas MD Anderson Cancer Center (Houston, Texas) institutional review board and in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. MDA-PATC53 cells were harvested and $2X10^6$ cells in 100 μl PBS were mixed with equal volume of matrigel (Invitrogen, Carlsbad, CA) and implanted into nude mice. When tumors reach the size of ~100 mm^3, mice were randomly divided into 4 groups with 5 mice in each group. Mice were treated with PBS or Delta-24-RGD adenovirus 3 times weekly by intratumoral injection of $1X10^8$ plaque-forming units (pfu) virus. Treatment with 1N11 antibody was started after the 3rd virus treatment by intraperitonial injection of 100 μl antibody with the dose of 1mg/kg. A total of 3 doses of 1N11 were administrated. The tumor volumes were calculated using the formula, length × $width^2$ × 0.52. When tumors reached the size of 12 mm in diameter, all mice were sacrificed, and tumors were harvested for histochemical analysis.

Statistical analysis

The significance of differences between different treatment groups was analyzed by Two-way ANOVA or *t*-test (two tails), and P value < 0.05 was considered as significance. Correlation of Cylin D1 expression with sensitivity of cell lines to Delta-24-RGD virus was analyzed with Pearson correlation method. All statistical analyses were done with GraphPad Prism 6.0 (Graphpad Software Inc. La Jolla, CA).

Results

Oncolytic virus Delta-24-RGD induced dramatic anticancer activity in pancreatic cancer cells

To test the cytotoxicity of Delta-24-RGD in pancreatic cancer cells, four cell lines, BxPC3, PANC1, MiaPaCa2, and MDA-PATC53, a primary cell line established in our laboratory, were infected with Ad-GFP-RGD control and Delta-24-RGD at different MOIs followed by Crystal violet staining. Infection of cells with Delta-24-RGD virus induced dramatic cytotoxicity effects in PANC1, MiaPaCa2, and MDA-PATC53 cells but not in BxPC3 cells (Fig. 1A). We then used the cell viability assay to test cytotoxicity of Delta-24-RGD in 6 classic and 6 primary pancreatic cancer cell lines derived from PDAC PDX models (Fig. 1B and C). Six out 12 of tested cell lines were sensitive to Delta-24-RGD. Notably, PANC1, MiaPac2, and AsPC1 have similar sensitivity as human glioma cell line (U251), which was used as a positive control as it has been previously shown to be sensitive to Delta-24-RGD

(5). The IC50 of Delta-24-RGD in each of the cell lines were calculated (Supplemental-table 1). We used the IC_{50} less than 10 MOIs as a sensitivity cutoff. Based on this cutoff, of the 12 lines tested, MDA-PATC53, MiaPaCa2, PANC1, MDA-PATC108, AsPC1, and MDA-PATC118 are sensitive to Delta-24-RGD. These results suggest that Delta-24-RGD induced dramatic cytotoxicity in a subset of pancreatic cancer cells.

CyclinD1 expression is associated with sensitivity of PDAC cells to Delta-24-RGD

To determine the molecular markers that correlate with sensitivity of pancreatic cancer cells to Delta-24-RGD (Supplemental-table 1). We analyzed the protein expressions of the major components of p16/RB/Cyclin D1/CDK4 pathway using Western blotting assay. The expression of p16 was low in all of the tested cells, except MDA-PATC43 (Fig. 2A), which is consistent with that high frequency deletion and promoter methylation of p16 gene in pancreatic cancer. This result indicate that the low or no expression of p16 is not a predictor of sensitivity to Delata-24-RGD. The expression of RB gene was low in all the sensitive cells, while 2 out of 5 resistant cells also have low RB expression, suggesting that low RB expression alone is not a predictor of sensitivity either (Fig. 2A). Overall, the expression of Cyclin D1 and CDK4 was higher in sensitive cells than in resistant cells (Fig. 2B). Using Pearson correlation analysis, we found that Cyclin D1 expression is reversely correlated with the IC50 of Dela-24-RGD (R=-0.8506) (Fig. 2C). To examine for differences in virus replication between sensitive and resistant cells, virus copy number was analyzed with a PCR-based assay. As was shown in Fig. 2D, virus copy number increased dramatically over time in two sensitive cell lines, MiaPaCa2 and MDA-PATC53, but not in the resistant BxPC3 cells, suggesting that replication of Delta-24-RGD in infected cells is associated with its cytotoxicity effects.

Delta-24-RGD induced autophagy in pancreatic cancer cells

Previous studies have found that oncolytic virus induces autophagy-mediated cancer cells death (19). To investigate if the cytotoxicity induced by Delta-24-RGD in pancreatic cancer cells is also mediated by autophagy, LC3B-GFP expression reporter was introduced into the cells after 5 days infection with Delta-24-RGD. Infection of Delta-24-RGD indeed dramatically induced autophagsome formation evidenced by LC3B-GFP punctae in Delta-24-RGD sensitive not resistant cells (Fig. 3A and B). Acridine Orange staining further confirmed that acidic vesicular organelles (AVO) were significantly increased in sensitive cells after infection with Delta-24-RGD (Fig. 3C). In addition, Delta-24-RGD induced dramatic expression of LC3B-II, an autophagy marker, in sensitive cells (Fig. 3D). The result also showed that high MOI of Delta-24-RGD induced cleaved PARP, Caspases 7, and Caspase 9, indicating caspase activation was induced (Fig. 3D).

Delta-24-RGD adenovirus induced PS exposure in pancreatic cancer cells

Phosphatidylserine (PS) is an aminophospholipid distributed in inner leaflet of cell membrane. It is externalized to the outer leaflet when cells undergo apoptosis. Exposure of PS is a signaling for phagocytosis of apoptotic cells. PS exposure on a virus's envelop or vesicle is a strategy called apoptotic mimicry for virus entry into host cells (20–22). Previous studies have revealed that some viruses can induce exposure of PS on the membrane of infected cells, which has been explored as a strategy for antivirus therapy (10). PS exposure

on cancer specific endothelial cells and cancer cells was also reported and plays a role in cancer related immune suppression (8, 23–25). Targeting PS with an anti-PS antibody has been under clinical investigation for cancer therapy (26–28). To check whether Delta-24-RGD can also induce PS exposure in infected pancreatic cancer cells, immunofluorescence staining followed by flow cytometry analysis of Detla-24-RGD-infected cells was performed with a PS specific antibody. As was shown in Fig. 4A, infection with Delta-24-RGD virus induced PS exposure in both resistant and sensitive pancreatic cancer cells. Further microscopic analysis also confirmed that Delta-24-RGD induced PS externalization to the outer leaflet of the cell membrane in three PDAC cell lines (Fig. 4B). These results suggest that Delta-24-RGD can induce PS exposure, which may not depend on the sensitivity of infected cells.

Combination of Delta-24-RGD and PS targeting antibody induced enhanced antitumor activity *in vivo*

Previous studies have shown that PS exposure in tumor cells induces immune suppression, and targeting PS promotes anticancer immune response (11, 26, 27). We hypothesize that combination of Delta-24-RGD oncolytic virus with PS targeting antibody may further enhance anticancer effects through both antibody-dependent cell-mediated cytotoxicity (ADCC) and enhanced anticancer immune response. To confirm this hypothesis, mice bearing tumors derived from a sensitive cell line MDA-PATC53 were first treated with $1X10^8$ pfu of Delta-24-RGD for 3 times by intratumoral injections. Then mice were treated with intraperitoneal injections of PS targeting antibody 1N11, a fully human PS antibody. The detailed treatment schedule was shown in Fig. 5A.). The result showed that treatment with Delta-24-RGD alone significantly inhibited tumor growth compared with nontreated control ($p<0.0001$). PS targeting antibody 1N11 had a moderate antitumor effect, that was less effective than Delta-24-RGD alone (Fig. 5B and C). However, the combination of Delta-24-RGD virus and PS targeting antibody was more effective than each agent alone (Delta-24-RGD plus 1N11 vs 1N11 alone, $p<0.0001$; Delta-24-RGD plus 1N11 *vs* Delta-24-RGD, $p<0.01$). Expression of adenovirus antigen E1A was confirmed in Delta-24-RGD or combination treated tumors, indicating efficient virus infection and caspase-related cell death induced by Delta-24-RGD *in vivo* (Fig. 5C). Consistent with previous studies that PS targeting antibody induces microphage activation (28), our study shows enhanced staining of CD68, a marker of macrophages, after treatment with 1N11 alone or after the combination treatment (Fig. 6). In addition, we also observed that infiltration of activated NK cells were enhanced in the tumor tissue with single or combined treatments (Fig.6)

Discussion

A recent clinical trial of Delta-24-RGD in patients with glioblastoma demonstrated favorable toxicity profile and remarkable clinical efficacy (7). This prompted us to evaluate its anticancer activity in pancreatic cancer, since 85% of PDACs have an incomplete p16/RB/E2F pathway due to promoter methylation and homozygous deletion of CDKN2A locus encoding p16 protein (29). In our study, Delta-24-RGD induced dramatic cytotoxicity in more than 50% of the tested cell lines. Specifically, three of 6 tested pancreatic cancer cell lines have sensitivity comparable to that of the human glioma cell line U251 (Fig. 1A),

which has been shown, in previous study, to be a sensitive cell line to Delta-24-RGD *in vitro* and *in vivo* (19). One primary pancreatic cancer cell line, MDA-PATC53, which was established in our laboratory from a liver metastasis of pancreatic cancer (15), was particularly sensitive to Delta-24-RGD (Fig. 1A and C). Because most of metastatic pancreatic cancer cells are usually resistant to chemotherapies, this result suggests that Delta-24-RGD might be a promising agent for pancreatic cancer therapy. Importantly, a telomerase reverse transcriptase (TERT)-immortalized human pancreatic duct epithelia cell line, HPNE-tert, was not sensitive to Delta-24-RGD, suggesting that Delta-24-RGD may not be toxic to normal pancreatic cells.

In this study, two conventional cell lines, BxPC3 and Hs776T were not sensitive to Delta-24-RGD, and these two cell lines, based on previous studies, have wild type of p16 gene (30). However, the genetic status of p16 seems not to be the principle driver of cell sensitivity to Delta-24-RGD, since p16 was very low in almost all tested cells lines (Fig. 2B). Likewise, RB expression was not found to be the determinant of sensitivity, although all the sensitive cells have low expression of RB (Fig. 2B). These results suggest that although Delta-24-RGD was originally engineered to preferably replicate in cancer cells with abnormality in p16/RB pathway, genetic or molecular changes of other factors might also affect the sensitivity of cancer cells to Delta-24-RGD. We found that among the tested components of p16/RB pathway, the expression level of Cyclin D1 was most correlated with the sensitivity of PDAC cells to Delta-24-RGD. Cyclin D1 can bind to and activate CDK4/CDK6, which phosphorylates RB protein and promotes the release of E2F and cell cycle progression. This pathway is negatively regulated by p16, however, in cells with deletion or suppressed expression of p16, Cyclin D1 becomes the key driver for the activation of this pathway. This could explain why, in our study, high expression of Cyclin D1 was closely linked to Delta-24-RGD sensitivity. Considering that about 40–80% of pancreatic cancer have Cyclin D1 overexpression or gene amplification (31, 32), we speculate that Delta-24-RGD could be active in a large fraction of pancreatic cancers.

Autophagy or autophagy-mediated apoptosis are a described mechanism of oncolytic virus induced cytotoxicity (19). In our study, we used multiple approaches, including autophagy reporter LC3-GFP, Acridine Orange staining, and Western blotting to confirm autophagy induction by Delta-24-RGD in pancreatic cancer cells (Fig.3). Exposure of PS on enveloped viruses or on the vesicular membrane of unenveloped viruses, a strategy called apoptosis mimicry, is a described method of virus infection (33). In addition, some viruses induce PS exposure in infected cells, opening the possibility of using anti-PS antibodies as antiviral therapy (10). However, the mechanism of virus induced PS exposure in host cells has not been well studied. We found that Delta-24-RGD induces PS exposure in both sensitive and resistance cells (Fig. 4). We speculate that oncolytic virus-induced PS exposure could be caused by specific viral genes, as in reports of E1B 19 Kd protein-induced apoptotic mimicry in host cells in order to modulate innate immune responses (34). Alternatively, PS exposure could be related with metabolic changes induced by oncolytic virus (35). Further study is necessary to determine the mechanism of adenovirus-induced PS exposure in order to develop further rationale treatment combinations with virus-mediated gene therapy.

PS exposure induced by oncolytic viruses can help the clearance of infected cells by immune system. This offers an opportunity for combination therapy with PS-targeting antibodies. PS targeting antibodies, including Bavituximab and 1N11 have been evaluated in metastatic breast and lung cancers in phase 1 clinical trials showing promising results (12, 13). In this study, we tested the combination of Delta-24-RGD and 1N11, a fully human PS targeting antibody similar to Bavituximab, by using a sequential combination strategy. The major consideration for this sequential combination strategy is that PS targeting antibody may suppress virus production and spreading if given concurrently. In a mouse pancreatic cancer model, sequential combination of Delta-24-RGD and 1N11 was superior to each single treatment alone (Fig. 5B). Mechanistically, combination treatment induced macrophage-mediated-anticancer immune response, which was evidenced by increased staining of microphage marker, CD68, in the tumors treated with single and combination regimen. Stimulating of differentiation of M2 tumor associated microphage (TAM) to M1 TAM by PS targeting antibody as has been reported previously (26, 28). In addition, modulating anticancer immune response by Delta-24-RGD has been observed in patients with glioblastoma and in immunocompetent mouse brain tumor models (7, 36–38). Notably, single treatment with Delta-24-RGD also induced strong anticancer effects, which may involve direct tumor lyses, autophagy as well as immune responses, since the nude mice used for the tumor model keep nature killer cells and macrophages (39). Nevertheless, more pancreatic tumor animal models, including immunocompetent mouse models, might be used to evaluate the anticancer effects induced by Delta-24-RGD as a single agent and in combination with other agents, such as PS targeting antibodies.

In summary, in this study, we evaluated the anticancer activity of oncolytic virus Delta-24-RGD in pancreatic cancer cells *in vitro* and in preclinical animal models *in vivo*. We demonstrate that Delta-24-RGD is active against pancreatic cancer cells, and Cyclin D1 expression is associated with the vulnerability of pancreatic cancer cells to oncolytic adenovirus treatment. In addition, we demonstrate Delta-24-RGD induced PS exposure, and combination of PS targeting antibody and Delta-24-RGD viral therapy induced enhanced antitumor activity. Further studies are needed for full understanding of the mechanism of oncolytic virus induced cytotoxicity, PS exposure, and oncolytic virus induced immune response in order to develop rational combination therapies as well as predictive biomarkers for patients' selection. Nevertheless, our study demonstrates that Delta-24-RGD is a promising agent alone or in combination, for the treatment of pancreatic cancer, a devastating disease with limited therapeutic regimens available. Given that Delta-24-RGD and PS targeting antibodies are being evaluated in clinical trials and have favorable toxicity profile, combination of these two agents could be quickly translated into clinical testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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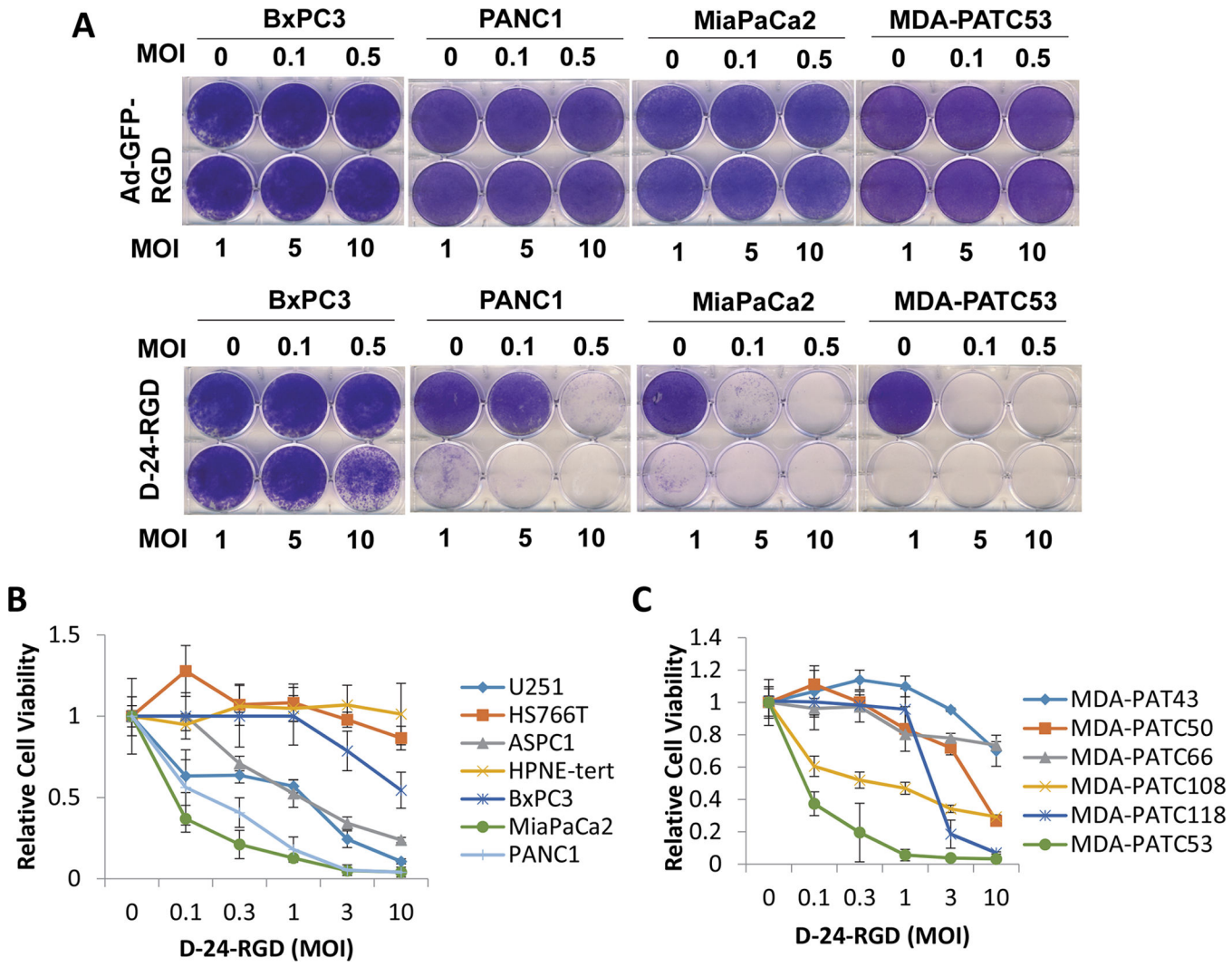

Figure 1.

Oncolytic virus Delta-24-RGD (D-24-RGD) induced cytotoxicity in pancreatic cancer cells. (A) Cytotoxic crystal violets staining. Cells ($1X10^4$) were seed in 6-well plate and infected with D-24-RGD or Ad-GFP-RGD control at different MOIs (multiplicity of infection), then 10 days post infection, cells were fixed with 4% formalin and stained with Crystal violets. (B-C) MTT cell viability assays in multiple conventional and primary pancreatic cancer cell lines. Cells were seed at $1X10^3$ in 96 well plates and infected with D-24-RGD with indicated MOIs, then 10 days after infections, cells viability were analyzed with MTT assay.

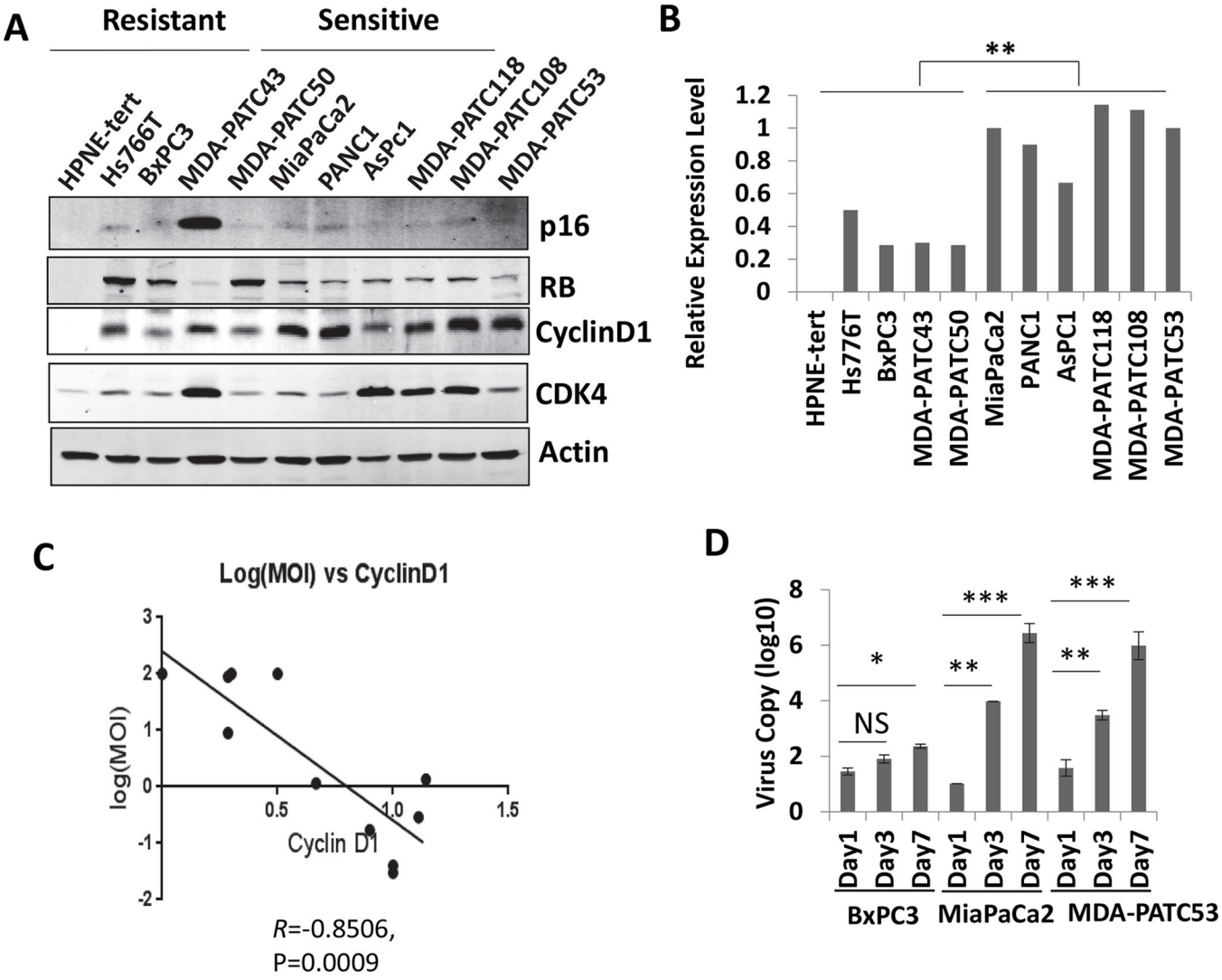

Figure 2.

CyclinD1 expression is associated with the sensitivity of pancreatic cancer cells to Delta-24-RGD (D-24-RGD). (A) Western blotting assay. The expression of the major components in p16/RB pathways, including p16, RB, Cyclin D1, and CDk4 were analyzed with western blotting. (B) The relative expression level of Cyclin D1 in resistant and sensitive pancreatic cell lines was analyzed using the ratio of the density of Cyclin D1 over beta-Actin. (C) The correlation of Cyclin D1 expression with cells' sensitivity was analyzed with Pearson correlation. (D) D-24-RGD virus replication. D-24-RGD virus replications in the cells after infection were analyzed using PCR-based assay to check virus copy number in BxPC3, MiaPaCa2 and MDA-PATC53 cells as described in the method section. NS, not significant; *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

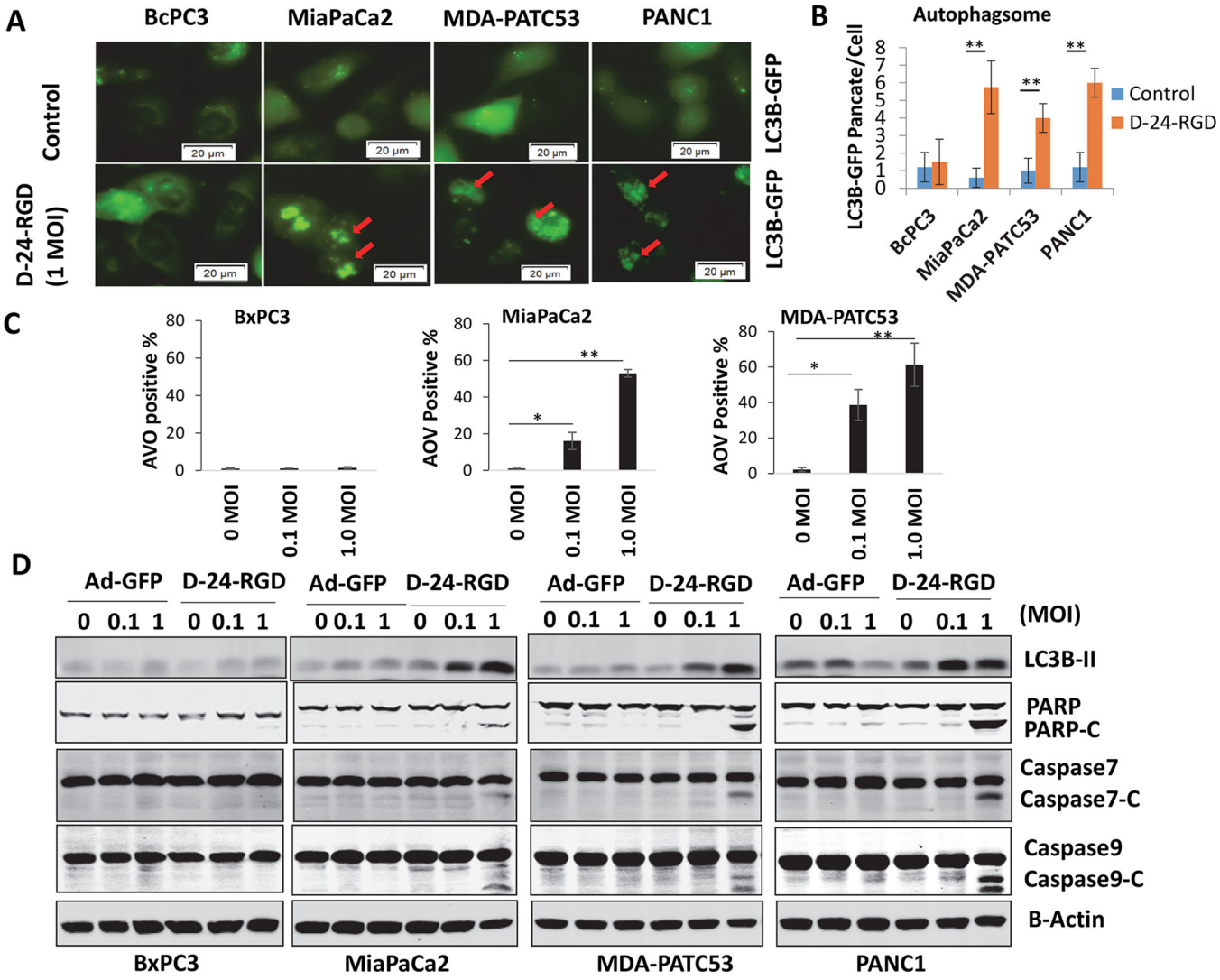

Figure 3.

Delta-24-RGD (D-24-RGD) induced autophagy in pancreatic cancer cells. (A) Autophagy assay with LC3B-GFP fusion protein. Cells were infected with D-24-GDR (1MOI) for 5 days followed by infection with LC3-GFP expression baculovirus for 24 hours, and then LC3B-GFP punctae (arrow indicated) were checked under microscope. (B) Quantification of LC3B-GFP punctae. Punctae in 10 random views were counted under microscope. Results are mean +/− SD, **, p<0.01. (C) Acridine Orange staining. Cells were infected with D-24-RGD virus at different MOIs, and 5 days post infection, cells were harvested and stained with acridine orange followed by flow cytometric analysis; AVO, acidic vesicular organelle; results are mean +/− SD, *, p<0.5; **, p<0.01. (D) Western blotting assay. Cells were infected with or Ad-GFP-RGD (Ad-GFP) control or D-24-RGD for 5 days, and cell lysates were harvested with RIPA buffer for western blotting assay using the indicated antibodies.

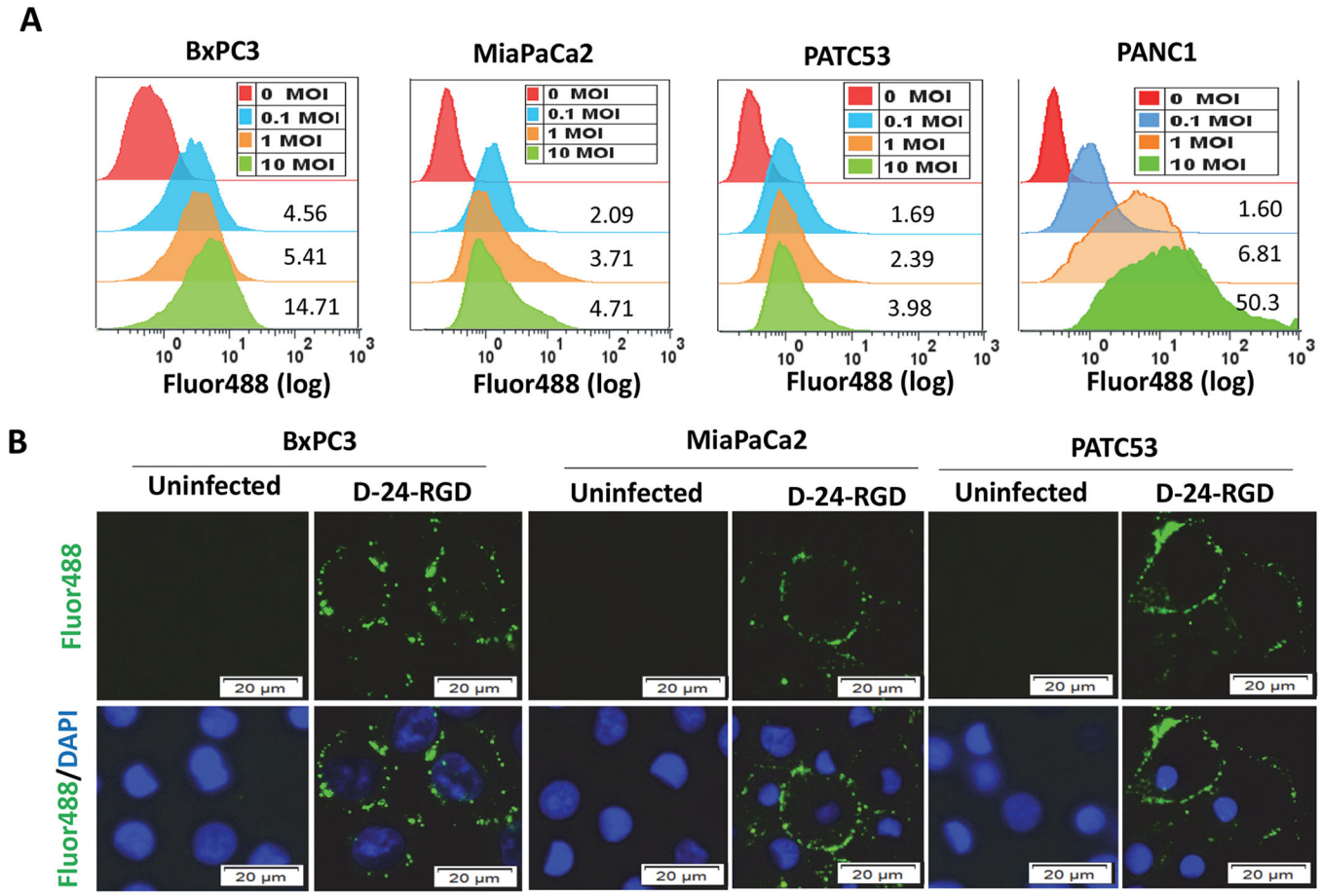

Figure 4.

Delta-24-RGD (D-24-RGD) induced PS exposure in pancreatic cancer cells. (A) Flow cytometry analysis of PS. Cells were infected with D-24-RGD in 6-well plates for 5 days, and cells will be harvested and stained with PS antibody and Alexa Fluor-488-conjugated second antibody followed by flow cytometry analysis. (B) Microscopic analysis of PS exposure. Cells were grown and infected with D-24-RGD (1MOI) in chamber slide for 3 days. Cells were fixed with 4% formadehyde and stained with PS antibody. The cells were permeabilized with 0.1% Triton X-100, and the nuclei were stained with Hoechst 33342. Images were taken under fluorescence microscope (40X, bar=50 μm).

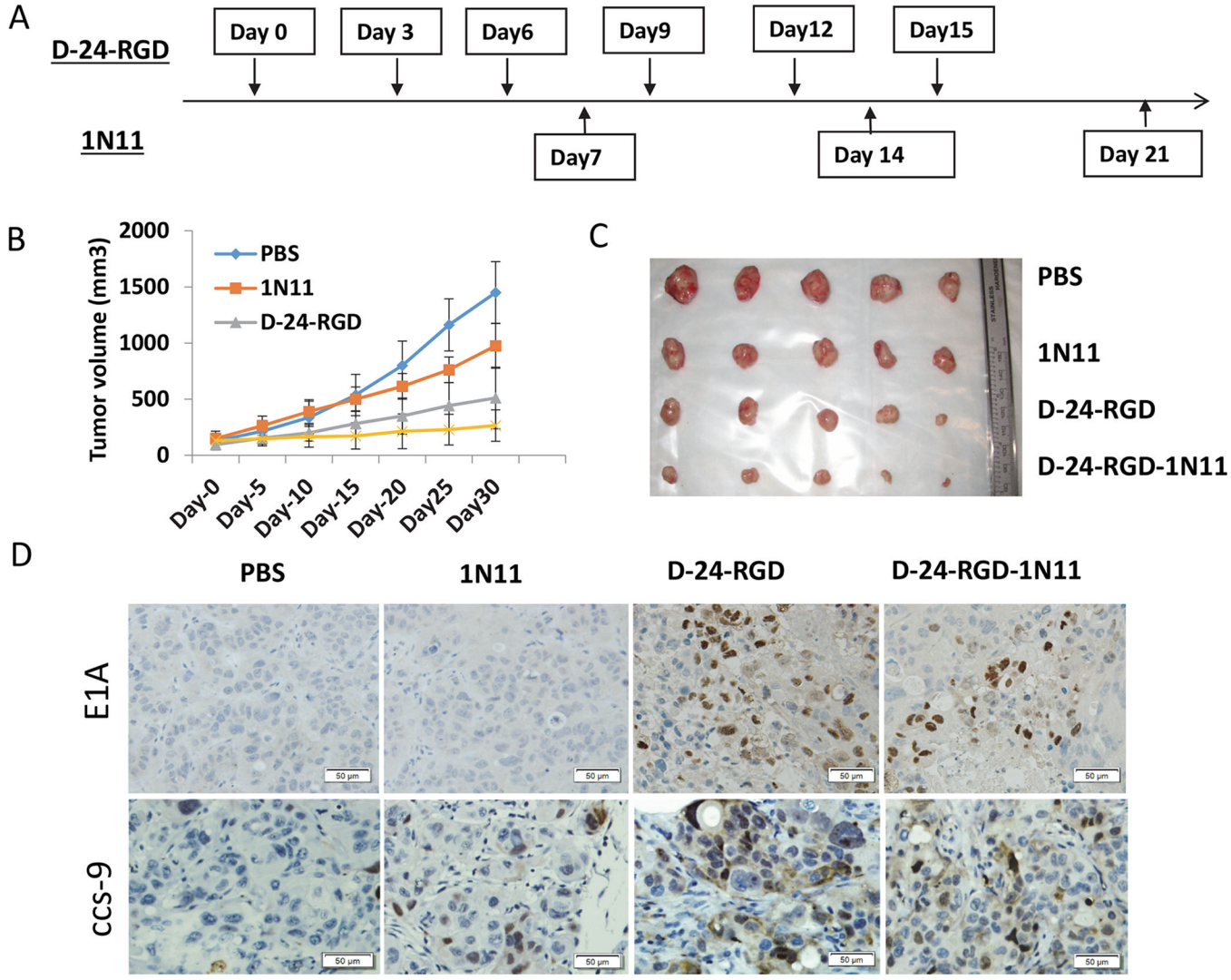

Figure 5.

Evaluation of the combination of Delta-24-RGD (D-24-RGD) and 1N11 *in vivo*. (A) The schedule of administration of D-24-RGD and 1N11 antibody. When tumor reach the size of about 100 mm3, mice were divided into 4 treatment groups and treated with D-24-RGD, 1N11, alone or in combination as indicated schedule. (B) Tumor growth curve. Tumor sizes were measured with caliper every 5 days and calculated with the formula of dxl2x0.52. Two-way ANOVA was used for significance between two treatment groups. (Combo vs 1N11, $p<0.0001$; combo *vs* D-24-RGD, $p<0.01$; 1N11 *vs* control, $p<0.01$; D-24-RGD *vs* control, $p<0.0001$) (C) Tumors at the end of the experiments were harvested. (D) immunohistochemical staining were performed in tumor tissues with antibodies against adenovirus E1A.

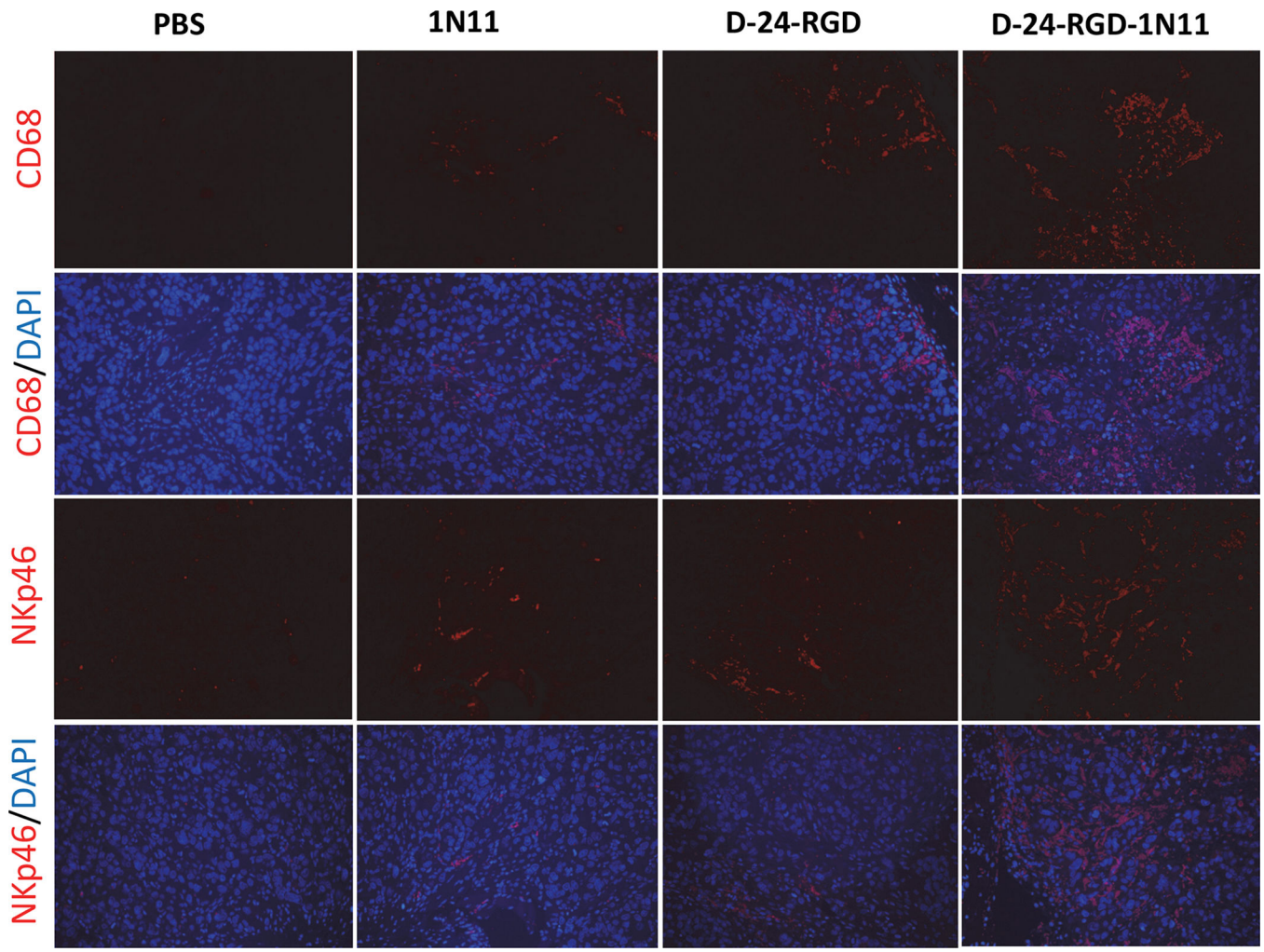

Figure 6.

Combination of D-24-RGD and 1N11 enhanced antitumor immunoresponse. Immunofluorescence staining was performed with tumor tissues from treated or untreated mice. Microphage marker CD68 and NK cell marker NKp46 were used to check microphage infiltrations in tumor tissues. Image were taken under fluorescence microscope (20x).