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14. ABSTRACT Prostate cancer is the most commonly diagnosed non-skin carcinoma, and one of the leading causes of cancer-related deaths in North American men. Presently there are no curative therapies available for advanced metastatic prostate cancer. Oncolytic viral therapy provides an opportunity to efficiently kill primary and metastatic cancer cells while sparing normal cells. Vesicular Stomatitis Virus (VSV) is an oncolytic virus which is able to replicate in cells with a defective interferon (INF) response. Here, we examined the effect of a mutated VSV (AV3), which expresses luciferase and has an enhanced INF-sensitivity, on the viability of prostate tumours that develop in prostate-specific PTEN null transgenic mice. Prostates of PTEN knockout and control mice were injected with 5x10 ⁸ pfu/ml of VSV(AV3) and monitored for luminescence over a 96h time period using the IVIS-Xenogen machine to track the virus distribution. Plaque analyses for live virus in tissues extracted at various time points revealed that VSV(AV3) predominantly replicated in the prostates of transgenic PTEN knockout mice. Additionally, using TUNNEL staining of paraffin embedded tissues, we demonstrated that VSV(AV3) is capable of selectively infecting and killing malignant prostate cells while sparing normal cells. This cancer-specific cell death was not due to infiltration of neutrophils into the prostate tumours of PTEN null mice as has been reported for other tumour models. However, there was an increase in macrophage and B-lymphocyte infiltration into the prostates of PTEN null mice compared to control mice. In conclusion, VSV(AV3) is able to replicate and selectively kill the prostate cancer cells that develop in the PTEN null mouse and hence prove clinically useful for treating locally advanced prostate cancer while sparing normal prostate tissue.						
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Introduction:

Prostate cancer is the most prevalent malignancy and the second leading cause of cancer related mortality in North American men. It is estimated that this year over 186,000 men will be diagnosed with prostate cancer and approximately 28,000 will die of this disease. Approximately half of all men with clinically localized disease are not cured by surgery or radiotherapy (1, 2) Survival prospects of patients with locally advanced and metastatic prostate cancer are low and current therapies are riddled with harsh side effects that cause a decrease patient's quality of life (1-5). The high incidence of prostate cancer in men over 50, in addition to the rapidly aging North American male demographic, highlights the need to make the management and treatment of this cancer a priority (6).

In recent years, oncolytic viruses have been tested in various clinical trials (7, 8) for their anti-tumor targeting capabilities, as they are often able to exploit tumor specific genetic defects (9-11). Some of these viruses are fully replication-competent and rapidly spread through and kill cancerous cells. Vesicular Stomatitis Virus (VSV) is an oncolytic virus which can infect and kill cells which have defects in their cellular anti-viral immune response (12-14). One of the key cellular antiviral immune reactions involves the interferon (IFN) response pathway (13). Typically most cancer cells display a faulty interferon (IFN) response and therefore are susceptible to infection by VSV. Previous studies have demonstrated that selectivity of VSV for malignant cells in comparison to normal cells can be enhanced either by pre-treatment with IFNs or by using a mutated form of VSV- Δ M51 (14); referred to as VSV(AV3), which better induces IFN production in infected cells. It has been shown that deletion of methionine at position 51 in the matrix (M) protein of VSV renders it more susceptible to host innate immune response. This enhanced susceptibility is important since the virus is safely cleared from normal cells but destroys malignant cells, thereby making VSV (AV3) more cancer specific (14-17).

Currently there are a variety of tumor models, principally transplantable human xenografts, and transgenic mouse models that can be used to test new strategies for prevention and treatment of prostate cancer. Each type of model has clear advantages and disadvantages. Some of the advantages of using a transgenic mouse model for pre-clinical testing are that the cancer arises *in situ* in the target tissue with the appropriate microenvironment, that the mice possess intact immune systems, that the cancers are frequently heterogeneous, and that arising *de novo*, they undergo neoplastic progression similar to that seen in human cancers (18-20).

In this study, we used the prostate specific PTEN (phosphatase and tensin homolog deleted on chromosome 10) null transgenic mouse as a model system to test the therapeutic effects of the AV3 strain of VSV. Deletion or mutation of the tumor suppressor PTEN gene has been implicated in many human cancers (19-22), and has been seen in up to 30% of primary prostate cancers and over 64% of prostate metastases, making PTEN an important candidate gene for prostate cancer development and progression. Not surprisingly, selective deletion of PTEN in mouse prostate leads to *de novo* formation of prostate tumors. The progression of prostate specific PTEN null mice to prostate cancer is very similar to that seen in the humans (19, 20).

The goal of this study was to determine whether VSV(AV3) can infect and kill the prostate tumors of PTEN null mouse. Our results indicate that this virus is able to selectively infect, replicate, and increase apoptosis in malignant tissue while sparing normal tissue. This occurs through a mechanism involving direct oncolysis rather than neutrophil infiltration as has been previously reported in other cancer models (20).

OVERALL HYPOTHESIS:

The overall hypothesis of this research project is to create a viral based gene therapy that will target and destroy malignant prostate cells while sparing normal cells.

MODIFICATION TO SPECIFIC AIMS AND RESONS:

In the original proposal the specific aims were as follows:

- Aim 1.** Construct viral vectors that have exogenous cellular promoters and an eIF4E UTR recognition sequence. This will enable us to test differential tumor expression of a therapeutic protein.
- Aim 2.** Test these vectors for prostate and tumor specific targeting *in vivo* utilizing prostate-restricted PTEN knock-out mice.
- Aim 3.** Enhance the targeting of both lentiviral and VSV viral vectors to prostate cells by modifying envelope proteins of these viruses.

Why modify the specific aims:

As previously stated (in original grant proposal), the lentivirus system is used as a proof of principle for prostate tumor targeting. Lentiviruses propagated in the laboratory are replication incompetent to ensure safety for viral handling. Additionally, during their life cycle, lentiviruses integrate randomly into host genome, which can lead to other unpredictable phenotypes. These shortcomings of the lentivirus system hinder its usage as a gene therapy vehicle in the clinical setting. Alternatively, VSV is a replication competent oncolytic virus capable of enhanced infection of cancer tissue. Furthermore, VSV seldom infects humans and in rare cases of infection there is only flu-like symptoms (35, 36). This makes VSV, especially VSV (AV3) (the more IFN sensitive strain) a better therapeutic candidate.

Thus, a closer look at our original specific aims revealed that prior to further modification of the VSV, we first need to make sure that this virus is capable of targeted infection in our prostate specific PTEN null *in vivo* model. For this reason we modified our aim to determine whether VSV (AV3) can infect and selectively kill the prostate tumors of PTEN null mice while sparing control prostate tissue. Having said this we are still interested and will be perusing aim 2 with lentivirus, but due to limitations of propagating the transgenic mice, we initially decided to address the effect of VSV (AV3) on the prostates of tumor bearing PTEN knockout mice in comparison to control mice.

RESULTS:

The effect of mutant VSV (AV3) on cytotoxicity of prostate cancer cells *in vitro*.

The MTS assay was used to assess the effect of the VSV (AV3) on cell viability of prostate cells propagated *in vitro*. Three cell lines, two human cell lines representing normal human prostate epithelial (RWPE-1) and human prostate cancer cells (LNCaP) as well as a mouse cell line (MPPK-1) derived from prostatic tissue of PTEN transgenic mice, were tested. The results demonstrated that there was a parallel relationship between cell death and increasing viral titer over a 24h period for all three cell lines. However, VSV (AV3)'s induction of cell death at a given viral titer was generally significantly higher in MPPK-1 and LNCaP cells compared to RWPE-1 normal prostate epithelial cells, suggesting preferential killing of cancer cells by this virus (Fig.1A).

VSV is known to be sensitive to IFN and the mutant AV3 strain has been shown to be even more IFN-sensitive (14-16). To test this in our system, the three cell lines (LNCaP, RWPE-1 and MPPK-1) were pre-incubated for 16h with different amounts of universal type I IFN prior to being challenged with VSV(AV3) (10^8 pfu/ml). Pre-incubation of cells with (10,000 IU/ml) IFN led to increased cell viability in approximately 35% of RWPE-1 non-malignant cells. However, in MPPK-1 and LNCaP prostate cancer cells, there was little or no difference in cell death followed pre-treatment with INF (Fig. 1B). This demonstrated that mutant AV3 strain of VSV is highly sensitive to IFN and in cells with intact an IFN pathway, such as RWPE-1 cells.

Viral distribution after intra-prostatic injections of VSV (AV3) virus into PTEN null and control mice.

In vivo studies were performed to determine the viral distribution of VSV (AV3) in both prostate-specific PTEN knock out transgenic mice and control mice (30). Tumor bearing PTEN knock out and control mice were injected intra-prostatically with 5×10^8 pfu/ml of VSV (AV3). Monitoring of the viral distribution was carried out by intra-peritoneal (i.p.) injection of luciferin followed by bioluminescence measurement over a 3 to 96 hour time period (Fig. 2C). Notably, after VSV(AV3) infection of tumor bearing mice, bioluminescence was sustained up to 72h post injection (Fig. 2B), considerably longer than in control mice, which displayed a signal only up to 24-48h post infection (Fig. 2A). Additionally, mice had much higher levels of bioluminescence compared to control mice. These observations indicate a greater initial infection and a higher containment of VSV (AV3) infection in the tumor-bearing mice relative to control mice. Systemic administration of VSV (AV3) by intra-venous (i.v.) injection showed that the virus was sequestered in the spleen in 100% of animals tested in both control and tumor bearing animals (data not shown). Although the virus was visualized by bioluminescence in approximately half of the prostate tumors, the amount of virus was highly diluted and barely detectable. However, by injecting virus directly into the prostate tumors, there was an apparent amplification of viral load. Additionally, even though VSV (AV3) was administered intra-prostatically, there was a broad distribution of virus throughout the body (Fig. 2A and 2B).

Presence of live virus in prostatic tissue of PTEN null mice.

To determine whether the bioluminescence data correlated with the presence of live virus, mice at each time point were sacrificed and their organs (kidney, liver, lung, prostate and spleen) were harvested. Homogenized tissues were titrated and plaque assays (23) were performed in order to quantify viral delivery and replication within various organs of PTEN null and control mice (Fig. 3). In both PTEN null and control mice, ultraviolet inactivated (UVI) treated virus, which is dead virus, was used as control.

With in 3h after VSV (AV3) injection, in PTEN null tumor-bearing mice (Fig. 3A) viral titer was highest in prostate (13.0 ± 1.53 log Pfu/g) followed by spleen (4.3 ± 0.67 log Pfu/g) and lung (2.3 ± 0.33 log Pfu/g). Live virus in lung reached its highest point (3.7 ± 0.88 log Pfu/g) at 24h post intra-prostatic injection, while in spleen the viral titer declined and was no longer detectable by 48h. Viral titer in prostate at 24h post infection was slightly reduced (11.7 ± 0.89 log Pfu/g) but dramatically increased at 48h (30.3 ± 1.45 log Pfu/g), suggesting amplified viral replication in the prostate tissue. After 72h, there was a marked decline of VSV (AV3) live virus (1.30 ± 0.33 log Pfu/g) in the prostate. In the liver and kidney, there were negligible levels of virus detected at all time points, suggesting that these organs were not readily infected.

In comparison to the PTEN-null mice, in control mice (Fig.3B), initially VSV (AV3) was present in both prostate (9.52 ± 0.89 log Pfu/g) and spleen (8.67 ± 1.67 log Pfu/g). However by 24h, infection is only sustained in the prostate tissue (9.13 ± 1.76 log Pfu/g). This infection in the prostate declined at 48h (4.37 ± 1.2 log Pfu/g) and further declined at 72h (1.87 ± 0.88 log Pfu/g) until eventually no virus detected. This decline in prostate infection is seen earlier in control mice in comparison to tumor bearing PTEN null mice.

These results demonstrate that there was enhanced selectivity and persistence of VSV (AV3) infection of tumor-bearing PTEN null mice prostates relative to control mice, after treatment with the same viral input (5×10^8 pfu/ml). This plaque assay was validated using real time quantitative PCR analysis of viral RNA in the organs of both PTEN null (Fig 3C) and control mice (Fig 3D) relative to UVI VSV (AV3) treated tissues.

VSV (AV3) causes preferential killing of prostate cancer cells *in vivo* while sparing normal prostate tissue.

The difference between levels and persistence of virus in the prostates of PTEN null and control mice led us to assess whether the virus was preferentially killing infected tumor cells. To test this, VSV (AV3) infected prostate tissue from both PTEN null and control mice were collected at various time points, embedded in paraffin and evaluated for apoptosis by TUNEL assay (Fig. 4).

Fig. 4 shows an example of TUNEL stained prostates from both PTEN null and control mice. There was a dramatic increase in apoptotic cell bodies observed in the prostates of VSV (AV3)-treated PTEN null mice compared to controls (Fig. 4B). This increase in apoptosis in the tumor-bearing mice correlated with increased viral titer (Fig. 3); suggesting that active infection with oncolytic VSV (AV3) is the cause of this increased apoptosis. Very little apoptosis was observed in PTEN null mice treated with UV-inactivated virus.

In vivo cancer cell death is not associated with neutrophil infiltration in PTEN null mice prostates.

Previous reports on VSV infection using a non-transgenic mouse model indicated that viral delivery was blocked by vasoconstriction and that cell death was associated with neutrophil infiltration (23), suggesting that tumor cell death was a consequence of a neutrophil-mediated immune response rather than viral oncolysis. To test whether this was also the case in our endogenous transgenic prostate cancer model, prostate tissue was collected post-VSV (AV3) infection at various time points, stained for the presence of a repertoire of host immune cells, and then evaluated by a pathologist (Fig. 5).

Fig. 5A shows that in both PTEN null and control mice prostates, there was an initial rise in neutrophil cells as compared with prostates treated with UV-inactivated virus. However, this peak in neutrophil penetration at the 6h time point was not significantly different between tumor bearing (23.00 ± 6.03 cells/time point) and control mice (30.00 ± 6.81 cells/time point) and that this peak quickly diminished by 24h in both control (9.67 ± 2.91 cells/time point) and PTEN null mice (9.33 ± 3.53 cells/time point). These results suggest that the differences seen in apoptosis (Fig. 4) are not correlated with neutrophil infiltration (Fig. 5A).

VSV (AV3) infection leads to an increase in macrophage and B-lymphocyte response.

When prostate tissues of PTEN null and control mice were stained for the presence of macrophages, which like neutrophils are phagocytic, there was a marked increase in their number at 24h (78.67 ± 21.33 cells/time point) and at 72h (72.30 ± 27.67 cells/time point) in PTEN null mice, but an almost undetectable and unchanged number of neutrophils was detected in the control prostate tissue. This macrophage increase may reflect an increase in dead cells initially after 6h and again at 48h post infection at which time macrophages are recruited to carry out phagocytosis (Fig 5B). It has been documented that VSV infection leads to activation of the innate immune response (17). VSV (AV3) infected prostate tissues were thus also, stained for both B-lymphocytes (Fig 5C) and T-lymphocytes (Fig. 5D). There is a significant increase in B-lymphocyte count in CRE (+) prostate tissue (39.67 ± 7.27 cells/time point) at 48h, while there is no marked increase in control CRE (-) at the same time point (7.0 ± 1.0 cells/time point). Also, there is an increase in T-cell infiltration in prostates of both tumor bearing PTEN knock out and control mice. However, there is no significant difference between T-cell infiltration in prostate tissue of PTEN null and control mice.

In vivo effects of VSV (AV3) on IFN receptor-1 alpha (IFNR-1 α).

Since VSV infection leads to activation of the IFN response pathway expression, prostates of control and PTEN null mice challenged with VSV (AV3) infection were immunohistochemically stained for Interferon Receptor 1 alpha (IFNR1 α). As predicted, in normal control mice the IFN response pathway is intact and as the infection period increases, so does the level of IFNR-1 α to protect the host cells against VSV infection and oncolysis. However, PTEN null mice did not display an increase in IFNR-1 α , suggesting that there is a faulty IFN mechanism in these PTEN null, tumor bearing prostate tissues. This permits further viral infection of PTEN null prostate tumor cells and subsequently increased cell death due to viral oncolysis (Fig. 6 A and B).

KEY RESEARCH ACCOMPLISHMENTS:

1. *In vitro* infection with VSV (AV3) leads to cancer specific cell death.
2. Cancer cell lines, MPPK-1 and LNCaP, display a faulty IFN response. This is evident since pre-incubation of these cells with IFN prior to VSV (AV3) challenge does NOT protect them from cell death.
3. Intra-prostatic injections of VSV (AV3) leads prolonged infection of the prostate in PTEN null mice. However, this infection is not as extensive in control mice. Both qPCR analyses for presence of virus and plaque assay demonstrates presence of live virus in prostate of PTEN null mice as compared to control.
4. Plaque assay also demonstrates that there is an amplification of virus seen in the prostates of tumor bearing PTEN null mice.
5. TUNNEL staining of prostate tissue shows increased levels of apoptotic bodies in the prostate tissue of tumor bearing PTEN null mice. In contrast, there is almost no apoptotic bodies visualized in control mice.
6. Cellular immunity seems to be predominantly due to Macrophage infiltration and increase in B-lymphocytes in tumor bearing PTEN null mice.
7. IFN receptor alpha staining showed no change in activity upon infection in the tumor bearing mice. However, in control prostates there is an increase in IFN α receptor levels post VSV (AV3) injection.

REPORTABLE OUTCOME:

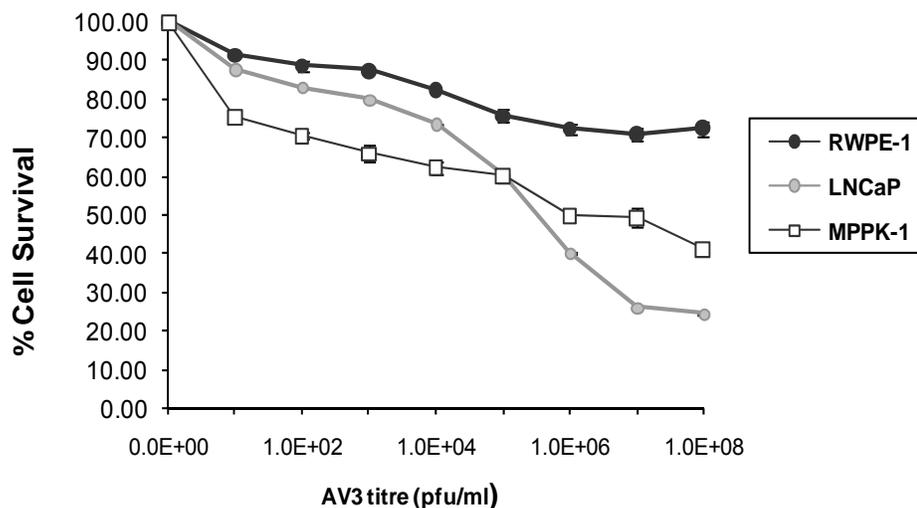
1. A summary of the above data has been presented in the following meetings in chronological order:

#	Conference Name	Date	Location	Type of Presentation
1	American Association for Cancer Research (AACR)	April 12-16 (2008)	San Diego, CA (USA)	Poster
2	4th PacRim Breast and Prostate Cancer Meeting	August 12-16 (2008)	Whistler, British Columbia (Canada)	Poster
3	The 5th International Meeting on Replicating Oncolytic Virus Therapeutics	March 18-21 (2009)	Banff, Alberta (Canada)	Oral
4	Bridging the Gulf between BC Cancer Clinicians and Scientists	April 3-5 (2009)	Squamish, British Columbia (Canada)	Oral
5	American Association for Cancer Research (AACR)	April 18-22 (2009)	Denver, CO (USA)	Poster

2. Manuscript in preparation for journal of “Cancer Research” to be submitted in May/June 2009.
Maryam Moussavi, Ladan Fazli, Howard Tearle, Yubin Guo, Michael Cox, John Bell, Christopher Ong, William Jia, and Paul S. Rennie. “Infection with Vesicular Stomatitis Virus (VSV) leads to differential cell death in prostate tumors of transgenic PTEN knockout mice”

FIGURES:

(A)



(B)

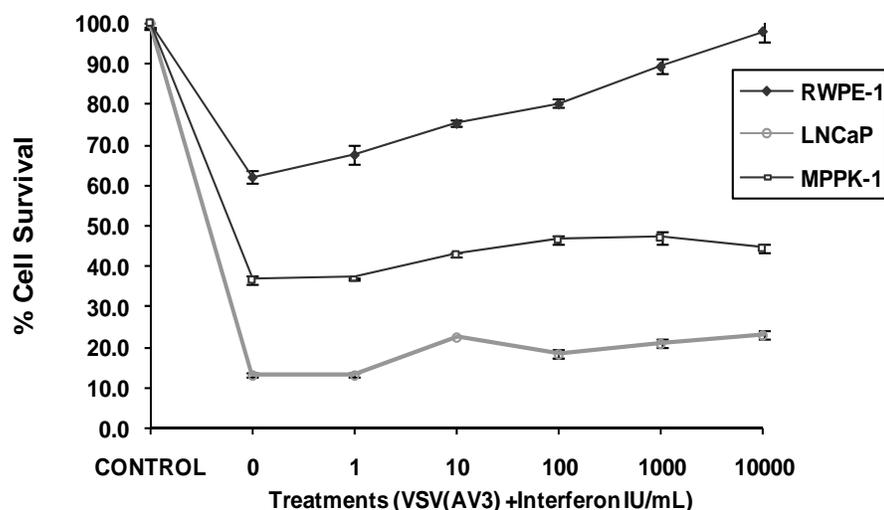


Figure 1. (A) Effects of VSV (AV3) infection on cell survival. Cells were infected with increments of viral titer from 0 to 10⁸ pfu/ml. MTS assays were performed 24h after viral infection. (B) Effects of universal type I IFN on cell survival of prostate epithelial control and neoplastic cell lines. Cells were pre-incubated with increasing concentrations of IFN, from 0-10,000 IU/ml, for 16h and then challenged with 10⁸ pfu/ml of VSV (AV3) for 24h. Cell viability was measured by MTS assays 24h post infection. Each graph is representative of three independent experiments with five replicates.

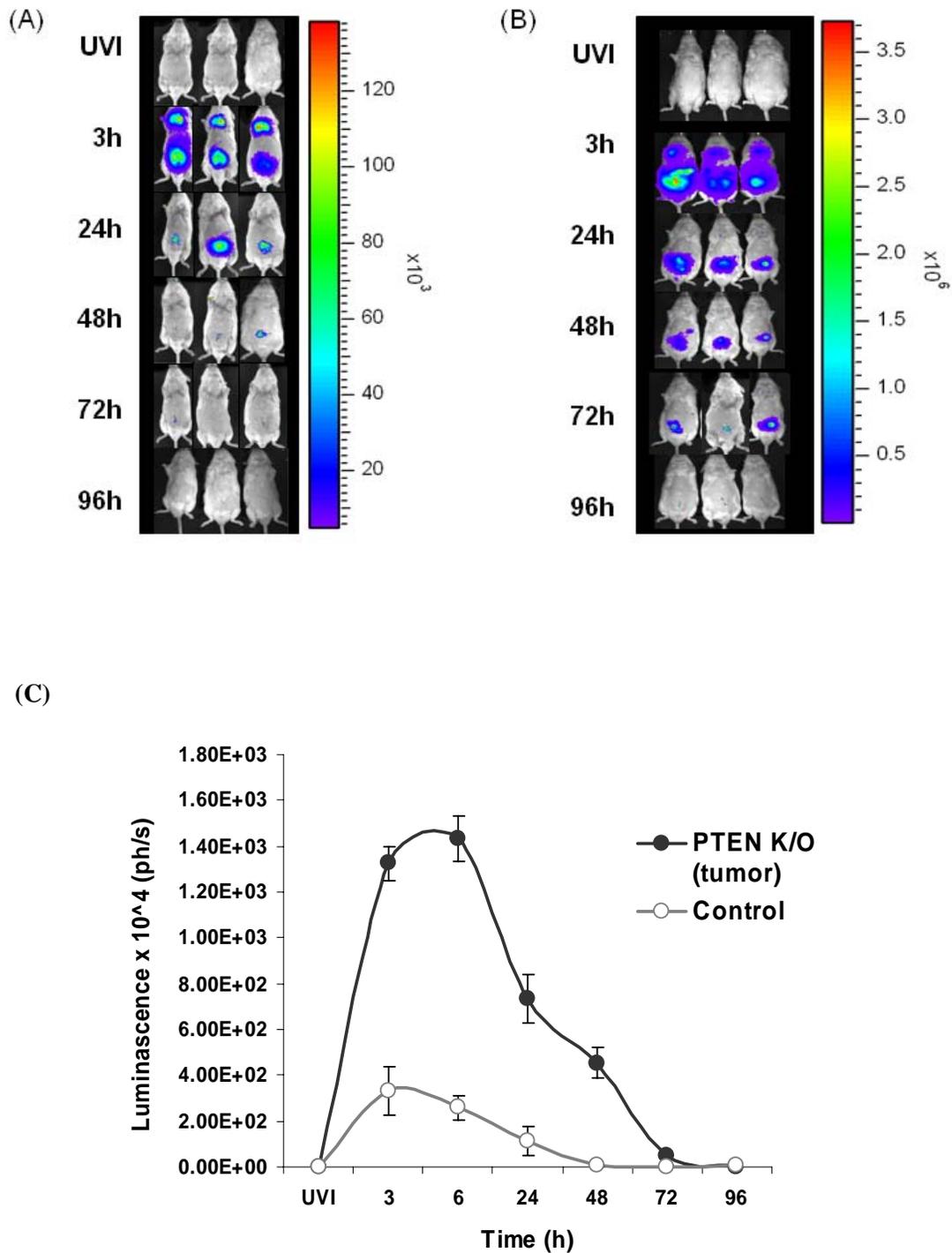
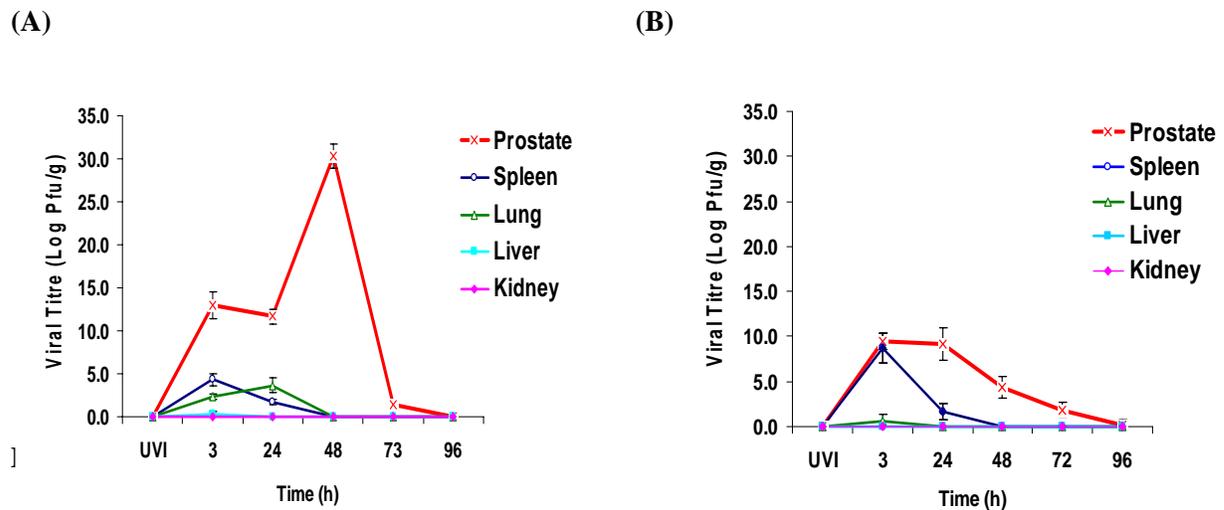


Figure 2. Measurement of viral distribution in the mice body after viral injection. 5×10^8 pfu/ml of VSV(AV3) were injected directly into prostates of control (A) and PTEN null (B) mice. Viral distribution was tracked by i.p. injections of luciferin (150mg/kg) over time (3-96h). (C) Bioluminescence was recorded using Xenogen-IVIS 100 imaging system and average bioluminescence of each time point in prostates of transgenic PTEN null and control mice were graphed. Data demonstrated as average \pm standard error, n=3.



(C)

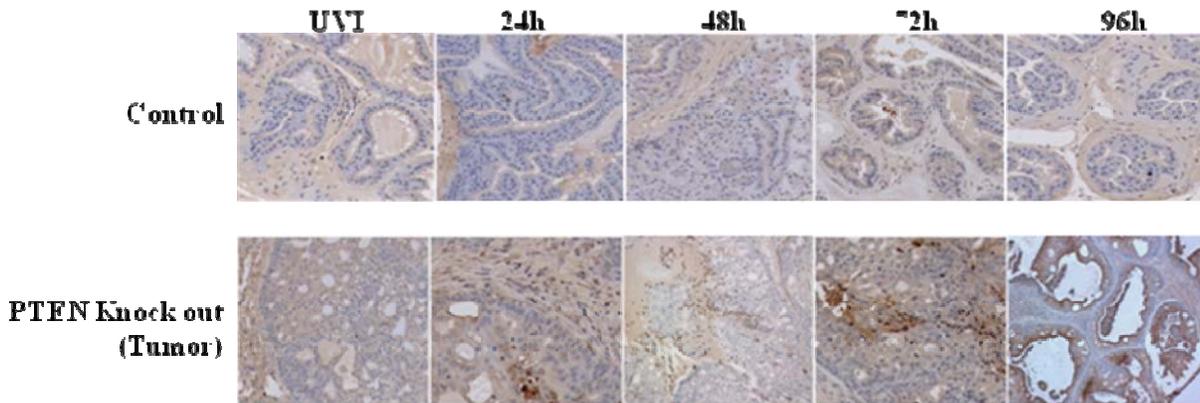
PTEN k/o Tumor bearing Tissues	UVI	3h	24h	48h	72h	96h	p-Value
Kidney	1 ± 0.00	0.50 ± 0.02	0.48 ± 0.03	0.48 ± 0.07	0.40 ± 0.13	0.61 ± 0.06	p=0.0007
Liver	1 ± 0.00	4.49 ± 0.69	1.04 ± 0.58	2.147 ± 0.89	1.119 ± 0.32	1.15 ± 0.45	p= 0.0053
Lung	1 ± 0.00	2.98 ± 1.25	6.67 ± 0.80	1.60 ± 0.26	0.609 ± 0.21	0.481 ± 0.08	p<0.0001
Prostate	1 ± 0.00	357.95 ± 141.22	94.16 ± 54.52	2163.4 ± 884.70	5.97 ± 1.82	1.77 ± 0.36	p=0.0075
Spleen	1 ± 0.00	2.96 ± 0.15	2.65 ± 0.20	2.95 ± 0.94	1.70 ± 0.44	1.01 ± 0.09	p=0.0150

(D)

Control Tissues	UVI	3h	24h	48h	72h	96h	p-Value
Kidney	1 ± 0.00	0.148 ± 0.13	0.35 ± 0.16	0.53 ± 0.27	0.18 ± 0.02	0.19 ± 0.03	p<0.0001
Liver	1 ± 0.00	0.21 ± 0.11	0.13 ± 0.03	0.18 ± 0.04	0.10 ± 0.03	0.27 ± 0.09	p<0.0001
Lung	1 ± 0.00	1.68 ± 0.40	1.78 ± 0.69	0.46 ± 0.04	1.51 ± 0.12	0.43 ± 0.25	p=0.0463
Prostate	1 ± 0.00	4.43 ± 0.35	2.96 ± 1.42	3.63 ± 0.31	1.06 ± 0.40	0.90 ± 0.53	p=0.0080
Spleen	1 ± 0.00	0.99 ± 0.17	2.52 ± 0.69	3.05 ± 0.51	3.13 ± 0.37	2.31 ± 0.75	p=0.0275

Figure 3. Tissue distribution of live virus. PTEN knockout mice (A) and control (B) were injected with 5×10^8 pfu/ml of VSV(AV3). Mice were sacrificed at time points of 3, 24, 48, 72 and 96h post viral injection. Organs (kidney, liver, lung, prostate and spleen) were frozen and virus titers were subsequently determined by plaque assay. Data are represented as means \pm standard error; n=3. qPCR analysis of VSV RNA levels in (C) PTEN knockout mice and (D) control mice tissues. Total RNA was extracted and relative GFP gene, which is embedded in viral backbone, was compared to UV-inactivated virus.

(A)



(B)

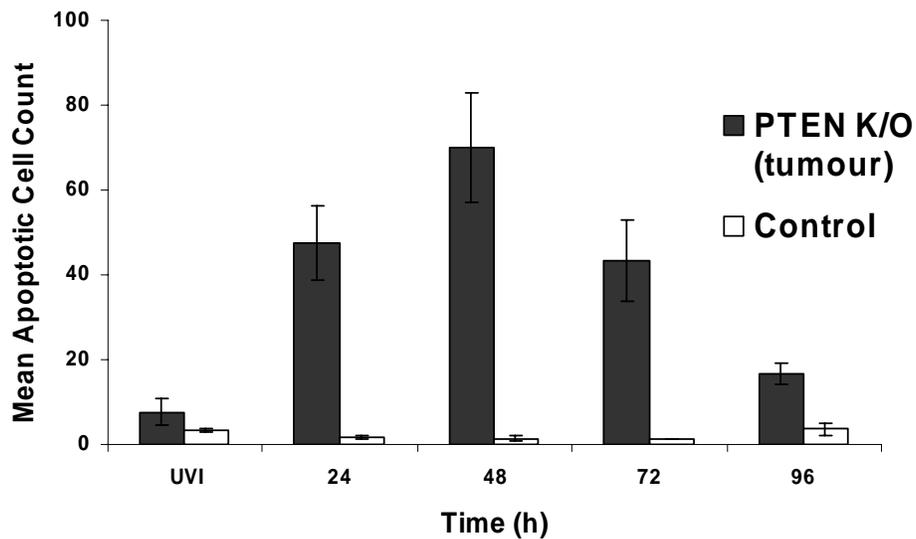


Figure 4. Apoptotic cell death visualized in prostates of PTEN knockout and control mice. Each mouse was injected with 5×10^8 pfu/ml of VSV(AV3). Mice were euthanized at 24, 48, 72 and 96h post viral injection. (A) Paraffin embedded prostates tissues were stained with TUNEL. Representative slides at 40x magnification show presence of apoptotic bodies (stained brown) in prostates of PTEN knockout mice, with little to no apoptosis seen in control mice. (B) Apoptotic cells were counted in ten fields of view and average number apoptotic bodies \pm standard error was plotted.

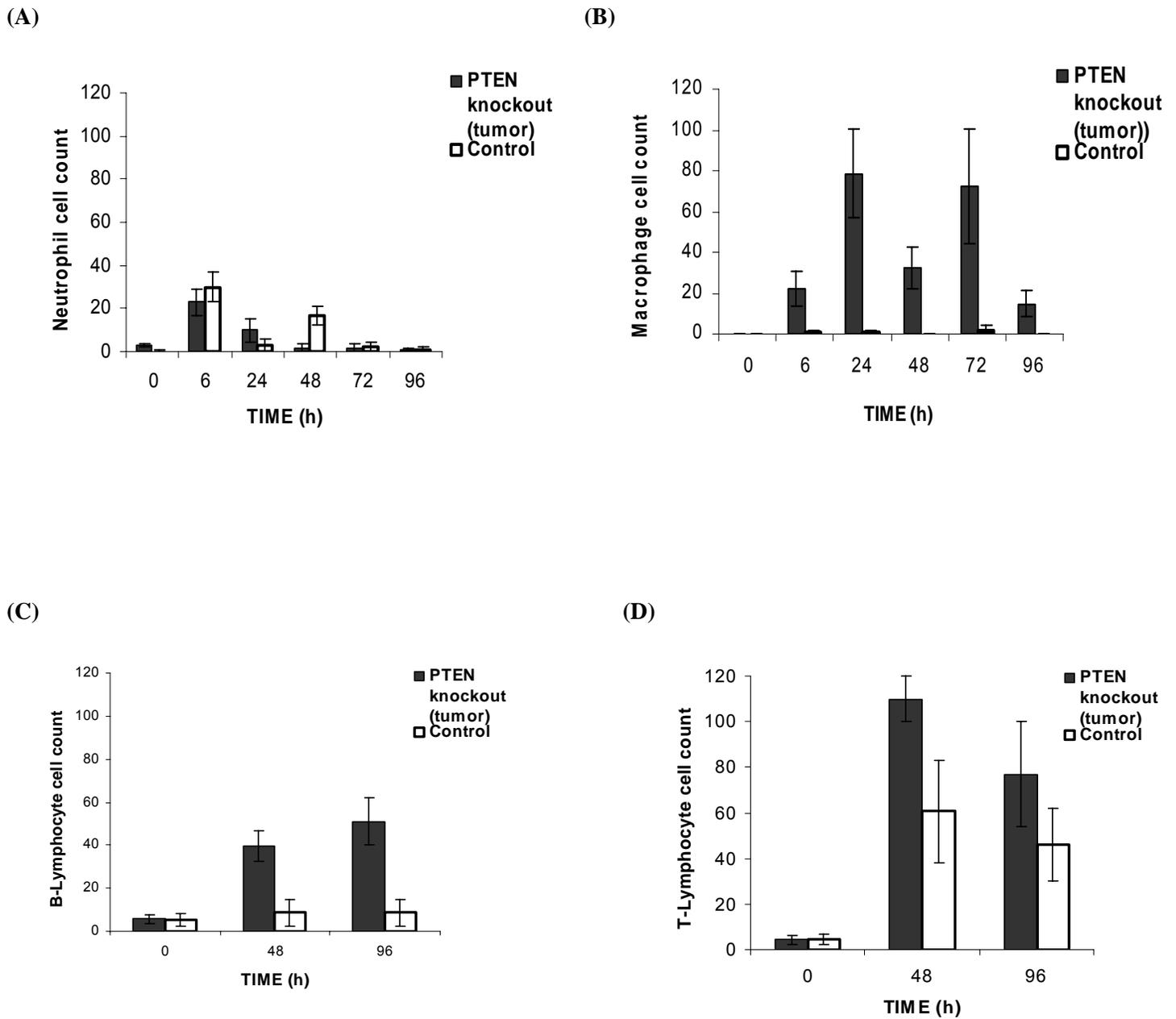
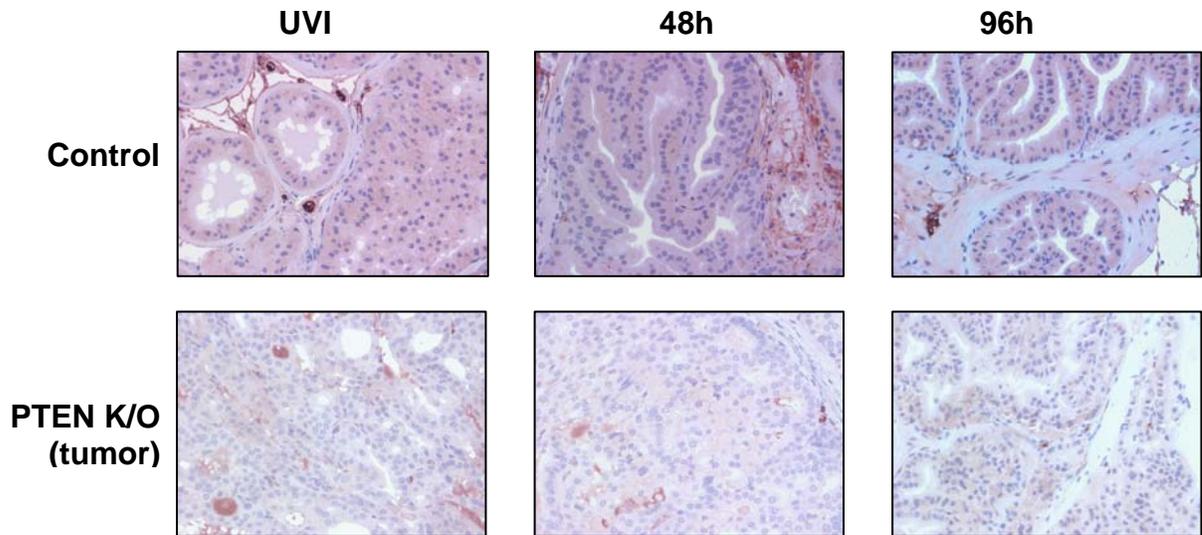


Figure 5. Effects of viral infection on immune response parameters. Immune cells were counted and presented as average of total cell count \pm SEM, $n=3$ mice. There is significantly no difference between total neutrophil (A) and T-lymphocyte (D) count in PTEN knockout tumor bearing and control prostates ($p>0.05$). There is a significant difference between total macrophage (B) and B-lymphocyte (C) infiltration of prostate tissue between PTEN null tumor bearing and control mice ($P<0.05$).

(A)



(B)

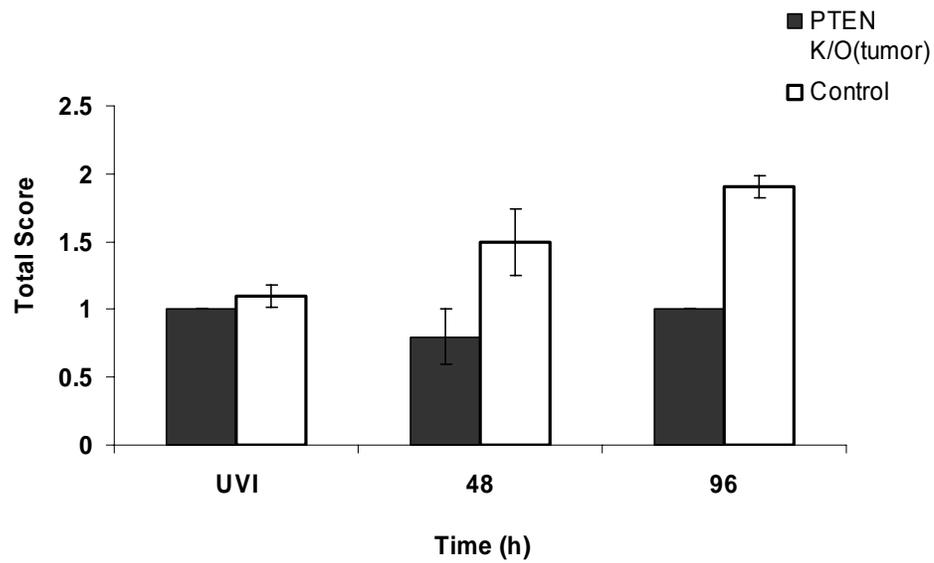


Figure 6. INFR-1 (α/β) receptor levels after injection with VSV (AV3). Paraffin embedded tissues were stained with IFNR-1 α . Slides were prepared and visualized at 40x magnification. Results are representative of three experiments and scored as presented in table (B) by a pathologist.

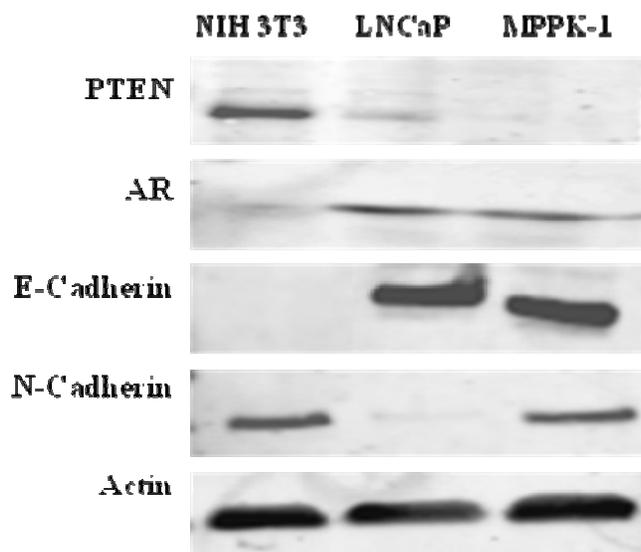


Figure 7. VALIDATION OF MPPK-1 CELLS AS PROSTATE EPITHELIAL CELLS: Western blot analysis of NIH3T3, LNCaP and MPPK-1 cells, demonstrating that MPPK-1 mouse derived cells (N-cadherin positive) from prostate transgenic PTEN knockout mice (lack of PTEN and presence of AR) and over express E-cadherin similar to LNCaP, a human neoplastic cell line.

CONCLUSION:

The goal of any viral therapy for treatment of cancer is to provide an opportunity to efficiently direct cell killing to primary and metastatic cancer cells, while sparing normal cells (31, 32). Prostate cancer is a good candidate for viral therapy since there are various technically easy routes (e.g. trans-urethral, trans-perineal and trans-rectal) of viral delivery (33). Oncolytic viruses, such as VSV, have shown promising results in killing other cancer cells in various animal models. Using an animal model that emulates prostate cancer disease progression as seen in humans, such as the prostate specific PTEN knockout mouse model (19), is key, and to date such studies have not been exploited. Oncolytic viruses typically act by recognizing a common genetic defects occurring during tumor evolution. VSV replicates poorly in normal tissues, but is very efficient in infection and killing tumor cells, which frequently have acquired defects in IFN signaling. It has been shown that approximately 75% of tumor cells lack a normal IFN response, making VSV a useful tumor-selective tool (13, 34). Additionally, since the primary hosts of VSV are rodents, cattle, horses and swine, patients living in non-endemic areas do not have neutralizing titers to the virus. In terms of toxicity, VSV rarely infects humans and in rare cases of infection, there is usually only mild flu-like symptoms observed. Hence, VSV is a relatively safe candidate as a therapeutic agent, particularly the AV3 strain which is mutated to be even more susceptible to the IFN response that protects normal cells (35, 36).

To show that VSV (AV3) was in fact able to infect human and mouse prostate cell lines, three cell lines representing normal prostate epithelial cells (RWPE-1), human prostate cancer cells (LNCaP) and mouse prostate cancer cells, (MPPK-1) were tested. MPPK-1 cells were derived from our PTEN knockout mice prostates (Supplemental data). As shown in Fig. 1, there was a direct correlation between increased viral infection titer and decreased cell survival. Importantly, cell viability at a given titer was always lower in neoplastic cells than in non- neoplastic control cells. These results are consistent with findings of others (25, 37), though effects of VSV virus on RWPE-1 and MPPK-1 cells have not been previously shown.

An underlying assumption for using VSV (AV3) for viral therapy is that tumor cells have a defective antiviral host response, specifically faulty interferon pathway activation. To test this both prostate cancer cell lines (LNCaP and MPPK-1) and non-cancerous prostatic cells (RWPE-1) were pre-treated with IFN. Our results (Fig.1B) indicated that due to a defective INF response, there was no change in the death of prostate cancer cells (LNCaP and MPPK-1), whereas, pre-treatment of RWPE-1 normal prostate epithelial cells with IFN led to an increase in cell survival. These results confirm that VSV (AV3) is capable of infecting cells which have a defective IFN response and supports previous reports that the mutated M protein in VSV can restrict its infectivity to cancer cells with a malfunctioning IFN response (14, 37).

Using the xenogen-IVIS imaging system, it was evident that after administration of VSV (AV3) *in vivo* via direct injection into the prostate, the virus is quickly distributed through out the body (Fig. 2). This phenomenon is seen in both control and PTEN null mice, and as expected there is a higher viral infection in the prostate by 24h, the original site of viral injection. However, based on both luminescence and plaque data (Fig.

3) the level of VSV (AV3) infection and replication in PTEN knockout mice is considerably higher than that of non tumor bearing control mice.

TUNEL staining of paraffin embedded prostate tissue from control and PTEN knockout mice post infection indicated high levels of apoptosis in tumor bearing mice, with very low levels of apoptosis observed in control mice (Fig.4). Apoptotic cell counts were high between 24 to 72h, with a peak in apoptotic cell death at 48h, in the prostates of VSV (AV3) injected PTEN null mice. However by 96h, presence of apoptotic cells were much less apparent with some evidence of morphological changes in the prostate tissues of the tumor bearing mice.

It has previously been shown that intra-tumor injection of VSV in BALB/c mice with subcutaneous tumors leads to tumor cell death through an indirect mechanism. This cell death was shown to be a by-product of neutrophil infiltration. These cells originate from white blood cells, are phagocytic, and typically act as first response to pathogens (23). To test this in our model, paraffin embedded prostate tissues from both control and prostate specific PTEN null mice at various times post VSV injection were stained for neutrophils. Our results (Fig.5A) indicated that there was some initial neutrophil infiltration in both control and tumor-bearing prostates, but, there was no difference seen in neutrophil concentrations between tumor and control prostates.

Though with respect to other immune system parameters, there was a significant difference detected in macrophage (monocyte derived) numbers in these prostates after VSV (AV3) injection. In the PTEN null prostate tumor tissues, macrophage counts increased after peaks of viral infection and cell death, specifically at the 24h and 72h time points. Previous studies have shown that macrophages play an inhibitory role in viral replication by phagocytic clearance of virus (i.e. Herpes simplex virus) (38, 39). In the PTEN null model, an increase in macrophage infiltration occurred after a surge of viral replication which takes place at 3h and then again at 48h after viral administration (Fig. 3A). The cell death level is highest at 48h post infection. Since one of the main jobs of macrophages is to remove dead cells, this likely explains the increase in macrophage count between 48-72h post viral injection (Fig 5B).

Typically upon VSV infection, normal cells mount an initial IFN (α/β) response that prevent viral replication. IFN (α/β) act through the IFN-receptor α/β (IFN-R α/β). Therefore, it was expected to see an increase in IFNR α upon VSV infection, which was the case in control mice that were injected with VSV (AV3) virus (Fig. 6).

Conversely in PTEN null mice, as viral replication (Fig. 3) and cell death (Fig.4) peak at 48h post viral injection, the IFNR- α levels did not change. This indicated that in cells with intact IFN systems (*in vivo*), IFNR- α levels increased post infection, and did not allow for VSV (AV3) replication in prostate tissue.

Since IFN response is the initial antiviral cellular response, in a faulty system as in the PTEN knockout mice, viral replication continued. PTEN knockout mice are immunocompetent, therefore innate immune response of T-lymphocyte and B-lymphocytes were activated. In the case of control mice, due to presence of IFN possibly only a T-cell response was needed. Conversely, in prostate tissue of conditionally PTEN knock out

mice, there was also an increase infiltration of B-lymphocytes. This increase in B-lymphocytes could be due to a faulty initial IFN response that does not occur and allows for viral replication. Therefore, a secondary B cell response is needed to eradicate the virus as is the case and seen in figure 3 by 96h post viral infection (40). Additionally, Bach *et al* (2007) have demonstrated that VSV infection leads to increase in B-cell response in IFNR- α deficient mice (41).

Overall, we have described a mechanism of action for VSV (AV3) oncolytic property in prostates of conditionally PTEN knockout mice. This data has clinical relevance due to the endogenous nature of the tumors, and similar disease progression of prostate specific PTEN null mice and prostate cancer in humans. Therefore, we can speculate that VSV (AV3) is able to selectively kill prostate cancer cells, due to their faulty anti-viral IFN response, while sparing normal cells. A major advantage of this viral therapy is the ability of viral clearance by the host immune response after targeted killing of cancer cells. Furthermore, the fact that VSV rarely is capable of infecting humans and in rare cases of infection there is only flu-like symptoms (35, 36), make VSV and specially AV3 (the more IFN sensitive strain) a great therapeutic candidate. Currently there are various oncolytic viruses, such as Measles, G207, Vaccinia Seneca Valley virus, Coxsackievirus and New Casstel Virus [NVD], undergoing phase I and II clinical trials (42) . The results from this study suggest that VSV (AV3) is a good candidate to be further exploited for clinical trials.

Future Direction:

The limitation of prostate specific PTEN null mouse model is that it has very low levels of metastatic lesions reported. Therefore to see whether VSV (AV3) is capable of finding metastatic cells, we purpose to look at a more aggressive prostate cancer tumor model. The transgenic adenocarcinoma of the mouse prostate (TRAMP) has high frequency of metastatic lesions particularly to lungs and pelvic lymph nodes.

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