A Vaccinia virus armed with interleukin-10 is a promising therapeutic agent for treatment of murine pancreatic cancer

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Short title: Oncolytic Vaccinia virus for treatment of pancreatic cancer

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Grant support: The UK Charity Pancreatic Cancer Research Fund, National Natural Science Foundation of China (81101608, 81201792), Ministry of Sciences and Technology, China (2013DFG32080), Henan Provincial Department of Science and Technology as well as Department of Health, Henan Province, China (124200510018 and 104300510008).

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Disclosures: The authors declare no competing financial interests.

Abbreviations: VV-Vaccinia virus; PaCa-Pancreatic Cancer; OV-Oncolytic virotherapy; IL-10-Interleukin-10.

Word Count: 4,539
Translational Relevance

Oncolytic virotherapy is beginning to show promise as a realistic alternative to standard cancer therapeutics. To date, clinical trials have proved this strategy safe and well tolerated by patients, however clinical responses after treatment with virus alone have been modest. A new generation of oncolytic viruses that engage the host immune system in the attack against the tumor are providing more encouraging clinical results.

This study demonstrates that Vaccinia virus armed with the cytokine IL-10 is a novel and extremely promising therapeutic for treatment of pancreatic tumors and prevention of disease recurrence. Understanding the mechanisms by which IL-10 improves oncolytic virotherapy provides a foundation for the rational design of clinical trials for treatment of pancreatic cancer and other solid tumors with this virus and provides valuable information for the design of future anti-tumor strategies that aim to combine oncolytic virotherapy with immunotherapeutic approaches.

Abstract

Purpose Vaccinia virus (VV) has strong potential as a novel therapeutic agent for treatment of pancreatic cancer (PaCa). We investigated whether arming VV with IL10 could enhance the antitumor efficacy with the view that IL10 might dampen the host immunity to the virus, increasing viral persistence thus maximising the oncolytic effect and antitumor immunity associated with VV.

Experimental Design The antitumor efficacy of IL10-armed VV (VVL∆TK-IL10) and control VVΔTK was assessed in pancreatic cancer cell lines, mice bearing subcutaneous PaCa tumors and a PaCa transgenic mouse model. Viral persistence within the tumors was examined and immune depletion experiments as well as immunophenotyping of splenocytes were carried out to dissect the functional mechanisms associated with the viral efficacy.
Results  Compared to unarmed VVLΔTK, VVLΔTK-IL10 had a similar level of cytotoxicity and replication in vitro in murine pancreatic cancer cell lines, but rendered a superior anti-tumor efficacy in the subcutaneous pancreatic cancer model and a K-ras-p53 mutant-transgenic PaCa model after systemic delivery, with induction of long-term anti-tumor immunity. The antitumor efficacy of VVLΔTK-IL10 was dependent on CD4+ and CD8+, but not NK cells. Clearance of VVLΔTK-IL10 was reduced at early time points compared to the control virus. Treatment with VVLΔTK-IL10 resulted in a reduction in virus-specific, but not tumor-specific CD8+ cells compared to VVLΔTK.

Conclusions These results suggest that VVLΔTK-IL10 has strong potential as an anti-tumor therapeutic for PaCa.

Introduction

Pancreatic cancer (PaCa) is the fourth leading cause of cancer-related death worldwide (1) and remains consistently lethal with a five-year survival rate of less than 5%. This situation signifies a need for radically new therapeutic strategies that are not subject to cross-resistance with conventional therapies.

Oncolytic viruses have emerged as attractive therapeutic candidates for cancer treatment due to their inherent ability to specifically target and lyse tumor cells and induce anti-tumor effects. An engineered replication-competent Adenovirus, dl1520 (ONYX-015) was the first of these viruses to be tested for human PaCa treatment. The treatments were well tolerated, but no objective responses with virus therapy alone were seen in any of the patients(2).

Vaccina virus (VV) has strong potential for exploitation as both an oncolytic agent and vector for therapeutic gene delivery to tumors. Extremely promising clinical trial data have recently emerged in which GMCSF-armed VV induced objective responses in liver, colon, kidney and lung cancer and
melanoma patients (3, 4). VV has several inherent features that make it particularly suitable for use as an oncolytic agent, including fast and efficient replication with rapid cell-to-cell spread, natural tropism for tumors, a well-documented safety record and an ability to replicate in many different cell types, a feature not shared by adenoviruses. We recently demonstrated that hypoxia, which contributes to the aggressive and treatment-resistant phenotype of pancreatic ductal adenocarcinoma (5), does not inhibit and may even enhance the potency of oncolytic VV (6). In addition, VV has recently been shown to be effective at human tumor-targeting after intravenous delivery (4).

Interleukin-10 (IL-10), first described as a factor produced by Th2 clones capable of inhibiting Th1 cytokine production (7), is a potent inhibitor of T cell-mediated anti-viral responses by prevention of dendritic cell (DC) activation of the CD4+Th1 inflammatory pathway (8, 9). IL-10 is a key player in the establishment and perpetuation of viral persistence in vivo (10, 11). Therefore, arming VV with IL-10 may prolong viral persistence and enhance the antitumor efficacy. IL-10 has historically been regarded as an immunosuppressive cytokine that has extensively been described in association with cancer, including PaCa (12, 13) as a mechanism of tumor escape from immunosurveillance (14, 15). However, accumulating evidence demonstrates that IL-10 also has immunostimulatory and anti-tumor properties (16). Functional mechanisms investigated include activation of natural killer (NK) cells (17) that have been associated with tumor clearance in murine models of breast and colorectal cancer (18); inhibition of angiogenesis; enhancement of macrophage infiltration into tumors (19); and prevention of metastasis by inhibition of matrix metalloproteinase-2 (20). A number of preclinical (21) and clinical trials have consistently demonstrated safety of IL-10 administration in treatment of diseases including psoriasis (22), Crohn’s disease (23) and chronic hepatitis C infection (24), which make a strong case for its use as a therapeutic modality in cancer. IL-10 has been reported to enhance the therapeutic effectiveness of a VV-based vaccine against murine cancer cells (25), which may be connected to its ability to enhance the growth and proliferation of T cells (26) or
its role as a chemotactic agent for CD8+ T cells. Unfortunately the half-life of IL-10 is only approximately 20 minutes and it is difficult to maintain a high concentration after administration of recombinant protein (27). Non-replicating adenovirus-mediated delivery has shown promise in retaining therapeutically effective levels of IL-10 \textit{in vivo} (28). Given its pleiotropic effects, IL-10 may be an effective agent with which to improve the anti-tumor potential of VV.

In this study, we have tested a Lister strain, TK-deleted replicating VV armed with murine IL-10 (VVL\Delta TK-IL-10) in subcutaneous and transgenic murine models of PaCa and demonstrated that VVL\Delta TK-IL-10 has far superior anti-tumor activity compared to unarmed VV (VVL\Delta TK), resulting in almost complete tumor clearance, significantly increased survival times and the production of long-term tumor immunity in the host. Our results suggest that VVL\Delta TK-IL-10 has strong potential as an effective treatment for PaCa and lay the foundation for translation of this therapeutic into a clinical setting.

\section*{Materials and Methods}

\subsection*{Cell lines and viruses}

The murine pancreatic ductal adenocarcinoma (PDAC) cell line DT6606 and the pre-invasive PaCa (PanIN) cell line DT4994 were cultured from LSL-Kras$^{G12D/+}$; Pdx-1-Cre mice that had developed PDAC (29). These were kindly provided by David Tuveson (Cancer Research UK Cambridge Research Institute, now at Cold Spring Harbor Laboratory). The DT6606-ovalbumin (OVA) stable cell line was created by transfection of DT6606 cells at with pCI-neo-cOVA (Addgene) using Effectene transfection reagent (Qiagen) according to the manufacturers’ protocol. CV1 (African monkey kidney) cells and PT45 (human pancreatic carcinoma) cells were obtained from American Type Culture Collection (ATCC, VA, USA).
Construction and production of recombinant VV Lister strains VVLΔTK-IL10 (rVV-IL10, armed with murine IL-10) and VVLΔTK (rVV-L15) was previously described (30, 31).

**Vaccinia virus replication assay**

Appropriate cell lines were seeded in triplicate and infected 16 hours later with VVLΔTK or VVLΔTK-IL-10 at a multiplicity of infection (MOI) of 1 PFU/cell. Cells and supernatant were collected at 24, 48 and 72 hours post-infection and titres were determined by measuring the median tissue culture infective dose (TCID50) on indicator CV1 cells. The Reed–Muench mathematical method was used to calculate the TCID50 value for each sample (32). Viral burst titres were converted to PFU per cell based on the number of cells present at viral infection. One-way ANOVA followed by Bonferroni post-test was used to assess significance.

**Cell cytotoxicity assay**

The cytotoxicity of the viruses in each cell line was assessed 6 days after infection with virus using an MTS non-radioactive cell proliferation assay kit (Promega) according to the manufacturers’ instructions, which allowed determination of an EC50 value (dose required to kill 50% of cells).

**Real-time quantitative PCR**

Subcutaneous tumors collected from treated mice were homogenised before DNA was extracted using the QIAamp DNA blood mini kit (QIAGEN Ltd, Crawley, UK) according to the manufacturers’ instructions. TaqMan® system primers and probes (Supplementary Table 1) were designed using Primer Express® v3.0 software (Applied Biosystems, New Jersey, USA) and constructed by Sigma-Aldrich and Applied Biosystems respectively. Samples, controls and standards were tested in triplicate by quantitative polymerase chain reaction (qPCR) using 7500 Real-time PCR System. Results were normalised to Nanodrop readings and expressed as genome copy number/0.01g tumour tissue. One-way ANOVA followed by Bonferroni post-test was used to assess significance.
IL-10 and Interferon-γ (IFN-γ) ELISA

IL-10 or IFN-γ protein levels were quantified using an IL-10-specific or IFN-γ-specific ELISA (R&D Systems) according to the manufacturers’ instructions. Where appropriate, data were normalized to cell number present at time of infection.

Splenocyte preparation

Spleens were extracted from mice, combined with complete T cell medium (RPMI medium, 10% FBS, 1 % penicillin/streptomycin, 1% sodium pyruvate) and cells separated using a 70µm cell strainer.

Cells were re-suspended in red blood cell lysis buffer (Sigma-Aldrich), washed in PBS and the pellet re-suspended in T cell medium.

In Vitro splenocyte restimulation

2x10⁶ cells were aliquoted into each well of a 96-well plate in duplicate. Cells were restimulated with either a VV-specific B8R peptide (TSYKFESV) (Proimmune) at a final concentration of 20 µg/ml or 5x10⁵ mitomycin C-treated DT6606-OVA cells. Restimulated splenocytes were incubated at 37°C/5% CO₂ for 72 hours and the supernatant collected.

Tumor cell preparation

Tumor cell suspensions were prepared by incubation with 1x collagenase/hyaluronidase (Stemcell) for 30 minutes at 37°C. Cells were separated using a 70 µm cell strainer and resuspended in complete T cell medium.
Immunophenotyping of splenocytes and tumors

All fluorochrome-conjugated antibodies were supplied by EBiosciences and used at a 1:200 dilution. The B8R and OVA H-2Kb restricted, MHC class I pentamers were synthesized by Proimmune and used at a 1:20 dilution.

Splenocytes and tumors were prepared and aliquoted into 96-well U-bottom plates. Pentamer staining was carried out by resuspending cells in FACS buffer (FB) (PBS+1% heat inactivated BCS+0.1%NaN₃) plus pentamer and incubating at room temperature for 10 minutes. Cells were washed before being incubated in FB plus appropriate fluorescent marker-conjugated anti-immune cell marker antibodies for 30 minutes on ice. Cells were washed and fixed in 2% formalin prior to analysis using a BD LSR Fortessa flow cytometer. Data were analyzed using FlowJo software (Tree Star Inc).

In Vivo studies

All animal studies were carried out under the terms of the Home Office Project Licence PPL 70/6030 and subject to Queen Mary University of London ethical review, according to the guidelines for the welfare and use of animals in cancer research (33).

The C57/BL6 mouse is H-2 haplotype-identical to the injected DT6606 cells thus DT6606 allografts could be established in the right flank of 3-4-week male C57/BL6 mice by injecting 3x10⁶ DT6606 cells. When tumors reached around 0.6 cm in diameter, mice were stratified by tumor size into groups of 8 and received 100 µl intratumoral (IT) injections of 1x10⁶ PFU of VVLΔTK, VVLΔTK-IL-10 or PBS daily for 5 days. Tumor size was measured twice weekly until the death of the first animal in each group and volume estimated (Volume = (length x width² x π)/6). Survival analysis was carried out using Kaplan Meier survival curves with log rank (Mantel Cox) tests employed to assess significance. Mice that had cleared tumor after treatment were re-challenged 4 weeks post-clearance in the opposite flank with 4x10⁶ DT6606 cells and tumor volume estimated as previously.

For immune depletion studies, DT6606 subcutaneous tumors were established as described and 1
day prior to commencement of viral treatment 200 µg of anti-CD4 IgG (antibody clone GK1.5), anti-
CD8 IgG (antibody clone TIB210), anti-NK IgG (antibody clone PK136) or control rat IgG was injected
intraperitoneally (IP) in 200 µl PBS. Injections were continued twice weekly for the duration of the
experiment and FACS analysis was used to verify depletion for the duration of the experiment. Five
mice per group were treated and the experiment carried out twice.

Transgenic Mice

LSL-KrasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre (KPC) mice were kindly provided by David Tuveson (Cancer
Research UK Cambridge Research Institute) and have been described previously (29). Mice were
treated when they reached 2.5 months, previously demonstrated to be the mean age at which pre-
vasive (PanIN) PaCa has progressed to pancreatic ductal adenocarcinoma (PDAC) (29). Mice were
treated IP with 2x10⁸ PFU/injection VVLΔTK or VVLΔTK-IL-10 on days 1, 3 and 5. Mice were
examined daily for signs of disease progression and culled when they showed symptoms of sickness.
Survival data were compared using Prism® (GraphPad Software, CA, USA) and a log rank (Mantel
Cox) test was used to determine significance of survival differences.

In vivo imaging

Seven days after treatment of KPC or KP mice, the biodistribution of VVLΔTK was determined in
anesthetized animals (2% isofluorane inhalation) after IP injection of D-Luciferin (150 mg/kg)
(Xenogen) and fluorescence measured with the IVIS camera (Xenogen Corp).

Histopathological examination and immunohistochemistry for viral proteins

2.5-month old KPC or control KP mice were treated IP with 2x10⁸ PFU/injection VVLΔTK or VVLΔTK-
IL-10 on days 1, 3 and 5. On relevant days, animals were sacrificed, the pancreas removed, snap-
frozen and stored at -80°C. Frozen tissue was processed for immunohistochemistry analysis of
Vaccinia virus coat protein (1:50, rabbit anti-vaccinia virus coat protein polyclonal antibody
Results

**VVLΔTK-IL-10 replicates efficiently *in vitro* in murine cancer cell lines derived from a transgenic mouse model of PaCa**

To determine whether inclusion of IL-10 impacted on characteristics of VVLΔTK *in vitro*, replication and cytotoxicity in three cell lines were examined; DT6606, representing late-stage invasive pancreatic ductal adenocarcinoma (PDAC) and DT4994, representing pre-invasive pancreatic cancer (PanIN) were both derived from the K-ras transgenic mouse model of PaCa (29). DT6606-OVA, in which the ovalbumin antigen is over-expressed in DT6606 cells, was also examined. All cell lines supported production of infectious virions of VVLΔTK and VVLΔTK-IL-10 (Figure 1 A-C) and IL-10 did not act to inhibit nor promote viral replication. The dose required to kill 50% of cells (EC50) was comparable between VVLΔTK and VVLΔTK-IL-10 (Figure 1D). Furthermore, IL-10 was expressed in all three cell lines over 72 hours after infection (Figure 1E). Thus, arming VVLΔTK with IL-10 does not adversely affect the *in vitro* oncolytic effect desired for our virotherapy strategy.

VVLΔTK-IL-10 infection was also assessed in the human pancreatic cancer cell line PT45 to demonstrate potential translation of this therapy into human cells. VVΔTK-IL-10 showed efficient replication, cytotoxicity and IL-10 expression in this cell line (Supplementary Figure 1).
**VVLΔTK-IL-10 shows superior anti-tumor efficacy compared to VVLΔTK in immunocompetent mouse models of PaCa**

*In vivo* efficacy of VVLΔTK-IL-10 was examined using a subcutaneously established PaCa model. DT6606 subcutaneous tumors were established in male C57/Bl6 mice and the animals received IT injections of 1x10^8 PFU of VVLΔTK, VVLΔTK-IL-10 or PBS daily for 5 days. The selected viral dose was ten times lower than the most commonly reported 1x10^9 PFU/dose in the literature(34). Both VVLΔTK and VVLΔTK-IL-10 demonstrated anti-tumor efficacy (Figure 2A). However, treatment with VVLΔTK-IL-10 resulted in a superior antitumor efficacy by day 44, with 87.5% of mice showing tumor clearance and significantly improved overall survival rates compared to both VVLΔTK- and PBS-treated animals (Figure 2B). The C57/Bl6 mouse is H-2 haplotype-identical to the injected DT6606 cells. Growth of tumors in PBS-treated animals confirmed that there was no immunological rejection of the DT6606 cell line due to MHC or minor antigen mismatches.

To determine whether VVLΔTK-IL-10 remained efficacious in a more pathologically relevant model of PaCa, KPC transgenic mice were used. In these mice, pancreas-specific expression of mutant *Kras*<sup>G12D</sup> and *Trp53*<sup>R172H</sup> results in progressive development of PDAC (35). Three doses of virus (2x10<sup>8</sup> Pfu/day) were given IP to 2.5-months old, PDAC-bearing mice. To confirm the specificity of virus for pancreatic tumors after IP injection, VVLΔTK, which expresses a luciferase transgene in the viral TK region, was injected into either experimental KPC mice or control KP mice. Two days later mice were imaged for luciferase expression (Figure 2C). Strong luciferase signals were obtained specifically in the pancreatic area of KPC transgenic mice (Figure 2C, left panel), while no signal was obtained from control mice (Figure 2C, right panel). The VV proteins were expressed in cancer cells and proliferative acinar cells in KPC mice (Figure 2C, left panel bottom), whereas no viral protein expression was observed in the ductal epithelial cells and acinar cells in KP mice (Fig 2C, right panel bottom) confirming specificity of replication of TK-deleted VV for pancreatic tumor cells. Efficacy of viral treatment in this model was assessed by survival (Figure 2D). Treatment with VVLΔTK-IL-10
resulted in significantly improved survival rates compared to treatment with VVLΔTK. Mean survival time for VVLΔTK-IL-10 treated animals after commencement of treatment was 138.5 days compared to 69.7 days for VVLΔTK-treated animals, suggesting VVLΔTK-IL-10 as an extremely effective treatment for pancreatic ductal adenocarcinomas even in the most complex murine models of the disease.

**Treatment with VVLΔTK-IL-10 results in long-term protection against disease recurrence**

Successful OV strategies aim not only to eradicate the primary tumor, but also to induce long term anti-tumor immunity to prevent disease recurrence. Thus animals were rechallenged with 4x10^6 DT6606 cells four weeks after complete regression of the primary tumor (Figure 3A). Treatment with both viruses resulted in long-term immunity to DT6606 tumor cells as evidenced by rapid clearance of these cells that necessitated no further viral treatments. Interestingly, VVLΔTK-IL-10 - treated animals were able to clear the secondary tumor more quickly and more consistently than VVLΔTK-treated animals.

**CD8+ and CD4+, but not NK cells are required for VVLΔTK-IL-10 efficacy in vivo**

Long term immunity suggests an activation of specific anti-tumor immune responses after treatment. To assess the contribution of different immune cells to treatment efficacy, CD8+, CD4+ or NK immune subsets were depleted from mice before treatment of subcutaneous DT6606 tumors with VVLΔTK-IL-10 (Figure 3B). Depletion of CD4+ or CD8+ cells both had a significantly detrimental effect on the efficacy of treatment, suggesting VVLΔTK-IL-10 is acting via these immune subsets to eliminate the tumor. Surprisingly, given previous reports that IL-10 can activate NK cells to mediate tumor clearance (17), depletion of NK cells in our experiment had no effect on treatment efficacy.
Tumor-associated activated T cell and macrophage populations are altered after treatment with VVLΔTK-IL-10 compared to VVLΔTK, which impacts on viral persistence.

Given the involvement of T cells in VVLΔTK-IL-10 treatment efficacy, tumor T cell populations were analyzed in more detail. Pancreatic tumours of KPC transgenic mice treated as previously were harvested post-treatment and T cell populations analyzed by IHC. We noted a significant increase in CD3+ CD8+ infiltrate after treatment with both viruses compared to PBS (Supplementary Figure 2) and a significant increase in CD3+ CD8+ cells in VVLΔTK-IL-10-treated animals at day 22 post-infection compared to VVLΔTK-treated animals.

DT6606-subcutaneous tumors were also harvested for analysis of T cell populations by FACS. In accordance with data obtained from KPC mice, we found a significant increase in tumor T cell infiltrate after treatment with both viruses, with a significant increase in CD8+ infiltrate into tumors of VVLΔTK-IL-10-treated animals (Figure 4A). However, most interesting was that in CD4+ (data not shown) and, more significantly, CD8+ populations (Figure 4B) the proportion of activated (CD45RBlo/CD44hi) T cells in tumors treated with VVLΔTK was higher than those treated with VVLΔTK-IL-10. Interferon-γ (IFN-γ) expression within VVLΔTK-IL-10-treated tumors was also significantly reduced compared with VVLΔTK-treated tumours (Figure 4C).

Tumor-associated macrophage populations were also assessed in KPC (Supplementary Figure 3) and DT6606-tumor-bearing mice (Figure 4D) after infection. We found that treatment with either virus increased macrophage infiltrate into tumors of KPC mice compared to PBS, but that treatment with VVLΔTK-IL-10 resulted in a reduced macrophage tumor infiltrate compared to treatment with VVLΔTK. This result was mirrored in DT6606-tumor-bearing mice. Further assessment of macrophage activation status in the DT6606 subcutaneous model revealed that VVLΔTK-IL-10 induces a downregulation of MHCII expression compared to VVLΔTK (Figure 4E).
To assess the impact of these phenomena on viral persistence, viral DNA load in the tumors (6 mice/group/timepoint) was analyzed after IT treatment at days 8, 16 and 24 post-infection using qPCR (Figure 4Fi) and TCID50 (Figure 4Fii). We found that by day 24, both viruses had been cleared from the tumor to the same extent, but at days 12 and 16 significantly more VVLΔTK-IL-10 was recovered from tumors than VVLΔTK, indicating a delay in clearance of VVLΔTK-IL-10 compared to VVLΔTK. These results were confirmed by IHC analysis of viral load in pancreatic tumors of KPC mice (Supplementary Figure 4).

The splenic CD4+ and CD8+ cell populations are altered after treatment with VVLΔTK-IL-10 compared to treatment with VVLΔTK

It is clear that VVLΔTK-IL-10 treatment efficacy involves modulation of the immune system, thus splenic immune cell population dynamics in response to treatment were assessed in greater detail. DT6606 tumor-bearing mice were treated as described and their spleens collected and assessed for presence of various immune cell subsets. No differences were found in splenic B cell (B220+ cells), Treg (CD4+, CD25hi cells), NK (CD3-, CD49b+ cells) or NKT populations (CD3+, CD49b+ cells) after treatment with either virus compared to PBS treated animals (Supplementary Figure 5).

Analysis of CD4+ and CD8+ populations revealed that frequencies of these populations were altered at early timepoints (Figure 5 A and D; Supplementary Figure 6). At days 8 and 16, a significant increase in the frequency of total CD8+ cells was seen after treatment with either virus, however VVLΔTK-IL-10 treatment resulted in fewer total CD8+ cells than treatment with VVLΔTK (Figure 5D). This phenomenon was also observed in the CD4+ populations at day 16 post treatment (Figure 5A).

Further examination revealed that after treatment with VVLΔTK-IL-10 or VVLΔTK, T cell populations shifted towards an effector/memory phenotype (Figure 5 B-F) at days 8 and 16. However, VVLΔTK-
IL-10 induced statistically fewer activated CD4+ and CD8+ T cells than VVLΔTK at days 8 and 16 post infection (Figure 5 B-F), as noted previously within the tumor.

**VVLΔTK-IL-10 treatment results in reduced anti-viral immune responses compared to treatment with VVLΔTK, but an increased frequency of tumor-specific T cells**

To clarify the proportions of virus-specific and tumor-specific splenic effector CD8+ cells elicited after treatment with VVLΔTK and VVLΔTK-IL-10, splenocytes from DT6606-OVA tumor-bearing animals were analysed. For virus-specific T cells an MHCI-specific pentamer against an immunogenic VV antigen, B8R, was used (Figure 6A; Supplementary Figure 7A). As expected, viral treatment resulted in detection of B8R-specific CD8+ cells in both treatment groups. However, VVLΔTK-treated animals had a significantly higher proportion of B8R-specific T cells than VVLΔTK-IL-10-treated animals at all timepoints, suggesting a decreased virus-specific immune response after treatment with VVLΔTK-IL-10, which could account for the fewer effector CD8+ cells noted after VVLΔTK-IL-10 treatment. We confirmed the decreased frequency of anti-virus-specific T cells using an *in vitro* restimulation assay, in which IFN-γ production from splenocytes in response to B8R peptide restimulation was measured (Figure 6B). At all timepoints, significantly less IFN-γ was detected from VVLΔTK-IL-10 treatment groups compared to VVLΔTK treatment groups.

To assess T cell reaction to tumor antigens, an MHCI OVA-specific pentamer was used in FACS staining (Figure 6C; Supplementary Figure 7B). At day 8, no differences in OVA-specific CD8+ T cells was observed after treatment with either virus when compared to PBS, however by day 16, VVLΔTK-IL-10 treated animals showed an increase in production of OVA-specific antigens compared to VVLΔTK-treated animals. This result was reflected in restimulation assays (Figure 6D).
Taken together, these results indicate that although VVLΔTK-IL-10 treatment resulted in a reduction in antiviral T cell production, the frequency of anti-tumor specific CD8+ T cells was comparable or even increased compared to VVLΔTK-treated mice.

Discussion

Efficacy of oncolytic virotherapy is dependent on both the oncolytic action of the virus itself and the effective stimulation of a local immune response to viral infection (36, 37). Oncolytic viruses may represent a method of achieving vaccination in situ, enabling the adaptive arm of the immune system to clear residual disease and provide long-term surveillance against relapse. To date however, the use of oncolytic viruses alone has proved unsuccessful in clinical trials and this is likely due to their early clearance preventing their oncolytic effects and an effective immune-stimulating release of TAAs. Many viruses encode homologues of the cytokine IL-10, generally considered immunosuppressive, in order to dampen the anti-viral immune response and circumvent early viral clearance (11, 38). We aimed to adopt this natural strategy of viruses by arming VV with IL-10, which has been reported to be effective at prevention of VV clearance (39). We hypothesized that prolonging viral persistence in the host would improve the anti-tumor efficacy by enhancing both the direct oncolytic effect and release of TAAs.

The pancreatic cancer subcutaneous tumour model we developed was based on the use of a DT6606 cell line, which was originally derived from the transgenic KPC spontaneous model of pancreatic cancer (29) and therefore accurately reflect the PDAC populations of cells within these mice. Previous study has demonstrated that these cancer cells resemble human PDAC in many respects, including their expression of oncogenic KrasG12D and the tumor-associated antigen mesothelin, and both spontaneous and subcutaneous tumors show similar histopathological features such as the presence of FAP+ stromal cells (40).
The long-held paradigm of IL-10 function suggests it as an immunosuppressive cytokine, commonly investigated therapeutically in the context of treatment for inflammatory autoimmune conditions and allograft survival (41, 42). However, using these two different murine models of PaCa we observed significantly enhanced therapeutic responses after treatment with our IL-10-armed-VV compared to unarmed virus. In both models, low doses of the virus were sufficient to induce objective responses and in agreement with previous reports, no IL-10-related toxicity was observed (43). Treatment also resulted in rejection of tumors after rechallenge, confirming the development of effective long-term immunity against tumor antigens. These results are consistent with those of others investigating the anti-tumor properties of IL-10 in which systemic administration of recombinant protein or tumor cells transfected with IL-10 induced tumor clearance and long-term memory responses in mice bearing sarcoma (16), melanoma (16, 44), colorectal cancers (16), breast cancers (45) and prostate cancers (20).

*In vitro* studies indicated that IL-10 did not alter VV replication or cytotoxicity and no effect on cell proliferation was observed. To determine other possible mechanisms for the superior efficacy associated with this virus, viral persistence within tumors was assessed. While both IL-10-armed and unarmed viruses were effectively cleared from animals, greater titers of VVLΔTK-IL-10 were recovered at days 12 and 16 compared to VVLΔTK in both the transgenic and subcutaneous models of pancreatic cancer, suggesting that IL-10 could significantly delay viral clearance.

Given previous reports of the ability of IL-10 to stimulate NK cells (17) and as a cytotoxic T cell differentiation factor (46), we examined reliance of our treatment on these immune subsets. Depletion of NK cells had no effect on treatment efficacy *in vivo* and we found no evidence of altered splenic or tumor (data not shown) NK populations after treatment with VVLΔTK-IL-10. By contrast, depletion of CD4+ and CD8+ T cell populations had a negative impact on treatment efficacy. It has previously been reported that progression from PanIN to PDAC is accompanied by progressive infiltration of T cells into the tumor in KPC transgenic mice (47, 48), however no anti-
tumor response is induced by this infiltrate. Our analysis of T cell populations in spleens and tumors revealed that treatment with both unarmed and IL-10-armed viruses induced a high level of adaptive immunity in mice compared to untreated mice. However, an interesting finding was that the magnitude of the activated splenic CD4+ and CD8+ population response in VVLΔTK-IL-10 treated mice was lower compared to the unarmed virus. This difference correlated with a reduction in virus-specific CD8+ T cells and IFN-γ recovery from tumors after VVLΔTK-IL-10 treatment, which accounted for the delayed viral clearance from tumors. Interestingly, although VVLΔTK-IL-10 treatment reduced anti-viral CD8+ populations, IL-10 had no inhibitory effect on production of anti-tumor CD8+ cells. Indeed, at day 16 post-injection, an increase in anti-OVA CD8+ cells was observed, which we postulate is a result of the increased oncolysis occurring with VVLΔTK-IL-10 treatment, which improves TAAs release.

These results suggest that IL-10 improves the efficacy of OV by modulation of the early immune response to infection, resulting in dampening of antiviral, but not antitumor immunity. However, the mechanism by which IL-10 elicits this alteration remains unclear. Our investigations revealed that local IL-10 expression results in modification of the tumor macrophage population, which is highly sensitive to IL-10 exposure (49). Numerous investigators have reported that IL-10 can negatively regulate macrophages by i) inhibiting their infiltration into tumors and ii) downregulating MHCII expression and suppressing production of pro-inflammatory cytokines and reactive nitrogen oxides (50). Whilst we found that VVLΔTK-IL-10 treatment increased macrophage infiltrate into tumors in both the spontaneous and subcutaneous models of pancreatic cancer, we found that in accordance with previous data, VVLΔTK-IL-10 treatment results in a significant downregulation of MHCII expression. Thus, it is feasible that in our model, tumor macrophages are responsible for viral antigen presentation to T cells and a reduction in macrophage activation by IL-10 leads to reduced cross-priming of the anti-viral immune response. A further consideration is that this model suggests distinct pathways of viral and tumor antigen presentation, which are the subject of ongoing investigation in our laboratory.
These findings demonstrate that IL-10 armed VV shows great promise as a novel therapeutic for PaCa and that IL-10 in combination with oncolytic virotherapy is clearly able to enhance tumor rejection through modulation of the innate and adaptive immune responses.

References


Figure Legends

Figure 1. Replication, potency and protein expression of IL-10 by VVLΔTK and VVLΔTK-IL-10 in vitro. (A-C) Production of infectious virions in murine DT6606 (A), DT6606-0VA (B) and DT4994 (C) cells. Mean viral replication ± SEM was determined by TCID50 assay on CV1 cells. Statistical analysis was carried out using a Students unpaired T test at each time point. **P<.01. (D) Cytotoxicity of VVLΔTK and VVLΔTK-IL-10 against DT6606, DT6606-0VA and DT4994 cells. Cell death was determined by MTS assay 144 hours post-infection. Mean EC50 values ± SEM are shown. (E) Expression of IL-10 by VVLΔTK-IL-10. Cells were infected with VVLΔTK-IL-10 at an MOI of 1 PFU/cell. Supernatant was collected every 24 hours for 72 hours and assayed for IL-10 by ELISA. Data were normalized to cell number infected and are displayed as pg IL-10/1x10^4 cells.

Figure 2. Efficacy of VVLΔTK and VVLΔTK-IL-10 against pancreatic tumors in vivo. DT6606 cells were injected into the right flank of male C57/BL6 mice. Eight mice/group were injected IT with 1x10^8 PFU VVLΔTK, VVLΔTK-IL-10 or PBS daily for 5 days. Mean tumor size ± SEM are displayed until the death of the first mouse in each group and compared by one-way ANOVA with post-hoc Bonferroni testing. (A) Tumor growth curve of DT6606 tumors treated by IT injection. Both treatments significantly reduce tumor growth compared to PBS at day 19 (***P<.001) and VVLΔTK-IL-10 significantly reduces tumor growth compared to VVLΔTK by day 44 (*P<.05, ***P<.001). (B) Kaplan-Meier survival analysis of mice bearing DT6606 tumors after IT treatment. Log rank (Mantel-Cox) tests indicate that both treatments significantly improve survival compared to PBS (P<.0001). *P=.03(VVLΔTK versus VVLΔTK-IL-10). (C) 2.5-months old KPC (LSL-Kras G12D+/− ; LSL-p53R172H+/-; Pdx-1-Cre) (left panel) or KP (LSL-Kras G12D+/- ; LSL-p53R172H+/- ; Cre-), which do not express Cre (right panel) transgenic mice were injected IP with 2x10^8 pfu/ml VVKΔTK on days 1, 3 and 5. On day 7, mice were imaged using the IVIS imaging system (n=2/group). Pancreatic tissue was also harvested for each mouse, sectioned, immunohistochemical staining was performed for detection of VV coat protein (brown), which co-localized within tumor cells in the pancreas of KPC mice but was absent from KP mice. (D) Kaplan-Meier survival analysis of KPC transgenic mice (n=10/group).
injected IP with $2 \times 10^8$ PFU VVLΔTK or VVLΔTK-IL-10 on days 1, 3 and 5 after the animals reached 2.5 months. Significance was assessed using Log rank (Mantel-Cox) tests. *$P<.05$ **$P<.01$.

Figure 3. Immune system involvement in the efficacy of VVLΔTK-IL-10 in vivo. (A) Mice that had cleared tumors after IT treatment with VVLΔTK or VVLΔTK-IL-10 during efficacy experiments were rechallenged four weeks later in the opposite flank with $4 \times 10^6$ DT6606 cells and tumor growth measured as previously. VVLΔTK n=3, VVLΔTK-IL-10 n=6. Mean tumor size ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. *$P<.05$, **$P<.01$. (B) DT6606 tumors were established in male C57/BL6 mice as described previously and 1 day prior to commencement of viral treatment, rat anti-mouse CD4, CD8, NK or control monoclonal antibodies were injected IP. Injections were continued twice weekly for the duration of the experiment and FACS analysis used to confirm depletion. Mean tumor size ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. *$P<.05$, **$P<.01$. (n=5/group).

Figure 4. Analysis of activated T cells, IFN-γ expression, macrophage populations and viral persistence in tumors. DT6606-OVA tumors were established and mice treated IT with VVLΔTK or VVLΔTK-IL-10 following the same regime as described for efficacy experiments. At days 10 and 15, tumors were harvested and analyzed using FACS analysis (n=3/group). (A) CD8+ T cells as assessed by analysis of CD3+/CD8+ populations within CD45+ populations. Mean populations ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing (B) Activated CD8+ cells as assessed by analyzing CD44RBloCD44hpopulations within the CD8+ population. Mean populations ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. *$P<.05$, **$P<.01$. (C) IFN-γ expression within tumors was assessed by ELISA using tumor homogenates after treatment. Mean concentration/0.1g tumor ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. (D) DT6606-OVA tumors were established and mice treated IT with VVLΔTK or VVLΔTK-IL-10 following the same regime as described for efficacy experiments. At days 8 and 16, tumors were harvested and analysed for total macrophage

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populations using FACS analysis (n=3/group). Macrophage activation status was also assessed using an MHCII marker, with MHCIIhi populations regarded as activated macrophages (Ei) and MHCIIlo populations regarded as naive macrophages (Eii). Mean populations ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. (F) To assess viral persistence, DT6606 tumors were established and 18 mice per group treated IT with VVLΔTK or VVLΔTK-IL-10 following the same regime as described previously. At days 8, 16 and 24, tumors were harvested, viral DNA extracted and viral DNA levels quantified in relation to a standard curve using qPCR (i). Mean VV copy number ± SEM is displayed and analyzed at each time point using a Student’s unpaired T test. **P<.01. (n=6/group). Infectious virus recovered from homogenized tumors was also analyzed (ii). Mean viral replication ± SEM was determined by TCID50 assay on CV1 cells. Statistical analysis was carried out using a Student’s unpaired T test at each time point. *P<.05.

**Figure 5. Analysis of CD4+ and CD8+ populations and activation status in splenocytes of VVLΔTK-IL-10 or VVLΔTK treated mice.** DT6606-OVA tumors were established and mice treated IT with VVLΔTK or VVLΔTK-IL-10 following the same regime as described for efficacy experiments. At days 8, 16 and 24 spleens were harvested and analyzed using FACS analysis (n=6/group). (A) CD4 populations as a percentage of live cells in splenocytes of treated mice assessed by gating on CD3+CD4+ populations. (B) Naive CD4 cells as assessed by analyzing CD44RBhiCD44lo populations within the CD4+ population. (C) Activated CD4 cells as assessed by analyzing CD44RBloCD44hi populations within the CD4+ population. (D) CD8 populations in splenocytes of treated mice assessed by gating on CD3+CD8+ populations. (E) Naive CD8 cells as assessed by analyzing CD44RBhiCD44lo populations within the CD8+ population. (F) Activated CD8 cells as assessed by analysing CD44RBloCD44hi populations within the CD8+ population. Mean populations ± SEM are displayed and compared by one-way ANOVA with post-hoc Bonferroni testing. *P<.05, **P<.01, ***P<.001. Representative FACS profiles with gating criteria are shown in supplementary Figure 6.
Figure 6. Analysis of anti-viral and anti-tumor specific CD8 cells in splenocytes of VVLΔTK-IL-10 or VVLΔTK treated mice. DT6606-OVA tumors were established and mice treated IT with VVLΔTK or VVLΔTK-IL-10 following the same regime as described for efficacy experiments. At days 8, 16 and 24 spleens were harvested for analysis (n=6/group). (A) Harvested splenocytes were stained with anti-CD3, anti-CD8 and an H-2Kb restricted, MHC class I anti-B8R pentamer (Proimmune). Mean B8R+CD8+CD3+ cells from virus-treated animals are expressed relative to PBS-treated animals. Mean ratios ± SEM are shown for each group. (B) Splenocytes were incubated for 72 hours with a B8R peptide (Proimmune) and IFN-γ production in response to stimulation measured by ELISA. Mean IFN-γ levels ± SEM are shown. (C) Harvested splenocytes were stained with anti-CD3, anti-CD8 and an H-2Kb restricted, MHC class I anti OVA pentamer (Proimmune). Mean OVA+CD8+CD3+ cells from virus-treated animals are expressed relative to PBS-treated animals. Mean ratios ± SEM are shown for each group. (D) Splenocytes were incubated for 72 hours with mitomycin-treated DT6606-OVA cells and IFN-γ production in response to stimulation measured by ELISA. Mean IFN-γ levels ± SEM are shown. Statistical analysis was carried out using a Student’s unpaired T test. *P<.05, **P<.01, ***P<.001. Representative FACS profiles are shown in supplementary Figure 7.
Figure 1
Figure 3

A

B

Day post first injection

Tumour volume (mm$^3$)

Days after rechallenge

Tumour volume (mm$^3$)

mlgG
α-CD4
α-CD8
α-NK
Figure 4

A

B

C

D

E

F

Author Manuscript Published OnlineFirst on November 21, 2014; DOI: 10.1158/1078-0432.CCR-14-0464
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5

CD4+

(A) Total CD4+CD44+ (live cells) across different treatments.

(B) Naïve CD44+ of CD4+ cells across different treatments.

(C) Activated CD69+CD4+ cells across different treatments.

CD8+

(D) Total CD8+CD44+ (live cells) across different treatments.

(E) Naïve CD44+ of CD8+ cells across different treatments.

(F) Activated CD69+CD84+ cells across different treatments.
Figure 6
A Vaccinia virus armed with interleukin-10 is a promising therapeutic agent for treatment of murine pancreatic cancer

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*Clin Cancer Res* Published OnlineFirst November 21, 2014.

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doi:10.1158/1078-0432.CCR-14-0464

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