Repurposing Sunitinib with Oncolytic Reovirus as a Novel Immunotherapeutic Strategy for Renal Cell Carcinoma

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Translational relevance

In this study, we demonstrate the ability to repurpose sunitinib, a multi-tyrosine kinase inhibitor and first-line metastatic renal cell carcinoma (RCC) agent, to potentiate the immunotherapeutic efficacy of the oncolytic virus, reovirus. This novel therapeutic strategy not only improved tumor responses and overall survival in a murine model of RCC, but also, resulted in the generation of a systemic protective anti-tumor immune response. We validated our findings in a murine model of lung squamous cell carcinoma (SCC), highlighting the potential broad applicability of this treatment approach. Taken together, these results provide clear rationale to investigate this promising viroimmunotherapeutic strategy in early phase clinical trials for a broad range of tumor histologies.
Abstract

Purpose: In addition to their direct cytopathic effects, oncolytic viruses are capable of priming anti-tumor immune responses. However, strategies to enhance the immunotherapeutic potential of these agents are lacking. Here, we investigated the ability of the multi-tyrosine kinase inhibitor and first-line metastatic renal cell carcinoma (RCC) agent, sunitinib, to augment the anti-tumor immune response generated by oncolytic reovirus.

Experimental design: In vitro, oncolysis and chemokine production were assessed in a panel of human and murine RCC cell lines following exposure to reovirus, sunitinib or their combination. In vivo, the RENCA syngeneic murine model of RCC was employed to determine therapeutic and tumor-specific immune responses following treatment with reovirus (intra-tumoral), sunitinib or their combination. Parallel investigations employing the KLN205 syngeneic murine model of lung squamous cell carcinoma (NSCLC) were conducted for further validation.

Results: Reovirus mediated oncolysis and chemokine production was observed following RCC infection. Reovirus monotherapy reduced tumor burden and was capable of generating a systemic adaptive anti-tumor immune response evidenced by increased numbers of tumor-specific CD8+ IFN-γ producing cells. Co-administration of sunitinib with reovirus further reduced tumor burden resulting in improved survival, decreased accumulation of immune suppressor cells and the establishment of protective immunity upon tumor re-challenge. Similar results were observed for KLN205 tumor bearing mice, highlighting the potential broad applicability of this approach.

Conclusion: The ability to repurpose sunitinib for augmentation of reovirus' immunotherapeutic efficacy positions this novel combination therapy as an attractive strategy ready for clinical testing against a range of histologies, including RCC and NSCLC.
Introduction

Despite the improvements in progression free (PFS) and overall survival (OS) associated with the use of targeted therapy, metastatic renal cell carcinoma (mRCC) remains an incurable disease. With a five-year survival rate of less than 10% this malignancy remains a significant health issue [1]. As such, the need for the development of novel therapeutic strategies for this disease is obvious. Sunitinib, a multi-tyrosine kinase inhibitor targeting VEGFR, PDGFR, C-KIT, RET, CSF-1R and FLT-3 is currently a first line therapy for mRCC [2]. While this drug has historically been associated with potent anti-angiogenic activity, recent clinical studies have highlighted its immune modulatory effects. Indeed, following two cycles of oral sunitinib therapy at 50 mg daily for 4 weeks every 6 weeks, mRCC patients display an increase in the percentage of IFN-γ producing T-cells relative to treatment naive patients [3]. Moreover, the immune suppressive type-2 T-cell cytokine response and accumulation of T regulatory cells (Treg) and myeloid derived suppressor cells (MDSC) that are characteristic of mRCC patients is reversed following sunitinib therapy [3,4]. Accordingly, this evidence has generated significant interest in targeting Tregs and MDSC with sunitinib to reverse the mRCC induced immunosuppressive microenvironment and enhance the anti-tumor immune response generated by immunotherapeutics [5]. The feasibility of this approach has been demonstrated in an immunocompetent syngeneic murine model of RCC (RENCA) in which the downregulation of MDSC and Treg by sunitinib enhanced intratumoral infiltration and activation of adoptively transferred CD8 T-cells, leading to tumor regression [6]. Furthermore, sunitinib has also been demonstrated to reduce tumor burden and
improve OS in mouse models of melanoma, hepatocellular carcinoma and colorectal metastasis in immunotherapeutic models, highlighting the efficacy and widespread utility of this approach against multiple histologies [7-9].

In the current study, we investigated the ability of sunitinib to enhance the anti-tumor immune response generated by the dsRNA oncolytic reovirus. To date, reovirus is one of the most clinically advanced oncolytic viruses (OV), demonstrating modest efficacy in phase II clinical trials as a monotherapy and in combination with platinum and taxane based chemotherapy across multiple solid malignancies [10-11]. Of the over 1,500 patients treated thus far with this agent, the maximum tolerated dose has not been reached, highlighting its tolerability and safety. As such, reovirus is one of the most promising OV currently being investigated in the clinic. Despite this however, early phase monotherapy clinical trials have failed to demonstrate appreciable clinical effectiveness in the form of objective and durable complete responses, highlighting the need to augment reovirus’ therapeutic potency. Interestingly, in experimental murine models, reovirus administration is capable of priming innate and adaptive anti-tumor immune responses [12-14]. This anti-tumor immunity has not only been shown to contribute to reovirus’ therapeutic efficacy, but has also been demonstrated to result in the generation of long-term tumor immunosurveillance [15-17]. Moreover, reovirus-mediated anti-tumor immunity has been demonstrated clinically in a phase I trial of a single intra-prostatic injection of reovirus that involved 6 patients with localized prostate cancer [18]. In this study, despite the robust anti-viral neutralizing antibody response that was seen, a significant number of tumor infiltrating CD8+ T-cells were present following reovirus injection. Likewise, in a phase I clinical trial of intravenous reovirus
administration involving patients who had previously received cytotoxic chemotherapy and radiotherapy, increased CD8+ T- and NK-cell (CD3-/CD56+) numbers were observed in the peripheral blood [19]. Hence, in addition to its direct oncolytic effects, reovirus is also a potent immunotherapeutic. While a considerable amount of literature supports reovirus’ immunotherapeutic potential, most pre-clinical investigations have instead focused on improving the systemic delivery of the virus with immunosuppression or histology relevant cytotoxic chemotherapy, respectively [20, 21]. As such, strategies to augment reovirus mediated anti-tumor immune responses for generation of long-term protective immunity capable of improving clinical objective responses are warranted.

Herein, the ability to augment reovirus’s immunotherapeutic efficacy through combination with sunitinib was explored utilizing clinically relevant syngeneic murine models. Collectively, this work highlights the promise of combining reovirus with sunitinib as a novel viroimmunotherapeutic approach, supporting the investigation of this strategy in phase II clinical trials.

**Methods**

**Cell Lines and Virus.** ACHN, A498, 786-0, L-929, KLN205 and RENCA cell lines were obtained from the American Type Tissue Collection (ATCC; Manassas, VA) in July 2010. Cell lines were authenticated by the ATCC by short tandem repeat analysis prior to purchase. No further authentication was done by the authors. For all experiments early passage cells were utilized. ACHN, A498 and KLN205 were cultured in MEM; 786-0 and RENCA were cultured in RPMI; and L-929 was cultured in DMEM; (Invitrogen,
Grand Island, NY). All media was supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Grand Island, NY). Cultures were free of antibiotics and negative for mycoplasma as determined by routine testing. Reovirus, Dearing strain serotype 3 was grown in L-929 cells then purified and tittered as previously described [18]. Sunitinib was purchased from Selleck chemicals (Houston, TX).

**Cell Viability Assay.** Cells were seeded at a density of $3 \times 10^3$ (A498, 786-0) or $8 \times 10^3$ (ACHN, RENCA) cells/well into 96 well microtitre plates and incubated for 24 hours in 10% FCS containing media. Reovirus, sunitinib or their combination were then added to each well for 48 hours. Subsequently, drug containing media was replaced with media containing WST-1 (diluted 10:1) and absorbance was quantified utilizing a BioTek® plate reader (Winooski, VT). Percent viability was calculated as the absorbance ratio of treated/untreated cells multiplied by 100. Additionally, photomicrographs of reovirus cytopathic effects at 48 hours post-infection were captured with a Zeiss® (Jena, Thuringia, Germany) Axiovert 200M microscope at 10x zoom.

**Viral Progeny Assay.** ACHN, 786-0, A498 and RENCA cells were treated with reovirus in 12 well microtitre plates in their respective media for up to 72 hours. The cultures were then freeze-thawed 3 times and to quantify progeny production, supernatants were harvested and plaque titrated on monolayers of L-929 cells in semi-solid medium for 72 hours as previously described [18].

**Chemokine Expression Assay.** Supernatants were harvested from RENCA cells infected with live (LV) or UV-inactivated reovirus (DV) for 12 or 24 hours in 6 well plates. The multiplexing analysis was performed using the Luminex® 100 system (Luminex,
Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Thirty-two markers were simultaneously measured in the serum using a MILLIPLEX® Mouse Cytokine/Chemokine 32-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. Assay sensitivities for these analytes ranged from 0.2 – 63.6 pg/ml.

**In Vitro Synergy Assay.** Dose response curves for all cell lines to reovirus and sunitinib were generated. Calcusyn® software (Biosoft, Great Shelford, Cambridge, UK) was utilized to generate effective dose for 50 percent cytotoxicity (ED$_{50}$) values for each cell line to reovirus and sunitinib from the dose response data. Cell lines were then treated with escalating doses of reovirus and sunitinib concurrently at a fixed ratio of ED$_{50}$:ED$_{50}$. Cell viability was quantified as per the cell viability assay and combination index values were generated using calcusyn® software with a CI value < 1 denoting an synergistic response, > 1 denoting an antagonistic response and = 1 denoting an additive response.

**In Vivo Studies in Immunocompetent Syngeneic Murine Mouse Models.** All mice in these studies were housed in pathogen-free conditions with food and water *ad libitum* and treated within procedural guidelines that were approved by the University of Calgary Animal Care Committee. 2.5 x 10$^6$ RENCA or 5 x 10$^5$ KLN205 cells were implanted into the right hind-flank of Balb/C or DBA/2 mice, respectively (Charles River, Montreal, QC) on day 0. Once tumors were palpable (approximately day 5), therapy was initiated. Mice were grouped into cohorts and treated with intraperitoneal (i.p) PBS, intratumoral (i.t) UV-irradiated reovirus (5x10$^8$ PFU), i.t live reovirus (5x10$^8$ PFU), i.p sunitinib (40 mg/kg) or a combination of these agents as indicated on figure legends. Bi-
weekly caliper measurements were taken to monitor tumor burden. Tumor volume was calculated using the formula, volume = 0.52 x (width)$^2$ x length.

For the adoptive transfer studies, spleens were extracted from mice treated as above 19 days after RENCA tumor implantation (3 mice/group). After pooled single cell suspensions were generated by passing mechanically separated spleens through 100 µm cell strainers (Becton Dickinson®, Franklin Lakes, NJ), mononuclear splenocytes were isolated by centrifugation over a Ficoll-Hypaque gradient (GE Healthcare®, Uppsala, Sweden). 1 x 10$^7$ splenocytes in 100 µl PBS were then administered intravenously via tail-vein into each of 6 recipient Balb/C mice for each group. 7 days later, all recipient mice were challenged with an implantation of 2.5 x 10$^6$ RENCA cells into the right hind-flank then measured bi-weekly for tumor burden.

For immune depletion studies, CD8$^+$ or CD4$^+$ lymphocyte depletion was accomplished by i.p injection of 0.25 mg depleting anti-CD8a monoclonal antibody (BioXcell, West Lebanon, NH; CD8 Clone: 2.43; CD4 Clone GK1.5) on day 5, followed by 0.1 mg on day 8, 12 and 19.

For tumor re-challenge studies, Balb/C RENCA tumor bearing mice successfully treated with reovirus + sunitinib combination therapy were re-challenged with 1 x 10$^6$ RENCA cells in the contralateral hind flank and tumor burden was assessed by caliper measurement. Treatment naïve Balb/C mice were challenged in the same manner to serve as a control.

**CD8$^+$ Enrichment.** Spleens were processed as described above from Balb/C or DBA/2 mice bearing RENCA or KLN205 tumors, respectively, and enriched for CD8$^+$ T-cells
using an EasySep® Mouse CD8⁺ Selection Kit via an EasySep® magnet as per manufacturer’s protocol (Stem Cell Technologies, Vancouver, BC).

**Flow Cytometry.** Flow Cytometry was performed as previously described [22]. Briefly, cell staining was done using FITC, Alexa Flour 488, PE, APC, PE-Cy7 or PerCP Cy5.5 conjugated rat monoclonal antibodies against CD4, CD8, Foxp3, CCR7, CD62L, CD11b and GR1 (Lyc6G+/Lyc6C)(BD Biosci ence®). APC conjugated hamster IgG against CD3e (eBioscience®). Appropriate mouse IgG isotypes were used as controls (BD Bioscience®). Intracellular Foxp3 staining was done using Mouse Foxp3 Buffer Set (BD Bioscience®) according to the manufacture protocol. Intratumoral lymphocytes were stained and analysed by flow cytometry following collagenase type I (Gibco, cat #17100-017) treatment according to the manufacture protocol. Briefly, tumor tissue were washed with PBS, cut into small piece (>1 × 1 mm) and incubated with collagenase type I (100 U/µl) for 4 h at 37°C. Tumor fragments were then disaggregated through a 100 µm cell strainers, single cells were collected and enumerated. All Samples were run using BD accuri flow cytometry (BD Bioscience®) and analyzed by flowJo software (FlowJo Enterprise®).

**ELISA Assay.** 1 x 10⁶ CD8⁺ splenocytes were isolated as above and co-incubated in 1 ml of complete RPMI in 24 well plates with 1 x 10⁵ RENCA cells either untreated of infected with 1 MOI of reovirus for the previous 12 hours. At 48 hours, supernatants were harvested for IFN-γ ELISA as per manufacturer’s protocol (R&D systems, Minneapolis, MN) at room temperature. Briefly, capture antibody at 8 µg/ml; biotinylated detection antibody at 0.8 µg/ml; and avidin peroxidise at 1.5 µg/ml were utilized with ABTS substrate. The sensitivity of the assay was 31.25 pg/ml.
**ELISPOT Assay.** CD8⁺ splenocytes from RENCA tumor bearing Balb/C mice were isolated as above from pooled cell suspension and 2 x 10⁵ cells were subsequently co-cultured with RENCA cells (typically 1 x 10⁴ cells) for 48 hours in 96-well nitrocellulose membrane plates pre-coated with anti-mouse IFN-γ monoclonal antibody (BD Bioscience, Cat # 552569). Detection antibody solution at 100 μL/well was added for 2 hours followed by Streptavidin-HRP solution at 100 μL/well for a 1 hour incubation period. AEC substrate was then added and spots were scanned and counted using the CTL-ImmunoSpot® S6 Macro Analyzer and BioSpot® Software, respectively (C.T.L, Shaker Heights, OH). The frequency of RENCA-specific CD8⁺ splenocytes was calculated based on the percentage of CD8⁺ T cells present in the responding population.

**Lactate dehydrogenase (LDH) Cytotoxicity Assay.** CD8⁺ splenocytes from KLN205 tumor bearing DBA/2 mice were isolated as above from pooled cell suspension and subsequently co-cultured with 1 x 10⁴ KLN205 cells in a 96 well plate for 24 hours. Supernatant was collected and cytotoxicity was measured by CytoTox Non-Radioactive Cytotoxicity Assay kit according to the manufacture protocol (Promega, Madison, WI). Cytotoxicity was calculated using the formula: effector (experimental) – effector (spontaneous) – target (spontaneous)/ target (maximum) – target (spontaneous) multiplied by 100.

**Statistics.** Statistical analysis was preformed utilizing Graph Pad version 6. Unpaired two-tailed t-tests and two-way ANOVA followed by Bonferroni Post Hoc tests were used to determine significance between experimental groups. Kaplan-Meier analysis together with log-rank sum test was utilized to determine significant for *in vivo* survival benefits.
Statistical significance was defined as p-values being < 0.05 unless otherwise stated.

Results

Reovirus replicates in human and murine RCC cell lines resulting in oncolysis, chemokine production and synergistic cytotoxicity when combined with sunitinib.

Reovirus is a non-enveloped dsRNA virus that has demonstrated oncolytic activity against a wide variety of malignancies in both preclinical and clinical studies. As this virus has not been investigated to date against RCC the first objective was to determine its oncolytic activity in a panel of human RCC cell lines with differing von Hippel Lindau (VHL) gene status (786-O VHL+/−, A498 VHL+/−, ACHN VHL+/+). Treatment of 786-O, ACHN and A498 cell lines with reovirus resulted in a dose dependent decrease in cell viability (Figure. 1A). Light microscopy of all cell lines exposed to virus confirmed cytopathic effect, highlighted by plasma membrane blebbing, cell surface detachment and rounding up of the RCC cell lines (Figure. 1B). A rise in viral titre was also observed following reovirus treatment, confirming a productive lytic infection of the RCC cells (Figure. 1C).

Reovirus has been demonstrated to initiate innate immune responses characterized by the production of pro-inflammatory cytokines including RANTES, MIP-1-α, MCP-1, KC, IP-10 and MIG across a variety of melanoma and prostate cancer cell lines in addition to its direct oncolytic effects [14, 17]. Based on these findings we characterized the ability of reovirus to stimulate production of these chemokines during infection of the murine RCC RENCA cell line. This cell line was studied to provide proof-
of-principle of chemokine generation during reovirus oncolysis of RCC in vitro to support subsequent in vivo experiments utilizing this cell line. Similar to the human RCC cell lines, infection of RENCA cells with reovirus resulted in viral replication and cytotoxicity (Figures. 1A-C). Furthermore, following 24 hours of reovirus infection at a concentration of both 0.007 MOI (ED$_{50}$) and 1 MOI increased chemokine expression was observed, highlighting the potential of utilizing this agent to generate an inflammatory oncolytic response against RCC (Figure. 1D). Importantly, chemokine production was observed in viable RENCA cells 12 hours post-infection suggesting chemokine production was a product of viral replication rather than cell lysis (Supplemental Figure. 1).

To assess in vitro synergy between reovirus and sunitinib, combination index (CI) values as per the Chou and Talalay method were determined [23]. Dose response data for reovirus and sunitinib on RCC cells was used to calculate ED$_{50}$ values (Figure. 1E, Supplemental Figure. 2A), with median effect plots demonstrating acceptable linear correlation coefficients (r > 0.9). Treatment of cells with increasing doses of reovirus and sunitinib at fixed ratios (ED$_{50}$:ED$_{50}$) revealed a synergistic or additive cytotoxic response (CI ≤ 1) for both the ACHN and A498 cell lines across all doses studied (Figure. 1F). Reovirus-sunitinib synergy was also observed in RENCA and 786-O cells; however, the magnitudes of effect were highly dependent on concentration (Figure. 1F).

Reovirus demonstrates in vivo therapeutic efficacy as a monotherapy and in combination with sunitinib.
The RENCA murine model of RCC is an established immunocompetent syngeneic model for studying novel therapeutics including immunotherapeutics against this disease preclinically [24]. This model was employed to investigate whether or not reovirus has therapeutic efficacy against RCC in vivo and determine the ability of sunitinib to augment this activity. Sunitinib dose response studies were conducted in vivo to determine the optimal dose to administer in combination with reovirus. A dose range of 20 to 60 mg/kg was chosen as it is clinically relevant and has previously been utilized in the RENCA model [6]. A dose of 40 mg/kg of sunitinib significantly decreased tumor burden while not eradicating tumors (Supplemental Figure. 2B) and as such was chosen for subsequent experiments. Reovirus was administered at a dose of $5 \times 10^8$ plaque forming units (PFU) based on previous reports in immunocompetent murine models [13].

In combination experiments, sunitinib was administered prior to reovirus and on a continuous basis as this approach has been demonstrated to improve immunotherapeutic efficacy by allowing sunitinib to precondition the tumor microenvironment through downregulation of immune suppressor cells, including MDSC [25]. RENCA tumor bearing balb/C mice were treated with monotherapy sunitinib [i.p], reovirus [i.t] or a combination of these agents. UV-irradiated non-replicating reovirus (DV) [i.t] was administered as a control. Sunitinib treatment was initiated once palpable tumors were present (approximately day 5) and continued for 14 days while reovirus was administered on day 8, 11 and 14. Although, relative to DV and PBS controls, reovirus given as a monotherapy significantly reduced tumor burden, the use of this agent in combination with sunitinib resulted in a significantly increased reduction in
tumor volume (P < 0.001 by two-way ANOVA) (Figure. 2A). Tumor burden was not plotted beyond 18 days as considerable central necrosis in large tumors occurred limiting accurate measurement. Instead, overall survival was followed to establish long-term therapeutic efficacy. Kaplan-Meier analysis of two independent experiments with pooled results (8 mice overall) revealed an overall survival benefit (P < 0.05, log-rank sum test) for those mice receiving reovirus in combination with sunitinib versus either agent used as a monotherapy (Figure. 2B). Death in all cases was a result of tumor progression as determined by tumor size greater than 2 centimeters in a single dimension or greater than 20 percent loss of body weight as per Animal Care institutional guidelines. Notably, animal body weights remained reasonably stable during treatment and no therapy specific toxicities were observed (Supplemental Figure. 3).

**Sunitinib augments reovirus mediated anti-tumor immune response through reversal of tumor-induced immunosuppression.**

As reovirus infection results in the production of pro-inflammatory cytokines and consequent priming of innate and adaptive immunity [14-17], the anti-tumor immune response generated by reovirus treatment of RCC in vivo was determined. Mice were treated as in Figure 2A with sunitinib, reovirus or combination therapy and CD8+ splenocytes were harvested and co-cultured with RENCA cells or reovirus infected RENCA cells for antigenic stimulation prior to IFN-γ ELISA. In mice receiving reovirus monotherapy a significant IFN-γ response was demonstrated from harvested CD8+ splenocytes following stimulation with reovirus infected RENCA cells (Figure. 3A). Interestingly, sunitinib therapy significantly augmented this reovirus generated anti-tumor immunity, as a robust increase in IFN-γ production (4-fold) in those mice that
received reovirus in combination with sunitinib was observed (Figure. 3A). In mice receiving PBS, DV or sunitinib, minimal or no IFN-γ response was seen. Notably, while CD8+ reactive splenocytes were not observed in reovirus or combination therapy treated mice by ELISA when RENCA cells were used alone as an antigenic stimulus, their presence was confirmed by the higher sensitivity ELISPOT assay (Figure. 3B-C). To further establish an immunotherapeutic mechanism of reovirus and combination therapy, CD8 depletion studies were conducted (>98% depletion confirmed; Supplementary Figure. 4) confirming overall survival benefit observed with reovirus or combination therapy was CD8 mediated (Figure. 3D). Interestingly, we observed that CD4 depletion (>98% depletion confirmed; Supplementary Figure. 4) as a monotherapy resulted in a therapeutic benefit, consistent with recent reports highlighting the ability to enhance tumor immunogenicity with this approach through Treg depletion (Figure. 3E) [26]. However, as expected, CD4 depletion did not abrogate the therapeutic effects of reovirus or combination therapy. Taken together, these results suggest that reovirus generates a systemic adaptive anti-tumor immune response against RCC that is augmented by sunitinib.

As sunitinib is known to reverse tumor induced immunosuppression [4], we next immunophenotyped splenocytes and tumor-infiltrating immune populations in mice receiving reovirus, sunitinib or combination therapy. Consistent with a viroimmunotherapeutic effect, reovirus monotherapy resulted in an increased accumulation of CD8+ splenocytes, as well as, CD8+ tumor-infiltrating lymphocytes (Figure. 4A-C). This was associated with a concomitant accumulation of splenic and intra-tumoral MDSC as well as intra-tumoral Tregs (Figure. 4D-F). As hypothesized,
sunitinib reversed this reovirus-induced immunosuppressive effect preventing the accumulation of splenic MDSC and intratumoral MDSC and Tregs (Figure 4D-F), highlighting this as a potential mechanism explaining sunitinib’s ability to augment reovirus-mediated adaptive immune responses (Figure 3A-C).

To determine whether reovirus or combination therapy resulted in the establishment of a protective immune response, adoptive transfer experiments were conducted. Here, splenocytes from mice treated with reovirus, sunitinib, or combination therapy were isolated and intravenously transferred into treatment-naive mice (Figure 5A). These mice were then challenged with a s.c injection of RENCA cells and followed for tumor initiation and burden. Interestingly, only those mice receiving splenocytes from mice treated with combination therapy demonstrated reduced tumor growth rate relative to controls, highlighting an established protective immune response in this group (P < 0.05 between all groups by two-way ANOVA) (Figure 5B). To further support these findings tumor re-challenge experiments were conducted with mice that had achieved a complete response to combination therapy versus mice that were treatment naïve. Indeed, this demonstrated that pre-treated cured animals were protected against tumor re-challenge whereas naïve mice formed rapidly growing tumors (Figure 5C). Overall, these results confirmed establishment of protective immunity following combination therapy.

Sunitinib augments reovirus immunotherapeutic effect in a murine model of lung SCC.
To confirm a broader utility of reovirus-sunitinib viroimmunotherapy as a novel therapeutic strategy we conducted parallel investigations in a syngeneic murine lung squamous cell carcinoma model employing the KLN205 cell line. Consistent with the results in the RENCA model, KLN205 cells demonstrated sensitivity to reovirus oncolysis in vitro and displayed a dose-variable synergistic response when treated with combination therapy (Supplementary Figure. 5A-C). In vivo, combination therapy also resulted in decreased KLN205 tumor burden as well as improved overall survival relative to monotherapy (Figure. 6A-B). Moreover, this effect was associated with increased splenic CD8+ lymphocytes with a memory phenotype [CD8+/CCR7+/CD62L] (Figure. 6C-D) and a concomitant prevention of reovirus-induced MDSC accumulation (Figure. 6E). Furthermore, isolated CD8+ splenocytes from mice treated with combination therapy demonstrated increased effector function in co-culture cytotoxicity assays with KLN205 cells, confirming enhanced adaptive immunity (Figure. 6F). Taken together, these results establish a viroimmunotherapeutic benefit for reovirus-sunitinib combination therapy against murine lung SCC, highlighting the broad potential of this novel treatment paradigm.

Discussion

Metastatic renal cell carcinoma remains an incurable disease with an urgent need for novel therapeutics to be developed that impact overall survival. Immunotherapeutic strategies against RCC are of particular interest given that durable complete responses have only been demonstrated in patients receiving cytokine (IL-2) immunotherapy, as well as recent evidence highlighting the efficacy of immune checkpoint inhibition of PD-1 [27-29]. The current study represents the first preclinical...
evidence of reovirus efficacy as both a direct oncolytic and immunotherapeutic agent for use against RCC. Beyond this, the ability to repurpose sunitinib, a first-line approved mRCC agent, for augmentation of reovirus’ immunotherapeutic efficacy was confirmed. This not only positions reovirus-sunitinib combination therapy as an attractive novel treatment strategy, but further supports the growing body of literature highlighting the benefit of harnessing the immune system to improve oncolytic virotherapy.

The conducted in vitro studies demonstrate the ability of reovirus to induce oncolysis against RCC, as has been described for multiple tumor histologies (Figure. 1A-C). Oncolysis of human RCC cell lines was seen within 48 hours of infection with all ED$_{50}$ values being less than 40 MOI, which is comparable to that seen with other solid malignancies [18, 30-32]. Notably, the RENCA cell line demonstrated significantly greater in vitro sensitivity relative to human RCC cells, consistent with previous reports highlighting increased murine cell line sensitivity to reovirus [33]. While the precise mechanism for the enhanced sensitivity remains unknown, this observation suggested a direct oncolytic in vivo response could be achieved. As such, the established sensitivity of the RENCA cell line coupled with the observed production of pro-inflammatory cytokines known to be involved in the priming of reovirus mediated innate and adaptive immunity (RANTES, MIP-1α, MCP-1, KC, IP-10 and MIG) supported its use for in vivo investigation of reovirus immunotherapeutic efficacy against RCC as a model to establish pre-clinical proof-of-principle [14, 17] (Figure. 1D). Moreover, these results also highlight that similar to infection of melanoma and prostate cancer cells, reovirus replication induces an inflammatory cell death response against RCC [14, 18].
The therapeutic utility of the in vitro studies was confirmed in vivo utilizing the RENCA immunocompetent murine model of RCC. Treatment of mice with reovirus and sunitinib resulted in a reduction in tumor burden relative to both PBS and DV controls (Figure. 2A). Furthermore, in those mice receiving reovirus and sunitinib, tumor specific CD8+ splenocytes could be isolated (Figure. 3A-B), highlighting the ability to generate a systemic adaptive anti-tumor immune response.

The combination of OV with relevant standard-of care treatments will likely be essential for their successful translation into clinical practice. The observation that the combination of sunitinib, a first-line mRCC therapeutic, with reovirus resulted in a significant regression in tumor burden and improved overall survival relative to either of these agents given as monotherapies, providing proof-of-principle to support the use of these agents in combination for superior anti-tumor activity (Figure. 2A-B). The potential to broaden this treatment paradigm to other histologies was confirmed by demonstrating combination therapy efficacy in the KLN205 murine lung SCC model (Figure. 6A-B). While the observed benefit in this model was significant, it was not as robust as seen with the RENCA model possibly due to disparities in sensitivity to reovirus oncolysis or differences in immunogenicity.

To better understand the direct cytotoxic effects of reovirus-sunitinib combination therapy on RCC, the Chou and Talalay method was utilized [23]. This line of experimentation demonstrated the potential for reovirus and sunitinib to synergistically induce cell death, highlighting enhanced direct cytotoxicity as a plausible mechanism explaining the superior therapeutic efficacy seen with these agents in combination in vivo (Figure. 1F). Interestingly a recent report has demonstrated the ability to sunitinib
to enhance viral replication through targeting innate immune pathways such as double stranded RNA protein Kinase R (PKR) and RNaseL, shedding light on a possible mechanism for the observed in vitro synergy [3]. Moreover, as reovirus mediated apoptosis and autophagy have previously been well characterized in other cancer cell lines and ex vivo human tumor specimens [35-39], we hypothesize the observed synergy may reside in sunitinib’s ability to sensitize RCC cells to these alternate modes of cell death.

In addition to direct synergistic effects on cell viability these results also support augmentation of anti-tumor immunity as a contributing mechanism mediating the observed in vivo synergy between reovirus and sunitinib. This is evidenced by a significant increase in the production of IFN-γ from tumor specific CD8⁺ splenocytes isolated from mice treated with reovirus-sunitinib combination therapy (Figure 3A-B). Augmentation of a therapeutic immune response by sunitinib is further established through our CD8 depletion (Figure 3D-E), adoptive transfer (Figure. 5B) and tumor re-challenge experiments (Figure. 5C), which confirm the ability to generate a systemic protective immune response with combination therapy. This finding has significant translational potential given the recent results published by Zamarin et al. who established that intratumoral treatment of primary tumors with immunogenic oncolytic viruses has the ability to clear metastatic disease once protective immunity has been established; known as the abscopal effect [40]. As such, our work supports the potential of circumventing longstanding issues with intravenous OV administration and viral immune clearance by application of intratumoral approaches to generate systemic immune responses for treatment of metastatic disease. Notably, destruction of tumor
vasculature has also been observed through intratumoral endothelial cell sensitization to oncolytic virotherapy following resolution of sunitinib therapy [41]. As such, it is likely that sunitinib and reovirus work through multiple independent mechanisms to achieve the observed in vivo synergy including both immune and non-immune mediated.

Sunitinib also has been reported to enhance therapeutic immune responses through reversing tumor induced immune suppression in patients with mRCC [3]. This activity resides partly in its ability to downregulate the levels of circulating and intratumoral MDSC, which have been demonstrated to orchestrate mRCC immune suppression through direct T-cell inhibition as well as stimulating the upregulation of Tregs [3-4]. Given this, mechanistic investigations were focused on correlating MDSC and Treg levels with tumor specific CD8\(^+\) splenocyte IFN-\(\gamma\) production between mono- and combination-therapy groups in vivo. Consistent with an inflammatory cell death, reovirus monotherapy administration induced a marked rise in both immune stimulatory (CD8\(^+\) and CD4\(^+\) lymphocytes) and suppressor (MDSC and Treg) populations within the spleen and tumor, similar to recent reports in the IP8 ovarian peritoneal carcinomatosis model [33] (Figure. 4A-F). In keeping with our hypothesis, the accumulation of immune suppressor cells could be prevented by combination of reovirus with sunitinib, correlating with the observation that mice treated with combination therapy produce more tumor specific CD8\(^+\) splenocytes. These results were consistent with our findings in the KLN205 murine lung squamous cell carcinoma model, where mice treated with reovirus-sunitinib combination therapy had increased numbers of tumor reactive CD8\(^+\) cells and increased memory T cells, while splenic MDSC accumulation was prevented (Figure. 6C-F). Given the previously documented ability of these immune suppressor
cell populations in mediating resistance to reovirus-mediated immunity [42], our results suggest sunitinib prevention of MDSC and Tregs accumulation as a potential mechanism for enhancing reovirus mediated adaptive immunity. Our laboratory is currently focused on elucidating the precise immune mechanism underlying the observed immunotherapeutic efficacy of reovirus-sunitinib combination therapy.

Taken together, we believe these findings provide proof-of-principle for the study of this novel viroimmunotherapeutic paradigm against a broad range of malignancies, including RCC. Beyond this, as sunitinib is currently a first line mRCC approved therapeutic and reovirus is in advanced phase III clinical trials, we believe the rapid translation of these findings in the setting of a clinical trial for patients with this incurable cancer is warranted.
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Figure Legends

Figure 1. Reovirus has direct oncolytic effects against human and murine RCC. A) Cell viability of human (786-O, A498, ACHN) and murine (RENCA) RCC cell lines treated with escalating doses of reovirus (RV) determined by WST-1 assay. B) Pictures of RCC cells infected with 40 (786-O, A498, ACHN) or 0.007 (RENCA) MOI of UV-irradiated virus (DV) or RV for 48 hours, taken with a Zeiss Axiovert 200M microscope at 10x zoom. C) RV titre over 72 hours following infection of RCC cell lines (40 MOI) determined by plaque titration assay. D) Chemokine levels in supernatants from RENCA cells infected with RV at ED50 and 1 MOI for 24 hours determined by luminex analysis. E) Sunitinib and reovirus ED50 doses after RCC cell line treatment for 48 hours calculated by calcsyn software. F) Combination index (CI) values (+/- SEM) for RCC cell lines treated for 48 hours with sunitinib and reovirus at a fixed ratio of ED50:ED50 calculated by calcsyn software. Synergistic values in bold. In all panels error bars = SEM for at least three independent experiments. P < 0.001 (***)) by two-tailed students t-test.

Figure 2. Reovirus combined with sunitinib results in decreased tumor burden and improved overall survival in the RENCA murine model. Balb/C mice were implanted with RENCA (2.5 x 10^6 cells) s.c tumors and treated with PBS, sunitinib (S) [40 mg/kg i.p], UV-irradiated reovirus (DV) [5 x 10^6 pfu i.t], live reovirus (RV) [5 x 10^8 pfu i.t], or a combination of these agents. Sunitinib was given daily for 14 consecutive days starting on day 5 post RENCA implantation. Reovirus was administered three times (day 8, 11, 14). A) Tumor size was followed with caliper measurements. N = 6 mice per group. Error bars = SEM of tumors within each group. P < 0.001 (**), 0.01 (**), 0.05 (*) by two-way ANOVA. Data representative of two independent experiments. B) Kaplan-Meier plot of mice treated as per panel A. N = 8 mice per group. Analysis represents data pooled from two independent experiments involving 3 and 5 mice, respectively. P < 0.001 (***)) by log-rank between PBS and RV or Sunitinib + RV. P < 0.05 (*) by log-rank between RV and Sunitinib + RV.

Figure 3. Reovirus induced anti-tumor immune response is augmented by sunitinib. A) CD8^+ cells were separated from pooled spleens of RENCA tumor-bearing mice within each treatment group from figure 2A and stimulated with reovirus infected RENCA cells. IFN-γ production was quantified by IFN-γ ELISA. N = 3 mice. B-C) CD8^+ cells were separated from pooled spleens of RENCA tumor-bearing mice treated as per figure 2A and stimulated with RENCA cells. Percentage of RENCA-specific IFN-γ^+ cells determined by ELISPOT assay. N = 5 mice. Representative wells B) and their quantitation C) shown. In both panels error bars = SEM of experimental triplicate. P < 0.001 (**), by two-tailed students t-test. D, E) Kaplan-Meier plot demonstrating overall survival for mice pre-treated with depleting anti-CD8a (D) or anti-CD4a (E) antibodies.
(i.p) followed by treatment as in figure 2A. P < 0.01 (**), by log-rank test between Sunitinib+RV vs. Sunitinib+RV+CD8 Ab. N = 5 mice.

**Figure 4.** Reovirus-sunitinib therapy increases tumor and splenic immune stimulatory cells while preventing accumulation of immune suppressor cells. Pooled splenocytes (A, B, D) and tumor single-cell suspensions (C, E, F) from RENCA tumor-bearing mice treated as per figure 2A were immunophenotyped by flow cytometry. In all panels error bars = SEM of experimental triplicate. P < 0.001 (***) , 0.01 (**), by two-tailed students t-test. Source of cells indicated in parentheses.

**Figure 5.** Combination therapy induces a protective immune response against tumor re-challenge. A) Adoptive transfer experimental scheme adapted from Gujar et al. 2011, Molecular Therapy [17]. Briefly, mice (donor) were implanted with RENCA tumors and treated as shown in figure 2A. On day 19, splenocytes were harvested and transferred to treatment naïve mice (recipient) by tail vein injection, followed by RENCA (2.5 x 10^6 cells s.c.) tumor challenge. Arrow indicates treatment start date, X indicates treatment end date. B) Tumor size in recipient mice challenged with RENCA cells s.c. N = 6 mice/group. Error bars = SEM of tumors within each group. P < 0.01 (**), by two-way ANOVA. Data representative of two independent experiments. C) Pre-treated (reovirus + sunitinib) mice demonstrating curative response (N = 3) and a cohort of treatment naïve mice (N = 5) were challenged with RENCA tumors (1 x 10^6 cells, s.c). Tumor burden over time is displayed. P < 0.001 (***) , by two-tailed students t-test.

**Figure 6.** Sunitinib augments reovirus immunotherapeutic efficacy in a syngeneic immunocompetent murine model of lung squamous cell carcinoma. DBA/2 mice were implanted with KLN205 (5 x 10^5 cells) s.c tumors and treated as in figure 2A. Sunitinib was given daily for 14 consecutive days starting on day 6 post KLN205 implantation. Reovirus was administered four times (day 9, 12, 15, 18). A) Tumor size was followed with caliper measurements. N = 8 mice per group. Error bars = SEM of tumors within each group. P < 0.001 (***) , 0.01 (**), 0.05 (*) by two-way ANOVA. B) Kaplan-Meier plot of mice treated as per panel A. N = 4 mice per group. P < 0.05 (*) by log-rank between PBS or RV or Sunitinib and Sunitinib + RV. C-E) Splenocytes from KLN205 tumor-bearing mice treated as per panel A (sacrificed day 23) were immunophenotyped by flow cytometry. In all panels error bars = SEM of experimental triplicate. P < 0.001 (***) , 0.01 (**), 0.05 (*) by two-tailed students t-test. F) CD8^+ splenocytes were isolated from KLN205 tumor-bearing mice treated as per panel A and co-cultured with KLN205 cells. % KLN205 cytotoxicity was determined by LDH assay. Error bars = SEM of experimental triplicate. P < 0.05 (*) by two-tailed students t-test.
Figure 1

A

% Viability vs Reovirus [MOI]

B

DV

RV

C

Log 10 Viral Titer (pfu/ml)

D

Chemokine Levels (pg/ml)

E

ED50 Values

| Cell Line | Reovirus | Sunitinib |
|-----------|----------|-----------|
| 786-O     | 35 MOI   | 7 μM      |
| ACHN      | 11 MOI   | 8 μM      |
| A498      | 37 MOI   | 4 μM      |
| RENCA     | 0.007 MOI| 4 μM      |

F

Combination Index

<1: Synergistic  =1: Additive  >1: Antagonistic

| Cell Line | ED50/8 | ED50/4 | ED50/2 | ED50  | 2 x ED50 |
|-----------|--------|--------|--------|-------|--------|
| ACHN      | 0.48 +/- | 0.59 +/- | 0.93 +/- | 0.55 +/- | 0.21 +/- |
|           | 0.01   | 0.02   | 0.06   | 0.20  | 0.01   |
| A498      | 0.99 +/- | 0.73 +/- | 0.69 +/- | 0.63 +/- | 0.27 +/- |
|           | 0.07   | 0.07   | 0.07   | 0.12  | 0.05   |
| RENCA     | 0.65 +/- | 0.67 +/- | 1.03 +/- | 1.48 +/- | 1.18 +/- |
|           | 0.10   | 0.09   | 0.06   | 0.06  | 0.33   |
| 786-O     | 5.97 +/- | 2.65 +/- | 1.83 +/- | 0.33 +/- | 0.23 +/- |
|           | 0.63   | 0.43   | 0.17   | 0.09  | 0.03   |
Figure 2

A

![Graph showing tumour volume (mm³) over days post RENCA implantation.](image)

- PBS
- DV
- Sunitinib
- Live-RV
- Sunitinib + DV
- Sunitinib + RV

B

![Graph showing percent survival over days post RENCA implantation.](image)

- PBS
- DV
- Sunitinib
- RV
- Sunitinib + DV
- Sunitinib + RV

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Figure 3

A

![Graph showing IFN-γ levels after different treatments](image)

B

CD8+ cells + RENCA

|   | CD8+ cells + RENCA |
|---|---------------------|
| 1 | PBS                 |
| 2 | Sun                 |
| 3 | RV                  |
| 4 | RV + Sun            |
| 5 | Blank               |
| 6 | Media               |

C

%CD8+ IFN Spots/205 Cells

![Bar graph showing %CD8+ IFN Spots/205 Cells](image)

D

Percent survival vs. Days post RENCA Implantation

E

Percent survival vs. Days post RENCA Implantation

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Figure 6

A

Tumour Volume (mm\(^3\))

- PBS
- DV
- Sunitinib
- RV
- Sunitinib + DV
- Sunitinib + RV

Days post KLN205 implantation

B

Percent survival

- PBS
- DV
- Sunitinib
- RV
- Sunitinib + DV
- Sunitinib + RV

Days post KLN205 implantation

C

%CD8\(^+\) (Spleen)

- PBS
- Sunitinib
- RV
- Sunitinib + RV

E

%CD11b\(^+\)LY6C/LY6G\(^+\) (Spleen)

- PBS
- Sunitinib
- RV
- Sunitinib + RV

D

%CD8\(^+\)CCR7\(^-\)CD62L\(^-\) (Spleen)

- PBS
- Sunitinib
- RV
- Sunitinib + RV

F

% LDH Cytotoxicity (Spleen)

- PBS
- Sunitinib
- RV
- Sunitinib + RV
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