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

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Abstract

Purpose: In addition to their direct cytotoxic effects, oncolytic viruses are capable of priming antitumor 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 antitumor 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 after 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 after treatment with reovirus (intratumoral), 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 antitumor 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 rechallenge. Similar results were observed for KLN205 tumor-bearing mice, highlighting the potential broad applicability of this approach.

Conclusions: 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. Clin Cancer Res; 22(23): 5839–50. ©2016 AACR.

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 antiangiogenic activity, recent clinical studies have highlighted its immune-modulatory effects. Indeed, after 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-naïve 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), which are characteristic of mRCC patients, are reversed after 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 antitumor 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 antitumor 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.
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 antitumor 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.

Methods

Cell lines and virus

ACHN, A498, 786-0, L-929, KLN205, and RENCA cell lines were obtained from the ATCC 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 minimum essential medium; 786-0 and RENCA were cultured in RPMI; and L-929 was cultured in DMEM (Invitrogen). All media were supplemented with 10% heat-inactivated FCS (Invitrogen). 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 titrated as described previously (18). Sunitinib was purchased from Selleck Chemicals.

Cell viability assay

Cells were seeded at a density of $3 \times 10^5$ (A498, 786-0) or $8 \times 10^5$ (ACHN, RENCA) cells/well into 96-well microtiter plates and incubated for 24 hours in 10% FCS-containing media. Reovirus, sunitinib, or their combination was then added to each well for 48 hours. Subsequently, drug-containing media were replaced with media containing WST-1 (diluted 10:1) and absorbance was quantified utilizing a BioTek plate reader. Percent viability was calculated as the absorbance ratio of treated/untreated cells multiplied by 100. Additionally, photomicrographs of reovirus cytopathic effects at 48 hours after infection were captured with a Zeiss Axiovert 200M microscope at $10 \times$ zoom.

Viral progeny assay

ACHN, 786-0, A498, and RENCA cells were treated with reovirus in 12-well microtiter 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 described previously (18).

Chemokine expression assay

Supernatants were harvested from RENCA cells infected with live (RV) 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) by Eve Technologies Corp. Thirty-two markers were simultaneously measured in the serum using a MILLIPLEX Mouse Cytokine/Chemokine 32-plex kit (Millipore) according to the manufacturer’s protocol. Assay sensitivities for these analytes ranged from 0.2 to 63.6 pg/mL.

In vitro synergy assay

Dose–response curves for all cell lines to reovirus and sunitinib were generated. CalcuSyn software (Biosoft) was utilized to generate effective dose for 50 percent cytotoxicity (ED50) 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 ED50:ED50. Cell viability was quantified as per the cell viability assay and combination index (CI) 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. A total of $2.5 \times 10^6$ RENCA or $5 \times 10^5$ KLN205 cells were implanted into the right hind-flank of Balb/C or DBA/2 mice, respectively (Charles River Laboratories) on day 0. Once tumors were palpable (approximately day 5), therapy was initiated. Mice were grouped into cohorts and treated with intraarterial injection (i.p) PBS, intratumoral UV-irradiated reovirus ($3 \times 10^5$ PFU), intratumoral live reovirus ($5 \times 10^5$ PFU), sunitinib (40 mg/kg, i.p.), or a combination of these agents as indicated in the figure legends. Biweekly caliper measurements were taken to monitor tumor burden. Tumor volume was calculated using the formula, volume = $0.52 \times (width)^2 \times length$.

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), mononuclear splenocytes were isolated by centrifugation over a Ficoll–Hypaque gradient (GE Healthcare). A total of $1 \times 10^7$ splenocytes in 100-µL PBS were then administered intravenously via tail-vein into each of 6 recipient Balb/C mice for each group. Seven days later, all recipient mice were challenged with an implantation of $2.5 \times 10^5$ RENCA cells into the right hind flank and then measured biweekly for tumor burden.

For immune depletion studies, CD8$^+$ or CD4$^+$ lymphocyte depletion was accomplished by intraperitoneal injection of 0.25 mg depleting anti-CD8α mAb (BioXcell; CD8 Clone: 2.43; CD4 Clone GK1.5) on day 5, followed by 0.1 mg on day 8, 12, and 19.

For tumor rechallenge studies, Balb/C RENCA tumor–bearing mice successfully treated with reovirus-sunitinib combination therapy were rechallenged with $1 \times 10^5$ 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).

Flow cytometry

Flow cytometry was performed as described previously (22). Briefly, cell staining was done using FITC, Alexa Flour 488, PE, APC, PE-Cy7, or PerCP Cy5.5 conjugated rat mAbs against CD4, CD8, Foxp3, CCR7, CD62L, CD11b, and GR1 (Lyc6G+/Lyc6C; BD Biosciences). APC-conjugated hamster IgG against CD3ε (eBioscience). Appropriate mouse IgG isotypes were used as controls (BD Biosciences). Intracellular Foxp3 staining was done using Mouse Foxp3 Buffer Set (BD Biosciences) according to the manufacturer's protocol. Intratumoral lymphocytes were stained and analyzed by flow cytometry following collagenase type I (Gibco, cat #17100-017) treatment according to the manufacturer's protocol. Briefly, tumor tissue was washed with PBS, cut into a small piece (>1 x 1 mm), and incubated with collagenase type I (100 U/µL) for 4 hours at 37°C. Tumor fragments were then disaggregated through 100-µm cell strainers, and single cells were collected and enumerated. All Samples were run using BD Accuri Flow Cytometry (BD Biosciences) and analyzed by FlowJo software (FlowJo Enterprise).

ELISA

A total of $1 \times 10^6$ CD8$^+$ splenocytes were isolated as above and co incubated in 1 ml of complete RPMI in 24-well plates with $1 \times 10^5$ RENCA cells either untreated or infected with 1 MOI (multiplicity of infection) of reovirus for the previous 12 hours. At 48 hours, supernatants were harvested for IFγ ELISA as per the manufacturer's protocol (R&D Systems) 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 $2 \times 10^5$ cells were subsequently cocultured with RENCA cells (typically $1 \times 10^5$ cells) for 48 hours in 96-well nitrocellulose membrane plates precoated with anti-mouse IFγ mAb (BD Biosciences, 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.). The frequency of RENCA-specific CD8$^+$ splenocytes was calculated on the basis of the percentage of CD8$^+$ T cells present in the corresponding population.

Lactate dehydrogenase cytotoxicity assay

CD8$^+$ splenocytes from KLN205 tumor–bearing DBA/2 mice were isolated as above from pooled cell suspension and subsequently cocultured with $1 \times 10^5$ 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 manufacturer's protocol (Promega). Cytotoxicity was calculated using the formula: effector (experimental) – effector (spontaneous) – target (spontaneous)/target (maximum) – target (spontaneous) × 100.

Statistical analysis

Statistical analysis was performed utilizing GraphPad 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 significance 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 nonenveloped 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 (Fig. 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 (Fig. 1B). A rise in viral titer was also observed after reovirus treatment, confirming a productive lytic infection of the RCC cells (Fig. 1C).

Reovirus has been demonstrated to initiate innate immune responses characterized by the production of proinflammatory chemokines including RANTES, MIP-1α, 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). On the basis of 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 used to provide proof-of-principle of chemokine generation during reovirus oncology 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 cytoxicity (Fig. 1A–C). Furthermore, after 24 hours of reovirus infection at a concentration of both 0.007 MOI (ED50) and 1 MOI increased chemokine expression was observed, highlighting the potential of utilizing this agent to generate an inflammatory oncolytic response against RCC (Fig. 1D). Importantly, chemokine production was observed in viable RENCA cells 12 hours after infection suggesting chemokine production was a product of viral replication rather than cell lysis (Supplementary Fig. S1).

To assess in vitro synergy between reovirus and sunitinib, CI values as per the Chou and Talalay method were determined (23). Dose response data for reovirus and sunitinib on RCC cells were used to calculate ED10 values (Fig. 1E and Supplementary Fig. S2A), 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 (ED50:ED50) revealed a synergistic or additive cytoxic response (CI ≤ 1) for both the ACHN and A498 cell lines across all doses studied (Fig. 1F). Reovirus–sunitinib synergy was also observed in RENCA and 786-O cells; however, the magnitudes of effect were highly dependent on concentration (Fig. 1F).

Reovirus demonstrates in vivo therapeutic efficacy as a mono- and in combination with sunitinib

The RENCA murine model of RCC is an established immuno-competent syngeneic model for studying novel immunotherapeautics 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–reovirus 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 (Supplementary Fig. S2B) and as such was chosen for subsequent experiments. Reovirus was administered at a dose of 5 × 10⁶ 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 MDSCs (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 nonmultiplying reovirus (DV;1/2) 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; Fig. 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 test) for those mice receiving reovirus in combination with sunitinib versus either agent used as a monotherapy (Fig. 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% 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 (Supplementary Fig. S3).

Sunitinib augments reovirus-mediated antitumor immune response through reversal of tumor-induced immunosuppression

As reovirus infection results in the production of proinflammatory chemokines and consequent priming of innate and adaptive immunity (14–17), the antitumor immune response generated by reovirus treatment of RCC in vivo was determined. Mice were treated as in Fig. 2A with sunitinib, reovirus, or combination therapy and CDS− splenocytes were harvested and cocultured with RENCA cells or reovirus-infected RENCA cells for antigenic stimulation prior to IFNγ ELISA. In mice receiving reovirus monotherapy, a significant IFNγ response was demonstrated from

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**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 (RENC) MOI of UV-irradiated virus (DV) or RV for 48 hours, taken with a Zeiss Axiosvert 200M microscope at ×10 (zoom). C, RV titer over 72 hours after 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 CalcuSyn software. F, CI values (±SEM) for RCC cell lines treated for 48 hours with sunitinib and reovirus at a fixed ratio of ED50:ED50 calculated by CalcuSyn software. Synergistic values in bold. In all panels error bars = SEM for at least three independent experiments. P < 0.001 (“**”) by two-tailed Student t test.
bars \( \frac{1}{4} \) was followed with caliper measurements. Sunitinib \(+\) live reovirus (RV; 5\(^{10^8}\) pfu i.t), or a combination of these agents. Sunitinib was given daily for 14 consecutive days starting on day 5 after 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. \( * * * , P < 0.001 ; * * , P < 0.01 ; * , P < 0.05 \) by two-way ANOVA. Data representative of two independent experiments. B, Kaplan-Meier plot of mice treated as per 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. 

harvested CD8\(^+\) splenocytes after stimulation with reovirus-infected RENCA cells (Fig. 3A). Interestingly, sunitinib therapy significantly augmented this reovirus-generated antitumor immunity, as a robust increase in IFN-g 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 (Fig. 3B–C). To further establish an immunotherapeutic mechanism of reovirus and combination therapy, CD8\(^-\) depletion studies were conducted (>98% depletion confirmed; Supplementary Fig. S4) confirming OS benefit observed with reovirus or combination therapy was CD8\(^-\) mediated (Fig. 3D). Interestingly, we observed that CD4 depletion (>98% depletion confirmed; Supplementary Fig. S4) 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 (Fig. 3E; ref. 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 antitumor 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 (Fig. 4A–C). This was associated with a concomitant accumulation of splenic and intratumoral MDSCs as well as intratumoral Tregs (Fig. 4D–F). As hypothesized, sunitinib reversed this reovirus-induced immunosuppressive effect preventing the accumulation of splenic MDSCs and intratumoral MDSCs and Tregs (Fig. 4D–F), highlighting this as a potential mechanism explaining sunitinib’s ability to augment reovirus-mediated adaptive immune responses (Fig. 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-naïve mice (Fig. 5A). These mice were then challenged with a subcutaneous 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; Fig. 5B). To further support these findings, tumor rechallenge experiments were conducted with mice that had achieved a complete response to combination therapy versus mice that were treatment naïve. Indeed, this demonstrated that pretreated cured animals were protected against tumor rechallenge, whereas naïve mice formed rapidly growing tumors (Fig. 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 \( \text{in vitro} \) and displayed a dose-variable synergistic response when treated with combination therapy (Supplementary Fig. S5A–C). \text{In vivo}, combination therapy also resulted in decreased KLN205 tumor burden as well as improved OS relative to monotherapy (Fig. 6A and B). Moreover, this effect was associated with increased splenic CD8\(^-\) lymphocytes with a memory phenotype (CD8\(^-\)CCR7\(^-\)CD62L\(^-\); Fig. 6C and D) and a concomitant prevention of reovirus-induced MDSC accumulation (Fig. 6E). Furthermore, isolated CD8\(^+\) splenocytes from mice treated with combination therapy demonstrated increased effector function in coculture cytotoxicity assays with KLN205 cells, confirming enhanced adaptive immunity (Fig. 6F). Taken together, these results establish a viroimmunotherapeutic benefit for...
Reovirus induced antitumor immune response is augmented by sunitinib. A, CD8\(^+\) cells were separated from pooled spleens of RENCA tumor-bearing mice within each treatment group from Fig. 2A and stimulated with reovirus-infected RENCA cells. IFN\(\gamma\) production was quantified by IFN\(\gamma\) ELISA. N = 3 mice. B and C, CD8\(^+\) cells were separated from pooled spleens of RENCA tumor-bearing mice treated as per Fig. 2A and stimulated with RENCA cells. Percentage of RENCA-specific IFN\(\gamma\)^+ cells determined by ELISPOT assay. N = 5 mice. Representative wells (B) and their quantitation (C) is shown. In both panels, error bars = SEM of experimental triplicate. \(***\), \(P < 0.001\), by two-tailed Student t test. D and E, Kaplan–Meier plot demonstrating OS for mice pretreated with depleting anti-CD8a (D) or anti-CD4a (E) antibodies (i.p) followed by treatment as in Fig. 2A. \(***\), \(P < 0.01\), by log-rank test between sunitinib + RV versus sunitinib + RV + CD8 Ab. N = 5 mice. Data from panel D and E Represent a single experiment and are presented separately for clarity.
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 Fig. 2A were immunophenotyped by flow cytometry. N = 5 mice/group. In all panels, error bars = SEM of experimental triplicate. ***, P < 0.001, **, P < 0.01, by two-tailed Student t test. Source of cells indicated in parentheses.
Viroimmunotherapy for Renal Cell Carcinoma

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 (Fig. 1A–C). Oncolysis of human RCC cell lines was seen within 48 hours of infection with all ED50 values being less than 40 MOI, which is comparable with 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 proinflammatory chemokines 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 preclinical proof-of-principle (14, 17) (Fig. 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 (Fig. 2A). Furthermore, in those mice receiving reovirus and sunitinib, tumor-specific CD8$^+$ splenocytes could be isolated (Fig. 3A-B), highlighting the ability to generate a systemic adaptive antitumor 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 results in a significant regression in tumor burden and improved OS relative to either of these agents given as monotherapies provides proof-of-principle to investigate these agents in combination for superior antitumor activity (Fig. 2A and 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 (Fig. 6A and B). While the observed benefit in this model was significant, it was not as robust as seen with the RENCA model possibly due to

Figure 5.

Combination therapy induces a protective immune response against tumor rechallenge. A, Adoptive transfer experimental scheme adapted from Gujar and colleagues (17). Briefly, mice (donor) were implanted with RENCA tumors and treated as shown in Fig. 2A. On day 19, splenocytes were harvested and transferred to treatment-naïve mice (recipient) by tail vein injection, followed by RENCA (2.5 $\times$ 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, Pretreated (reovirus + sunitinib) mice demonstrating curative response (N = 3) and a cohort of treatment-naïve mice (N = 5) were challenged with RENCA tumors (1 $\times$ 10$^7$ cells, s.c). Tumor burden over time is displayed. ***, P < 0.001, by two-tailed Student t test.

reovirus–sunitinib combination therapy against murine lung SCC, highlighting the broad potential of this novel treatment paradigm.
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 (Fig. 1F). Interestingly, a recent report has demonstrated the ability of 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 (34). Moreover, as reovirus-mediated apoptosis 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 (Fig. 1F). Interestingly, a recent report has demonstrated the ability of 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 (34). 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 antitumor 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 IFNγ from tumor specific CD8+ splenocytes isolated from mice treated with reovirus-sunitinib combination therapy (Fig. 3A and B). Augmentation of a therapeutic immune response by sunitinib is further established through our CD8 depletion (Fig. 3D and E), adoptive transfer (Fig. 3B), and tumor rechallenge experiments (Fig. 3C), 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 and colleagues 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 after 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 nonimmune 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 IFNγ 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 carcinoma metastasis model (ref. 33; Fig. 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 (Fig. 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by a graduate studentship award from the Alberta Cancer Foundation (to K.A. Lawson) and by operating grants from the Alberta Cancer Foundation and Alberta Innovates Health Solutions (awarded to D.G. Morris).

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Received January 18, 2016, revised April 11, 2016, accepted April 18, 2016, published OnlineFirst May 24, 2016.

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