Reovirus as a Viable Therapeutic Option for the Treatment of Multiple Myeloma

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Abstract

Purpose: Despite the recent advances made in the treatment of multiple myeloma, the disease still remains incurable. The oncolytic potential of reovirus has previously been shown and is currently in phase III clinical trials for solid tumors. We tested the hypothesis that reovirus can successfully target human multiple myeloma in vitro, ex vivo, and in vivo without affecting human hematopoietic stem cell (HHSC) repopulation/differentiation in a murine model that partially recapitulates human multiple myeloma.

Experimental Design: Human myeloma cell lines and ex vivo tumor specimens were exposed to reovirus and oncolysis and mechanisms of cell death were assessed. RPMI 8226GFP+ cells were injected intravenously to non-obese diabetic/severe combined immune deficient (NOD/SCID) mice and treated with live reovirus (LV) or dead virus (DV). Multiple myeloma disease progression was evaluated via whole-body fluorescence and bone marrow infiltration. HHSCs exposed to LV/DV were injected to NOD/SCID mice and repopulation/differentiation was monitored.

Results: A total of six of seven myeloma cell lines and five of seven patient tumor specimens exposed to reovirus showed significant in vitro sensitivity. Tumor response of multiple myeloma by LV, but not DV, was confirmed by comparison of total tumor weights (P = 0.05), and bone marrow infiltration (1/6, LV; 5/6, DV). Mice injected with LV- or DV-exposed HHSCs maintained in vivo re-population/lineage differentiation showing a lack of viral effect on the stem cell compartment. Reovirus oncolysis was mediated primarily by activation of the apoptotic pathways.

Conclusions: The unique ability of reovirus to selectively kill multiple myeloma while sparing HHSCs places it as a promising systemic multiple myeloma therapeutic for clinical testing. Clin Cancer Res; 1–11. ©2012 AACR.

Introduction

Multiple myeloma is a clonal neoplasm of plasma cells that accounts for approximately 10% of all hematological malignancies (1). The incidence of multiple myeloma has doubled in the last 5 decades (2), with a current actuarial average of 17 years life lost per diagnosis (3). Despite recent advances in the understanding of multiple myeloma biology and the availability of newer therapeutics such as thalidomide, revlimid, and bortezomib, which have improved responses and survival (4), the disease remains incurable and treatment resistance/toxicities associated with these agents ultimately result in patient demise (5, 6). Thus, it is imperative to find novel, more effective, better-tolerated therapeutics for multiple myeloma and of equal importance is the elucidation of these novel therapeutics mechanisms of action.

Oncolytic viruses are a group of therapeutics that exhibit an extensive spectrum of anticancer activity with minimal human toxicity. One such virus, the reovirus, is an ubiquitous, non-enveloped double-stranded RNA virus with negligible pathogenicity in humans (7). The reovirus’s extensive preclinical efficacy has been documented by our group and others and it is presently undergoing phase III clinical trial testing for head and neck tumors. The viral sensitivity of a wide array of tumor histologies is possibly mediated via multiple permissive oncogenic signaling pathways that allow the reovirus to target malignancies while sparing normal cells (8–10). Thus, the “oncogenic addiction” of multiple myeloma associated with its survival and proliferation creates an environment conducive to reovirus therapy.

Recently, it has been shown that mutated ras and constitutively active Akt delineate distinctive oncogenic
Translational Relevance

This study examines the potential use of the oncolytic virus, the reovirus, as a therapeutic agent for multiple myeloma, a disease for which there is no cure at present despite several new therapeutics being brought to the clinic. This work utilizes human multiple myeloma cell lines, *ex vivo* patient tumors, and *in vivo* animal models to ensure that the observations are as clinically relevant to a translational clinical setting. It lays the foundation to support an early-phase clinical trial for multiple myeloma with reovirus.

The reovirus, serotype 3 (strain Dearing), has been previously shown to purge multiple myeloma xenografts (SCID-hu), and in *ex vivo* patient specimens and provide evidence that the reovirus orchestrates cells death via apoptosis, we showed for the first time that reovirus infection of multiple myeloma leads to the modulation of autophagy which is of further clinical relevance as understanding mechanisms of reovirus oncolysis of multiple myeloma will lead to optimal use of this therapeutic, especially for the treatment of therapy-resistant disease.

Materials and Methods

**Cell lines**

RPMI 8226, U266, MM1S, and H929 were obtained from the American Type Culture Collection (ATCC, Virginia). OPM2 was obtained from the German collection of microorganisms and cell cultures (Braunschweig, Germany). KMS-11, INA-6, and RPMI 8226 GFP+ cells were kindly provided by Drs. Irene Ghobrial (Dana Farber Cancer Institute, Boston, MA), Renate Burger (University of Erlangen-Nuernberg, Erlangen, Germany), and Linda Pilarsky (University of Alberta, Canada), respectively. RPMI 8226, U266, MM1S, H929, KMS11, and OPM2 were maintained in RPMI 1640 medium (Gibco BRL) containing 10% FBS. In addition, the medium was supplemented with either G148 (100 μg/mL), interleukin 6 (IL-6; 2.5 ng/mL), or 0.05 mmol/L 2-mercaptoethanol for RPMI 8226 GFP+, IN-6A, and H929 cells, respectively. Multiple myeloma primary tumor cells were obtained from BM aspirate/biopsies. Diagnosis was based on histopathology, immunohistochemistry, and immunophenotype. All procedures involving patients were approved by the Conjoint Health Research Ethics Board, University of Calgary, Calgary, Alberta, Canada.

Reovirus

Reovirus serotype 3 (strain Dearing) was propagated in L929 cells and purified as previously described (10). Human multiple myeloma cell lines (HMCL) grown to subconfluence were infected with either no virus (NV), live reovirus (LV), or UV-inactivated reovirus (dead reovirus, DV) at a multiplicity of infection (MOI) of 40 plaque-forming units (PFU)/cell for 24, 48, and 72 hours. Reovirus cytotoxicity was monitored using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (24). Similarly, patient BM samples that were found to contain more than 3% multiple myeloma cells were washed in PBS and cultured in RPMI 1640 medium containing 20% FBS and antibiotics and exposed to 40 or 100 MOI of LV or DV for 24 hours. The higher reovirus MOI was used as the marrow samples were not enriched for large cohort of human multiple myeloma cell lines (HMCL) and *ex vivo* patient specimens and provide evidence that intravenous treatment of reovirus is effective in targeting disseminated human multiple myeloma *in vivo* and that reovirus exposure does not abrogate human hematopoietic stem cell re-population/differentiation in the BM compartment. This is the first report to show the efficacy of reovirus targeting of human multiple myeloma in the BM milieu and human stem cell re-population post-reovirus treatment in an *in vivo* setting, and thus support the rapid translation of preclinical data to a future phase I clinical trial. In addition to confirming that reovirus orchestrates cells death via apoptosis, we showed for the first time that reovirus infection of multiple myeloma leads to the modulation of autophagy which is of further clinical relevance as understanding mechanisms of reovirus oncolysis of multiple myeloma will lead to optimal use of this therapeutic, especially for the treatment of therapy-resistant disease.

**Translational Relevance**

This study examines the potential use of the oncolytic virus, the reovirus, as a therapeutic agent for multiple myeloma, a disease for which there is no cure at present despite several new therapeutics being brought to the clinic. This work utilizes human multiple myeloma cell lines, *ex vivo* patient tumors, and *in vivo* animal models to ensure that the observations are as clinically relevant to a translational clinical setting. It lays the foundation to support an early-phase clinical trial for multiple myeloma with reovirus.

pathways in this malignancy, which independently contributes to multiple myeloma survival (11). Activating mutations in the ras gene family are common in multiple myeloma, with N- and K-ras mutations being the most frequent (12, 13). In addition, multiple other cancer-promoting signaling pathways have been shown to be active in multiple myeloma such as the MEK/ERK, phosphoinositide 3-kinase (PI3K)/Akt, mTOR/p70S6-kinase, and NF-kB pathways (14–16) that may be permissive to reovirus lytic infection, thereby increasing the spectrum of potentially treatable patients. In susceptible tumor cells, reovirus is thought to exploit these activated neoplastic signaling pathways in a p53-independent/-dependent manner (17, 18) and involves NF-kB activation/trafficking (17, 19), autocrine release of the TNF-related apoptosis-inducing ligand (TRAIL; refs. 20, 21), and activation of endoplasmic reticular (ER) stress (22).

To date, only 2 studies have examined the preclinical effects of reovirus on multiple myeloma. Our group has previously shown the ability of the reovirus to purge multiple myeloma successfully during autologous transplantations (23) and Kelly and colleagues (22) have investigated the potential of using reovirus in combination therapy with bortezomib on subcutaneous multiple myeloma tumor established in a syngeneic murine model as well as in a 5TG1 syngeneic murine system. Although there is a lack of consensus about the best *in vivo* preclinical model of multiple myeloma, that is, immunocompetent syngeneic or human xenograft murine model or fetal bone marrow (BM) implant-multiple myeloma xenograft (SCID-hu), a relatively straightforward intravenous/intracardiac approach to the delivery of multiple myeloma should be considered. Subcutaneous models fail to consistently home to the bone marrow (22), and murine multiple myeloma cell lines involved in syngeneic models fail to authentically replicate human genetic lesions/aberrations found in human multiple myeloma. The SCID-hu model, although it mimics the human BM environment, does not produce disseminated disease as seen in advanced human multiple myeloma.

To represent clinical relevance, it is imperative to study the effects of reovirus given systemically on human multiple myeloma in vivo in the BM microenvironment. In this study, we showed the oncolytic potential of the reovirus against a
the multiple myeloma cells, thus diluting the effective concentration of reovirus.

Flow cytometric analysis of apoptosis and autophagy
HMC-Ls were infected with either 40 MOI of DV, LV, or NV for 24, 48, and 72 hours and harvested. Following centrifugation, the cell pellets were washed in PBS twice before subsequent staining with propidium iodide (PI)/RNase A or Annexin-V-Alexa Fluor 488 (A13201; Invitrogen)/7AAD (A07704; Beckman Coulter) as previously described (10). Flow cytometric analysis was carried out to assess DNA fragmentation and phosphatidyl serine expression using a Navios flow cytometer (Beckman Coulter) and FCS Express software (De Novo Software).

For caspase inhibition, cells were pretreated with Z-VAD-FMK-001 (R&D systems), 50 μmol/L for 1 hour before infection with reovirus. Harvested cells were stained for rabbit anti-active caspase 3 V450 (Becton Dickinson Biosciences) using Beckman Coulter’s IntraPrep intracellular staining kit according to the manufacturer’s instructions. Autophagy was detected using the Cyto-ID Autophagy detection kit (ENZ-51031-k200; Enzo Life Sciences) in NV-, DV-, or LV-treated RPMI 8226 cells at 0, 24, and 48 hours. An autophagy inhibitor, 3-MA (SC-205596; Santa Cruz), was added to cells at 10 mmol/L for 1 hour before infection with reovirus. To identify vesicles colocalizing with LC3-II, a marker of autophagosomes, 1 × 10⁶ cells were centrifuged and resuspended in 1 mL of Assay Buffer. Then, 0.5 mL of a 1:2,000 dilution of the Cyto-ID Green Detection Reagent was added to the cell suspension, and the mixture was incubated at 37°C for 30 minutes in the dark and analyzed immediately in the LFL1 detector.

Apheresis product and reovirus effects on human CD34 + stem cells
All apheresis products (AP) used in the present study were obtained from patients registered at the Tom Baker Cancer Centre, Calgary, Canada, after informed consent. AP cells were washed in PBS and treated with ammonium chloride for 10 minutes to induce lysis of red blood cells. Mononuclear cells resuspended in Robosep buffer (cat # 20104; Stem Cell Technologies) were depleted of lineage-committed cells using the Robosep automated immunomagnetic cell-separation system (cat # 20000; Stem Cell Technologies). The RoboSep progenitor enrichment antibody cocktail (Catalog # 18056; Stem Cell Technologies) was used to enrich for CD34+ cells. The isolated cells were seeded at a density of 2 to 3 × 10⁵/mL in StemSpan SFEM (Stem Cell Technologies) containing cytokines and growth factors (23) and then incubated in the presence of LV or DV (40 MOI) for 72 hours at 37°C in a humidified incubator with 5% CO₂. Harvested cells were assayed for viability and purity (CD34+ and CD45+ cells) using flow cytometry as previously described (23). We have previously shown no significant difference between DV and NV treatments (23, 25) in different tumors as well as CD34+ stem cells and AP. Therefore, DV was selected as the most stringent control for all reovirus experiments.

PCR for detection of human “Alu” sequences
To confirm “human cell” re-population in vivo, DNA was isolated from mouse bone marrow and BM using QIAamp DNA mini kits (Qiagen) and PCR was carried out against human-specific “Alu” primers (AAGTGCAGGCGGCTCAGTGAGCCAGAT; ref. 26). The PCR product was separated in 2% agarose gels containing ethidium bromide and “human Alu” bands were visualized.

Animals
Six- to 8-week-old female severe combined immune deficient/non-obese diabetic (SCID/NOD) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. The animals were housed in a biocontainment animal facility and provided food and water ad libitum. All procedures were reviewed and approved by the University of Calgary Animal Care Committee.

Evaluation of reovirus efficacy in human multiple myeloma under in vivo settings
Twelve SCID/NOD mice were sublethally irradiated (1.25 Gy total body γ-irradiation from a 137Cs source) and administered an intraperitoneal injection of 50 μL of anti-asialo GM1 antibody for elimination of natural killer (NK) cells. Mice were injected with 2.5 × 10⁶ RPMI 8226-GFP+ cells via tail vein for tumor establishment. On day 7, mice in 2 groups were given a single injection of either LV or DV (1 × 10⁶ PFU) intravenously. Animals were assessed for weight loss, grooming performance, and signs of visible tumor 3 times per week. On day 35, when animals exhibited greater than 20% weight loss or required euthanasia, all animals were sacrificed and assessed for multiple myeloma disease prevalence.

Sacrificed animals were subjected to whole-body fluorescence imaging for detection of GFP+ multiple myeloma lesions in the skeleton and subcutaneous tissues. Liver, spleen, lung, brain, heart, small, and large intestines were analyzed for metastatic deposits by GFP fluorescence. Femurs from animals were flushed in PBS + 10% FBS and the collected BM cells were analyzed for GFP +/CD38+ staining.

Human stem cell re-populating ability in vivo
Sixteen NOD/SCID mice were sublethally irradiated and treated with anti-asialo GM1 antibody as described above. Animals were divided into 3 groups, and the first 2 groups (n = 6) were administered 200 μL of LV- or DV-treated CD34+ stem cells (1–5 × 10⁶, isolated from patient AP) mixed with 2 × 10⁶ of AP cells that have been impaired for proliferation through irradiation (2 Gy; carrier cells) via the tail vein. The 3rd group (n = 4) was given only carrier cells (2 × 10⁶). To prevent reovirus-related morbidity and inflammatory vasculitis in the extremities seen in immunocompromised animals beyond day 30 (never seen in nude mice or immunocompetent mice), all animals were treated with purified polyclonal rabbit antireovirus serum (100 μg tail vein) on days 14, 21, and 28. Body weights and grooming abilities of animals were recorded. On days 25 and 40, 3 animals in
each group were subjected to cardiac puncture, and peripheral blood was collected in tubes containing heparin (Sigma-Aldrich). Femurs and tibia were removed from the sacrificed mice and BM cells were flushed out into saline and combined for each mouse. Using a CD45/LSS gating strategy and a CD19 gate, harvested BM and blood were immunophenotyped by flow cytometry with the following 2- and 5-color antibody combinations: CD45/CD34, CD8/CD4/CD45/CD16+56/CD3, Kappa/Lambda/CD19/CD5/CD20, and CD64/CD13/CD45/CD33/CD16 (Beckman Coulter).

**Statistical Analysis**

All analyses were carried out using the statistical software program R (Boston, MA; ref. 27). For the WST assay experiments, 3 independent experiments were carried out with each cell line in triplicate. The resulting colorimetric readings were averaged and background readings were subtracted to provide the corresponding number of viable cells. The data is presented as the mean with their 95% confidence intervals (CI).

To assess reovirus treatment on multiple myeloma tumor, harvested tumor from DV- and LV-treated animals was subjected to an exact Wilcoxon Mann–Whitney Rank Sum test, a nonparametric, robust alternative to the 2 sample t-test, appropriate when data may be non-normally distributed. To explore the significance of reovirus-induced DNA fragmentation and annexin V expression, we calculated 2-factor Analysis of Variance (ANOVA) models within each of the 3 cell lines. Levene’s test for variance homogeneity based on the median values was used to check this assumption.

To confirm caspase and autophagy activation during reovirus infection of multiple myeloma, DV- and LV-treated multiple myeloma cells with and without caspase and autophagy inhibitors were also compared using their mean
and 95% CIs, with planned t-test comparisons for specific questions of interest.

Results

Reovirus sensitivity of HMCLs and ex vivo specimens

All HMCLs displayed significant sensitivity toward reovirus, showing less than 12% viability at 72 hours, except for OPM2 (Fig. 1A). Reovirus sensitivity could not be correlated with immunoglobulin H (IgH) translocations or ras mutational status of HMCLs (Supplementary Table S1). Interestingly, the only reovirus-resistant HMCL, OPM2, harbored a PTEN mutation.

When reovirus sensitivity on ex vivo patient-derived tumor was investigated, 5 out of 7 patient multiple myeloma cells exhibited reovirus sensitivity, highlighting its potential to be used as a systemic therapeutic (Fig. 1B). It was difficult to grow the ex vivo tumor in RPMI medium beyond 48 hours and, therefore, only data for 24 hours are presented. Supplementary Table S2 presents data indicating that reovirus sensitivity does not correlate with the prognostic characteristics of the 7 patient tumors [individual t (4:14), 13q(-) and TP53]. Flow cytometric scatterplots representative of sensitive, moderately sensitive, and resistant patients are shown in Fig. 1C.

Reovirus oncolysis is mediated through apoptosis

To investigate the mechanism of reovirus-mediated cell death, RPMI8226, NCI-H929, U266, and OPM2 cells

![Figure 2. HMCLs were exposed to 40 MOI of LV or DV for 24, 48, and 72 hours and apoptosis was assayed. A, DNA fragmentation assessed by PI incorporation using flow cytometry. B, Annexin V and 7AAD expression in DV- and LV-treated HMCLs. C, Annexin V expression by flow cytometry for the multiple myeloma (MM) cell line U266.](image-url)
were treated with DV or LV and assessed for apoptosis. All 3 reovirus-sensitive HMCLs exposed to LV showed significantly elevated features of apoptosis compared with DV-treated cells (i.e., membrane phosphatidyl serine flipping and DNA fragmentation). Significant temporal changes on DNA fragmentation with LV treatment were seen in all 3 HMCLs (F-tests for interaction; P-values < 0.01; Fig. 2A) and in annexin V binding in U266 cells (P < 0.006; Fig. 2B). Significant differences were seen between LV and DV at all time points in RPMI8226 and NCI-H929 cells (F-tests for treated cells; P < 0.0001); these collective results confirm the presence of reovirus oncolysis.

Figure 2C represents flow cytometric analysis of U266 apoptosis with nearly 90% of the LV-treated cells showing annexin V and 7-AAD positivity by 72 hours. In significant contrast to these HMCLs, no evidence of any apoptotic activity was detected in reovirus-resistant OPM2 when treated with either LV or DV (data not shown). To confirm that reovirus-induced apoptosis is mediated via terminal caspase 3, multiple myeloma cells were treated with caspase inhibitor Z-VAD-FMK-001 and DNA fragmentation was assessed. As depicted in Figs. 3A and B, treatment with Z-VAD-FMK-001 led to a significant downregulation of fragmented DNA in the sub-G0/G1 peak in LV-treated cells at 24 hours (t-test P = 0.0012).

Reovirus modulates autophagy induction in multiple myeloma

Although apoptosis induction was prominent in reovirus-infected HMCLs, complete reovirus oncolysis could not
be attributable to this process. We, therefore, examined autophagy involvement post-reovirus infection of myeloma. Autophagy activity was similar in DV- and LV-treated RPMI 8226 cells at 0 hours (Fig. 3C). Autophagy induction was evident at 24 hours in LV-treated cells and, at 48 hours, this was more pronounced with a distinctive shift in median channel fluorescence of stained cells with Cyto-ID. To confirm the autophagy process, we treated RPMI 8226 cells with autophagy inhibitor 3-MA and verified autophagy activity post virus infection. Distinct temporal increases in autophagy induction were evident in LV-treated cells in comparison to DV-treated cells (Fig. 3D). Treatment with 3-MA considerably downregulated this increase in autophagy caused by LV, resulting in a 4-fold reduction by 48 hours, thus indicating that autophagy might also contribute to cell death.

**Intravenous reovirus treatment successfully targets disseminated human multiple myeloma**

To explore whether intravenous reovirus treatment would target disseminated human multiple myeloma in a mouse model, animals in 2 groups with established disease were treated with a single intravenous injection of DV or LV. All of the 6 mice injected with DV showed symptoms of malignancy (weight loss, neurological deficits, etc.) beyond days 20 and, therefore, all animals were sacrificed at day 35. Multiple GFP + multiple myeloma lesions were seen in 5 out of 6 DV-treated mice (Fig. 4A), with the majority of lesions seen in the axial skeleton, skull, and plasmacytoma-like lesions. In contrast, 4 of the 6 mice treated with LV appeared to be disease free with no visible GFP+ lesions detected. Comparison of total tumor weights between the 2 groups of mice showed significant differences ($P = 0.05$, exact Mann–Whitney Rank Sum test; Fig. 4B). Mouse BM analyzed for GFP+/CD38+/CD138+ multiple myeloma cells showed tumor in 5 of 6 DV-treated mice and only in 1 LV-treated mouse (Table 1). Figure 4C presents the flow cytometric scatterplots of 2 mice from each group that showed the highest amount of tumor burden in BM. The tumor burden seen in DV-treated mouse was $>10$-fold than that of the only LV-treated mouse detected with human multiple myeloma in BM. These mice were treated only with a single injection of reovirus and, if multiple LV injections were given, complete eradication of disease would be anticipated.

**Reovirus exposure of human CD34+ hematopoietic stem cells does not abrogate re-population/differentiation in mice**

Mice of both groups (DV- or LV-treated) showed human stem cell re-population at 25 to 30 days post infusion (Fig. 5A). In addition, DV- or LV-treated stem cells showed lineage differentiation in mouse BM. As lymphoid differentiation preceeds myeloid differentiation, the
majority of differentiated cells were noted to be of the myeloid lineage as expected. In significant contrast to LV- or DV-treated stem cells-infused mice, no human stem cells were seen in mouse BM in recipients that received carrier cells alone (Figs. 5A and 5B). To further confirm the presence of human cells, DNA was extracted from mouse BM and blood. PCR conducted against human “Alu” sequences confirmed human cell re-population only in animals that received DV- or LV-treated stem cells. (Fig. 5C).

Discussion

The present study evaluated reovirus as a viable option for systemic therapy of human multiple myeloma. The broad-spectrum activity of reovirus on HMCLs was confirmed under in vitro conditions where 6 of the 7 HMCLs showed sensitivity to reovirus. Four of these harbored ras mutations, but this alone was not predictive of virus oncolysis, as U266 and KMS11 were ras wild type. Interestingly, only OPM2 harbored a PTEN mutation and was reovirus resistant. Testing of 3 reovirus-sensitive HMCLs confirmed that cell
death is manifested through apoptosis (annexin V expression and DNA fragmentation) and corroborate our previous findings with breast and prostate cancer (10, 17). Inhibition of caspase 3 with Z-VAD-FMK-001 significantly reduced DNA fragmentation induced by reovirus confirming the active role of caspase 3 in reovirus-induced apoptosis.

The novel finding that reovirus infection of multiple myeloma leads to significant induction of autophagy, a catabolic process involved in routine turnover of proteins and intracellular organelles is of interest (28). Reovirus infection of RPMI8226 cells lead to a time-dependent increase in autophagy and this was suppressed by 3-MA, a specific inhibitor of autophagy. Although autophagy could serve a dual purpose, either to protect against cancer or contribute to cancer by promoting the survival of nutrient-starved cells (29), many studies have indicated its role as

Table 1. Reovirus treatment abrogates human multiple myeloma dissemination in mouse bone marrow or in vivo

<table>
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<tr>
<th>LV-treated mice</th>
<th>Presence of RPMI 8226 myeloma cells</th>
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Figure 5. Reovirus treatment does not abrogate hematopoietic engraftment in mice. Human CD34⁺ stem cells were isolated from AP and treated with LV or DV up to 72 hours. 1 to 5 x 10⁶ CD34⁺ cells were mixed with carrier cells and injected into sublethally irradiated SCID/NOD mice. A, mouse bone marrow was harvested and analyzed for human hematopoietic stem cells enumeration using flow cytometry. B, evidence of differentiation by flow cytometric immunophenotyping carried out on leukocyte populations from representative animals treated with carrier cells, DV or LV treatment. C, DNA was extracted from blood and BM of animals that received human CD34⁺ stem cells treated with DV or LV or from mice that received only carrier cells. PCR was carried out for human “Alu” sequences.
a pro-death pathway post treatment (30–35). Several lines of evidence indicate that ER stress leads to autophagy (36–38), and recent studies have highlighted a prominent role between autophagic cell death induced by Akt–mTOR signaling triggered by ER stress (37, 38). Recently, Kelly and colleagues (22) showed that reovirus infection of multiple myeloma leads to ER-stress-induced apoptosis. Therefore, it is plausible to hypothesize that viral replication within multiple myeloma, in addition to induction of apoptosis, also promotes autophagic cell death initiated via a signaling mechanism of the ER stress pathway.

Analysis of reovirus-treated multiple myeloma ex vivo tumors revealed that reovirus sensitivity is not restricted to multiple myeloma cancer cell lines in vitro. Further, 5 of the 7 samples showed sensitivity to reovirus, highlighting the applicability of this virus as a possible systemic therapeutic strategy. A more robust assessment of reovirus efficacy is to examine its ability to target disseminated multiple myeloma under in vivo conditions. All DV-treated mice showed signs of disease and required euthanasia due to tumor burden; however, 5 of the 6 mice treated with LV maintained health for the duration of the experiment (35 days). Mice treated with DV showed evidence of GFP+ lesions in bone including the appendicular and axial skeletons, mimicking the osteotropic nature of multiple myeloma in patients. Mice showing hind-leg paralysis was likely due to vertebral bone lesions and spinal cord involvement, which was the primary reason for euthanization. In addition to the multiple myeloma tumor detected in the bodies of animals, multiple myeloma tumor homing to the bone marrow milieu was confirmed with human multiple myeloma detection in 5 of the 6 mice subjected to DV treatment. In comparison, a single injection of 1 × 10^7 PFU of reovirus was able to target bone marrow infiltration of multiple myeloma in 5 of the 6 mice, highlighting its potential as a systemic agent. We hypothesize that multiple injections of reovirus would completely eradicate disease, but our NOD/SCID/irradiated/NK cell-depleted model does not support multiple reovirus injections due to development of vasculitis beyond day 25.

Another important objective of this study was to prove that reovirus treatment does not abrogate human stem cell re-population and differentiation in vivo. Previously, we have shown negligible adverse effects of reovirus on purified CD34+ human stem cells or AP under in vitro conditions (23, 25). Human stem cells isolated from AP, treated with LV for 3 days, and transplanted into NOD/SCID mice showed similar re-population and lineage differentiation in comparison to the DV controls. In contrast, control mice that received only carrier cells did not show human stem cell engraftment. Flow cytometric analysis of mouse bone marrow showed distinct CD45+ leukocytes and CD45+CD34+ human stem cell clusters in all DV- or LV-treated mice. The CD45+CD34+ stem cells were viable as shown by the lack of staining with the viability dye, 7-AAD. CD45-positive cells in the CD45^dim (Blast), monocyte (Mono), and granulocyte (Gran) regions showed mainly myeloid lineage commitment on the basis of their co-expression of CD13 and CD33. Additional myelomonocytic lineage-associated markers, including CD64 and CD45^dim, were expressed on CD13/33+ cells in the monocyte and granulocyte regions. Cells in the lymphocyte gate (Lymph) did not show expression of any antigens other than CD45, confirming that myeloid differentiation precedes lymphoid differentiation.

To further confirm that cells of human origin were present in mice, DNA extracted from blood and BM of LV- and DV-treated mice was subjected to PCR to detect human specific "Alu" sequences. Indications of "Alu" sequences were identified only in mice that received human CD 34+ cells (irrespective of their treatment) and not in control mice or commercially available mouse DNA, further confirming the capability of the reovirus as a successful systemic agent to treat human multiple myeloma.

The reovirus fulfills many of the attributes of a viable treatment option for multiple myeloma: selective targeting of disease without affecting the stem cell compartment, enhanced efficacy, and relative ease of manufacturing and storage. Reovirus is not associated with toxicities in immunocompetent animals nor in the over 560 patients treated with reovirus to date.

In conclusion, this study represents the first utilization of reovirus as a clinically relevant systemic agent for the treatment of human multiple myeloma that could lead to rapid bench-to-bedside translation. In an era where a personalized approach to medicine is sought, multiple myeloma may represent a malignancy where bone marrow aspirates could easily be withdrawn from patients and tested for reovirus sensitivity before initiating a treatment regimen. In addition, this study constitutes a framework for the possible extension of these findings to other hematological malignancies as well.

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

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