Selective Purging of Human Multiple Myeloma Cells from Autologous Stem Cell Transplantation Grafts using Oncolytic Myxoma Virus

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Autologous stem cell transplantation and novel therapies have improved overall survival of patients with multiple myeloma; however, most patients relapse and eventually succumb to their disease. Evidence indicates that residual cancer cells contaminate autologous grafts and may contribute to early relapses after autologous stem cell transplantation. Here, we demonstrate that ex vivo treatment with an oncolytic poxvirus called myxoma virus results in specific elimination of human myeloma cells by inducing rapid cellular apoptosis while fully sparing normal hematopoietic stem and progenitor cells. The specificity of this elimination is based on strong binding of the virus to myeloma cells coupled with an inability of the virus to bind or infect CD34+ hematopoietic stem and progenitor cells. These 2 features allow myxoma to readily identify and distinguish even low levels of myeloma cells in complex mixtures. This ex vivo rabbit-specific oncolytic poxvirus called myxoma virus treatment also effectively inhibits systemic in vivo engraftment of human myeloma cells into immunodeficient mice and results in efficient elimination of primary CD138+ myeloma cells contaminating patient hematopoietic cell products. We conclude that ex vivo myxoma treatment represents a safe and effective method to selectively eliminate myeloma cells from hematopoietic autografts before reinfusion.


KEY WORDS: Ex vivo purging, Myxoma virus, Viral oncolytics, Multiple myeloma, Autologous hematopoietic stem cell transplantation

INTRODUCTION

Multiple myeloma (MM) is a clonal plasma cell malignancy that is most prevalent in adults over the age of 65 and accounts for 10% to 15% of newly diagnosed hematopoietic cancers with patients losing an average of 17 years of life expectancy per diagnosis [1]. Although recent clinical advances have improved the prognosis for patients with MM, life expectancy at diagnosis remains 2 to 5 years with a 5-year survival rate of only 34% [2,3]. Currently, the standard of care for patients with MM is treatment with high-dose chemotherapy followed by autologous stem cell transplantation (ASCT). In North America alone, ASCT is used to treat approximately 5,000 patients with MM annually. This treatment results in improved rates of disease remission as well as significantly prolonged event-free survival time compared with patients treated with conventional chemotherapy [4,5]. However, despite the improved prognosis associated with myeloablative chemotherapy followed by ASCT, the treatment is generally not curative, and a large majority of all patients with MM will suffer from relapsed disease.

The malignant cells that cause relapse are thought to originate from 2 sources: residual MM cells, which escape ablative therapy in bone marrow (BM) niches and low levels of MM cells that contaminate the autografts. To date, the relative impact of each of these 2 sources in causing MM relapse remains unclear; however, several observations suggest that residual plasma cell contamination plays a significant role. First, it has been repeatedly reported that patients with MM with lower levels of autograft contamination achieve greater benefit from transplantation than patients with higher levels of contamination [6-8]. Second, patients undergoing syngeneic transplantation, using uncontaminated BM from an identical twin donor, routinely achieve better rates of complete remission, improved long-term survival, and possibly even complete cures [9-11]. These data, particularly the
syngeneic transplantation studies, support the conclusion that even low levels of MM cells contaminating the autograft can play a significant role in disease relapse and suggest that ex vivo manipulation of the autograft before infusion to remove all contaminating malignant cells, a process known as purging [12], could improve the outcomes of patients with MM.

Proposed MM purging procedures must meet 2 important criteria: (1) they must effectively remove all contaminating cancer cells from the grafts and (2) they must fully spare the normal hematopoietic stem/progenitor cells (HSPCs) in the autograft allowing for successful reconstitution of the patient’s hematopoietic system. Several purging methods have been explored in ASCT [13-16], including a recent study focusing on ex vivo culture conditions that favor survival of HSPCs [17]. For MM, most of the focus has been placed on CD34+ stem cell enrichment [18-20], which can reduce the level of MM contamination within the graft by 2 to 3 logs [20]. Unfortunately, clinical trials have demonstrated that this CD34-based purging does not improve clinical outcomes for patients with MM [19,21]. The results of these trials were initially interpreted as proof that myeloma relapse was primarily caused by residual disease persisting in the patient after ablative chemotherapy; however, subsequent molecular studies have shown that low levels of contaminating CD138+ MM cells remain in ASCT samples even after multiple rounds of CD34+ cell enrichment [22-24]. Moreover, CD34+ malignant MM clones have been identified in patients which calls into question the utility of CD34 enrichment in these patients [25,26]. Together, these data suggest that CD34+ stem cell enrichment might fail to improve the prognosis of patients with MM because disease-causing MM cells remain in the autografts after positive CD34+ cell selection of peripheral blood stem cells. Therefore, alternative means of ex vivo purging must be explored [12].

Previously, our laboratory demonstrated that a rabbit-specific oncolytic poxvirus called myxoma virus (MYXV) can eliminate primary acute myeloid leukemia cells from primary human BM samples while sparing normal HSPCs [27]. MYXV is an attractive virotherapeutic to target and eliminate human cancer cells for several reasons. First, the virus does not elicit detectable disease in any non-rabbit species, including humans or severely immunocompromised mice [28,29]. Second, the ex vivo therapeutic application of MYXV is not dependent on expression of transgenes or addition of chemotherapeutic agents and requires only a brief ex vivo incubation of the graft with MYXV before transplantation, thus making it an attractive strategy for clinical administration that minimally deviates from standard ASCT clinical practice [27,30]. Due to our previous success using MYXV to purge primary human acute myeloid leukemia cells, the virus’ safety for the engraftment of normal human HSPCs, and the high rate of MM relapse after AHCT, we hypothesized that ex vivo MYXV treatment might represent an improved method for clinical elimination of MM cells contaminating patient autograft samples before reinfusion.

**MATERIALS AND METHODS**

**Cells and Reagents**

U266 (American Type Culture Collection [ATCC] #TB-196), RPMI-8266 (ATCC# CCL-155), MM.1S (ATCC# CRL-2974), and HuNS1 (ATCC# CRL-8644) human myeloma cells as well as HL60 acute myeloid leukemia cells (ATCC# CCL-240) were obtained from ATCC and were maintained below 2 x 10^7 cells/mL in RPMI media supplemented with 1 x pen/strep, 2 mM L-glutamine, and 20% FBS. The following Abs were used: HLA-A, HLA-B, HLA-C-APC, CD45-PE, CD45-FitC, poly ADP-ribose polymerase, HLA-A2.1-PE (BD Biosciences, San Jose, CA), caspase 3 and cleaved caspase 3 (Cell Signaling, Danvers, MA), and B actin (Ambion, Grand Island, NY). Clinical-grade heparin (1,000 USP U/mL) was a kind gift from Dr. Alexandra Lucas. Primary MM cells were obtained by patient donation under the approval of the University of Florida Institutional Review Board.

**MYXV and Viral Infections**

vMyx-green fluorescent protein (GFP) has been previously described [31]. Unless indicated, infections were carried out by exposing cells to vMyx-GFP at a multiplicity of infection (MOI) of 10 for 1 hour in PBS + 10% FBS in a humidified chamber at 37°C and 5% CO2. Mock-treated cells were incubated in PBS + 10% FBS containing no virus under the same incubation conditions. Treatment with inactivated virus was performed using the same incubation conditions, but with inactivated vMyx-GFP prepared by exposing virus to UV light for 2 hours (UV inactivated) or incubating virus at 55°C for 2 hours (heat inactivated). Fluorescently tagged MYXV virions (vMyx-M093L-Venus) were created by fusing the GFP variant “Venus” protein in frame to the N-terminus of the M093 open reading frame (to be described elsewhere).

**Analysis of MYXV Infection in Cultured Myeloma Cells**

To measure initiation of early viral gene expression, human myeloma cells were analyzed 24 hours after infection with vMyx-GFP for expression of GFP using flow cytometry. To measure completion
of the viral replication cycle and production of new infectious progeny virus, myeloma cells were harvested at the indicated time points, pelleted, and frozen. After harvesting, infectious virus was released by sequential freeze-thaw, and the amount of virus in each sample was determined as previously described [32]. To measure the binding of MYXV to the cell surface by flow cytometry, 1E9 vMyx-GFP virions were incubated with 25 micrograms of cyclophosphamide (Cy5)-NHS ester for 30 minutes at room temperature. Unbound Cy5-NHS was removed by pelleting virions at 13,000 rpm for 5 minutes and washing the resulting pellet extensively with PBS. Cells were incubated with MYXV-Cy5 at MOI = 10 for 1 hour, washed extensively, and analyzed by flow cytometry. For the binding of MYXV to primary MM patient BM samples, vMyx-M093L-Venus was used as described in the text. To measure the binding of MYXV to the cell surface, myeloma cells were exposed to vMYX-GFP at MOI = 10 for 1 hour at 37°C. Cells were then washed 4 times with PBS + 10% FBS. The contents of the resulting pellet (virus) as well as the supernatant from the last wash were then acid precipitated using trichloroacetic acid (final concentration 30%). Samples were resuspended in Laemmli buffer, separated on a 15% acrylamide gel, and transferred to polyvinylidene fluoride membrane. The presence of viral proteins derived from MYXV virions was then analyzed by standard immunoblot analysis using an aMYXV rabbit polyclonal serum [33].

Myeloma Cell Viability and Proliferation Assays

Human myeloma cells were mock-treated or infected with vMyx-GFP as previously mentioned. For viability studies, 1 × 10^5 myeloma cells were plated in triplicate into 96-well plates. Twenty-four hours after treatment, cellular adenosine triphosphate concentration of viral proteins derived from MYXV virions was then analyzed by standard immunoblot analysis using an aMYXV rabbit polyclonal serum [33].

MM Xenografts

For in vivo systemic engraftment studies, NOD/Scid/IL2Rγ^−/− (NSG) mice were sublethally irradiated using 175 cGy total body irradiation from a Cs^{137} source. Within 24 hours after irradiation, mice were injected through the tail vein with 100 uL PBS + 10% FBS containing 1 × 10^6 MM cells, which had been either mock-treated or infected with vMyx-GFP at MOI = 10. Prophylactic antibiotics were administered to animals in the drinking water for 2 weeks after transplantation to prevent opportunistic bacterial infection. Two to 6 weeks after transplantation, mice were euthanized, and BM was harvested from the right hind femur using a tuberculin syringe, stained with Abs against human HLA-A, HLA-B, and HLA-C, and analyzed using flow cytometry. Data are presented as “level of engraftment,” which corresponds to the percentage of HLA-A, HLA-B, and HLA-C+ cells in the BM of each mouse. Mice displaying any level of HLA-A, HLA-B, and HLA-C+ cells were scored as engrafted (●), whereas mice without any detectable HLA-A, HLA-B, or HLA-C+ cells were scored as nonengrafted (○). All animal experiments were carried out under approved University of Florida Institutional Animal Care and Use Committee protocol #2010-5023.

Mixing Experiments with Human Myeloma into Normal Donor BM Samples

Fresh human BM aspirates were obtained commercially from Lonza (Walkersville, Maryland). BM mononuclear cells were then enriched over a Ficoll gradient using a clinical Sepax device (Biosafe Inc., Lincolnshire, IL) as per manufacturer’s recommendations. CD34+ human cells were fractionated from Sepax-purified normal BM aspirates using the CD34+ microbead separation kit (Miltenyi Biotec, Cambridge, MA) as per manufacturer’s recommendations. Cells were then separated on an autoMACS pro separator (Miltenyi Biotec, Cambridge, MA) as per manufacturer’s recommendations. The relative purity of the fractionated population was confirmed after separation using flow cytometry. The total number of fractionated cells was determined after separation using a hemocytometer. The 1 × 10^5 CD34+ cells were then mixed with 1 × 10^6 U266 myeloma cells. U266 cells could be distinguished from normal hematopoietic cells based on their expression of HLA-A2.1 and CD138 as well as their lack of expression of CD45 and CD34.

RESULTS

MYXV Infects and Kills Human MM Cell Lines In Vitro

The therapeutic potential of oncolytic viruses in vivo frequently correlates with their ability to selectively infect and kill target cancer cells in vitro. We therefore tested whether MYXV infected and killed 4 established human MM cell lines (U266, HuNS1, MM.1S, and RPMI-8266) in vitro. Each cell line was incubated with vMyx-GFP, which expresses GFP from a viral synthetic early/late promoter, at an MOI of 10. After 24 hours of incubation, all 4 cell lines displayed significant numbers of GFP+ cells (Figure 1A),
demonstrating that MYXV efficiently initiated infections in all 4 cell lines. Additionally, treatment with vMyx-GFP significantly reduced the cellular viability, as measured by the MTT mitochondrial function assay, of all 4 cell lines (Figure 1B) and completely abrogated subsequent cellular proliferation in culture (Figure 1C).

**Ex Vivo MYXV Treatment Inhibits In Vivo Systemic Engraftment of MM Cell Lines into NSG Mice**

To determine if ex vivo MYXV treatment could inhibit systemic engraftment of MM cells in vivo, 4 human MM cell lines were mock-treated or treated with vMyx-GFP ex vivo and then transplanted i.v. into sublethally irradiated immunodeficient NSG mice. Two to 6 weeks after transplantation, the animals were analyzed for human MM in femurs. Engrafted human MM cells were readily observed in the BM of mice transplanted with mock-treated RPMI-8266 cells (9 of 10 mice positive, average human myeloma engraftment in mouse BM = 6.2%), MM.1S cells (all of 10 mice positive, average = 75.7%), HuNS1 cells (all of 10 mice positive, average = 34.5%), and U266 cells (9 of 10 mice positive, average = 35.0%), demonstrating that all 4 MM cell lines effectively xenografted into NSG mouse BM (Figure 2). In contrast, no human MM cells were detected in the BM of mice transplanted with MYXV-treated RPMI-8266, HuNS1, or U266 cells and were only observed at a very low level (0.1%) in 1 of the 10 mice injected with MYXV-treated MM.1S cells. Additionally, mice transplanted with MYXV-treated cells did not develop any symptoms of clinical MM, which were routinely observed 2 weeks after injection of mock-treated HuNS1 and 5 weeks after injection of mock-treated U266 cells (our unpublished observations).

**MYXV Specifically Eliminates MM Cells while Sparing Normal Hematopoietic Stem Cells**

One of the major challenges associated with autograft purging is specifically eliminating contaminating cancer cells while sparing the normal HSPCs required for reconstitution of the patient's hematopoietic system. Therefore, to determine if MYXV treatment distinguished human MM cells from normal HSPCs, we treated mixtures of U266 cells and primary human HSPCs with vMyx-GFP and then analyzed GFP expression in both populations of cells after 24 hours. In these experiments, U266 cells can be distinguished from HSPCs based on expression of the surface markers...
HLA-A, HLA-B, HLA-C, HLA-A2.1, CD45, and CD34 (U266 cells are HLA-A, HLA-B, and HLA-C where human HSPCs are HLA-A, HLA-B, and HLA-C). Expression of GFP, indicating the initiation of viral infection, was observed in a significant percentage of HLA-A2.1 U266 cells (45.9%). In contrast, expression of GFP was observed in less than 5% of the CD34 cell population (Figure 3A).

To confirm that MYXV treatment functionally discriminated MM cells from normal HSPCs, mixtures of U266 cells and primary human CD34+ HSPCs were mock-treated or treated with vMyx-GFP and then transplanted into sublethally irradiated NSG mice. Six weeks after transplantation, engraftment of both U266 cells and repopulating human leukocytes derived from HSPCs were analyzed using flow cytometry. In mice transplanted with mock-treated mixtures, coengraftment of both U266 cells as well as HSPCs was readily observed (Figure 3B). In contrast, mice injected with MYXV-treated mixtures failed to display detectable engraftment of U266 cells, while exhibiting engraftment of HSPCs at levels identical to mock-treated samples. Together, these results indicate that MYXV effectively discriminates between MM cells and normal HSPCs found in the same graft sample and purges the MM cells while sparing the normal HSPCs allowing efficient hematopoiesis.

**MYXV Elimination of Human MM Cells Requires Direct Virion Binding**

We recently discovered that the ability of MYXV to purge acute myeloid leukemia cells necessitates viral binding to the cell surface [34]. We therefore tested whether MYXV also required direct adsorption of the virus particles in order to eliminate MM cells. U266 cells were treated with vMyx-GFP in either the presence or absence of soluble heparin, which inhibits poxvirus binding to mammalian cells by coating the positively charged virion [35] (and our unpublished observations). After 24 hours, a high percentage of U266 cells incubated in the absence of heparin displayed GFP expression, whereas virtually no GFP cells could be identified in samples incubated in the presence of heparin (Figure 4A). The presence of heparin during infection also largely abrogated the ability of MYXV to reduce cellular viability (data not shown) and prevent subsequent cellular proliferation (Figure 4B). Finally, to determine if virion binding was required for MYXV to inhibit engraftment of MM cells in vivo, U266 cells were incubated with vMyx-M093L-Venus in either the presence or absence of soluble heparin and then transplanted i.v. into NSG mice. Consistent with our previous results, mice transplanted with mock-treated U266 cells displayed a high number of human cells engrafted in the BM 6 weeks after transplantation, whereas these cells could not be detected in mice transplanted with ex vivo MYXV-treated cells. In contrast, engrafted human cells were readily detectable in the BM of mice transplanted with cells treated with MYXV in the presence of soluble heparin (Figure 4C) suggesting that effective purging requires MYXV binding to the target MM cancer cell.

**MYXV does not Bind to Normal Human HSPCs**

Due to this result, we hypothesized that MYXV might spare normal human HSPCs due to a failure of these cells to support virion binding. To test this, primary human BM was mixed with U266 cells or HL60 cells, an acute myeloid leukemia cell line, which is unable to support MYXV binding (our unpublished observations) and then incubated with MYXV-Cy5.
virions. After washing, the amount of Cy5 fluorescence on U266 cells, HL60 cells, or primary CD34^+ HSPCs was analyzed using flow cytometry (Figure 4D). While the MYXV-purgeable U266 cells displayed a high level of Cy5 fluorescence, corresponding to efficient MYXV virion binding, only low levels of Cy5 fluorescence were detected on the MYXV-nonpurgeable HL60 cells and CD34^+ HSPCs supporting the hypothesis that

Figure 3. Rabbit-specific oncolytic poxvirus called myxoma virus (MYXV) eliminates human multiple myeloma (MM) cells while sparing normal primary human hematopoietic stem cells. (A) U266 cells were mixed at a 10:1 ratio with donor human bone marrow (BM)-derived CD34^+ hematopoietic stem and progenitor cells (HSPCs). Mixtures were then mock-treated or infected with vMyx-green fluorescent protein (GFP) at multiplicity of infection (MOI) = 10 for 24 hours, and expression of GFP was analyzed using flow cytometry. Events shown are gated on live HLA-A2.1^+ (Top) or CD34^+ (Bottom) cells. Inset number indicates the percent of GFP^+ and GFP^- cells. (B) U266 cells were mixed at a 10:1 ratio with purified human BM derived CD34^+ HSPCs. Mixtures were then mock-treated or infected with vMyx-GFP at MOI = 10 and injected i.v. into NOD/Scid/IL2R^γc^-/- (NSG) mice. Six weeks after injection, BM was harvested from each mouse, stained with Abs against human HLA-A, HLA-B, HLA-C, human HLA-A2.1, and human CD45 and analyzed using flow cytometry. U266 cells were identified as staining HLA-A, HLA-B, HLA-C^-/HLA-A2.1^-/CD45^-, while progeny derived from normal HSPCs were identified as staining HLA-A, HLA-B, HLA-C^-/HLA-A2.1^-/CD45^- . Data are presented as “level of engraftment,” which corresponds to the percent of HLA-A, HLA-B, HLA-C^-/HLA-A2.1^-/CD45^- cells in the BM of each mouse. Mice displaying any level of positive cells were scored as engrafted (●), while mice without any detectable positive cells were scored as nonengrafted (○).
MYXV spares normal HSPCs because these cells do not support MYXV virion binding.

MYXV Kills Human MM Cells by Rapidly Inducing Apoptosis

Frequently, oncolytic viruses eradicate their targets through direct lytic replication; we therefore asked whether this mechanism was responsible for MYXV-based elimination of MM cells. Each of our 4 human MM cell lines was incubated with MYXV at MOI = 1, and the replication of new infectious viral progeny was measured using single-step viral growth analysis. Surprisingly, we observed that even though MYXV effectively initiated infection and killed all 4 MM cell lines, no new viral progeny were created during this process (Figure 5A). Additionally, treatment of human MM cells with replication-incompetent MYXV, which had been UV-inactivated, reduced cellular viability and proliferation comparably to treatment with live virus (Figure 5B, C). In contrast, treatment with heat-inactivated MYXV did not affect MM cell viability or proliferation.

Because our data suggested that MYXV kills human MM cells via a mechanism that is not dependent on lytic viral replication, we next determined the cause of MYXV-induced MM cell death. A phenotypic examination of MM cells treated with MYXV revealed that these cells rapidly displayed membrane blebbing, a process frequently associated with
apoptosis (Figure 5D). Additionally, U266 cells treated with either live or UV-inactivated MYXV displayed cleavage of the apoptotic effector, caspase-3, as well as cleavage of poly (ADP-ribose) polymerase, a marker for late-stage apoptosis (Figure 5E). We therefore conclude that MYXV kills human MM cells by inducing rapid cellular apoptosis that is triggered so quickly that it aborts the virus replication cycle before the generation of progeny virus.

**MYXV Infects Primary Human CD138^+ Myeloma Cells Contaminating Patient BM**

Immortalized cell lines frequently exhibit phenotypes significantly different from primary cancer cells analyzed directly ex vivo. We therefore tested whether MYXV targeted and eliminated primary CD138^+ MM cells contaminating primary patient BM samples. Three BM samples from different patients with myeloma were mock-treated or incubated with vMyx-GFP, heat-inactivated vMyx-GFP, or UV-inactivated vMyx-GFP at an apparent MOI = 10. After 24 hours, cellular adenosine triphosphate generation, which correlates with the number of living cells, was measured using the commercial MTT assay. U266 cells were mock-treated or infected with live, heat, or UV-inactivated vMyx-GFP, as described above, and then cellular morphology was observed 24 hours after infection using a Leica DMi 6000B light microscope. Cells were harvested at the indicated time points, and whole cell lysates were assayed with the indicated Abs via immunoblot.

**Figure 5.** Rabbit-specific oncolytic poxvirus called *myxoma virus* (MYXV) kills human multiple myeloma (MM) cells in vitro by inducing rapid apoptosis. (A) Each human MM cell line was infected with vMyx-green fluorescent protein (GFP) at multiplicity of infection (MOI) = 1. At the indicated times, cells were harvested and frozen. After all time points had been collected, cells were lysed using repeated freeze-thaw, and the amount of infectious virus in each sample was quantitated using foci formation on BSC40 cells. (B) U266 cells were either mock-treated or infected with live vMyx-GFP, heat-inactivated vMyx-GFP, or UV-inactivated vMyx-GFP at an apparent MOI = 10. After 24 hours, cellular adenosine triphosphate generation, which correlates with the number of living cells, was measured using the commercial MTT assay. (C) U266 cells were mock-treated or infected with live, heat, or UV-inactivated vMyx-GFP, as described above, and the number of trypan blue-excluding cells was determined at the indicated times using a hemocytometer. (D) U266 cells were mock-treated or infected with live, heat, or UV-inactivated vMyx-GFP, as described above. Cells were harvested at the indicated time points, and whole cell lysates were assayed with the indicated Abs via immunoblot.
patient samples (Figure 6B Top). After 24 hours, high numbers of CD138\(^+\) MM cells could still be found in all 3 mock-treated samples, whereas virtually no contaminating CD138\(^+\) cells remained in any patient sample, which had been treated with MYXV (Figure 6A Bottom). Significantly, the few CD138\(^+\) cells that still persisted after 24 hours displayed high levels of Venus fluorescence indicating they were undergoing active MYXV infection. In contrast, the number of CD34\(^+\) stem cells was unaffected by MYXV treatment, and relatively few of these cells displayed any evidence of viral infection (Figure 6B Bottom). Unfortunately, our repeated attempts to engraft primary human MM samples into NSG mice resulted in little or no stable engraftment even in mock-treated controls, and so we were unable to document the in vivo efficacy of

Figure 6. Rabbit-specific oncolytic poxvirus called myxoma virus (MYXV) effectively eliminates CD138\(^+\) cells from primary multiple myeloma (MM) patient samples. (A) Primary bone marrow (BM) from 3 patients with MM was mock-treated or incubated with vMyx-M093L-Venus at multiplicity of infection (MOI) = 10 for 1 hour, stained with Abs against either CD138 (A) or CD34 (B), and then washed extensively. Virus produced by vMYX-M093L-Venus incorporates high levels of Venus into the virion and can be used to quantify virion binding by flow cytometry. Venus florescence from mock-treated (black line) as well as vMyx-M093L-Venus treated (red line) samples was then analyzed using flow cytometry at 0 hours and 24 hours after adsorption. Data from normal BM mixed with U266 cells are shown as a comparison. B
MYXV treatment at inhibiting engraftment of primary myeloma. However, our data clearly demonstrate that MYXV infects and eliminates U266 cells and primary CD138+ MM cells in vitro with similar efficacy in the context of BM samples from patients with myeloma.

DISCUSSION

We describe a novel method to eliminate human MM cells from autologous HSPC samples ex vivo using an oncolytic virus, MYXV, which selectively targets MM cells but does not bind or infect normal human CD34+ stem cells. MM disease relapse after ASCT may arise from 2 sources: residual disease sequestered in bone niches that failed to be eliminated by the myeloablative chemotherapy and/or low levels of MM cells that contaminate the HSPC graft. However, previous studies have shown that the prognosis for patients with MM does correlate with the level of contamination in stem cell product used for transplantation [6-8] and that patients treated with syngeneic transplantations from identical twin donors achieve considerably better rates of disease remission and prolonged survival [9-11]. This strongly indicates that clinical outcomes will be improved if a method can be developed that quantitatively eliminates all contaminating MM cells from autologous HSPC samples. These observations have led to a variety of other methods being explored for purging MM cells from autograft samples, most notably positive selection of CD34+ HSPCs. Unfortunately, CD34+ positive selection-based purging strategies have not proven clinically effective [18,19]. Although the reason for these failures is not fully known, it has been demonstrated that CD34+ stem cell selection is unable to completely eliminate all MM cells contaminating ASCT products [22]. Additionally, patient samples can contain a CD34+ clonal cell population related to the patient with MM that cannot be eliminated by CD34+ enrichment. These data suggest that, although MM purging in general might be clinically beneficial, CD34+ stem cell enrichment is unable to completely eradicate disease-causing MM cells from HSPC samples. Alternatively, it is possible that the major cause of myeloma relapse after ASCT is residual disease persisting in the patient after ablative chemotherapy. Therefore, novel MM purging strategies that are not based on positive CD34+ stem cell selection, particularly a procedure also capable of treating residual disease, are needed.

Our data demonstrate the effectiveness of oncolytic MYXV as a selective purging agent for human MM cells both in vitro and in vivo. Several other groups have recently published purging strategies for various malignancies based on other oncolytic viruses, including adenovirus, vesicular stomatitis virus, herpes simplex virus, and reovirus (reviewed in [36,37]). Although many of these viral treatments were effective in vitro, to date, only 3 have demonstrated purging efficacy in vivo [38-40]. Unfortunately, all 3 of these previously described viral purging strategies required the stem cell transplantation graft to be incubated ex vivo for long periods of time and then treated with cytotoxic chemotherapeutics before transplantation, limiting applicability to clinical translation. In contrast, the MYXV-based ex vivo purging strategy described here demonstrates excellent efficacy against human MM both in vitro and in vivo, did not harm the engraftment potential of normal HSPCs, and required minimal ex vivo manipulation of the autograft before transplantation. Therefore, this study represents the first report of an effective viral ex vivo purging strategy that demonstrates efficacy at preventing relapse in the in vivo models and can be easily translated to the clinic. Additionally, the absence of documented cases of MYXV infection in any non-rabbit species, including humans or mice, suggests that MYXV possesses an excellent safety profile for in vivo use.

Interestingly, although MYXV treatment had a profound effect on long-term MM growth both in vitro and in vivo, evidence of direct viral infection, as measured by GFP expression from an encoded transgene, was only observed in 50% to 70% of treated cells (Figure 1). This could be related to the limitations in detecting GFP fluorescence or to the ability of MYXV to eliminate MM cells through a mechanism independent of viral replication. For example, epitopes on the MYXV virion may act as functional ligands that trigger the rapid activation of a cell surface death receptor on MM cells, such as those of the TNF-receptor superfamily. This second direct- killing hypothesis is supported by the observation that U266 cells treated with MYXV at MOIs as low as 0.1 universally undergo apoptosis and fail to engraft into NSG mice (data not shown). Importantly, the observed MYXV-induced reductions in cellular viability and levels of direct infection were similar in both the 4 established human MM cell lines as well as all 3 primary patient samples, and these levels of MM cell depletion translated into significant clinical benefit in vivo in terms of the ability to prevent engraftment of the MYXV-treated MM cell lines. These data collectively suggest that ex vivo MYXV treatment will be efficacious in reducing relapse rates of MM caused by infusion of contaminated ASCT samples. It is believed that residual MM cells persisting in the patient’s BM despite myeloablative chemotherapy also play a major role in patient disease relapse. Interestingly, systemic injection of oncolytic vaccinia virus
has been shown to decrease levels of established MM in humans [41], whereas injection of oncolytic measles virus bound to MM carrier cells has been shown to effectively treat established MM in murine models [42,43]. Additionally, it has been demonstrated that treatment with a variety of oncolytic viruses can result in elimination of residual cancer cells from therapy-resistant niches by redirecting the immune system to more effectively recognize tumor antigens [44,45]. While still untested, it should be noted that because of the potent MM cell death induced by cell contact with this virus, the MYXV-based purging strategy described here also has the potential to treat established MM, which is resistant to current therapy through all these mechanisms. Alternatively, systemic injection of MYXV might also be used to directly treat residual myeloma that has resisted the primary myeloablative chemotherapy. In our preliminary experiments, such treatments can have promising efficacy in mouse models of pre-established myeloma (our unpublished observations). Significant issues, such as developing an optimal strategy to deliver virus to all potential myeloma niches in vivo, remain to be addressed. Despite these limitations, however, we propose that MYXV-based virotherapy, either as an ex vivo MYXV-based purging protocol or possibly augmented by systemic viral injection, has the potential to treat both MM-contaminated autografts as well as in vivo residual MM, which persists after ablative therapy. Interestingly, since UV-inactivated and live-MYXV showed similar in vitro efficacy at inducing apoptosis in MM cells, it is possible that completely replication-incompetent virus could also be used to eliminate MM cells contaminating the ASCT grafts. This would create an even safer clinical purging agent and alleviate any ethical concerns associated with injection of live virus.

Significantly, we demonstrate that when normal primary CD34+ human HSPCs are admixed with human MM cells, the resulting mixture causes engraftment of both normal HSPCs and MM cells in the BM of NSG mice. However, in stark contrast, when these same mixtures are treated ex vivo with MYXV, only the normal HSPCs successfully engraft into the recipients, while the contaminating MM cells are selectively purged. This specificity seems to be due to the selective binding of MYXV to MM cells but not to primary CD34+ HSPCs. Interestingly, poxviruses like MYXV are extremely promiscuous in their binding to relatively conserved glycosaminoglycans, effectively attaching to a broad spectrum of mammalian cells [46].

In conclusion, our data show that ex vivo MYXV treatment of autografts contaminated with MM is safe and effective in preclinical models of human MM. Results from this work support the translation of this novel purging method to the clinic.

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