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Ex Vivo Virotherapy With Myxoma Virus Does Not Impair Hematopoietic Stem and Progenitor Cells

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Abstract

Background—Relapsing disease is a major challenge after hematopoietic cell transplant for hematological malignancies. Myxoma virus (MYXV) is an oncolytic virus that can target and eliminate contaminating cancer cells from auto-transplant grafts. The aims of this study were to examine the impact of MYXV on normal hematopoietic stem and progenitor cells, and define the optimal treatment conditions for *ex vivo* virotherapy.

Methods—Bone marrow (BM) and mobilized peripheral blood stem cells (mPBSCs) from patients with hematological malignancies were treated with MYXV at various time, temperature and incubation media conditions. Treated BM cells from healthy normal donors were evaluated by flow cytometry for MYXV infection, LTC-IC assay, and CFC assay.

Results—MYXV initiated infection in up to 45% of antigen presenting monocytes, B cells and natural killer cells; however, these infections were uniformly aborted in > 95% of all cells. Fresh graft sources showed higher levels of MYXV infection initiation than cryopreserved specimens but all cases, less than 10% of CD34⁺ cells could be infected after *ex vivo* MYXV treatment. MYXV did not impair LTC-IC colony numbers compared to mock treatment. CFC colony types and numbers were also not impaired by MYXV treatment. MYXV incubation time, temperature or culture media did not significantly change percentage of infected cells, LTC-IC colony formation or CFC colony formation.

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DISCLOSURES

GM and CRC hold IP on the MYXV technology to prevent GVHD, which is licenced to DNatrix Corp. All other authors report no conflicts of interest.

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Conclusions—Human hematopoietic cells are non-permissive for MYXV. Human hematopoietic stem and progenitor cells were not infected and thus unaffected by MYXV *ex vivo* treatment.

Keywords

bone marrow; mobilized peripheral stem cell blood; myxoma virus; purging; virotherapy

INTRODUCTION

A major challenge in treating patients with hematological malignancies, such as multiple myeloma, is disease relapse even despite high dose chemotherapy and autologous stem cell transplant (ASCT) ¹⁻³. Relapse disease occurs due to persistence of minimal residual disease from two potential sources: (1) contaminating cancer cells in the ASCT graft, and (2) protective niches in the transplant recipient that resist conditioning chemotherapy⁴⁻⁷. To address the first of these, an *ex vivo* strategy that selectively removes or “purges” any contaminating cancer cells prior to transplant may decrease the rate of relapse observed clinically.

Ideal purging strategies should specifically target contaminating cancer cells in the ASCT graft yet spare the normal hematopoietic stem and progenitor cells (HSPCs), which are required for reconstitution of hematopoiesis. In addition, the ideal purging strategy should be applied quickly, so that the transplant process is not unduly delayed.

Oncolytic viruses may meet the criteria as ideal purging agents for ASCT⁸. We have demonstrated that myxoma virus (MYXV), an oncolytic poxvirus, selectively targets human leukemia, myeloma and lymphoma cells while sparing normal HSPCs ^{6,9,10}.

Although MYXV selectively purged human cancer cells while sparing normal human hematopoietic cells in animal models of disease, the preclinical safety of MYXV with respect to HSPC must be critically examined before proceeding to the transplant clinic.

METHODS

Cell Sources

The University of Florida Institutional Review Board approved this study. HSPC were collected by bone marrow aspiration and G-CSF mobilization from patients with hematological malignancies. Whole bone marrow (BM) and mobilized peripheral blood stem cells (mPBSCs) from patients with hematological malignancies were obtained from patients treated at UF Health Shands Hospital under UF IRB-01 approval. Bone marrow from healthy donors were obtained from Lonza. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (LifeSouth). Cryopreserved cells were thawed according to the protocol reported by Fry et al., ¹¹ with some modifications. Briefly, aliquots of each cryopreserved graft source were thawed rapidly in a water bath at 37°C. Three times volume of 1X-PBS supplemented 20% FCS was added dropwise, mixed gently, and followed by centrifugation at 1,000 RPM for 5 minutes. Supernatants were carefully aspirated and cell pellets were re-suspended in the indicated growth media.

Colony Assays

For the CFC assay, MNCs derived from healthy BM samples ($n = 3$) were incubated with vMyx-green fluorescent protein (GFP)¹² at MOI of 10 for 3 hours at different temperatures including 4°C, room temperature (20–25°C), and 37°C; and different media such as Plasma-lyte A + 10% anticoagulant citrate dextrose solution A (ACDA), Plasma-lyte A + 10% heparin sodium (Wockhardt), and M199 (Gibco) + 15% heparin sodium (Wockhardt). Cells were then assayed for normal hematopoietic progenitor cell differentiation using an *in vitro* methylcellulose-based colony forming cell (CFC) assay, (MethoCult® H4435 Enriched, StemCell Technologies) as per manufacture's instructions.

For long-term culture-initiating cell (LTC-IC) assay, fresh or post-thaw BM samples ($n = 3$) were mock-treated or vMyx-GFP-treated (MOI of 10) at room temperature (20–25°C) for 1–3 hours. LTC-IC assay (Stem Cell Technologies) was performed according to manufacturer's instructions. In brief, cells were seeded onto established stromal cell feeders of irradiated (8000cGy) human fibroblasts (M2-10B4, StemCell Technologies). Mock-treated BM cells were used as positive controls. Mock- or MYXV-treated cells were suspended in triplicate in myeloid long-term culture medium for primitive human hematopoietic cells (MyeloCult H5100; StemCell Technologies) containing 10–6 M freshly dissolved hydrocortisone (in aMEM according to manufacturer's protocol; StemCell Technologies). Following 6 weeks of culture at 37°C with weekly half media exchanges, the contents of each well were harvested and plated into methylcellulose (MethoCult GF+ H4435; StemCell Technologies). The hematopoietic stem cell content was determined by enumerating the number and variety of colonies present in the methylcellulose 14 days of culture.

MYXV Treatment And Culture Conditions Of Hematopoietic Graft Sources

BM or mPBSCs suspended in plain Iscove's Modified Dulbecco's (IMDM) medium or Plasma-lyte A supplemented with ACDA were incubated with vMyx-GFP at MOI of 10 for 1 hour at room temperature to allow virus adsorption. An MOI of 10 was selected to ensure that hematopoietic cells would be exposed to an abundance of virus for infection. After this, cells were incubated for either 2 or 24 hours at 37°C, 5% CO₂ to allow for virus infection. For both time points, cells were suspended in either complete IMDM (supplemented with 10% FBS, 2 mM L-glucosamine and 100 U/mL penicillin-streptomycin) or Plasma-lyte A + 10% ACDA for 2 hours. Mock-treated cells were used as controls and subjected to the same incubation conditions as the MYXV-treated cells.

Immunophenotyping Analysis Using Flow Cytometry

To determine the levels of MYXV infection of different lineage subsets, 1×10^6 cells were suspended in 100 µL of staining buffer followed by staining with monoclonal antibodies coupled with allophycocyanin fluorochrome (APC) or with phycoerythrin fluorochrome (PE) (BD Biosciences) and against HLA-ABC, CD45, CD34 CD14, CD19, CD3, CD15, CD235a. The 7-Aminoactinomycin D (7-AAD) viability dye (Life Technologies) was used to discriminate live from dead cells. Cells were incubated with the antibodies for 1 hour at 4°C, washed and fixed with paraformaldehyde. Unless otherwise indicated, all cell frequencies and the percentages of MYXV infection (GFP⁺) refer to 7AAD-negative

(viable) cells only. Immunophenotyping analysis was performed using flow cytometry, and data analysis was carried out with FCS Express, version 4.0 Software.

MYXV Permissiveness Testing

PBMCs from three healthy individuals were incubated with vMyx-GFP/tomato red fluorescent protein (TrFP) at an MOI of 10 at 4 °C for 1 hr. After washing to remove unbound virions, cells were further incubated at 37 °C for 24 hr. Cells were then harvested for staining with anti-CD14 (monocytes), anti-CD19 (B cells), anti-CD3 (lymphocytes), anti-CD4 (lymphocytes), anti-CD8 (lymphocytes), and anti-CD56 (natural killer cells) antibodies conjugated to various fluorophores (BD Biosciences). Next, the stained cells were analyzed by flow cytometry to measure the proportion of cells expressing GFP or TrFP in each hematopoietic subset. Experiments were performed in triplicate.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). The reported values represent the mean $\pm$ standard error of the mean (SEM) of at least three independent experiments. Statistical differences between different experimental conditions were determined using unpaired Student's t-test. A *P* value of less than 0.05 is considered statistically significant.

RESULTS

MYXV Infection in Human Bone Marrow Cells After Ex Vivo Virotherapy

Because our goal is to use MYXV as adjunctive therapy to more effectively treat hematological malignancies, we sought to define optimal conditions for *ex vivo* MYXV treatment of hematopoietic graft sources in terms of temperature, media, and incubation time¹³. Fresh or cryopreserved bone marrow (BM) samples from patients with hematological malignancies were suspended in IMDM or Plasma-lyte A + 10% ACDA and then either mock-treated (vehicle only) or treated with vMyx-GFP at MOI of 10 for 1 hour at room temperature to allow virus adsorption but not enough time to proceed further in the infection. Cells were then incubated at 37°C and 5% CO₂ in their respective media (IMDM or Plasma-lyte A + 10% ACDA) for 2 hours or 24 hours to allow virus infection to proceed (as monitored by GFP expression). MYXV infection was then analyzed by flow cytometry to quantify the percent of cells expressing GFP in various hematopoietic cell subsets. Representative 2D-plots for the 2-hour time point are shown (Figure 1A–B). Similar results were obtained for the 24-hour time point (data not shown). Overall, higher levels of MYXV infection initiation were detected in fresh BM cells compared to cryopreserved BM cells (Table 1 and Figure 1C, E, G, I vs. Figure 1D, F, H, J, respectively). For each of the two media tested (IMDM and Plasma-lyte A + 10% ACDA), the percentage of infection of fresh BM-derived CD45, CD3, CD34, HLA-ABC, CD15, and CD235a after 2 hours was not significantly different (Table 2). However, significant differences in infection after 2 hours were found for cells that are CD19⁺, (17.13 $\pm$ 1.80 in IMDM vs. 24.36 $\pm$ 3.02 in Plasma-lyte A + 10% ACDA; *P* = 0.020) and CD14⁺ (44.70 $\pm$ 3.74 in IMDM vs. 79.21 $\pm$ 4.00 in Plasma-lyte A + 10% ACDA; *P* = 0.040). Similarly, the percentage of infection of fresh BM-derived cell lineages after 24 hours was not significantly different (Table 2). The

initiation of MYXV infection was highest in fresh CD14⁺ monocytes (45% vs. 32% in IMDM and Plasma-lyte A, respectively at 2 hours; 79% vs. 72% in IMDM and Plasma-lyte A, respectively at 24 hours).

When comparing infection of the cryopreserved BM in IMDM vs. Plasma-lyte A + 10% ACDA, no significant differences were observed in almost all the cell lineages, except CD19⁺ at 2 hours (Table 2). In contrast, significant differences in the percentage of infection were observed in almost all the cell lineages, at 24 hours (Table 2). Importantly, < 10% of CD34⁺ HPSCs showed evidence of any MYXV infection for all the experimental conditions (Table 1). Overall, there was no significant difference in MYXV infection between incubation times of 2 hours versus 24 hours in either IMDM, or Plasma-lyte A + 10% ACDA media (Tables 1 and 2, and Figure 1C–J), with some exceptions that are shown in Table 2. Together the data, suggest feasibility of a short incubation time of 2 hours to evaluate whether MYXV could initiate infection in various leukocyte subtypes.

MYXV Infection in Mobilized Peripheral Blood Stem Cells After Ex Vivo Virotherapy

Because mobilized peripheral blood stem cells (mPBSCs) are the most common source for ASCT transplants, we examined the effects of MYXV treatment of fresh and cryopreserved mPBSCs from patients with hematological malignancies in identical fashion as BM. Similar to BM samples, higher levels of MYXV infection were observed in fresh cells compared to cryopreserved cells (Table 3 and Figure 2). Also similar to BM, MYXV infection was highest in mPBSC monocytes, and < 10% in mPB CD34⁺ cells (Table 3). In regards to incubation time (2 hours versus 24 hours) and media type (IMDM versus Plasma-lyteA + 10% ACDA), there were no significant differences in levels of MYXV infection for the majority of hematopoietic cell subsets, with some exceptions that are shown in Table 4.

Healthy Human Peripheral Blood Cells Are All Non-Permissive to MYXV Infection

Given the evidence of at least the initiation of MYXV infection in various hematopoietic subsets, we next investigated if the viral infection could progress to completion and generate progeny virus. Human peripheral blood mononuclear cells (PBMCs) were incubated with the vMyx-GFP/TrFP construct that expresses GFP at both early and late times of infection and TrFP at late times of infection only. In prior work, we have used this viral construct to determine the extent of permissiveness of various classes of test cells to MYXV^{14,15}. Whereas, permissive cells show double positive fluorescence (GFP⁺TrFP⁺) as a prelude to the synthesis of progeny virus that is indicative of a permissive infection, non-permissive cells may be revealed as either GFP⁺TrFP⁻ or GFP⁻TrFP⁻. After incubation, the hematopoietic subsets were analyzed by flow cytometry for GFP and TrFP expression. Like BM specimens, a sizeable proportion of monocytes showed the initiation of MYXV infection (mean 34.3%±16.1%) as evidenced by GFP⁺ expression (Figure 3). However, in all hematopoietic subsets, considerably less than 5% of cells expressed any detectable TrFP expression (Figure 3). These results indicate that while early MYXV gene expression may have initiated in some subsets of healthy human hematopoietic cells, like antigen presenting monocytes, but these examples of MYXV infection were all aborted and essentially all of the cells in the transplant samples tested were ultimately non-permissive to MYXV.

Hematopoietic Stem and Progenitor Colony Formation Is Unaffected by MYXV Treatment

To investigate the effect of MYXV *ex vivo* virotherapy on stem or progenitor hematopoietic cells, we first used the LTC-IC assay. Fresh bone marrow or cryopreserved bone marrow from healthy donors were treated with either mock-treatment or vMyx-GFP in various culture media, including IMDM, Plasma-lyteA + 10% ACDA, heparin and M199. Differentiated colonies were generated from all conditions tested (Figure 4A). The colonies did not express GFP, indicating that none were infected by MYXV. Colony counts were not significantly different between mock-treated and MYXV-treated samples (Figure 4 B&C), suggesting that MYXV did not impair hematopoietic stem cell numbers. In regards to media type, there were no significant differences in colony number or variety among the four media tested (IMDM, Plasma-lyte A + 10% ACDA, Plasma-lyteA + 15% heparin, M199 + 15% heparin).

To examine the effect of MYXV *ex vivo* treatment on hematopoietic stem or progenitor cell differentiation, we performed Colony Forming Cell (CFC) assays. Bone marrow samples from healthy donors were treated with mock treatment or vMyx-GFP under different incubation conditions, including various media (i.e., Plasma-lyte A + 10% ACDA, heparin or M199) and various temperatures (i.e., 4°C, room temperature (RT), and 37°C). Multiple colony types were observed after 14 days of culture in either MYXV-treated or mock-treated conditions (Figure 5A). The colonies did not express GFP and there was no evidence for virus in any of them. The number and types of colonies were not significantly different between mock-treated and MYXV-treated bone marrow (Figure 5 B&C), suggesting that MYXV did not adversely affect hematopoietic stem or progenitor cell differentiation. These results also indicate feasibility of using Plasma-lyte A + 10% ACDA, Plasma-lyte A + 15% heparin, M199 + 15% heparin, and any of the three incubation temperatures tested (4°C, RT or 37°C) for *ex vivo* MYXV treatment of HPSC grafts.

DISCUSSION

In this study, we report that *ex vivo* treatment with MYXV can initiate infection in low levels of primary human hematopoietic cells from BM and mPB graft sources, such as antigen presenting monocytes and NK cells, but only initiated early infection in these cells. Indeed, essentially all of the cells that permitted the initiation of MYXV infection successfully aborted the infection prior to the stage of late virus gene expression. On the other hand, MYXV did not infect the vast majority of cells in the CD34⁺ compartment and did not impair primitive hematopoietic stem and progenitor cell colony formation. Before this study, we reported that *ex vivo* MYXV treatment of human BM or PBMC xenografts fully spared normal human hematopoietic cell engraftment in immunocompromised mice¹⁶. Therefore, our findings indicate that MYXV spares normal human HSPCs for all conditions tested. This is the first documentation of MYXV sparing healthy human HSPCs in all potential human transplant specimens tested and is important because of the ultimate goal in developing MYXV as an *ex vivo* virotherapeutic for hematopoietic cell transplant patients.

The use of oncolytic viruses for selectively targeting and eliminating cancer cells is a powerful therapeutic strategy that has some unique requirements for successful translation into clinical practice. To serve the needs of patients with cancer, efficacy and safety should

complement each other. One of these requirements is that the *ex vivo* virotherapy should be simple and quick, especially when using cryopreserved stem cell products. In this study, we provide *in vitro* data that MYXV is expected to be safe for immune engraftment following *ex vivo* virotherapy against hematological malignancies. We demonstrate that either fresh or cryopreserved grafts (BM or mPBSCs) maybe used safely for *ex vivo* treatment with MYXV. Also, the use of a clinically approved transplant solutions like Plasma-lyte A + 10% ACDA showed similar outcomes to the IMDM research media. Early stages of MYXV infection were detected after 2 and 24 hours of incubation in some differentiated hematopoietic cells. However, >95% of all infections that initiate MYXV infection in human hematopoietic cells (i.e., cells that turn green after infection with vMyx-GFP) abort the virus replication cycle prior to the late stage of virus replication, and therefore cannot make progeny virus and are considered functionally nonpermissive for MYXV. Fresh mPBSCs showed slightly higher levels of MYXV infection initiation in differentiated cells like monocytes and NK cells, most likely due to manipulation-induced activation of some differentiated cells, as we have previously shown¹⁵. When considering our recent report of primary human T lymphocytes serving as transport vehicles for MYXV to target cancer cells¹⁵, the limited infection found in this study are interpreted as a potentially positive finding in assisting MYXV delivery to cancer cells post-transplant.

Although our original intent with MYXV was to safely eliminate contaminating cancer cells from ASCT grafts^{17,18}, our more recent work found that *ex vivo* MYXV treatment of allogeneic transplant grafts had the additional property of mitigating graft-versus-host disease^{15,16}. Thus, we now predict that MYXV *ex vivo* virotherapy will be applicable to both auto- and allo-transplant methods. In this report, demonstrating safety and feasibility of MYXV *ex vivo* treatment on normal HSPCs is critical towards advancing this virotherapy to the clinic. The safety data reported in this paper support a first-in-human clinical trial, where MYXV will be used as an adjunctive *ex vivo* therapy for hematopoietic cell transplant to treat patients with hematologic malignancies.

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Figure 1. MYXV treatment of fresh and cryopreserved bone marrow in various incubation conditions

MYXV treatment of fresh or cryopreserved BM samples from patients with hematological malignancies was performed using a vMyx-GFP construct (MOI of 10) at 37°C. Incubation was carried out in either IMDM, or Plasma-lyte A + 10% ACDA culture media for 2 hours or 24 hours. At the indicated time points, cells were collected and stained with monoclonal antibodies against cell lineages including CD45, HLA-ABC, CD19, CD3, CD34, CD14, CD15, and CD235a. Virus infection of these cell lineages was quantified using flow cytometry. (A and B), Representative 2D-plots showing the percentage of MYXV infection (GFP⁺) for 2 hours in IMDM and in Plasma-lyte A + 10% ACDA, respectively. (C–J), The percentage of MYXV infection (GFP⁺) of fresh vs. cryopreserved BM samples, for 2 hours

or 24 hours, in IMDM or in Plasma-lyte A + 10% ACDA are shown. Values are mean $\pm$ SEM of at least four independent experiments with three independent specimens.

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Figure 2. MYXV treatment of fresh and cryopreserved mobilized peripheral blood stem cells in various incubation conditions

MYXV treatment of fresh or cryopreserved mobilized peripheral blood stem cell samples (mPBSCs) from patients with hematological malignancies was performed using a vMyx-GFP construct (MOI of 10) at 37°C. Incubation was carried out in either IMDM, or Plasma-lyte A + 10% ACDA culture media for 2 hours or 24 hours. At the indicated time points, cells were collected and stained with monoclonal antibodies against cell lineages including CD45, HLA-ABC, CD19, CD3, CD34, CD14, CD15, and CD235a. Virus infection of these cell lineages was quantified using flow cytometry. (A and B), Representative 2D-plots showing the percentage of MYXV infection (GFP⁺) for 2 hours in IMDM and in Plasma-lyte A + 10% ACDA, respectively. (C–J), The percentage of MYXV infection (GFP⁺) of fresh vs. cryopreserved BM samples, for 2 hours or 24 hours, in IMDM or in Plasma-lyte A + 10% ACDA are shown. Values are mean $\pm$ SEM of at least four independent experiments with three independent specimens.

Figure 3. Human peripheral blood mononuclear cells are nonpermissive to MYXV

To determine whether MYXV is permissive to the cell subsets, CD14⁺ (monocytes), CD3⁺, CD4⁺ and CD8⁺ (T cells), CD19⁺ (B cells) and CD56⁺ (NK cells), PBMCs were isolated from healthy donors and then incubated with vMyx-GFP/TrFR (MOI =10) at 4°C for 1 hour. Unbound virus was washed and cells were incubated at 37°C for 24 hours. Next cells stained with the respective fluorochrome-conjugated antibodies, and the proportion of cells expressing GFP⁺ or TrFP⁺ in each hematopoietic cell subset was quantified using flow cytometry. Although a high proportion of monocytes expressed GFP⁺, in all hematopoietic subsets, greater than 95% of cells lacked TrFP expression, indicating that even though early MYXV infection may have initiated in some healthy human hematopoietic cells, the MYXV infection was aborted and the cells were non-permissive to the virus.

A**CFU-G** **BFU-E**

CFU-M

CFU-GEMM

CFU-GM

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Figure 4. Effect of MYXV treatment on LTC-IC from human bone marrow

(A) Micrographs of LTC-ICs after treatment with MYXV. (B) Fresh or (C) cryopreserved BM was treated with vMyx-GFP (MOI of 10) in various culture media including IMDM, Plasma-lyte A + 10% ACDA, heparin, and M199. The hematopoietic stem cell content was quantified by long-term culture-initiating cell (LTC-IC) assay. Data shown correspond to the mean $\pm$ SEM of at least three independent specimens, each one performed in duplicate.

Figure 5. Effect of MYXV treatment on CFC from human bone marrow
 (A) Micrographs of CFC after treatment with MYXV. (B) Colony formations observed after 14 days according to various MYXV incubation media conditions. (C) CFC colony formations observed after 14 days according to various MYXV incubation temperature conditions. Data shown are the mean $\pm$ SEM of at least three independent specimens, each one performed in duplicate.

TABLE 1
Infection of fresh versus cryopreserved bone marrow samples from patients with hematological malignancies were performed for 2 hours and 24 hours at 37°C in IMDM or in Plasma-lyte A + 10% ACDA

Percentage levels of MYXV in hematopoietic cell subsets are mean $\pm$ standard error of the mean.

Cell lineages	Fresh BM % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 2 hpi	Cryopreserved BM % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 2 hpi	Fresh BM % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A +10 % ACDA, 2 hpi	Cryopreserved BM % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A+10 % ACDA, 2 hpi
CD45	12.81 $\pm$ 1.05	6.79 $\pm$ 1.44	14.88 $\pm$ 2.24	8.86 $\pm$ 2.24
CD19	17.13 $\pm$ 1.80	19.80 $\pm$ 0.27	9.98 $\pm$ 1.16	2.06 $\pm$ 0.75
CD34	2.00 $\pm$ 0.24	1.57 $\pm$ 0.23	1.88 $\pm$ 0.23	1.38 $\pm$ 0.23
CD3	5.15 $\pm$ 1.46	2.54 $\pm$ 0.43	5.46 $\pm$ 0.31	2.58 $\pm$ 0.25
HLA-ABC	15.37 $\pm$ 2.66	15.95 $\pm$ 6.25	17.11 $\pm$ 6.00	10.97 $\pm$ 1.27
CD15	10.40 $\pm$ 2.34	6.42 $\pm$ 2.18	22.96 $\pm$ 10.05	2.17 $\pm$ 1.10
CD14	44.70 $\pm$ 3.74	9.53 $\pm$ 4.72	31.98 $\pm$ 2.80	13.25 $\pm$ 1.01
CD235a	2.27 $\pm$ 0.61	0.76 $\pm$ 0.21	2.01 $\pm$ 0.30	0.76 $\pm$ 0.21
Cell lineages	Fresh BM % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 24 hpi	Cryopreserved BM % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 24 hpi	Fresh BM % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A + 10% ACDA, 24 hpi	Cryopreserved BM % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A + 10% ACDA, 24 hpi
CD45	30.09 $\pm$ 5.75	19.81 $\pm$ 2.71	29.76 $\pm$ 10.08	8.82 $\pm$ 2.49
CD19	24.36 $\pm$ 3.02	26.75 $\pm$ 5.27	19.83 $\pm$ 4.83	6.80 $\pm$ 0.76
CD34	6.86 $\pm$ 2.28	9.15 $\pm$ 0.57	2.33 $\pm$ 0.66	2.80 $\pm$ 0.96
CD3	4.69 $\pm$ 2.30	6.55 $\pm$ 0.37	6.00 $\pm$ 2.2	5.53 $\pm$ 0.63
HLA-ABC	35.03 $\pm$ 1.84	22.38 $\pm$ 1.01	20.23 $\pm$ 9.77	7.17 $\pm$ 2.46
CD15	34.73 $\pm$ 7.17	6.62 $\pm$ 4.00	13.23 $\pm$ 7.23	4.42 $\pm$ 0.40
CD14	79.21 $\pm$ 4.00	76.56 $\pm$ 6.60	72.23 $\pm$ 0.01	11.09 $\pm$ 7.4
CD235a	2.62 $\pm$ 0.39	2.77 $\pm$ 0.43	4.49 $\pm$ 2.0	2.63 $\pm$ 0.89

BM, bone marrow

IMDM, Iscove's modified Dulbecco's medium

MYXV, myxoma virus

GFP, green fluorescent protein

CD, cluster of differentiation

HLA, human leukocyte antigen

ACDA, anticoagulant citrate dextrose solution A

hpi, hours post incubation

TABLE 2
***P* values calculated for BM samples derived from patients with hematological malignancies, at different experimental conditions using GraphPad Prism software through the use of unpaired Student's t-test**

Effect of media, incubation time, and cryopreservation of BM on the percentage of MYXV infection.

Effect of different media on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh BM in IMDM vs. Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Cryopreserved BM in IMDM vs. Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Fresh BM in IMDM vs. Plasma-lyte A + 10% ACDA, 24 hpi	<i>P</i> values: Cryopreserved BM in IMDM vs. Plasma-lyte A + 10% ACDA, 24 hpi
CD45	0.449, N.S.	0.682, N.S.	0.954, N.S.	0.029
CD19	0.020	0.002	0.503, N.S.	0.016
CD34	0.093, N.S.	0.622, N.S.	0.074, N.S.	0.017
CD3	0.814, N.S.	0.941, N.S.	0.723, N.S.	0.322, N.S.
HLA-ABC	0.659, N.S.	0.517, N.S.	0.644, N.S.	0.019
CD15	0.218, N.S.	0.245, N.S.	0.138, N.S.	0.523, N.S.
CD14	0.040	0.522, N.S.	0.319, N.S.	0.010
CD235a	0.684, N.S.	1.000, N.S.	0.387, N.S.	0.913, N.S.

Effect of the incubation time on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh BM in IMDM, 2 hpi vs. 24 hpi	<i>P</i> values: Cryopreserved BM in IMDM, 2 hpi vs. 24 hpi	<i>P</i> values: Fresh BM in Plasma-lyte A + 10% ACDA, 2 hpi vs. 24 hpi	<i>P</i> values: Cryopreserved BM in Plasma-lyte A + 10% ACDA, 2 hpi vs. 24 hpi
CD45	0.104, N.S.	0.232, N.S.	0.337, N.S.	0.989, N.S.
CD19	0.111, N.S.	0.319, N.S.	0.259, N.S.	0.025
CD34	0.159, N.S.	0.010	0.651, N.S.	0.405, N.S.
CD3	0.552, N.S.	0.019	0.831, N.S.	0.926, N.S.
HLA-ABC	0.402, N.S.	0.228, N.S.	0.789, N.S.	0.335, N.S.
CD15	0.128, N.S.	0.964, N.S.	0.752, N.S.	0.417, N.S.
CD14	0.002	0.056, N.S.	0.076, N.S.	0.837, N.S.
CD235a	0.379, N.S.	0.054, N.S.	0.280, N.S.	0.205, N.S.

Effect of cryopreservation of BM on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh BM vs. Cryopreserved BM in IMDM, 2 hpi	<i>P</i> values: Fresh BM vs. Cryopreserved BM in IMDM, 24hpi	<i>P</i> values: Fresh BM vs. Cryopreserved BM in Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Fresh BM vs. Cryopreserved BM in Plasma-lyte A + 10% ACDA, 24 hpi
CD45	0.707, N.S.	0.274, N.S.	0.130, N.S.	0.114, N.S.
CD19	0.336, N.S.	0.732, N.S.	0.016	0.063, N.S.
CD34	0.324, N.S.	0.406, N.S.	0.209, N.S.	0.868, N.S.
CD3	0.265, N.S.	0.510, N.S.	0.002	0.704, N.S.
HLA-ABC	0.928, N.S.	0.027	0.489, N.S.	0.199, N.S.
CD15	0.281, N.S.	0.063, N.S.	0.004	0.202, N.S.
CD14	0.010	0.733, N.S.	0.015	0.008
CD235a	0.165, N.S.	0.814, N.S.	0.052, N.S.	0.445, N.S.

$P > 0.05$ is considered statistically not significant

$P < 0.05$ is considered statistically significant

N.S., not significant

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TABLE 3
Infection of fresh vs. cryopreserved mPBSC derived from patients with hematological malignancies, were performed for 2 hours and 24 hours at 37°C in IMDM or in plasma-lyte A + 10% ACDA

Percentage levels of MYXV in hematopoietic cell subsets are mean $\pm$ standard error of the mean.

Cell lineages	Fresh mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 2 hpi	Cryopreserved mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 2 hpi	Fresh mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A +10 % ACDA, 2 hpi	Cryopreserved mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A+10 % ACDA, 2 hpi
CD45	45.10 $\pm$ 2.96	12.34 $\pm$ 2.58	69.67 $\pm$ 0.57	12.19 $\pm$ 3.57
CD19	25.51 $\pm$ 2.89	11.16 $\pm$ 2.75	38.07 $\pm$ 9.31	4.60 $\pm$ 1.28
CD34	2.49 $\pm$ 1.40	1.00 $\pm$ 0.30	4.6 $\pm$ 1.76	6.07 $\pm$ 1.88
CD3	34.51 $\pm$ 2.15	4.17 $\pm$ 1.28	21.46 $\pm$ 4.42	8.93 $\pm$ 1.07
HLA-ABC	57.90 $\pm$ 1.40	9.52 $\pm$ 2.13	68.62 $\pm$ 3.14	6.39 $\pm$ 0.92
CD15	46.06 $\pm$ 7.54	5.03 $\pm$ 1.30	62.90 $\pm$ 8.05	8.76 $\pm$ 0.39
CD14	74.27 $\pm$ 2.05	29.78 $\pm$ 3.02	77.83 $\pm$ 5.08	10.48 $\pm$ 0.16
CD235a	5.38 $\pm$ 1.49	0.94 $\pm$ 0.30	2.84 $\pm$ 0.45	3.42 $\pm$ 0.68
Cell lineages	Fresh mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 24 hpi	Cryopreserved mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: IMDM, 24 hpi	Fresh mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A +10 % ACDA, 24 hpi	Cryopreserved mPBSC % MYXV infection (GFP ⁺), (n = 4) Culture media: Plasma-lyte A+10 % ACDA, 24 hpi
CD45	62.17 $\pm$ 2.13	11.88 $\pm$ 1.65	53.16 $\pm$ 1.19	10.11 $\pm$ 2.33
CD19	22.92 $\pm$ 3.56	21.95 $\pm$ 3.21	28.57 $\pm$ 8.42	10.09 $\pm$ 3.01
CD34	5.59 $\pm$ 1.75	6.04 $\pm$ 0.72	6.07 $\pm$ 0.49	4.93 $\pm$ 1.34
CD3	15.32 $\pm$ 4.14	4.18 $\pm$ 0.59	4.29 $\pm$ 0.30	4.0 $\pm$ 1.1
HLA-ABC	42.51 $\pm$ 3.20	8.90 $\pm$ 1.21	52.80 $\pm$ 5.22	5.62 $\pm$ 0.07
CD15	46.80 $\pm$ 7.98	4.80 $\pm$ 1.93	64.80 $\pm$ 6.29	4.50 $\pm$ 0.72
CD14	76.07 $\pm$ 5.16	51.37 $\pm$ 8.53	57.86 $\pm$ 4.54	28.18 $\pm$ 11.0
CD235a	2.86 $\pm$ 0.49	2.25 $\pm$ 0.44	3.59 $\pm$ 1.06	2.68 $\pm$ 0.19

mPBSC, mobilized peripheral blood stem cells

IMDM, Iscove's modified Dulbecco's medium

MYXV, myxoma virus

GFP, green fluorescent protein

CD, cluster of differentiation

HLA, human leukocyte antigen

ACDA, anticoagulant citrate dextrose solution A

hpi, hours post incubation

TABLE 4

***P* values calculated for mPBSC samples from patients with hematological malignancies, at different experimental conditions using GraphPad Prism software through the use of unpaired Student's t-test**

Effect of media, incubation time, and cryopreservation of mPBSC on the percentage of MYXV infection.

Effect of different media on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh mPBSC in IMDM vs. Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Cryopreserved mPBSC in IMDM vs. Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Fresh mPBSC in IMDM vs. Plasma-lyte A + 10% ACDA, 24 hpi	<i>P</i> values: Cryopreserved mPBSC in IMDM vs. Plasma-lyte A + 10% ACDA, 24 hpi
CD45	0.0002	0.973, N.S.	0.013	0.556, N.S.
CD19	0.316, N.S.	0.134, N.S.	0.504, N.S.	0.042
CD34	0.017	0.134, N.S.	0.858, N.S.	0.441, N.S.
CD3	0.065, N.S.	0.029	0.151, N.S.	0.749, N.S.
HLA-ABC	0.001	0.288, N.S.	0.289, N.S.	0.187, N.S.
CD15	0.249, N.S.	0.079, N.S.	0.157, N.S.	0.884, N.S.
CD14	0.594, N.S.	0.003	0.060, N.S.	0.160, N.S.
CD235a	0.136, N.S.	0.030	0.502, N.S.	0.610, N.S.

Effect of different incubation times on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh mPBSC in IMDM, 2 hpi vs. 24 hpi	<i>P</i> values: Cryopreserved mPBSC in IMDM, 2 hpi vs. 24 hpi	<i>P</i> values: Fresh mPBSC in Plasma-lyte A + 10% ACDA, 2 hpi vs. 24 hpi	<i>P</i> values: Cryopreserved mPBSC in Plasma-lyte A + 10% ACDA, 2 hpi vs. 24 hpi
CD45	0.002	0.878, N.S.	< 0.0001	0.642, N.S.
CD19	0.173, N.S.	0.040	0.500, N.S.	0.201, N.S.
CD34	0.292, N.S.	0.010	0.518, N.S.	0.628, N.S.
CD3	0.014	0.998, N.S.	0.061, N.S.	0.014
HLA-ABC	0.013	0.792, N.S.	0.041	0.563, N.S.
CD15	0.957, N.S.	0.920, N.S.	0.862, N.S.	0.006
CD14	0.822, N.S.	0.056, N.S.	0.038	0.213, N.S.
CD235a	0.067, N.S.	0.096, N.S.	0.590, N.S.	0.467, N.S.

Effect of cryopreservation of mPBSC on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh mPBSC vs. Cryopreserved mPBSC in IMDM, 2 hpi	<i>P</i> values: Fresh mPBSC vs. Cryopreserved mPBSC in IMDM, 24hpi	<i>P</i> values: Fresh mPBSC vs. Cryopreserved mPBSC in Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Fresh mPBSC vs. Cryopreserved mPBSC in Plasma-lyte A + 10% ACDA, 24 hpi
CD45	0.0002	< 0.0001	< 0.0001	< 0.0001
CD19	0.015	0.003	0.029	0.067, N.S.
CD34	0.418, N.S.	0.499, N.S.	0.589, N.S.	0.553, N.S.
CD3	< 0.0001	0.020	0.033	0.772, N.S.
HLA-ABC	< 0.0001	< 0.0001	< 0.0001	0.004
CD15	0.0003	0.007	0.003	0.001
CD14	< 0.0001	< 0.0001	< 0.0001	0.081, N.S.

Effect of cryopreservation of mPBSC on the percentage of MYXV infection (GFP ⁺)				
Cell lineages	<i>P</i> values: Fresh mPBSCvs. Cryopreserved mPBSC in IMDM, 2 hpi	<i>P</i> values: Fresh mPBSCvs. Cryopreserved mPBSC in IMDM, 24hpi	<i>P</i> values: Fresh mPBSCvs. Cryopreserved mPBSC in Plasma-lyte A + 10% ACDA, 2 hpi	<i>P</i> values: Fresh mPBSCvs. Cryopreserved mPBSC in Plasma-lyte A + 10% ACDA, 24 hpi
CD235a	0.034	0.373, N.S.	0.511, N.S.	0.601, N.S.

$P > 0.05$ is considered statistically not significant

$P < 0.05$ is considered statistically significant

N.S., not significant