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Prevention of EBV lymphoma development by oncolytic myxoma virus in a murine xenograft model of post-transplant lymphoproliferative disease

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ABSTRACT

Epstein–Barr virus (EBV) has been associated with a variety of epithelial and hematologic malignancies, including B-, T- and NK cell-lymphomas, Hodgkin's disease (HD), post-transplant lymphoproliferative diseases (LPDs), nasopharyngeal and gastric carcinomas, smooth muscle tumors, and HIV-associated lymphomas. Currently, treatment options for EBV-associated malignancies are limited. We have previously shown that myxoma virus specifically targets various human solid tumors and leukemia cells in a variety of animal models, while sparing normal human or murine tissues. Since transplant recipients of bone marrow or solid organs often develop EBV-associated post-transplant LPDs and lymphoma, myxoma virus may be of utility to prevent EBV-associated malignancies in immunocompromised transplant patients where treatment options are frequently limited. In this report, we demonstrate the safety and efficacy of myxoma virus purging as a prophylactic strategy for preventing post-transplant EBV-transformed human lymphomas, using a highly immunosuppressed mouse xenotransplantation model. This provides support for developing myxoma virus as a potential oncolytic therapy for preventing EBV-associated LPDs following transplantation of bone marrow or solid organ allografts.

1. Introduction

Oncolytic viruses are live replication-proficient viruses preferentially infecting human cancer cell while sparing normal counterpart. Replication-proficient viruses provide a series of potentially viable anti-cancer therapeutic approaches. Such oncolytic viruses have many advantages over conventional chemotherapy/radiotherapy or replication-incompetent viral vectors. First, they generally target cancer cells because of their natural or engineered reduced ability to replicate in normal cells, while replicating vigorously in and killing transformed cells. Second, as compared to replication-incompetent viral vectors, they can propagate from initially infected cancer cells to surrounding or distant cancer cells, thereby achieving a large volume of distribution and potent anti-cancer effects [1,2].

Because of these unique features of the replicating nature of oncolytic viruses, they are highly dependent on the host cell physiology for optimal performance as viral cancer-targeting agents. Several naturally occurring viruses including myxoma virus (MYXV) have shown a great potential as cancer targeting agents by exploiting various oncogene signaling pathways that are established by host cancer cells during tumorigenesis [3,4]. Because carcinogenesis is a multi-step process involving accumulation of not only oncogene abnormalities but also tumor suppressor gene abnormalities, we have recently shown that cellular tumor suppressor genes are also important in determining viral oncotropism including MYXV [5]. Thus, mechanistically replicating viral oncolysis can be established by both cellular oncogene/tumor suppressor gene abnormalities. Importantly, we have shown that MYXV specifically eliminated contaminating AML patients-derived cancer stem cells in hematologic explants by *ex vivo* viral purging prior to re-engraftment [6].

MYXV's natural host tropism in nature is highly restricted to European rabbits (*Oryctolagus cuniculus*), and it is nonpathogenic

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for all other vertebrate species tested, including humans [7,8]. Despite its narrow host specificity, we have previously shown that MYXV is capable of selectively infecting and killing a variety of human cancers while sparing normal tissues and cells, including primary hematopoietic stem cells, *in vitro* and *in vivo* [5,6,9].

Epstein–Barr virus (EBV) is associated with a variety of human epithelial and hematologic malignancies, including B-, T- and NK cell-lymphomas, Hodgkin's disease (HD), post-transplant lymphoproliferative diseases (LPDs), nasopharyngeal and gastric carcinomas, smooth muscle tumors, and HIV-associated lymphomas [10]. Currently, treatment options for EBV- or KSHV-associated post-transplant LPDs are limited due to a high level of host immunodeficiency [11]. Since the majority of LPDs arises from the graft donor lymphocytes, a selective method of purging potentially tumorigenic EBV- or KSHV-infected cells from allograft samples prior to transplantation would provide significant benefits in decreasing the incidence of post-transplant LPDs.

In this study, we report that MYXV can prevent EBV-transformed human B-lymphoma engraftment in a highly immunosuppressed mouse xenotransplantation model.

2. Materials and methods

2.1. Cells and viruses

Namalwa (an EBV$^+$ Burkitt lymphoma cell line), BJAB (an EBV$^-$ Burkitt lymphoma cell line), BCBL-1 (a KSHV$^+$ primary effusion lymphoma cell line), and B-LCL (a B lymphoblastoid cell line derived by *in vitro* EBV transformation of primary human B lymphocytes) were provided by Dr. Sankar Swaminathan in University of Florida. vMyx-GFP and vMyx-RFP stocks were propagated on permissive Vero or BGMK cells and titrated by focus formation on Vero or BGMK monolayers as described previously [4].

2.2. In vitro incubation and western blotting

Cell viability of MYXV-infected cells was evaluated by WST-1 assay kit (Roche, Mannheim, Germany). Phospho-Akt (serine 473) levels were evaluated by immunoblot analysis of cell lysates as described previously [4]. Blots were probed with either rabbit polyclonal anti-phospho-Akt antibody (Cell Signaling, Danvers, MA) or rabbit polyclonal anti-total Akt antibody (Cell Signaling, Danvers, MA) at 1:1000 dilution. Protein-antibody complexes were visualized by an enhanced chemiluminescence (ECL) method (Amersham).

2.3. Flow cytometry

Myx-RFP-infected cells were washed, re-suspended in DPBS +2% FBS containing Viaprobe and viable human cell content was measured using a Beckton Dickinson LSR II flow cytometer. Detection of phosphatidyl serine on the surfaces of apoptotic cells was performed using the Annexin-V detection kit (PharMingen, San Diego, CA, USA).

2.4. Myxoma virus ex vivo incubation and xenotransplantation

B-lymphoma cells were incubated with vMyx-GFP or vMyx-RFP at an MOI of 10 pfu per cell for 3 h. All animal studies were performed after receiving approval from the University of Florida Institutional Animal Care and Use Committee. B-LCL cells were either mock-treated or exposed to MYXV for 3 h *ex vivo*, and subsequently transplanted into irradiated (325 cGy) NOD/scid mice (Charles River) via retro-orbital sinus injection. 5×10^6 B-LCL cells were transplanted per mouse. Tumors were measured by calipers every other day after becoming palpable and survival of mice was recorded on a daily basis. Animals exhibiting signs of excessive tumor burden (tumor size 1.5 cm), were euthanized, necropsied and analyzed for signs of lymphoproliferative disease and viral infection. At 120 days, all living mice were euthanized and also analyzed for signs of lymphoproliferative disease and viral infection.

2.5. Immunohistochemistry

Organs and lymphomas from all euthanized mice were harvested, fixed in 10% normal buffered formalin at room temperature and stained with hematoxylin and eosin. To detect human lymphoid cells, tumor and brain tissues were examined by staining with anti-human CD45 antibodies (1:100, DAKO Corporation, Carpinteria, CA) at 4 °C overnight by M.O.M kit (Vector Laboratories, Burlingame, CA) and visualized by Vector ABC-Peroxidase using DAB. The sections were immunostained with a solution containing 0.1% human CD45 antiserum, 50 mM Tris (pH 7.5), 120 mM NaCl, 0.2% Tween 20, 0.1% Triton X-100 and 2% normal goat serum for 2hr. As a secondary antibody, biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was used at 1:100 in a solution containing 50 mM Tris (pH 7.5), 120 mM NaCl, 0.2% Tween 20, 0.1% Triton X-100 and 2% normal goat serum for 2 h at room temperature. Detection was monitored by a diaminobenzidine tetrahydrochloride-based immunohistochemistry protocol according to the suggestions of the manufacturer (Vector Laboratories, Burlingame, CA). Dehydration was carried out in a series of graded ethanol solutions, followed by clarification in xylene. Slides were mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI) and images acquired with an Olympus BX51 microscope using Image ProMC 6.0 software (Media Cybernetics).

3. Results

3.1. MYXV in vitro oncolytic effects on EBV lymphoma cells

In order to test the susceptibilities of human B cell lymphomas to infection and oncolysis by MYXV, Namalwa (an EBV$^+$ Burkitt lymphoma cell line), BJAB (an EBV$^-$ Burkitt lymphoma cell line), BCBL-1 (a KSHV$^+$ primary effusion lymphoma cell line), and B-LCL (a B lymphoblastoid cell line derived by *in vitro* EBV transformation of primary human B lymphocytes) cells were infected *in vitro* with MYXV. Each cell line was infected with vMyx-GFP (GFP expressed under viral early/late promoter) or vMyx-RFP (RFP expressed under viral late promoter only) at an MOI of 10. At 3 days post-infection, all the human B cell lines were efficiently infected by MYXV as evidenced by GFP or RFP expression (Fig. 1A). These infections reduced cell viabilities *in vitro* as monitored by a mitochondrial function assay (Fig. 1B).

We have previously shown that activated AKT signaling facilitates MYXV permissiveness in human cancer cells (Wang et al., 2006). Constitutive Akt activation has been described in numerous human lymphoma cell lines and also varies among lymphoma lines [12,13]. As shown in Fig. 1C, endogenous Akt activation (Ser473 phosphorylation) was detected in three of the lines. Also, B-LCL cells were induced into apoptosis by the virus infection, as evidenced by Annexin V co-staining over a 3-day period (Fig. 1D).

3.2. MYXV in vivo oncolytic effects on EBV lymphoma cells

EBV-transformed B-LCL cells derived from primary human PBMCs are known to form tumors in immunosuppressed mice that histologically resemble post-transplant LPDs and have similar expression patterns of EBV and cellular genes [14]. Accordingly,

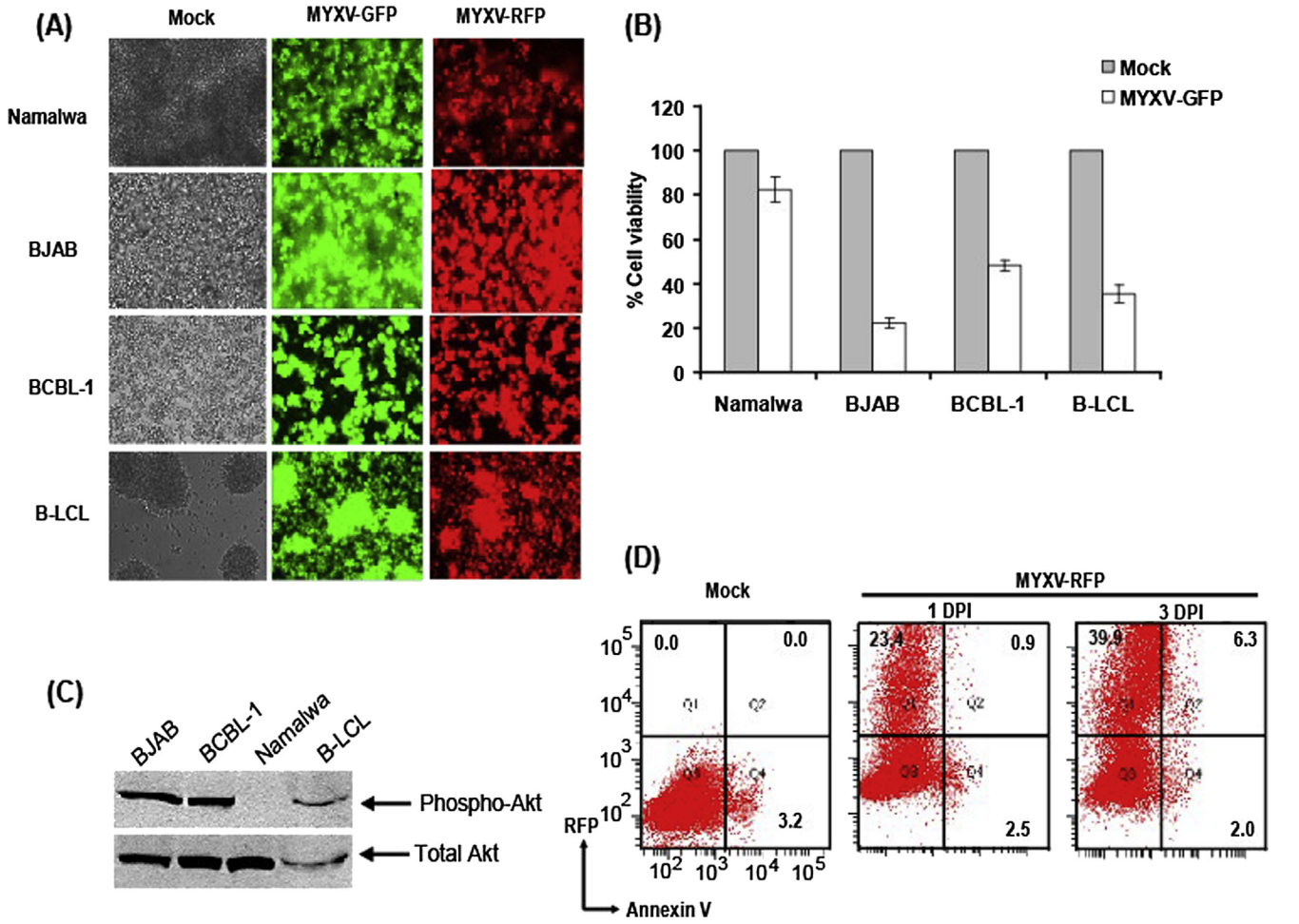

Fig. 1. Susceptibility of various human B-lymphoma cells *in vitro* to MYXV Infection. (A) MYXV infection of human B-cell lymphomas. Various human B-cell lymphomas (Namalwa, BJAB, BCBL-1) and an EBV-transformed B lymphoblastoid cell line (B-LCL) were incubated *in vitro* with either vMyx-GFP or vMyx-RFP at 10 MOI. At 3 days, cells were visualized and photographed under a fluorescent microscope. vMyx-GFP expresses EGFP (green) under a synthetic viral early/late promoter, and vMyx-RFP expresses Ds-Red (red) under a viral late P11 promoter. (B) Viability of MYXV-infected human B-cell lymphomas. Each cell line was infected with vMyx-GFP or mock-infected as shown and percent cell viability was calculated 72 h post-infection. Cell viability of vMyx-GFP-infected cells was evaluated by WST-1 assay. (C) Akt activation and total Akt levels in lymphomas and B-LCLs. Phospho-Akt (serine 473) and total Akt levels were evaluated by immunoblot analysis of cell lysates from lymphoma and B-LCL cells. (D) Apoptosis in MYXV-infected B-LCL. B-LCL cells were incubated with vMyx-RFP at 10 MOI for 3 days. Analysis of apoptotic events was performed by flow cytometry at 1 or 3 days, and positive Annexin V staining was measured in the RFP + infected cell population.

B-LCL cells were treated *ex-vivo* with MYXV for 3-h and transplanted into sublethally irradiated Nod/SCID mice by the retro-orbital sinus route [6,15]. As shown in Fig. 2A (upper panel), 100% (5/5) of the control mice engrafted with B-LCL cells that had received no virus treatment developed excessive tumor burdens in the cervical region, exhibited severe morbidity (such as hunched back, scruffy/ruffled fur, and decreased mobility) and had to be sacrificed prior to 35 days post-transplantation. In sharp contrast, none (0/5) of the mice transplanted with B-LCL cells that had been treated with MYXV *ex vivo* prior to engraftment showed any symptoms, and these MYXV-purged mice survived tumor-free for 3 months post-transplantation, when the experiment was terminated (Fig. 2A, lower panel).

Mice engrafted with mock-treated B-LCL cells developed lymphoid tumors in the neck, and in brain tissues, whereas in contrast, MYXV-purged mice exhibited no evidence of B-LCL tumor development in any of these tissues (Fig. 2B). Immunostaining with anti-huCD45 was used to verify that the neck and brain tumors in the mock-treated mice were derived from injected human B-LCL cells (Fig. 2B). Taken together, these data indicate that *ex vivo* infection with MYXV completely prevented B-LCL mediated tumor development *in vivo* but caused no effects in any other nontumor tissues such as heart, liver and lung (data not shown).

4. Discussion

LPDs commonly occur in transplant patients with severe immunodeficiency and EBV is frequently involved in their etiology [11]. B-cell lymphomas have been mainly reported after allogeneic bone marrow transplantation, with few cases described after autologous transplants [16]. EBV is a gamma-herpesvirus that infects over 90% of healthy individuals and persists lifelong in oral epithelial cells and B cells [17]. In healthy individuals, B cells that express these proteins are eradicated by EBV-specific T cells [17]. After allograft transplantation, however, these T cells can be absent or dysfunctional, allowing EBV-infected B cells to proliferate uncontrollably in some patients [16–20]. In solid organ transplantation patients, simply withdrawing immunosuppressive therapy has proven effective, but carries a high risk of graft rejection [15,21]. Patients undergoing ABMT using unmanipulated bone marrow samples, post-transplant lymphoid malignancy relapse is a serious clinical complication [22]. Since bone marrow transplant

recipients and solid organ transplant patients all can develop EBV-associated LPDs and lymphomas, MYXV may be of great utility to target EBV-associated malignancy and lymphomas in immunocompromised patients.

We have previously shown that hyper-active AKT oncogene signaling facilitates MYX permissiveness in a broad variety of human cancer cells [4]. It is important to note that two EBV proteins, LMP1 and LMP2A, which are expressed during certain types of

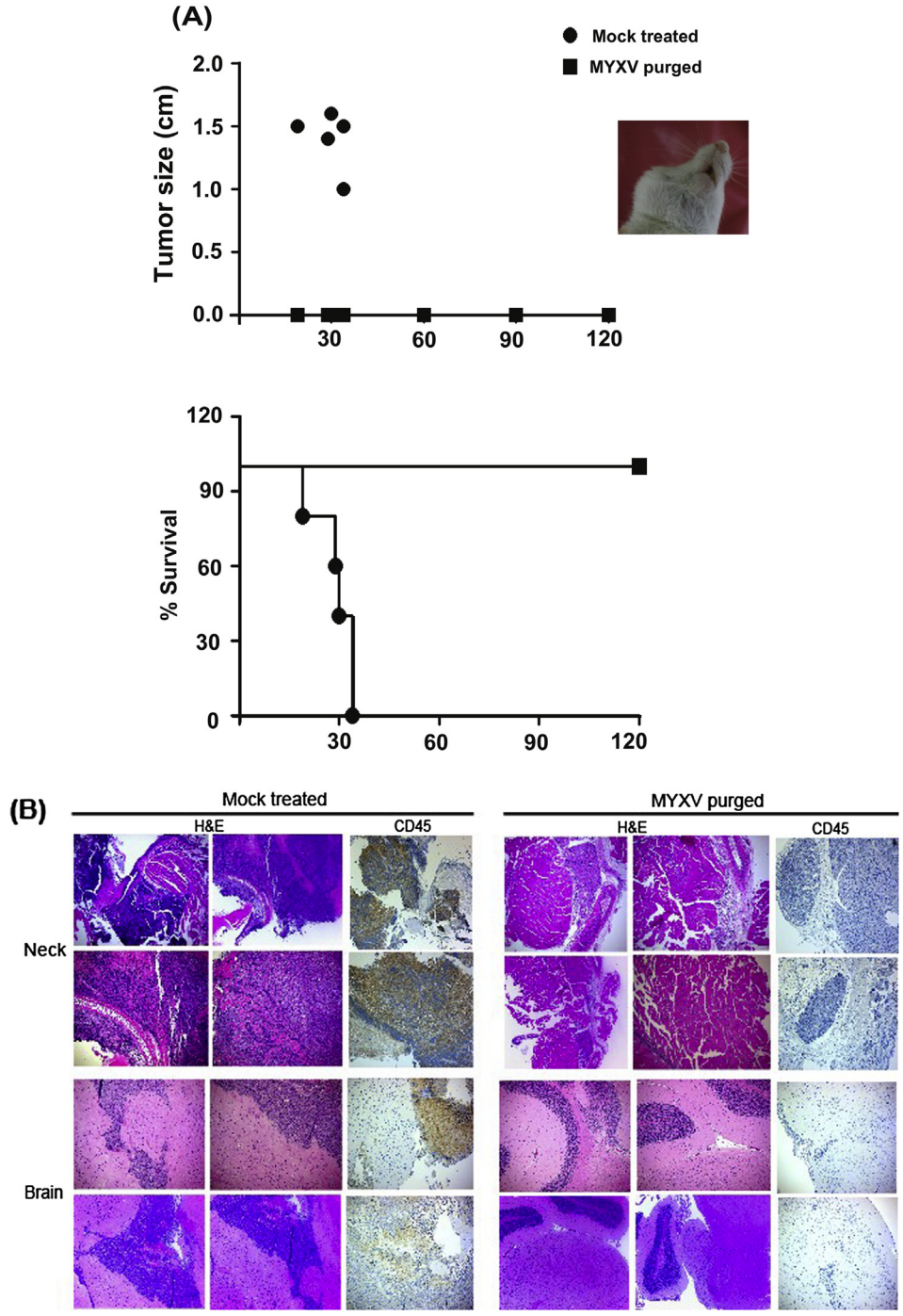

Fig. 2. *Ex vivo* treatment of B-LCL xenografts with MYXV prevents B-LCL lymphoid tumor formation. (A) Inhibition of cervical B-LCL tumor growth by MYXV *ex vivo* purging. B-LCL cells were either mock-treated (control) or exposed to MYXV at 10 MOI for 3-h *ex vivo*, and subsequently transplanted into sub-lethally irradiated NOD/scid mice. Cervical tumors developed only in the mock-treated cohort (n = 5) whereas no tumors were observed in the MYXV- treated cohort (n = 5). The gross appearance of a representative cervical tumor in a control mouse is shown in the right inset. Tumor size of the cervical area of mice was measured (upper panel) and survival of mice (lower panel) was followed up to 120 days. (B) Pathology and cellular origin of tumors arising in xenotransplanted mice. Histologic and immunostaining analysis of cervical lymphoma and brain sections were performed on samples from mice injected with B-LCLs treated or mock-treated with MYXV. Mice were euthanized when they exhibited excessive lymphoma burden or when cancer cachexia reached a predetermined endpoint. Lymphomas and organs were then excised, fixed and stained with hematoxylin and eosin. To detect human lymphoma cells, tumor and brain tissues were also examined by staining with anti-human CD45 antibodies (brown).

latent infection, can activate phosphatidylinositol 3′-OH kinase (PI3K) and its target Akt in epithelial cells [23]. LMP2A has been shown to activate PI3K and Akt in B lymphocytes as well [24]. More importantly, during the multi-step process of immortalization and tumorigenesis of human B-lymphoblastoid cells caused by Epstein–Barr virus, p53 or Rb host genes can be mutated or down-regulated [25]. Thus not only oncogene-induced but also tumor suppressor dysfunctional EBV transformed cells could be targeted by MYXV.

In summary, our data strongly support the clinical utility of MYXV for *ex vivo* purging of EBV-induced tumor precursors from allogeneic donor tissue or from autografts prior to various classes of transplantation.

Disclosure

G.M. has a patent held by UWO, London, ON issued to DNAtrix.

Conflict of interest

None

Acknowledgments

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