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Removal of lactate dehydrogenase-elevating virus from human-in-mouse breast tumor xenografts by cell-sorting

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Abstract

Lactate dehydrogenase-elevating virus (LDV) can infect transplantable mouse tumors or xenograft tumors in mice through LDV-contaminated mouse biological materials, such as Matrigel, or through mice infected with LDV. LDV specifically infects mouse macrophages and alters immune system and tumor phenotype. The traditional approaches to remove LDV from tumor cells, by transplanting tumors into rats or culturing tumor cells *in vitro*, are inefficient, labor-intensive and time-consuming. Furthermore, these approaches are not feasible for primary tumor cells that cannot survive tissue culture conditions or that may change phenotype in rats. This study reports that fluorescence-activated cell sorting (FACS) is a simple and efficient approach for purifying living primary human breast tumor cells from LDV⁺ mouse stromal cells, which can be completed in a few hours. When purified from Matrigel contaminated LDV⁺ tumors, sorted human breast tumor cells, as well as tumors grown from sorted cells, were shown to be LDV-free, as tested by PCR. Our data demonstrate that cell sorting is effective, much faster and less likely to alter tumor cell phenotype than traditional methods for removing LDV from xenograft models. This approach may also be used to remove other rodent-specific viruses from models derived from distinct tissues or species with sortable markers, where virus does not replicate in the cells to be purified.

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AUTHOR CONTRIBUTIONS

H.L. designed experiments, analyzed data and wrote the manuscript. H.L., J.B., R.D., Y-F.C., and D.Q. performed experiments in maintaining and labeling human-in-mouse tumor models, tumor dissociation and staining, sorting tumor cells and organizing samples. L.A.Z. provided technical support and assisted the animal protocol. M.F.C. and G.L.G designed and revised the manuscript.

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Keywords

LDV removal; cell sorting; human-in-mouse tumor models; breast cancer

1. Introduction

Current cancer research approaches take advantage of mouse models, including mouse tumors and human tumor xenografts in mice, to elucidate regulatory mechanisms of tumors through interactions between tumor cells and their microenvironment *in vivo*. In cancer stem cell (CSC) transplantation studies, mouse biological materials, such as the basement membrane matrix (Matrigel), are used to facilitate tumor initiation and progression in mice (Al-Hajj et al., 2003; Liu et al., 2010; Quintana et al., 2008). However, the species *lactate dehydrogenase-elevating virus* in the order Nidovirales, family Arteriviridae, genus Arterivirus (LDV, LDEV, or LDH virus), a natural mouse virus that infects macrophages, is one of the common organisms that contaminate mouse tumor models and cell byproducts like Matrigel (Peterson, 2008). LDV can significantly affect the immune system and tumor behavior (Ammann et al., 2009; Baker, 2003; Riley et al., 1978). It interferes with the mouse immune system by trapping macrophages and activating lymphocytes, which cause changes in immune response (Ammann et al., 2009; Riley et al., 1978). In addition, LDV alters tumor behavior by increasing or decreasing tumor incidence and growth rate, regulating oncogenic virus expression, and modifying responses to cancer therapies (Riley et al., 1978). In general, LDV is persistent and subclinical except for transient elevation of plasma lactate dehydrogenase levels, although certain laboratory mutants of LDV can induce secondary polioencephalomyelitis and flaccid paralysis in conditioned immunosuppressed mice (Anderson et al., 1995; Li et al., 1999). As macrophages and other immune cells play an important role in cancer development or progression, LDV contamination is a potential problem for mouse tumor studies or human cancer and cancer stem cell models in immune-compromised mice.

Detection of LDV in tumor models or mouse tissue can be achieved by polymerase chain reaction (PCR) with specific primers that recognize the single-strand virus RNA (Chen and Plagemann, 1997; Wagner et al., 2004). In some of laboratory-established human-in-mouse breast tumor models (Liu et al., 2010), LDV contamination was detected by PCR. However, there is currently no therapeutic approach to clear LDV from the host mice, where it produces lifelong persistent viremia (1991; Baker, 2003; Riley et al., 1978). Since primary tumor models do not survive in tissue culture or may change phenotype in rats, it is not realistic or appropriate to eliminate LDV from these tumors through prolonged selective culturing of tumor cells or by tumor transplantation into rats (1991; Baker, 2003; Riley et al., 1978). Based on the fact that LDV only infects a subpopulation of mouse macrophages (Kowalchuk and Plagemann, 1985; Plagemann et al., 1995; Ritzi et al., 1982), fluorescence-activated cell sorter (FACS)-based cell sorting was used as a fast and efficient approach to remove mouse stromal cells and LDV from tumor cells within a few hours, with minimal risk of tumor feature alterations.

2. Materials and Methods

2.1. Materials and protocols

Antibodies (H2K^d-biotin, streptavidin-PE-Cy5, streptavidin-PE-Cy7, anti-hCD44-APC and lineage antibodies) and Matrigel were purchased from BD Biosciences (San Jose, CA, USA), the nucleic acid stain DAPI (the dilactate form), ACK lysis buffer and fetal bovine serum from Invitrogen (Carlsbad, CA, USA), Liberase Blendzymes from Roche (Palo Alto, CA, USA), Collagenase type III from Worthington Biomedical (Lakewood, NJ, USA), IgG

blocking antibody, DNase I from Sigma (St. Louis, MO, USA). Research involving human tumor and mouse models was approved by the Institutional Review Board (IRB), Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) of the University of Chicago, in accordance with an assurance filed with and approved by the Office of Laboratory Animal Welfare, the Department of Health and Human Services.

2.2. Human breast tumor models

Bypassing cell culture *in vitro*, the human-in-mouse breast tumor models were generated from patient tumors mixed with Matrigel (BD, 2007) and maintained by continuous passaging in non-obese, diabetic/severe combined immunodeficiency (NOD/SCID) mice *via* orthotopic implantation of tumor chunks or sorted CD44⁺ breast cancer stem cells (BCSCs) with Matrigel (BD, 2009) as described (Al-Hajj et al., 2003; Liu et al., 2010). Tumor chunks (fine minced pieces) or living tumor cells were stored in RPMI/10% DMSO/50% fetal bovine serum (FBS) at -135°C or liquid nitrogen tank. To generate tdTomato or eGFP-labeled tumor models, BCSCs were transduced with optical reporters in a lentiviral vector pFU-tdTomato or pFU-eGFP (Liu et al., 2010). Mice bearing LDV⁺ tumors or tumor cells isolated from these models were housed in a quarantined animal facility.

2.3. Tumor cell isolation and staining

Tumors were dissociated with Liberase Blendzyme 2 and 4 or Collagenase III as described (Al-Hajj et al., 2003; Liu et al., 2010). After red blood cell lysis with ACK buffer, single cell suspensions were filtered and re-suspended in HBSS/2% FBS for further staining. Following mouse IgG blocking (100ug/ml) bulk tumor cells were stained with anti-H2K^d-biotin antibody and then the secondary Streptavidin-PE-Cy5 or Streptavidin-PE-Cy7 for labeling mouse stromal cells. In order to enrich human CD44⁺ BCSCs, cells were stained with anti-CD44-APC antibody. DAPI was added into cell suspension at 1 nM (viability marker) prior to flow analysis and sorting. In addition, single color controls were prepared for color compensation settings on flow cytometer.

2.4. Tumor cell purification by double sorting on flow cytometer

A FACSAria I or II (BD) BD flow cytometer (BD, San Jose, CA, USA) was washed with 10% Contrad and then sterile H₂O, prior to and after each experiment. Single color controls (blank, DAPI, PE-Cy5/7, and APC, eGFP, tdTomato) were then analyzed for compensation settings. For sample sorting, all gates for human tumor cells were set up based on single DAPI-negative (viable) and H2K^d-negative tumor cells (P1 to P2, to P3, and then to P4), excluding debris, duplets or multiplets, dead cells (DAPI⁺) or mouse stromal cells (H2K^{d+}) (P4). Additional markers for human tumor cells included the hCD44 surface marker, or eGFP or tdTomato as optical reporters. Sorted cells were collected in HBSS/10%FCS, concentrated by centrifugation and re-suspended in a small volume of HBSS/2%FCS prior to a second sorting. Double-sorted cells were used for LDV PCR tests and mouse injection. The double sorted cells were used for LDV PCR tests and mouse injection.

2.5. PCR test of LDV and murine viruses by RADIL

Tumor samples or sorted cells were prepared in 10 μl PBS solution and shipped on dry ice (overnight delivery) to the University of Missouri-Columbia Research Animal Diagnostic Laboratory (<http://radil.missouri.edu/>) (RADIL, Columbia, MO, USA) for PCR tests (the full list of IMPACT I mouse viruses or individual LDV tests). The initial screen for the full list of mouse viruses was carried out with pooled tumor samples and BD Matrigel (purchased after 2009). To identify or remove LDV, individual PCR tests were performed to examine the LDV status of Matrigel, unsorted bulk tumor cells from distinct tumor models,

double sorted tumor cells for each tumor model, and tumors grown from these sorted or unsorted cells upon implantation in mice.

Statistical Analysis—Two-tailed student T tests were used to evaluate the significant difference or p values between samples. Standard deviations (SD) of mean values were calculated.

3. Results

3.1. Detection of LDV from human breast tumor models

Following IACUC requirements, and before passaging human-in-mouse tumors derived from clinical breast tumor specimens and old batches of Matrigel purchased in 2007, tumor samples and new Matrigel (purchased in 2009) were analyzed by PCR for the presence of 18 murine viruses and Mycoplasma (RADIL IMPACT I PCR panel). LDV was found in pooled samples containing tumors and Matrigel (2009) (Table 1). The new batch of Matrigel (2009) was excluded as the source of virus, and LDV contamination was confirmed in three breast tumors (unlabeled, eGFP-labeled and td-Tomato-labeled, Table 1). Because these human-in-mouse tumor models are only maintained in mice *in vivo*, LDV appeared to transfer to the next series of tumor generations following orthotopic implantation, even after being frozen for a couple of years in liquid nitrogen (Table 2), suggesting that it persists in transplantable tumors unless it is removed. LDV specifically infects and propagates in mouse macrophages and may severely affect the immune system and tumor phenotype. Therefore, it was necessary to remove LDV from these tumors. Because tissue culture or rat transplantation were not appropriate for LDV removal from these human-in-mouse models, cell sorting was tested as an approach to efficiently remove LDV⁺ infected mouse cells from human tumor cells

3.2. Purification of LDV-free human tumor cells by double sorting

First introduced in 1970s, Fluorescence-activated cell sorting (FACS) is an effective method for purifying living cells (Herzenberg et al., 1976; Shapiro, 2004). Therefore, FACS was used to purify human breast tumor cells. Dissociated tumor cells were stained with Biotin-conjugated antibody against mouse stromal cell marker H2K^d and the secondary Streptavidin-PE-Cy5 (or -PE-Cy7). The nucleic acid stain DAPI was added to cell suspensions to stain dead or dying cells with compromised cell membrane permeability. Single living tumor cells were sorted based on light scattering characteristics and negative selection of H2K^d and DAPI signals (P4 Figure 1). To further distinguish human tumor cells from mouse cells, an additional marker was used for positive selection of tumor cells from LDV⁺ tumor models: human BCSC marker CD44 for the unlabeled tumor (P5 in Figure 2A), eGFP from the eGFP-labeled tumor (P6 in Figure 2B), or tdTomato from the tdTomato-labeled tumor (P7 on Figure 2C), respectively. To maximize the efficiency of removing LDV⁺ mouse cells, cells were double sorted on gated populations (P5, P6 and P7). The average purity for sorted populations post sorting was very high (99.3±0.3%, n=3, Figure 2), reaching 100% in the P4 population (n=3, Figure 1). Double sorted tumor cells (166 to 250,000 cells) were implanted into NOD/SCID mice.

Sorted tumor cells from H2K^d-DAPI⁻eGFP⁺ (P6) or H2K^d-DAPI⁻ tdTomato⁺ (P7) populations were found to be free of LDV (Table 2) by PCR analysis. In addition, tumors grown from P5, P6 or P7 cell populations that were passaged in mice were also LDV-negative (samples of B1, B2, C3, and D3 in Table 2). These data demonstrate that FACS-based sorting is an efficient and effective approach to remove LDV from tumors.

4. Discussion

LDV causes persistent viremia in host mice with significant effects on the immune system and tumor behavior (Ammann et al., 2009; Baker, 2003; Riley et al., 1978), while sometimes inducing secondary infections and flaccid paralysis in immune-suppressed mice (Anderson et al., 1995; Li et al., 1999).

By recognizing a trypsin-sensitive Ia-related receptor (Kowalchuk and Plagemann, 1985), LDV can specifically infect and propagate in a small population of murine macrophages responsible for clearing extracellular lactate dehydrogenase (LDH) (Plagemann et al., 1995; Riley et al., 1960; Ritzi et al., 1982; Smit et al., 1990). Removal of LDV from tumors by traditional approaches, namely through tissue culture *in vitro* or by rat transplantation, is based on the assumption that mouse macrophages cannot survive under these conditions and thus LDV disappears over time. However, complete removal will not occur if mouse macrophages or LDV continue to propagate permissively (Kowalchuk and Plagemann, 1985). In this study, LDV was removed quickly and efficiently from human tumor xenograft cells by sorting prior to transplantation. Because cell purity is essential, double sorting was necessary, as single sorting did not achieve 100% LDV elimination. Compared to the lengthy traditional approaches of culturing cells *in vitro*, or using tumor transplantation in rats that may alter tumor phenotype, cell sorting can be completed in a few hours and poses minimal risk to the tumor phenotype. Sorting is especially valuable for primary tumor-derived models that do not survive in tissue culture or may lose tumorigenicity in rats.

The spread of LDV in a mouse barrier facility has several likely sources, such as direct mouse-mouse contact with infected laboratory mice. However, this source is probably rare in the laboratory setting and usually does not spread across cages. A more likely source is the injection of contaminated tumors, cells, serum, or mouse biological material used to facilitate tumor initiation and progression in mice, such as Matrigel. Matrigel is made from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Kleinman and Martin, 2005) and the BD product made before the summer of 2008 was not guaranteed to be LDV-free by PCR (BD, 2010). While the basement membrane matrix Matrigel effectively facilitates CSC-mediated tumor growth in mice (Quintana et al., 2008), it can also introduce murine viruses to transplantable tumors where macrophages play a critical role (Patsialou et al., 2009). Because these viruses can alter tumor behavior by increasing or decreasing tumor incidence and growth rate, regulating oncogenic virus expression, and modifying cancer therapy, all animal researchers should perform mouse virus screens prior to using mouse biological materials in mouse models.

FACS based tumor cell purification is effective for removing LDV from human tumor models, and it may be applied to other mouse-specific (or host cell-specific) viruses in human tumor models or any models/tissues, where viruses do not propagate in the cells to be sorted.

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Abbreviations

LDV	lactate dehydrogenase-elevating virus
FACS	fluorescence-activated cell sorter, CSC, cancer stem cells
PCR	polymerase chain reaction, BCSC, breast cancer stem cells
NOD/SCID	non-obese diabetic/severe combined immunodeficiency
IACUC	Institutional Animal Care and Use Committee

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Figure 1. Flow cytometry analysis of human tumor cells upon and post sorting

A: Dot plot of bulk tumor cells profiled by the cell size/volume channels, forward scatter area (FSC-A, X axis) and the side scatter area (SSC-A, Y axis). The gate of P1 population excluded cell debris (<50). **B:** Top panel is a dot plot of P1 cells by side scatter height (SSC-H, X axis) and width (SSC-W, Y axis) to gate potential single cells (P2 population), while bottom panel profiles P2 cells by forward scatter height (FSC-H, X axis) and width (FSC-W, Y axis) to further gate single cells (P3 population). **C:** Dot plot of P3 cells in the fluorescent channels of DAPI (a viability marker) and H2K^d-PE-cy5 (a mouse stromal cell marker). The P4 population was gated for viable (DAPI⁻) human tumor cells (H2K-PEcy5⁻) for sorting. **D:** The purity examination of P4 population by flow cytometer after double sorting.

Figure 2. Flow cytometry analysis of sorted human breast tumor cells

Cells were sorted with an extra marker based on single DAPI⁻H2K^d⁻ cells as P4 in Fig 1. **A:** Representative flow profile of sorted CD44⁺H2K^d⁻ breast tumor cells (P5), by CD44-APC and H2K^d-PE-Cy5 channels (99.4% pure). **B:** Representative flow profile of sorted tdTomato-labeled tumor cells (98.8% pure). **C:** Representative flow profile of sorted tdTomato-labeled tumor cells (98.9% pure).

Table 1
Detection of LDV in human-in-mouse breast tumors by PCR

The first sample, containing pooled tumor cells (in 10 µl PBS) mixed with 10 µl of Matrigel (purchased in 2009 from BD), was submitted for PCR tests for the full list of mouse viruses included in RADIL IMPACT I. Once LDV was detected, the second batch of submitted samples included tumor cells (mixture of three models in 10 µl of PBS) and Matrigel (10 µl). The third batch of samples contained individual tumor cell models (unlabeled, eGFP-labeled and tdTomato-labeled) in 10 µl PBS.

Samples	LDV PCR evaluation
Pooled tumor suspension and Matrigel	+
Matrigel	–
Mix of three tumor models	+
1) unlabeled tumor	+
2) eGFP-labeled tumor	+
3) tdTomato-labeled tumor	+

Other viruses detected free by RADIL include Ectromedia, Mouse rotavirus (EDIM), Hantaan, K virus, Lymphocytic choriomeningitis virus (LCMV), Mouse adenovirus MAD1/MAD2, Mouse cytomegalovirus (mCMV), Mouse hepatitis virus (MHV), Murine norovirus (MNV), Mouse parvovirus (MPV1/2/3), Mouse thymic virus (MTV), Minute virus of mice (MVM), *Mycoplasma* sp., Polyoma, Pneumonia virus of mice (PVM), Reovirus 3 (REO3), Sendai, and Theiler's murine encephalomyelitis virus (TMEV GDVII). (Symbols: "–" Negative, "+" Positive)

Table 2
PCR evaluation of LDV without or post cell sorting

LDV PCR results of unsorted tumors, sorted populations or grown tumors from sorted populations (P5, P6 and P7). Tumor samples were submitted to RADIL for LDV PCR evaluation, including tumors grown from LDV⁺ tumor cells after 3–5 passages or from frozen tumor cells in liquid nitrogen tank (n=8, A1–A8), double sorted P6 or P7 populations of tumor cells (C1, C2, D1 and D2), and tumors propagated in mice following *in vivo* implantation of double sorted P5, P6 or P7 cells (B1, C3 and D3 respectively), or serially passaged tumors (B2).

Sample	Description	LDV evaluation
A1–A8	Tumors grown from unsorted LDV ⁺ tumor cells after 3–5 generations or frozen tumor cells (n=8)	+
B	H2Kd⁺CD44⁺ (P5)	
B1	Tumor grown from 166 sorted cells	–
B2	Tumor after 3 serial passages from B1	–
C	H2Kd⁺tdTomato⁺ (P6)	
C1	Double-sorted cells (1K)	–
C2	Double-sorted cells (250K)	–
C3	Tumor grown from 250K sorted cells	–
D	H2Kd⁺eGFP⁺ (P7)	
D1	Double-sorted cells (1K)	–
D2	Double-sorted cells (250K)	–
D3	Tumor grown from 250K sorted cells	–

(Symbols: “–” Negative, “+” Positive, “K” 1000)