Specific Elimination of CD133+ Tumor Cells with Targeted Oncolytic Measles Virus

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Abstract

Tumor-initiating cells (TICs) are critical yet evasive targets for the development of more effective antitumoral strategies. The cell surface marker CD133 is frequently used to identify TICs of various tumor entities, including hepatocellular cancer and glioblastoma. Here, we describe oncolytic measles viruses (MV) retargeted to CD133. The viruses, termed MV-141.7 and MV-AC133, infected and selectively lysed CD133+ tumor cells. Both viruses exerted strong antitumoral effects on human hepatocellular carcinoma growing subcutaneously or multifocally in the peritoneal cavity of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Notably, the CD133-targeted viruses were more effective in prolonging survival than the parental MV-NSe, which is currently assessed as oncolytic agent in clinical trials. Interestingly, target receptor overexpression or increased spreading kinetics through tumor cells were excluded as being causative for the enhanced oncolytic activity of CD133-targeted viruses. MV-141.7 was also effective in mouse models of orthotopic glioma tumor spheres and primary colon cancer. Our results indicate that CD133-targeted measles viruses selectively eliminate CD133+ cells from tumor tissue, offering a key tool for research in tumor biology and cancer therapy.

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Introduction

Tumor-initiating cells (TICs) form an attractive target for novel antitumoral strategies as they differentiate into cancer cells forming the tumor mass and were shown to be resistant toward conventional therapies, such as radio- and chemotherapy (for review see ref. 1). A putative marker for TICs is CD133, also known as prominin-1. In the healthy organism, CD133 is mainly expressed on stem cells of the hematopoietic and the endothelial system with a yet unknown physiologic function (2). Two epitopes, CD133/1 and CD133/2, both located in the extracellular domains of CD133, are often used for the detection of CD133+ cells using specific monoclonal antibodies AC133 and AC141, respectively. The expression of CD133 underlies a complex regulation at different levels (3).

In glioma, small subpopulations of CD133+ cells extracted from tumors were shown to be considerably more tumorigenic than the tumor bulk population. As few as a hundred CD133+ tumor cells were sufficient to induce malignant tumors in immunodeficient mice, whereas 1 × 105 cells of the primary tumor material are required for tumor formation (4). Moreover, poor prognosis correlates with CD133 expression in glioma biopsies (5). Yet, the relevance of CD133 as a sole marker for TICs in glioma is under debate, as CD133+ cells have been reported to exhibit TIC properties as well (6). It has been suggested that these cells constitute a lineage hierarchy with primordial CD133+ tumor stem cells (TSC), which can give rise to CD133+ progenitors (7). Other publications have shown that CD133 expression depends on oxygen, as a portion of glioma cells shows large phenotypic plasticity and can acquire stem-like features and AC133-positivity in response to a hypoxic microenvironment (8). In line with this, a subset of endothelial cells lining the tumor vessels was recently shown to be derived from CD133+ tumor cells extracted from tumor tissue, offering a target for research in tumor biology and cancer therapy.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Selective killing of defined cell types of interest can be achieved by virotherapy, which is based on oncolytic viruses engineered to preferentially infect and destroy tumor cells. Because of their promising safety profile (vector generation on the basis of viral vaccine strains) and their potential to overcome resistance against irradiation or chemotherapy, oncolytic viruses are currently investigated in numerous clinical trials (15, 16). Measles virus (MV) vaccine strains were shown to be oncolytically active against various types of human cancer, including HCC, glioma, breast cancer, ovarian cancer, lymphoma, and mesothelioma (17).

Measles virus entry into cells is dependent on attachment of the hemagglutinin envelope glycoprotein (H) to its cellular receptors, CD46, CD150/SLAM, and nectin-4, followed by fusion with the cell membrane via the envelope fusion (F) glycoprotein. CD46 is expressed ubiquitously on nucleated cells including hematopoietic stem cells (HSC), whereas signalling lymphocyte activation molecule expression is confined to immune cells (18). Nectin-4, which is selectively expressed on lung epithelial cells, was very recently identified as a host exit receptor mediating horizontal transfer of measles virus (19, 20). We and others have shown that the envelope protein complex of measles virus can be engineered to restrict infection to cell types defined by a cell surface marker of choice (21–23). For this purpose, residues interacting with the measles virus receptors are mutated and a single-chain antibody (scFv) specific for the target receptor of interest is genetically fused to H. This way measles virus glycoproteins specific for a variety of cell surface receptors, such as CD20, Her2/neu, CD105, glutamate receptor, and also CD133 have been generated and were used to target lentiviral vectors (21, 22, 24) and oncolytic measles viruses (25).

Here, we present oncolytic measles viruses retargeted to the CD133 molecule. We show that these viruses are absolutely specific for CD133+ cells and highly efficient in target cell killing. We assess their oncolytic activity in 3 different cancer types, that is, glioma, colon cancer, and hepatocellular cancer by applying mouse tumor models. Our data suggest that CD133-targeted measles virus can serve as a novel tool not only to improve our understanding on the role of CD133+ cells in oncology, but also providing an innovative strategy for tumor therapy supplementing current treatment modalities.

Materials and Methods

Generation of CD133 measles viruses
Cloning of the 141.7 scFv cDNA has been described (21). The AC133 scFv was PCR-assembled from the hybridoma line AC133 (Miltenyi-Biotec) as described for other scFv (21, 22). Both scFv reading frames were cloned into pCG-HmutXcdCD20 (26) via SfiI/NotI resulting in pCG-HmutCD133streptacyscFv-(AC133) and pCG-HmutCD133scFv-(141.7), respectively. The PackI/SpeI-digested fragment of pCG-HmutCD133scFv-(141.7) was inserted into the corresponding sites of pMV-eGFP(SpeI; refs. 27, 28), whereas the PackI/SpeI fragment of pCG-HmutCD133scFv-(AC133) was transferred into p(+)-PoLiH-MVNS-eGFP(N; ref. 29).

MV-141.7 was rescued using the hexa-histidine (His6) retargeting system (23) and MV-AC133 as described by Martin and colleagues with modifications (29). In short, the plasmid encoding the MV-AC133 genome was cotransfected with expression plasmids pCA-MV-N, pCA-MV-P, and pCA-MV-L into 293T cells using Lipofectamine 2000. Two days posttransfection, 293T cells were overlayed onto Vero-αHis cells to amplify rescued virus.

Virus stocks were produced upon infection of Vero-αHis cells at a multiplicity of infection (MOI) of 0.03. Two to 3 days after infection, cells were harvested and lysed by 3 freeze-thaw cycles. Virus stocks were titered on Vero-αHis cells and the tissue culture infective dose (TCID50) was determined.

Cells
Vero and HT1080 cell lines were purchased from the American Type Culture Collection and HuH7 cells from JCRB (Japan). HT1080-CD133 and Vero-αHis cells have been described (21, 23). Luciferase (pGL4.50, Promega) or red fluorescent protein (RFP) gene (TurboFP635-C, Evrogen) expressing cell lines were generated by lentiviral transduction using pSEW derived transfer vectors (21, 22) followed by bulk cell sorting (MoFlow cell sorter, Beckmann Coulter) for homogenous gene expression. All cell lines were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal calf serum (FCS), 2 mmol/L glutamine and passaged for less than 4 months before another vial from the master cell bank was taken into culture. All cell lines were regularly tested for mycoplasma and found to be negative.

Primary glioblastoma cells NCh646 were obtained from patients undergoing surgical resection as approved by the Institutional Review Board of the Medical Faculty Heidelberg (Heidelberg, Germany). Tumor tissue was enzymatically dissociated and short-term cultures were established as floating tumor spheres in DMEM/F-12 medium containing 20% bovine insulin transferrin serum-free supplement, basic fibroblast growth factor (bFGF), and EGF at 20 ng/mL each (all Provitro). The TSC-03 tumor sphere culture was established from a primary colorectal cancer lung metastasis, which was obtained at Heidelberg University Hospital (Heidelberg, Germany) in accordance with the declaration of Helsinki and under an Institutional Review Board–approved protocol following written informed consent. Sphere cultures were established and grown as previously described (30).

Human HSCs were obtained from apheresis products after mobilization with granulocyte colony-stimulating factor (G-CSF). CD133+ HSCs were purified and cultivated as described (21). The scientific use of anonymous samples no longer required for stem cell transplantation from deceased individuals or from a remaining sample has been approved by the University Hospital Ethics Committee (Frankfurt, Germany; 163/03).

Immunoblotting
Virus particles harvested from cell supernatant were denatured for 5 minutes at 95 °C in 2× urea sample buffer (5% SDS, 8 mol/L urea, 200 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.05% bromphenol blue, 2.5% di-thiothreitol, pH 8.0), separated on a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (GE Healthcare). After blocking of membranes with...
5% skim milk powder in TBS supplemented with 0.05% Triton X-100 for 30 minutes at room temperature, they were incubated with rabbit antimesiales measles nucleoprotein N antibody (Abcam) diluted 1:5,000 or anti-MV-Hcyt antiserum 1:5,000 (Eurogentec), which were both detected by a peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO) diluted 1:2,000 as secondary antibody.

**Virus spreading assay**

HuH7 cells (5 × 10^5) were cultivated in triplicates in 24-well plates and infected at an MOI of 0.005. Twenty-four, 40, and 60 hours after infection, cells were fixed with 0.25% glutaraldehyde in PBS. Twenty-five adjacent tiles (total area: 8.35 mm²; resolution: 1.34 pcm/μm) were acquired at ×10 magnification using a motorized Axio-Observer-Z1 microscope. Infected area was determined by computational analysis using the CellProfiler software 2.0 (31). For quantification of GFP-expression, acquired images were converted to binary files, using threshold levels as determined on uninfected control cells. The GFP⁺ area was normalized to the total area for quantification.

**Flow cytometry**

Cells were trypsinized and staining was conducted using anti-human CD133/2-PE antibody (clone AC133, MiltenyiBiotec), anti-human CD133/2-PE antibody (clone 293C3, MiltenyiBiotec), anti-CD46-PE antibody (AbD Serotec), or anti-CD46-FITC (Biolegend), anti-human CD133/2-PE (clone 293C3, MiltenyiBiotec), and the BD FACS DIVA Software (Becton Dickinson) according to the manufacturer’s instructions. Appropriate isotype controls were used according to the manufacturer’s instructions. Cells were subjected to flow-cytometry analysis using a FACSRSII cytometer (Becton Dickinson) and the BD FACS DIVA Software (Becton Dickinson). Only cells with staining intensities above the maximal level of isotype controls were defined as CD133⁺ cells. Receptor densities of CD133 and CD46 on HuH7 cells were determined using QantiBRITE-PE beads (Becton Dickinson) according to the manufacturer's instructions.

**Cell viability assay**

The MTT (Cell Proliferation Kit I, Roche) assay was used to measure cell viability in quadruplicate as recommended by the manufacturer. Viability was calculated as ratio of mean values measured for infected and uninfected cells.

**Animal experiments**

All animal experiments were carried out according to the German animal protection law. Animals in tumor experiments were regularly checked for health condition and sacrificed if they had lost 20% of body weight or showed severe signs of illness.

For the subcutaneous tumor model, HuH7 cells (5 × 10⁶/100μL) were injected into the right flank of 6-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Harlan). Tumor diameters were measured and the volume calculated (0.5 × length × width).

For the multifocal tumor model, 6- to 8-week-old athymic nude mice (HSD; Athymic Nude-FoxN1NL, Harlan) were intra-peritoneally injected with 5 × 10⁶ HuH7-luc cells. Tumor formation was analyzed by in vivo imaging using the IVIS Spectrum optical imaging system equipped with the Living Image 4.1 analysis software (Caliper Life Sciences). For imaging, mice were intraperitoneally injected with 250 μl luciferin (15 mg/ml) in PBS and anesthetized with 2.5% isoflurane. Images were acquired ventral, within 10- to 20 minutes after luciferin injection. For quantification of luciferase activity, total light emission per mouse was determined after background subtraction.

A total of 1 × 10³ TSC-03 sphere culture cells were mixed in a 1:1 ratio with Matrigel (BD Biosciences) 24 hours postinfection and transplanted under the kidney capsule of NOD.Cg-Pkd₁flox flox Prkd₁fl/– (B6.129S4–Il2rgtm1Wjl/J, NOD.Cg-Pkd₁flox flox Il2rgtm1Wjl/J (IL2rg<sup>+</sup>/–)) mice (The Jackson Laboratory). Mice were sacrificed and tumors were compared 58 days after transplantation. For retransplantation experiments 1 × 10⁵ cells were transplanted under the kidney capsule of secondary mice.

A total of 1 × 10³ NCH644 tumor sphere cells were infected at an MOI of 0.5, dissociated 16 hours later and stereotactically implanted into the right hemispheres of NOD/SCID mice as described (32).

**Statistical analysis**

Evaluation of curves describing tumor volumes was conducted comparing treatment groups versus control groups by means of the area under the curve (AUC) for each single mouse. Survival data were depicted with Kaplan–Meier curves. Comparisons between different virus types and the control group were conducted by a log-rank test, adjusted for multiple comparisons according to Bonferroni or Dunnet method. P < 0.05 was regarded significant.

**Results**

**Cell specificity of CD133⁻retargeted oncolytic measles viruses**

To generate CD133-specific measles viruses, scFv-reading frames cloned from the hybridoma cell lines AC141.7 or AC133 were fused to that of the measles virus H protein mutant deficient in receptor recognition. Virus genomes encoding an additional GFP-reading frame for easy detection of virus infection were then assembled and the corresponding viruses MV-AC133 and MV-141.7 rescued (23). Western blot analysis of virus stocks confirmed that the anti-CD133 scFv displaying H proteins were properly expressed and incorporated into virus particles (Fig. 1A). Next, we determined the spreading kinetics of the viruses in CD133⁺ HuH7 hepatoma cells upon infection at a low MOI. There was no significant difference between the spreading kinetics of targeted MV-CD133 viruses and the parental MV-NSe, showing that the retargeted entry via CD133 had neither impaired nor enhanced virus spreading and measles virus-induced syncytia formation (Fig. 1B).

Selectivity of the MV-CD133 viruses for CD133⁻ cells was determined on HuH7 (CD133⁻), Vero (CD133⁻), and HT1080 (CD133⁻) cells. While MV-NSe infected all cell types, MV-141.7 and MV-AC133 infected HuH7 cells only (Fig. 1C; Supplementary Fig. S1A). To easily discriminate between the infection of CD133⁻ and CD133⁺ cells and to exclude any influence by cell type specific differences, we generated HT1080-CD133 cells stably expressing CD133 and HT1080-RFP cells, which are...
CD133⁺ and express a RFP (Supplementary Fig. S2). Infection with GFP-encoding measles virus was thus expected to result in green fluorescence when CD133⁺ cells, and yellow fluorescence (resulting from the overlay of red and green) when CD133⁻ cells became infected. Both cell lines were mixed in equal amounts and infected with MV-141.7, MV-AC133, or MV-NSe, respectively. While MV-NSe infection resulted in green and yellow fluorescence, only green fluorescence was detected upon infection with MV-141.7 or MV-AC133 (Fig. 1D). Moreover, MV-141.7- and MV-AC133-infected cells formed large green colored syncytia, completely sparing out all red-labeled CD133⁻ cells (Fig. 1D). Thus, MV-141.7 and MV-AC133 are also highly selective for CD133⁺ cells even when these are in close contact to CD133⁻ cells.

Enhanced oncolytic activity of CD133-specific measles viruses
We next assessed the oncolytic activity of MV-141.7 by infection of HuH7 hepatoma cells at different MOIs. Compared with mock-treated HuH7 cells, infected cells were substantially reduced in their viability. Interestingly, MV-141.7-infected cells were significantly less viable than MV-NSe infected cells (Fig. 2A). To determine the in vitro oncolytic activity, HuH7 cells were subcutaneously implanted into NOD/SCID mice. We then followed tumor growth upon intratumoral injection of viruses MV-NSe, MV-AC133, and MV-141.7, respectively. Compared with mock-treated animals, tumor growth was significantly reduced in all virus-treated animals. However, there were substantial differences between the respective groups. Remarkably, both groups treated with CD133-targeted measles viruses showed a slower tumor growth and a considerably prolonged survival than the MV-NSe–treated group (Fig. 2B and C). Among the CD133-targeted viruses, the oncolytic activity and overall survival were most pronounced in MV-141.7–treated animals (P = 0.0002). Further studies therefore focused on MV-141.7.

Oncolytic activity of MV-141.7 in primary tumor spheres
To test the oncolytic activity of MV-141.7 on primary tumor cells, we cultivated tumor cells derived from colon cancer and glioblastoma, 2 cancer types that have been described to contain CD133⁺ TICs (3, 7, 33, 34). Both formed spheres under serum-free conditions (Figs. 3 and 4) and contained about 90% of CD133⁺ cells (Supplementary Figs. S3 and S5). Both, MV-NSe and MV-141.7, efficiently infected the primary colon tumor sphere culture and induced syncytia formation.

Figure 1. Basic characterization of CD133-targeted viruses. A, Western blot analysis of virus particles (5 × 10⁷ TCID₅₀). Signals derived from H proteins and N protein are indicated. B, spreading kinetics of MV-NSe, MV-141.7, and MV-AC133 through HuH7 hepatoma cells. There were no statistically significant differences between the curves (P = 1.00; N = 3). C, specificity of infection. Vero, HT1080 and HuH7 cells were infected with the indicated viruses at an MOI of 1, respectively. At 72 hours, after infection cells were analyzed by fluorescence microscopy. Scale bar, 200 μm. D, cell specificity in mixed cultures. CD133⁺ HT1080 cells (HT1080-CD133) and CD133⁻ HT1080-red cells were cocultivated and infected with MV-141.7, MV-AC133, and MV-NSe at an MOI of 0.5, respectively. In the uninfected control culture (top left) HT1080-red cells appear red (labeled with arrow head CD133⁻), whereas HT1080-CD133 are invisible (labeled with arrow head CD133⁺). While MV-141.7 (bottom left) and MV-AC133 (bottom right) infection resulted in green cells only, MV-NSe infected both cell types, resulting in green and yellow color (top right).
Targeting CD133+ Tumor Cells

Moreover, reisolated tumors from both groups contained about 70% CD133+ cells (Supplementary Fig. S4).

Of note, also the glioma tumor spheres became readily infected upon incubation with MV-141.7 and MV-NSe, respectively. Especially tumor spheres infected with MV-141.7 showed strong syncytia formation (Fig. 4), which resulted in loss of CD133+ cells (Supplementary Fig. S5). To follow tumor cell killing in vivo, we injected infected tumor spheres into the brains of NOD/SCID mice. Mice that had received control-treated tumor cells had to be sacrificed before day 35 due to cachexia, gasping, and anergy, whereas animals that had obtained MV-141.7 or MV-NSe–treated spheres survived up to 70 to 90 days after implantation (Fig. 4B). Interestingly, the survival period of MV-NSe–injected mice was heterogeneous with half of the mice showing reduced survival times compared with MV-141.7–treated mice. Overall, no significant difference was evident between both groups. However, also in this primary orthotopic tumor model for glioma, MV-141.7 was at least as effective as MV-NSe in prolonging survival times (Fig. 4B).

**MV-141.7 does not infect hematopoietic stem cells**

Besides TSCs, CD133 is a marker for early progenitors of the hematopoietic system (35, 36). It is therefore theoretically possible that MV-141.7 infects and lyases HSCs. Fluorescence-activated cell sorting (FACS) analysis confirmed that HSCs expressed similar high cell surface levels of CD133 and CD46 as HuH7 cells (Fig. 5A). HSCs were incubated with different doses of MV-141.7 or MV-NSe. While no GFP+ cells were detected at low dose of MV-141.7 (MOI = 0.1), single GFP+ cells were detected at MOI = 1 (0.08%) and MOI = 10 (0.17%; mean values of 2 samples). Notably, the percentages of GFP+ cells were slightly higher for MV-NSe (0.14% at MOI = 1 and 0.3% at MOI = 10; mean values of 2 samples; Fig. 5B). Importantly, the fraction of GFP+ cells did not increase over time. Also when cocultivated with infected HuH7 cells, we could not detect GFP+ HSCs (Fig. 5B). The data confirm that the tropism of MV-141.7 is restricted to tumor cells and that its safety profile does not differ from that of measles virus vaccine strains currently in clinical use as oncolytic viruses.

**Oncolytic activity of MV-141.7 in a multifocal tumor model**

To further assess the therapeutic potential of MV-141.7, we established a multifocal cancer model in which labeled HuH7 hepatoma cells form many tumor foci scattered through the peritoneal cavity and allow quantitative monitoring of tumor growth over time. For this purpose, HuH7 hepatoma cells were stably transduced with the firefly luciferase (luc) gene generating HuH7-luc cells. Athymic nude mice injected intraperitoneally with these cells developed multiple tumor foci covering most parts of the peritoneal cavity, which became detectable by *in vivo* imaging 7 days after injection (Supplementary Fig. S6). From then on, luciferase activity slowly but steadily increased. This was reflected by steady tumor growth resulting in an increasing tumor burden that finally caused significant body weight loss between days 80 and 200 after tumor cell implantation so that the animals had to be...
sacrificed. Gross pathology of the animals revealed perfect colocalization of bioluminescent signals and tumor foci (Supplementary Fig. S6).

The oncolytic activity of MV-141.7 was then compared with that of MV-NSe upon intraperitoneal injection of virus into mice harboring multiple tumor foci in the peritoneal cavity. At the time point of virus injection, mice from all groups showed an almost identical luciferase activity. This changed dramatically after virus injection. While the signal steadily increased in the control group, it slowly decreased in the MV-NSe–treated group until day 50 (Fig. 6A and B). From then on, signals in this group rapidly increased and became as high as in the control group by day 95 (Fig. 6B; Supplementary Fig. S6). Accordingly, overall survival of control and MV-NSe–treated mice did not differ significantly (Fig. 6C). In sharp contrast, the luciferase signals in MV-141.7–treated mice declined rapidly immediately after virus injection (Fig. 6B). Later on, only in 1 animal signals increased reaching a similar level as those of the MV-NSe–treated group (Supplementary Fig. S7). All other MV-141.7–treated animals remained healthy over the whole observation period, which correlated nicely with a low or even undetectable luciferase activity (Fig. 6C). In sharp contrast, the luciferase signals in MV-141.7–treated mice declined rapidly immediately after virus injection (Fig. 6B). Later on, only in 1 animal signals increased reaching a similar level as those of the MV-NSe–treated group (Supplementary Fig. S7). All other MV-141.7–treated animals remained healthy over the whole observation period, which correlated nicely with a low or even undetectable luciferase activity (Fig. 6B; Supplementary Fig. S7). These long-term survivors were sacrificed after 247 days of observation. One of the animals showed a few distinct signals of low intensity, whereas no luminescence was emitted from the other 2 animals (Supplementary Fig. S7). Gross pathology was negative for all 3 animals (Supplementary Fig. S8A). Microscopic examination of histologic sections of the luminescent tissue showed presence of a single tumor site located in the intraperitoneal adipose tissue of 1 animal (Supplementary Fig. S8B). These data showed that MV-141.7 treatment had fully cleared 2 animals from tumor cells in the peritoneal cavity.

Discussion

CD133 is currently among the most intensely investigated molecules in molecular medicine being an important surface marker not only for HSCs but also for TICs in a variety of tumor entities. Here, we describe oncolytic viruses retargeted...
to CD133. Our data show that targeting to the CD133 epitopes AC133 or AC141.7 is equally well possible with recombinant measles viruses. While these epitopes have not been mapped exactly, antibody binding to these epitopes is glycosylation-dependent, and cell differentiation results in loss of epitope recognition (37). Both viruses showed very similar spreading kinetics through CD133 \(^+\) tumor cells but did not infect CD133 \(^-\) cells. Their oncolytic activity was shown on 3 different tumor entities including primary tumor material.

A principle concern when targeting CD133 with oncolytic viruses is spreading to extratumoral cells, such as HSC or endothelial progenitors. In this context, nontargeted MV-NSe, being already in clinical testing, does not differ from CD133 \(^-\) targeted measles virus-based viruses. We therefore analyzed infection of cultivated HSC for both, MV-141.7 and MV-NSe, but could not detect any infected cells. At high virus dose a few reporter-positive cells were detected for both virus types but these did not give rise to productive infection. This is in line with the safety data obtained from ongoing clinical trials with oncolytic measles viruses, where for most patients spreading to nontumor tissue has not been reported (38–40). Our data confirm that receptor attachment is not the crucial step for vaccine-derived measles viruses to infect HSC. It is rather likely that the innate immune response of these cells, similarly as that of other nontumor cells, controls infection by measles virus vaccine strains, which carry mutations in the V protein, a viral inhibitor of the \(\beta\)-interferon response (28, 41). Accordingly, a low level of HSC infection with clinical measles virus isolates encoding a fully active V protein counteracting innate immunity has been previously reported (42, 43). CD133-retargeted measles viruses can therefore be expected to be at least as safe as nontargeted oncolytic measles viruses currently investigated in clinical trials, whereas spreading through tumor cells that are often deficient in innate immunity proceeds with wild-type efficiency (37).

An unexpected outcome of this study was the substantially enhanced oncolytic activity of CD133-specific viruses as compared with the unmodified MV-NSe, which is closely related to an oncolytic measles virus currently in clinical trials. This effect was especially pronounced on the HCC-derived HuH7 cell line but also evident on primary colon cancer tumor spheres in which a high number of cells expressed CD133. We determined CD46 as the relevant entry receptor for MV-NSe on HuH-7 cells, as nectin-4, the recently identified alternative measles virus receptor on epithelial cells (19), could not be detected by FACS analysis using a mouse anti-human nectin-4 (PVRL4) antibody (own unpublished data and ref. 20). Quantification of the receptor densities on HuH7 cells revealed a higher density of CD46 compared with that of CD133 (Supplementary Fig. S1B). Reduced expression of the MV-NSe receptor or increased CD133 expression was therefore excluded as potential reasons for the enhanced oncolytic activity of MV-141.7. Moreover, spreading kinetics through tumor cells did not differ between MV-141.7 and MV-NSe and were therefore excluded as causative as well.

Alternative explanations must thus be based on the interaction of the particle-exposed scFv with CD133. We have previously shown that a lentiviral vector pseudotyped with the measles virus glycoproteins displaying a CD20-specific scFv forms multiple contacts with CD20, thereby triggering mitotic activation in resting lymphocytes (44, 45). It is well conceivable that also MV-141.7 establishes multiple scFv-CD133 contacts on the tumor cell surface. Such contacts can also be formed by noninfectious particles that do usually build the vast majority of particles in virus stocks. This could substantially contribute to the oncolytic activity of MV-141.7 provided that this interaction triggers apoptosis in tumor cells.

Figure 5. MV-141.7 does not infect HSCs. A, FACS analysis of CD34 \(^+\) human HSCs and HuH7 cells for CD133 and CD46 expression. Cells were stained with the CD133-specific antibody CD133/2-PE (top) or the CD46-specific phycoerythrin (PE)-labeled antibody (bottom). B, fluorescent microscopic pictures of HSC or mixed HuH7/HSC cultures infected with MV-NSe (top) or MV-141.7 (bottom) at the indicated MOI, respectively. Pictures were taken 72 hours after infection. Scale bar, 100 \(\mu\)m.
However, such properties have not yet been reported for the monoclonal antibodies AC133 or AC141.7. Moreover, HSCs were unimpaired in growth and differentiation into all hematopoietic lineages when transduced with lentiviral vectors pseudotyped with the same 141.7-specific measles virus glycoproteins as used here on oncolytic measles viruses (21). It is therefore rather unlikely that CD133-scFv contact enhanced the oncolytic activity of MV-141.7.

In an alternative scenario, MV-141.7 infects a cell population that is protected from infection by MV-NSe but highly relevant for tumor growth. Friedman and colleagues compared the oncolytic activity of herpes virus on glioblastoma-derived CD133+/CD133− cells (46). They observed a substantially reduced sensitivity of CD133+ glioma cells toward virus treatment as compared with CD133− cells, which correlated with a reduced CD111 (nectin-1) expression, a cell entry receptor for HSV. Transferred to oncolytic measles virus, this suggests downmodulation of CD46 expression in some CD133+ TICs. Future work will have to test both hypotheses by, for example, characterizing HuH7 cells that survived MV-NSe infection for CD133 expression and infectability by MV-141.7.

The enhanced oncolytic activity of MV-141.7 was also evident on primary colon cancer cells in which we observed a significantly reduced growth of MV-141.7 versus MV-NSe on the initially infected tumor cells, although both viruses eliminated all spheres in long-term cultures. We did, however, not detect any effect of MV-141.7 on secondary transplants. While formally it cannot be excluded that in vivo not all CD133+ cells have been eliminated by the virus, it is more likely that similarly as in glioblastoma (7), CD133− TSCs were among the colon cancer cells of this patient, which gave rise to CD133+ cells. This is in line with previous publications showing that colon cancer–derived CD133− cells can possess tumor-initiating properties (30, 47). A final conclusion on this issue will require testing of many more patient samples, which is now possible with the approach described here.

MV-141.7 and MV-AC133 are to our knowledge the first oncolytic viruses rationally designed to target putative TICs or
CSC. Their primary application will be in basic research addressing the relevance of CD133 as marker for TICs. They will allow a systematic investigation on how tumors react to the selective elimination of CD133+ cells. Importantly, this will be possible not only in colon cancer but basically in any cancer entity for which CD133 has been suggested as marker of TICs and/or to be of prognostic value. Moreover, the same approach can be used to determine the relevance of other putative TIC markers, such as CD44, EpCam, or CD13 (48–50) by generating oncolytic measles viruses retargeted to these markers.

Beyond that, MV-141.7 also warrants further investigation as a highly target specific virotherapeutic agent for cancer therapy. The antitumoral effect we observed in the multifocal tumor model of HuH7 with MV-141.7 was remarkable. MV-141.7 did not only prevent further tumor spreading through the peritoneum but even cleared 2 animals from tumor cells. Future work will have to define the optimal therapeutic setting for clinical studies on MV-141.7.

Disclosure of Potential Conflicts of Interest
I.C.D. Johnston is an employee of Miltenyi Biotec GmbH. No potential conflicts of interest were disclosed by the other authors.

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References

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Specific Elimination of CD133+ Tumor Cells with Targeted Oncolytic Measles Virus

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