

# Targeted BiTE Expression by an Oncolytic Vector Augments Therapeutic Efficacy Against Solid Tumors



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## Abstract

**Purpose:** Immunotherapy with bispecific T-cell engagers has achieved striking success against hematologic malignancies, but efficacy against solid tumors has been limited. We hypothesized that oncolytic measles viruses encoding bispecific T-cell engagers (MV-BiTEs) represent a safe and effective treatment against solid tumors through local BiTE expression, direct tumor cell lysis and *in situ* tumor vaccination.

**Experimental Design:** To test this hypothesis, we generated MV-BiTEs from the Edmonston B vaccine strain to target two model antigens. Replicative and oncolytic potential were assessed by infection and cell viability assays, respectively. Functionality of virus-derived BiTEs was tested *in vitro* by complementary binding and cytotoxicity assays. *In vivo* efficacy of MV-BiTE was investigated using both syngeneic and xenograft mouse models of solid cancers.

**Results:** We verified secretion of functional BiTE antibodies by MV-BiTE-infected cells. Further, we demonstrated therapeutic efficacy of MV-BiTE against established tumors in fully immunocompetent mice. MV-BiTE efficacy was associated with increased intratumoral T-cell infiltration and induction of protective anti-tumor immunity. In addition, we showed therapeutic efficacy of MV-BiTE in xenograft models of patient-derived primary colorectal carcinoma spheroids with transfer of peripheral blood mononuclear cells.

**Conclusions:** MV-BiTE treatment was effective in two distinct models of solid tumors without signs of toxicity. This provides strong evidence for therapeutic benefits of tumor-targeted BiTE expression by oncolytic MV. Thus, this study represents proof of concept for an effective strategy to treat solid tumors with BiTEs. *Clin Cancer Res*; 24(9); 2128–37. ©2018 AACR.

## Introduction

With the major progress made in recent years, immunotherapy has become an integral part of cancer treatment. Bispecific T-cell engagers (BiTEs) are one promising approach to enhance cellular antitumor immunity. BiTEs consist of tandem single-chain variable fragments (scFv), targeting CD3 on T cells and a tumor-associated antigen, respectively (1). Thus, T cells are recruited to tumor cells irrespective of T-cell receptor specificity, antigen presentation, and costimulation (2). BiTE-mediated T-cell–tumor cell interaction triggers the formation of immunological synapses, which ultimately results in tumor-specific cell lysis and release of T<sub>H</sub>1 effector cytokines (2–4).

Blinatumomab (Blinicyto<sup>®</sup>), a CD19-targeting BiTE for treatment of B-cell leukemia, gained accelerated approval by the FDA based on encouraging results in clinical trials (5–7). However, continuous infusion is required due to its short half-life and dose-limiting side effects and nevertheless, systemic administration can cause severe, in some cases even fatal treatment-related toxicities (5). For treatment of nonhematologic malignancies, BiTEs targeting CEA (NCT02291614), EpCAM (NCT00635596), and PSMA (NCT01723475) are in clinical development. However, engaging these antigens may cause on-target off-tumor toxicities and successful BiTE therapy of solid tumors has not been reported so far (8).

Oncolytic viruses (OVs) selectively infect and replicate in malignant cells, inducing immunogenic lytic cell death (9). We and others have hypothesized that tumor-targeted expression of BiTEs by oncolytic viral vectors can improve BiTE therapy of solid tumors (10–12). This approach should allow for tumor-restricted BiTE expression, thereby increasing local concentrations and reducing systemic exposure, which results in a higher therapeutic index. In addition, oncolysis induces release of tumor antigens in an immunostimulatory context, enabling *in situ* tumor vaccination (13–17). Thus, we further hypothesized that immunomodulatory effects of oncolysis in combination with local BiTE-mediated T-cell recruitment can induce a sustained antitumor immune response (Fig. 1A). To test these hypotheses, we generated oncolytic measles viruses (MVs) of the Edmonston B vaccine strain encoding bispecific T-cell engagers (MV-BiTEs) and assessed their efficacy *in vitro* and *in vivo*.

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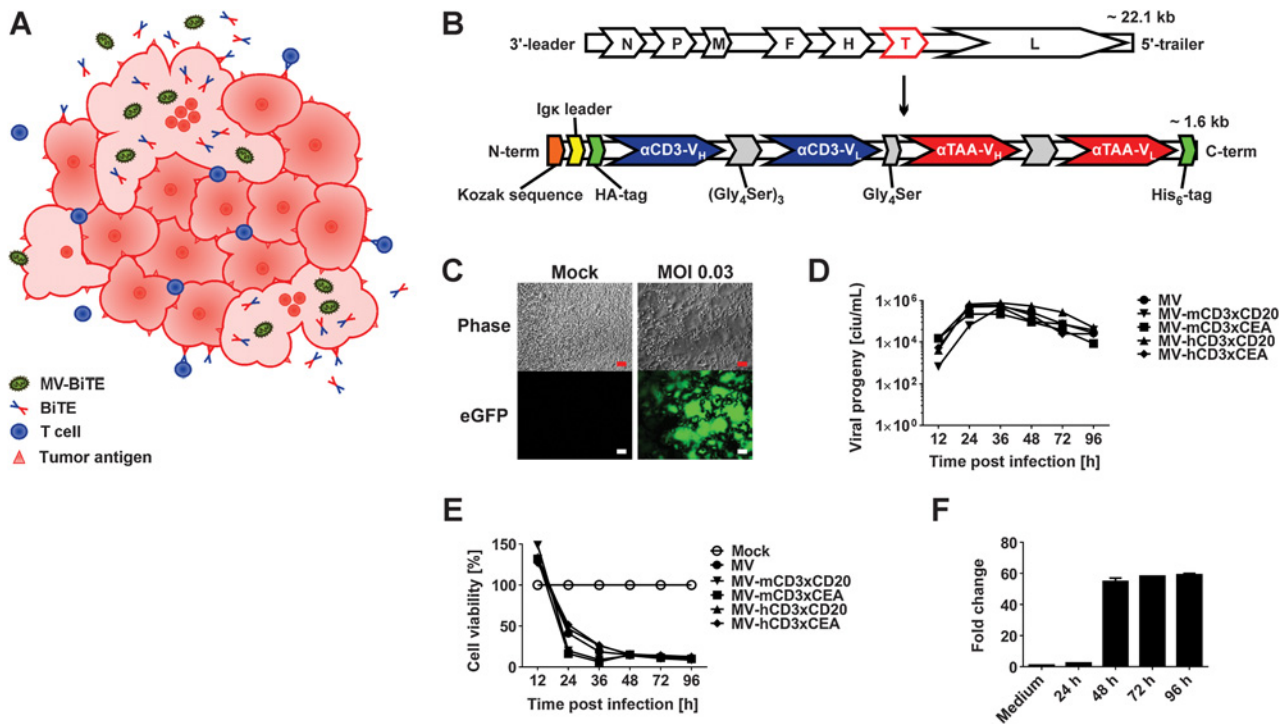
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**Translational relevance**

Bispecific T-cell engagers (BiTEs) are antibodies, which recruit T cells to mediate cytotoxicity against crosslinked tumor cells. Despite compelling success in hematologic malignancies, BiTEs have so far failed to achieve meaningful benefits against solid cancers. Moreover, continuous infusions are necessary and severe, even fatal toxicities can occur. To address these limitations, we have generated oncolytic measles viruses encoding BiTEs (MV-BiTEs) to achieve tumor-restricted continuous BiTE expression and *in situ* vaccination effects. We hypothesized that the combined effects of local BiTE-mediated cytotoxicity and oncolytic immune modulation would be safe and effective against solid tumors. In this study, we show that MV-BiTEs lead to durable remissions and protective antitumor immunity in an immunocompetent mouse model as well as long-term tumor control in patient-derived xenografts. Our study provides proof of concept for a strategy to augment BiTE efficacy against solid tumors.

**Materials and Methods**

A detailed description of the protocols applied in this study is provided in the Supporting Information. MV vectors were designed based on the established reverse genetics system and vector platform (18, 19). MVs were generated to encode BiTEs targeting either human or murine CD3 and human CEA or CD20, respectively. Virus stock titers were determined in octuplicate from three individual aliquots in parallel. MV-BiTEs were characterized in terms of growth kinetics by titration of viral progeny and oncolytic capacities by cell viability assays. BiTEs were purified from MV-BiTE-infected cells (vpBiTEs) by affinity chromatography to assess binding specificity and functionality. vpBiTEs from eight different batches were used throughout the study. Quality control of vpBiTEs included Coomassie R-250 staining of SDS-PAGE gels, Western blot analysis of purification steps, and flow cytometry binding assays. Relative quantification of vpBiTEs normalized to BSA standards was carried out with a BCA protein assay. vpBiTE binding specificity was investigated by ELISA, flow cytometry, and magnetic pull-down of BiTE-labeled cells. *In vitro* co-culture assays to assess vpBiTE-mediated T-cell cytotoxicity were performed with at least three biological



**Figure 1.** Oncolytic measles viruses encoding bispecific T-cell engagers (MV-BiTEs). **A**, Proposed mechanism of action of MV-BiTE. MV-BiTE-infected tumor cells express and secrete BiTE antibodies. Infected cells form multinucleated syncytia, which ultimately leads to tumor cell lysis. BiTEs recruit T cells and mediate T-cell cytotoxicity against non infected tumor cells as a bystander effect. **B**, Schematic of recombinant MV-BiTE genomes. T, transgene;  $\alpha$ -CD3-V<sub>H/L</sub>, anti-murine/human-CD3 variable heavy/light domain;  $\alpha$ -TAA-V<sub>H/L</sub>, antitumor-associated antigen (CD20/CEA) variable heavy/light domain. **C**, Infection with MV-eGFP-BiTE. Vero cells were inoculated with MV-eGFP-mCD3xCEA at a multiplicity of infection (MOI) of 0.03. Images were taken 48 hours post infection. Scale bars: 200  $\mu$ m. **D**, Replication kinetics of MV-BiTEs. Vero cells were infected with MV-BiTEs or unmodified MV at MOI 1. Viral progeny at indicated time points were quantified by titration assay to generate one-step growth curves. **E**, Cytotoxic effects of MV-BiTEs. Vero cells were infected at MOI 1 and cell viability was determined by XTT assay at indicated time points. **F**, BiTE expression kinetics. Vero cells were infected with MV-BiTE (mCD3xCEA) at MOI 0.03. Supernatants were collected at indicated time points and relative BiTE concentrations were determined by ELISA with recombinant human CEA. Data were normalized to background control (medium) and is presented as fold change over background. Error bars represent SD.

replicates (splenocytes: individual mice; human PBMCs: healthy donors). *In vitro* experiments were performed in at least three technical replicates, unless otherwise indicated. *In vivo* efficacy studies were set up with 10 mice per group. Prof. U. Abel, Heidelberg, was consulted for statistical guidance. Sample size was calculated using nquery advisor 6.01 (Statistical Solutions) to detect differences in tumor size with an effect size of  $P(X > Y) = 75\%$  with a power of 80% at a significance level of 5%, where  $P$  is defined as the probability that a randomly chosen subject from one group shows a higher tumor volume than a randomly chosen subject from another group. Mice were stratified into groups according to average tumor volume and SD before treatment initiation. Mice were excluded from the analysis if they failed to develop tumors; group sizes are indicated for the respective experiments. Investigators who conducted treatment and monitored tumor volumes were blinded to intervention. Mice were sacrificed according to the pre-defined termination criteria detailed below. Tumor-infiltrating lymphocytes were analyzed by flow cytometry with markers for differentiation and activation ( $n = 10$  mice per group). For flow cytometric analyses, isotype and fluorescence-minus-one (FMO) controls were used. Animals with complete tumor remissions (67–291 days posttumor implantation) were challenged with tumor cells to assess protective anti-tumor immunity. Efficacy studies with syngeneic mouse models were performed twice. Representative results from the second experiments are shown. Efficacy studies with xenograft mouse models were performed with three biological replicates [three different tumor spheroid cultures (TSCs)].

#### Primary human colorectal cancer spheroid cultures

Primary human colorectal cancer tissue or derived metastases were obtained from the University Hospital Heidelberg in accordance with the Declaration of Helsinki. From each patient, informed consent as approved by the Heidelberg University Ethics Review Board was received. Tumor tissue was mechanically dissociated and digested with Dispase (BD Biosciences). Resulting single-cell suspensions were cultured under non-adhesive serum-free spheroid conditions in advanced DMEM/F-12 medium supplemented with 0.6% glucose, 1% penicillin/streptomycin, 2 mmol/L L-glutamine (Invitrogen, Thermo Fisher Scientific), 4  $\mu$ g/mL heparin, 5 mmol/L HEPES, 4 mg/mL BSA (Sigma-Aldrich), 10 ng/mL FGF basic, and 20 ng/mL EGF (R&D Systems). Primary spheroid cultures were tested for authenticity and contamination by multiplex cell line authentication (MCA) and cell contamination test analyses (McCT, both provided by Multiplexion).

#### Animal experiments

All animal experimental procedures were approved by the Animal Protection Officer at the German Cancer Research Center (DKFZ, Heidelberg, Germany) and by the regional council according to the German Animal Protection Law. Mice were housed in individually ventilated cages with four or five individuals under specific pathogen-free conditions in the Center for Preclinical Research of the DKFZ.

#### Immunocompetent models

CD46, the major entry receptor for vaccine strain MVs, is not expressed on murine cells. To establish syngeneic mouse models in C57BL/6J mice, B16-CD20 and MC38-CEA tumor cells were transduced to stably express CD46. While B16-CD20-CD46

tumors engrafted, MC38-CEA-CD46 tumors were rejected by C57BL/6J mice. Therefore, the MC38-CEA model was used in this study. A total of  $1 \times 10^6$  B16-CD20-CD46 or MC38-CEA cells were implanted subcutaneously into the right flanks of 6- to 8-week-old female C57BL/6J mice (Charles River) in 100  $\mu$ L PBS ( $n = 10$  mice per group). Treatment was initiated when average tumor volumes reached 40 mm<sup>3</sup> (survival analysis) or 60 to 70 mm<sup>3</sup> (TIL analysis, RNA and cytokine profiling). Mice received intra-/peritumoral injections of 100  $\mu$ L Opti-MEM (mock) or  $10^6$  cell infectious units (ciu) of MV-BiTE in 100  $\mu$ L Opti-MEM. Mice received treatment on four (MC38-CEA) and five (B16-CD20-CD46) consecutive days, respectively, according to the treatment schedules established previously (19–21). Tumor diameters were measured with digital calipers three times per week and tumor volumes were determined using the following formula: largest diameter  $\times$  smallest diameter<sup>2</sup>  $\times$  0.5. Mice were sacrificed when tumor volume exceeded 1,000 mm<sup>3</sup>, largest diameter exceeded 15 mm, ulceration occurred, or mice showed signs of severe illness, such as apathy, heavy breathing, or weight loss >20%. For B16 challenge,  $1 \times 10^5$  cells in 100  $\mu$ L PBS were injected subcutaneously into the left flanks and mice were monitored as described above.

#### Xenograft models

Human primary colorectal cancer-derived spheroids were treated with 0.25% trypsin to prepare single-cell suspensions. A total of  $1 \times 10^6$  cells in 100  $\mu$ L matrigel (Corning Inc.) were implanted subcutaneously into the right flanks of 6- to 8-week-old female NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice ( $n = 10$  mice per group; Charles River). Tumor volumes were measured daily and treatment was initiated when average tumor volumes reached 100 mm<sup>3</sup>. Mice received intratumoral injections of  $1 \times 10^6$  ciu of MV-BiTE in 50  $\mu$ L Opti-MEM on four consecutive days. On the first day of treatment, mice additionally received an intratumoral injection of  $1 \times 10^7$  freshly isolated, unstimulated human PBMCs in 50  $\mu$ L PBS 3 h after virus administration. PBMCs were isolated from healthy donor blood by standard Ficoll–Paque cell separation. Control mice received injections of 50  $\mu$ L carrier fluid (virus: Opti-MEM; PBMCs: PBS). Tumor volumes were measured and calculated as described above. Mice were sacrificed according to the termination criteria described above.

#### Statistical analyses

Statistical analyses were performed with GraphPad Prism software (v6.04; GraphPad Software). Results show mean values with SD. Statistical analyses of column data with one independent variable were performed by one-way ANOVA, and  $P$  values were adjusted for multiple comparisons by Tukey test. For competitive ELISAs, the mean of each column was compared to the mean of one control column, and  $P$  values were adjusted for multiple comparisons by Dunnett test. Statistical analyses of grouped data with two independent variables were performed by two-way ANOVA, and  $P$  values were adjusted for multiple comparisons by Sidak test. Curve comparison of two groups for survival analyses were performed by log-rank (Mantle–Cox) test, and  $P$  values were adjusted for multiple comparison by Bonferroni correction.

## Results

### Oncolytic vectors for expression of bispecific T-cell engagers

Recombinant measles virus vaccine vectors were cloned to encode secretable bispecific T-cell engagers (MV-BiTEs) in an

additional transcription unit (ATU) downstream of the *H* open reading frame (ORF; Fig. 1B). BiTEs were designed to target either human or murine CD3 and human CEA or CD20, respectively. Variable regions of heavy ( $V_H$ ) and light chains ( $V_L$ ) of the scFvs were arranged in the sequence  $V_H - V_L$  from N- to C-terminus. In addition, MV-BiTEs encoding enhanced GFP (eGFP) in an ATU upstream of the *N* ORF were generated to monitor viral infection and spread. Replicating viruses were generated from MV-BiTE plasmids and caused the typical cytopathic effect of MV, syncytia formation (Fig. 1C). Replication kinetics of MV-BiTEs and unmodified MV were compared by titration of infectious progeny after inoculation of Vero producer cells (Fig. 1D). Direct cytotoxic effects of MVs were assessed using a metabolic cell viability assay (Fig. 1E). Neither viral replication nor oncolytic potential was impaired by insertion of BiTE transgenes. Secretion of BiTEs by infected cells was demonstrated by ELISA of cell-free supernatants (Fig. 1F).

### Functionality of MV-encoded BiTEs

For *in vitro* characterization, BiTEs were purified from supernatants of MV-BiTE-infected Vero cells (vpBiTE; Supplementary Fig. S1A). In a typical infection experiment, BiTE protein yield was between 1 and 3  $\mu\text{g}$  BiTE per milliliter supernatant, as estimated by comparison of Coomassie staining and Western blot analysis bands to protein standards as well as by ELISA. Specific binding of vpBiTEs to CD3 on human or murine immune cells and to target tumor antigens was confirmed by flow cytometry, magnetic pull-down assays, competitive and sandwich ELISAs (Fig. 2A and B; Supplementary Fig. S1B–S1D). LDH release assays were conducted to assess vpBiTE-mediated T-cell cytotoxicity (Fig. 2C). Co-cultures were prepared with either varying effector to target cell (E:T) ratios or varying vpBiTE concentrations. Effector cells lysed tumor cells expressing the respective target antigen in the presence of relevant vpBiTE (i.e., directed against the target antigen) in a concentration-dependent manner. Only baseline tumor cell lysis was detected in controls with non-relevant BiTE or tumor cells lacking the target antigen. Both resting, unstimulated effector cells from human PBMCs or murine splenocytes and activated, Trp-2-specific murine CD8<sup>+</sup> T cells showed specific cytotoxicity against tumor cells (Fig. 2C). Interestingly, Trp-2-specific murine T cells were engaged to lyse Trp-2-negative tumor cells (MC38-CEA), demonstrating the ability of BiTEs to redirect T cells independent of their T-cell receptor specificity. Cytokine secretion was characterized by cytometric bead arrays, revealing elevated levels of IFN $\gamma$ , TNF, and IL2 in co-cultures with tumor cells expressing the relevant BiTE target antigen (Fig. 2D).

### Therapeutic efficacy of MV-BiTE in immunocompetent mouse models

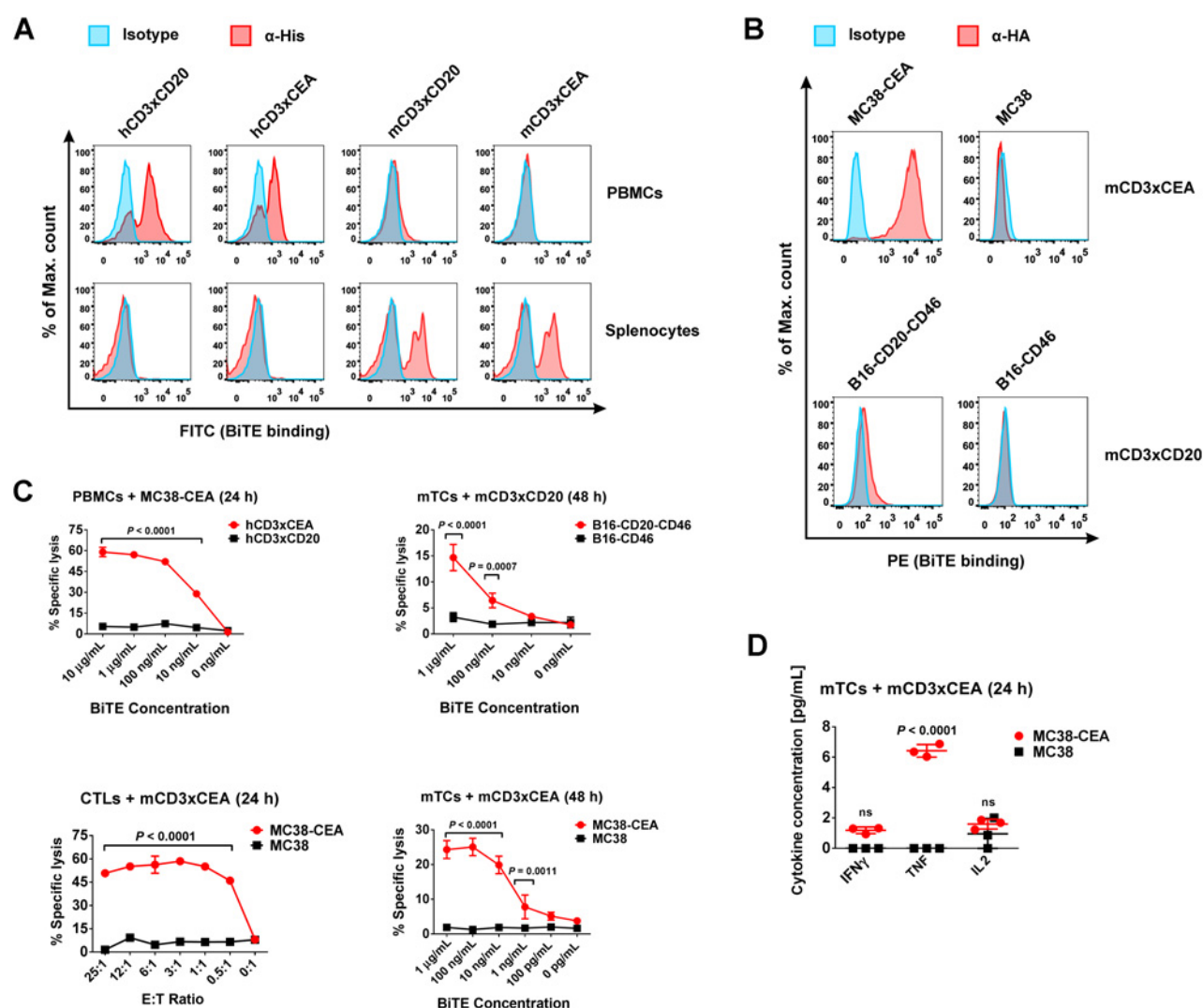
The therapeutic potential of MV-BiTE in the presence of an endogenous immune system was investigated in two established syngeneic tumor models of MV oncolysis, MC38-CEA and B16-CD20-CD46 (19, 22). Consistent with limited sensitivity of murine cells to MV oncolysis, infection of MC38-CEA and B16-CD20-CD46 cells *in vitro* decreased cell viability by only 40% to 60% compared to mock treatment (Supplementary Fig. S2A and S2B). Both cell lines supported productive MV-BiTE replication, while progeny titers were two orders of magnitude higher in B16-CD20-CD46 compared to MC38-CEA cells (Supplementary Fig. S2). ELISA of infected cell super-

natants confirmed *de novo* BiTE synthesis after MV-BiTE infection (Supplementary Figs. S2E and S3B).

Therapeutic efficacy *in vivo* was assessed in C57BL/6J mice harboring subcutaneous MC38-CEA or B16-CD20-CD46 tumors (Fig. 3). In the MC38-CEA model, MV-BiTE significantly prolonged survival and delayed tumor growth compared to mock treatment (Fig. 3A; Supplementary Fig. S3). However, there was no significant difference between mice treated with MV encoding relevant (mCD3xCEA) and non-relevant (mCD3xCD20) BiTE, respectively. In the B16-CD20-CD46 model, MV encoding relevant BiTE (mCD3xCD20) significantly prolonged survival compared to MV encoding a non-relevant BiTE (mCD3xCEA) or unmodified MV (Fig. 3B; Supplementary Fig. S4A). Levels of intratumoral *MV nucleoprotein* (MV-N) mRNA and *BiTE* mRNA demonstrated viral gene expression until 48 h posttreatment (Supplementary Fig. S5). To further discriminate between oncolytic and BiTE-mediated antitumor effects in the B16-CD20-CD46 model, therapeutic efficacy of MV-BiTE was compared to UV-irradiated MV-BiTE (Fig. 3C; Supplementary Fig. S4B and S6C). "Complete UV inactivation" (cUV) completely abrogated viral replication without compromising BiTE function. "Partial UV inactivation" (pUV), which decreased viral progeny by two orders of magnitude (Supplementary Fig. S6A), was used to mimic a less permissive tumor model, such as MC38-CEA. Interestingly, there was no significant difference between UV-irradiated and non-irradiated MV-BiTE (Fig. 3C; Supplementary Fig. S4B and S6C). Also, no significant difference was detected in therapeutic efficacy of UV-inactivated, unmodified MV compared to non-irradiated MV (Supplementary Fig. S6D). To test whether local application of BiTE alone has antitumor activity, mice bearing B16-CD20-CD46 tumors were treated with intratumoral injections of mCD3xCD20 vpBiTE at a dose equivalent to the amount of BiTE delivered by MV-BiTE injections (Supplementary Fig. S6E). Local vpBiTE injections only showed minimal therapeutic effects compared to mock treatment and were significantly less effective than MV-BiTE or unmodified MV (Fig. 3C). BiTE serum levels after MV-BiTE treatment remained below detection limit (Supplementary Fig. S7). Notably, therapeutic efficacy of MV-BiTE was not abrogated in MV-vaccinated mice (Supplementary Fig. S8).

### Immunological effects of MV-BiTE treatment

Tumor-infiltrating lymphocytes (TILs) were analyzed by flow cytometry. In the MC38-CEA model, MV-mCD3xCEA treatment led to only a modest increase in total T cells and effector T cells compared to mock controls, with no significant difference between MV-mCD3xCEA and non-relevant MV-BiTE (Supplementary Fig. S9A). Notably, mock-treated MC38-CEA tumors already harbored high numbers of T cells, with approximately 30% CD3<sup>+</sup> TILs among all live cells, a high percentage of which were activated (CD69<sup>+</sup>; Supplementary Fig. S9A). In contrast, B16-CD20-CD46 tumors featured low numbers of CD3<sup>+</sup> TILs, albeit a considerable percentage of these showed an activated phenotype (CD69<sup>+</sup>; Fig. 3D; Supplementary Fig. S9B). Importantly, in the B16-CD20-CD46 model, treatment with MV-mCD3xCD20 led to significantly increased ratios of T cells to tumor cells (CD3<sup>+</sup>/CD46<sup>+</sup>) and of CD8<sup>+</sup> to CD4<sup>+</sup> T cells, compared to mock and non-relevant MV-BiTE treatment (Fig. 3D; Supplementary Fig. S9B). Furthermore, ratios of intratumoral *CD8* and *Foxp3* mRNA increased, indicating elevated T effector to regulatory T-cell ratios (Fig. 3E). Although intratumoral

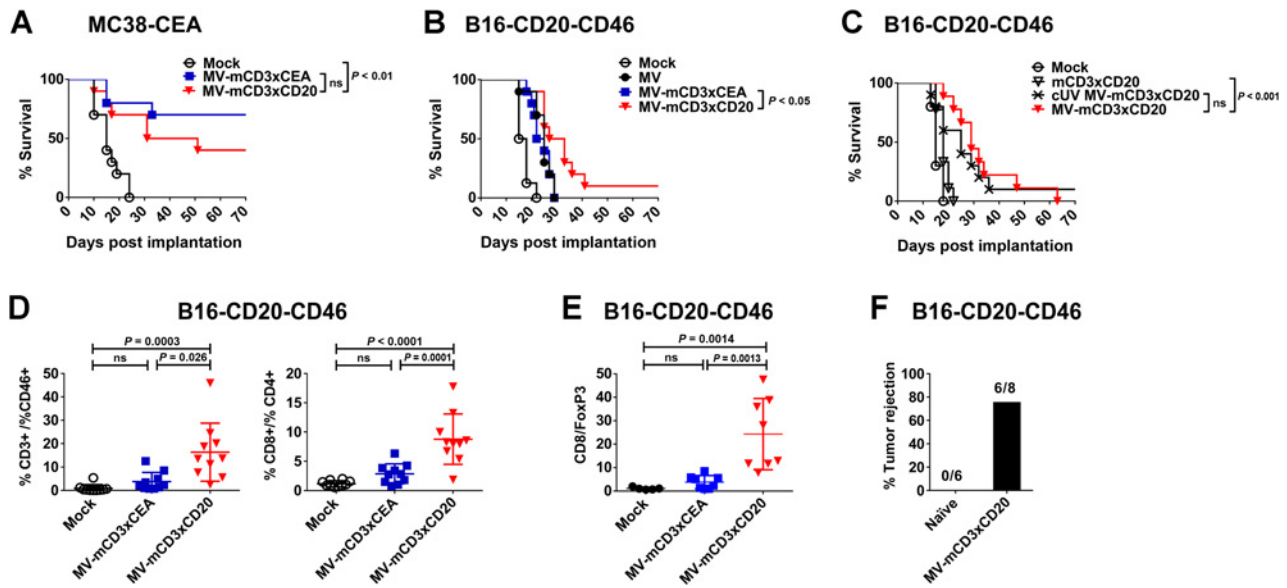


**Figure 2.** Functional characterization of MV-encoded BiTEs. BiTEs were purified from supernatants of infected cells (vpBiTEs). **A**, Binding to CD3<sup>+</sup> T cells. Human PBMCs or murine splenocytes were incubated with vpBiTEs. vpBiTE binding was detected by flow cytometry with a His<sub>6</sub>-specific antibody. **B**, Binding to tumor cells. Tumor cells expressing or lacking BiTE target antigens were incubated with vpBiTEs. vpBiTE binding was detected by flow cytometry with an antibody specific for the BiTE HA-tag. **C**, T-cell cytotoxicity. Tumor cells were co-cultured with human or murine immune effector cells and vpBiTEs with either varying BiTE concentrations or effector to target cell (E:T) ratios. LDH release assays were performed at indicated time points and specific tumor cell lysis was calculated in percent. **D**, BiTE-induced cytokine secretion. Tumor cells were co-cultured with vpBiTE and murine T cells purified from splenocytes for 24 hours. Cytokine concentrations in cell culture supernatants were determined using a cytometric bead array. **C** and **D**, Statistical analysis was performed by two-way ANOVA and *P* values were adjusted for multiple comparisons by Sidak test. Error bars represent SD. mTC, murine T cells; CTLs, murine cytotoxic T lymphocytes; ns, not significant.

*Foxp3* mRNA levels increased twofold after MV-mCD3xCD20 treatment, mRNA levels of the T<sub>H</sub>1-characteristic transcription factor *T-bet* increased 100-fold (Supplementary Fig. S9C). Gene expression analysis revealed differential expression of T-cell-associated genes in the different treatment groups (Supplementary Fig. S10). Tumors from MV-mCD3xCD20-treated mice showed the highest relative expression of genes associated with T-cell activation, proliferation, and differentiation, as compared to mock- and MV-mCD3xCEA-treated mice. Tumors from the mock-treated group formed separate clusters exhibiting the lowest relative expression of T-cell-associated genes. Differential

expression patterns were observed for several classes of immune-related genes, e.g., chemokines and chemokine receptors such as *Cxcl12* and *Cxcr4*, respectively, the transcription factor *T-bet*, the adhesion molecule *CD48*, genes encoding the T-cell receptor complex and downstream signaling molecules such as *CD3*, *CD8*, *CD28*, and *Lck*. Notably, genes associated with T-cell exhaustion and inhibition, such as *Pdcd1* and *Ctla4*, were also increased after treatment with MV-mCD3xCD20, as compared to mock and MV-mCD3xCEA treatment.

Naïve mice and mice in complete remission after MV-BiTE treatment were challenged with parental B16 cells. While tumors



**Figure 3.** Immunovirotherapy with MV-BiTE in syngeneic tumor models. **A**, Established subcutaneous MC38-CEA tumors were subjected to mock treatment (carrier fluid), treated with MV-BiTE encoding relevant BiTE (MV-mCD3xCEA) or non-relevant BiTE (MV-mCD3xCD20) on four consecutive days by intratumoral injections ( $n = 10$  mice per group). **B** and **C**, Established subcutaneous B16-CD20-CD46 tumors were treated by intratumoral injections on five consecutive days: mock, carrier fluid only; MV, unmodified oncolytic measles virus; MV-mCD3xCEA, MV vector encoding non-relevant BiTE targeting CEA; MV-mCD3xCD20, MV vector encoding relevant BiTE targeting CD20; mCD3xCD20 - vpBiTE only; cUV MV-mCD3xCD20, inactivated, replication-deficient virus ( $n = 9-10$  mice per group). **A-C**, Kaplan-Meier survival analysis is shown. Log-rank (Mantel-Cox) test was performed for statistical comparison of survival curves, and  $P$  values were corrected for multiple comparisons by the Bonferroni method. **D** and **E**, Analysis of tumor-infiltrating lymphocytes. B16-CD20-CD46 tumors were explanted 1 day after the last treatment and each tumor was divided for flow cytometric analysis (**D**) and targeted transcriptome analysis (**E**), respectively. **D**, Left panel: Ratios of infiltrating T cells ( $CD3^+$ ) to tumor cells ( $CD46^+$ ) gated on all live cells. Right panel: Ratios of  $CD8^+$  to  $CD4^+$  T cells gated on all live  $CD3^+$  cells. **E**, CD8 to FoxP3 ratios calculated from mRNA expression levels.  $n = 8$  tumors for mock,  $n = 10$  tumors for both MV-mCD3xCEA and MV-mCD3xCD20, respectively. Mean values and SD are shown. Statistical analysis was performed by one-way ANOVA and  $P$  values were adjusted for multiple comparisons by Tukey test. **F**, Protective antitumor immunity. Mice in long-term remission after treatment of B16-CD20-CD46 tumors with MV-BiTE were challenged with B16 cells. In parallel, B16 cells were injected subcutaneously into naïve mice. Tumor rejection rates are shown. ns, not significant.

grew in 6/6 naïve mice, 6/8 MV-BiTE-treated mice rejected tumor engraftment (Fig. 3F), indicating protective antitumor immunity after successful MV-BiTE therapy.

**Efficacy of MV-BiTE against patient-derived colon cancer spheroids**

Primary colorectal carcinoma cells were used to assess MV-BiTE efficacy against patient-derived tumors. Tumor-initiating cells can be enriched in serum-free 3D TSCs and upon xenotransplantation reliably form tumors in immunodeficient mice. These models faithfully reflect the original tumor histology and are functionally and genetically heterogeneous (23, 24). Three TSCs with low, intermediate and high levels of CEA expression were tested for MV susceptibility (Fig. 4A; Supplementary Fig. S11A). eGFP expression after inoculation with MV-eGFP-BiTE indicated productive infection and viral spread in TSCs. Titration of viral progeny confirmed low-level MV replication in TSCs (Supplementary Fig. S11B) and ELISA confirmed production of BiTEs after MV-BiTE infection (Supplementary Fig. S11C). vpBiTE from MV-hCD3xCEA-infected cells mediated TSC lysis by unstimulated PBMCs (Fig. 4B) and induced secretion of IL6 and  $T_H1$  cytokines (Fig. 4C).

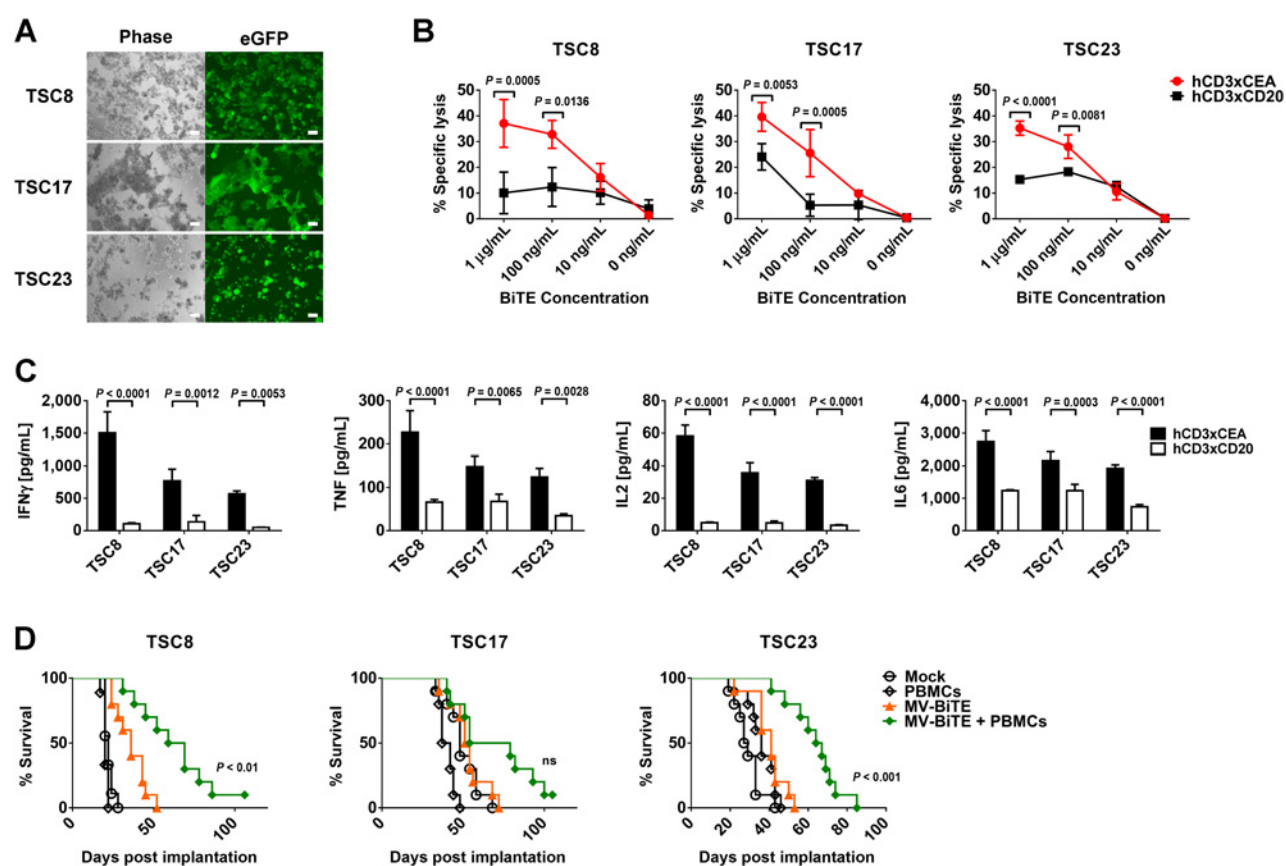
Patient-derived xenografts were generated by subcutaneous transplantation of TSCs into NSG mice. Upon MV-BiTE treatment, BiTE serum levels remained below detection limit (Supplementary Fig. S12A). Interestingly, intratumoral injection of MV-BiTE

did not result in immediate systemic exposure to BiTE present in the virus suspension, in contrast to intravenous MV-BiTE application (Supplementary Fig. S12B). After intravenous injection, no viral (MV-N) or BiTE mRNA was detected intratumorally (data not shown), whereas after intratumoral injection, levels of MV-N mRNA and BiTE mRNA increased intratumorally during the treatment of TSC8-bearing mice and reached a maximum at 24 h after the last treatment (Supplementary Fig. S13). In contrast to the murine B16-CD20-CD46 model, viral gene expression in the xenograft model remained stable until 240 h posttreatment.

PBMCs or MV-BiTE treatment alone showed only a moderate impact on survival of xenografted NSG mice. In contrast, MV-BiTE treatment combined with transfer of human PBMCs showed synergistic effects and significantly prolonged survival compared to treatment with PBMCs only or MV-BiTE only (Fig. 4D; Supplementary Fig. S14). Notably, the percentage of intratumoral lymphocytes decreased by 66% 3 days post injection, and no intratumoral lymphocytes were detectable at 11 days post injection (Supplementary Fig. S15A). Furthermore, MV-BiTE therapy did not induce negative selection of the BiTE target tumor antigen CEA (Supplementary Fig. S15B).

**Discussion**

This study demonstrates that therapeutic efficacy of BiTEs against solid tumors can be achieved by means of an oncolytic



**Figure 4.**

MV-BiTE efficacy against patient-derived colorectal carcinoma xenografts. **A**, Low-passage 3D cultures from human primary colorectal cancers (TSCs) were inoculated with MV-eGFP-BiTE (hCD3xCEA) at MOI 1. Images were acquired 24 hours post infection. Scale bars: 200  $\mu$ m. **B**, BiTE-mediated T-cell cytotoxicity against TSCs. TSCs were co-cultured with human PBMCs (E:T 50:1) and vpBiTE at indicated concentrations. LDH release was measured after 24 hours and specific tumor cell lysis was calculated. **C**, BiTE-induced cytokine secretion. TSCs were co-cultured with human PBMCs and vpBiTEs for 24 hours. Cytokine concentrations in culture supernatants were quantified using a cytometric bead array. **B** and **C**, Statistical analysis was performed by two-way ANOVA and *P* values were adjusted for multiple comparisons by Sidak test. Mean values of triplicates and SD are shown. **D**, Efficacy of MV-BiTE against TSCs *in vivo*. NSG mice harboring subcutaneous TSC xenografts were treated with MV-BiTE (hCD3xCEA) on four consecutive days and PBMCs on the first day of treatment. Mice receiving either carrier fluid (mock), PBMCs only or MV-BiTE only served as controls (*n* = 9–10 mice per group). Survival was assessed by Kaplan–Meier analysis with long-rank (Mantel–Cox) test, and *P* values were corrected for multiple comparisons by Bonferroni's correction. *P* values for comparison of MV-BiTE + PBMCs to MV-BiTE only are shown. ns, not significant.

viral vector (MV-BiTE). With the approval of T-VEC (Imlytic) by the FDA and EMA in 2015 (25), Pexa-Vec in phase III (NCT02562755), and promising results in phase I/II trials of MV (26, 27) as well as many other OV (28), oncolytic virotherapy is gradually entering clinical practice. Many of these OV vectors encode additional therapeutic genes, including immunomodulators such as GM-CSF (28) or CD40L/4-1BB (LOAd703; NCT03225989; ref. 29).

Insertion of BiTE cassettes into MV does not compromise replicative or oncolytic capacities. MV-encoded BiTEs are functional in terms of antigen binding, target-specific T-cell activation and induction of T-cell cytotoxicity. Thus, MV-BiTEs further add to the repertoire of immunomodulatory MV vectors (19–21). While recruitment of T cells was achieved by targeting CEA, and CD20 served as model target tumor antigens in this study. Within the MV vector platform, the scFvs are readily exchangeable by a targeting domain of choice. Thus, MV-BiTEs can be directed against any tumor surface antigen, provided that an appropriate binding moiety is available. This enables application to additional tumor

entities and concomitant targeting of several tumor antigens to prevent potential antigen escape. Moreover, the anti-CD3 scFv could be replaced, for example, by an NK-cell-specific scFv to generate MV-"BiKEs" (bispecific killer cell engagers; ref. 30).

Therapeutic efficacy of MV-BiTE was assessed in complementary mouse models. While syngeneic models are necessary to study effects in the context of an autochthonous immune system, mice are not susceptible to MV infection and murine tumors show limited permissiveness for the primate-adapted virus MV (31). Although they do not account for natural immune responses, humanized models more adequately reflect the extent of oncolysis. Therefore, we chose both established syngeneic models of MV oncolysis and patient-derived xenografts of early-passage patient-derived spheroid cells with transfer of unstimulated PBMCs to test efficacy of MV-BiTE.

In the syngeneic B16-CD20-CD46 model, treatment with MV-BiTE augmented the number of tumor-infiltrating T cells as well as their activation status and conferred protective anti-tumor immunity. Furthermore, mRNA levels of the transcription factor

*T-bet* were significantly increased, indicating T-cell polarization towards a  $T_H1$  phenotype. Increased infiltration and activation of T cells was not only associated with upregulation of T-cell activation, differentiation, and proliferation markers, but also with upregulation of T-cell exhaustion markers and inhibitory molecules. This provides a rationale for combination with immune checkpoint inhibition. Recently, a case report on combining blinatumomab with anti-PD-1, as well as promising data from a phase Ib trial combining T-VEC with anti-PD-1 have been published (32, 33).

In the B16-CD20-CD46 model, therapeutic effects did not depend on viral replication, but could not be achieved by local injection of BiTEs only. Thus, the immunostimulatory properties of the MV vector appear essential for efficacy. Furthermore, in this model, viral replication and thus virus-mediated BiTE expression were limited, as shown by analysis of intratumoral MV-N mRNA and BiTE mRNA levels. Therefore, in a more permissive tumor, stronger viral replication may add to a favorable treatment outcome. Most patients have been vaccinated against measles and thus have MV-neutralizing antibodies. Importantly, in the B16-CD20-CD46 model, therapeutic efficacy of MV-BiTE was not compromised in MV-immune mice. Of note, mice were treated with intratumoral injections of MV-BiTE, probably limiting accessibility of MV-BiTE for neutralizing antibodies. Noteworthy, intraperitoneal administration of oncolytic MV has been successfully applied in measles-immune ovarian cancer patients (26). Furthermore, the recently developed Tupaia paramyxovirus vector platform may represent an alternative to MV, as no cross-neutralizing antibodies exist (34).

Therapeutic benefit of relevant MV-BiTE in the MC38-CEA model was modest. Given the results obtained for UV-inactivated MV-BiTE in the B16-CD20-CD46 model, permissiveness for MV does not seem to be the limiting factor. Rather, even untreated MC38-CEA tumors harbor many activated T cells. Thus, there seems little additional benefit of BiTE-mediated T-cell recruitment in this tumor model. Previous studies have shown that MV encoding GM-CSF, anti-PD-L1, or IL12 are effective against MC38-CEA, indicating that overcoming T-cell exhaustion and activating further immune effector mechanisms is more relevant in this model than the recruitment of additional T cells (20, 21). These findings reflect that the specific immune environment determines whether a certain immunotherapy is effective in a given tumor, demanding a personalized approach to immunotherapy.

Treatment of patient-derived spheroid xenografts with PBMC transfer demonstrated efficacy of MV-BiTE (MV-hCD3xCEA) against genetically and functionally heterogeneous tumor cells which closely mimic clinical reality. Interestingly, MV-hCD3xCEA therapy did not induce negative selection of CEA-expressing tumor cells. Analysis of intratumoral lymphocytes revealed limited persistence of transferred PBMCs. Thus, in this model, temporary BiTE-mediated tumor cell lysis might have mitigated negative selection of CEA-expressing target cells. Moreover, MV-BiTE treatment in the B16-CD20-CD46 model induced protective immunity against the parental cell line B16, indicating protection also against tumor cells lacking the BiTE target antigen. Although BiTEs have achieved compelling efficacy in hematologic malignancies, both preclinical and clinical studies have so far failed to demonstrate lasting responses at an acceptable level of toxicity in solid tumors (8). In preclinical studies, short-term reduction of tumor volume as well as prophylactic effects in lung

colonization models have been reported (35, 36). Other BiTE molecules and different formats of T-cell engaging bispecific antibodies are currently under clinical investigation for treatment of melanoma and colon cancer. Examples are IMCgp100 (ImmTAC targeting gp100, NCT03070392), MT-110 (BiTE targeting EpCAM, NCT00635596), catumaxomab (TrioMab targeting EpCAM, NCT01504256), RO6958688 (CrossMab targeting CEA, NCT02650713), and MGD007 (DART-Fc targeting gpA33, NCT02248805).

MV-BiTEs address two main challenges in BiTE therapy for solid tumors: safety and delivery. Both in syngeneic and patient-derived models, BiTE serum levels two to 24 hours after MV-BiTE treatment remained below detection limit, indicating a safety advantage of MV-encoded BiTEs. Furthermore, intratumoral injection of MV-BiTE did not result in immediate systemic exposure to BiTEs. However, intravenous injection may be the most desirable route of administration in many clinical situations. In the xenograft model, intravenous injection of MV-BiTE resulted in high systemic and insufficient intratumoral BiTE levels. In clinical trials, tumor-restricted MV replication and protein expression after intratumoral, intraperitoneal and also intravenous administration have been demonstrated (26, 37, 38). This reflects the limitations of mouse models in the assessment of MV oncolysis. In human subjects, more efficient MV-BiTE replication and spread can be anticipated. With respect to the narrow therapeutic window of T-cell engaging antibodies, MV-BiTE vectors could be equipped with artificial riboswitches (39) to control viral gene expression.

In terms of BiTE delivery, a single treatment cycle of four to five intratumoral (i.t.) MV-BiTE injections was sufficient to achieve durable responses. A recent study reported that injections with BiTE mRNA reduced to once weekly still achieved efficacy against xenograft tumors (40). In contrast to non-immunogenic mRNA, MV-BiTEs have additional immunostimulatory properties, as virus-associated molecular patterns activate innate immunity and oncolysis constitutes an *in situ* tumor vaccination, enabling adaptive, long-term antitumor immunity. Previous approaches to encode BiTEs in oncolytic vaccinia virus and adenovirus yielded transient effects on tumor volume in xenograft models and *ex vivo* tumor cell killing with patient-derived specimens (10–12). Advantages of measles vaccine strains include their excellent safety record (41) and high immunogenicity (42). Currently, systematic comparisons of different oncolytic vectors are lacking and should be pursued in the future to identify relevant biomarkers for the choice of therapeutic vector and optimal treatment options for individual cancer patients.

Remarkably, UV-irradiated MV-BiTE showed comparable efficacy to non-irradiated MV-BiTE. To rule out that UV irradiation improved MV immunogenicity by altered "danger signals", we compared efficacy of irradiated and non-irradiated unmodified MV, yielding comparable survival. These results confirm the dominance of immunotherapeutic effects over direct oncolysis in MV immunovirotherapy. The possibility to use an inactivated, non-replicating virus for cancer therapy can further add to the safety advantage of MV vectors in oncolytic therapy.

To our knowledge, this is the first report of *in vivo* efficacy of an oncolytic virus encoding BiTEs in both an immunocompetent mouse model and patient-derived xenografts. We demonstrate long-term tumor remissions without relapse and induction of



protective immunity after MV-BiTE treatment. These data provide proof of concept for efficacy against solid tumors by targeted BiTE expression using an oncolytic vector. Thus, this approach could circumvent limitations in current BiTE therapy and may translate into meaningful therapeutic effects in treatment of solid cancers.

### Disclosure of Potential Conflicts of Interest

T. Speck and C.E. Engeland are listed as co-inventors of a patent regarding RNA Viruses for Cancer Immunotherapy owned by the German Cancer Research Center and Heidelberg University. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** T. Speck, J.P.W. Heidbuechel

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