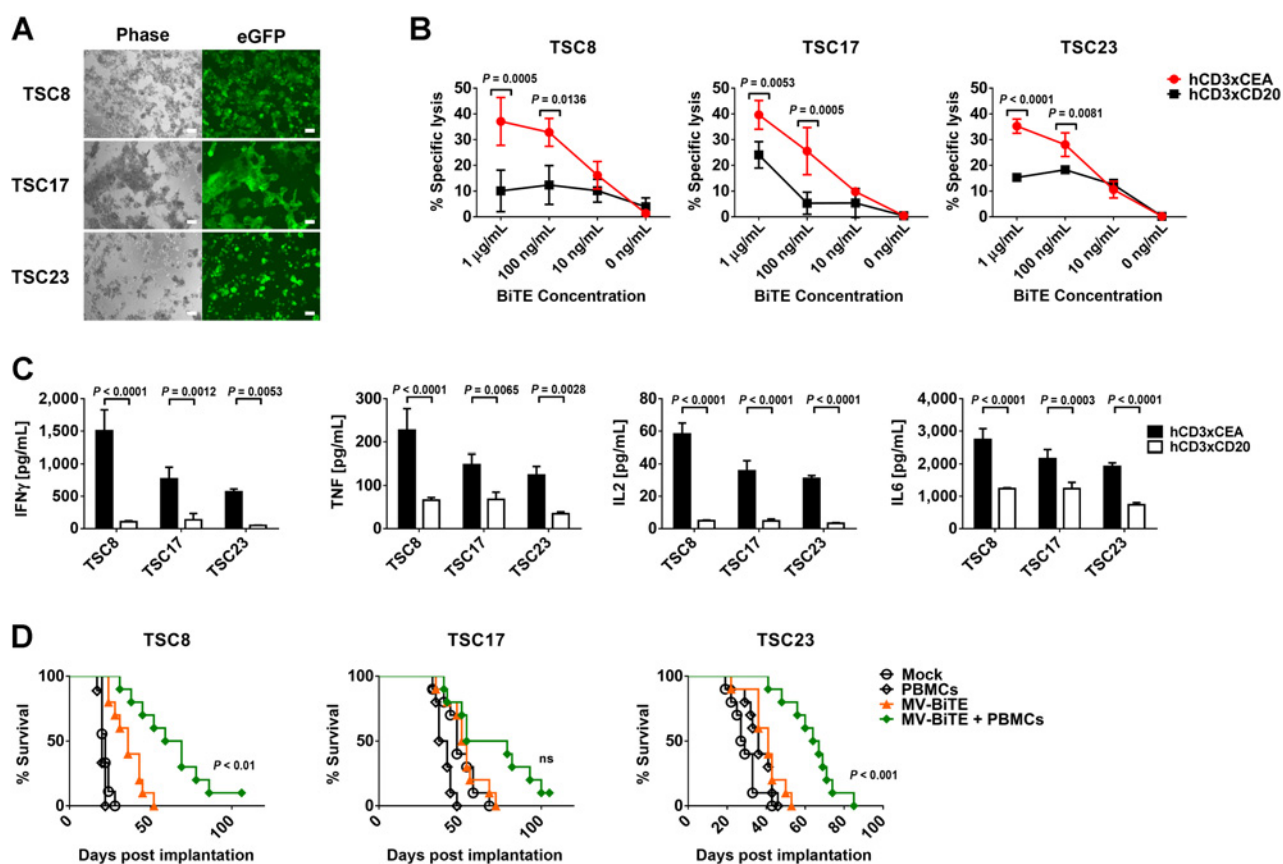Speck et al.

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 μm. **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 (Imlygic) 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 OVs (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

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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 Immunovirotherapy owned by the German Cancer Research Center and Heildelberg University. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Speck, J.P.W. Heidbuechel
Writing, review, and/or revision of the manuscript: T. Speck, J.P.W. Heidbuechel, D. Jaeger, C. von Kalle, G. Ungerechts, C.E. Engeland

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

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