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ORIGINAL ARTICLE

Targeted release of oncolytic measles virus by blood outgrowth endothelial cells in situ inhibits orthotopic gliomas

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Malignant gliomas remain largely incurable despite intensive efforts to develop novel therapies. Replicating oncolytic viruses have shown great promise, among them attenuated measles viruses of the Edmonston B strain (MV-Edm). However, host immune response and the infiltrative nature of gliomas limit their efficacy. We show that human blood outgrowth endothelial cells (BOECs), readily expandable from peripheral blood, are easily infected by MV-Edm and allow replication of MV-Edm while surviving long enough after infection to serve as vehicles for MV-Edm (BOEC/MV-Edm). After intravenous and peritumoral injection, BOEC/MV-Edm deliver the viruses selectively to irradiated orthotopic U87 gliomas in mice. At the tumor, MV-Edm produced by the BOECs infect glioma cells. Subsequent spread from tumor cell to tumor cell leads to focal infection and cytopathic effects that decrease tumor size and, in the case of peritumoral injection, prolong survival of mice. Since MV-Edm within BOECs are not readily neutralized and because BOEC/MV-Edm search and destroy glioma cells, BOEC/MV-Edm constitute a promising novel approach for glioma therapy.
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Introduction

Nearly all patients with malignant gliomas die from their disease and prognosis has improved only little over decades. The efficacy of locoregional therapies such as a surgical resection, irradiation, radioimmunotherapy and gene therapy is modest at best. One major reason is the limited delivery of the therapeutic moiety to the glioma cells. This is exemplified by the failure of gene therapy, which, when directly aimed against glioma cells, is hampered by low gene transfer efficacy requiring a complete bystander effect impossible to achieve. A second major reason for the failure of existing local therapies is the infiltrative growth of malignant glioma leading to the formation of glioma cell clusters isolated within normal brain tissue. These are inaccessible without risking destruction of normal brain tissue. Systemic therapy is also inefficient in glioma patients despite recent advances. This is in part due to the blood–brain barrier, which, although impaired, remains partially functional in malignant gliomas and hinders their systemic therapy by drugs, DNA or viruses.

Replicating oncolytic viruses are emerging as a promising modality for the treatment of malignant gliomas and other malignancies. They promise to overcome the problem of limited delivery of the therapeutic agent since, in principle, the successful infection of only a few tumor cells should suffice for subsequent spread to most tumor cells. A variety of replicating oncolytic viruses are being investigated. A recent addition to this arsenal is attenuated measles virus of the Edmonston B vaccine strain (MV-Edm; reviewed in Nakamura and Russell[1] and Fielding[2]). MV-Edm infects cells by preferentially binding to CD46[3,4] rather than to CD150, as wild-type MV does. The higher density of CD46 on malignant cells, including malignant gliomas,[5] compared to non-malignant cells contributes to the preferential killing of tumor cells by MV-Edm.[6] Once MV-Edm has infected tumor cells it spreads to noninfected tumor bystander cells by cell-to-cell contact.[7,8] MV-Edm-infected tumor cells form syncytia[9] induced by the F-protein of the virus, eventually leading to cell death that includes programmed cell death.[10] MV-Edm has been shown in animal models to have oncolytic activity against human lymphoma,[9] multiple myeloma,[11] ovarian cancer,[12,13] malignant glioma,[5] fibrosarcoma[14] and against cutaneous T-cell lymphoma in patients.[15]

As for other replicating oncolytic viruses, host immune response and cellular barriers limit infection by and intratumoral spread of MV-Edm, respectively. MV is

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readily neutralized by serum antibodies from humans immune to measles, which is true for most adults. Although cancer patients are usually immunocompromised and the brain may be an 'immune-privileged' site, an immune response against MV-Edm may still be mounted, limiting infection of gliomas by MV-Edm. As the spread of MV-Edm depends on tumor-cell-to-tumor-cell contact, noninfectable cells such as most nonmalignant cells will impede the spread of MV-Edm. This problem is accentuated in malignant gliomas because of their propensity for infiltrative growth.

Various cellular vehicles can be envisioned to deliver therapeutic agents to experimental brain tumors. These include neural progenitors derived from the patient, from cadavers or fetuses, or generated from embryonic stem cells. However, clinical, logistical, immunological and ethical considerations will limit their use (reviewed in Jarmy *et al*.[16]). In contrast, bone marrow-derived cells, known to target experimental tumors including gliomas,[17–19] are more easy to procure. Blood outgrowth endothelial cells (BOECs), in particular, are readily obtained from human peripheral blood.[20] They can be massively expanded *ex vivo* and lend themselves to genetic manipulation. BOECs are progenitor cells with a microvascular endothelial phenotype[20,21] expressing VEGFR2, VEGFR1, vWF, CD31, VE-cadherin, $a_v\beta_3$, $a_v\beta_5$ and CD105. They do not or only marginally express CD133, c-kit, CD45, CXCR4, ESL (E-selectin ligand), CD162, cutaneous lymphocyte-associated Ag, CD49d, CD11a and CD11b. The expression of CD34 varies. BOECs take up acetylated low-density lipoprotein and build vessel-like structures on Matrigel. BOECs have been shown to seek experimental tumors, invade them and incorporate into tumor structures.[21] However, when armed with the cytosine deaminase gene and administered intravenously (i.v.), they did not prolong the life of mice bearing disseminated metastases of Lewis lung carcinoma.[21] A major reason for this failure was the temporary and spatially limited bystander effect on tumor cells, the limitation being caused by suicide of the BOECs upon prodrug administration.

We reasoned that combining the tumor-seeking and protective ability of BOECs with the sustained and far reaching oncolytic 'lateral effect' of attenuated measles virus might control experimental brain tumors. We show in this study that MV-Edm within BOECs are protected against MV-neutralizing serum antibodies and are carried in mice through normal brain tissue to orthotopic malignant glioma cells, infect them, decrease tumor size and increase survival.

Results

BOECs express CD46 and can be infected by MV-Edm while being resistant to MV-Edm-mediated cell death

To employ BOECs as tumor-targeting cellular vehicles for MV-Edm, we first had to verify whether BOECs can be infected by MV-Edm. Expression of CD46, the preferred receptor for MV-Edm, on BOECs was strong and comparable with U87 glioma cells, A549 lung carcinoma cells and the myelomonocytic leukemia cell line K562, and was markedly stronger than on monocytes (Figure 1a).

We then determined whether MV-Edm infect and replicate within BOECs. Indeed, the virus titer ($TCID_{50}$) of lysates of BOECs infected with MV-Edm increased 139-fold as compared to the virus inoculum within 3 days after infection (Figure 1b). Human monocytes, another potential cellular vehicle for MV, also supported MV replication, whereas replication in human lymphocytes was limited (Figure 1b). Despite efficient replication, the majority of BOECs did not succumb to MV 3 days after infection (Figure 1c), in contrast to monocytes. Thus, BOECs, but not monocytes or lymphocytes, support replication of MV for several days without dying.

While BOECs initially resisted MV-induced cell death, human cancer cell lines, such as A549 lung carcinoma and U87 glioma cells, were readily killed by MV-Edm (Figure 1d).

Infection with MV-Edm may change homing-associated surface molecules of BOECs. However, no change in the expression of VEGFR2 (KDR), CXCR4, VE-Cadherin, $a_v\beta_5$, CD11a, CD11b, CD49d, CD162 and cutaneous lymphocyte-associated Ag was detected 1 and 24 h after infection (data not shown).

Taken together, BOECs could be infected by MV-Edm without being quickly killed or downregulating homing-associated surface molecules. This suggests that they are suitable cellular vehicles for MV-Edm.

BOECs do not carry loosely adsorbed MV-Edm-eGFP on their surface after infection with MV-Edm-eGFP

We investigated whether MV-Edm added to BOECs *in vitro* just adhere to the BOECs' surface and subsequently become released to infect surrounding cells. To this end, Vero indicator cells were incubated with cell-free washes of BOEC/MV-Edm-eGFP. While BOECs were clearly infected with MV-Edm-eGFP, as seen by the green fluorescence of BOECs (Figure 2, left panel), no infectious MV-Edm-eGFP could be detached by washing and trypsinizing after infection as no Vero cells became infected (Figure 2, right panel).

MV-Edm spread from BOECs to neighboring U87 glioma cells and exert a strong bystander effect

It is crucial for any therapeutic vehicle to effectively release its payload to the target cells. As determined by fluorescence microscopy, MV-Edm-eGFP spread rapidly from single BOEC/MV-Edm cells to surrounding U87 cells (Figures 3Aa–c) and then to more distant U87 cells (Figures 3Ad–f). The few GFP-positive foci of U87 cells that were not adjacent to the BOEC/MV-Edm may have originated from MV-Edm-eGFP released into the culture medium by dying tumor cells. The spread of MV-Edm-eGFP induced multinuclear syncytia of U87 cells (Figures 3Ag and h) and caused their death (Figure 3B). Low numbers of BOEC/MV-Edm sufficed to cause a strong bystander effect on U87 glioma cells (Figure 3B). These data show that MV-Edm efficiently spread from BOECs to tumor cells and kill them.

MV-Edm protected within BOECs from measles antibodies infect bystander tumor cells in the presence of measles immune serum

Clinical application of MV-Edm as an oncolytic virus faces the potential hurdle of pre-existing measles antibodies in

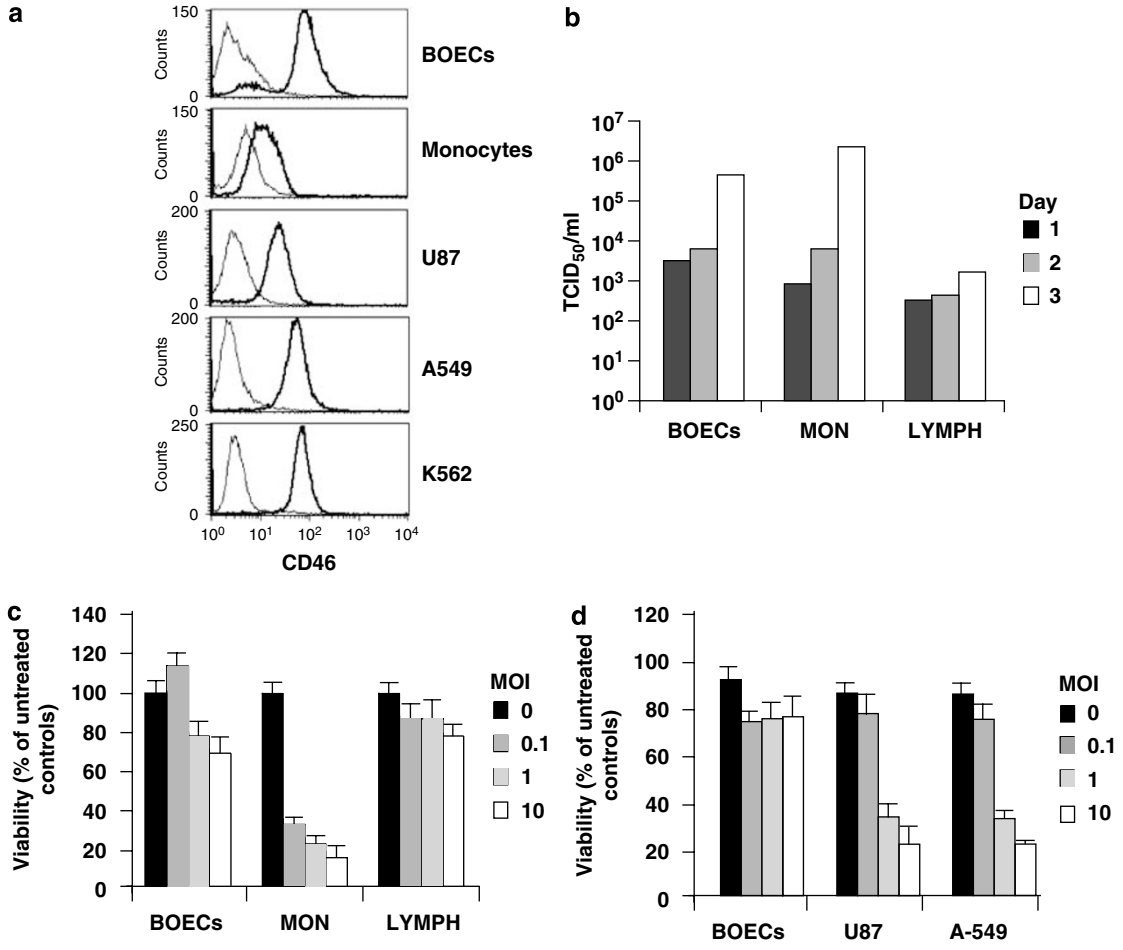


Figure 1 BOECs (blood outgrowth endothelial cells) allow efficient replication of MV-Edm, while being transiently resistant to measles viruses of the Edmonston B strain (MV-Edm)-induced cell death. (**a**) BOECs strongly express CD46, as do human U87 glioma, A549 lung carcinoma and K562 myelomonocytic leukemia cells. Cell surface expression of CD46 was determined by fluorescence-activated cell sorting analysis using anti-CD46 and isotype-matched control antibodies (bold and thin lines, respectively). Similar results were obtained in two independent experiments. (**b**) MV-Edm efficiently replicate within BOECs. BOECs, human monocytes (MON) and human lymphocytes (LYMPH) were infected with MV-Edm at a MOI of 2. At the time points indicated, MV-Edm was procured from cell lysates and Vero indicator cells were infected. Shown is the $TCID_{50}$ per ml of cell lysate supernatant. (**c**) BOECs are initially resistant to MV-Edm-induced cell death. BOECs, human monocytes and human lymphocytes were seeded in 96-well plates (2×10^3 BOECs, 2×10^4 monocytes and 2×10^5 lymphocytes per well) and infected with MV-Edm at increasing MOIs. Cell viability was determined 3 days after infection by the MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. Results are means of six duplicates and are expressed as percentage of untreated controls. (**d**) U87 glioma cells are susceptible to MV-Edm-induced cell death. BOECs, U87 glioma cells and A549 lung carcinoma cells were seeded at 2×10^3 cells per well in 96-well plates. Cell viability was determined by MTT assay 3 days after infection by MV-Edm with increasing MOIs. Results are means of six duplicates and are expressed as percentage of untreated controls. Similar results were obtained in two independent experiments.

most patients. Indeed, infection of U87 by MV-Edm was almost completely abrogated by human MV immune serum (Figure 4Aa), which rescued the viability of U87 cells (Figure 4Ac, upper panels). Of note, just 1% immune serum sufficed to prevent MV-Edm-induced cell death (Figure 4Ac). In contrast, MV antibody-negative serum did not prevent infection (Figure 4Ab) and cell death (Figure 4Ac, lower panels) of U87 cells by MV-Edm.

We reasoned that BOECs should protect MV-Edm from neutralization by human measles antibodies. To simulate the situation that would occur in immune hosts immediately after i.v. injection of BOEC/MV-Edm, we first exposed BOEC/MV-Edm to human measles immune serum for a short period of time (30 min) before adding them to U87 cells. As shown in Figure 4B, 48 h later MV-Edm had spread to surrounding U87 cells and formed syncytia, even when BOEC/MV-Edm had been preincubated with measles immune serum at concentrations as high as 90% (Figure 4B, right panel). In contrast, when MV-Edm was exposed to immune serum, the infectious capability of MV-Edm on tumor cells was neutralized (Figure 4B, left panel).

Next we wanted to know whether the protection of MV-Edm provided by the BOECs would allow the spread of MV-Edm during continuous exposure to human measles immune serum. After a 30 min preincubation with measles immune serum, BOEC/MV-Edm were added to U87 glioma cells in the presence of 90% measles immune serum. Indeed, despite the continuous presence of measles antibodies, MV-Edm-eGFP spread from a single BOEC/MV-Edm-eGFP (Figure 4Cb, arrow) to surrounding U87 cells and to more distant tumor cells, eventually causing the formation of a syncytium (Figure 4Cd). This suggests that the spread of MV-Edm from

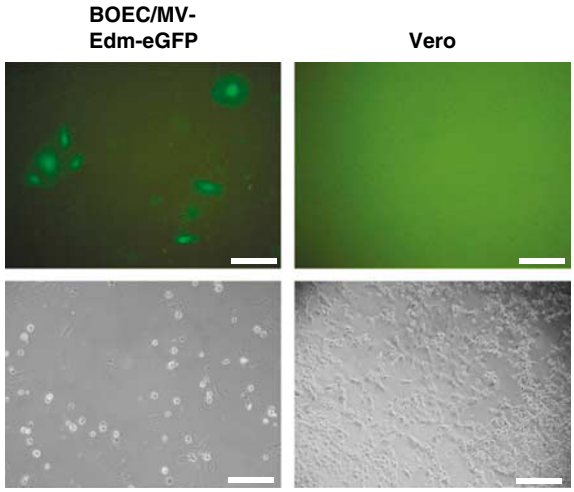


Figure 2 BOECs do not carry adsorbed MV-Edm-eGFP on their surface shortly after infection with MV-Edm-eGFP. BOECs were infected with MV-Edm-eGFP at a MOI of 2 for 3 h in OptiMEM and washed with phosphate-buffered saline. After incubation for 1 h in growth medium, BOECs were trypsinized and resuspended in OptiMEM. The cell suspension was centrifuged and the cell-free supernatant was added to Vero cells. The infected BOEC/MV-Edm-eGFP were replated in complete growth medium. The left panels show adherent BOEC/MV-Edm-eGFP 48 h after infection; the right panel, Vero cells after 72 h of incubation with supernatant. Scale bars represent 100 μm. BOECs, blood outgrowth endothelial cells; MV-Edm, measles viruses of the Edmonston B strain.

BOECs to cancer cells and from cancer cells to cancer cells is protected against human measles antibodies because spread proceeds by cell-to-cell contact.

Intratumoral administration of BOEC/MV-Edm significantly prolongs the life of mice with orthotopic U87 gliomas

To determine whether BOEC/MV-Edm exert a tumor inhibitory effect *in vivo*, we injected BOEC/MV-Edm stereotactically into U87 gliomas growing in the striatum of mice. Survival of mice was significantly prolonged compared to controls, which received intratumoral injections of phosphate-buffered saline (PBS) (Figure 5, red and black lines, respectively). The therapeutic efficacy of BOEC/MV-Edm was similar to MV-Edm injected intratumorally (blue line). Since noninfected BOECs do not kill tumor cells[21] this indicates that MV-Edm spread from BOEC/MV-Edm to tumor cells and killed them.

I.v. injected BOECs home to orthotopic U87 gliomas and, when armed with MV-Edm, decrease tumor size

We first confirmed that BOECs target orthotopic gliomas. Since irradiation has been shown to promote homing of hematopoietic progenitors toward gliomas,[19] cranial irradiation was employed prior to i.v. injection of BOECs. Because the autofluorescence of U87 gliomas complicates the interpretation of fluorescent signals, BOECs were labeled *ex vivo* with BrdU. Anti-BrdU staining showed i.v. injected BOECs within the gliomas but not in surrounding normal brain tissue (Figures 6Aa–c).

Next, we determined whether BOEC/MV-Edm given i.v. prolong the life of mice with orthotopic U87 gliomas. After five i.v. injections of BOEC/MV-Edm (denoted by arrows in Figure 6B), there was only a slight, albeit statistically significant, survival advantage (Figure 6B, red line) compared to control mice treated with uninfected BOECs or PBS (green and black lines, respectively). Mice treated with cell-free MV-Edm i.v. (blue line) survived significantly longer than both the control and the BOEC/MV-Edm-treated groups.

Magnetic resonance imaging (MRI) images of fixed brains of mice that died on day 28 revealed significantly smaller tumors in mice treated with BOEC/MV-Edm when compared to nontreated mice (Figure 6C). When tumor volumes were calculated from the MRI images, the volume of gliomas treated with BOEC/MV-Edm was clearly smaller than that of untreated gliomas (Figure 6C).

Peritumorally injected BOEC/MV-Edm migrate to orthotopic U87 gliomas

Given the infiltrative nature of gliomas leading to small clusters of glioma cells that initially lack vessels, an efficient cellular vehicle has to search for glioma cells navigating through normal brain tissue. Since a robust infiltrative human glioma mouse model is lacking, we investigated cell migration by injecting BrdU-labeled BOEC/MV-Edm into normal brain tissue surrounding U87 gliomas. Two days after BOEC injection, BOECs were visualized by anti-BrdU staining. While the majority of BOECs remained at the injection site (Figure 7Aa), numerous BOECs migrated toward the tumor (Figure 7Ab) and accumulated there (Figure 7Ac). To determine the presence of measles virus in the glioma, adjacent sections were stained separately with anti-measles hemagglutinin antibody and anti-BrdU, thus avoiding the pitfalls of immunohistochemical double staining. BrdU-positive BOECs were seen at sites where MV were detected (compare the MV-containing cells in Figure 7Bc with the BOECs in Figure 7Bd). This indicates that the peritumorally injected BOEC/MV-Edm carried MV-Edm to the tumor mass.

We then investigated whether BOECs target orthotopic gliomas in mice passively immunized against measles virus. Intraperitoneal injection of human measles antibodies generated levels of measles-specific immunoglobin G (IgG) antibodies in the blood known to prevent measles in humans (ELISA index greater than 1.1). Such preventive levels were found at 1 h, increased at 6 h and were still present 28 h after injection (data not shown). The presence of antibodies against measles virus did not preclude that many BOEC/MV-Edm injected peritumorally migrated into the tumor (Figures 7Ca–c). Only very few BOEC/MV-Edm migrated into the normal brain tissue (Figures 7Ca–c).

To address the specificity of homing of BOECs, we injected BrdU-labeled human fibroblasts peritumorally into glioma-bearing mice (Figure 7Da). The fibroblasts did not migrate into the gliomas (Figure 7Db) nor into the surrounding normal brain tissue (Figure 7Dc). Thus, the ability of BOECs to target gliomas is specific.

Peritumorally injected BOEC/MV-Edm significantly prolong survival of mice with orthotopic U87 gliomas by focal infection

Having shown the tumor searching capability of BOEC/MV-Edm navigating through the brain, we wanted to clarify whether peritumorally applied BOEC/MV-Edm prolong survival of mice with orthotopic U87 gliomas.

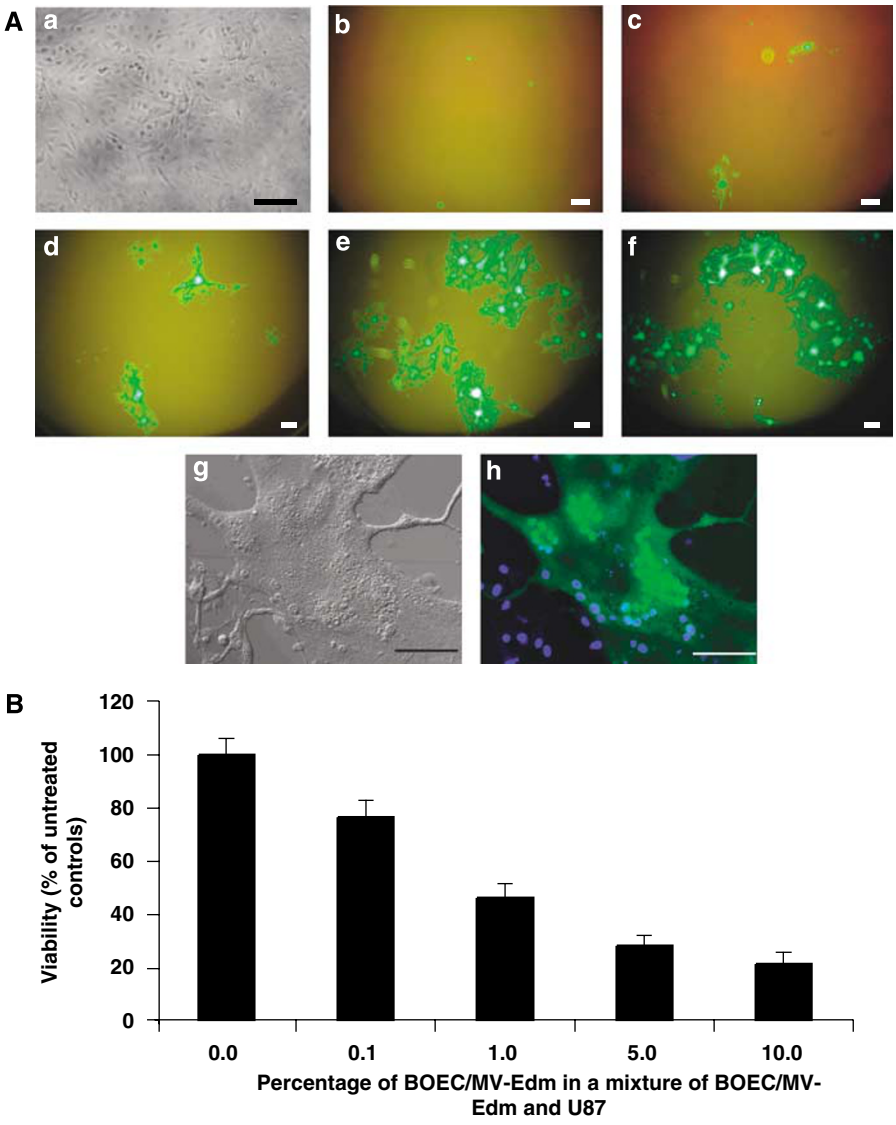


Figure 3 MV-Edm spread from BOEC/MV-Edm to surrounding U87 glioma cells and exert a strong bystander effect. BOECs were infected with MV-Edm-eGFP at a MOI of 2 for 24 h (BOEC/MV-Edm-eGFP) and added to U87 cells seeded at 2×10^3 cells per well in collagen I-coated 96-well plates in pentaplicates. (**A**) MV-Edm-eGFP spread from BOEC/MV-Edm-eGFP to U87 cells. BOEC/MV-Edm-eGFP were added to U87 cells (a; phase contrast). Spread of MV-Edm-eGFP was monitored by fluorescence microscopy at time points 2 h (b), 24 h (c), 48 h (d), 72 h (e) and 96 h (f) after the addition of MV-Edm-eGFP. A syncytium of U87 cells induced by a single BOEC/MV-Edm-eGFP is seen at 72 h after the addition of BOEC/MV-Edm-eGFP (g and h). Nuclei were stained by Hoechst 33258. Scale bars = 200 μm (a–f) and 100 μm (g and h). (**B**) BOEC/MV-Edm exert a strong bystander effect on U87 glioma cells *in vitro*. BOEC/MV-Edm-eGFP were added to U87 cells at shares indicated and viability was determined by MTT assay after 4 days. Results are expressed as percentage of untreated controls. Means and standard deviations of pentaplicates are shown. Similar results were obtained in three independent experiments. MV-Edm, measles viruses of the Edmonston B strain; BOECs, blood outgrowth endothelial cells.

After three sequential peritumoral injections at alternating sites (Figure 8A), mice treated with BOEC/MV-Edm (Figure 8B, red line) survived significantly longer than those injected with BOECs (green line) or with PBS (black line). Importantly, mice treated with BOEC/MV-Edm survived longer than MV-Edm-treated mice (blue line). This shows that tumor control is enhanced when the immobile MV-Edm are delivered by tumor targeting BOECs to the tumor.

The extent of intratumoral spread of MV-Edm after peritumoral injection of BOEC/MV-Edm was investigated in a subset of mice at the time of death. Syncytium formation by MV-Edm and persistent infection by MV-Edm without formation of syncytia was clearly focal, with large areas of unaffected tumor tissue surrounding islands of affected tissue (Figure 8C).

Discussion

Inefficient delivery of therapeutic modalities and isolation of glioma cells due to infiltrative growth are major hurdles toward improving the poor prognosis of malignant gliomas. We show that replicating oncolytic

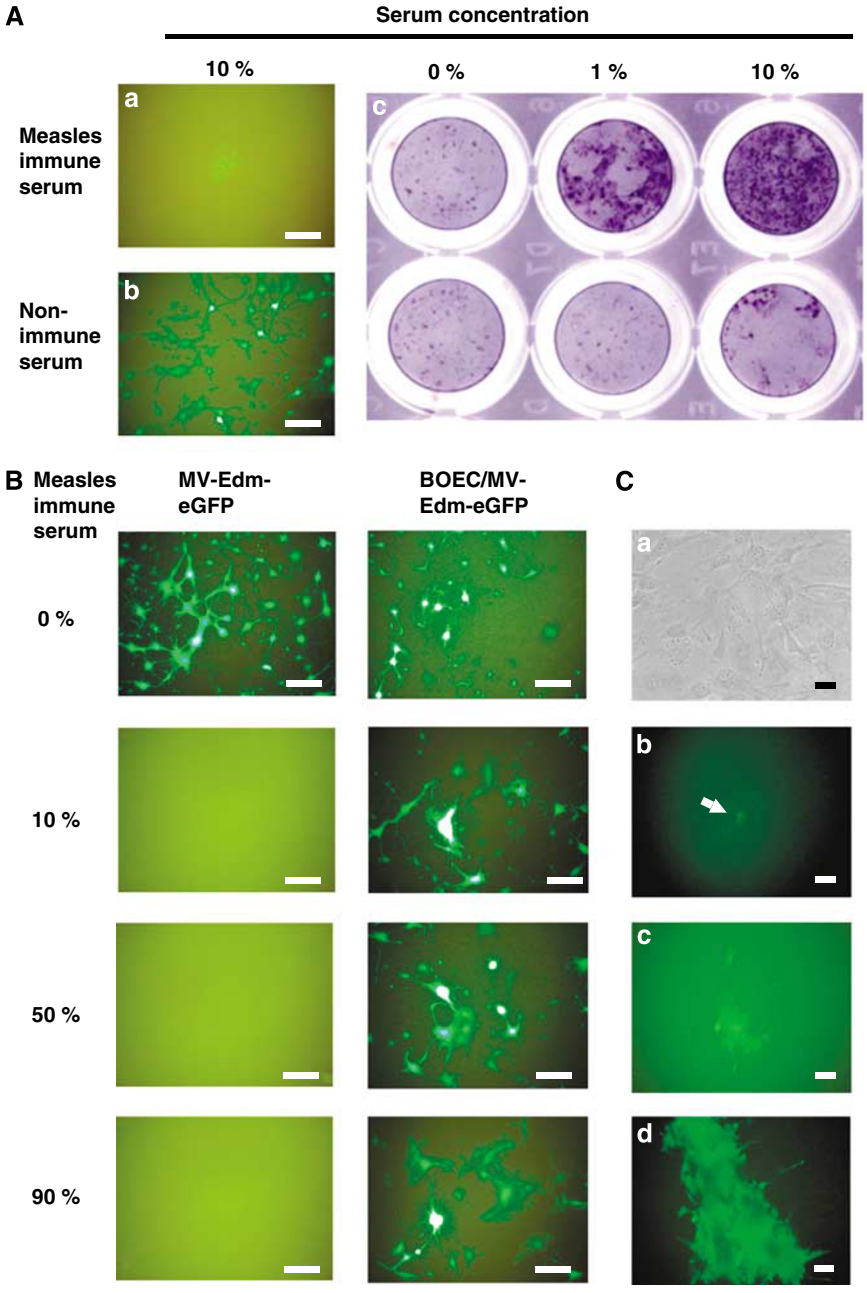


Figure 4 MV-Edm protected within BOECs from human measles immune serum spread to and kill bystander tumor cells. (**A**) Human measles immune serum prevents MV-Edm-eGFP from infecting and killing U87 cells. MV-Edm-eGFP were incubated with human measles immune serum or with human nonimmune serum at concentrations of 0, 1 and 10% for 30 min at 37 °C and then added at a MOI of 5 to U87 cells seeded at 2×10^3 cells per well on 96-well plates. The spread of MV-Edm-eGFP and syncytia formation was monitored by fluorescence microscopy at 72 h after infection (a and b, scale bars = 500 μm). Cell viability was determined by crystal violet staining at 96 h after infection (c). (**B**) BOECs protect MV-Edm from exposure to human measles immune serum, thus allowing their spread to U87 cells. BOEC/MV-Edm-eGFP and MV-Edm-eGFP were incubated with measles immune serum for 30 min at concentrations indicated before being added to U87 cells. MV-Edm-eGFP at a MOI of 5 (left panel) or 2×10^2 BOEC/MV-Edm-eGFP cells (right panel) were added to 2×10^3 U87 cells per well in a 96-well plate. Spread of MV-Edm-eGFP and syncytia formation was observed by fluorescence microscopy at 48 h after infection (scale bars = 500 μm). (**C**) MV-Edm can spread from BOECs to U87 cells in the continuous presence of human measles immune serum. 2×10^2 BOEC/MV-Edm-eGFP preincubated for 30 min with 90% measles immune serum were transferred to 2×10^3 U87 cells per well in a 96-well plate containing medium with 90% measles immune serum (a, phase contrast, scale bar = 25 μm). The spread of MV-Edm-eGFP and syncytia formation was monitored by fluorescence microscopy at 18 h (b), 48 h (c) and 72 h (d). Scale bar = 100 μm for (b–d). MV-Edm, measles viruses of the Edmonston B strain; BOECs, blood outgrowth endothelial cells.

MV-Edm carried within BOECs to malignant gliomas may be one approach to overcome these hurdles. BOECs protected MV-Edm from human measles serum antibodies, homed to brain tumors and carried MV-Edm through normal brain tissue to the tumors. Subsequently, MV-Edm released from BOECs infected brain tumor cells causing shrinkage of tumors, which prolonged the life of tumor-bearing mice.

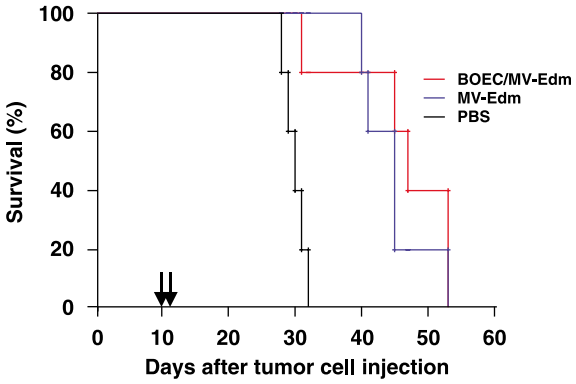


Figure 5 Intratumorally injected BOEC/MV-Edm significantly prolong the life of mice with orthotopic U87 gliomas. 5×10^5 U87 glioma cells were stereotactically injected into the right striatum of 10- to 12-week-old male Rag2$^{-/-}$cγc$^{-/-}$ mice. Ten days later, mice were randomized into three groups ($n = 5$ for each group). The treatment groups received two intratumoral injections (denoted by arrows) spaced 1 day apart of either 2×10^5 BOEC/MV-Edm or 3×10^5 PFU MV-Edm, while the control group received PBS. Survival was determined and plotted for Kaplan–Meier survival analysis and analyzed by log-rank test. Compared to PBS (black line), survival of both BOEC/MV-Edm (red line) and MV-Edm (blue line) was prolonged ($P = 0.0088$ and 0.0018, respectively). BOECs, blood outgrowth endothelial cells; MV-Edm, measles viruses of the Edmonston B strain; PBS, phosphate-buffered saline.

For this approach to be successful, several prerequisites have to be met in sequence: easy procurement of BOECs, efficient infection of BOECs by MV-Edm, replication of MV-Edm within the BOECs, resistance of BOECs to MV-Edm-induced early cell death, protection of MV-Edm against MV-neutralizing serum activity, efficient homing and specific migration of MV-Edm-infected BOECs to glioma cells, proficient infection of glioma cells by MV-Edm from BOECs, intratumoral spread of MV-Edm within the glioma and finally killing of the infected glioma cells.

The ease of BOEC generation as well as determinants of efficacy concerning their use in gene therapy of peripheral metastases has been established previously.[20,21]

MV-Edm infected and efficiently replicated within BOECs without killing them during the first few days after infection. This predisposes BOECs to be carriers for MV-Edm as compared to other blood-derived cells such as monocytes, which were rapidly killed by replicating MV-Edm, and lymphocytes, which did not allow MV-Edm to replicate sufficiently. BOECs were resistant to MV-Edm-induced cell death despite the strong expression of CD46, known not only to function as the entry receptor for MV but also to determine the degree of cell death.[6] The multitude of antiapoptotic mechanisms operative in endothelial cells most likely accounts for this resistance. These mechanisms include VEGF-induced PI3K-mediated signaling, nuclear factor-κB activation and strong expression of Bcl-2, survivin and XIAP, and of antioxidative enzymes such as MnSOD, catalase and glutathione peroxidase (reviewed in Jarmy *et al*.[16]).

Many cell types infected with MV-Edm are killed by complement-mediated lysis, which is facilitated by downregulation of CD46, an inhibitor of complement-mediated lysis, after MV-Edm infection.[22] Of note, BOEC/MV-Edm were not killed when subjected to human measles immune serum containing complement. CD46 may not have been downregulated yet and MV proteins may not have appeared yet on the surface of infected BOECs during the time of transit to the tumor. Furthermore, the degree of downregulation of CD46 may be limited in BOECs. Further studies will elucidate the mechanisms involved.

Homing of BOEC/MV-Edm into gliomas also occurred when measles virus antibodies were present at levels sufficient to protect humans from measles. It remains to be shown whether BOEC/MV-Edm are susceptible to T cell-mediated destruction. The low number of T cells in cancer patients secondary to disease and previous chemotherapy may reduce this potential problem. T cells may also exert a beneficial effect by reacting against MV-Edm-infected tumor cells, thus enhancing the oncolytic effect of MV-Edm.

Specific and efficient homing of BOEC/MV-Edm to gliomas is crucial for their therapeutic efficacy. Intravenously injected BOECs homed into irradiated orthotopic U87 malignant gliomas, but did not sequester into normal brain tissue. More importantly, when BOEC/MV-Edm were injected peritumorally to simulate the situation in the patient, where BOEC/MV-Edm have to reach isolated glioma cells not yet connected to blood vessels, BOEC/MV-Edm navigated through normal brain tissue to the glioma mass. Migration appeared to be directional, because far more BOEC/MV-Edm were seen within the glioma than in the normal brain tissue surrounding the injection site and since human fibroblasts injected in the same manner did not target gliomas. It is important to note that infection with MV-Edm did not preclude migration of peritumorally injected BOECs, even in mice immune against measles virus.

Cranial irradiation was employed assuming that it would increase migration of BOECs to gliomas as has been described for hematopoietic stem cells. However, we could not demonstrate increased infiltration of BOECs into irradiated tumor spheroids;[23] thus the role of irradiation in the homing of BOECs remains to be elucidated.

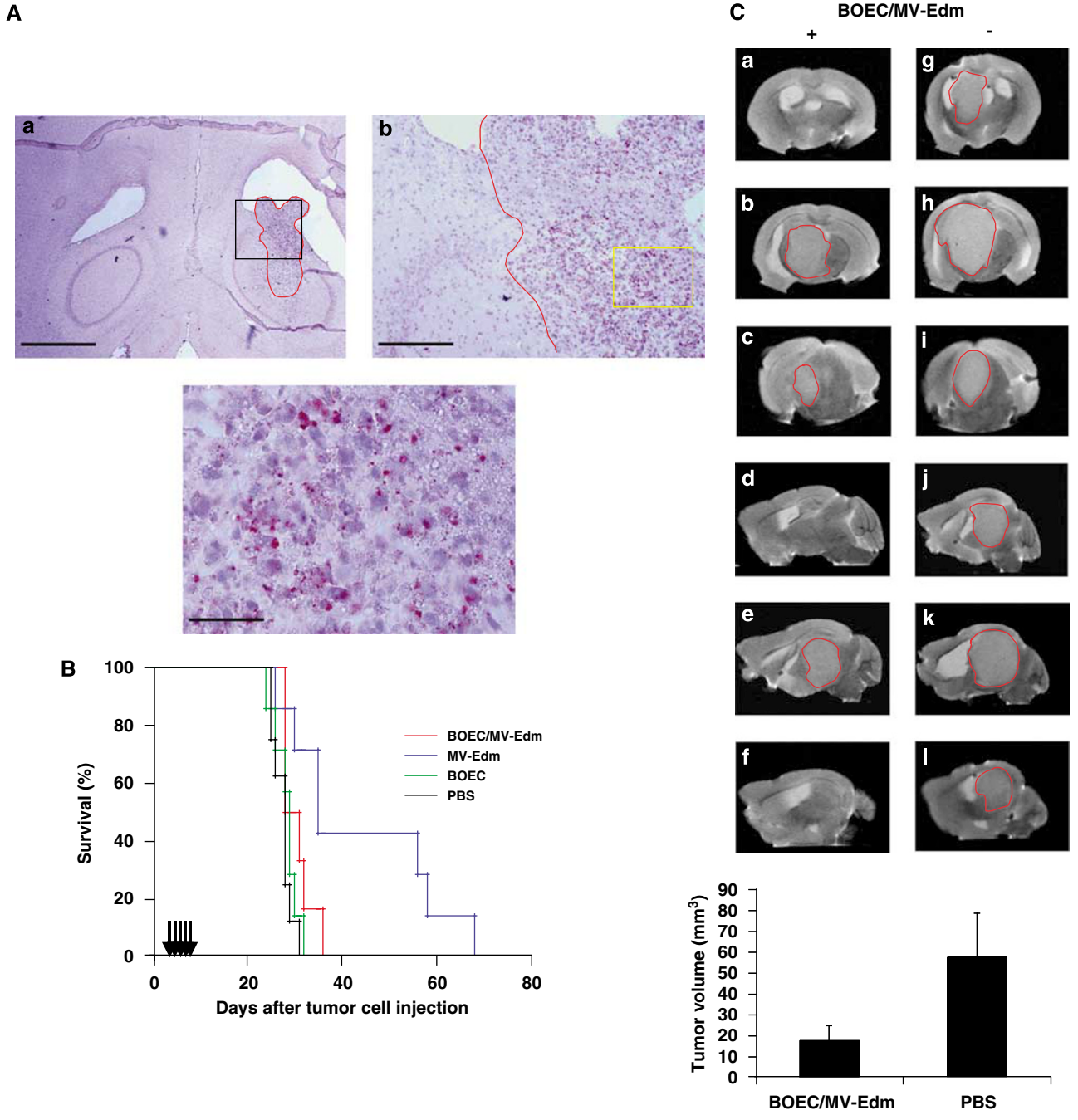
Once at the glioma cells, MV-Edm residing within the BOECs have to infect the glioma cells. This was indeed the case, despite the heterologous nature of BOECs and glioma cells. We have no evidence that infection of surrounding cells occurred by MV-Edm-eGFP that remained loosely adsorbed on the surface of BOECs following their infection with MV-Edm-eGFP *ex vivo*. However, it remains possible that some MV-Edm-eGFP strongly tethered to the BOECs surface may have contributed to the infection of tumor bystander cells.

After infection of glioma cells surrounding the BOEC/MV-Edm, the virus spread to more distal glioma cells and killed them. *In vitro*, a few BOEC/MV-Edm sufficed to infect a far larger number of glioma cells and to kill most of them. *In vivo*, glioma cells were infected and killed by intratumorally injected BOEC/MV-Edm leading to a significant survival benefit of glioma-bearing mice indistinguishable from those receiving intratumorally injected MV-Edm. Of note, despite the presence of MV-neutralizing serum antibodies *in vitro*, MV-Edm spread from BOECs to surrounding and then to more distant glioma cells inducing syncytia. This shows that U87 glioma cells can be controlled by BOEC/MV-Edm in the face of natural MV-neutralizing activity. As a corollary, spread by tumor-cell-to-tumor-cell contact, as

has been described for MV-Edm in other tumors,[7,8] appears to be the relevant route MV-Edm can spread in U87 gliomas.

We show that i.v. injected BOEC/MV-Edm home specifically to orthotopic U87 gliomas and that BOEC/MV-Edm decrease the size of the tumors, as detected by MRI. However, because the responses were partial (with some tumors continuing to grow into the brain stem, as in Figure 6Ce), the survival benefit was minute. In contrast, i.v. injected cell-free MV prolonged survival more pronounced than i.v. injected BOEC/MV-Edm. It is known that some i.v. injected BOECs are sequestered in the lung and the spleen.[21] Sequestration of i.v. injected MV-Edm may be less, which could explain their increased efficacy. In addition, the blood–brain barrier is less permeable for large cells such as BOECs than for viruses with their smaller size. This may also have led to increased extravasation of MV-Edm into the cerebrospinal fluid and thus to increased infection of tumors abutting the ventricle.

I.v. injection of MV-Edm will not be feasible in most patients, given their immunity against measles. BOEC/MV-Edm are not affected by measles antibodies; however, they show limited efficacy against gliomas when given i.v. Thus, intracranial injection of BOEC/MV-Edm is an obvious route to ensure high-level and consistent delivery. We injected BOEC/MV-Edm peritumorally to mimic the clinical situation, where BOECs would have to



migrate to isolated clusters of glioma cells. BOEC/MV-Edm navigated through normal brain tissue, infected glioma cells and killed them, thus significantly prolonging the life of glioma-bearing mice. Therefore, BOEC/MV-Edm have the search-and-destroy capability to target infiltrative gliomas. Surprisingly, there was a survival benefit of peritumorally injected MV-Edm. Thus, some of the MV must have found their way into the tumor despite their immobility. This most likely occurred by convection of the small MV-Edm to the tumor by increased hydrostatic pressure due to the large carrier volume applied during the injections of MV-Edm. This factitious convection-enhanced delivery of MV-Edm was nevertheless inferior to delivery via the actively tumor searching BOECs, since there was a clear trend toward increased survival for BOEC/MV-Edm. Again, in the measles immune glioma patient, cell-free MV-Edm may be subjected to neutralization, in contrast to MV-Edm protected within BOECs, adding to the superiority of BOEC/MV-Edm over MV-Edm when applied intracranially.

For glioma abutting or growing into the ventricles (as in Figure 6Aa), intrathecal injection of BOEC/MV-Edm may be the most efficient route of delivery.

While tumor size decreased and survival was prolonged upon administration of BOEC/MV-Edm, no cures were achieved since responses were partial, as shown by MRI. Intratumoral heterogeneity of response was reflected on the cellular level. After peritumoral injection, BOEC/MV-Edm infiltrated and infected large parts of the tumors. At the time of death, however, the distribution of syncytia and MV-Edm-infected cells was focal. There are several explanations for this finding. First, proliferation of uninfected tumor cells must have outpaced the spread of MV, in line with recent findings in experimental ovarian carcinoma.[24] It remains to be determined whether attenuation of the spread of MV-Edm contributes to this. Second, the presence of foci of persistent MV-Edm infection without cytopathic effect suggests resistance of tumor cells to MV-Edm-induced cell death. Finally, the migratory function of BOECV/MV-Edm within the tumor must have subsided, most likely because MV-Edm finally overcame the resistance of BOECs to early cell death. Elucidating the mechanisms of intratumoral virus spread and of MV-Edm-induced cell death, and modulating them, such as by arming MV-Edm with additional tumor cytotoxic effectors,[11,25,26] will address the challenge of increasing the efficacy of BOEC/MV-Edm for therapy of gliomas and other tumors.

Several oncolytic viruses in diverse cellular vehicles have been successful in preclinical studies. These encompass herpes simplex virus-1 in human teratocarcinoma cells,[27] retrovirus in outgrowth endothelial cells,[28] retrovirus on antigen-specifc T cells[29] and modified vaccinia virus in cytokine-induced killer cells.[30] Their relative merit for glioma therapy has yet to be defined. The nonintegrating MV do not carry the risk of insertional mutagenesis, unlike retroviruses. Not all the positive features of BOECs are shared by the other cellular vehicles that have been used to carry oncolytic viruses to experimental tumors. Tumor antigen-specific T cells, for example, are complex to generate,[29] a feature they share with neural progenitors that target experimental brain tumors,[31] and may lack the ability to navigate through normal brain tissue. The latter may also apply to cytokine-induced killer cells.[30]

In conclusion, BOEC/MV-Edm warrant to be included in future studies of cellular vehicles carrying oncolytic viruses for the therapy of gliomas.

Materials and methods

Cell culture

Tissue culture media and supplements were obtained from Life Technologies (Eggenstein, Germany) unless stated otherwise. BOECs were isolated from human adult peripheral blood, propagated as described[16] and cultured in ECBM-2 medium (PromoCell, Heidelberg, Germany) supplemented with SupplementPack (PromoCell) and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany). Human mononuclear cells were isolated from peripheral blood cells and separated into monocytes and lymphocytes by adherence to plastic. Monocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 15% human serum. Lymphocytes were cultivated in RPMI supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 1 mM pyruvate and 30 U ml^{-1} interleukin-2 (Pepro Tech, Rocky Hill, NJ, USA). U87, K-562 cells and Vero cells were

Figure 6 I.v. applied BOECs target orthotopic U87 gliomas and, when carrying MV-Edm, decrease tumor size. 5×10^5 U87 glioma cells were injected stereotactically into the right striatum of 8- to 12-week-old male $Rag2^{-/-}c\gamma c^{-/-}$ mice. (**A**) BOECs home to U87 gliomas in the brain when given by tail vein injection. Fourteen days after tumor cell injection, the brain of U87 glioma-bearing mice was γ-irradiated with a dose of 6 Gy. Three days later, the mice received tail vein injection of BOECs pulsed with BrdU (BOEC-BrdU, $n = 3$). Organs were procured 1 day later, cryosectioned, immunohistochemically stained using anti-BrdU antibody to detect BOEC-BrdU and counterstained with hematoxylin. A U87 glioma outlined in red is seen in the right striatum (a, scale bar = 1 mm). Higher magnification of the black frame in (a) shows BrdU-positive (red-stained) BOECs in the glioma, but not in the surrounding normal brain tissue (b, scale bar = 200 μm). Higher magnification of the yellow frame in (b) demonstrates accumulation of BOECs in the glioma (c, scale bar = 50 μm). (**B**) I.v. injected BOEC/MV-Edm just slightly prolong the life of mice with orthotopic U87 gliomas. Four days after tumor cell injection, mice received cranial γ-irradiation with a dose of 6 Gy and were randomized into four groups on day 6 followed by daily i.v. injections for 5 days (indicated by arrows). The treatment groups received BOEC/MV-Edm or MV-Edm and the control groups BOECs or PBS. Compared to control mice treated with PBS (black line) or BOECs (green line), survival of mice injected with BOEC/MV-Edm (red line) was slightly prolonged ($P = 0.0498$), whereas mice treated with MV-Edm (blue line) had a more pronounced survival benefit ($P = 0.0035$). Statistical testing was by log-rank test. (**C**) I.v. injected BOEC/MV-Edm decrease the size of orthotopic gliomas. Three mice treated with BOEC/MV-Edm and three untreated control mice died on day 28. The brains of these mice were procured, fixed in 4% paraformaldehyde and subjected to serial MRI. The upper panel shows corresponding axial planes in an anterior to posterior order (a–c and g–i) and sagittal planes in a left to right order (d–f and j–l) of two representative mice that either received or did not receive BOEC/MV-Edm (+ or −). The gliomas are outlined in red. Smaller size of the gliomas treated with BOEC/MV-Edm is discernible as absence or smaller area of tumor on corresponding planes. The lower panel depicts the means and standard deviations of the tumor volumes calculated from the MRI scans. I.v. intravenously; BOECs, blood outgrowth endothelial cells; MV-Edm, measles viruses of the Edmonston B strain; PBS, phosphate-buffered saline; MRI, magnetic resonance imaging.

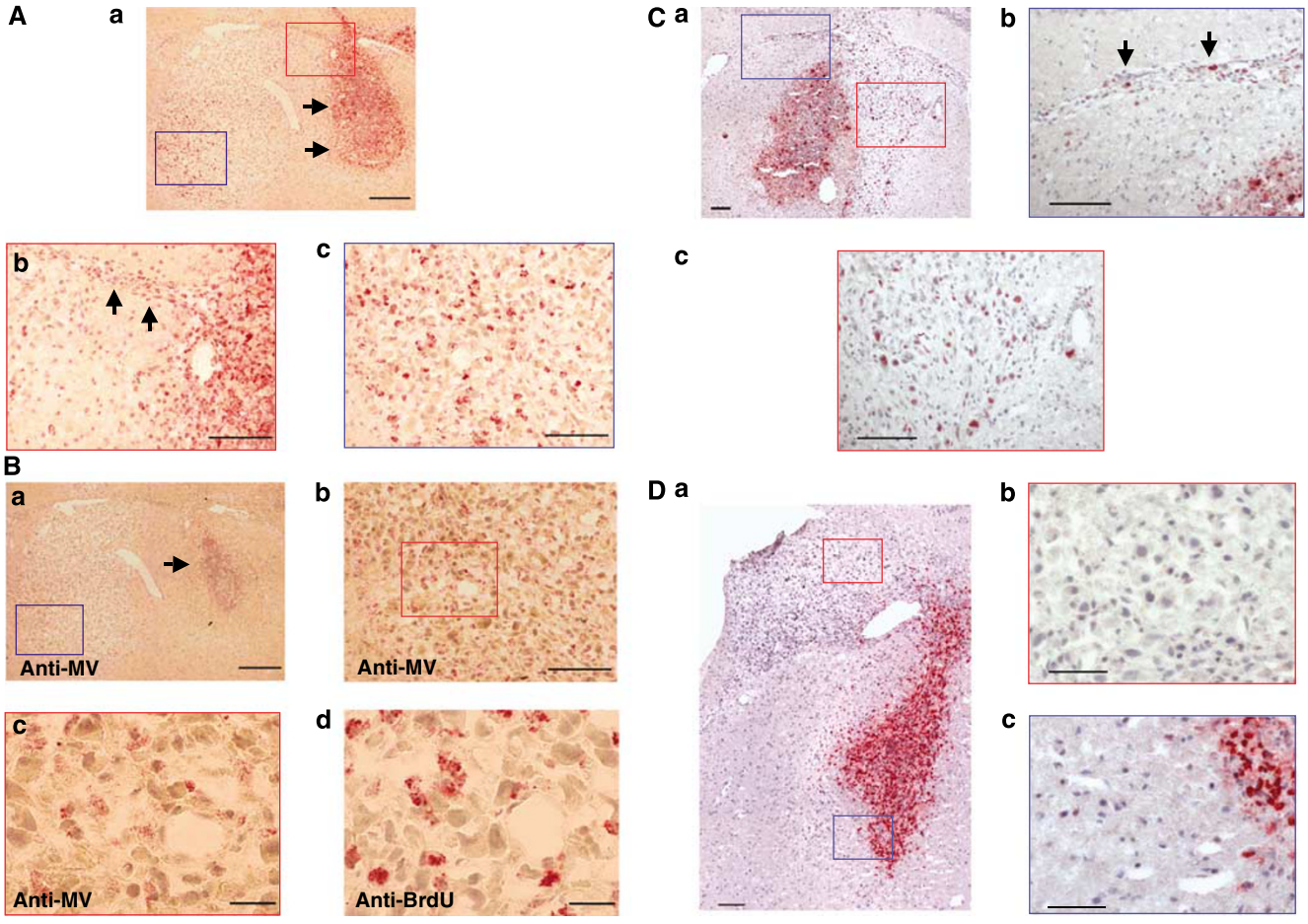


Figure 7 Peritumorally injected BOEC/MV-Edm specifically target orthotopic U87 gliomas in nonimmune mice and in mice passively immunized against measles. 5×10^5 U87 glioma cells were stereotactically injected into the right striatum of 8- to 10-week-old male Rag2$^{-/-}$cγc$^{-/-}$ mice. Ten days after tumor cell injection, mice received 6 Gy of γ-irradiation to the brain. Three days later, BOECs pulsed with BrdU and infected with MV-Edm were injected peritumorally into mice that were not immunized (**A**, **B**; $n = 3$) or immunized by intraperitoneal injection of 500 µl human measles immune serum 18 h prior to injection of BOEC/MV-Edm (**C**; $n = 3$). (**A**) BOEC/MV-Edm navigate within the brain of nonimmunized mice to U87 gliomas. Brains were procured 2 days later and cryosectioned horizontally. BOECs were detected by anti-BrdU immunohistochemical staining. Counterstaining was by hematoxylin. In (a) (scale bar = 1 mm), BrdU-positive BOECs (red-stained nuclei) are seen deposited at the end of the injection canal (arrows) lateral of the hypercellular glioma. Higher magnification of the red-framed area in (a) shows a stream of BrdU-positive BOECs migrating (arrows) toward the tumor (b, scale bar = 100 µm). Higher magnification of the blue frame in (a) depicts BrdU-positive cells accumulating within the tumor (c, scale bar = 100 µm). (**B**) The distribution of intratumoral MV-Edm mirrors the intratumoral spread of BOEC/MV-Edm. Adjacent cryosections were stained by anti-BrdU for BOEC/MV-Edm and by anti-measles hemagglutinin for MV-Edm. Low magnification (a, scale bar = 250 µm) shows MV-Edm-containing cells stained red at the injection site (indicated by an arrow). Higher magnification of the blue-framed area reveals MV-positive cells within the tumor (b, scale bar = 100 µm), which are clearly delineated at higher magnification of the red-framed area (c, scale bar = 25 µm). A section adjacent to (c) shows BrdU-positive BOECs (d, scale bar = 25 µm). (**C**) BOEC/MV-Edm navigate to U87 gliomas in passively immunized mice. In these coronal sections, BrdU-positive BOECs (red) are seen at the injection site and in the tumor (a, scale bar = 100 µm). Higher magnification of the blue-framed area in (a) shows BrdU-positive BOECs following a sheath of tumor cells (arrow) without migrating into the normal brain tissue (b, scale bar = 100 µm). Higher magnification of the red frame in (a) delineates BrdU-positive cells spreading within the main tumor mass (c, scale bar = 100 µm). (**D**) Human fibroblasts injected peritumorally do not migrate to U87 gliomas. Human foreskin fibroblasts were injected peritumorally into U87-bearing, cranially irradiated mice. Two days later the brains were procured, stained by anti-BrdU and counterstained by hematoxylin (a, scale bar = 100 µm). Higher magnification of the red- and blue-framed areas shows no migration of the fibroblasts into the glioma (b, scale bar = 50 µm) or the normal brain (c, scale bar = 50 µm), respectively. BOECs, blood outgrowth endothelial cells; MV-Edm, measles viruses of the Edmonston B strain.

obtained from ATCC (Manassas, VA, USA) and A549 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). K-562 cells were maintained in RPMI and all other tumor cell lines were grown in DMEM. Cell culture media for cell lines were supplemented with 10% FCS, 2 mM glutamine, 2 mM nonessential amino acids, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin.

Virus production and infection

The construction of MV-Edm-eGFP has been described elsewhere.[32] MV-Edm and MV-Edm-eGFP were propagated by Vero cells with a MOI of 0.02 in 2 ml OptiMEM (Life Technologies) at 37 °C for 3 h. The medium was changed to DMEM supplemented with 5% FCS and cells were incubated at 37 °C for 1 day before being transferred to 32 °C for another day. Cells were harvested

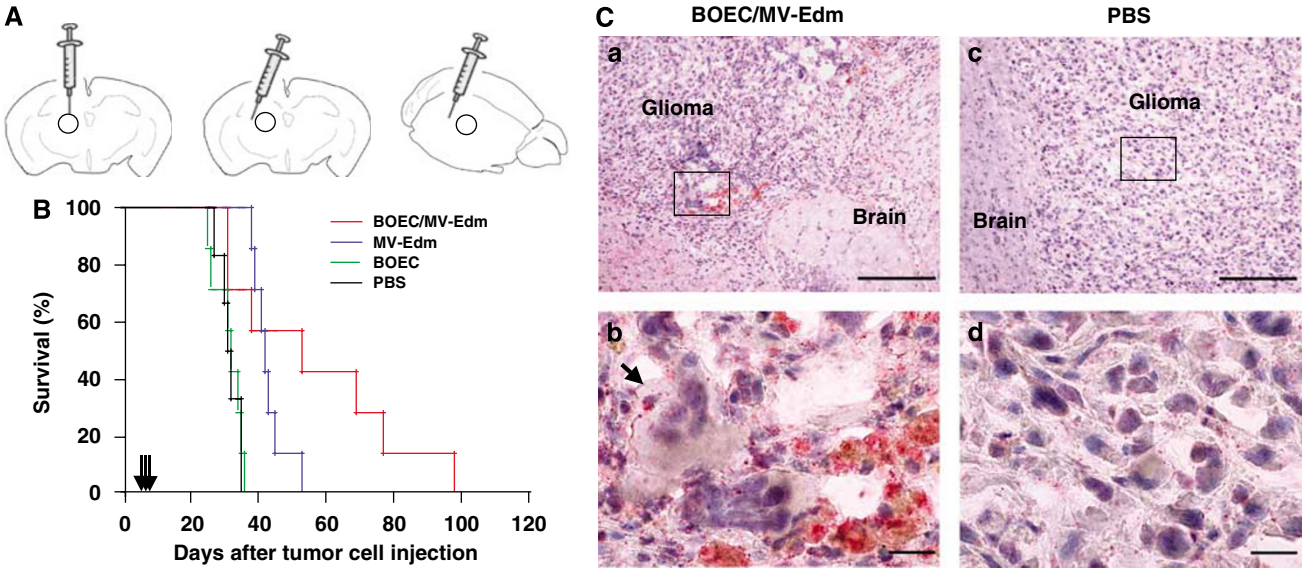


Figure 8 Peritumorally injected BOEC/MV-Edm significantly prolong survival of mice with orthotopic U87 gliomas by focal infection. 5×10^5 U87 glioma cells were stereotactically injected into the right striatum of 8- to 10-week-old male Rag2$^{-/-}$cγc$^{-/-}$ mice. Four days after tumor cell injection, the mice received 6 Gy of cranial γ-irradiation. On day 6, the mice were randomized into four groups and received daily peritumoral injections for 3 days at alternating sites. (**A**) Schematic of peritumoral injections. Injections were placed 1 mm superior, 0.5 mm lateral and 0.5 mm posterior to the tumor mass. (**B**) Mice treated with peritumoral injections of BOEC/MV-Edm survive longer. The treatment groups received BOEC/MV-Edm ($n=7$) or MV-Edm ($n=7$) and the control groups BOECs ($n=7$) or PBS ($n=6$). Arrows denote injections. Survival of the mice was determined and plotted for Kaplan–Meier survival analysis. Mice treated with BOEC/MV-Edm (red line) survived longer than control mice receiving BOECs (green line, $P=0.0018$), PBS (black line, $P=0.0031$) or MV-Edm (blue line, $P=0.174$). Statistical testing was performed by log-rank test. (**C**) Persistent infection by MV-Edm and formation of syncytia is focal. Brains of mice that had received BOEC/MV-Edm ($n=3$) or PBS ($n=3$) were analyzed by immunohistochemistry for expression of MV hemagglutinin when they had to be killed. The images of a representative BOEC/MV-Edm-treated mouse that was killed on day 69 (a and b) and of a PBS-treated mouse killed on day 30 (c and d) are shown. Higher magnification of the area framed in (a) reveals red-stained MV-Edm-infected tumor surrounding a syncytium (b, arrow), whereas no MV-Edm can be detected in tumors treated with PBS (c and d). Scale Bars = 200 μm (a and c) and 20 μm (b and d). BOECs, blood outgrowth endothelial cells; MV-Edm, measles viruses of the Edmonston B strain; PBS, phosphate-buffered saline.

and viral particles released by two cycles of snap freezing in liquid nitrogen and subsequent thawing. Viral titers were determined by 50% end-point dilution assays ($TCID_{50}$) on Vero cells.

Infection by MV-Edm or MV-Edm-eGFP in vitro

Tumor cells or BOECs were seeded on tissue culture flasks or plates. The medium was removed and the cells were infected with MV-Edm in OptiMEM for 3 h at 37 °C. The medium was then replaced with appropriate tissue culture media. BOECs infected with MV-Edm are called BOEC/MV-Edm and those infected with MV-Edm-eGFP are called BOEC/MV-Edm-eGFP.

Lymphocytes were infected in suspension for 3 h at 37 °C. After centrifugation the medium was replaced with the appropriate culture medium described above.

MV-Edm replication

BOECs and monocytes were seeded at 2×10^5 cells per well (six-well plate) and infected the following day with MV-Edm at a MOI of 2 for 3 h. Lymphocytes were infected in suspension at a MOI of 2 for 3 h and than seeded at 2×10^5 cells per well. At 24, 48 and 72 h after infection, the cells were scraped into 1 ml OptiMEM (Invitrogen, Karlsruhe, Germany) and the cell-associated viruses were released by two freeze–thaw cycles. Viral titers were determined as $TCID_{50}$ on Vero cells.

Determination of bystander infection by MV-Edm bound to the surface of BOECs

2×10^5 BOECs per well in a six-well collagen I-coated plate were infected with MV-Edm-eGFP at a MOI of 2 in OptiMEM for 3 h at 37 °C. BOECs were washed with PBS and the medium was changed to ECBM-2 with 10% FCS. After 1 h, BOECs were washed twice with PBS, trypsinized for 5 min, resuspended in OptiMEM and separated from the supernatant by centrifugation. The cell-free supernatant was used to infect 2×10^5 Vero cells per well in six-well plates. The infected BOECs were replated in growth medium to follow their expression of eGFP. Vero cells were monitored for eGFP expression 24, 48 and 72 h after infection.

Determination of viability in vitro

Prior to determination of viability, cell culture plates were ultraviolet irradiated to inactivate MV. Viability of cells was determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide) assay as described.[33] To determine viability by crystal violet staining, cells were incubated in 0.75% crystal violet ethanol solution at room temperature for 15 min, washed, dried and photographed.

Bystander effect assays

BOECs infected with MV-Edm were added to U87 glioma cells seeded at 2×10^3 cells per well in DMEM

on collagen I-coated 96-well plates at ratios of 0, 0.1, 1.0 and 10.0% for 4 days. Cell death was determined by the MTT assay.

MV neutralization assays

Serum was obtained from a healthy donor with immunity against measles as defined by high levels of measles-specific IgG antibodies using an appropriate ELISA. Nonimmune serum (as determined by ELISA) was obtained from nonimmune humans. Sera were centrifuged at 14 000 r.p.m. for 10 min at 4 °C before use. 5×10^4 PFU MV-Edm-eGFP were incubated with MV immune serum or with nonimmune serum at concentrations of 0.0, 1.0 and 10.0% in OptiMEM at 37 °C for 30 min. MV-Edm-eGFP at a MOI of 5 in 50 µl OptiMEM were added to 2×10^3 U87 glioma cells per well growing in collagen I-coated 96-well plates 2 h after infection, the infectious medium was removed, cells were washed once with PBS and then cultivated in supplemented DMEM. Cell viability was determined by crystal violet staining as described above. The spread of MV-Edm-eGFP and syncytia formation was monitored by fluorescence microscopy.

MV protection by BOECs in vitro

To investigate protection by BOECs of MV-Edm against the neutralizing effect of measles immune serum, BOECs were infected with MV-Edm-eGFP at a MOI of 2 in OptiMEM for 3 h at 37 °C and then maintained in supplemented ECBM-2 medium. Twenty-four hours after infection, BOEC/MV-Edm-eGFP were harvested and MV-Edm-eGFP were incubated in OptiMEM containing 0.0, 10.0, 50.0 and 90.0% measles immune serum at 37 °C for 30 min. Hundred BOEC/MV-Edm-eGFP or 1×10^4 MV-Edm-eGFP per well were then added to U87 glioma cells seeded at 2×10^3 cells per well on collagen I-coated 96-well plates. Two hours after the addition of BOEC/MV-Edm-eGFP or MV-Edm-eGFP, U87 cells were washed once with PBS followed by incubation in supplemented DMEM.

To investigate the spread of MV-Edm from BOECs to U87 cells in the presence of measles immune serum, BOEC/MV-Edm were preincubated with 90% measles immune serum as above and then cocultivated with U87 cells in the presence of 90% measles immune serum in supplemented DMEM for 4 days.

Fluorescence-activated cell sorting analysis

Cells were incubated with phycoerythrin-conjugated monoclonal antibodies against CD11a, CD11b, CD49d, CD162 and CXCR4 (all from BD Biosciences Pharmingen, Heidelberg, Germany), with fluorescein isothiocyanate-conjugated monoclonal antibodies against cutaneous lymphocyte-associated Ag (BD Biosciences Pharmingen), VE-cadherin (Bender MedSystems, Vienna, Austria) and with unconjugated antibodies against VEGFR2 (Sigma, Munich, Germany), CD46 and integrin $a_v\beta_5$ (both from BD Biosciences Pharmingen) followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (DAKO, Hamburg, Germany). All incubations were performed for 30 min at 4 °C. Isotype-matched antibodies (BD Biosciences Pharmingen) served as controls. Analyses were performed using a FACScan flow cytometer and CellQuest software (BD Biosciences).

Immunohistochemistry

Organs were perfused with PBS and 4% paraformaldehyde, further fixed for 2 h in 4% paraformaldehyde, cryoprotected in 20% sucrose/PBS (Roth, Karlsruhe, Germany), embedded in optimal cutting temperature compound (Sakura Finetek, Zoeterwoude, The Netherlands) at −80°C and serially cut into 7-µm-thick sections. Slides were microwaved in 0.01 M citrate acid solution (pH 6.0) twice for 5 min and incubated with 1% bovine serum albumin (Serva, Heidelberg, Germany). To detect BrdU-incorporating cells, slides were incubated with anti-BrdU monoclonal antibody (1:20; Roche, Mannheim, Germany) followed by alkaline phosphatase-conjugated anti-mouse IgG (Roche) and visualized by Fast Red (DAKO). To detect MV-Edm-infected cells, slides were blocked with MOM mouse Ig blocking reagent (Vector Laboratories Inc. Burlingame, CA, USA) and then incubated with mouse anti-measles hemagglutinin monoclonal antibody (Chemicon, Temecula, CA, USA) followed by alkaline phosphatase-conjugated anti-mouse IgG (DAKO) and visualized by Fast Red (DAKO). Hematoxylin was used for subsequent counterstaining.

Animal studies

Intracranial xenografting of human glioma cells. 5×10^5 U87 glioma cells in 5 µl DMEM were stereotactically injected into the right striatum of Rag2$^{-/-}$cγc$^{-/-}$ mice. Mice were anesthetized with xylazine and ketamine during the procedure. All animal work was carried out in accordance with state guidelines for animal care and use.

In vivo *homing.* Fourteen days after injection of U87 cells, when tumors had formed, the mice received 6 Gy of cranial γ-irradiation (^{137}Cs; Gammacell 40, Nordion, Kanata, Canada). Three days later, 1×10^5 BOECs pulsed with BrdU at a concentration of 10 µM for 5 days were injected into the tail vein once ($n = 3$). Tumor-bearing mice not receiving BOECs were used as controls ($n = 3$). One day later, brains were procured, fixed in 4% paraformaldehyde (PFA), cryosectioned and examined by immunohistochemistry for BrdU-incorporating cells as described above.

Transfer of antibodies against measles virus into mice. Serum was isolated from a healthy donor with immunity against measles as determined by high levels of measles-specific IgG antibodies (index 7.247) using an ELISA (Enzygnost Anti-MeaslesVirus/IgG, DADE Behring GmbH, Marburg, Germany). The serum was inactivated at 56 °C for 30 min, centrifuged at 14 000 r.p.m. for 10 min and proven to completely neutralize MV-Edm *in vitro* (data not shown).

To determine the duration of protection against measles, 500, 750 and 1000 µl of measles immune serum was injected intraperitoneally into two mice for each dose and blood was drawn at 1, 6, 18 and 28 h. The levels of measles-specific human IgG antibodies were determined by ELISA (Enzygnost Anti-Measles-Virus/IgG, DADE Behring GmbH), previously validated to perform correctly after dilution in mouse serum.

Intracranial migration of BOECs and fibroblasts. Mice with striatal U87 gliomas received 6 Gy of cranial γ-irradiation. Two days later, mice were immunized with

500 μl human measles immune serum injected intraperitoneally or were not immunized. Eighteen hours later, 2×10^5 BOECs pulsed with BrdU and infected with MV-Edm were injected peritumorally ($n = 4$ for the immunized and nonimmunized group). A nonimmunized control group with irradiated gliomas received human foreskin fibroblasts pulsed with BrdU ($n = 4$). Two days after BOEC injection, mouse brains were procured, fixed in 4% PFA, cryosectioned and examined by immunohistochemistry for BrdU incorporation and for the presence of MV-Edm.

In vivo *survival studies.* To determine the efficacy of intratumoral injections, mice with established striatal gliomas generated by the growth of 5×10^5 U87 cells for 10 days received daily intratumoral injections given for 2 days. Tumors of the treatment groups were injected with 3×10^5 PFU MV-Edm in 5 μl OptiMEM ($n = 5$) or with 2×10^5 BOEC/MV-Edm harvested 1 h after infection with MV-Edm at a MOI of 1.5 ($n = 5$). Tumors of the control group ($n = 5$) were injected with 5 μl PBS.

To investigate the effects of intravenous injections, mice with established striatal U87 gliomas generated by the growth of 5×10^5 human U87 glioma cells for 4 days were subjected to 6 Gy of cranial γ-irradiation. Two days later, mice were randomized into three groups to receive daily tail vein injections for 5 days. The treatment groups were injected with 4×10^5 PFU MV-Edm in 200 μl PBS ($n = 7$) or with 2×10^5 BOEC/MV-Edm harvested 1 h after infection with MV-Edm at an MOI of 2 ($n = 6$). The control group was injected with 200 μl PBS ($n = 8$).

To determine the efficacy of peritumoral injections, mice with striatal U87 gliomas established by the growth of 5×10^5 human U87 glioma cells for 4 days received 6 Gy of cranial γ-irradiation. Two days later, daily peritumoral injections of BOEC/MV-Edm were given for 3 days. Each day, injections were applied to a different peritumoral location, that is approximately 1 mm superior, 0.5 mm lateral and 0.5 mm posterior to the glioma. The treatment groups received 3×10^5 PFU MV-Edm in 5 μl OptiMEM ($n = 7$) or 2×10^5 BOEC/MV-Edm harvested 1 h after infection with MV-Edm at an MOI of 1.5 ($n = 7$). Control groups received 2×10^5 BOECs ($n = 7$) or 5 μl PBS ($n = 6$).

Animals were followed until they were moribund, at which time they were killed.

MRI scanning procedure and image analysis

MRI was performed with formalin-fixed mouse brains on a 4.7-T BRUKER Biospec scanner (Bruker Biospin MRI, Ettlingen, Germany) by generating high-resolution T_2-weighted spin echo images. Twenty sagittal and 20 axial slices were acquired covering the entire brain (slice thickness: 0.5 mm). Images were analyzed using ParaVision software (Bruker Biospin MRI). Borderlines of tumors were delineated using a tracking function, the volume of tumor tissue was calculated for each slice and then added from all slices to obtain the total tumor volume.

Statistical analysis

Survival data were analyzed by Kaplan–Meier estimator analysis and compared using the log-rank test. $P < 0.05$ were considered significant.

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