PolySia-Specific Retargeting of Oncolytic Viruses Triggers Tumor-Specific Immune Responses and Facilitates Therapy of Disseminated Lung Cancer

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Abstract

Polysialic acid (polySia) is expressed on several malignant tumors of neuroendocrine origin, including small cell lung cancer. In this study, we investigated the therapeutic efficacy of tumor-directed T-cell responses, elicited by polySia-retargeted oncolytic adenovirus infection, in an orthotopic murine model of disseminated polySia-positive lung cancer. In several cell lines, we demonstrated highly polySia-selective retargeting of adenoviral infection using a bispecific adapter comprising the ectodomain of the coxsackievirus/adenovirus receptor and a polySia-recognizing single-chain antibody domain. PolySia-dependent systemic infection in vivo facilitated effective uptake of viruses in subcutaneous polySia-expressing human tumors, whereas hepatic viral load and hepatotoxicity were significantly reduced. The impact and nature of antitumoral immune responses triggered by systemic delivery of polySia-retargeted oncolytic adenoviruses were investigated in an orthotopic model of disseminated lung cancer. Interestingly, improved transduction by polySia-retargeted oncolytic adenoviruses led to CD45-positive cell infiltrates in close association with large lytic areas. Consistently, enhanced tumor regression and prolonged survival was only observed in immunocompetent mice, but not in T-cell-deficient mice. To investigate whether improved systemic infection by polySia retargeting would elicit a tumor-specific T-cell response, we screened the used lung cancer cells for mutated oncogenes by complete exon sequencing. In agreement with our other results, only retargeted oncolysis was able to induce a significant response specific for the tumor-associated neoepitope Gst2-Y9H. In conclusion, we demonstrated that effective retargeting of oncolytic adenovirus against polySia-expressing tumors elicits an effective tumor-directed T-cell response after systemic virus delivery and facilitates therapy of disseminated lung cancer.

Introduction

Among neuroendocrine tumors, small cell lung cancer (SCLC) is a severe disease accounting for about 15% of all pulmonary malignancies (1, 2). Treatment of SCLC remains extremely challenging because of its rapid growth and early dissemination. SCLC responds well to first-line therapy, usually platinum-based chemotherapy (3), eventually combined with radiotherapy. Nevertheless, the 5-year survival rate is below 5% due to frequent disease relapse and drug resistance. Targeted molecular therapies are being investigated, but clinical progress has not been achieved so far (4). Therefore, alternative strategies for treatment of SCLC are urgently needed.

Tumor-selective replicating (oncolytic) viruses are a promising therapeutic option (5). Recently, oncolytic virotherapy has shown the first therapeutic success in late-stage clinical trials in advanced melanoma (6). Apart from immediate tumor cell lysis, virotherapy exerts further antitumoral activities, including local inflammation and modulation of the tumor vasculature (7, 8). In addition, collateral induction of innate and adaptive immune responses against the tumor essentially contribute to therapeutic efficacy of virotherapy (9). Oncolytic adenoviruses are highly immunogenic and cause massive tumor cell lysis and local inflammation after intratumoral application. Therefore, adenoviral oncolysis is a promising trigger for effective cross-priming of tumor-specific T-cell responses (10). However, the majority of human tumors, including lung tumors, are not easily accessible for local virus applications, and systemically administered oncolytic adenoviruses poorly infect disseminated tumor nodules but may cause hepatotoxicity (11). A convenient method for adenovirus retargeting to tumor cells is the use of bispecific adapters. These usually contain a tumor-binding ligand and the ectodomain of the coxackievirus/adenovirus-receptor (CAR), which binds to adenoviral fiber knob, thus facilitating CAR-independent infection of tumor cells (12, 13). In vivo, binding of factor X to adenoviral hexon has been identified to mediate liver uptake (14). Nevertheless, bispecific adapters containing the CAR-ectodomain reduce adenoviral liver load, suggesting that fiber knob is involved in adenoviral hepatotropism (15, 16).

PolySia is a homopolymer of the negatively charged nona-sugar sialic acid, a posttranslational modification predominantly found on the neural-cell adhesion molecule, NCAM. PolySia-expression is high during embryonic development (17, 18) but absent from...
responses are major determinants for therapeutic benefit. Consistently, we demonstrate that polySia-retargeting of oncolytic adenoviruses mounted a CD8 T-cell response against the mutated tumor epitope Gsta2-Y9H. Our data suggest that critical levels of polySia expression on the cell surface promotes intercellular repulsion. Accordingly, tumor cell contacts because the presence of this negatively charged molecule around gene sequences were analyzed using the SYPFEITHI-algorithm (27) to determine CD8 T-cell–specific epitope candidates with high affinity for H2-Db. Using ELISPOT analyses as described elsewhere (10), the candidate pool was screened following adenoviral oncolysis of CMT64 tumors on C57BL/6 mice, thus facilitating the identification of Gsta2-Y9H as a tumor-specific immunoeptope.

Plasmids and genetic construction

To generate a polySia-specific scFv (sc-pSia), cDNA was prepared from mAb735-hybridoma cells. Then, the light chain (V\textsubscript{l}, C\textsubscript{l}) and corresponding domains of the heavy chain (V\textsubscript{H}, C\textsubscript{H1}) were amplified by PCR using oligonucleotides directed against the conserved termini of murine IgG2a chains. The oligonucleotides 5'-AAAACTAAGATGTTGATGACCCAGACTCC-3' and 5'-TTCTCGAGGTTGAAGCTCTTGACAATGGGTG-3' were used to amplify the (V\textsubscript{l}, C\textsubscript{l})-fragment and 5'-AAAGACAGCTGACCTACAAGCTCTTGACCAGCCTCCACCTTTTATTTC-3' and 5'-TTGGTACCCTTGTCCACCTTGGT-3' were used for V\textsubscript{H}, C\textsubscript{H1}, respectively. Resulting fragments served as templates for amplification and fusion of V\textsubscript{l} and V\textsubscript{H} via a (Gly\_Ser), linker using the oligonucleotides 5'-AACGGGCGCCGGATGTTGATGACCCAGACTCC-3' and 5'-TCCCCCA-CGGGATCCGGCCCCACCCCGCCACCTCCGCCACCTTTTATTTC-3' for V\textsubscript{l} and 5'-GGGAGCTCGGCGCTGACCTACGAGGACGGCTGGACCCACCCACCGGCGGGTCACCTCCGCCACCTCCGCCACCTTTTATTTC-3' and 5'-AATCTAGACCCGCTAGGACAGCTGACCCACCGGCGGGTCACCTCCGCCACCTCCGCCACCTTTTATTTC-3' for V\textsubscript{H}, respectively. A fusion with the soluble human ectodomain of CAR was generated by inserting the scFv-fragment into the plasmid pCARex (28). In addition, an N-terminally located His\_tag was positioned 3' of the leader peptide cleavage site. To complete the CARsc-pSia adapter, a T4-fibritin trimerization motif and a hinge region were inserted as described by Kashetseva and colleagues (13).

The retroviral plasmids pQXIN-pSia and pQXIN-PST were generated by insertion of the CARsc-pSia sequence and a sequence coding for murine polysialyltransferase ST8SialIV (PST) into pQXIN (BD Biosciences). Stable PolySia-expressing CMT-PST and CARsc-pSia–expressing 293-pSia cells were generated by retroviral transduction of CMT64 and 293 cells, respectively, followed by 2-week selection with 0.8 mg/mL G418 (Calbiochem).

Recombinant adenoviruses

The replication-incompetent marker virus AdLacZ and the oncolytic virus hTERT-Ad (which expresses GFP) have been previously described (28, 29). A recombinant luciferase-expressing variant of hTERT-Ad was constructed according to the method by Mizuguchi and Kay (30). The required pH3M-based shuttle plasmid pTERT-Luc was generated from pTERT(GFP) (29) by replacement of the IRES-GFP sequence by the CMV-promoter-luciferase cassette from pGL3 (Promega). The shuttle plasmid was digested with P1-Sce I/1-CeuI, and the resulting fragment was cloned into pAdHM4. Infectious particles were produced in 293 cells and purified by CsCl density gradient centrifugation. Virus preparations were titered using the Rapid-Titer-Kit (Takara/Clontech).
Preparation of recombinant CAR adapter proteins
CARsc-pSia protein was purified using Ni-NTA-Agarose (Qiagen). Supernatants and freeze/thaw extracts from 293-pSia cells were collected, and debris was removed by centrifugation and subsequent filtration (0.2 μm). Filtrates were supplemented with Na-phosphate buffer pH 8.0 (50 mM/L final concentration) and NaCl (150 mM/L final). Protein binding to Ni-NTA-Agarose was carried out by shaking overnight at 4°C. Beads were pelleted and washed twice with phosphate buffer pH 8.0 (50 mM/L final), 3 mM/L imidazol. Elution was performed with Na-phosphate buffer pH 8.0 (50 mM/L final) containing 300 mM/L NaCl and 100 mM/L l-histidine. The eluate was dialyzed against PBS and protein concentration was determined by the Bio-Rad Protein Assay.

Cell infection assays
Ad-LacZ particles (MOI10) were preincubated with supernatants (2 mL) from pCARsc-pSia, pCARex-Tat or pBluescript-transfected cells. Alternatively, viruses were preincubated with purified CARsc-pSia protein as indicated in the figure legends in a total volume of 150 μL DMEM (2% FCS). Preincubation was carried out for 1 hour at 4°C in an overhead shaker. Target cells were infected for 15 minutes before medium was aspirated and cells were washed two times to remove excess virus. Cells were then incubated for 48 hours. Adenoviral infection was visualized by X-Gal staining and infection efficacy was measured by β-gal activity in cell extracts.

Determination of viral infection in vivo
C57BL/6 and NMRI-nu/nu mice were obtained from Charles River. All animal experimentation was performed according to the local rules for animal experimentation (TierSchG). To analyze hepatic detargeting, nude mice were injected intravenously with hTERT-AdvLuc particles (1 × 10⁶ pfu/mouse) pre-treated with purified CARsc-pSia (15 μg/mouse) or PBS in a total volume of 250 μL. Forty-eight hours after virus administration, mice were injected intraperitoneally with ketamine/xylazine (100 mg/kg; 10 mg/kg) and β-luciferin (30 mg/kg) and investigated for bioluminescence using an Optical Imaging System (Xenogen). Sera were prepared and analyzed for asparagine-aminotransferase (AST) and alanine aminotransferase (ALT) assay (Catachem).

Subcutaneous tumor xenografts were established by injection of 1 × 10⁶ cells into the flanks of nude mice. Once tumor nodules had reached approximately 0.5 cm in diameter, hTERT-AdvLuc (5 × 10⁸ pfu/mouse) or PBS were administered intravenously. On day 3 after virotherapy, mice were intravenously injected with β-luciferin (30 mg/kg), sacrificed, and tumors were explanted for optical imaging.

Orthotopic lung cancers were established by intravenous injection of 4 × 10⁶ CMT-PST cells in C57BL/6 mice. Seven days later, mice received intravenous injections of hTERT-AdvLuc, hTERT-AdvGFP, or Ad-LacZ (7 × 10⁸ pfu/mouse) with or without CARsc-pSia-pretreatment. Three days after virotherapy, mice were injected intravenously with β-luciferin (30 mg/kg) and sacrificed. Lungs from hTERT-AdvGFP-treated mice were inflated with 4% paraformaldehyde and paraffin sections were prepared for fluorescence microscopy. Lungs from hTERT-AdvLuc–infected mice were inflated with PBS and subjected to bioluminescence analyses. Bioluminescence quantitation was calculated from regions of interest (ROI) covering a significant area of the investigated tissue by measurement of total photons using the Living Image 3D software (Xenogen). To further confirm ROI evaluation, extracts were prepared from isolated tumors and lung tissue. Reporter activity was measured by luciferase assays and normalized by protein content (Bio-Rad Protein Assay).

Quantitation of adenoviral infection by hexon quantitative PCR (qPCR) has been described elsewhere (31).

Monitoring therapeutic efficacy
NMRI-nu/nu and C57BL/6 mice with established CMT-PST lung tumors were treated twice by intravenous injection of oncolytic hTERT-Ad (5 × 10⁸ pfu/mouse each injection) with or without CARsc-pSia–pretreatment. CD8 depletion was performed using LEAF anti-mouse CD8 antibody, clone 53-6.7, (Biologend) that was intravenously injected on days 1 and 5 following initial virus treatment using 75 μg antibody per injection. To investigate antitumor efficacy, mice were sacrificed after initial virotherapy as described in the corresponding figure legends. Sections of lung tissue were prepared and subjected to hematoxylin and eosin (H&E) staining. To visualize leukocyte infiltration, sections were treated with 3% H₂O₂ and stained with CD45 antibody (ab25386, Abcam), a secondary biotin-anti-rat-antibody (Invirogen), streptavidin-HRP (Invirogen), DAB (Zytomed), and hematoxylin for nuclear counterstaining. For quantitative evaluation of tumor tissue lysis and leukocytic tumor infiltration, 6 representative sections per group were microscopically surveyed and the sizes of lytic and vital tumor areas as well as the number of leukocytes were calculated using CellStar Software.

Using the peptides LHYPNARGRM and LHIFNARGRM as wt-control, (Proimmune), Gsta2-Y9H–specific CD8 T-cell immune responses were analyzed by determination of IFNγ release from peptide-stimulated splenocytes of treated mice using ELISpot assays as previously described (10).

Statistical analysis
Results of two treatment groups were compared for statistical significance by an unpaired, two-tailed t-test and survival curves were analyzed by the log-rank test using GraphPad Prism V5. P < 0.05 was considered statistically significant.

Results
Retargeting of adenovirus infection to polysialylated tumor cells by CARsc-pSia
To facilitate infection of polySia-expressing tumors by oncolytic adenoviruses, we generated the adapter CARsc-pSia by fusing the CAR-ectodomain to a polySia-specific scFv as described in Materials and Methods. Adapter construction and its function to mediate virus binding to polySia-expressing cells are illustrated in Fig. 1A. For effective trimerization, CARsc-pSia was provided with a T4-fibrinogen motif as described by Kashentsseva and colleagues (13).

First, we investigated whether CARsc-pSia enables adenoviral infection in a polySia-specific manner. We used cell lines with defined levels of polySia that are resistant to infection by adenovirus under normal conditions. To address polySia-specific infection in an isogenic setting we compared polySia-expressing
To investigate polySia-specific adenoviral infection in human tumors, IMR32 (neuroblastoma), TE671 (rhabdomyosarcoma), and H146 (SCLC) cells were selected to reflect clinical relevant tumor entities with high polySia expression. PolySia levels were investigated by FACS analysis (Fig. 1B). High levels of polySia were observed in IMR32 and TE671 cells, while H146 cells showed lower polySia expression. To enable polySia-specific adenoviral infection, a recombinant bispecific adapter CARsc-pSia was used. This adapter acts as a molecular bridge redirecting oncolytic adenovirus infection to CAR-deficient, polySia-expressing tumor cells. The genetic construction and function of the recombinant bispecific adapter CARsc-pSia are shown in Fig. 1A. The trimeric CARsc-pSia acts as a molecular bridge redirecting oncolytic adenovirus infection to CAR-deficient, polySia-expressing tumor cells.

Figure 1.
The molecular adapter CARsc-pSia enables polySia-specific adenoviral infection of CAR-deficient tumor cells. A, the genetic construction and function of the recombinant bispecific adapter CARsc-pSia. The trimeric CARsc-pSia acts as a molecular bridge redirecting oncolytic adenovirus infection to CAR-deficient, polySia-expressing tumor cells. B, expression levels of polysialic acid in CHO cells (CAR+/polySia+), the polySia-deficient mutant CHO-2A10, the human neuroendocrine tumor cell lines TE671 (rhabdomyosarcoma), IMR32 (neuroblastoma), and H146 (SCLC) cells were investigated by flow cytometric analyses using the α-polySia mAb735 as primary antibody (black line) in comparison with isotype (gray line). C, AdLacZ particles (MOI 10) were mixed with supernatants from 293 cells after transfection with a CARsc-pSia–expressing plasmid. Supernatants from CAR-Tat or pBluescript-transfected cells were used as positive and negative controls, respectively. Efficacy of viral infection was determined by X-Gal staining of fixed cells. D, results of β-galactosidase (β-gal) measurements in corresponding cell extracts. E, Ad-LacZ (MOI 10) was coated with increasing amounts of purified CARsc-pSia for 1 hour at 4°C and then subjected to target cells as indicated. Infection efficacy was determined by β-galactosidase assays in cell extracts.
detectable on human tumor cells and CHO cells, but not on CHO-2A10 cells. Infection efficacy was investigated using a marker adenovirus (AdLacZ) pretreated with CARsc-pSia, or CAR-Tat, respectively, a similar adapter containing the Tat protein transduction domain that was used as infection control (28). Infection efficacy was determined by X-gal staining of treated cells (Fig. 1C) and β-galactosidase assays (Fig. 1D). The results showed that CHO, CHO-2A10, IMR32, TE671, and H146 cells were refractory to untreated virus. CAR-Tat enabled efficient infection in all cells, indicating the correct function of integrin-mediated virus uptake, which is essential for CAR-independent retargeting approaches. Pretreatment of adenovirus with CARsc-pSia led to effective transduction of all polySia-positive cells, but not in CHO-2A10 cells, demonstrating that CARsc-pSia facilitates adenoviral infection in a polySia-specific manner. Using increasing amounts of recombinant, purified CARsc-pSia for virus pretreatment, we observed that CARsc-pSia promoted infection efficacy in a concentration-dependent manner (Fig. 1E). In summary, these experiments showed that CARsc-pSia enables effective polySia-specific adenoviral infection in human tumor cells.

CARsc-pSia-mediated hepatic detargeting of adenovirus reduces liver toxicity

After systemic delivery of human adenoviruses, the vast majority of viral particles end up in the liver, which may cause significant hepatotoxicity. It has been shown that factor X binding to adenoviral hexon is a major determinant of adenovirus delivery to hepatocytes in vivo (14, 32). Therefore, we wanted to investigate the influence of CARsc-pSia retargeting of adenoviruses on adenoviral liver infection. First, we performed a competition assay in HepG2 cells, a CAR-expressing hepatocarcinoma cell line that is permissive to adenoviral infection. Cells were pulse infected with AdLacZ after pretreatment with increasing amounts of CARsc-pSia to investigate whether adapter-mediated masking of fiberknob inhibits CAR-dependent cell entry (Fig. 2A). Adenoviral infection of HepG2 cells was strongly inhibited by CARsc-pSia in a concentration-dependent manner, confirming that masking of fiberknob interferes with CAR-dependent infection. To evaluate the effect of CARsc-pSia retargeting on adenoviral hepatotropism in vivo, we used hTERT-AdLuc, to facilitate sensitive detection of infection in living animals by bioluminescence imaging. Three days after intravenous injection of viruses, mice were imaged to examine hepatic luciferase activity. As shown in Fig. 2B, we observed strong luciferase activity in the livers of all mice that received untreated hTERT-AdLuc. In contrast, hepatic luciferase activity was significantly reduced in mice infected with CARsc-pSia–treated hTERT-AdLuc. In addition, relative amounts of adenoviral DNA were determined from total liver DNA (Fig. 2C) and confirmed the significant decrease of adenoviral liver load when adenovirus was pretreated with CARsc-pSia. These results demonstrate successful inhibition of adenoviral hepatotropism by CARsc-pSia and support previous reports in which CAR ectodomain containing adapters with alternative tumor-binding moieties were used (15, 16, 33). To evaluate whether CARsc-pSia–dependent hepatic detargeting can prevent toxicity in vivo, we infected mice with repeated high-dose applications of an oncolytic adenovirus with or without CARsc-pSia pretreatment. After 48 hours, mice were investigated for signs of liver damage by macroscopic inspection (Fig. 2D) and by transaminase measurements in sera (Fig. 2E). Application of untreated adenovirus caused severe liver damage as confirmed by elevated transaminases (AST, ALT). In contrast, livers from mice infected with CARsc-pSia–pretreated adenoviruses appeared normal and almost no transaminases were observed. The results demonstrate that CARsc-pSia pretreatment of oncolytic adenovirus facilitates effective liver detargeting and protects from adenovirus-induced liver toxicity.

CARsc-pSia mediates adenovirus retargeting to polySia-expressing human tumors in vivo

A central goal of retargeting strategies is to enable vector delivery to the target tumor even after systemic delivery. To address this question, we established subcutaneous xenografts of human SCLC (H146) and rhabdomyosarcoma (TE671) in nude mice. Tumor-bearing mice were treated intravenously with hTERT-AdLuc with or without CARsc-pSia. Two days after adenoviral application, mice were sacrificed and whole tumors as well as tumor slices were prepared for bioluminescence imaging (Fig. 3). In both models (Fig. 3A: H146; Fig. 3B: TE671), delivery of untreated hTERT-AdLuc resulted in negligible luciferase levels indicating ineffective uptake by these tumors. In contrast, tumors from mice that had received CARsc-pSia–pretreated particles revealed a spotted pattern of bioluminescence indicating successful uptake. These observations were further confirmed by ROI analyses and luciferase assays in tissue extracts (Fig. 3, bottom panels). The results clearly show that CARsc-pSia facilitates retargeting of systemically administered oncolytic adenovirus to polySia-expressing peripheral tumors in mouse models of human SCLC and rhabdomyosarcoma.

CARsc-pSia facilitates effective oncolytic adenovirus infection of disseminated lung cancer

It is known that immune-mediated effects essentially contribute to the therapeutic activity of oncolytic viruses. Therefore, we wanted to investigate the therapeutic efficacy of polySia-specific retargeting of oncolytic adenoviruses in an orthotopic model in immunocompetent mice. To establish a corresponding model of polySia-positive lung cancer, we used the murine lung cancer cell line CMT64 (34). These cells support replication of human adenoviruses (35–37) and establish lung colonies after intravenous injection in mice. Because CMT64 cells do not endogenously express polySia, we introduced the polysialyltransferase ST8SiaIV by retroviral transduction to yield the cell line CMT-PST. This approach provided us with two isogenic cell lines with or without polySia expression for functional tests. In FACS analysis, CMT-PST cells showed polySia expression comparable with human tumor cell lines (Figs. 1B and 4A). Furthermore, the CMT-PST cell line showed an equivalent production of human adenovirus particles compared with that of the maternal CMT64 cells. PolySia-specific retargeting was confirmed by infection assays in CMT-PST and CMT64 (Fig. 4B). To evaluate polySia retargeting of oncolytic adenoviruses in orthotopic lung cancer, CARsc-pSia–pretreated hTERT-AdLuc was intravenously administered in C57BL/6 mice with established CMT-PST lung colonies. Three days after virotherapy, only weak bioluminescence signals were detected in lungs from animals that received untreated virus. In contrast, CARsc-pSia retargeting led to significantly increased infection in the lung (Fig. 4C). Neither untreated nor pretreated hTERT-AdLuc infected normal lung tissue because bioluminescence was absent in tumor-free mice.
These observations indicate effective and specific retargeting to polySia-expressing lung colonies in vivo. To further validate the bioluminescence imaging results, luciferase activity was quantified by ROI analyses (Fig. 4D). Furthermore, luciferase activity was determined in protein extract and relative adenoviral DNA contents were determined. Up to 5-fold increased adenoviral infection efficacy by CARsc-pSia-modified particles was observed in the lungs of tumor-bearing mice (Fig. 4D). To directly visualize infection events, the GFP-encoding hTERT-Ad (CARsc-pSia) was intravenously delivered. Microscopic comparison of fluorescence and histology in serial sections demonstrated that infection with CARsc-pSia–pretreated oncolytic adenovirus was restricted to lung tumor colonies but was absent from the adjacent normal lung tissue (Fig. 4E). Our observations indicate effective and specific retargeting to polySia-expressing orthotopic lung colonies in vivo.

CARsc-pSia retargeting elicits therapeutically relevant antitumor immune responses in orthotopic disseminated lung cancer

To examine the therapeutic benefit of CARsc-pSia–mediated infection on disseminated lung cancer, C57BL/6 mice with established CMT-PST lung tumors were treated with CARsc-pSia–retargeted hTERT-Ad, or unmodified virus according to the experimental timeline in Fig. 5A. The experiment was similarly carried...
Figure 3.
CARsc-pSia facilitates successful retargeting of oncolytic adenoviruses to polySia-expressing human tumors in vivo. A, nude mice bearing subcutaneous H146 SCLCs were injected intravenously with oncolytic hTERT-AdLuc (7 × 10⁷ pfu/mouse) pretreatment with CARsc-pSia (15 μg/dose). Forty-eight hours after virus administration, mice were sacrificed, tumors were explanted, and adenovirus infection was monitored by bioluminescence imaging in entire tumors and in tumor slices (top). Luciferase activity was determined from ROIs of dissected whole tumors and further quantified by a luciferase assay of corresponding tumor extracts (bottom). B, the same experimental setup and analysis was performed in nude mice bearing subcutaneous TE671 rhabdomyosarcomas.

out in nude mice to investigate the contribution of adaptive immune responses. Antitumor activity was examined by histopathologic inspection of lung sections 10 days after initial virotherapy (Fig. 6B). Tumor load was slightly reduced in lungs of both mouse strains after delivery of untreated hTERT-Ad. In immunocompetent mice, CARsc-pSia–modified hTERT-Ad resulted in a strong reduction of tumor burden predominantly in the pericentral lung epithelium, whereas the lung periphery still contained tumor tissue. This antitumor activity after polySia retargeting was not observed in immunodeficient animals, suggesting a role of adaptive antitumor immune response raised by oncolytic tumor infection. We also demonstrated improved survival in immunocompetent animals after virotherapy alone that was significantly prolonged by polySia retargeting. In line with microscopic investigations, no statistically significant therapeutic benefit was obtained with virotherapy in immunodeficient mice (Fig. 5C). Because these observations suggest a significant contribution of immune-related effects to therapeutic efficacy, we investigated oncolytic infection of tumor tissue at different time points after virotherapy by microscopic examination. Lung sections were analyzed from mice that underwent the same treatments as described in Fig. 5A. Samples were prepared on day 3 for inspection of tissue damage after an initial round of viral replication and cell lysis. Comparison on day 7 was performed to allow for discrimination of adaptive immune effects. Three days after initial virotherapy, microscopic images showed weak adenoviral tumor infections, characterized by small lytic foci within tumor nodules. Those were detectable in both mouse strains, because CMT-PST cells are moderately permissive to adenoviral infection. Adenovirus pretreatment with CARsc-pSia improved tumor infection, resulting in increased oncolysis in both mouse strains. A small number of cells with a mononuclear phenotype could also be observed in lytic tumor areas, partially infiltrating vital tumor tissue (Fig. 6A). Day 7 following adenovirus administration in C57BL/6 mice, CARsc-pSia–pretreated virotherapy caused enlarged lytic tumor areas containing infiltrating mononuclear cells. Lytic areas were larger compared with those in mice treated with the virus alone. Furthermore, this difference was not observed in nude mice that received hTERT-Ad alone, or CARsc-pSia–pretreated virotherapy caused enlarged lytic tumor areas containing infiltrating mononuclear cells. Lytic areas were larger compared with those in mice treated with the virus alone. Therefore, this difference was not observed in nude mice that received hTERT-Ad alone, or CARsc-pSia–pretreated virotherapy caused enlarged lytic tumor areas containing infiltrating mononuclear cells. Lytic areas were larger compared with those in mice treated with the virus alone. Moreover, this difference was not observed in nude mice that received hTERT-Ad alone, or CARsc-pSia–pretreated virotherapy caused enlarged lytic tumor areas containing infiltrating mononuclear cells. Lytic areas were larger compared with those in mice treated with the virus alone. Furthermore, this difference was not observed in nude mice that received hTERT-Ad alone, or CARsc-pSia–pretreated virotherapy caused enlarged lytic tumor areas containing infiltrating mononuclear cells. Lytic areas were larger compared with those in mice treated with the virus alone.
with CARsc-pSia–retargeted virotherapy compared with virotherapy alone. In contrast, ratios of lytic/vital tumor area in immunodeficient nude mice did not show a significant difference between virotherapy alone and polySia-retargeted virus. These results indicate that adaptive immune responses are involved in clearance of infected tumor cells by CARsc-pSia–mediated virotherapy. In addition, large accumulations of infiltrating immune cells in lytic tumor areas and adjacent vital tumor, histologically

Figure 4.
CARsc-pSia effectively redirects oncolytic adenovirus to polySia-expressing tumors in an orthotopic murine model of disseminated lung cancer. A, PolySia-expressing CMT-PST cells were investigated for expression of polySia in comparison with maternal CMT-64 cells using FACS analysis (left). CMT-PST or CMT64, respectively, were infected with hTERT-Ad at MOI 10. After 96 hours, cells were lysed by repeated freeze/thaw cycles and infectious particles were determined by the Rapid Titer Assay and calculated per infected cell (right). B, CMT-PST, or CMT64, respectively, were infected with Ad-LacZ (MOI 10), pretreated with increasing amounts of CARsc-pSia, before infection was stopped after 30 minutes by extensive washing. After 48 hours, infection efficacy was determined by the β-galactosidase assay in cell extracts. C, lung colonies of CMT-PST cells were established in syngeneic C57BL/6 mice by intravenous injection of 4×10⁵ cells per mouse. Control animals were kept tumor free. Mice were treated with CARsc-pSia–coated oncolytic adenovirus hTERT-AdLuc (intravenous injection of 7×10⁸ pfu coated with 15 µg/dose) or unmodified virus. Lungs were isolated after 48 hours and efficacy of hTERT-AdLuc infection was monitored by bioluminescence imaging. D, luciferase activity was quantified by ROI analysis and by the luciferase assay using tissue extracts. In addition, relative contents of viral DNA (hexon gene) were determined by real-time qPCR in total DNA extracted from corresponding lung samples. E, according to the procedure described above, mice were infected with a GFP-expressing variant of hTERT-Ad to directly visualize adenoviral infection of lung colonies. After infection, lung tissue was obtained and investigated by histology and fluorescence microscopy using serial sections.
defined as CD45^+ leukocytes, were observed in the CARsc-pSia-pretreated virotherapy group in immunocompetent mice (Fig. 6C). A quantitative determination of leukocytes confirmed significantly enhanced leukocyte infiltrates in tumors of mice that received CARsc-pSia–treated adenovirus compared with that in mice that received virus alone (Fig. 6D). Focusing on CD8 T cells, we therefore investigated the contribution of tumor-directed lymphocyte responses that are triggered by CARsc-pSia retargeting of oncolytic adenovirus. To identify mutated antigens in our model, CMT64 cells were investigated by whole exome sequencing for nonsynonymous SNVs. Immunogenic mutant epitopes were identified using the SYFPEITHI algorithm and functionally validated by ELISpot analysis following adenoviral oncolysis in CMT64 tumor-bearing C57BL/6 mice. This screen led to identification of the Y9H mutation of Glutathion-S-Transferase 2 (Gsta2). We then investigated the Gsta2-Y9H–specific immune response following polySia-retargeted virotherapy in orthotopic, disseminated lung cancer. Splenocytes from tumor-bearing C57BL/6 mice after systemic delivery of polySia-retargeted virotherapy or virotherapy alone were analyzed by the IFN\(\gamma\)-ELISpot assay. Isolated splenocytes were stimulated with a CD8-specific, mutated Gsta2-peptide to investigate responses directed against this mutated tumor antigen. A peptide for adenoviral E1B was used as infection control. Strong antiviral immune response could be detected in mice treated with hTERT-Ad or CARsc-pSia–pretreated virus (Fig. 6E), indicating equivalent infection of mice. However, in mice that received polySia-retargeted virotherapy we detected a significantly higher Gsta2-Y9H–specific response compared with that in mice after virotherapy alone. These data provide strong evidence that sufficient tumor infection by successful retargeting can trigger substantial tumor-specific mediated immune responses. To evaluate the therapeutic significance of CD8 T-cell–mediated antitumor responses in this model, we monitored survival after using a CD8-depleting antibody during therapeutic application of CARsc-pSia–pretreated hTERT-Ad (Fig. 6F). To further investigate the role of virus replication for therapeutic success in our model, we included a group of mice that received a CARsc-pSia retargeted but replication-incompetent adenoviral vector (Ad-LacZ). Despite CARsc-pSia retargeting, the application of the replication-incompetent adenovirus did not improve survival. CD8 depletion in mice that received CARsc-pSia–retargeted hTERT-Ad completely abrogated the therapeutic efficacy finally confirming the significance of CD8 T-cell responses for the therapeutic outcomes described in our experiments. Together, our results showed that the bispecific adapter CARsc-pSia facilitates successful infection of orthotopic polySia-expressing tumors. Our observations...
Figure 6.
PolySia retargeting promotes oncolytic inflammation of lung colonies and triggers tumor-directed responses. Lung tumors were established by i.v. injection of 4 x 10^5 CMT-PST cells in C57BL/6 and in nude mice, respectively. Mice were treated twice with hTERT-Ad (5 x 10^8 pfu/mouse per injection) with or without CARsc-pSia-pretreatment. A, the histologic examination of representative H&E-stained lung sections obtained from treated mice at day 3 (top) and day 7 (bottom) after virus administration. Lytic areas as indicated by rectangles (left) are shown at higher magnification (right) to allow for detection of infiltrating immune cells. B, seven days after initial virotherapy, the extent of lytic tumor area was quantified in relation to vital tumor area. C, lung sections of treated mice at day 7 after virotherapy were stained for CD45 to allow the visualization of infiltrating leukocytes. D, lung sections were used to determine the absolute numbers of infiltrating leukocytes in a standardized tumor area of 1 x 10^6 \( \mu \text{m}^2 \). E, mice were treated as described above. At day 7 after virotherapy, mice were sacrificed, splenocytes were isolated and antigen-specific responses were investigated. Virus-specific (E1B) and tumor-specific (Gsta2-Y9H) immune responses were determined by ELISpot assays. F, mice with established lung tumors were treated twice with CARsc-pSia–pretreated hTERT-Ad as described above and additionally received a CD8-depleting antibody as described in the methods section. Furthermore, a group was included that was treated twice with CARsc-pSia–pretreated Ad-LacZ, a replication-incompetent adenovirus. Survival of mice was monitored. ns, not statistically significant.
further suggest that effective tumor retargeting of systemically administered oncolytic virus can be essential to overcome critical thresholds of oncolytic inflammation in tumor tissue required for relevant therapeutic effects and induction of tumor-directed responses.

Discussion

Therapy of polySia-expressing tumors, such as SCLC, urgently needs effective new treatment options. Oncolytic virotherapy is a promising cancer treatment combining multimodal anti-tumor activities, including elimination of cancer cells by innate and adaptive tumor-directed immune responses. Recently, statistically significant clinical results have been achieved in a phase III study with intratumoral infection of a GM-CSF-expressing oncolytic herpesvirus in advanced melanoma (6). Sustained responses were also observable in uninjected tumor nodules, indicating tumor growth control by oncolysis-triggered antitumor immune responses. Unlike melanoma, the majority of human tumors are not accessible to percutaneous virus infiltrations so that effective retargeting strategies are mandatory to achieve significant infection and inflammation of the tumor. In our study, we have developed an adapter-based strategy to redirect oncolytic adenovirus infection to tumors, which express polySia, an excellent molecular target on the cell surface of clinically relevant tumors. For this purpose, we adapted the concept of bispecific adapters that has already been used for retargeting of adenoviral vectors to tumor enriched structures such as Her2-Neu, EGF-R, or CEA (12, 13, 15). As targeting ligand, we generated a polySia-binding scFv to establish the bispecific adapter CARsc-pSia. In adenoviral infection assays in CHO cells and a polySia-deficient mutant thereof, we demonstrated that CARsc-pSia facilitated cell infection in a polySia-specific manner. PolySia-specific retargeting also resulted in effective adenoviral infection of polySia-expressing human tumors in vitro and in vivo (e.g., SCLC, neuroblastoma, and rhabdomyosarcoma). PolySia is also an attractive molecular target because it is associated with malignant features (20–22). Furthermore, the high degree of tumor selectivity promises a lower risk for off-target infection. It has been shown that T-cell progenitors transiently express polySia (38). However, lymphatic cells are resistant to adenoviral infection due to the lack of integrins. Off-target infection might affect regions in the brain that show a phenomenon called plasticity and are characterized by polySia expression (39). However, in case of systemic application of polySia-retargeted virotherapy, brain infection is unlikely because adenovirus is unable to pass the blood–brain barrier. Off-target infection may play a role once polySia-retargeted oncolytic adenovirus is being considered for direct intracranial treatments of glioblastoma or medulloblastoma.

Although it has been demonstrated that factor X binding to hexon in viral capsids is a major determinant of adenovirus delivery to hepatocytes in vivo (14, 32), we showed a significantly reduced hepatotropism of oncolytic adenovirus after CARsc-pSia pretreatment. Our observations are consistent with previous reports showing that the use of adapters containing the CAR ectodomain led to reduced hepatic uptake of adenoviral vectors after systemic delivery, which is an important aspect for future application of adenoviral vectors in the clinic (15, 16, 33). Using the oncolytic adenovirus hTERT-Ad, which may cause liver damage after systemic high-dose administration, we showed that hepatotoxicity was prevented by CARsc-pSia pretreatment. The reduced hepatic uptake of adenoviruses after treatment with CAR-derived adapters independent from the used targeting ligand suggests that masking fiberknob interferes with liver infection. To explain almost complete prevention of liver hepatotoxicity by adapters, not only reduced hepatocyte infection but also masking of fiberknob has to be considered. Certain motifs in fiberknob have been shown to serve as danger-associated molecular pattern for recognition by innate immune receptors (40–42).

Viral infections, including those within tumor tissue, are rapidly cleared by the immune system. Therefore, therapeutic results of oncolytic virotherapy in xenograft models are of limited significance. We therefore investigated polySia-specific retargeting in an immunocompetent, orthotopic murine model of lung cancer using ST8SiaIV-transgenic CMT64 cells that are susceptible for replication of human adenovirus (37) and reflect polySia-expression levels of human SCLC. We could show that only polySia-retargeted, oncolytic adenovirus achieved successful infection of disseminated lung nodules after systemic virus application. In this aggressively growing tumor model, polySia retargeting of oncolytic adenovirus significantly improved survival of treated animals. Interestingly, no therapeutic benefit was observed in immunodeficient mice, indicating an essential role of immune-mediated mechanisms.

Antitumor T-cell immune responses are crucial for long-lasting therapeutic effects in cancer, which has been impressively demonstrated by durable responses by PD-1 and CTLA-4 immune checkpoint blockade in advanced melanoma and lung cancer (43–45). Although the exact mechanisms of these immunotherapies are not fully clear, it has been reported that CTLA-4 inhibition led to expansion of CD8 T cells directed against mutated tumor epitopes (46, 47). CD8 T-cell responses are also important mediators of antitumor cytotoxicity following virotherapy whereby effective oncolytic inflammation in the tumor tissue is an important precondition to fully exploit these promising functions (10, 48, 49). The sum of protein-encoded mutations of a single tumor, the mutanome, provides an attractive pool of neoantigenic targets that can be rationally predicted by tumor exome sequencing and algorithm-based search (46, 50). To characterize the role of polySia retargeting on triggering of tumor-directed CD8 T cells responses in our syngeneic lung cancer model, we used Gsta2-Y9H, an immunogenic neoepitope that we have identified in CMT64 cells. When we investigated the role of Gsta2-Y9H–specific responses in our model we were able to prove a strong triggering of tumor-directed CD8 T cells that was only observed in mice treated with polySia-retargeted oncolytic adenovirus but not with unmodified virus. Our data demonstrate that only effective retargeting enabled sufficient tumor infection and inflammation that is necessary to elicit a response against a tumor neoepitope. Furthermore, survival monitoring after CD8 depletion confirmed the significant role of CD8 T-cell responses in our experiments.

In summary, we developed an effective strategy for targeted delivery of oncolytic adenoviruses to polySia-expressing neuroendocrine tumors. The results of our study show that effective retargeting is an important prerequisite for eliciting therapeutically relevant immune responses against mutated tumor-associated antigens by systemic virotherapy applications.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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