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Increasing the odds: antibody-mediated delivery of two distinct immunogenic T-cell epitopes with one antibody

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ABSTRACT

Antibody-epitope conjugates (AECs) proved to be a promising new therapeutic strategy to redirect virus-specific CD8$^+$ T cells toward cancer cells by delivering T-cell epitopes. To be able to redirect a larger fraction of the virus-specific T-cell population, it is beneficial to deliver a broader selection of T-cell epitopes. We investigated two different methods to generate AECs with two distinct virus-specific T-cell epitopes fused to one antibody. Epitopes were either placed in a tandem-like fashion at the C-terminus of the AEC (t-AEC) or bispecific-AECs (bs-AECs) were generated via controlled Fab-arm exchange to generate bs-AECs with two identical antigen binding domains, but two distinct epitopes on each Fab-arm. Our study revealed that maintaining a free epitope terminus was required for efficient delivery of the virus-specific T-cell epitopes. Consequently, viral-epitope delivery using t-AECs was suboptimal as the concatenated epitopes were less effectively delivered to the target cells. However, well-defined bs-AECs containing both CMV and EBV epitopes were successfully generated and both *in vitro* and *in vivo* efficacy was evaluated. Our results demonstrate that bispecific-AECs can efficiently deliver EBV and CMV epitopes simultaneously to multiple cancer cell lines from different origins, thereby redirecting and activating two distinct populations of virus-specific T cells. Furthermore, our *in vivo* findings indicate that when both virus-specific T-cell populations are present and tumor cells express the proteases required for efficient epitope delivery, bs-AECs exhibit similar efficacy in reducing tumor burden compared to AECs. To conclude, our study demonstrates the feasibility of redirecting two groups of virus-specific T cells using a single antibody and highlights the potential of bs-AECs both *in vitro* and *in vivo*.

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Introduction

Antibody-epitope conjugates (AECs) can deliver immunogenic MHC-class I epitopes to redirect virus-specific CD8$^+$ T cells[1–4] and increase the immunogenicity of the tumor while bypassing the need for tumor-specific T cells.[5,6] Viruses like cytomegalovirus (CMV) and Epstein–Barr virus (EBV) are widely distributed among the human population and can trigger a potent memory T-cell response.[7,8] These virus-specific T cells are also present as bystanders in the tumor microenvironment (TME) and are therefore attractive candidates for cancer therapies based on redirecting T cells.[9–11] While individuals can have prominent levels of T cells recognizing virus-derived T-cell epitopes, not every viral epitope is recognized by each patient, due to seroprevalence and differences in HLA types.[12,13] Moreover, in AECs, variation in processing efficiency of the protease cleavage site connecting the antibody to the viral epitope may also result in variable delivery of the T-cell epitope to different types of cancer cells.[4,14] Therefore, the likelihood of a broader group of cancer patients responding to AEC treatment may be increased by using a broadly applicable AEC or to deliver a wider selection of epitopes and/or protease cleavage sites by the AECs.

We previously established that we could generate well-defined genetically fused AECs by introducing an EBV epitope, preceded by a protease cleavage site, to the C-terminus of the heavy chains of cetuximab (CTX) and trastuzumab (TRS). These AECs demonstrated efficient delivery of the EBV epitope to a range of cancer cell lines *in vitro* and to a multiple myeloma xenograft model *in vivo*.[3,13] The EBV epitope, an MHC-class I binding peptide, is released by proteases within the extracellular environment and binds to the HLA molecules expressed on the tumor cells.[4,13,14] One of the benefits of having a protease cleavage site mediating the release of the viral epitope is the additional safety mechanism as tumor cells are known to overexpress and secrete multiple proteases

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within the tumor microenvironment (TME).[15–17] Introduction of a viral epitope to the C-terminus of the light chain of an antibody, however, did not result in efficient T-cell activation, limiting the design to the C-terminus of the heavy chain of the genetically fused AECs.[13]

In this study, we aimed to improve and expand the AEC approach, by exploring two distinct methods to attach different viral epitopes from EBV and CMV to the C-terminus of the heavy chain of a single antibody. In the first approach, we arranged the two epitopes in a tandem-like fashion (t-AECs) and for the second approach we utilized controlled Fab-arm exchange to generate bispecific-AECs (bs-AECs) with two identical antigen binding domains but two distinct epitopes on each Fab-arm. Our results demonstrate that in the t-AEC approach, the terminal viral epitope of the t-AEC can be efficiently delivered to the tumor cells. The enclosed viral epitope, however, is delivered inefficiently. In contrast, the bs-AECs approach is successful, since both the EBV and CMV epitopes on these bs-AECs are efficiently delivered to tumor cells both *in vitro* and *in vivo*.

Materials and methods

Antibodies and peptides

Antibodies used for flowcytometry experiments were cetuximab, trastuzumab, goat anti-human IgG-A488 (Jackson immunoResearch, UK, Cambridgeshire) or -PE (Jackson immunoResearch), goat anti-mouse-FITC (Jackson immunoResearch), mouse anti-HLA-A2 (produced inhouse from clone BB7.2).[18] Peptides were synthesized with Fmoc chemistry, and the identity was confirmed with mass-spectrometry. The peptide sequences are listed in Table 1 and all peptides were dissolved at 20 mg/ml in DMSO before usage.

Generation of the AECs and bs-AECs

All AECs, genetically modified antibodies and wt antibodies used in coculture assays were produced as described before at Genmab via transient expression in ExpiHEK293 FreeStyle cells.[19] The produced antibodies were purified by Protein A affinity chromatography, and if required, aggregates were removed via Size Exclusion Chromatography (SEC) to yield a product with a > 95% monomeric content as analyzed on HPLC-SEC. To silence effector functions and prevent interactions with Fc gamma-receptors in *in vivo* experiments, LALAPG mutations were introduced in the Fc domain of the AECs; P329G, L234A and L235A.

Table 1. List of peptides used. The sequence in bold is derived from the BRLF1/A2 epitope and the underlined epitope from the NLV/A2 epitope. The peptides used for sortase a conjugation contained the following sequence in front of the indicated sequence: GGGGG-PEG11-. The first cleavage site (CI-1) was designed for the proteases matriptase, urokinase plasminogen activator (uPa) and legumain, the second cleavage site (CI-2) for matrix metalloproteinase (MMP)-2, –7 and – 9, and the third (CI-3) for MMP-14.

Abbreviation	Sequence
CI-1-EBV	LSGRSDNH-**YVLDHLIVV**
CI-2-CMV	VPLSLYSG-NLVPMVATV
CI-1-EBV-CI-2-CMV	LSGRSDNH-**YVLDHLIVV**-VPLSLYSG-NLVPMVATV
CI-1-EBV-CI-3-CMV	LSGRSDNH-**YVLDHLIVV**-PRSAKELR-NLVPMVATV
CI-2-CMV-CI-1-EBV	VPLSLYSG-NLVPMVATV-LSGRSDNH-**YVLDHLIVV**
CI-2-CMV-CI-2-EBV	VPLSLYSG-NLVPMVATV-VPLSLYSG-**YVLDHLIVV**
CI-2-CMV-CI-3-EBV	VPLSLYSG-NLVPMVATV-PRSAKELR-**YVLDHLIVV**

Table 2. Overview of the bs-mutations used and sequences attached to the heavy chain of the parental AECs. The EBV and CMV epitope in the sequence are indicated in bold.

Abbreviation	Mutation	Sequence added to the heavy chain
CTX-EBV TRS-EBV	F405L	GGSGLSGRSDNH**YVLDHLIVV**
CTX-CMV TRS-CMV	K409R	GGSGVPLSLYSG**NLVPMVATV**
b12	K409R	-
b12	F405L	

To generate the t-AECs, the sortase A conjugation method was used as described before.[3] For the bs-AECs, first AECs with the K409R or F405L mutations were produced (Table 2 provides the details of the AECs). Fab-arm exchange was performed as described in Labrijn et al. (Nat Protocols 2014).[20] In short, parental homodimers containing the DuoBody mutations (F405L and K409R in human IgG1 or equivalent positions in mouse IgG) were mixed in equimolar ratios, in the presence of the reductant 2-Mercaptoethylamine (2-MEA) to reduce the hinge disulfides and generate half-molecules. Next, the mixture was incubated for 5 h at 30°C to allow for the formation of DuoBody molecules. Efficiency of the exchange is monitored by intact mass spectrometry. When additional, nonstandard (N270) glycosylations are present, such as in Cetuximab, the m/z signals are heterogeneous and MS quantification is complicated. In those cases, successful exchange was derived from retention time changes between DuoBody and parental homodimers in Cation Exchange Chromatography (CEX) and/or Hydrophobic Interaction Chromatography (HIC). DuoBody content should be at least 80–85% for samples to be used in further studies. Example data for both assays (MS and CEX) are provided in the supplementary data. All AECs, t-AECs and bs-AECs were stored in PBS at −80°C.

Cell lines and cell culture

All adherent cell lines were cultured in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Biowest, France, Nuaillé) and 1% Pen/Strep (Gibco). Cell lines without endogenous HLA-A2 expression were transduced with a pEF1α lentiviral vector encoding the cDNA of HLA-A2. HeLa-A2 EGFR and Her2 KO cell lines were generated with an expression cassette encoding the guide RNAs (Sigma, clone ID 123,759,703 and 244,226,520) in vector plv-u6g-ppb. Cells were simultaneously transfected with the plasmids containing the gRNA and Cas9. EGFR and Her2 KO populations were enriched with puromycin selection (2 µg/mL for 48 h, InvivoGen, USA, San Diego), followed by FACS sorting using an Aria III cell sorter (BD Bioscience, USA, Franklin Lakes). KO clones were isolated by limiting dilution. One of the HeLa-A2 Her2 KO clones was transduced with a truncated-Her2 receptor encoding cDNA in the MP71 retroviral vector as described before.[21] Cell cultures were enriched for transduced cells by FACS sorting using an Aria III cell sorter (BD Bioscience).

The T cells used for the coculture assays were $CD8^+$ T cells derived from peripheral blood mononuclear cells (PBMCs) of healthy donors and transduced with the indicated virus-specific TCR as described before.[13] The T cells were either specific for the EBV derived BRLF1 epitope (YVLDHLIVV) or the CMV derived pp65 epitope (NLVPMVATV). Both epitopes are presented in HLA-A *02:01 and when both the EBV- and CMV-specific T-cells were mixed for a coculture, the TCR transduced T cells used were derived from the same donor. All T cells were cultured in Iscove's modified Dulbecco`s medium (IMDM, Gibco) containing 5% FCS (Gibco), 5% human serum, 3 mm L-glutamine (Lonza), 1% Pen/Strep, and 200 IU/ml IL-2. T cells were stimulated every 10–16 d with PHA and irradiated allogeneic peripheral blood mononuclear cells in a 1:3 ratio. Before being used in coculture assays expansion-related cytokines were removed, by washing the T cells three times with IMDM supplemented with 0.5% human serum albumin (HSA, Albuman, Sanquin, The Netherlands, Amsterdam).

T-cell activation and cytotoxicity assays

The coculture assays with antibody titrations to measure T-cell activation were performed as described before.[3,13] For the T-cell titrations, 5,000 target cells/well were plated in a 384-well flat-bottom tissue culture plate and cultured overnight to allow them to adhere. Target cells were exposed to an AEC dilution of 16 nM in IMDM supplemented with 0.5% HSA for 1 h at 37°C. The T cells were titrated in IMDM supplemented with 0.5% HSA and 100 IU/ml IL-2. The wells were washed 3× to remove non-bound AECs, and subsequently the titrated T cells were added to the wells. The highest concentration of individually titrated T-cells was 4,000 T cells/well and all the T-cell mixtures contained a total of 4,000 T cells/well.

For the U266 cell line, which is a cell line cultured in suspension, the tumor cells were exposed to the AECs diluted in IMDM supplemented with 0.5% HSA for 1 h at 37°C. Next, cells were washed 3× with IMDM supplemented with 0.5% HSA and 40,000 cells/well were transferred to a 384-well flat-bottom tissue culture plate to which 4 000 T-cells/well were added in IMDM supplemented with 0.5% HSA and 100 IU/ml IL-2. After an overnight coculture (18 h), IFN-γ production by the T cells was measured in the supernatant by means of ELISA (Diaclone, France, Besançon).

After harvesting of the supernatants from the cocultures, T-cell medium was added, and the cocultures were incubated for an additional 48 h. T cells were removed by gentle washing 2×, and DMEM culture medium supplemented with AlamarBlue HS cell viability reagent (ThermoFisher, USA, Massachusetts, Waltham) was added. Between every washing step, the cells were checked with light microscopy for loose or dissociated viable cells and the presence of T cells. Viability was measured in relative fluorescence units (RFU) according to the manufacturers protocol, and target cell killing was calculated as previously described.[13] For both the T-cell activation and target cell killing assay, target cells were also exposed to either titrated concentrations of the BRLF1 peptide epitope (YVLDHLIVV) or the CMV derived pp65 peptide epitope (NLVPMVATV) as a positive control.

In vivo experiments

Animal procedures were performed according to AVD116002017891 appendix 2, which was approved by the Central Committee of Animal Experiments (CCD, The Hague, The Netherlands) according to the European legislation (EU 2010/63/EU) and Animal Experiments Committee of Leiden University.

NOD-scid-IL2Rgammanul (NSG) mice were injected intravenously (i.v.) with 2×10^6 U266-tEGFR or subcutaneously (s.c.) injected with 6×10^6 SKOV3-A2 cells. Group sizes (8 mice per group) were determined based on variation observed in previous experiments. Mice were injected i.v. with the TCR transduced T cells on day 14 (2.5×10^6 BRLF1-specific and 2.5×10^6 pp65-specific T cells) after tumor injection. On day 15 and 18, and in the case of SKOV3-A2 also on day 21, 100 µg of AEC-EBV, AEC-CMV or bs-AEC-EBVxAEC-CMV diluted in PBS were administered intraperitoneal (i.p.) in combination with 100 µg pembrolizumab. The control groups receiving only antibody were injected i.p. with 100 µg bs-AEC-EBVxAEC-CMV and 100 µg pembrolizumab. The control groups receiving only T cells were in addition treated with 100 ug pembrolizumab on day 15 and 18 (U266) and day 15, 18 and 21 (SKOV3-A2).

To enable measurement of tumor outgrowth, both the U266 and SKOV3 cell lines were transduced with firefly luciferase (D-luciferase gene). Mice were injected subcutaneously (s.c.) with 150 µl 7.5 mm D-luciferine (Cayman chemical) and were measured with a CCD camera (IVIS Spectrum, PerkinElmer). For the SKOV3-A2 xenograft model, tumor outgrowth was also determined by means of caliper measurements and the T cells were additionally transduced with a renilla luciferase (R-luciferase gene). A selection of mice was injected retro-orbitally with 100 µl of coelenterazine in PBS with 10% DMSO 3 d after T-cell injection and measured with a CCD camera.

Statistical analysis

For statistical analyses, GraphPad Prism software (V.9.3.1) was used. The statistical tests used are indicated in the figure legends. The significance levels are indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Study approval

Healthy donor material from the Leiden University Medical Center Biobank for Hematological Diseases was used in this study. This research was approved by the Institutional Review Board of the Leiden University Medical Center (approval number B16.039). Materials were obtained after written informed consent in accordance with the Declaration of Helsinki.

Results

Free terminus required for efficient epitope processing and delivery

For attachment of two different epitopes to a single antibody using only the C-termini of the heavy chains of the antibody, two approaches were evaluated. The first approach places the two epitopes in tandem, separated by a protease cleavage site, at the

C-termini of the heavy chains, thereby increasing the epitope-to-antibody ratio (EAR) from 2 to 4. The second approach utilizes controlled Fab-arm exchange to obtain bispecific AECs (bs-AEC) with two identical antigen binding domains, but two distinct epitopes on each Fab-arm, maintaining a total EAR of 2: one EBV and one CMV epitope per bs-AEC. Since we previously demonstrated that AECs with the BRLF-1-YVL (EBV) epitope or the pp65-NLV (CMV) epitope efficiently activated their respective virus-specific T cells, we used these viral epitopes to test both approaches.[3,13]

As a first step, we investigated whether the EBV and CMV epitope in the tandem configuration were efficiently presented in HLA-A2. Therefore, multiple long peptides were generated with the EBV and CMV epitope either at the enclosed or C-terminal position in combination with varying protease cleavage site sequences (Table 1). The first cleavage site (Cl-1) was previously used for the EBV epitope and was designed for the proteases matriptase, urokinase plasminogen activator (uPa) and legumain.[3,13,22] Since we previously demonstrated that the Cl-1-CMV combination did not result in efficient presentation of the CMV epitope, this combination was not taken along.[3] The second cleavage site (Cl-2) was designed for matrix metalloproteinase (MMP)-2, −7 and −9, and the third (Cl-3) for MMP-14.[3,4,22] HeLa cells transduced with HLA-A2 (HeLa-A2) were exposed to titrated concentrations of various long peptides, followed by 18 hrs of coculture with either EBV-specific or CMV-specific T cells. IFN-γ levels in the supernatant of the cocultures were determined as a measure of T-cell activation. Unfortunately, for both the EBV- and the CMV-epitope, very limited T-cell activation was observed when T-cell epitopes were incorporated (enclosed) in the peptide chain instead of located at the C-terminal end (Figure 1(a)). T-cell epitopes concatenated in the peptide chain only demonstrated T-cell activation at the highest peptide concentrations with at least a 100-fold difference in efficiency compared to the C-terminally positioned epitopes.

We hypothesized that the efficiency of processing might improve when the peptides were conjugated to an antibody. To test this hypothesis, several tandem-AECs (t-AECs) were generated with the sortase A conjugation method.[3] However, due to the increased size and hydrophobic nature of the tandem peptides, strong precipitate formation or aggregation during the conjugation reaction was observed, with low yields of t-AECs. Similar as for free peptides, T-cell activation for the enclosed epitopes in the tandem configuration in t-AECs was absent or inefficient (Figure S1). Moreover, activation of the virus-specific T-cells was observed within the coculture assays when antibody target knockout cell lines were exposed to the t-AECs, which can be linked to instability of the t-AEC, as previously demonstrated.[3] From these observations, we concluded that epitopes with a free C-terminus in the tandem configuration in t-AECs are more efficiently processed and presented in the HLA molecules on the cell surface than enclosed epitopes.

Both viral epitopes of bispecific-AECs can efficiently activate T cells

Since internal peptide epitopes in the t-AECs were not efficiently presented, we next proceeded with generating bispecific-AECs (bs-AECs) with two identical antigen binding domains, but two distinct viral epitopes on each Fab-arm. Before the bs-AECs were generated, AECs of both cetuximab (CTX) and trastuzumab (TRS) with either the EBV BRLF1-YVL epitope (AEC-EBV) and the F405L mutation or the CMV pp65-NLV epitope and the K409R mutation (AEC-CMV) were produced separately. These mutations allow for controlled Fab-arm exchange when the two antibodies are recombined under specific conditions, resulting in bs-AECs (Figure 1b).[20] Successful Fab-arm exchange was confirmed with MS and CEX (Figure S2). This allowed for the delivery of both an EBV- and CMV-epitope by the AECs and therefore the redirection of these two different virus-specific T cells (Figure 1c).

CTX and TRS recognize the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (Her2), respectively. The HeLa-A2 cell line expresses high levels of EGFR and was transduced with Her2 lacking the intracellular signaling domain (HeLa-A2 tHer2) to increase Her2 expression levels. Expression levels of EGFR, Her2 and HLA-A2 have been previously quantified.[13] To demonstrate that the genetically fused AECs are both able to deliver their epitopes to the target cells, HeLa-A2 tHer2 were exposed to TRS-EBV or -CMV followed by a coculture with either EBV- or CMV-specific T cells (Figure 2a,b). The T cells used in the experiments were TCR transduced $CD8^+$ T cells isolated from healthy donors, therefore some variability between the different donors/experiments can be observed. Similar data were obtained for CTX-EBV and CTX-CMV (Figure S3) and we concluded that both AECs-EBV and -CMV were able to activate their respective T cells.

The bs-AECs with an EBV and a CMV epitope (bs-AEC-EBVxAEC-CMV) were generated for both CTX (bs-Cetuximab-EBVxCetuximab-CMV: bs-CTX-EBVxCTX-CMV) and TRS (bs-Trastuzumab-EBVxTrastuzumab-CMV: bs-TRS-EBVxTRS-CMV) by controlled Fab-arm exchange and the exchange efficiency was checked with intact MS analysis. Additionally, to check whether the reduction of the effective EAR from 2 to 1 for each individual epitope would result in a significant loss in efficiency, bispecific antibodies were generated with the Fab-arm of human monoclonal antibody b12 recognizing the human immunodeficiency virus type 1 (HIV-1) antigen gp120 without epitopes fused to the heavy chain (bs-TRS-EBVxb12 or bs-b12×TRS-CMV). To determine whether the different AECs and bs-AECs exhibited a similar level of antigen recognition, the antibodies were titrated on HeLa-A2 or HeLa-A2 tHer2 cells. As expected, no differences were observed at non-saturating antibody concentrations (Figure S4). Next, HeLa-A2 tHer2 cells were exposed to the different AECs and bs-AECs and subsequently cocultured overnight with either the EBV- or CMV-specific T cells (Figure 2c,d). All bs-AECs were able to deliver their epitopes and induced T-cell activation when cocultured with their respective virus-specific T cells. As expected, the efficiency of bs-TRS-EBVxTRS-CMV was slightly reduced compared to the respective parental AECs, which can be explained by the decreased epitope-specific EAR from 2 to 1. This was also observed for the bs-TRS-EBVxb12 and bs-b12×TRS-CMV.

To be able to compare the efficiency of bs-TRS-EBVxTRS-CMV to redirect both the EBV- and CMV-specific T cells, the coculture experiment was repeated for bs-TRS-EBVxTRS-

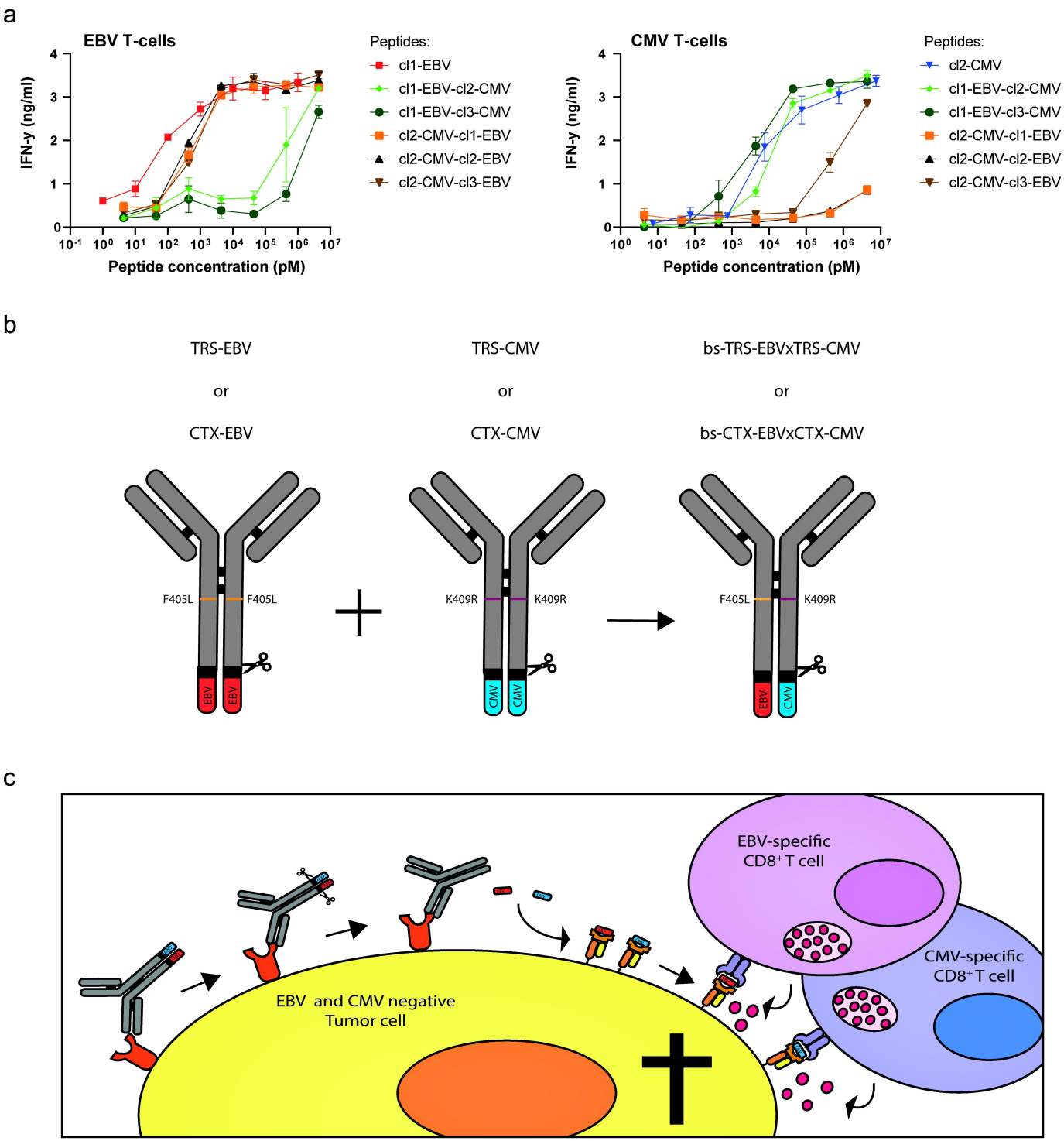

Figure 1. Epitopes in an enclosed position are not delivered properly (a) HeLa-A2 cells were exposed for 1 h to titrated concentrations of the different peptides. The sequences of the peptides used within the experiment are listed in Table 1. The exposed HeLa-A2 cells were cocultured for 18 hrs with either the EBV- or CMV-specific T cells as indicated, and T-cell activation was analyzed by measuring IFN-γ production of the T cells within the supernatant. Plotted values are the means of duplicates (SEM) and each graph shows a representative figure of three independently performed experiments. (b) An overview of the bs-AECs with two distinct epitopes attached to one antibody (CTX-EBV and CTX-CMV or TRS-EBV and TRS-CMV) where DuoBody technology was used to generate bispecific-AECs (bs-CTX-EBVxCTX-CMV or bs-TRS-EBVxTRS-CMV) without changing the specificity of the binding domain. (c) Schematic overview of the hypothesized mechanism of the bs-AECs, in which the bs-AEC first recognized the antibody target, followed by proteolytic release of the epitope by proteases secreted by the tumor cells. After release the epitopes eventually gets presented on the HLA molecules present on the tumor cells, which can be recognized by the two different virus specific $CD8^+$ T cells.

CMV with EBV-, CMV- or a 50%/50% mixture of EBV- and CMV-specific T cells (Figure 2e). A lower T-cell activation was observed for the cocultures with single CMV-specific T cells compared to single EBV-specific T cells. However, upon coculturing of the bs-AEC exposed target cells with a 50%/50% mixture of the virus-specific T cells, the T cells were activated to a similar extent as when cocultured with twice the amount of EBV-specific T cells, and activation was stronger

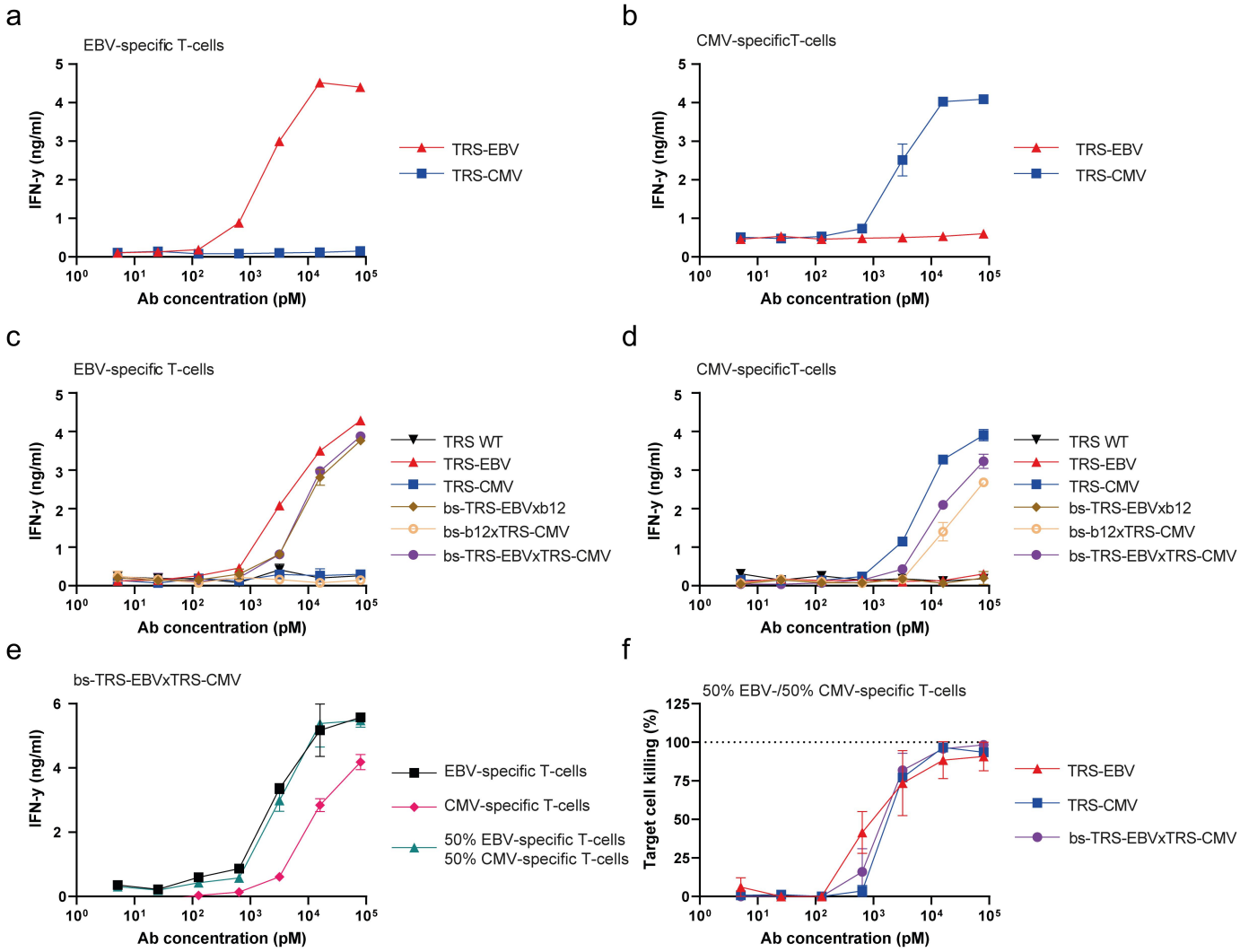

Figure 2. Both the EBV- and CMV-epitope of bs-TRS-EBVxTRS-CMV are delivered and induce T cell activation and target cell killing. (a–b) to determine whether both AECs were able to deliver their epitopes, HeLa-A2 tHer2 cells were exposed to TRS-EBV and -CMV and cocultured with either (a) EBV- or (b) CMV-specific T cells. (c–d) HeLa-A2 tHer2 cells were exposed to the wildtype (WT) of TRS, TRS-EBV, -CMV and different bs-AECs, and subsequently cocultured with (c) EBV-specific T cells or (d) CMV-specific T cells. (e) HeLa-A2 tHer2 cells were exposed to bs-TRS-EBVxTRS-CMV and cocultured with either EBV-, CMV- or a 50%/50% mixture of EBV- and CMV-specific T cells with a constant E:T ratio for all three T cell combinations (a-e). For all coculture assays, T cell activation was analyzed by measuring the IFN-γ production of the T cells within the supernatant after 18 hrs of coculture. (f) To measure specific cell killing HeLa-A2 tHer2 cells were exposed to TRS-EBV, -CMV, or bs-TRS-EBVxTRS-CMV, followed by a 72 hrs coculture with a 50%/50% mixture of EBV- and CMV-specific T cells. Tumor cell killing was measured with an AlamarBlue assay. (a-f) Plotted values are the means of duplicates (SEM) and each graph shows a representative figure of three independently performed experiments.

than coculturing with only CMV-specific T cells. From these data, we can conclude that bs-TRS-EBVxTRS-CMV can efficiently deliver both epitopes, thereby activating a mixture of EBV- and CMV-specific T cells.

To determine whether bs-TRS-EBVxTRS-CMV can also induce tumor cell killing, HeLa-A2 tHer2 cells were exposed to titrated concentrations of TRS-EBV, TRS-CMV or bs-TRS-EBVxTRS-CMV, followed by coculture with a 50%/50% mixture of EBV- and CMV-specific T-cells (Figure 2f). We observed similar kinetics in cytotoxic activity of the T cells against the HeLa-A2 tHer2 cells exposed to either TRS-EBV, -CMV or the bs-TRS-EBVxTRS-CMV. Consequently, we concluded that treatment with bs-AECs can result in equal levels of target cell killing compared to the parental AECs when cocultured with a mixture of both T-cell populations.

Bispecific-AECs induce Tcell activation and target cell killing on multiple different cancer cell lines

To investigate whether similar findings would be obtained with other cancer cell lines, the coculture experiments were repeated with cancer cell lines from different origins: SKOV3 (ovarian carcinoma), MDA-MB231 (breast cancer), and H292 (lung cancer). The SKOV3 and H292 cell lines do not express endogenous HLA-A2 and were therefore transduced with a lentiviral vector containing HLA-A2 (SKOV3-A2 and H292-A2). All three cell lines express high levels of EGFR, and SKOV3-A2 also expresses high levels of Her2 as previously quantified.[13]

The cell lines were exposed to titrated concentrations of CTX-EBV, -CMV and bs-CTX-EBVxCTX-CMV, followed by coculture with a 50%/50% mixture of EBV- and CMV-specific T cells. T-cell activation was evaluated by measuring

the IFN-γ levels in the supernatant after 18 hrs, while tumor cell killing was assessed after 3 d of coculture. The SKOV3-A2 demonstrated similar efficiencies for both CTX-EBV, CTX-CMV and bs-CTX-EBVxCTX-CMV in delivering the epitope and subsequently activating the virus-specific T cells (Figure 3a,b). As the SKOV3-A2 cell line also expresses high levels of Her2, the experiments were repeated with TRS-EBV, -CMV and bs-TRS-EBVxTRS-CMV. For TRS-EBV, TRS-CMV and bs-TRS-EBVxTRS-CMV again similar efficiencies in T-cell activation and target cell killing of SKOV3-A2 were observed. We conclude that bs-CTX-EBVxCTX-CMV and bs-TRS-EBVxTRS-CMV demonstrated the capacity to induce equivalent levels of T-cell activation and target cell killing compared to their parental AECs on the SKOV3-A2 cell line.

When the MDA-MB231 cells were exposed to CTX-EBV and bs-CTX-EBVxCTX-CMV efficient T-cell activation and tumor cell killing was observed. However, exposure to CTX-CMV induced limited T-cell activation and tumor cell killing (Figure 3c). To rule out that the CMV-epitope could not be presented by the MDA-MB231 cell line in the context of HLA-A2, the MDA-MB231 cells were loaded with the CMV peptide (Figure S5), which ruled out the inability of the CMV-epitope being presented by the target cells. In contrast, H292-A2 cells exposed to CTX-CMV and bs-CTX-EBVxCTX-CMV induced increased T-cell activation compared to CTX-EBV. Since H292-A2 cells appeared to be insensitive to T-cell cytotoxicity, which was also observed for peptide loaded H292-A2 cells (Figure S5), no conclusions on the efficiency of induction of cytotoxic capacity could be drawn (Figure 3d).

To exclude the possibility that differences in internalization speed between the cell lines might account for the observed variations in epitope delivery, the internalization speed of EGFR-CTX complexes on the cell surface of the cell lines was analyzed (Figure S6). Collectively, the data highlight the high variability in the efficiency of AECs to release and deliver their epitopes to cancer cells from different origins. These differences between the cell lines may be attributed to variations in their protease expression and activity levels. Utilizing bs-AECs can help to address this variability. Furthermore, the data underscore that AECs with an EAR of 1 can still effectively redirect virus-specific T cells to a variety of tumor cell lines.

An additive effect in T-cell activation is observed for the bispecific-AECs

Since in the tumor microenvironment the presence and ratio of virus-specific T cells will be variable, we determined whether this would influence the capacity to activate the virus-specific T cells. SKOV3-A2, HeLa-A2 tHer2 and H292-A2 were exposed to 16 nM bs-CTX-EBVxCTX-CMV or bs-TRS-EBVxTRS-CMV and exposed tumor cells were subsequently cocultured overnight with titrated amounts of either the EBV-specific T cells or CMV-specific T cells, or the same amount of total virus specific T cells with different ratios of CMV- and EBV-specific T cells. As demonstrated in Figure 4 the SKOV3-A2 cell line exposed to either bs-CTX-EBVxCTX-CMV or bs-TRS-EBVxTRS-CMV induced nearly identical levels of T-cell activation for both the EBV- and CMV-specific T cells. (Figure 4a,b). Furthermore, when the EBV- and CMV-specific T cells were combined at different ratios, an additive effect was observed for all the different T-cell mixtures. For the HeLa-A2 tHer2 cell line exposed to bs-TRS-EBVxTRS-CMV the T cell activation appeared to be predominantly derived from the EBV-specific T-cells (Figure 4c), while for the H292-A2 cell line exposed to bs-CTX-EBVxCTX-CMV this was mainly derived from the CMV-specific T cells (Figure 4d). Nevertheless, also here the mixtures of T cells demonstrated increased T-cell activation at all ratios compared to the single T-cell populations. Moreover, for all target cell lines cocultured with either the EBV-specific T cells or CMV-specific T cells, low effector-to-target ratio's already resulted in T-cell activation levels. We concluded from these results that when both epitopes are being delivered and both T-cell populations are present, there is an additive effect in T cell activation realized for the bs-AECs (Figure 4).

Bispecific-AECs enhance epitope delivery and efficacy in vivo

We previously demonstrated that CTX- and TRS-EBV AECs could result in a significant tumor reduction in the U266 multiple myeloma xenograft mouse model and that combination with immune checkpoint blockade (ICB) prolonged survival even further due to prolonged T-cell function as previously demonstrated.[13] In this study, we evaluated AECs-EBV, -CMV and bs-AECs for their ability to deliver both epitopes and assessed the capacity of bs-CTX-EBVxCTX-CMV to produce an additive effect of T-cell activation against the U266 cell line in vitro (Figure 5a,b and S7b).

To investigate this, NSG mice were injected with U266-tEGFR, and 14 d after tumor engraftment, mice were infused with a total of 5×10^6 T cells, consisting of a 1:1 mixture of $CD8^+$ T-cells transduced with EBV- and CMV- TCRs. Treatment with CTX-EBV, -CMV, or bs-CTX-EBVxCTX-CMV was administered on day 15 and 18, with all mice receiving ICB (Figure 5c). To specifically examine the redirection of the virus-specific T cells and exclude Fc-mediated interactions, the antibodies used contained the L234A, L235A and P239G (LALAPG) mutations. Directly after treatment with CTX-EBV and bs-CTX-EBVxCTX-CMV a reduction of tumor burden was observed, whereas no such reduction was seen in mice treated with CTX-CMV (Figure 5d).

Although the *in vitro* experiments demonstrated efficient delivery of the CMV epitope to U266 cells (Figure 5a), the *in vivo* data indicated a lack of effective delivery of the CMV-epitope to U266 cells within the bone marrow. To investigate this discrepancy, we harvested the bone marrow and plasma of U266 engrafted mice 24 h after injection of CTX-CMV or CTX-EBV, in the absence of T cells. The results presented in Figure S7C demonstrate that the U266 cells obtained from mice injected with CTX-CMV failed to stimulate CMV specific T cells, whereas U266 cells derived from mice injected with CTX-EBV successfully activated EBV-specific T cells. Since intact CTX-CMV was still detectable within the blood plasma (Figure S7D), we hypothesized that intrinsic differences in the

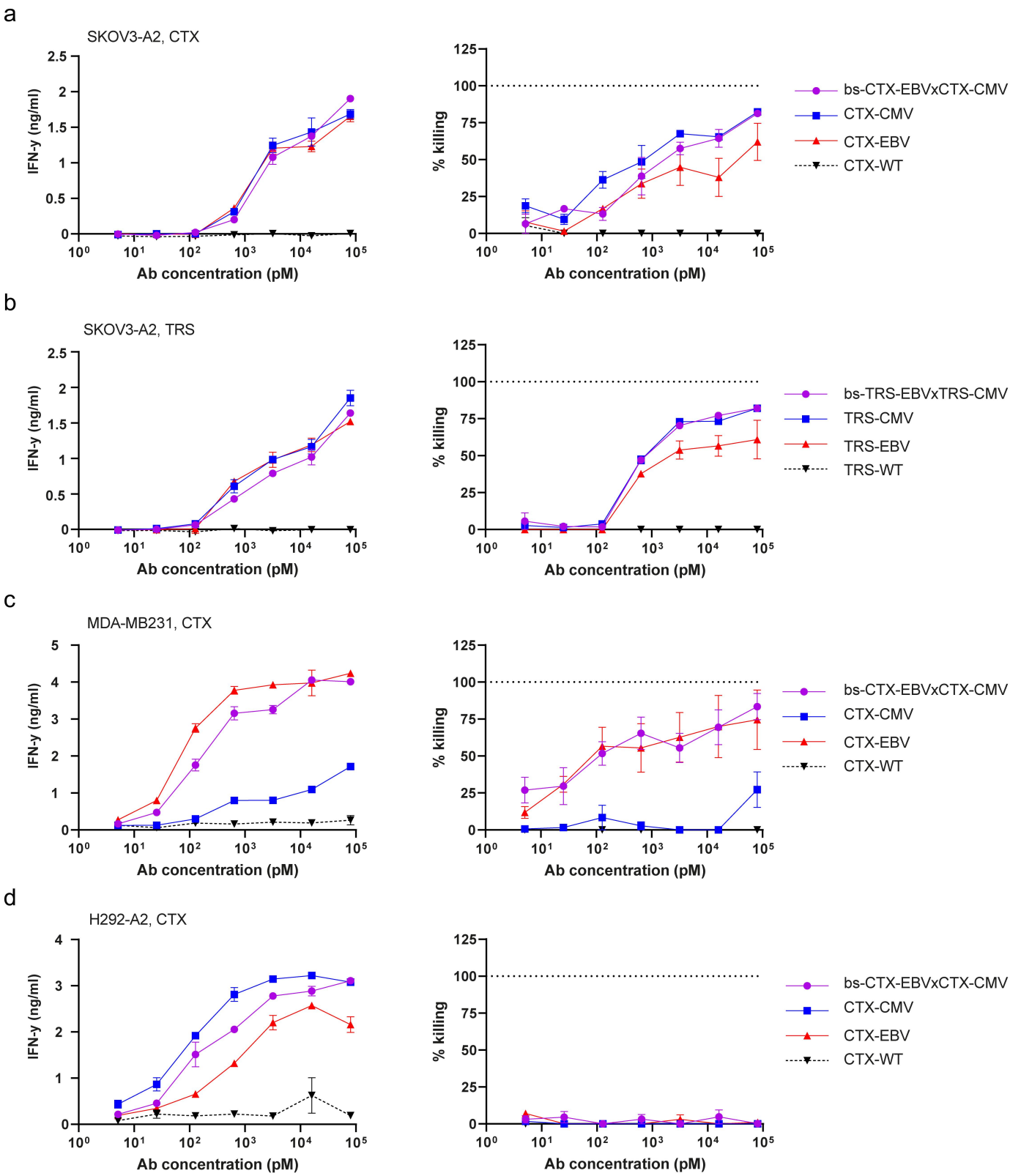

Figure 3. Tumor cells treated with bs-AECs efficiently activate virus-specific T cells. (a) SKOV3-A2, (c) H292-A2 and (d) MDA-MB231 were exposed for 1 h to wildtype CTX (CTX-WT), CTX-EBV, -CMV, or bs-CTX-EBVxCTX-CMV, and subsequently cocultured for 72 hrs with a 50%/50% mixture of EBV- and CMV-specific T cells. (b) SKOV3-A2 were exposed to TRS (WT), TRS-EBV, -CMV, or bs-TRS-EBVxTRS-CMV followed by a coculture with a 1:1 mixture of EBV- and CMV-specific T cells. (a-d) Supernatant was harvested after 18 hrs of coculture to determine IFN-γ production as a measure of T-cell activation. The tumor cell killing was measured after 72 hrs of coculture using the Alamar blue assay. Plotted values are the means of duplicates (SEM) and each graph shows a representative figure of three independently performed experiments.

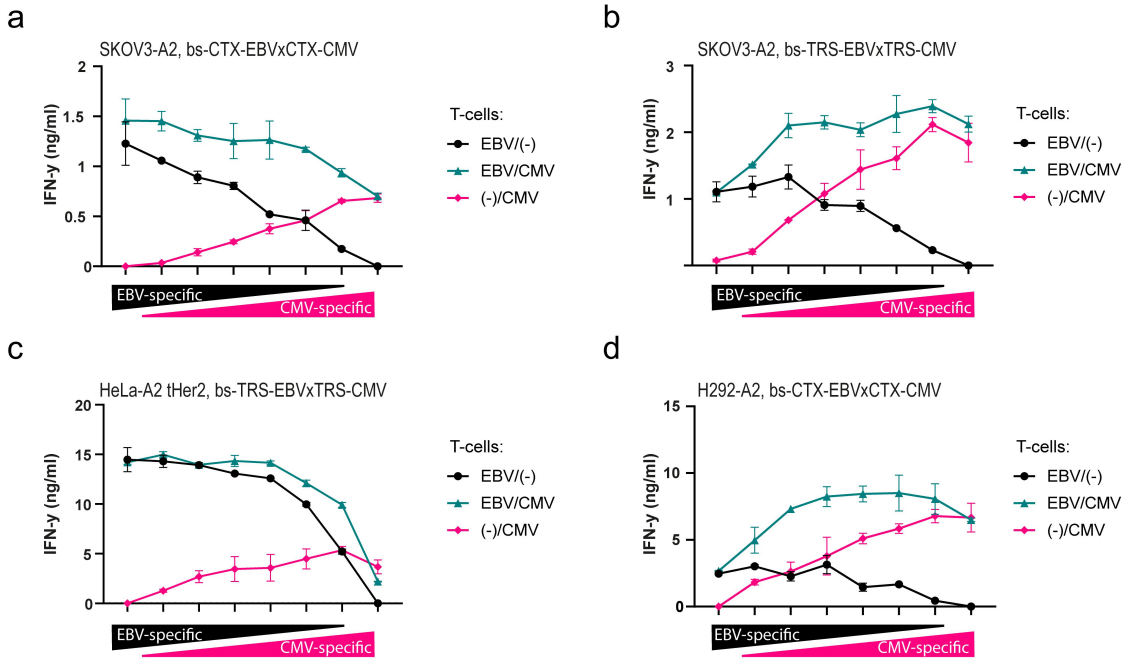

Figure 4. Bs-AECs induce additive T-cell activation when mixtures of EBV- and CMV-specific T cells are present. (a) SKOV3-A2 and (d) H292-A2 cells were exposed to 16 nM bs-CTX-EBVxCTX-CMV and (b) SKOV3-A2 and (c) HeLa-A2 tHer2 were exposed to 16 nM bs-TRS-EBVxTRS-CMV, and (a-d) subsequently cocultured with titrated amounts of EBV-specific T cells (EBV/(-)) or CMV-specific T cells ((-)/CMV) or the mixture of these titrated EBV- and CMV-specific T cells (EBV/CMV). The highest number of T cells in the EBV- and CMV-specific T-cell titration was 4.000 T cells per well and per titration step the number of T cells per well was reduced by 570. In the combination of titrated EBV- and CMV-specific T cells a total of 4.000 T-cells was present. The T-cell activation was analyzed by measuring the IFN-γ production of the T cells within the supernatant after 18 hrs of coculture. Plotted values are the means of duplicates (SEM) and each graph shows a representative figure of three independently performed experiments.

protease expression and activity levels between U266 cells *in vitro* and *in vivo* could explain this observation. Interestingly, despite the inability of CTX-CMV to deliver the CMV epitope efficiently *in vivo*, treatment with bs-CTX-EBVxCTX-CMV still resulted in a significant increase in overall survival (Figure 5e).

We extended our *in vivo* experiments with a solid xenograft model using the ovarian carcinoma cell line SKOV3-A2, which expresses both EGFR and Her2 and effectively delivers the CMV and EBV epitopes upon exposure to bs-TRS-EBVxTRS-CMV. NSG mice were s.c. injected with firefly luciferase-expressing SKOV3-A2 cells. Fourteen days after tumor engraftment, the mice were infused with a mixture of EBV- and CMV-specific T cells (1:1 ratio) transduced with Renilla luciferase. Subsequently, mice were treated with TRS-EBV, TRS-CMV or bs-TRS-EBVxTRS-CMV in combination with ICB on day 15, 18 and 21 (Figure 6a). On day 18, 4 d after T cell injection, bioluminescence imaging was performed to visualize the location of the T cells within four mice (Figure S8A). Results showed that T cells were homing to the tumor site independently of AEC treatment. We hypothesized that the human T-cells were attracted to tissues expressing human HLA, driving their migration to the tumor independently of AEC-mediated redirection.

Within all treatment groups, overall growth of the tumor was slowed down (Figure 6b), and survival was significantly increased compared to the control group (Figure 6c). This was also reflected in tumor growth as imaged with bioluminescence imaging (Figure S8B). On day 120, 2/8 mice treated with TRS-EBV, and 1/7 mice treated with bs-TRS-EBVxTRS-CMV were still alive with relatively low tumor burden (<500 mm^3). Using the SKOV3 xenograft model, we demonstrated that genetically fused AECs can redirect mixtures of virus-specific T cells toward solid tumors, leading to marked tumor reduction. Most importantly, we demonstrate that bs-AECs were as efficient as the parental AECs.

Discussion and conclusion

We previously demonstrated that genetically fused AECs represent a promising strategy for delivering immunogenic viral epitopes to redirect virus-specific $CD8^+$ T cells.[3,13] Here, we explored two methods to generate AECs with two distinct viral epitopes attached to the C-terminus of the heavy chains. In this study, we demonstrate that bs-AECs can successfully deliver both EBV and CMV epitopes to tumor cells *in vitro* and *in vivo*, offering a method to broaden the diversity of epitope payloads in genetically fused AECs. Moreover, we demonstrate that by using the already available knowledge of antibody engineering methods, new therapeutic modalities, such as the AECs, can be advanced further. Although the bs-AECs do not outperform single-epitope AECs, we demonstrate that bs-AECs can be effectively generated and utilized to increase the number of T cells redirected toward tumors in both *in vitro* and *in vivo* settings.

As previously demonstrated, genetically fused AECs proved to have a higher stability and a well-defined homogeneous EAR of 2 compared to other conjugation methods.[3] Therefore, we focused on advancing AECs through genetic fusions on the C-terminus of the heavy chain, rather than via chemical and/or

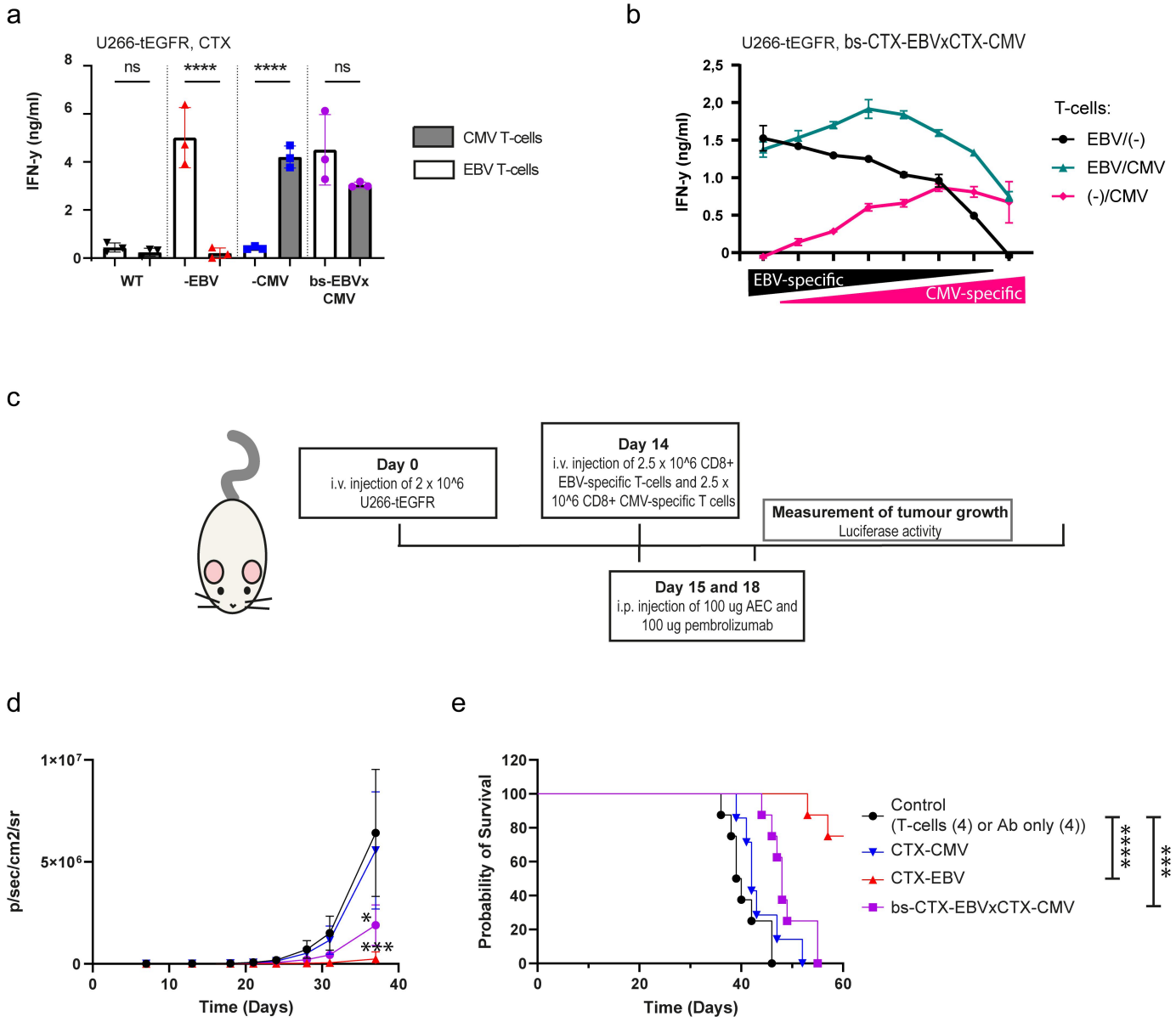

Figure 5. One EBV-epitope of bsAECs can still reduce tumor outgrowth in an U266 xenograft model. (a) U266-tEGFR cells were exposed to 16 nM of CTX-WT, -EBV, -CMV or bs-CTX-EBVxCTX-CMV and cocultured with either EBV- or CMV-specific T cells. Plotted values are the means of duplicates (SEM) of three independent experiments. (b) U266-tEGFR were exposed to 16 nM bs-CTX-EBVxCTX-CMV and subsequently cocultured with titrated amounts of EBV-specific T cells (EBV/(-)) or CMV-specific T cells ((-)/CMV) or the mixture of these titrated EBV- and CMV-specific T cells (EBV/CMV). The highest number of T cells in the EBV- and CMV-specific T-cell titration was 4.000 T cells per well and per titration step the number of T cells per well was reduced by 570. (c) Overview of the experimental set-up of the in vivo experiment. (d) NSG mice engrafted with 2×10^6 luciferase positive U266-tEGFR cells were i.V. injected with 2.5×10^6 EBV and 2.5 10^6 CMV TCR-transduced $CD8^+$ T cells at day 14. On day 15 and 18 after tumor injection mice were i.P. injected with 100 µg CTX-EBV, -CMV or bs-CTX-EBVxCTX-CMV and 100 µg pembrolizumab. Tumor outgrowth of the U266-tEGFR2 was visualized by bioluminescence imaging 1–2 times per week of the ventral side. Significance was determined with a two-way ANOVA, with a Tukey`s multiple comparisons on log-transformed data and only visualized within the graph day 36. (e) The survival analysis was performed with the Kaplan–Meier method and significance was assessed with the mantel cox method and corrected using the Bonferroni method. Treatment groups were compared to the combined control groups. The control group (Ab only) received bs-TRS-EBVxTRS-CMV and pembrolizumab, but no T cells, to demonstrate that binding of the antibody and delivery of the epitopes does not influence tumor outgrowth. Control group (T cells only) received the mixture of virus specific T cells and pembrolizumab. The endpoint was determined when the tumor outgrowth reached a bioluminescent signal of 1×10^7, which is considered the endpoint.

enzymatical conjugation methods.[3,13] To increase the overall epitope payload from 2 to 4, we explored the possibility of positioning epitopes in a concatenated, tandem-like fashion. However, as the enclosed epitope was not efficiently delivered to the cancer cells (Figure 1 and S1), this approach appeared to be disadvantageous. These results highlight the importance of a free terminus to facilitate efficient extracellular delivery of the viral epitopes, consistent with findings from other research groups.[4,14,23] Since the enclosed epitopes were unable to redirect their cognate T cells, our focus shifted toward the generation of bs-AECs without changing the specificity of the antigen recognition domains.

The need for a more diverse AEC format was demonstrated with the U266 xenograft model (Figure 5 and S7). While T-cell activation was observed *in vitro* following exposure of the U266 cells to CTX-CMV, this AEC failed to deliver the CMV epitope to the U266 cells *in vivo*. This discrepancy can potentially be explained by a shift in protease expression levels between

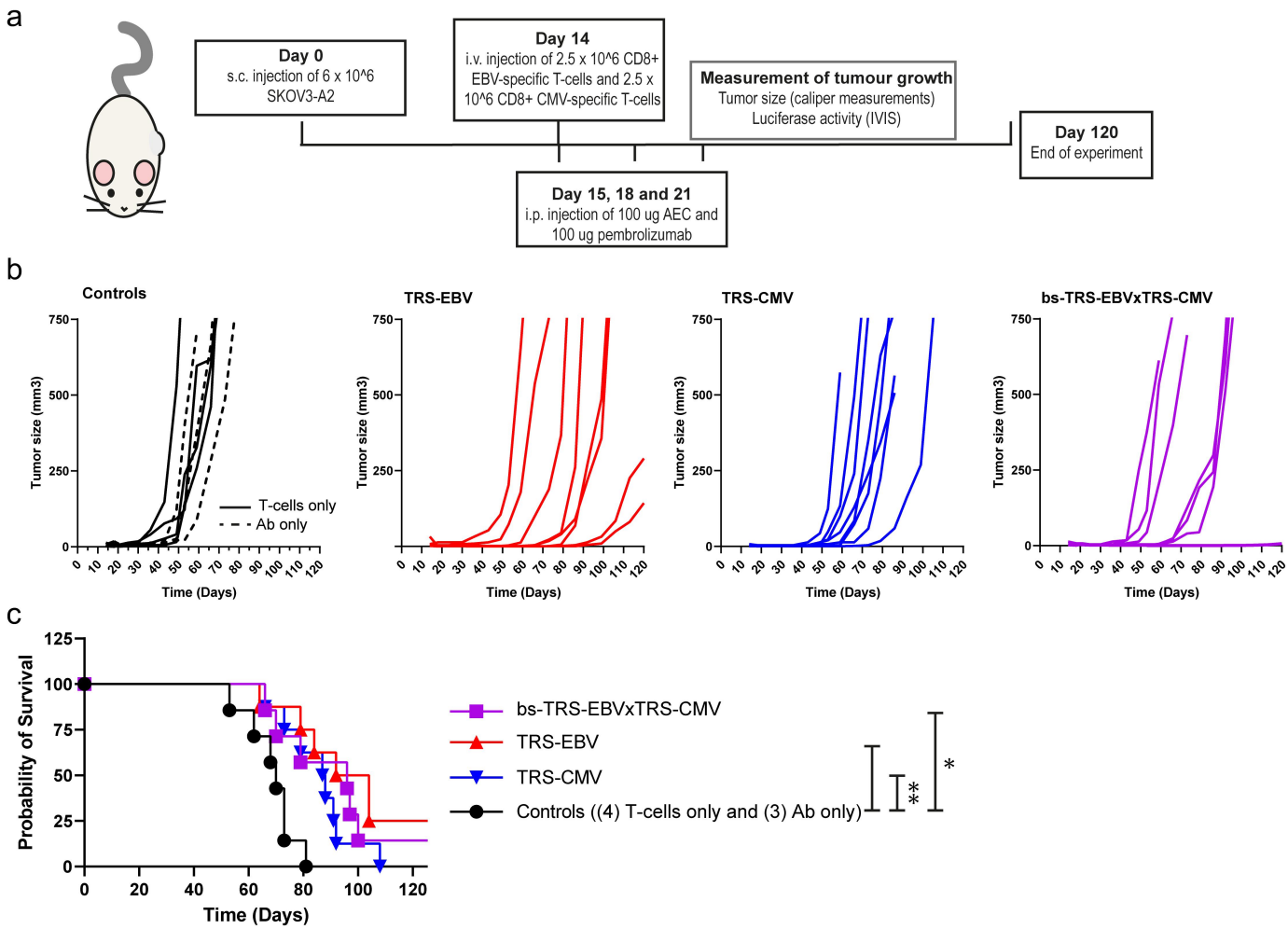

Figure 6. Both the EBV- and CMV-epitope of bs-AECs can reduce tumor outgrowth in an SKOV3-A2 xenograft model. (a) Overview of the experimental set-up of the in vivo experiment. (b) NSG mice engrafted with 6×10^6 luciferase positive SKOV3-A2 cells were subsequently i.V. injected with 2.5×10^6 EBV and 2.5 10^6 CMV TCR-transduced $CD8^+$ T cells at day 14. On day 15, 18 and 21 after tumor injection mice were i.P. injected with 100 µg TRS-EBV, -CMV or bs-TRS-EBVxTRS-CMV and 100 µg pembrolizumab. Tumor outgrowth was determined by means of caliper measurement. The control group (Ab only) received bs-TRS-EBVxTRS-CMV and pembrolizumab, but no T cells, to demonstrate that binding of the antibody and delivery of the epitopes does not influence tumor outgrowth. Control group (T cells only) received the mixture of virus specific T cells and pembrolizumab. (c) The survival analysis was performed with the Kaplan–Meier method and significance was assessed with the mantel cox method and corrected using the Bonferroni method. Treatment groups were compared to the combined control groups. The control group (Ab only) received bs-TRS-EBVxTRS-CMV and pembrolizumab, but no T cells, to demonstrate that binding of the antibody and delivery of the epitopes does not influence tumor outgrowth. The endpoint was defined at a tumor outgrowth of 1000 mm^3.

in vitro and *in vivo* conditions. Predicting the *in vivo* efficiency of a protease cleavage site–epitope combination is challenging due to the complexity of proteases, as well as the efficiency of correct epitope delivery. The complexity of the protease cleavage site lays in their broad specificity, (dys)regulation and mechanism in cancer, varying expression levels and therefore also the predictability of the proteases.[24,25] Therefore, to improve efficiency in delivering, presenting epitopes on HLA molecules, and engaging the corresponding T-cell populations, combining multiple protease cleavage site-epitope pairs could be a viable strategy.[14]

One of the advantages of the technology used to generate the bs-AECs is the possibility to mix-and-match AECs,[26] allowing for the broadening of the targeted population of T cells and adding a layer of personalization to the AECs. To broaden and improve the approach, further variations can be introduced within the attached T-cell epitopes, protease cleavage sites, as well as in the location of the epitope and the antigen specificity of the antibody itself.[4,14,27,28] However, to be able to broaden the approach and to accomplish a more personalized format, new epitopes and/or protease cleavage sites should be screened, tested, and evaluated to select the most favorable combinations.[4,14] Moreover, it is worth considering targeting other HLA class I molecules with viral epitopes derived from EBV or CMV[7,8] or potentially other viruses that are widely prevalent within the human population such as SARS-CoV2.[29,30] In addition, we recently demonstrated the feasibility of fusing an EBV-epitope to the N-terminus of the light chain, which would increase the possibilities even further.[23]

An important question is the precise mechanism by which epitopes are delivered and presented on cell surface-expressed HLA molecules. The slow internalization rate of CTX argues against intracellular delivery of the viral epitopes. Furthermore, we have previously shown that epitope delivery is more efficient when the epitopes are located closer to the cell membrane.[23] If intracellular release of the AEC were required for epitope presentation, the efficiency would not depend on the position of the epitope. These findings align with previously published data supporting a mechanism involving extracellular release of the viral epitope, mediated by

proteolytic cleavage near the cell membrane.[4] We hypothesize that when viral epitopes are released closer to the cell surface, the likelihood of their encountering and binding to HLA molecules increases. While it has been suggested that these HLA molecules may be empty, it is more plausible, given the inherent instability of empty HLA molecules, that they are occupied by low-affinity peptides.[4,31]

Interestingly, AECs have the potential to induce immune infiltration and promote both immediate tumor control and long-term antitumor immunity, as previously demonstrated by intratumoral injection of viral epitopes in immunocompetent mice.[32,33] In addition, AECs offer greater specificity and safety by precision, compared to direct intratumoral injection of viral epitopes. This specificity could be further enhanced by incorporating a functional Fc-domain or combining the AECs with immune checkpoint blockade. Nevertheless, additional studies and the use of more complex immunocompetent mouse models are necessary to investigate this in more detail.

In conclusion, we successfully generated bispecific AECs with two distinct viral epitopes positioned at the C-terminus of the heavy chain. These bs-AECs efficiently deliver both epitopes to tumor cells, redirecting EBV- and CMV-specific T-cells toward multiple cancer cell lines *in vitro* and suppressing outgrowth of SKOV3 tumors in an established xenograft mouse model. The generation of bs-AECs opens the door to an unlimited number of combinations and possibilities, making it an exciting new development for AECs as a therapeutic avenue in cancer treatment.

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Disclosure statement

BB, JS, and LG are (former) employees at Genmab BV and have ownership interests (including stocks, warrants, patents, etc.).

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Author contributions

W.W. conceived the research, designed the experiments, performed experiments, analyzed data, and performed manuscript writing and editing. D.F. G.R., C.S.K. and A.K.W. performed experiments and data analysis. M.E.R., J.S., S.I.v.K., B.B., R.C.H., L.G., and M.H.M.H. provided scientific guidance by experimental design, data analysis, and manuscript editing. R.C.H. and M.H.M.H. provided supervision. All authors reviewed the manuscript.

Data availability statement

Data are available upon request.

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