

## ORIGINAL ARTICLE

# Balanced secretion of anti-CEA × anti-CD3 diabody chains using the 2A self-cleaving peptide maximizes diabody assembly and tumor-specific cytotoxicity

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Adoptive transfer of genetically engineered human cells secreting bispecific T-cell engagers has shown encouraging therapeutic effects in preclinical models of cancer. However, reducing the toxicity and improving the effectiveness of this emerging immunotherapeutic strategy will be critical to its successful application. We have demonstrated that for gene-based bispecific antibody strategies, two-chain diabodies have a better safety profile than single-chain tandem scFvs (single-chain variable fragments), because their reduced tendency to form aggregates reduces the risk of inducing antigen-independent T-cell activation. Here, we demonstrate that the incorporation of a 2A self-processing peptide derived from foot-and-mouth disease virus conveying co-translational cleavage into a two-chain anti-CD3 × anti-CEA diabody gene enables near-equimolar expression of diabody chains 1 and 2, and thus increases the final amount of assembled diabody. This was found to maximize diabody-mediated T-cell activation and cytotoxicity against carcinoembryonic antigen-positive tumor cells.

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## INTRODUCTION

Novel immunotherapeutic strategies aiming to improve antitumor T-cell responses link the specificity of cancer-targeting antibodies with the efficient trafficking properties and effector functions of T cells.<sup>1</sup> Two such strategies are the adoptive transfer of T cells that are genetically engineered to express tumor-associated antigen-specific chimeric antigen receptors (CARs), and the infusion of engineered bispecific antibodies with one binding site recognizing a T-cell activation molecule and a second binding site recognizing a tumor-associated antigen.<sup>2</sup>

Despite the impressive clinical responses in patients with CD19-positive malignancies, both strategies have certain inherent limitations.<sup>3–8</sup> The therapeutic potential of exogenously administered engineered bispecific antibodies, such as tandem scFvs (single-chain variable fragments; otherwise known as bispecific T-cell engagers or BiTEs) or diabodies, is limited by their short half-lives and the general difficulty of delivering biopharmaceuticals to tumors.<sup>9</sup> In the case of adoptive transfer of CAR T cells, efficacy depends on *in vivo* expansion and long-term persistence that may not always be possible, especially in the immunosuppressive tumor environment.<sup>10</sup> In addition, adoptively transferred CAR T cells do not redirect resident T cells toward cancer cells.

Our group has pioneered the development and preclinical testing of a cancer immunotherapy strategy based on the adoptive transfer of genetically engineered cells secreting bispecific antibodies.<sup>11</sup> We have demonstrated that bispecific antibodies, secreted from intratumoral or tumor-distant gene-modified human cells, effectively recruit and activate T-cell cytotoxicity against tumor cells, and have potent antitumor activity in xenograft models.<sup>11–14</sup> Furthermore, we have recently demonstrated that two-chain diabodies are preferable to single-

chain BiTEs for genetic strategies based on secretion of bispecific antibodies for T-cell recruitment.<sup>15</sup> Whereas single-chain anti-CD3 × anti-CEA (αCD3 × αCEA) BiTEs induced human T-cell activation and proliferation in an antigen-independent manner, two-chain αCD3 × αCEA diabodies exerted almost no proliferative stimulus when human T cells were cultured alone or with carcinoembryonic antigen (CEA)-negative cells.<sup>15</sup>

The original two-chain αCD3 × αCEA diabody has been expressed in human cells using an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus.<sup>11,12</sup> When using an IRES to express multiple genes in one mRNA, the gene directly downstream of the promoter is translated by the canonical cap-dependent mechanism, whereas those downstream of the IRES are translated by a cap-independent mechanism. Because the cap-independent mechanism has lower translation efficiency than the cap-dependent mechanism, the first cap-dependent gene is translated up to sixfold higher than the second cap-independent gene.<sup>16</sup> This might be especially important for gene-based *in situ* secretion strategies of two-chain bispecific antibodies, as an excess of either chain might limit the interaction of the assembled diabody with the target antigen/s.

These limitations might be solved using the 2A peptides, small (18–22 amino acids) self-processing peptides first identified in the foot-and-mouth disease virus (FMDV) and later in other genera of the *Picornaviridae* family.<sup>17</sup> Also referred to as CHYSEL (*cis*-acting hydrolase element), 2A peptides process themselves during translation, resulting in the 'self-cleavage' of their primary 2A/2B polyproteins by interfering with the formation of the peptide bond between the C-terminal glycine residue of 2A and the N-terminal proline residue of 2B.<sup>18,19</sup> Here, we demonstrate that the incorporation of a 2A self-processing peptide derived from

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FMDV into the two-chain  $\alpha$ CD3 $\times\alpha$ CEA diabody significantly improves the balance between expressed diabody chain-1 and diabody chain-2. Furthermore, the balanced secretion of diabody chains facilitates the generation of functional assembled diabodies.

## RESULTS

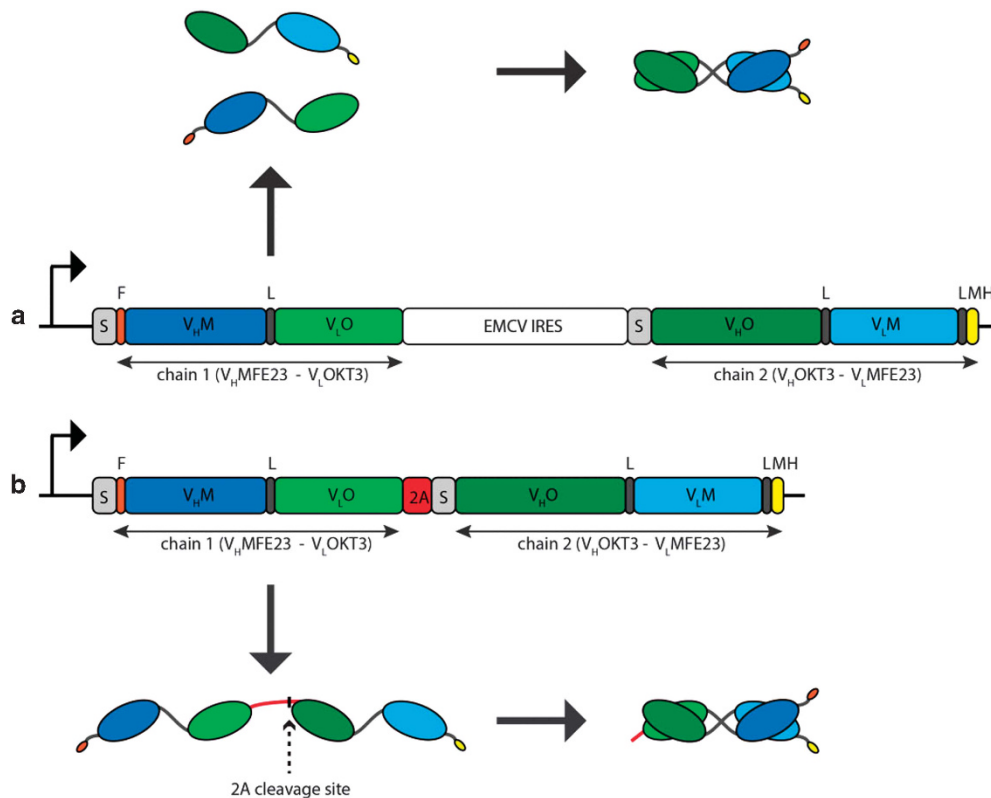
### Comparison of IRES and F2A for expression of a two-chain bispecific diabody

Two vectors were generated in order to compare the coexpression efficiency of the chain-1 ( $V_{H,MFE23}$ - $V_{L,OKT3}$ ) and the chain-2 ( $V_{H,OKT3}$ - $V_{L,MFE23}$ ) of the  $\alpha$ CEA $\times\alpha$ CD3 diabody from a single open reading frame using either an IRES sequence derived from encephalomyocarditis virus or a 2A self-cleaving peptide (APVKQLNFDLLKLAGDVESNPGP) derived from FMDV (F2A) (Figure 1). The diabody chain-1 bore a FLAG-tag at the N-terminus, whereas the diabody chain-2 bore a c-myc/His-6 tag at the C-terminus (Figure 1).

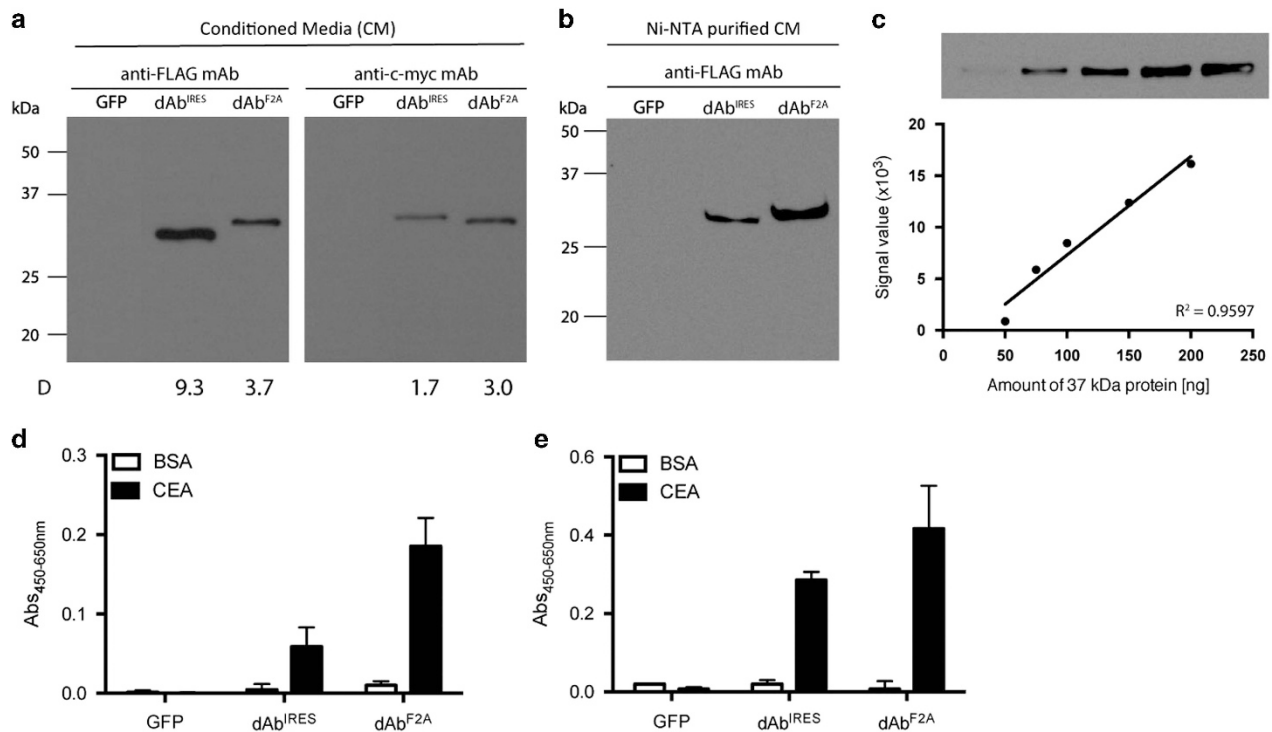
Liposome-mediated gene transfer of HEK-293 cells resulted in high transfection efficiencies, with 80–90% green fluorescent protein (GFP)-positive cells (Supplementary Figure 1). Conditioned media from HEK-293 cells transiently transfected with IRES- or F2A-based vectors were harvested to characterize expression levels and binding properties of secreted  $\alpha$ CEA $\times\alpha$ CD3 diabody. Western blot analysis, under reducing conditions, demonstrated that secreted chain-1 and chain-2 were single-chain-type molecules with a migration pattern consistent with the molecular weights calculated from their amino-acid sequences (Figure 2a). The molecular weight of the diabody chain-1 expressed from the

F2A-based plasmid was slightly higher (33.2 kDa) than that of the diabody chain-1 expressed from the IRES-based plasmid (30.7 kDa) because of a terminal segment of 23 amino-acid residues derived from the F2A sequence remnant after cleavage (Figure 1). Bands of 34.7 and 33.3 kDa, corresponding to the diabody chain-2, were detected in the cell culture supernatant from IRES-diabody- and F2A-diabody-transfected HEK-293 cells with the anti-c-myc monoclonal antibody mAb (Figure 2a). Comparing the relative intensities of the bands indicated that the IRES sequence gave expression of the two diabody chains at a ratio of ~5:1, whereas the F2A peptide allowed for equimolar coexpression of both diabody chains. Importantly, no high molecular weight bands that might correspond to unprocessed  $\alpha$ CEA $\times\alpha$ CD3 F2A diabody were observed, demonstrating high cleavage efficiency of F2A peptide in genetically modified human cells (Figure 2a). To determine the amount of assembled diabody, conditioned media from transfected HEK-293 cells were purified with Ni-NTA magnetic beads and the eluates were analyzed by western blotting using anti-FLAG mAb. As shown in Figure 2b, the amount of assembled  $\alpha$ CEA $\times\alpha$ CD3 diabody in conditioned media from F2A-diabody-transfected cells was higher than in conditioned media from IRES-diabody-transfected cells. In comparison with a standard curve obtained using an internally produced trimerbody N-terminally tagged with the FLAG tag<sup>20</sup> (Figure 2c), the amounts of assembled  $\alpha$ CEA $\times\alpha$ CD3 diabody were found to be  $0.15 \mu\text{g ml}^{-1} \times 10^6$  cells per 48 h and  $0.50 \mu\text{g ml}^{-1} \times 10^6$  cells per 48 h for IRES diabody and 2A diabody, respectively.

Secreted  $\alpha$ CEA $\times\alpha$ CD3 diabody from IRES-diabody- and F2A-diabody-transfected HEK-293 cells bound specifically to solid-phase CEA (Figures 2d and e). In agreement with the greater



**Figure 1.** Schematic representation of the gene constructs and the domain structure and assembly of the two-chain  $\alpha$ CD3 $\times\alpha$ CEA diabodies. Structure of the IRES-based (**a**) and the 2A-based (**b**) bicistronic cassettes, carrying both diabody chain-1 ( $V_{H,MFE23}$ - $V_{L,OKT3}$ ) and diabody chain-2 ( $V_{H,OKT3}$ - $V_{L,MFE23}$ ) under the control of the cytomegalovirus (CMV) promoter/enhancer and a heterologous signal peptide from the oncostatin M (S). The dark gray boxes represent ( $G_4S$ ) linker peptides (L), the orange boxes represent FLAG tags (F) and the yellow boxes represent c-myc/His-6 tags (MH). The white box represents the IRES sequence from the encephalomyocarditis virus (EMCV) and the red box the 2A peptide from the FMDV. Arrows indicate the direction of transcription.



**Figure 2.** Characterization of secreted two-chain  $\alpha$ CD3  $\times$   $\alpha$ CEA diabodies. The presence of secreted diabodies in the neat conditioned media (CM) from transfected HEK-293 cells was demonstrated by western blot analysis (a). Migration distances of molecular mass markers are indicated (kDa). The blots were developed with anti-FLAG or anti-c-myc mAb. Quantification of the assembled diabody by western blotting with anti-FLAG mAb after purification with Ni-NTA beads from neat conditioned media (CM) from transfected HEK-293 cells (b). Standard curves of signal values versus protein amount (in ng) were generated using data from the dilution series of a purified Flag-tagged 37 kDa protein (c). The functionality of secreted antibodies was demonstrated by ELISA against plastic immobilized bovine serum albumin (BSA) and CEA (d, e), as described in the Materials and methods section. Data shown are from a representative experiment out of three independent ones.

amount of the assembled diabody observed in conditioned media from F2A-diabody-transfected HEK-293 cells, more F2A diabody bound to solid-phase CEA than IRES diabody. Similarly, more F2A diabody bound to human CEA-positive MKN45 gastric carcinoma cells compared with IRES diabody (Figure 3). In contrast, secreted IRES and F2A diabodies bound similarly to CD3 expressed on the surface of Jurkat cells (Figure 3). Neither IRES nor F2A diabodies bound to CEA-negative and CD3-negative HeLa cells (Figure 3), and conditioned medium from HEK-293 cells transfected with GFP expression vectors did not bind to HeLa and MKN45 cells (data not shown).

#### Induction of cytotoxic activity by secreted IRES and F2A two-chain diabody

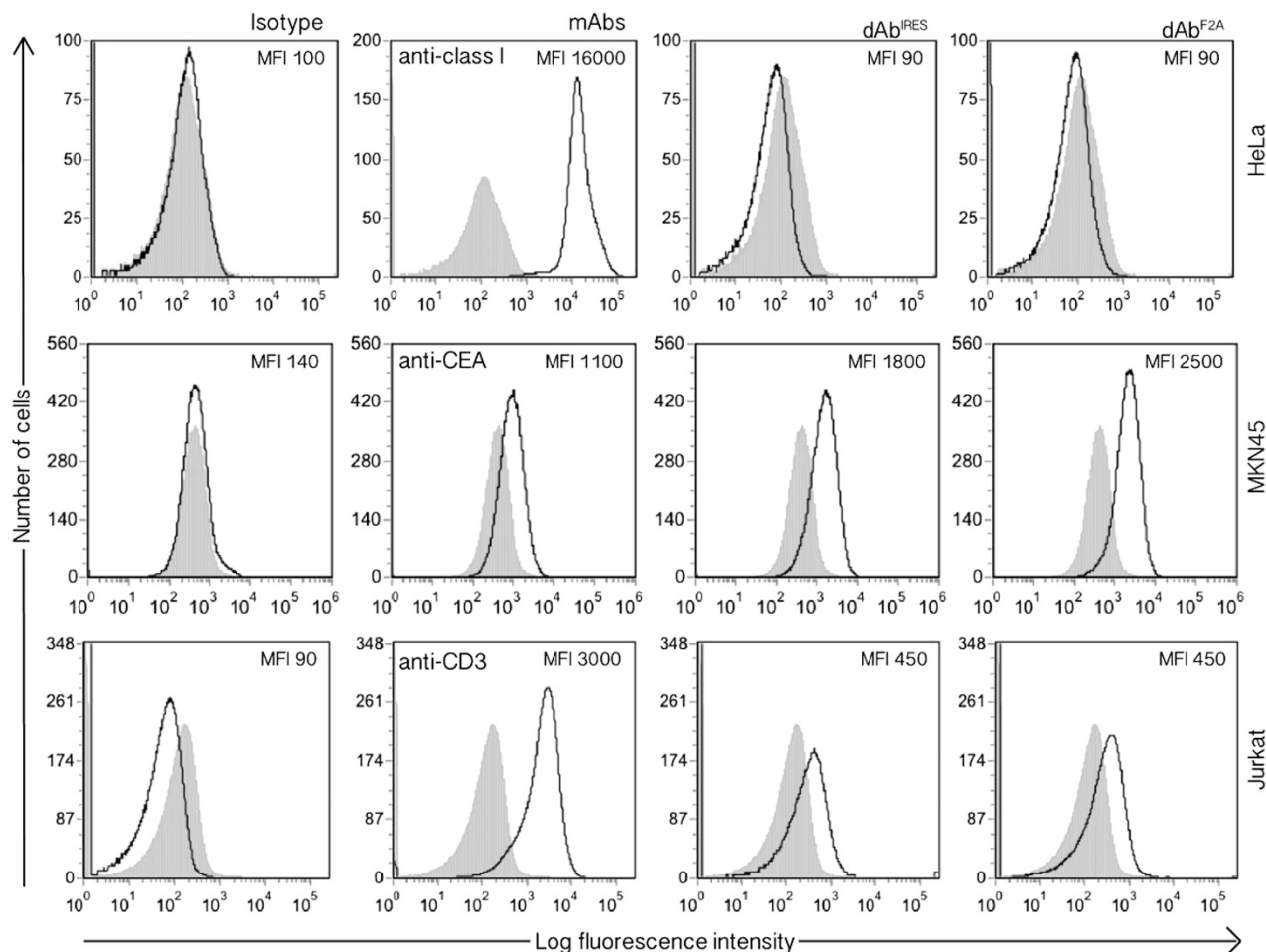
To demonstrate that secreted  $\alpha$ CEA  $\times$   $\alpha$ CD3 diabodies redirect human T cells to CEA-positive tumor cells, conditioned media from IRES- or F2A-diabody-transfected HEK-293 cells were mixed with luciferase-expressing CEA-negative HeLa<sup>Luc</sup> or CEA-positive MKN45<sup>Luc</sup> tumor cells (Supplementary Figure 2) and resting peripheral T cells derived from peripheral blood mononuclear cells (PBMCs) from healthy donors. After 24 h, interferon- $\gamma$  was measured in the cell culture supernatants. Both IRES and F2A diabodies redirected human primary T cells specifically to MKN45 cells (Figure 4a), but the interferon- $\gamma$  levels were significantly higher ( $P < 0.001$ ) when T cells were redirected to tumor by media from F2A-diabody-transfected HEK-293 cells ( $P < 0.001$ ) than by media from IRES-diabody-transfected HEK-293 cells ( $P < 0.01$ ). No secretion of interferon- $\gamma$  was detected when human T cells were cocultured with HeLa cells in the presence of IRES or F2A diabody in conditioned media, or when cocultured with HeLa or MKN45 cells in the presence of conditioned media from HEK-293 cells

transfected with an expression vector encoding an anti-laminin L36 N-terminal trimerbody (L36<sup>T</sup>; Figure 4a).

The ability of secreted  $\alpha$ CEA  $\times$   $\alpha$ CD3 diabodies to induce tumor cell cytotoxicity by redirecting T cell-mediated cytotoxicity was investigated using different *in vitro* systems. First, HeLa<sup>Luc</sup> or MKN45<sup>Luc</sup> tumor cells were cocultured with unstimulated human PBMCs at an effector/target ratio of 5:1 in the presence of conditioned media from transfected HEK-293 cells (Figure 4b). Both IRES- and F2A-diabody-containing media were found to specifically induce the death of CEA-positive tumor cells, but F2A diabody was more efficient than IRES diabody at triggering T-cell cytotoxic activity ( $P < 0.05$ ). No cytotoxic activity was observed using CEA-negative tumor cells as target cells (Figure 4b). To approximate *in vivo* conditions and to investigate the ability of locally produced  $\alpha$ CEA  $\times$   $\alpha$ CD3 diabodies to induce tumor cell lysis by unstimulated human peripheral T cells, we used transwell cell culture dishes. In this system, HeLa<sup>Luc</sup> or MKN45<sup>Luc</sup> tumor cells and freshly isolated primary T cells were cocultured in the bottom well and transfected HEK-293 cells were present in the insert well (Figure 4c). At an effector/target/producer ratio of 5:1:1, T cells activated with F2A or IRES diabody exhibited strong cytotoxicity toward MKN45 tumor cells (Figures 4c–e). No cell tumor killing was observed after cocultivation with L36<sup>T</sup> transfected HEK-293 cells or when HeLa cells were used as targets (Figures 4c–e). When T cells were omitted, no cytotoxicity was observed (data not shown).

#### DISCUSSION

Here, we demonstrate that the F2A self-processing peptide can be used for coexpression of the diabody chains at an equimolar ratio,



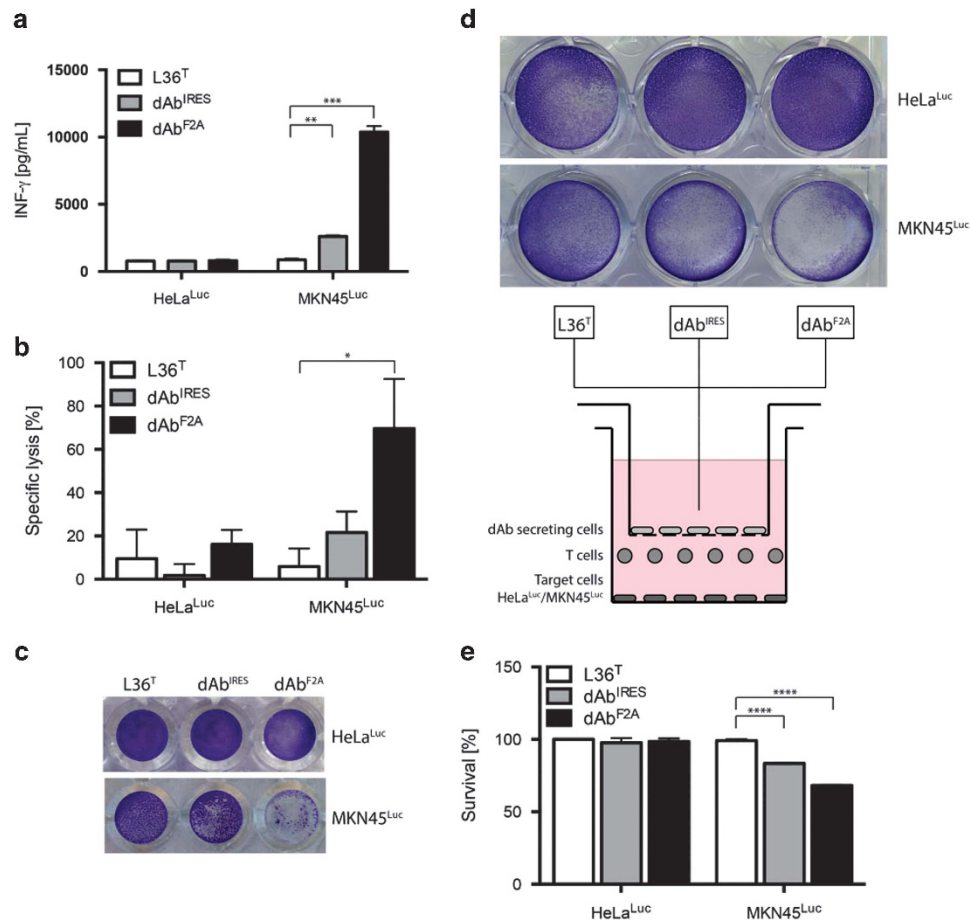
**Figure 3.** Flow cytometric analysis of binding of secreted two-chain  $\alpha$ CD3 $\times$  $\alpha$ CEA diabodies to the surface of HeLa, MKN45 and Jurkat cells, using neat conditioned media from transfected HEK-293 cells. The numbers in the right corners of each histogram indicate the mean fluorescence intensity (MFI). Anti-MHC class I (W6/32), anti-CD3 (OKT3) and anti-CEA (C6G9) mAbs were used as controls on fluorescence-activated cell sorting (FACS) studies. The y axis shows the number of cells and the x axis represents the intensity of fluorescence, expressed on a logarithmic scale. One representative experiment out of three independent experiments is shown.

whereas the IRES sequence allows expression of the diabody chains at a ratio of  $\sim$ 5:1. The F2A-containing diabody molecules were secreted in a functionally active form by gene-modified human cells and, importantly, the balanced production of the two diabody chains resulted in increased overall amounts of the assembled  $\alpha$ CEA $\times$  $\alpha$ CD3 diabody. The amount of assembled diabody in conditioned media from transfected HEK-293 cells using F2A was approximately threefold higher than that obtained using IRES, leading to enhanced cytotoxic T-cell responses against CEA-positive tumor cells. This successful co-translational 'cleavage' of diabody chains has important implications for therapeutic strategies based on the *in vivo* secretion of two-chain bispecific diabodies, as it maximizes the locally available, fully functional, assembled  $\alpha$ CEA $\times$  $\alpha$ CD3 diabody and minimizes free diabody chain-1 ( $V_H$ MFE23- $V_L$ OKT3) that might compete with the binding of assembled diabody to the antigen recognized by the  $V_H$ MFE23 (CEA), especially considering that  $V_H$  residues appear to contribute more to the free energy of binding than  $V_L$  residues.<sup>21</sup>

Although the use of F2A yielded more assembled diabody, the expression levels of the diabody chain-1 were higher in the IRES-containing construct, despite using identical vectors for both bicistronic cassettes. This was not clarified in the current study. Our findings suggest that the 23 additional amino acids derived from the F2A sequence at the C-terminus of the diabody chain-1 did not meaningfully affect the functionality of the secreted

$\alpha$ CEA $\times$  $\alpha$ CD3 diabody. If necessary, these residues could be removed by adding a furin cleavage site next to the F2A sequence.<sup>22</sup> A potential drawback of 2A peptides is the possibility of uncleaved protein.<sup>23</sup> This would be undesirable and could result in nonfunctional diabody, but we did not observe uncleaved  $\alpha$ CEA $\times$  $\alpha$ CD3 diabody when chain-1 and chain-2 were coexpressed from a single open reading frame using a viral 2A peptide. The use of viral-derived 2A sequences in constructs intended for human gene therapy is supported by a recent study demonstrating that these sequences do not produce unwanted T-cell responses in immunocompetent individuals.<sup>24</sup>

Our group has developed a cancer immunotherapy strategy based on the secretion of engineered anti-CD3 $\times$ antitumor bispecific antibodies by human cells.<sup>11</sup> We have shown that various types of human cell carriers, such as primary T cells, mesenchymal and hematopoietic stem cells and endothelial cells, can be genetically modified to secrete functionally active  $\alpha$ CEA $\times$  $\alpha$ CD3 diabody from tumor-resident cells or tumor-distant cells.<sup>12–14,25</sup> The secreted diabody is then able to redirect resident cytotoxic T cells to tumors. This strategy offers advantages over current antigen-specific immunotherapies, such as the systemic administration of purified bispecific antibodies and the grafting of T lymphocytes with CAR genes. The prolonged *in situ* secretion of engineered anti-CD3 $\times$ antitumor bispecific antibodies would provide time for tumor-infiltrating T lymphocytes to proliferate



**Figure 4.** Induction of T-cell activation and cytotoxicity by secreted two-chain  $\alpha$ CD3  $\times$   $\alpha$ CEA diabodies. Luciferase expressing CEA-negative HeLa<sup>Luc</sup> or CEA-positive MKN45<sup>Luc</sup> tumor cells were cocultured in 96-well plates with unstimulated human peripheral T cells in the effector/target (E/T) ratio of 5:1, and media from HEK-293 cells transfected with expression vector encoding an anti-laminin L36 N-terminal trimerbody (L36<sup>T</sup>), IRES diabody or F2A diabody. After 24 h, interferon- $\gamma$  (IFN- $\gamma$ ) production was determined by ELISA (mean  $\pm$  s.d.;  $n = 3$ ) (a), and after 72 h 20  $\mu$ g per well D-luciferin was added and bioluminescence detected via luminometry (b). Percent viability was calculated relative to the luminescence from an equal number of input control cells and used to calculate percent-specific lysis (mean  $\pm$  s.d.;  $n = 3$ ). Scheme of the transwell cell culture chamber used (c). In the lower chamber of a 24-well plate, monolayers of HeLa<sup>Luc</sup> or MKN45<sup>Luc</sup> cells were incubated (E/T cell ratio 5:1) with unstimulated human primary T cells. In the upper chamber, transfected HEK-293 cells (L36<sup>T</sup>, dAb<sup>IRES</sup> or dAb<sup>F2A</sup>) were added. After 72 h, adherent cells (lower chamber) were fixed, stained with crystal violet, photographed (d) and measured (e), as described in the Materials and methods section. The experiments were performed three times and results of one representative experiment are shown (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ).

and attack the cancer cells, and, importantly, recruitment is not restricted to the genetically engineered T cells, as in the CAR approach.<sup>2</sup> The polyclonal recruitment of both tumor-infiltrating gene-modified (*cis*-recruitment) and unmodified (*trans*-recruitment) T cells would amplify the effector response. Furthermore, spatially restricted expression of diabodies inside the tumor would decrease the toxicity inherent to systemic T-cell activation. This strategy could be implemented by engineering appropriate human cell carriers to secrete antibodies or fusion proteins blocking inhibitory receptors or triggering activator receptors.<sup>26,27</sup>

More recently, other groups have confirmed these early observations and have validated the effectiveness of this cancer immunotherapy strategy. Retrovirally transduced T cells secreting EphA2-specific or CD19-specific BiTEs have demonstrated potent antitumor activity in animal models.<sup>28,29</sup> Furthermore, it has been shown that electroporation of T cells with a CD19-specific BiTE mRNA completely eradicated leukemia cells *in vivo*.<sup>30</sup> Importantly, the T cells secreting CD19-specific BiTEs showed greater efficacy compared with T cells expressing CD19-specific CARs and resulted in complete leukemia remission. In addition, toxicities associated with genetically engineered T cells secreting bispecific antibodies

were controllable and did not include B-cell aplasia.<sup>30</sup> Engineered anti-CD16  $\times$  antitumor bispecific antibodies, aiming to recruit Fc-gamma receptors (Fc $\gamma$ R)-expressing effector cells, have also demonstrated their therapeutic utility in this context. Genetically engineered mesenchymal stem cell-like cells secreting a single-chain anti-CD16  $\times$  anti-HER2 antibody exhibited antitumor effect against human breast cancer tumors.<sup>31</sup>

Although most of these studies have been performed with bispecific antibodies in the BiTE format, our previous study has shown that two-chain bispecific diabodies are preferable for gene-based *in situ* secretion strategies.<sup>15</sup> Whereas single-chain  $\alpha$ CEA  $\times$   $\alpha$ CD3 BiTEs induced human T-cell activation and proliferation in an antigen-independent manner, secreted two-chain  $\alpha$ CEA  $\times$   $\alpha$ CD3 diabodies exerted almost no proliferative stimulus when human T cells were cultured alone or with CEA-negative cells.<sup>15</sup> Single-chain  $\alpha$ CEA  $\times$   $\alpha$ CD3 molecules have shown a greater tendency to form aggregates than diabody constructs and, importantly, there was a direct relationship between antibody aggregates and nonspecific activation of T cells. In this study, we demonstrate for the first time the usefulness of the FMDV 2A self-cleavable peptide for secretion of functionally active two-chain  $\alpha$ CEA  $\times$   $\alpha$ CD3

diabody by genetically engineered human cells. Importantly, we found that the balanced secretion of diabody chains resulted in an increase in the production of assembled diabodies, with a corresponding improvement in antigen binding and the promotion of cytotoxicity. The use of bispecific antibodies in a two-chain diabody format, with the equimolar coexpression of both diabody chains facilitated by internal self-processing peptides, may provide the efficacy and maintenance of antigen dependency that are necessary for this emerging immunotherapeutic strategy to advance to clinical use.

## MATERIALS AND METHODS

### General reagents and antibodies

Native human CEA was from Merck Millipore (Darmstadt, Germany) and bovine serum albumin was from Sigma-Aldrich (St Louis, MO, USA). The mAbs used were OKT3 (anti-human CD3 $\epsilon$ ; Orthoclone, Ortho Biotech, Bridgewater, NJ, USA), W6/32 (anti-human MHC class I; Sigma-Aldrich), C6G9 (anti-human CD66e/CEA; Sigma-Aldrich), M2 (anti-FLAG, Sigma-Aldrich) and fluorescein isothiocyanate-conjugated anti-human CD3 $\epsilon$  (clone UCHT1, ImmunoTools, Friesoythe, Germany). The polyclonal antibodies included: phycoerythrin-conjugated goat F(ab')<sub>2</sub> fragment anti-mouse IgG, Fc specific (Jackson Immuno Research, Newmarket, UK) and horseradish peroxidase-conjugated goat anti-mouse IgG (Fc specific, Sigma-Aldrich).

### Cell lines and culture conditions

HEK-293 cells (human embryo kidney epithelia, CRL-1573) and HeLa cells (human cervix adenocarcinoma; CCL-2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (HyClone, GE Healthcare Life Sciences, Little Chalfont, UK), 2 mM L-glutamine and penicillin/streptomycin (all from Life Technologies, Carlsbad, CA, USA). Jurkat (human acute T-cell leukemia; TIB-152) clone E6-1 cells were maintained in RPMI medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. All of these cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). MKN45 cells (human stomach adenocarcinoma; JCRB-0254) were obtained from the Japanese Collection of Research Bioresource (Tokyo, Japan) and were grown in complete Dulbecco's modified Eagle's medium. The generation of HeLa and MKN45 cells expressing the firefly luciferase (Luc) gene (HeLa<sup>Luc</sup> and MKN45<sup>Luc</sup>) has been described previously.<sup>15</sup> The cell lines were routinely screened for the absence of mycoplasma contamination by PCR using the Mycoplasma Plus TM Primer Set (Biotools, Jupiter, FL, USA).

### Construction of expression vectors

The construction of the IRES-containing  $\alpha$ CD3  $\times$   $\alpha$ CEA diabody expression vector pdAb3 has been previously described.<sup>11</sup> To construct the pdAb3<sup>FLAG</sup> expression vector the pdAb3 plasmid was digested with *NheI/BlnI* to remove the cytomegalovirus enhancer/promoter, the heterologous oncostatin M signal peptide and the partial V<sub>H</sub> MFE23 domain. A synthetic gene encoding the cytomegalovirus enhancer/promoter, the oncostatin M signal peptide, a N-terminal FLAG tag (DYKDDDDK) and the partial V<sub>H</sub> domain of MFE23 was synthesized by Geneart AG (Life Technologies) and cloned using *NheI/BlnI* restriction sites. The IRES sequence was removed using *NotI/XhoI* restriction sites and a synthetic gene encoding the 2A sequence derived from FMDV and the diabody chain 2 (V<sub>H</sub>-OKT3 and V<sub>L</sub>-MFE23) along with the C-terminal myc-tag and 6xHis-tag was synthesized by Geneart AG and cloned to construct the pdAb3<sup>FLAG</sup>-F2A expression vector. The pEGFP-N1 plasmid (Takara Bio Inc., Shiga, Japan) was used for evaluating transfection efficiency.

### Cell transfection

Transient transfection of HEK-293 cells was carried out using Lipofectamine 3000 following the manufacturer's protocol (Life Technologies). For each transfection,  $9 \times 10^5$  cells per well were plated in 6-well plates 1 day before to reach 70–80% confluence at the time of transfection. In each transfection, 5  $\mu$ g of the appropriate plasmid (pEGFP-N1, pCR3.1-L36<sup>T</sup>, pdAb3<sup>FLAG</sup> or pdAb3<sup>FLAG</sup>-F2A) was used. Transfections were carried out in duplicate. At 48 h post transfection, cells and supernatant were collected to measure GFP fluorescence intensity by flow cytometry and to study the

secreted  $\alpha$ CD3  $\times$   $\alpha$ CEA diabody by western blotting, enzyme-linked immunosorbent assay (ELISA) and flow cytometry.<sup>32</sup>

### Western blotting

Samples were separated under reducing conditions on 12% Tris-glycine SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Life Technologies) and probed with anti-c-myc mAb or anti-FLAG mAb, followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG. For Ni-NTA purification of His-tagged diabodies, 2 ml aliquots of filtered cell-free conditioned media from 72 h transfected HEK-293 cells were incubated with Ni-NTA magnetic beads (5 Prime GmbH, Hilden, Germany) overnight at 4 °C with rotation, washed and bound proteins were eluted by boiling in 1  $\times$  sample buffer and analyzed as described above with anti-FLAG mAb. Protein bands were detected using the ECL Plus western blot detection system-II (GE Healthcare Life Sciences). Densitometric analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### ELISA

The ability of secreted diabodies to bind purified CEA was studied by ELISA as previously described.<sup>33</sup> Briefly, Maxisorp (NUNC Brand Products, Roskilde, Denmark) plates were coated with CEA (0.3  $\mu$ g per well) and after washing and blocking with 200  $\mu$ l 5% bovine serum albumin in phosphate-buffered saline, 100  $\mu$ l of supernatant from transfected cells were added and incubated for 1 h at room temperature. After three washes, 100  $\mu$ l of anti-c-myc mAb or anti-FLAG mAb were added followed by 100  $\mu$ l of horseradish peroxidase-conjugated goat anti-mouse IgG, after which the plate was washed and developed.

### Flow cytometry

Binding of secreted diabodies to CEA and CD3 expressed on the cells surface was studied by fluorescence-activated cell sorting as described previously.<sup>15</sup> Briefly, HeLa, MKN45 or Jurkat cells were incubated on ice for 30 min with filtered cell-free conditioned media from 72 h of transfected HEK-293 cells, washed and incubated for 30 min with anti-c-myc mAb and phycoerythrin-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG antibody. C6G9 (anti-CEA), W6/32 (anti-MHC class I) and OKT3 (anti-CD3) mAbs were used as positive controls. The samples were analyzed using a Cell Sorter SH800 (Sony, Tokyo, Japan).

### T-cell activation assays

Human PBMCs were isolated from the buffy coat fraction of healthy volunteers' peripheral blood by density-gradient centrifugation. Unstimulated human PBMCs were cultured in RPMI complete medium and stimulated (in triplicate) in 96-well microtiter plates with gene-modified luciferase expressing HeLa (HeLa<sup>Luc</sup>) or MKN45 cells (MKN45<sup>Luc</sup>) target cells in a 5:1 effector/target cell ratio in the presence of cell-free conditioned media from 72 h of transfected HEK-293 cells. As controls, effector cells were cultured alone with the aforementioned cell-free conditioned medium. After 24 h, supernatants were harvested and the levels of interferon- $\gamma$  were measured by using a commercially available ELISA test kit (Diacclone, Besançon, France).

### Cytotoxicity assay

Gene-modified luciferase expressing HeLa<sup>Luc</sup> or MKN45<sup>Luc</sup> cells were plated in triplicate in 96-well plates at  $4 \times 10^4$  cells per well 1 day before the assay. Human PBMCs cells were added in a 5:1 effector/target ratio in the presence of cell-free conditioned medium from transfected HEK-293 cells. After 72 h of incubation, viability was measured by adding 20  $\mu$ g per well D-luciferin (Promega, Madison, WI, USA) and bioluminescence quantified in relative light units using an Infinite 200 luminometer (Tecan Trading AG, Männedorf, Switzerland). A 100% lysis control was included by treating the target cells with 1% Triton-X100 (Sigma-Aldrich), and the value for spontaneous lysis was obtained by incubating the target cells with effector cells only. Percent tumor cell viability was calculated as the mean bioluminescence of each sample divided by the mean bioluminescence of the input number of control target cells times 100. Specific lysis is the difference in tumor cell viability relative to control (0%). For cytotoxic studies in transwell systems, a polyethylene terephthalate filter insert (6.5 mm diameter) with 0.4  $\mu$ m pores (Falcon, BD Biosciences, San Jose, CA, USA) was used. HeLa<sup>Luc</sup> or MKN45<sup>Luc</sup> cells ( $5 \times 10^5$ ) were plated on

bottom wells of 24-well plate. After 24 h, human PBMCs ( $2.5 \times 10^6$ ) were added to bottom wells and transfected HEK-293 cells ( $1 \times 10^5$ ; L36<sup>T</sup>, dAb<sup>IR5</sup>, dAb<sup>F2A</sup>) were added to transwell insert wells. After 72 h, the transwell insert and the nonadherent cells were removed and the viable tumor cells detected by crystal violet staining. Briefly, after washing with phosphate-buffered saline, cells were incubated with crystal violet reactant (Sigma-Aldrich; 10 mg ml<sup>-1</sup> in a distilled water–0.5% glutaraldehyde solution) for 20 min at room temperature. The dye was washed in running water and colorant was recovered with 10% acetic acid and transferred to 96-well plates for optical density evaluation at 595 nm.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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