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Functional comparison of single-chain and two-chain anti-CD3-based bispecific antibodies in gene immunotherapy applications

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Abbreviations: CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; EMA, European Medicines Agency; EpCAM, epithelial cell adhesion molecule; EMCV, encephalomyocarditis virus; FcR, Fc receptor; mAb, monoclonal antibody; IRES, internal ribosomal entry site; PBMCs, peripheral blood mononuclear cells; scFv, single-chain variable fragment; ta-scFv, tandem scFv; TAA, tumor-associated antigens; TCR/CD3, T-cell antigen receptor/CD3 complex; V_H, immunoglobulin variable heavy chain; V_L, immunoglobulin variable light chain

Gene therapy to achieve in vivo secretion of recombinant anti-CD3 x anti-tumor bispecific antibodies in cancer patients is being explored as a strategy to counterbalance rapid renal elimination, thereby sustaining levels of bispecific antibodies in the therapeutic range. Here, we performed a comparative analysis between single- and two-chain configurations for anti-CD3 x anti-CEA (carcinoembryonic antigen) bispecific antibodies secreted by genetically-modified human cells. We demonstrate that tandem single-chain variable fragment (scFv) antibodies and two-chain diabodies are expressed as soluble secreted proteins with similar yields. However, we found significant differences in their biological functionality (i.e., antigen binding) and in their ability to induce non-specific T cell activation. Whereas single-chain tandem scFvs induced human T cell activation and proliferation in an antigen-independent manner, secreted two-chain diabodies exerted almost no proliferative stimulus when human T cells were cultured alone or in co-cultures with CEA negative cells. Thus, our data suggest that two-chain diabodies are preferable to single-chain tandem scFvs for immunotherapeutic strategies comprising in vivo secretion of bispecific antibodies aiming to recruit and activate anticancer specific lymphocytic effector T cells.

Introduction

The development of innovative immunotherapeutic strategies aiming to improve the efficacy of antitumor T cell responses has led the combination of the fine specificity of cancer-targeting antibodies with the efficient trafficking properties and effector functions of T lymphocytes.[1] These strategies are designed to convert tumor-associated antigens (TAA) expressed on the malignant cell surface into recruitment points for immune cells with effector functions, thereby fostering the major histocompatibility complex-independent activation of T lymphocytes. One such method is the genetic engineering of T cells through the introduction of a chimeric antigen receptor (CAR) composed of an extracellular antigen recognition domain, typically a single-chain variable fragment (scFv) antibody, fused to transmembrane and cytoplasmic signaling domains. Another approach involves the use of engineered bispecific antibodies, that link a TAA targeting component with a second binding site recognizing CD3 subunits.[1,2]

Presently, most bispecific antibodies are in early-phase clinical trials. An exception is catumaxomab, an anti-EpCAM (CD326, epithelial cell adhesion molecule) x anti-CD3 rat/mouse hybrid full-length IgG that has been approved by the European Medicines Agency (EMA) for intraperitoneal treatment of malignant ascites in patients with EpCAM$^+$ cancer.[3,4] However, in a Phase I/II study for the treatment of non-small cell lung cancer, it has been previously established that the maximum tolerated dose for multiple intravenous injections of catumaxomab was considerably low.[5] This is probably a consequence of the tumor cell independent cross-linking of T cells with Fc receptor (FcR)-bearing accessory cells, followed by cytokine release-related symptoms.[3]

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For this reason, it is advisable to utilize recombinant bispecific antibodies lacking the Fc portion of the antibody such as diabodies and tandem scFvs. Diabodies are bivalent dimers held together by associated immunoglobulin variable heavy chain (V_H) and immunoglobulin variable light chain (V_L) domains present on different polypeptide chains.[6] Bispecific diabodies are formed by the association of 2 V_HA-V_LB and V_HB-V_LA fragments concurrently expressed in the same cell, thereby leading to the formation of heterodimers with 2 different antigen binding sites.[7] Tandem scFvs (ta-scFvs), also known as $(scFv)_2$, are 2 scFv fragments connected by a peptide linker on a single protein chain.[8] Current ongoing clinical research studies of blinatumomab, an anti-CD3 x anti-CD19 ta-scFv, have revealed outstanding clinical results in relapsed B cell non-Hodgkin lymphoma and chronic lymphocytic leukemia.[9]

However, recombinant bispecific antibodies lacking Fc domains present a brief serum half-life and must be administered by continuous intravenous infusion by portable minipumps.[10] Furthermore, clinical-grade antibodies are extremely expensive to produce.[11] With this caveats in mind, we pioneered the combination of recombinant bispecific antibodies and gene transfer technologies to establish a new type of cancer immunotherapy.[12] We have demonstrated that in vivo secreted bispecific antibodies derived from intratumoral or tumor-distant gene modified cells, effectively recruit and activate T cell cytotoxicity against tumor cells, thereby significantly decreasing tumor burden.[13-15] Furthermore, we demonstrated that the in vivo secretion of recombinant bispecific antibodies may compensate for the rapid blood-pool clearance, resulting in therapeutically effective and persistent levels of antibody molecules.[14,15]

Although these studies have been conducted with a recombinant bispecific antibody in a diabody format, it is not yet clear under what conditions the diabody approach may be preferred to the ta-scFv strategy in gene immunotherapy applications. Here, in order to explore this important issue we set out to directly compare both antibody design formats using the same anti-CEA x anti-CD3 binding domains. Our side-by-side comparisons of single- and two-chain bispecific antibodies revealed that both of them were expressed as soluble secreted proteins with similar yields by genetically modified human cells. Nevertheless, we did find significant differences between the single-chain ta-scFv and the two-chain diabody in functionality and in their ability to induce antigen-dependent and antigen-independent T-cell activation, distinctions that may have clinical implications.

Results and Discussion

Secretion of recombinant bispecific antibodies by genetically-modified human cells

The V_H an V_L domains from 2 monoclonal antibodies (mAbs) MFE23[16] and OKT3,[17] directed against human carcinoembryonic antigen (CEA) and CD3 respectively, were used as the building blocks to construct vectors for the expression of recombinant bispecific antibodies in genetically-modified human cells. We designed one vector coding for a bispecific αCEA x αCD3 two-chain diabody with V_H an V_L domains linked via a 5 residues peptide linker (G_4S) (**Fig. 1A**), and

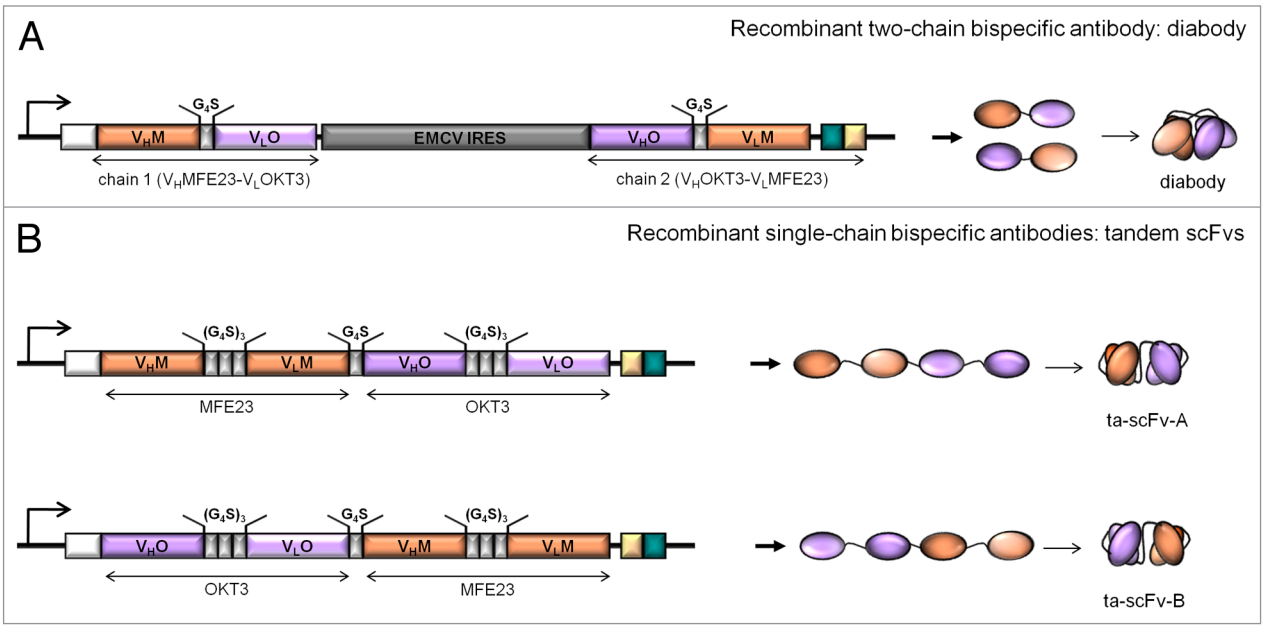

Figure 1. Schematic representation of the anti-CD3 x anti-CEA bispecific antibody gene constructs. (**A–B**) Recombinant bispecific antibodies, including the two-chain diabody (**A**) and tandem single-chain variable fragment (**B**) gene constructs were placed under the control of the cytomegalovirus (CMV) promoter/enhancer and a heterologous signal peptide from the Oncostatin M (white box) gene. The silver boxes represent the linker peptide (G_4S) and the dark-green and yellow boxes represent 6xHis tag and myc tag respectively. The internal ribosome entry site (IRES) sequence from the encephalomyocarditis virus (EMCV) provides for expression of both diabody chains from the same CMV promoter/enhancer element. Shown are the arrangements of immunoglobulin variable heavy chain (V_H) and immunoglobulin variable light chain (V_L) domains in the two-chain diabody (**A**) and in the single-chain tandem scFvs (ta-scFv-A and ta-scFv-B) (**B**).

2 vectors encoding 2 single-chain ta-scFv antibodies with inverse domain orders (ta-scFv-A [anti-CEA x anti-CD3] and ta-scFv-B [anti-CD3 x anti-CEA]), by fusing the MFE23 scFv and the OKT3 scFv via a five residues peptide linker (G_4S) (**Fig. 1B**).

Western blot analysis of conditioned medium from stably transfected HEK-293 cells ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, and $293^{ta\text{-}cFv\text{-}B}$) demonstrated that both diabody and ta-scFv were secreted at comparable levels (**Fig. 2A**). The migration patterns of the myc-tagged diabody chain 2 (31.1 kDa), single-chain ta-scFv-A and ta-scFv-B antibodies (57 kDa) were consistent with the predicted molecular weight.

However, we found significant differences in antigen binding between the different antibody formats. The diabody and the ta-scFv-A antibody bound similarly to CEA, both immobilized and expressed on the cell surface, and to $CD3^+$ Jurkat cells, (**Fig. 2B and C**). In contrast, the reverse ta-scFv-B antibody retained binding to CD3 while exhibiting reduced binding to CEA, both immobilized and expressed on the cell surface (**Fig. 2B and C**). Conditioned media from HEK-293 cells transfected with monospecific (anti-CEA or anti-CD3) N-terminal scFv-based trimerbody expression vectors[18,19] were used as positive controls (**Fig. 2**).

Differential human T cell activation in response to secreted recombinant bispecific antibodies

To study the biological effect of secreted, recombinant anti-CD3 x anti-CEA antibodies on T-cell activation, Jurkat cells were either cultured alone or co-cultured with CEA^- (HeLa) or CEA^+ (MKN45) tumor cells in the presence of fresh, cell-free conditioned medium derived from stable HEK-293 transfectant cell lines ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, or $293^{ta\text{-}scFv\text{-}B}$). The activation status of human T cells was measured by the cell surface expression of CD69. As shown in **Figure 3A**, the activation of Jurkat T cells in response to the secreted diabody was strictly antigen-dependent, such that CD69 was only expressed when Jurkat cells were co-cultured with CEA^+ tumor cells. In sharp contrast, nonspecific T cell activation occurred in response to either of the ta-scFv-A or ta-scFv-B single-chain bispecific antibodies, such that CD69 was upregulated in response to tandem single-chain variants even when Jurkat T cells were cultured alone or in co-culture with CEA^- tumor cells (**Fig. 3A**).

Next, we compared the efficacy of secreted bispecific antibodies to induce primary human T cell proliferation. Unstimulated peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured alone (**Fig. 3B**, left panel) or co-cultured with either CEA^- or CEA^+ tumor cells (**Fig. 3B**, right panel) in the presence of fresh cell-free conditioned medium from the stable HEK-293 cell lines ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, or

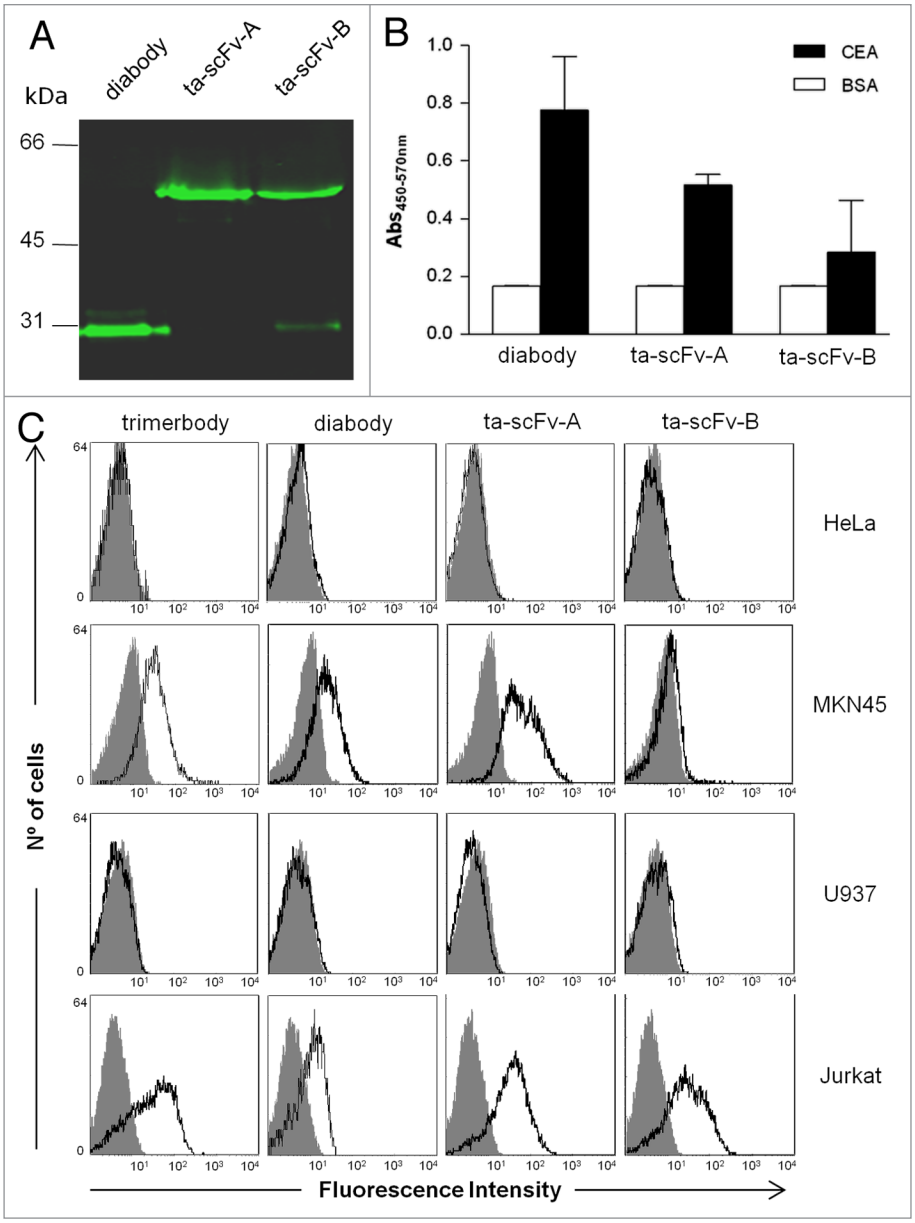

Figure 2. Characterization of secreted bispecific antibodies. (**A**) Engineered, bispecific antibodies secreted into the conditioned medium of stably transfected HEK293 cells ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$ or $293^{ta\text{-}scFv\text{-}B}$) were characterized for expression levels and binding properties. (**A**) western blot analysis. Migration distances of molecular mass markers are indicated (kDa). The blot was developed with anti-His tag mAb. (**B**) The functionality of secreted antibodies was demonstrated by ELISA against plastic immobilized BSA and human CEA. (**C**) Flow cytofluorimetric analysis of binding of secreted bispecific antibodies to the surface of MKN45 (CEA^+) and Jurkat ($CD3^+$) cells. HeLa (CEA^-) and U937 ($CD3^-$) cells were used as negative controls. The y-axis shows the number of cells and the x-axis represents the intensity of fluorescence, expressed on a logarithmic scale.

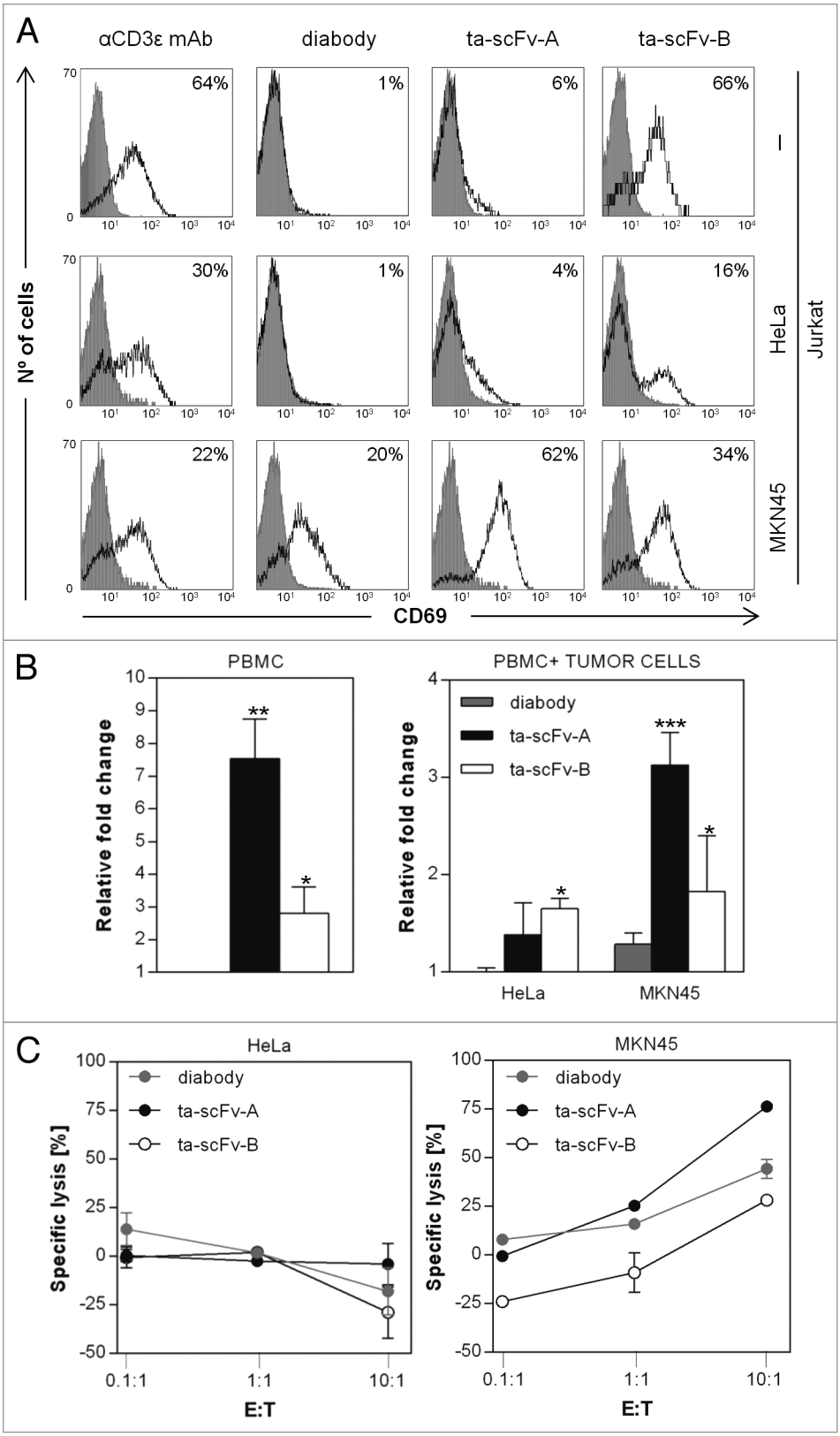

Figure 3. Activation of human T cells by secreted bispecific antibodies. (**A**) Flow cytofluorimetric analysis of CD69 expression on Jurkat cells cultured alone or co-cultured with target cells [HeLa (CEA^-) or MKN45 (CEA^+)] at an effector (E) to target (T) cell of 4:1, in the presence of purified anti-CD3 mAb OKT3, or fresh cell-free conditioned medium containing diabody or tandem single chain variable fragment (ta-scFv-A or ta-scFv-B) from $293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, $293^{ta\text{-}scFv\text{-}B}$, respectively. (**B**) ^{3}H-Thymidine proliferation assay with human peripheral blood mononucleated cells (PBMCs) cultured alone (left panel), or co-cultured (E:T= 4:1) with irradiated HeLa (CEA^-) or MKN45 (CEA^+) cells, in the presence of fresh cell-free conditioned medium containing diabody (gray column), ta-scFv-A (black column) or ta-scFv-B (white column). Data are reported as fold-change induction relative to the values obtained from unstimulated cells. (**C**) Specific lysis of tumor cells targeted by recombinant bispecific antibodies. Luciferase-tagged tumor cell lines ($MKN45^{Luc}$ and $Hela^{Luc}$) were incubated with PBMC at various E to T ratios (as indicated on the x-axis) in the presence of fresh cell-free conditioned medium containing diabody (gray circles), ta-scFv-A (black circles) or ta-scFv-B (white circles). After 48 h, 20 μg/well D-luciferin was added and bioluminescence detected via luminometry. Percent viability was calculated relative to the luminescence from an equal number of input control cells and used to calculate percent specific lysis. Results are expressed as a mean ± SD (n = 3) from 1 of at least 3 separate experiments. Significance was measured by Student's *t* test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

$293^{ta\text{-}scFv\text{-}B}$). In agreement with CD69 responses (**Fig. 3A**), single-chain bispecific antibodies (ta-scFv-A and ta-scFv-B) induced human T-cell proliferation in an antigen-independent manner. Both secreted ta-scFvs strongly activated unstimulated human T cell proliferation, irrespective of the presence of CEA^+ tumor cells (**Fig. 3B**). By contrast, the secreted two-chain diabody exerted almost no proliferative stimulus when primary T cells were cultured alone (**Fig. 3B**, left panel) or in co-culture with CEA^- HeLa tumor cells (**Fig. 3B**, right panel).

We also aimed to determine the capacity of fresh cell-free conditioned medium containing diabody, ta-scFv-A or ta-scFv-B to mediate human PBMC killing of tumor cells. As shown in **Figure 3C** (right panel), the death of CEA^+ cancer cells was specifically triggered by both diabody and ta-scFv-A containing media, but there was either no response, or an insubstantial response, to ta-scFv-B containing media. Also, regardless of the nature of the

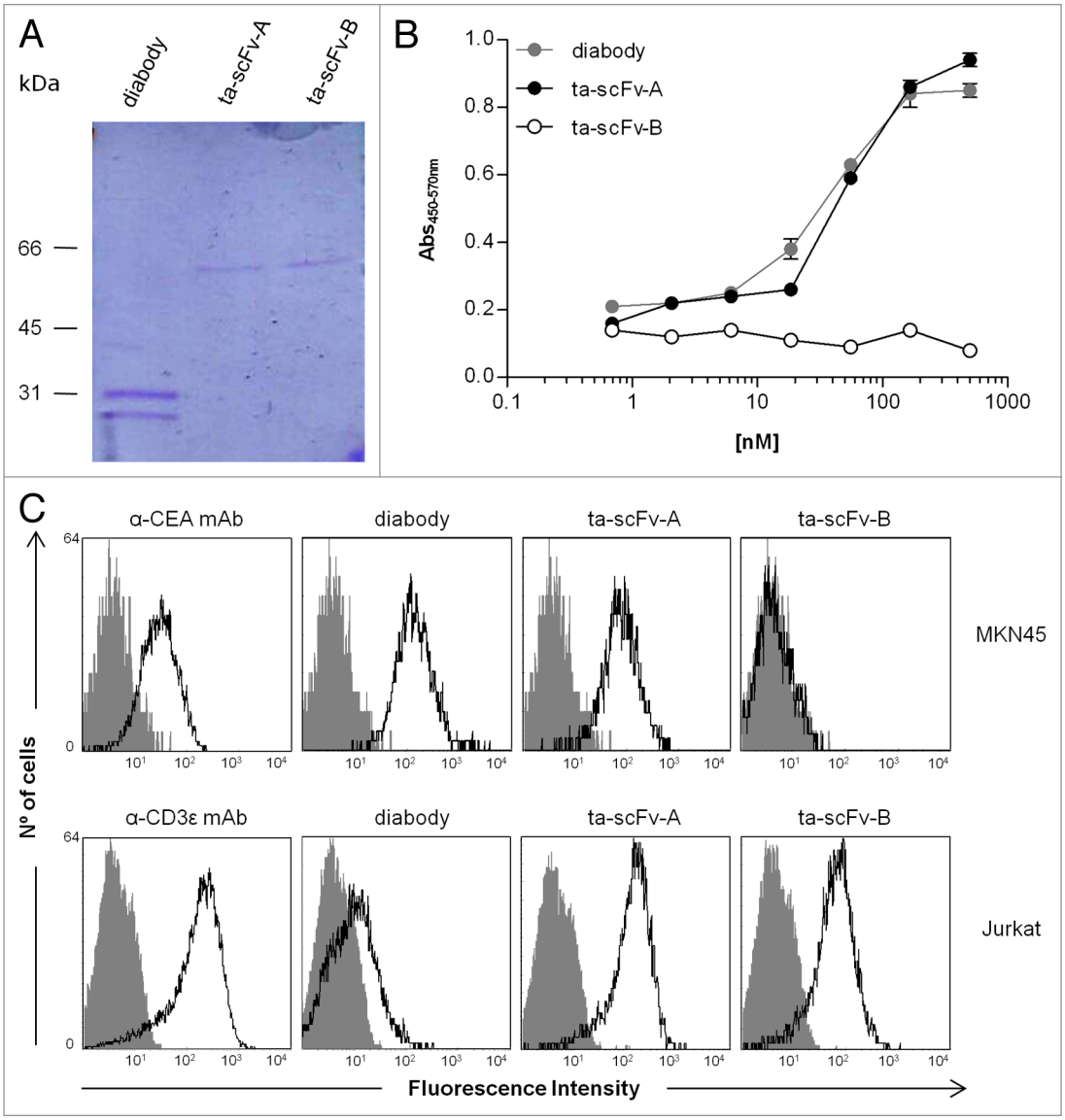

Figure 4. Characterization of purified bispecific antibodies. (**A–C**) Engineered, bispecific antibodies, including two-chain diabody and tandem, single-chain variable fragment (ta-sc-Fv), secreted into the conditioned medium of stably transfected HEK293 cells ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, or $293^{ta\text{-}scFv\text{-}B}$) were purified by affinity column chromatography and characterized for protein M.W. and antigen binding properties. (**A**) Reducing SDS-PAGE of purified diabody, ta-scFv-A and ta-scFv-B antibodies. (**B**) Antibody titration ELISA was performed against plastic immobilized human CEA using anti-c-myc mAb. (**C**) Specific binding of purified bispecific antibodies (at a concentration of 1 μg/mL) to CEA and CD3 antigens expressed on the cell surface (of MKN45 and Jurkat cells, respectively) was assessed by immunofluorescence staining and flow cytometry. Anti-human CD3ε and anti-human CD66e/CEA native mAbs were used as positive controls. The y-axis shows the number of cells, while the x-axis represents the intensity of fluorescence, expressed on a logarithmic scale.

recombinant antibody applied, no killing was observed when CEA^- tumor cells were used as target cells (**Fig. 3C**, left panel).

Purification and characterization of isolated recombinant bispecific antibodies

Recombinant bispecific antibodies were purified from conditioned medium harvested from stable HEK-293 cell lines by immobilized metal ion affinity chromatography, yielding proteins that were >95% pure as determined by reducing sodium dodecyl sulfate-PAGE (**Fig. 4A**). The diabody peptides resolved into 2 bands (28.3 kDa for diabody chain 1 and 31.1 kDa for diabody chain 2), whereas both ta-scFvs migrated as single bands with molecular weight of 57 kDa. No degradation products were observed. The functionality of the purified bispecific antibodies was demonstrated by enzyme-linked immunosorbant assay (ELISA) and fluorescence cytometry. As shown in **Figure 4B**, antibody titration ELISA analysis showed a dose-dependent binding of diabody and ta-scFv-A to plastic immobilized human CEA, whereas purified ta-scFv-B failed to bind (**Fig. 4B**). Furthermore, purified diabody and ta-scFv-A specifically interacted with both CEA^+ (MKN45) and $CD3^+$ (Jurkat) cells, as efficiently as the native mAbs C6G9 (anti-human CEA) and OKT3

(**Fig. 4C**). By contrast, purified ta-scFv-B showed excellent binding to cell surface CD3 while demonstrating no significant binding to CEA^+ cells.

Next, we analyzed the absolute molecular weight and oligomeric state of purified antibodies by size exclusion chomatography-multi-angle laser light scattering (SEC-MALLS). The two-chain diabody eluted from the column as a major peak at 10.5 mL. The mass calculated from the dispersed light at the center of the peak was 55 kDa, near the expected mass of 60 kDa for the dimer. Only a small proportion (~10%) of higher molecular mass multimer components were observed (**Fig. S1**). By contrast, both ta-scFvs showed a clear tendency to form multimeric aggregates. The ta-scFv-A preparation was estimated to contain approximately 50% of the purified protein in monomeric form while only 25% of the ta-scFv-B molecules were monomers (**Fig. S1**). Retention volume was about 10.19 mL for the monomeric peak of ta-scFv-A and about 10.31 mL for the monomeric form of ta-scFv-B (data not shown). The retention volumes of ta-scFv-A and ta-scFv-B aggregates ranged from 7.88 to 9.88 mL, representing dimeric and higher multimeric molecular aggregates.

In summary, we demonstrated that genetically modified human cells could efficiently secrete different formats of recombinant anti-CD3 x anti-CEA bispecific antibody fragments lacking Fc domains. However, the aggregation state of bispecific antibodies secreted into the conditioned medium of gene-modified human cells varied significantly between single- and two-chain antibodies. Both tandem scFvs have shown a greater tendency to form dimers, or even higher molecular weight species, than diabody constructs. Importantly, we have demonstrated that there is a direct relationship among the presence of antibody aggregates and non-specific activation of human T cells. This is likely a consequence of tumor cell independent TCR/CD3 (T cell receptor/CD3 complex) cross-linking via multimeric antibody aggregates, that in a clinical setting, might provoke cytokine-release syndrome.[3]

Other studies[20,21] have shown that overall, single-chain bispecific ta-scFv antibodies are preferable in therapeutic protocols based on the systemic injections of purified protein, principally due to their superior intrinsic stability. However, our data support the notion that two-chain bispecific diabodies are preferable for gene-based bispecific antibody strategies in vivo, as in a physiological context it is obviously not possible to 'purify' the protein. It has been previously reported that the non-covalent association of the 2 diabody chains could be sufficiently unstable to dissociate, thereby resulting in a reduction of binding capacity.[22] Various strategies have been used successfully to increase diabody stability.[23] Nevertheless, our data suggest that in an in vivo gene therapy context chain dissociation might not be considered a disadvantage, reducing the risk of antibody aggregation and cytokine release-related symptoms.

Materials and Methods

Antibodies and antigenic reactives

The monoclonal antibodies (mAbs) used included OKT3 (anti-human CD3ε; Orthoclone), C6G9 (anti-human CD66e/CEA; Sigma-Aldrich, C2331), 9E10 (anti-human c-myc; Abcam, ab32) and Tetra-His (Qiagen, 34670) specific for His-tagged proteins. For direct staining, the R-phycoerythrin (PE)-conjugated FN50 (anti-human CD69, BD Biosciences, 557745) was used. The polyclonal antibodies used included a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Fc specific, Sigma–Aldrich, A2554) and a PE-conjugated goat $F(ab')_2$ fragment anti-mouse IgG (Fc specific, Jackson ImmunoResearch, 115–116–071). Human carcinoembryonic antigen (CEA) was from (Calbiochem, 219368) and bovine serum albumin (BSA) was from (Sigma–Aldrich, A2153).

Cell lines and culture conditions

HEK-293 (human embryo kidney epithelia; CRL-1573), HeLa (human cervix adenocarcinoma; CCL-2) and MKN45 (human gastric adenocarcinoma; JCRB-0254) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, BE12–614F) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin (all from Invitrogen Life Technologies) referred as to DMEM complete medium. Jurkat clone E6–1 (human acute T cell leukemia; TIB-152) and U937 (human monocytic leukemia; CRL-1593.2) cells were maintained in RPMI-1640 (Lonza, BE12–115F) supplemented with heat inactivated 10% FCS, referred as to RPMI complete medium. All these cells lines were obtained from the American Type Culture Collection.

Construction of expression vectors

The construction of the pdAb3 expression vector has been described in previous work.[12] To construct the ta-scFv-A expression vector, the pCR3.1-MFE23-hXVNC1[19] plasmid was digested with NotI/SalI to remove the human collagen XV NC1 domain, resulting in pCR3.1-MFE23 vector. A single pair of complementary oligonucleotides, 1 and 2 (refer to **Table S1**) encoding a 5-residue linker (G_4S) was ligated into NotI/SalI restriction digested pCR3.1-MFE23 using T4 DNA ligase (New England Biolabs, M0202S) to derive pCR3.1-MFE23-(G_4S). A DNA fragment coding for the OKT3 scFv was synthesized by GeneArt AG, and subcloned via SalI-XbaI into the pCR3.1-MFE23-(G_4S) vector to give rise to the pta-scFv-A expression vector (pCR3.1-MFE23-(G_4S)-OKT3). To construct the pta-scFv-B expression vector, the pCR3.1-MFE23-(G_4S)-OKT3 plasmid was digested with ClaI/NotI and the MFE23 scFv removed. The ClaI/NotI fragment (OKT3-targeting scFv gene) from plasmid pCR3.1-OKT3-hXVNC1 was subsequently ligated into the ClaI/NotI digested backbone of plasmid pCR3.1-MFE23-(G_4S)-OKT3, to obtain the pCR3.1-OKT3-(G_4S)-OKT3 plasmid. The MFE-23 scFv gene was amplified by polymerase chain reaction (PCR) with oligonucleotides 3 and 4 (refer to **Table S1**) to introduce SalI and BglII restriction sites and the SalI/BglII cleaved fragment was ligated into the SalI/BglII digested backbone of plasmid pCR3.1-OKT3-(G_4S)-OKT3 to obtain the plasmid pCR3.1-OKT3-(G_4S)-MFE23, referred as pta-scFv-B. The sequences were verified using primers 5 and 6 (**Table S1**),

validating recombinant antibody construct structural integrity.

Cell transfections and purification of recombinant antibodies

HEK-293 cells were transfected with pdAb3, pta-scFv-A or pta-scFv-B expression vectors by calcium phosphate precipitation, as previously described.[13] To generate stable cell lines, HEK-293 cells transfected with pdAb3 plasmid ($293^{diabody}$) were selected in DMEM complete medium supplemented with 150 μg/mL hygromycin B (Invitrogen Life Technologies, 10687010). HEK-293 cells transfected with pta-scFv-A or pta-scFv-B plasmids ($293^{ta\text{-}scFv\text{-}A}$ and $293^{ta\text{-}scFv\text{-}B}$ respectively), were selected in DMEM complete medium supplemented with 750 μg/mL G418 (Invitrogen Life Technologies, 11811023). Conditioned medium was collected, centrifuged at 600 g for 10 min and analyzed by ELISA against plastic immobilized bovine serum albumin (BSA) and human carcinoembryonic antigen (CEA) using anti-c-myc mAb, as previously described.[13]

For western blot analysis, protein samples were transferred onto nitrocellulose membranes and reacted with anti-His tag mAb, followed by incubation with DyLight 488 conjugated-streptavidin (Rockland Immunochemicals Inc., S000–41). Visualization and quantitative analysis of protein bands were performed with the Odyssey® infrared imaging system (LI-COR Biosciences).

Stably transfected $293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, and $293^{ta\text{-}scFv\text{-}B}$ cells were used to collect serum-free conditioned media (approximately 1 L) and loaded onto a HisTrap HP 1 mL column (GE Healthcare, 17–5247–01) using an ÄKTA Prime plus system. The purified antibodies were dialyzed against phosphate buffered saline (PBS), analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE) under reducing conditions and stored at –20 °C. The ability of purified antibodies to bind human CEA and CD3 was analyzed by ELISA[13]and flow cytometry (see below).

Fluorescence cytometry

Binding of anti-CEA x anti-CD3 bispecific antibodies to MKN45 (CEA^+) and Jurkat ($CD3^+$) cells, was analyzed by indirect immunostaining and fluorescence cytometry. Briefly, cells were incubated for 30 min on ice with filtered cell-free conditioned media from stable transfected cells or 1 μg/mL purified diabody, ta-scFv-A or ta-scFv-B, washed and incubated for 30 min with anti-His mAb. Cells were washed and incubated with phycoerythrin (PE)-conjugated goat $F(ab')_2$ anti-mouse IgG antibody. C6G9 (anti-CEA) and OKT3 (anti-CD3) mAbs were used as positive controls. Hela and U937 cells were used as negative controls. Samples were analyzed with an EPICS XL flow cytometer (Coulter Electronics, Hialeah).

T-cell activation assay

Jurkat T cells were stimulated in triplicate under different conditions in 96-well microtiter plate with irradiated (25 Gy) target cells (HeLa or MKN45) at 4:1 effector:target (E:T) ratio. Purified recombinant bispecific antibodies (1 μg/mL) or filtered cell-free conditioned media from stable transfected cells ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, or $293^{ta\text{-}scFv\text{-}B}$) were added. As a control, effector cells were cultured with 1 μg/mL insoluble anti-CD3 mAb. After 24 h, cells were collected and the surface expression of CD69 was analyzed by fluorescence cytometry, as previously described.[24]

T-cell proliferation assays

Human peripheral blood mononuclear cells (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) under various conditions in 96-well microtiter plates with irradiated (25 Gy) target (HeLa or MKN45) cells at 4:1 effector (E) to target (T) cell ratio. Where indicated, filtered (i.e., cell-free) conditioned medium from untransfected (HEK-293) or stably transfected ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, or $293^{ta\text{-}scFv\text{-}B}$) cells was added. As controls, effector cells were cultured alone with the aforementioned cell-free conditioned medium. As a positive control, human PBMCs were cultured with 1 μg/mL plastic immobilized anti-CD3 mAb. Cells were incubated for 72 h and pulsed with 1 μCi/well [^{3}H]-thymidine (NEN Life Science Products, NET027Z005MC) for 16 h. The incorporation of [^{3}H]-thymidine was measured with a liquid scintillation β counter (1450 MicroBeta, WallacOy). Results are expressed as a mean ± SD (n = 3) from 1 of at least 3 separate experiments. Data are reported as fold change induction relative to the values obtained from un-stimulated cells. Significant differences (*P* value) were discriminated by Student's *t* test, with $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Cytotoxicity assay

Gene-modified luciferase expressing HeLa ($HeLa^{Luc}$) and MKN45 cells ($MKN45^{Luc}$)[14] were cultured in triplicate in 96-well microtiter plates with human PBMCs cells at different E:T ratios, in the presence of cell-free conditioned medium from either untransfected (HEK-293) or stably transfected ($293^{diabody}$, $293^{ta\text{-}scFv\text{-}A}$, $293^{ta\text{-}scFv\text{-}B}$) HEK-293 cells. After 48 h incubation, 20 μg/well D-luciferin (Promega, E1602) was added and bioluminescence quantified in relative light units (RLUs) using an Infinite 200 luminometer (Tecan). 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%).

Structural characterization of purified bispecific antibodies

Size-exclusion chromatography (SEC) was performed using PBS with 0.005% (v/v) P20 surfactant (GE Healthcare, BR100054) as running buffer on a Superdex-75 10/300 GL column (GE Healthcare, 17–5174–01) under the control of an ÄKTA FPLC (GE Healthcare). A Bio-Rad gel filtration standard was used as calibration standard for the SEC column. Samples of 100 μL at concentrations 313, 334, and 114 μg/mL were injected and chromatographed at a flow rate of 0.5 mL/min at room temperature.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: www.landesbioscience.com/journals/oncoimmunology/article/28810/

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