Cancer Immunology, Immunotherapy (2018) 67:1251–1260
https://doi.org/10.1007/s00262-018-2181-5

ORIGINAL ARTICLE

Bispecific light T-cell engagers for gene-based immunotherapy of epidermal growth factor receptor (EGFR)-positive malignancies

Kasper Mølgaard[1] · Seandean L. Harwood[1] · Marta Compte[2] · Nekane Merino[3] · Jaume Bonet[4] · Ana Alvarez-Cienfuegos[2] · Kasper Mikkelsen[1] · Natalia Nuñez-Prado[1] · Ana Alvarez-Mendez[5] · Laura Sanz[2] · Francisco J. Blanco[3,6] · Luis Alvarez-Vallina[1]

Received: 2 December 2017 / Accepted: 28 May 2018 / Published online: 4 June 2018
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Abstract

The recruitment of T-cells by bispecific antibodies secreted from adoptively transferred, gene-modified autologous cells has shown satisfactory results in preclinical cancer models. Even so, the approach's translation into the clinic will require incremental improvements to its efficacy and reduction of its toxicity. Here, we characterized a tandem T-cell recruiting bispecific antibody intended to benefit gene-based immunotherapy approaches, which we call the light T-cell engager (LiTE), consisting of an EGFR-specific single-domain V_{HH} antibody fused to a CD3-specific scFv. We generated two LiTEs with the anti-EGFR V_{HH} and the anti-CD3 scFv arranged in both possible orders. Both constructs were well expressed in mammalian cells as highly homogenous monomers in solution with molecular weights of 43 and 41 kDa, respectively. In situ secreted LiTEs bound the cognate antigens of both parental antibodies and triggered the specific cytolysis of EGFR-expressing cancer cells without inducing T-cell activation and cytotoxicity spontaneously or against EGFR-negative cells. Light T-cell engagers are, therefore, suitable for future applications in gene-based immunotherapy approaches.

Keywords Cancer immunotherapy · Bispecific antibody · T-cell recruitment · EGFR

Abbreviations

BiKE	Bispecific killer-cell engager
bsAb	Bispecific antibody
Fc	Fragment crystallizable
LiTE	Light T-cell engager
Luc	Luciferase
SEC-MALS	Size exclusion chromatography with multi-angle light scattering
T-bsAbs	T-cell recruiting bsAb
V_{HH}	Single-domain antibodies from camelid heavy-chain-only immunoglobulins

Introduction

Redirecting the activity of T-cells using bispecific antibodies (bsAbs) is a potent approach to cancer therapy [1]. T-cell recruiting bsAbs (T-bsAbs) combine the specificities of two antibodies into a single molecule, enabling the bridging of TCR-associated CD3 chains on effector T-cells with selected cell surface TAAs on cancer cells. As a consequence of TCR/CD3 engagement, T-cells undergo polyclonal activation that results in cytokine release and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-018-2181-5) contains supplementary material, which is available to authorized users.

✉ Luis Alvarez-Vallina
lav@eng.au.dk

1 Immunotherapy and Cell Engineering Laboratory, Department of Engineering, Aarhus University, Gustav Wieds Vej 10, 8000 C Aarhus, Denmark

2 Molecular Immunology Unit, Hospital Universitario Puerta de Hierro Majadahonda, Manuel de Falla 1, 28222 Madrid, Spain

3 CIC bioGUNE, Parque Tecnológico de Bizkaia 800, 48160 Derio, Spain

4 Laboratory of Protein Design and Immunoengineering, École Polytechnique Fédérale de Lausanne, Station 19, 1015 Lausanne, Switzerland

5 Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, 28040 Madrid, Spain

6 IKERBASQUE, Basque Foundation for Science, María Díaz de Haro 3, 48013 Bilbao, Spain

induction of cytotoxic responses. With the Food and Drug Administration (FDA) approval of blinatumomab, T-bsAbs have re-entered the limelight, and various T-bsAbs formats are in preclinical and clinical development [2, 3]. Blinatumomab is an anti-CD19 × anti-CD3 tandem scFv [otherwise known as a $(scFv)_2$ or bispecific T-cell engager or BiTE] [4]. Although its molecular weight (≈55 kDa), which is below the glomerular filtration threshold, might be advantageous with regards to tissue penetration, their short terminal elimination half-life necessitates continuous intravenous infusion via pumps [5].

We have developed a novel cancer immunotherapy strategy based on the in vivo secretion of T-bsAbs by engineered human cells [6]. We have previously shown that various types of human cells, such as terminally differentiated cells (primary T-cells and endothelial cells) or progenitor cells (mesenchymal and hematopoietic stem cells), can be genetically engineered to secrete functionally active anti-CEA × anti-CD3 (CEA×CD3) Fc-less antibodies from tumor-infiltrating or tumor-distant cells [6–11]. We have demonstrated that the long-term in vivo secretion of CEA×CD3 antibodies compensates for their short plasma half-lives, resulting in therapeutically effective blood levels [8]. The secreted T-bsAbs recruit and activate T-cells against TAA-expressing cancer cells, significantly decreasing tumor burden [7–9]. This strategy could benefit from smaller T-bsAb formats, which could facilitate the diffusion of antibodies throughout tumors, reaching areas not accessible to larger molecules.

In this article, we describe ≈40 kDa tandem T-bsAbs made by fusing an anti-EGFR single-domain V_{HH} and an anti-CD3 scFv [12]. We called these antibodies the *light T-cell engagers* (LiTEs). We generated and characterized two anti-EGFR LiTEs with the anti-EGFR V_{HH} and anti-CD3 scFv binding moieties arranged in both possible orders, i.e., as EGFR×CD3 LiTE and CD3×EGFR LiTE. Both constructs are expressed as non-aggregating, soluble proteins by human cells, bind the cognate antigens of both parental antibodies, and induce redirected T-cell-mediated cytolysis of EGFR-expressing cancer cells in an antigen-dependent manner.

Materials and methods

General reagents and antibodies

The human EGFR-Fc chimera (hEGFR) was from R&D Systems (Minneapolis, MN, USA) and BSA was from Sigma-Aldrich (St. Louis, MO, USA). The mouse mAbs used included: anti-His mAb (penta-His; Qiagen, Hilden, Germany), anti-c-myc clone 9E10 (Abcam, Cambridge, UK), anti-human kappa chain mAb clone SB81a (Abcam), anti-human CD3ε clone OKT3 (Ortho Biotech, Bridgewater, NJ, USA), PE-conjugated anti-human CD69 clone FN50 (BD Biosciences, San Jose, CA, USA), and FITC-conjugated anti-CD3 mAb (clone UCHT1, Abcam). The chimeric mouse/human anti-human epidermal growth factor receptor (EGFR) cetuximab was from Merck KGaA (Darmstadt, Germany). The F_{ab} fragments from cetuximab and OKT3 were generated using a Pierce Fab Micro Preparation Kit (Thermo Scientific, Rockford, IL, USA) [12].

Cells and culture conditions

HEK-293 (CRL-1573), HeLa (CCL-2), A431 (CRL-1555) and 3T3 (CRL-1658) cells were cultured in DMEM (Lonza, Walkersville, MD, USA) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated FCS, and antibiotics (Life Technologies, Carlsbad, CA, USA), referred to from here on as DMEM complete medium (DCM). Jurkat clone E6-1 (TIB-152) cells were maintained in RPMI-1640 (Lonza) supplemented with 2 mM L-glutamine, heat-inactivated 10% FCS. All these cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa and 3T3 cells expressing the firefly luciferase (Luc) gene ($HeLa^{Luc}$ and $3T3^{Luc}$) have been described previously [11]. Human A431 cells were infected with pRRL-Luc-IRES-EGFP lentivirus [8] to generate $A431^{Luc}$ cells. All cell lines were routinely screened for the absence of mycoplasma contamination using the Mycoplasma Plus TM Primer Set (Stratagene, Cedar Creek, TX, USA).

Construction of expression vectors

The mammalian expression vector pCR3.1-EGa1-(G_4S)-OKT3 encoding the anti-EGFR × anti-CD LiTE has been previously described [12]. To generate the inverse anti-CD3 × anti-EGFR LiTE expression plasmid pCR3.1-OKT3-(G_4S)-EGa1, the V_{HH} EGa1 gene was amplified by polymerase chain reaction (PCR) with oligonucleotides EGa1_2F and EGa1_2T (Supplementary Table 1) to introduce *Sal*I and *Bgl*II restriction sites and the *Sal*I/*Bgl*II cleaved fragment was ligated into the *Sal*I/*Bgl*II digested plasmid pCR3.1-OKT3-(G4S)-MFE23 [11]. The sequences were verified using primers FwCMV and RvBGH (Supplementary Table 1).

Expression and purification of recombinant antibodies

HEK-293 cells were transfected with the pCR3.1-EGa1-(G_4S)-OKT3 or pCR3.1-OKT3-(G_4S)-EGa1 plasmid using calcium phosphate [7] and selected in DCM with 500 µg/mL G-418 (Sigma-Aldrich) to generate stable cell lines. Collected medium was centrifuged, filtered, and purified using

HisTrap Excel columns (GE Healthcare, Uppsala, Sweden) on and ÄKTA Prime plus system (GE Healthcare), as previously described [12, 13]. Elution fractions containing EGFR×CD3 LiTE or CD3×EGFR LiTE were then pooled, diluted 10× in PBS, and further purified with HiTrap Protein A HP columns (GE Healthcare). The resulting fractions containing EGFR×CD3 LiTE or CD3×EGFR LiTE were pooled, dialyzed against PBS, and concentrated using 3 kDa MWCO centrifugal filters (Millipore, Temecula, CA, USA).

Western blotting

Samples were separated under reducing conditions on 12% Tris–glycine SDS polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes (Life Technologies) and probed with anti-c-myc mAb, followed by incubation with an IRDye800-conjugated donkey anti-mouse IgG (H&L) (Rockland Immunochemicals, Limerick, PA, USA). Analysis of protein bands was carried out with the Odyssey system (LI-COR Biosciences, Lincoln, NE, USA).

Size exclusion chromatography-multiangle light scattering (SEC-MALS)

Static light scattering measurements were performed at 25 °C using a Superdex 200 10/300 GL column (GE Healthcare) attached in-line to a DAWN-HELEOS light scattering detector and an Optilab rEX differential refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The column was equilibrated with PBS or PBS with 150 mM NaCl (0.1 µm filtered) and the SEC-MALS system was calibrated with a sample of BSA at 1 g/L in the same buffers. Then, a 100 µL sample of the EGFR×CD3 LiTE or CD3×EGFR LiTE at 1.8 or 0.3 g/L in PBS was injected into the column. Data acquisition and analysis were performed using the ASTRA software (Wyatt Technology). Based on numerous measurements on BSA samples at 1 g/L under the same or similar conditions, we estimate that the experimental error in the molar mass is around 5%.

Circular dichroism

Circular dichroism measurements were performed with a Jasco J-810 spectropolarimeter (JASCO, Tokyo, Japan). The spectra of EGFR×CD3 LiTE or CD3×EGFR LiTE were recorded on protein samples at 0.04 or 0.26 g/L in PBS or in PBS with 150 mM NaCl in a 0.2 cm path length quartz cuvette at 25 °C. The thermal denaturation from 5 to 95 °C was recorded on the same protein samples and cuvette by increasing temperature at a rate of 1 °C/min and measuring the change in ellipticity at 210 nm.

Mass spectrometry

A 2 µL protein sample of EGFR×CD3 LiTE at 1.8 g/L was desalted using ZipTip® C4 micro-columns (Millipore) and eluted with 0.5 mL SA (sinapinic acid, 10 mg/mL in [70:30] acetonitrile:trifluoroacetic acid 0.1%) matrix onto a GroundSteel massive 384 target (Bruker Daltonics, Billerica, MA, USA). An Autoflex III MALDI-TOF/TOF spectrometer (Bruker Daltonics) was used in linear mode with the following settings: 5000–40,000 Th window, linear positive mode, ion source 1: 20 kV, ion source 2: 18.5 kV, lens: 9 kV, pulsed ion extraction of 120 ns, and high gating ion suppression up to 1000 Mr. Mass calibration was performed externally with protein 1 standard calibration mixture (Bruker Daltonics). Data acquisition, peak peaking, and subsequent spectra analysis were performed using FlexControl 3.0 and FlexAnalysis 3.0 software (Bruker Daltonics).

Molecular modeling

The structure of the EGFR×CD3 LiTE and CD3×EGFR LiTE antibodies was built through comparative homology modeling with MODELLER [14] as previously described [15]. The structure of a homo-specific diabody (pdb:5GS1) [16] was used as a template for the anti-EFGR EGa1 V_{HH} domain. This template was found with a blast [17] e value of 3×10^{-81} and a 54% of sequence identity. The anti-CD3 OKT3 scFv domain was modeled after the MFE-23 recombinant antibody fragment (pdb:1QOK) [18]. This template has a 75% sequence identity with the scFv domain and was found with a blast e value of 1×10^{-107}. To mimic the putative repositioning of the two different domains according to their flexible linker, constraints in the linker were removed. 150 structures were generated representing the internal flexibility of the system.

ELISA

The ability of antibodies to bind purified hEGFR was studied by ELISA [13]. Briefly, Maxisorp plates (NUNC Brand Products, Roskilde, Denmark) were coated with hEGFR (0.3 µg/well) and after washing and blocking with 5% BSA in PBS, conditioned media from transfected HEK-293 cells or purified antibodies were added. After three washes, anti-c-myc mAb was added followed by HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich), after which the plates were washed and developed.

Flow cytometry

Binding of EGFR×CD3 LiTE and CD3×EGFR LiTE or control mAbs to EGFR and CD3 expressed on the cells surface was studied by FACS as described previously [12].

Briefly, HeLa, Jurkat, or 3T3 cells were incubated on ice for 30 min with filtered conditioned media from transfected HEK-293 cells or purified antibodies, and washed and incubated for 30 min with anti-c-myc mAb and PE-conjugated goat anti-mouse IgG, $F(ab')_2$ fragment (Jackson Immuno Research, West Grove, PA, USA). Cetuximab (anti-EGFR) and OKT3 (anti-CD3) mAbs were used as positive controls, and detected with PE-conjugated goat $F(ab')_2$ fragment anti-mouse IgG antibody and PE-conjugated goat $F(ab')_2$ fragment anti-human IgG antibody (H&L) (Abcam), respectively. The samples were analyzed using a Beckman Coulter FC-500 Analyzer (Coulter Electronics, Hialeah, FL, USA). The K_D of the interactions between EGFR×CD3 LiTE, cetuximab, and cetuximab-F_{ab} with EGFR on the surface of HeLa cells, as well as those of EGFR×CD3 LiTE, OKT3 mAb, and OKT3 F_{ab} with CD3 on the surface of Jurkat cells, were investigated using flow cytometry. By determining the shift in MFI of HeLa or Jurkat cells incubated with different concentrations of the previously mentioned antibodies, steady-state analysis can be used to find K_D values for the antibody:antigen interactions. HeLa cells were incubated with 0.1, 1, and 10 nM of EGFR×CD3 LiTE, cetuximab, or cetuximab-F_{ab}, after which anti-His mAb for EGFR×CD3 LiTE and anti-human kappa chain mAb for cetuximab and its F_{ab} derivative were added, followed by PE-conjugated goat $F(ab')_2$ fragment anti-mouse IgG. Similarly, Jurkat cells were incubated with 1, 10, and 100 nM of EGFR×CD3 LiTE, OKT3 mAb, or OKT3-F_{ab}, which was then detected with rabbit anti-mouse IgG (H&L) (Jackson Immuno Research) followed by PE-conjugated donkey $F(ab')_2$ fragment anti-rabbit IgG (H&L) (Abcam) for OKT3 and its F_{ab}-derivative, or anti-His mAb followed by PE-conjugated goat $F(ab')_2$ fragment anti-mouse IgG for EGFR×CD3 LiTE. The cells were then analyzed on a Cell Sorter SH800 (Sony, Tokyo, Japan), and K_Ds were determined by fitting to a single set of binding sites equation.

T-cell activation assay

HeLa or 3T3 cells were plated in triplicates in 96-well microtiter plates (4×10^4/well) 1 day before the assay. 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 target cells at a 5:1 E:T-cell ratio in the presence of purified anti-EGFR LiTEs. The study of CD69 expression was performed by FACS using PE-conjugated anti-CD69 mAb and FITC-conjugated anti-CD3 mAb. The samples were analyzed with a Beckman Coulter FC-500 Analyzer.

Cytotoxicity assay

Gene-modified luciferase (Luc) expressing HeLa ($HeLa^{Luc}$), A431 ($A431^{Luc}$) or 3T3 ($3T3^{Luc}$) cells were plated in triplicate in 96-well microtiter plates (4×10^4/well) 1 day before the assay. Human PBMCs cells were added in 5:1 E:T ratio in the presence of purified anti-EGFR LiTEs. After 72 h incubation, 20 µg/well D-luciferin (Promega, Madison, WI, USA) was added and bioluminescence quantified in relative light units (RLUs) using an Infinite 200 luminometer (Tecan Trading AG, Switzerland). For cytotoxic studies in transwell systems, a polyethylene terephthalate filter insert (6.5 mm diameter) with 0.4 µm pores (Falcon, BD Biosciences) was used. $HeLa^{Luc}$ or $3T3^{Luc}$ cells (5×10^5) were plated on bottom wells of 24-well plate. After 24 h, human PBMCs (2.5×10^6) were added to bottom wells and GFP-, EGFR×CD3- or CD3×EGFR-transfected HEK-293 cells (1×10^5) were added to transwell insert wells. After 72 h, the transwell insert and the nonadherent cells were removed and viable tumor cells quantified by bioluminescence as described above. A 100% lysis control with 1% Triton-X100, 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. Percent cell viability was then plotted against the effector molecule concentration and data were evaluated using Prism 5 (GraphPad Software, San Diego, CA) by fitting a sigmoidal dose–response (three parameter equation).

Results

Design and expression of anti-EGFR light T-cell engagers

In this study, we generated two bispecific tandem V_{HH}-scFv proteins by fusing the anti-human EGFR EGa1 V_{HH} [19] to the N- or C-terminal end of the anti-human CD3 OKT3 scFv (V_H–V_L orientation) [20], separated by a flexible GGGGS linker, in a format similar to previously described BiTEs or tandem scFvs (Fig. 1). Both anti-EGFR LiTEs were efficiently produced by transfected HEK-293 cells at similar levels (EGFR×CD3 LiTE, 0.9 µg/mL × 5×10^5 cells/48 h; CD3×EGFR LiTE, 1.2 µg/mL × 5×10^5 cells/48 h). Western blot analysis under reducing conditions of HEK-293 conditioned media showed a migration pattern consistent with the 44.3 kDa molecular weight calculated from the amino acid sequences excluding the signal sequence (Supplementary Fig. 1a). ELISA analysis demonstrated that the secreted LiTEs specifically recognize plastic-immobilized human EGFR-Fc chimera (from here on, hEGFR) (Supplementary

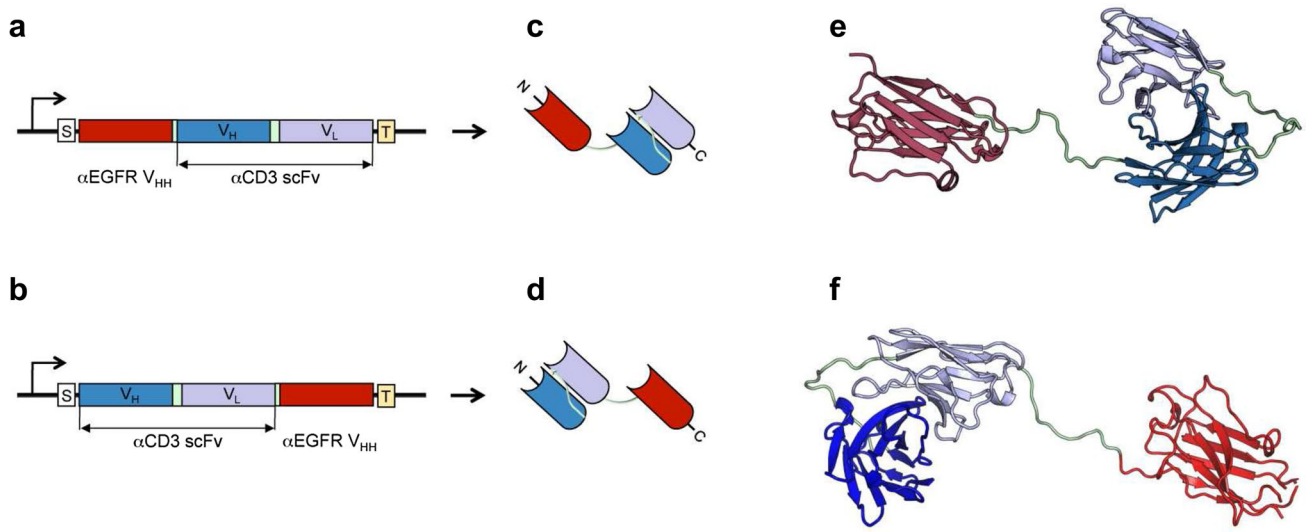

Fig. 1 Schematic representations and models of the bispecific LiTEs. Genetic structures of the tandem proteins formed by fusing the EGFR-specific EGa1 V_{HH} (red box) N- (**a**) or C-terminally (**b**) to the CD3-specific OKT3 scFv (V_H–V_L orientation, blue boxes). Arrow indicates the direction of transcription. The Oncostatin M signal peptide (white box) is used to direct secretion of recombinant antibody, and the myc/6xHis tags (yellow box) is appended for immunodetection and affinity purification, respectively. Schematic representations showing arrangement of V_{HH} and V_H and V_L domains (**c, d**) and three-dimensional models (**e, f**) of the EGFR×CD3 LiTE (**c, e**) and the CD3×EGFR LiTE (**d, f**)

Fig. 1b). The ability to detect antigens in a cellular context was studied by flow cytometry. Fluorescence staining was observed after incubation of EGFR-expressing HeLa cells and CD3-expressing human Jurkat T-cells with both EGFR×CD3 LiTE and CD3×EGFR LiTE, while no binding was detected with mouse 3T3 cells (Supplementary Fig. 1c).

Purification and structural characterization of the anti-EGFR LiTEs

Both EGFR×CD3 LiTE and CD3×EGFR LiTEs were purified in a two-step process from conditioned medium of transfected HEK-293 cells by metal-affinity chromatography, and then by protein A affinity chromatography which yielded protein that was > 90% pure, as evaluated by coomassie staining of reducing SDS-PAGE (Fig. 2a). The purified recombinant anti-EGFR LiTEs were obtained with yields in the range of 0.3–0.5 mg per liter of culture. The oligomeric status of both LITEs was investigated by SEC-MALS. The proteins eluted from the size exclusion column as major symmetric peaks, and the masses calculated from the dispersed light at the center of the peaks were 43 and 41 kDa for EGFR×CD3 LiTE and CD3×EGFR LiTE, respectively, close to the calculated value of 44.3 kDa (Fig. 2b). Mass spectrometry by matrix-assisted laser desorption ionization confirmed the absence of the first 25 N-terminal residues in the EGFR×CD3 LiTE sample, indicating that the signal sequence was cleaved during protein secretion. The circular dichroism spectra of the antibodies have a single minimum at 220 nm (Fig. 2c), which is consistent with a predominantly beta-sheet structure. The anti-EGFR LiTEs are folded into stable three-dimensional structures according to cooperative thermal denaturations (Fig. 2d), which show major denaturation events with mid-point temperatures of 52 and 54 °C. The width of the transition regions and the irreversibility of the denaturing processes (with partial protein precipitation in the cuvette after denaturation) likely prevent to observe the individual transitions for the V_{HH} and scFv globular moieties. These data are consistent with those measured for a tetravalent molecule reported recently [12] also containing V_{HH} and scFv parts.

Anti-EGFR LiTEs binds to T-cells and to tumor cells expressing EGFR

The binding properties of the purified LiTEs against plastic-immobilized hEGFR were characterized by ELISA, showing similar dose-dependent-binding curves with both constructs (Fig. 3a). Flow cytometry was used to confirm dual specificity of the purified EGFR×CD3 LiTE and CD3×EGFR LiTE against cells expressing the appropriate targets. Both antibodies were found to bind to $CD3^+$ human Jurkat T-cells and to $EGFR^+$ human HeLa cells, but not to mouse 3T3 cells (Fig. 3b). The binding affinity of purified EGFR×CD3 LiTE to both antigens, EGFR and CD3, expressed on the surface of HeLa and Jurkat cells respectively, was investigated by the steady-state analysis of data obtained from flow cytometry. This required

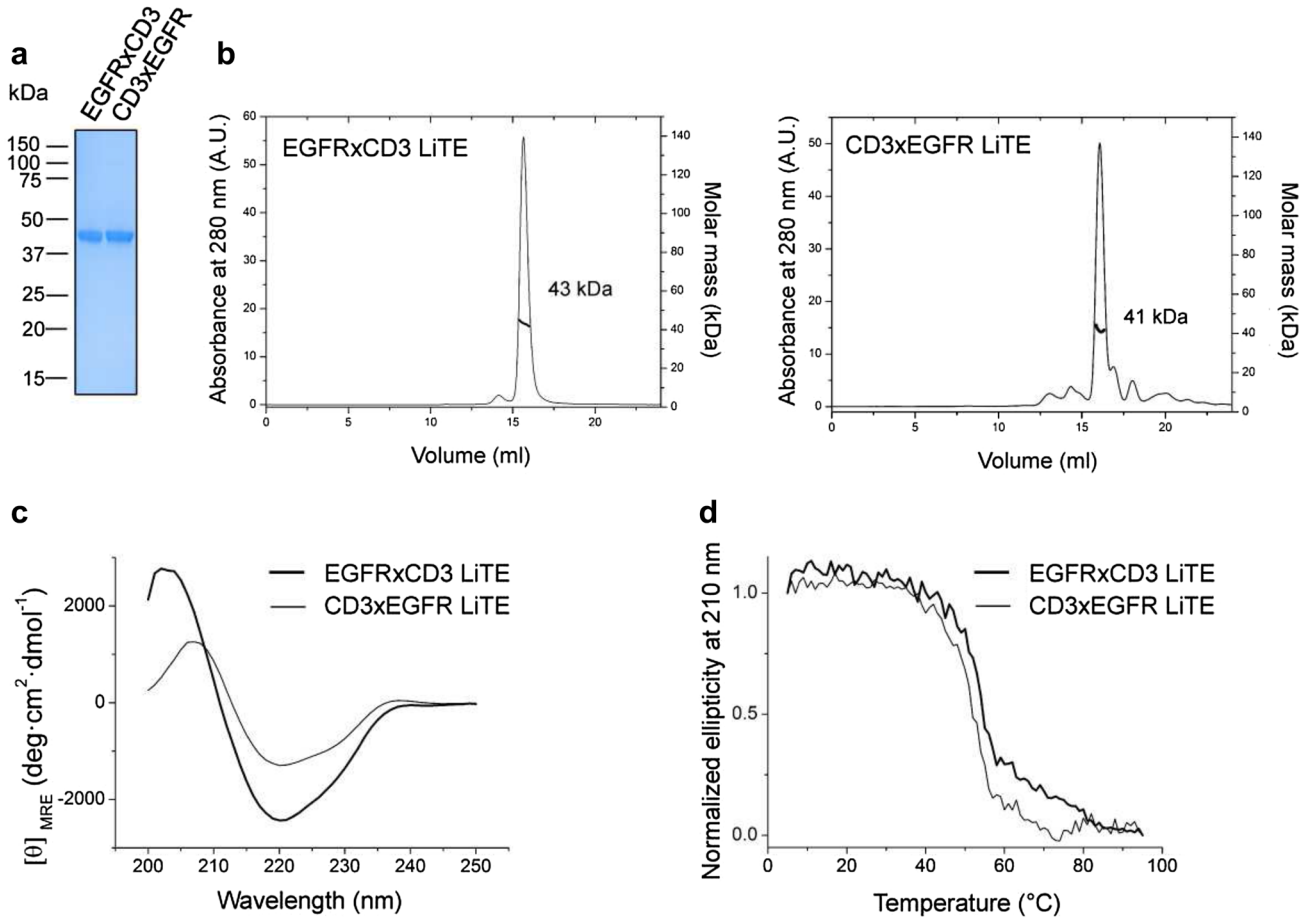

Fig. 2 Structural characterization of purified anti-EGFR LiTEs. Reducing SDS–PAGE (**a**) and oligomeric analysis by SEC-MALS (**b**) of the EGFR×CD3 LiTE and the CD3×EGFR LiTE. The molecular masses measured at the center of the chromatography peaks are indicated. The thin line corresponds to the UV absorbance (left axis) and the thick line to the measured molar mass (right axis). Circular dichroism spectrum (**c**) and tertiary structure analysis by thermal denaturation measured by the change in ellipticity at 210 nm for the two molecules (**d**)

measuring the equilibrium mean fluorescence intensity obtained from a given concentration of EGFR×CD3 LiTE for a range of concentrations in the vicinity of the anticipated K_D and fitting these values to a 1:1 binding model (Fig. 3c, d, Supplementary Table 2 and Supplementary Fig. 2). In addition to EGFR×CD3 LiTE, cetuximab and its derived F_{ab} fragment were included in the experiments involving HeLa cells, and OKT3 and its derived F_{ab} fragment were included with Jurkat cells. The mAbs show an improved functional affinity compared to their F_{abs}, as is anticipated due to their bivalence, although the K_D of the mAbs are not well resolved due to the saturated signal at all concentrations. EGFR×CD3 LiTE bound to EGFR with a K_D of ≈ 1 nM and to CD3 with a K_D of ≈ 8 nM. These are similar values to the included OKT3-F_{ab} and cetuximab-F_{ab}, supporting an intermediate affinity of EGFR×CD3 LiTE towards its antigens.

Anti-EGFR LiTEs induce activation of T-cells that kill EGFR-positive cancer cells

We next assayed purified antibodies for their ability to activate T-cells in vitro. In the presence of EGFR-positive HeLa cells (Fig. 4a), both anti-EGFR LiTEs induced a dose-dependent expression of the activation marker CD69 on human peripheral T-cells (Fig. 4b). Importantly, CD69 expression was not detected after co-culturing with EGFR-negative 3T3 cells (Fig. 4b). We next assessed the ability and specificity of anti-EGFR LiTEs to elicit cytotoxic responses against cancer cell lines expressing intermediate (HeLa) or high (A431) levels of EGFR (Fig. 4a). Luciferase expressing EGFR-positive cells ($HeLa^{Luc}$ or $A431^{Luc}$) or EGFR-negative 3T3 cells ($3T3^{Luc}$) were co-cultured with unstimulated PBMCs at an E:T ratio of 5:1 in the presence of different amounts of purified LiTE. Importantly, cytotoxicity was

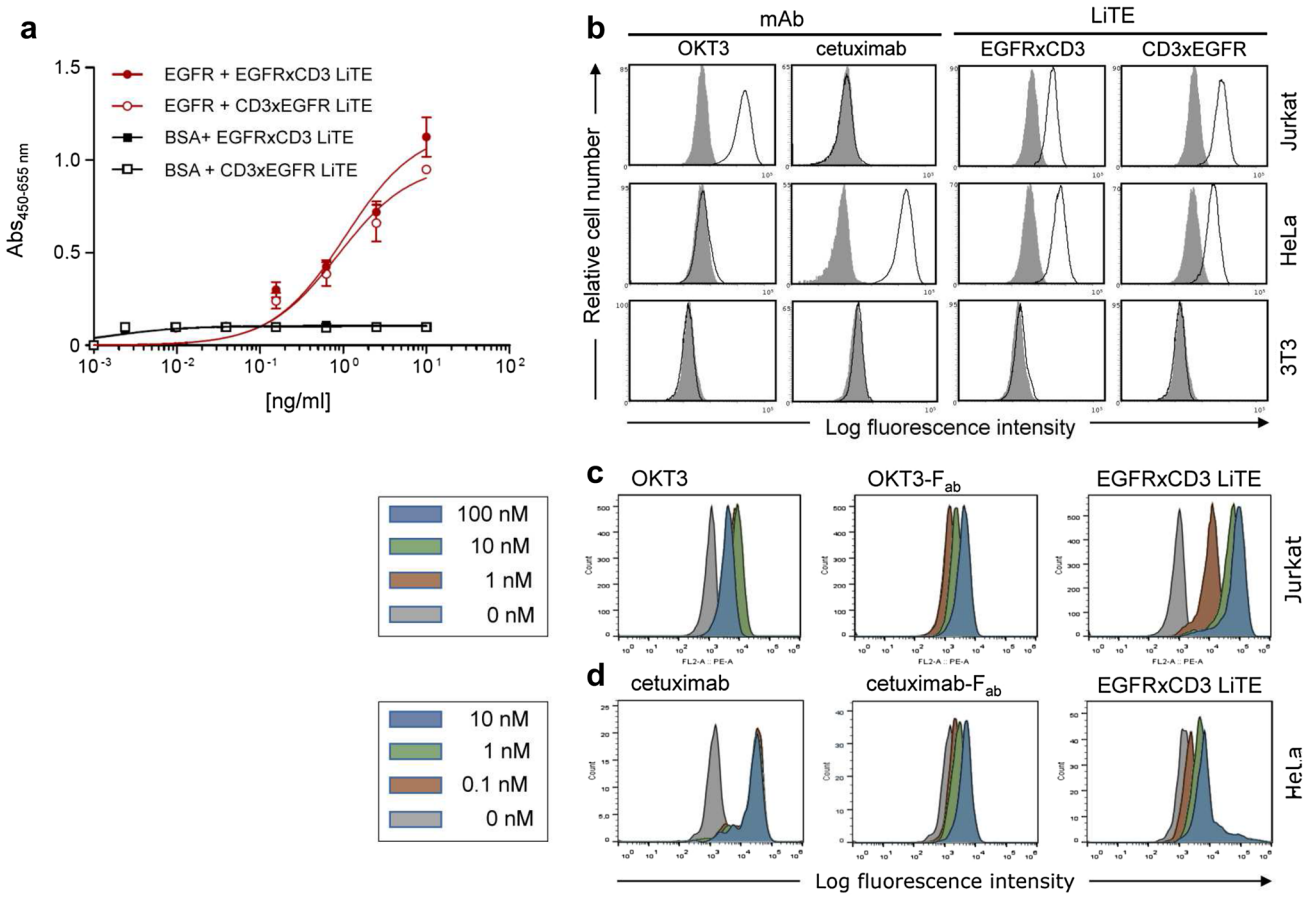

Fig. 3 Functional characterization of purified LiTEs. Titration ELISA (**a**) against plastic-immobilized human EGFR-Fc chimera (hEGFR) and BSA. FACS histograms (**b**) for Jurkat, HeLa, and 3T3 cells incubated with anti-CD3 mAb (OKT3), anti-EGFR mAb (cetuximab), EGFR×CD3 LiTE and CD3×EGFR LiTE. FACS histograms obtained for Jurkat cells incubated with OKT3, OKT3-F_{ab}, and EGFR×CD3 LiTE (**c**); and HeLa cells incubated with cetuximab, cetuximab-F_{ab}, and EGFR×CD3 LiTE (**d**). Each antibody was incubated at three different concentrations, represented with different colors. In FACS studies, fluorescence intensity (abscissa) is plotted against relative cell number (ordinate). All measurements were performed in triplicate; representative histograms are shown

strictly antigen-specific and dose-dependent. Figure 4c, d shows that the cytotoxicity profiles of the two anti-EGFR LiTEs were similar, with EC_{50} values of around 1 ng/mL for HeLa cells 0.5 ng/mL for A431 cells. Cytotoxicity was not observed against EGFR-negative 3T3 cells.

To investigate the ability of locally secreted anti-EGFR LiTEs to induce tumor cell lysis, we used transwell cell culture dishes (Fig. 4e). In this system, luciferase expressing target cells ($3T3^{Luc}$ or $HeLa^{Luc}$) and human PBMCs were co-cultured at an E:T ratio of 5:1 in the bottom and transfected HEK-293 cells were present in the insert well. Tumor cell killing was only seen when EGFR×CD3 LiTE- or CD3×EGFR LiTE-transfected HEK-293 cells were present in the insert well demonstrating the ability of a secreted anti-EGFR LiTEs to redirect T-cells to EGFR-expressing tumor cells (Fig. 4f). No cell killing was observed after co-culturing $HeLa^{Luc}$ cells and PBMCs with GFP-transfected HEK-293 cells, or co-culturing $3T3^{Luc}$ cells and PBMCs with HEK-293 cells transfected with GFP-, EGFR×CD3 LiTE or CD3×EGFR LiTE (Fig. 4f).

Discussion

In this study, we fully characterized a novel bispecific T-cell engager formed by fusing a single-domain anti-EGFR V_{HH} to a conventional anti-CD3 scFv [12]. Two anti-EGFR light T-cell engagers with the V_{HH} and the scFv arranged in both possible orientations were generated, and each of the two LiTEs was efficiently secreted as soluble and functional proteins by transfected mammalian cells. The purified EGFR×CD3 LiTE and CD3×EGFR LiTE were highly homogeneous non-aggregating molecules in solution, as was unambiguously shown by the light scattering measurements, and recognized cell surface-expressed EGFR and CD3 monovalently. Monovalent binding to CD3 is a key factor to

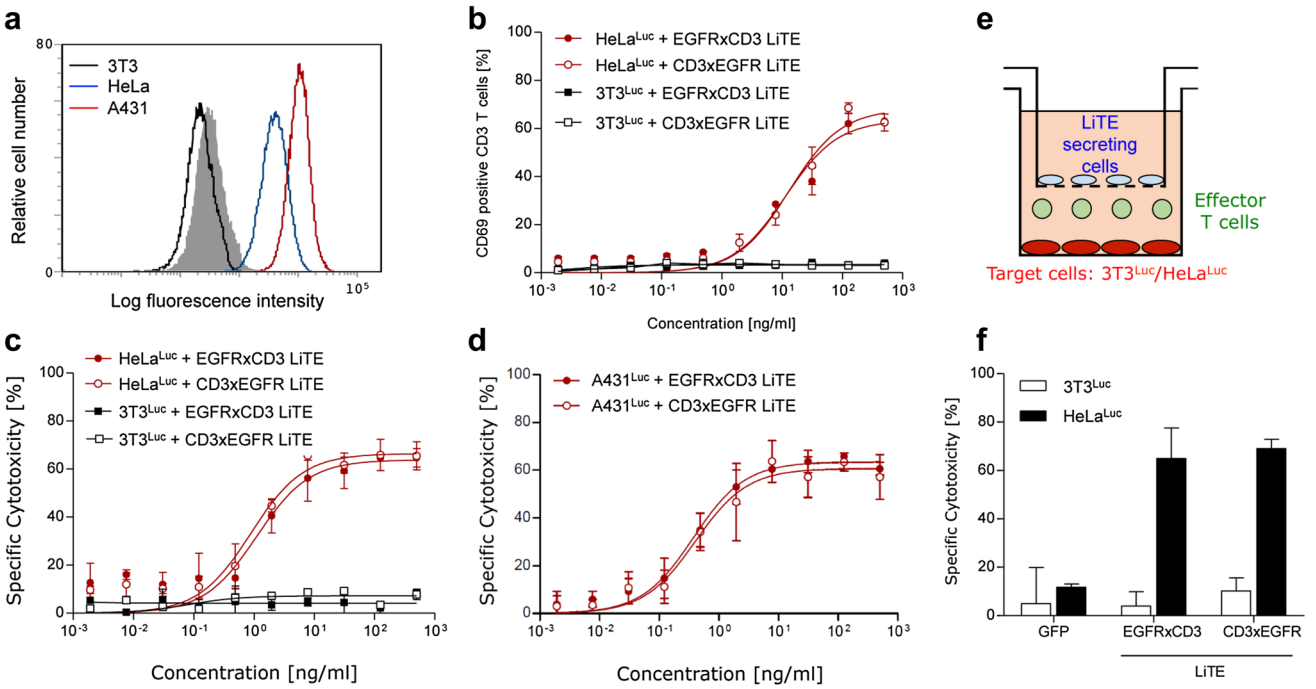

Fig. 4 Induction of T-cell activation and cytotoxicity by purified anti-EGFR LiTEs. Analysis of EGFR expression by flow cytometry on 3T3, HeLa, and A431 cells (**a**). Fluorescence intensity (abscissa) is plotted against relative cell number (ordinate). HeLa and 3T3 cells were co-cultured in 96-well plates with human PBMCs and purified anti-EGFR LiTEs. After 24 h, the surface expression of CD69 was determined by FACS (**b**). Specific lysis of $3T3^{Luc}$ (**c**), $HeLa^{Luc}$ (**c**), and $A431^{Luc}$ (**d**) cells was determined after 72 h. Results are expressed as a mean ± SD ($n = 3$) from one of at least three separate experiments. Scheme of the transwell cell culture chamber used for T-cell cytotoxicity studies by in situ secreted anti-EGFR LiTEs (**e**). In the lower chamber, $HeLa^{Luc}$ cells or $3T3^{Luc}$ cells were incubated with human PBMCs. In the upper chamber, transfected HEK-293 cells were added. After 72 h, the transwell insert was removed and viable tumor cells quantified by bioluminescence (**f**). Results are expressed as the mean ± SD ($n = 3$) from one of at least three separate experiments

avoid the cytokine-related side effects associated with TAA-independent crosslinking of CD3, and may also facilitate the quick disengagement of T-cells from tumor cells, which is necessary for serial cell killing. We demonstrated that both constructs selectively activated and recruited human T-cells to kill EGFR-positive cancer cells in vitro. Importantly, purified anti-EGFR LiTEs had no effect when human T-cells were cultured alone or with EGFR-negative cells.

Tandem scFv are a well-characterized and validated class of Fc-free T-bsAbs that can be expressed in bacteria or mammalian cells, and may show benefits over IgG-based T-bsAbs due to their improved pharmacokinetics for tissue penetration and perfect fit to the immunological synapse [21]. Single-domain V_{HH} antibodies, which are characterized by their smaller size and strictly monomeric behavior [22], have also been used to create various formats of multispecific antibodies by tandem cloning of two or more V_{HH} antibodies [23], and currently, several tandem V_{HH} molecules are in clinical trials [2]. To the best of our knowledge, bispecific tandem V_{HH} or V_{HH}-scFv binding CD3 on T-cells and a surface TAA on tumor cells to redirect T-cells to kill tumor cells has not yet been reported, although single-domain antibodies have been used to generate hybrid bispecific T-cell engagers by fusing a tumor-specific camel V_{HH} with an anti-CD3 Fab fragment [24, 25]. Another approach involved fusion of an anti-CD28-reshaped human V_H to a conventional CEA×CD3 tandem scFv, yielding a trispecific T-cell engager that provides an additional costimulatory signal [26].

One important characteristic of Fc-free T-bsAbs is their small size, which, in many cases, lies below the glomerular filtration threshold. While this may be advantageous with regard to tissue penetration, the short plasma half-lives necessitate frequent injections or continuous intravenous infusion using pumps [5]. Our group has developed a cancer immunotherapy approach based on the secretion of Fc-free T-bsAbs by human cells [6, 27]. We have shown that various types of human cells, such as T-cells, stem cells, and endothelial cells, can be genetically engineered to produce T-bsAbs [6–11]. The T-cell engagers secreted by tumor-infiltrating or tumor-distant cells are then able to redirect cytotoxic T-cells to cancer cells. Currently, strategies based on the redirection of T-cells towards cancer cells through targeting of a TAA can be broadly divided into two categories: the engineering of T-cells with additional TAA-specific

receptors (e.g., CARs), and the systemic administration of purified bispecific antibodies. The in situ expression of T-bsAbs could potentially combine the strengths of both approaches. Transduction of T-cells could circumvent the need for continuous antibody administration, and enable expression locally, at the tumor site [27, 28]. At the same time, the redirection of T-cells would not be limited to only those that have been transduced, as is the case with CAR transduction. Other groups have recently validated this approach demonstrating potent antitumor activity of TAA-specific T-bsAbs [29–33].

In this study, we demonstrate, for the first time, the potential of in situ secreted anti-EGFR LiTEs for effective induction of T-cell cytotoxicity of EGFR-expressing cancer cells. Importantly, the anti-EGFR LiTEs present in conditioned media did not induce objectionable levels of antigen-independent T-cell activation. While the small size of the LiTE antibodies (41–43 kDa) may discourage their use in therapeutic regimes involving the systemic administration of purified protein, it could be advantageous in a system where antibody is expressed locally in the tumor, as LiTE escaping the tumor will be quickly cleared, while other formats might circulate and cause off-tumor toxicity. Its small size and corresponding quicker diffusion may also allow it to reach tumor areas, which are effectively inaccessible to larger antibodies [34]. Single-domain V_{HH} antibodies recognizing several TAAs are available, and LiTEs combining these with well-characterized anti-CD3 or anti-CD16 scFvs could quickly be assembled into a broad battery of BiTE and BiKE analogues applicable to gene-mediated immunotherapy of human cancer [35, 36]. In this study, we focused on EGFR as model antigen, due to the availability of anti-EGFR antibodies and EGFR-positive tumor cell lines. However, a major limitation of EGFR as a target for T-cell redirection strategies is on-target off-tumor toxicity caused by EGFR expression on normal cells [37], as reported for both anti-EGFR IgG and anti-EGFR BiTEs [38, 39]. LiTEs targeting hematopoietic lineage-specific markers or receptors like CEA or PSMA [40] could be generated for minimizing the detrimental effects of cytokine-induced inflammatory responses.

Author contributions Luis Alvarez-Vallina was involved in the study conception and design. Kasper Mølgaard, Seandean L. Harwood, Marta Compte, Nekane Merino, Jaume Bonet, Ana Alvarez-Cienfuegos, Kasper Mikkelsen, Natalia Nuñez-Prado, Ana Alvarez-Méndez, Laura Sanz, and Francisco J. Blanco were involved in acquisition, analysis, and interpretation of data. Kasper Mølgaard, Seandean L. Harwood, and Luis Alvarez-Vallina drafted the manuscript, and all the authors were involved in critical revision of the manuscript.

Funding Luis Alvarez-Vallina was supported by grants from the Danish Council for Independent Research, Medical Sciences (DFF-6110-00533) and the Novo Nordisk Foundation (NNF14OC0011019). Jaume Bonet was supported by the 'EPFL Fellows' fellowship program co-funded by Marie Skłodowska-Curie, Horizon 2020 Grant agreement no. 665667. Francisco J. Blanco thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for support through grant CTQ2017-83810-R and Severo Ochoa Excellence Accreditation (SEV-2016-0644). Laura Sanz was supported by grants from the Fondo de Investigación Sanitaria/Instituto de Salud Carlos III (PI13/00090), co-funded by European Regional Development FEDER funds, and the Comunidad de Madrid (S2010/BMD-2312).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest related to this work.

Ethical approval All procedures involving human blood products were in accordance with the ethical standards of the Aarhus University Hospital Ethical Committee and with the 1964 Helsinki declaration and its later amendments. Human peripheral blood mononuclear cells were isolated from fresh peripheral blood of anonymized healthy volunteer donors.

Informed consent Blood donors were recruited to donate blood by standard phlebotomy. The investigational nature of the studies in which their blood would be used, and the risks and discomforts of the donation process were carefully explained to the donors, and a signed informed consent document was obtained.

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