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Highly flexible, IgG-shaped, trivalent antibodies effectively target tumor cells and induce T cell-mediated killing

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Abstract: A novel bispecific antibody format was applied to generate T cell-engaging antibodies. The TriFab format is a trivalent IgG-shaped entity composed of two Fab arms that bind to antigens on the surface of tumor cells, which are linked via flexible peptides to a CD3 binding moiety that replaces the CH2 domains of conventional IgGs. The distinctive feature of these T cell recruiting bispecifics is that their CD3 variable regions are incorporated between domains, rather than N- or C-terminally fused to an Fc or antibody fragments. T cell recruiting TriFabs resemble in size and shape, are expressed and show biophysical properties similar to regular IgGs. Transmission electron microscopy (TEM) demonstrates high flexibility of the cell surface binding arms as well as target antigen accessibility of the interspersed CD3 binding domain. Functional co-culturing assays of peripheral blood mononuclear cells (PBMCs) and different tumor cell lines (MCF7 and A431) revealed a dose-dependent T cell-mediated cytotoxicity that was induced by the TriFabs targeting either LeY or EGFR cell surface antigens.

Keywords: cytokine secretion; geometry; immunotherapy; PBMC; T cell recruiting; TriFab.

Introduction

Immunotherapy is a therapeutic concept that has gained growing focus within recent years. A major molecule class here are the T cell-engaging antibodies that cross-link target (tumor) and effector (T cells) and thereby lead to T cell activation, proliferation and tumor cell eradication (Kobold et al., 2018). T cell-engaging bispecific antibodies (TCBs for T cell bispecifics) can be generated in different formats and compositions. These include fused Fv-domains such as the BiTE formats which are already available as drugs (Wu et al., 2015; Kantarjian et al., 2017), as well as a large number of other compositions currently in clinical development (Spiess et al., 2015; Brinkmann and Kontermann, 2017; Almagro et al., 2018). Different formats define different properties of TCBs including valencies and geometry of binding to tumor and T cells, efficacy, biophysical characteristics and pharmacokinetics just to name a few.

The BiTE format as being the first-in-class molecule consists of cell surface targeting scFv and a CD3-binding scFv. These molecules are rather small and therefore display good tissue and tumor penetration. Their pharmacokinetics, on the other hand, are characterized by a very short serum half-life and necessitates continuous infusions (Stieglmaier et al., 2015; Wu et al., 2015). Other formats for generating TCBs include bsAbs with bivalent CD3 binding entities. Those may display higher overall potency but often report unspecific or non-targeted CD3-crosslinking and T cell activation (Kobold et al., 2018). This can lead to undesired secretion of pro-inflammatory cytokines at distinct sites (Moore et al., 2011; Bardwell et al., 2018). Additional formats in clinical development are IgG-derivatives with added mono-CD3-binding entities (Bacac et al., 2016; Bardwell et al., 2018).

Most currently available TCB formats are composed of N- or C-terminal fusions of CD3-binding entities to either antibody fragments or Fc-containing antibody derivatives (Kontermann and Brinkmann, 2015; Spiess et al., 2015; Harwood et al., 2017; Liu et al., 2017). To expand the format space for generating TCBs, we analyzed if the CD3-binding

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moiety can be introduced into a bsAb in an alternative manner, neither as N-terminal nor as C-terminal fusion. Here, we present the applicability of the TriFab format for the generation of TCBs. TriFabs are antibody derivatives that have their CH2 domains replaced by VH and VL, resulting in IgG-shaped and sized trivalent bsAbs (Mayer et al., 2015). We demonstrate that placing a CD3-binding VH/VL combination in the ‘stem-position’ replacing CH2 generates functional TriFab TCBs with avidity-enhanced bivalent tumor targeting and monovalent T cell engagement.

Results

Design, expression and characterization of TriFab TCBs

The goal was to generate T cell recruiting antibodies that harbor two cell surface binding Fab arms as well as one additional CD3 binding Fv in place of CH2 domains of an IgG. Based on the TriFab format, we substituted the CH2 domains of heterodimerizing knob-into-hole IgG H-chains (Spiess et al., 2015) with either the VH or the VL domains of a CD3 binding entity (VH on the ‘knob’ side, VL on the ‘hole’ side as previously described by Mayer et al., 2015). The ‘knob’ domain comprises the mutation T366W whereas the ‘hole’ part includes T366S, L368A, Y407V. The heterodimerization of the two heavy chains is enforced not only by KiH mutations but additionally by VH/VL association of the <CD3> entity. The <CD3> binding affinity was chosen to be in the mid nanomolar range in

order to ensure avidity-enhanced binding of T cells in the presence of tumor cells rather than inducing T cell activation in the periphery (Bacac et al., 2016; Leong et al., 2017). To enable access of this TriFab to the CD3 antigen on T cells, its hinge region was replaced by a flexible (G4S)4 peptide without a disulfide connection (Mayer et al., 2015) as shown in Figure 1.

To generate proof of concept molecules and for assessing targeted T cell recruitment by TriFab TCBs, two different target antigens were selected that are frequently found on the surface of tumor cells: LeY is a carbohydrate antigen found on various epithelial tumors and present on the breast tumor cell line MCF7 (Metz et al., 2011). EGFR is expressed on many tumors and in particular at high levels on A431 cells (Van de Vijver et al., 1991). In addition, a TriFab that binds an irrelevant antigen not present on target cells was selected to serve as control molecule.

Following transient expression in HEK293 cells, antibodies were purified via affinity chromatography applying resin that binds the kappa light chains (Methods section). The expression yields were found to be >30 mg/l expression volume for all constructs. The preparative size exclusion chromatography (SEC) profile displayed a clear product peak revealing a high purity and small amount of side products after affinity chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed proper removal of undesired impurities (Figure 2). Additionally, neither precipitation nor aggregation ($T_{agg} > 67^{\circ}\text{C}$) upon storage was observed for TriFabs indicating high stability of the molecules and good biophysical behavior. Molecule integrity at 37°C was

Figure 1: Design of TriFab TCBs.

Cell surface binding domains (blue) are regular Fab arms connected via (G4S)4 linkers with a central variable region (red) that substitutes the CH2 domains of IgGs. To achieve correct assembly of the two heavy chains and the respective variable region (red), knob-into-hole (KiH) mutations (Spiess et al., 2015) are introduced into the CH3 domains. Furthermore, correct chain assembly is supported by VH/VL assembly of the <CD3> binder. Accessibility of the central binding region is given due to high flexibility of the glycine-rich linker region (right). Loss of CH2 domains eliminates their capability to bind FcRs and thereby inactivates their capability to trigger antibody-dependent cell-mediated cytotoxicity (ADCC).

Figure 2: Characterization of TriFab TCBs by size exclusion chromatography and SDS-PAGE.

TriFab TCBs targeting LeY or EGFR were purified using HighTrap kappa select column and eluted at low pH. (A) exemplarily shows the preparative SEC profile for the <EGFR>-TriFab, indicating a sharp peak. (B) The fractions that represent the main peak (75–90 ml) contained pure TriFabs without further contaminations as shown by Coomassie-stained SDS-PAGE for all constructs including the control molecule.

confirmed in thermal stability measurements (Supplementary Figure S1).

Previously it was shown that the added variable region in the ‘stem region’ of TriFabs (Figure 1, red) is able to bind haptens such as digoxigenin, biotin or FITC (Mayer et al., 2015). However, haptens are small molecules which are able to diffuse to and in between protein domains without excessive steric hindrance. In contrast, protein antigens

including CD3 on T cells are positioned on cell surfaces in defined orientations and geometries. Therefore, it is required to have a sufficient degree of molecular flexibility to allow access of the third binding region of TriFabs. Such high flexibility of the binding arms and accessibility of the 3rd binding entity was visualized by negative stain-transmission electron microscopy (NS-TEM). Figure 3 shows various molecule conformations, class averages as

Figure 3: Negative stain transmission electron microscopy of TriFab TCB.

To demonstrate flexibility of the two regular Fab arms (blue), we performed NS-TEM (details see materials and methods). The images further illustrate Fab arm flexibility enabling effective accessibility of the CD3-binding Fv to its cognate antigen on the surface of T cells.

they derive from statistical analysis of raw micrographs recorded with NS-TEM. The cell surface binding Fab-arms of TriFabs (blue entities in the scheme) are connected to the ‘stem region’ of the TriFab in a highly flexible manner. Thereby, access of the 3rd binding site (red domains in the scheme) to CD3 on T cells is not expected to be subject to relevant steric hindrance. All three Fabs are condensed as described for other IgGs, top and side view orientations have been captured, as expected. The molecule is confirmed to behave like a flexible chain of three rigid and properly folded segments.

TriFab TCBs specifically bind target cells and activate effector T cells

Flow cytometry analyses were applied to prove that the generated TriFabs retained the capability to specifically

bind to the surface of cells. Therefore, MCF7 (LeY⁺) and A431 (EGFR⁺) cells were incubated with the respective TriFabs and subsequently detected with a secondary fluorescence-labeled anti-light chain antibody. As depicted in Figure 4, each of the molecules retained their cell surface binding capability upon exposure to the respective target cells. Binding of the <LeY> TriFab resulted in higher mean fluorescence intensity (MFI=864) compared to the <EGFR> TriFab (MFI=264).

To address whether TriFab TCBs can simultaneously bind to tumor and T cell – the prerequisite for the TriFab to induce T cell activation – we co-incubated a Jurkat reporter T cell line (see Materials and methods) with respective TriFabs and target cells. These Jurkat cells express luciferase upon CD3-mediated activation of the NFAT pathway. Thus, light emission after adding luciferin reveals T cell activation. For both molecules a dose-dependent activation of T cells could be observed. The

Figure 4: Target cell binding analysis by flow cytometry and T cell activation assay using a reporter cell line.

(A) Binding of TriFabs targeting either EGFR or LeY to the surface of the respective cell lines A431 and MCF7 was assessed via flow cytometry. Binding was detected by a FITC-labelled anti-kappa light chain antibody. (B) T cell activation capability of TriFab TCBs was analyzed in co-cultivation assays of target cells, TriFab and Jurkat NFAT-Luc2 reporter cell line (materials and methods). Light emission as readout was measured by photometer (RLU = relative light units). The results were confirmed in two independent experiments (see Supplementary Figure S2 for complete data sets). Results are expressed as mean and SD from triplicate wells and plotted as 4-parameter non-linear regression fitting using Graphpad Prism software.

highest absolute signal could be measured for the LeY-targeting TriFab.

TriFab TCB induce T cell-mediated tumor cell lysis and cytokine secretion in peripheral blood mononuclear cell (PBMC) co-culturing experiments

As additional proof for the successful T cell recruitment by CD3-binding TriFabs, we analyzed their capability to activate immune cells to initiate tumor cell killing in a targeted manner. Therefore, fresh PBMC were isolated from the blood of healthy human donors and co-incubated with TriFab-TCBs and tumor cells. We observed a dose-dependent tumor cell lysis as shown in Figure 5A for both TriFabs applied to the respective target cell line.

The *in vitro* EC_{50} values and all killing curves with PBMCs of three different donors are summarized in the Supplementary material, Figure S3, Table S1. As an example, we detected the *in vitro* EC_{50} between 0.1 and 1 nM which is in the same range (taking donor and assay variations into account) as recently reported in similar assays for TCBs (Bardwell et al., 2018). As additional evidence that the observed cytotoxicity is mediated by T cell recruitment, we analyzed the cytokine profile of supernatants from the co-cultivation experiments above. Figure 5B shows significantly elevated concentrations of interferon (IFN) γ , interleukin (IL)-2, tumor necrosis factor (TNF) α and granzyme B. This supports the notion that – as expected – targeted cytotoxicity is caused by antibody-mediated T cell activation. Notably, we used in all assays a <ctrl> TriFab with Fab arms that do not bind to antigens present on the applied target cells but carrying intact <CD3> in the ‘stem’ region as a control. Those controls did not elicit off-target

Figure 5: PBMC killing assays and cytokine profiles.

To assess the TriFab TCB-mediated activation of lymphocytes, PBMC were isolated from blood of healthy human donors [$n = 3$, (#1, #2, #3)] and co-cultivated for 48 h with respective antibody dilution and target cells. (A) Percentage of target cell killing was measured by LDH release of dead cells. Results are expressed as mean and SD from triplicate wells and plotted as 4-parameter non-linear regression fitting using Graphpad Prism software. Representative killing curve of each TriFab and cell line is shown. (B) Cytokine profile of supernatants at 2 nM reveals CD8⁺ T cell activation. Mean and SD of triplicate wells are shown [for additional data of other donors (#1, #2, #3) see Supplementary Figures S3 and S4].

activation of T cells, supporting the concept of a specifically targeted mode of action.

Discussion

Among the variety of T cell-engaging antibodies it turned out that different molecular designs and geometries define the pharmacokinetic and pharmacodynamical behavior in immunotherapy approaches (Le Gall et al., 2004; Spiess et al., 2015; Liu et al., 2017). Important parameters to be considered in that context include (i) mode of binding to targeted cell surface antigens that defines tumor cell specificity (Zhukovsky et al., 2016); (ii) accessibility and valency of agonistic binding to CD3 which affects the intensity of induced T cell signaling (Reusch et al., 2015; Bardwell et al., 2018); and (iii) format dependent geometry and flexibility of individual binding domains which influence the efficacy of crosslinking of target cells and effector cell, and the activation of effector cells. The latter parameter (geometry) also includes the distance between the targeting moiety and the effector cell binding moiety (Hoffmann et al., 2005; Bluemel et al., 2010). Additional parameters that influence the therapeutic application of T cell-recruiting antibodies include size (affect distribution and tissue/tumor penetration and pharmacokinetics (Reusch et al., 2015) and intra- and interdomain stability that ensures the maintenance of active conformation in serum and circulation (Correia, 2010).

Finally, the presence or absence of Fc-receptor target sites in Fc regions of TCBs determines their capability to trigger antibody-dependent cell-mediated cytotoxicity (ADCC) (Sathish et al., 2013). Taking the mentioned points into account, the design of a TCB needs to combine targeting entities and effector inducing entities in a manner that generates molecules with a good efficacy as well as acceptable safety. Because the bsAb format influences and modulates these parameters, this work was aimed at extending the format space for generating T cell-recruiting antibodies.

The TriFab format allows bivalent binding of a target cell and can benefit of avidity-mediated specificity gain (AMSG) by permitting cell surface binding with two Fab arms each of which with rather low affinity in case of monovalent antigen recognition. This concept aims to reduce antibody binding to cells with low antigen expression (Rudnick and Adams, 2009). In order to reduce T cell crosslinking in the absence of target antigens, a monovalent <CD3> in the central binding region benefits the TriFab setup. The close proximity of target and effector cell

binding moieties in combination with the flexible linker design may facilitate the formation of a tight immune synapse (Bacac et al., 2016). TriFabs are in size very similar to regular IgGs (150 kDa), hence above the molecular weight of entities for which rapid renal clearance would be expected (Meibohm and Zhou, 2012). On the other hand, because TriFabs have their CH2 domains substituted by the variable domains of a CD3-binder, TriFabs do not bind FcRn. This feature inactivates the FcRn-mediated recycling mechanism which causes regular IgGs to have very long serum half-lives. Thus, the pharmacokinetics behavior of TriFabs is expected to represent ‘medium-half-life’ characteristics: longer than small scFv fusions (e.g. BiTEs) and shorter than fully Fc-competent IgG fusions. Replacement of CH2 domains with the CD3-binding Fv also inactivates their FcγR-binding functionalities, including binding to FcγRIII. Because of that, TriFabs by themselves are not expected to induce ADCC. This may be a safety advantage of TriFabs as ADCC induction has been mentioned as a potential safety concern (off-target toxicity and systemic secretion of pro-inflammatory cytokines (Sathish et al., 2013)).

The results of this work show that the TriFab format can be applied to generate functional T cell-recruiting antibody derivatives. This rather new bsAb format that has a CD3-binding Fv interspersed in a monovalent manner between two cell surface Fab-binding arms provides sufficient flexibility and suitable geometry to direct T cells to tumor cells. High flexibility and novel geometry (CD3-binding in between cell binding arms facing in the same direction) enables effective formation of immune synapses to trigger targeted, T cell-mediated tumor cell killing.

Materials and methods

Design, expression and purification of TriFab TCB

Considering the parental targeting antibody sequences, we used the mAb B3 parental clone for <LeY> (Brinkmann et al., 1991) and the cetuximab parental clone C225 for <EGFR> (Huang et al., 1999). In regard to the knob-into-hole mutations we introduced knob: T366W; hole: T366S, L368A, Y407V into the respective CH3 domains (Ridgway et al., 1996). All constructs were expressed in HEK293 suspension cells that were grown at 37°C, in a humidified incubator with 8% CO₂ supply. HEK 293 cells were transfected with PEIpro® (Polyplus, Illkirch-Graffenstaden, France) according to the manufacturer’s recommendations. The transcription was driven by a CMV promotor. As signal peptide an IgG leader sequence of Ig heavy chain V region was used. Supernatants were harvested 7 days following transfection, spun down at 3500 g and sterile filtered through a 0.22 μm filter unit (Thermo Fisher Scientific, Waltham, MA, USA). For affinity chromatography we applied the supernatants to a 1×5 ml HiTrap™

KappaSelect SuRe™ (GE, 17-5458-11, Boston, MA, USA) and processed according to the manufacturer's recommendations. Bound proteins were eluted by applying a 50 mM sodium citrate buffer at pH 2.7. As neutralization buffer, 1 M TRIS (pH 9) was added to the fractions ($v=5\%$ of fractionation volume). Prior to SEC eluted solution was sterile filtered. The sample was then applied to a HiLoad® 26/600 Superdex® 200 pg (GE, 28989336, Boston, MA, USA) and processed as recommended by the manufacturer. As a running buffer a 20 mM histidine, 140 mM sodium chloride solution at pH 6.0 was used. Profiles of preparative SEC were extracted from UNICORN™. Purity was subsequently analyzed using the NuPAGE system (Invitrogen, Carlsbad, CA, USA) with 4–12% Bis-Tris-gels. The gel was stained with Instant-Blue™ Protein Stain (Expedeon, ISBIL, Heidelberg, Germany).

Negative stain transmission electron microscopy (NS-TEM)

For grid preparation, freshly thawed samples were diluted in D-phosphate buffered saline (PBS) to a concentration of ~ 5 mg/ml. Four microliters of the diluted sample were adsorbed to glow-discharged 400 mesh carbon coated Parlodion copper grids washed with three drops of water, incubated with 3 μ l of tobacco mosaic virus containing solution, further washed with two drops of water and finally stained with two drops of uranyl acetate 2%. Samples were subsequently imaged using a Tecnai12 transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV. Electron micrographs were recorded on a 2048 by 2048 pixel charged-coupled device camera (Gloor Instruments, Kloten, Switzerland) at a nominal magnification of $\times 195\,000$ yielding a final pixel size of 0.296 nm on the specimen level. Alternatively, samples were imaged using a FEI Tecnai G2 Spirit TEM (FEI, Eindhoven, The Netherlands) operating at 80 kV. Electron micrographs were then recorded on a 2048 by 2048 pixel charged-coupled device camera (Veleta Soft Imaging Systems, EMSIS, Münster, Germany) at a nominal magnification of $\times 135\,000$ yielding a final pixel size of 0.33 nm on the specimen level. Images were processed by reference-free alignment on manually selected particles from recorded images using the EMAN2 image processing package. The extracted particles were aligned and classified by multivariate statistical analysis yielding two-dimensional (2D) class averages. Additionally, when class averaging was not possible, raw images of particles were also manually stained for clarity using Photoshop (Adobe Systems, San José, CA, USA).

Cell culture and reagents

MCF7 (ATCC® HTB-22™) and A-431 (ATCC® CRL-1555™, Manassas, VA, USA) were cultivated in Roswell Park Memorial Institute (RPMI) Medium 1640 (Gibco 31870-025, Waltham, MA, USA) substituted with 10% fetal calf serum (FCS) (Biowest, S181B) and 2 mM L-glutamine (Gibco 25030-81, Waltham, MA, USA). For detaching cells Accutase® solution (Sigma, A6964) was used. Cells were counted using a Vi-CELL XR Analyzer (Beckman Coulter, Brea, CA, USA).

Binding studies

Binding to target cells was confirmed via flow cytometry. A total of 3×10^5 cells were incubated with 100 nM of TriFab TCB in FACS buffer [PBS DPBS (PAN Biotech, P04-36500) with 2% FCS] for 1 h at 37°C.

Then, cells were washed twice with PBS and incubated for an additional hour with 100 nM FITC-labeled Anti-Human kappa Light chain (Sigma, F3761) in FACS buffer on ice. Following two washes with PBS, the cells were analyzed for fluorescein isothiocyanate intensity using a FACS CantoII instrument (BD biosciences).

Reporter cell assay for the detection of effector cell activation

TriFab-induced activation of effector cells was measured using a T Cell Activation Bioassay (NFAT) (Promega, J1621) according to the manufacturer's recommendations. Target cells were seeded out the day before co-culturing to aim for 100% confluency at day 1. Respective antibody dilutions and Jurkat effector cells (E:T ratio 2:1) were added to a final volume of 75 μ l. After 6 h of incubation in a humid 37°C, 5% CO₂ incubator, 75 μ l of Bio-Glo™ reagent were added. Following 5 min incubation at room temperature, luminescence was measured using a Tecan Infinite F200 plate reader (Tecan Trading AG, Männedorf, Switzerland).

PBMC co-cultivation assays

A total of 1.5×10^4 cells in standard RPMI1640 (10% FCS, 2 mM glutamine) of the respective target cell line (MCF7 or A431) were seeded out the day before PBMC co-cultivation in 96-well plates (Day 0). On Day 1, fresh whole blood from healthy human donors was processed according to the manufacturer's recommendations using Ficoll® Paque Plus (GE Healthcare, Chicago, IL, USA) and Leucosep™ centrifuge tubes (Greiner Bio-one, Kremsmünster, Austria). Mononuclear cells were stained for viability with trypan blue and counted using a conventional Neubauer chamber. As assay media for co-culturing RPMI1640 (1% FCS, 2 mM glutamine) was used. A dilution series of respective antibodies was performed in assay media and added to target cells. A total of 1.5×10^5 PBMC were added to each well to end up with a total volume of 200 μ l. Lactate dehydrogenase (LDH) release was measured after 48 h using the Cytotoxicity-Detection Kit (from Sigma, by Roche 11644793001, Basel, Switzerland) according to the manufacturer's recommendations (including calculations and high control using 2% Triton-X100). The results were analyzed as mean and standard deviation (SD) from triplicate wells and plotted as 4-parameter non-linear regression fittings using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). Supernatants from the triplicate wells of the same assay as used for LDH release measurement were further analyzed for cytokine concentration using the flow cytometry-based CBA technology (BD, cat#558264, Franklin Lakes, NJ, USA) according to the recommended protocol. The assay was performed in a multiplexed manner using the following reagents: Human IFN- γ Flex Set (cat# 560111), Human IL-2 Flex Set (cat# 558270), Human TNF Flex Set (cat # 560112) and Human Granzyme B Flex Set (cat # 560304). Data was analyzed using the FCAP Array Software (BD bioscience) and plotted as interleaved bars (mean and SD) with GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

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