Design, selection and optimization of an anti-TRAIL-R2/anti-CD3 bispecific antibody able to educate T cells to recognize and destroy cancer cells

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ABSTRACT
Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or TRAIL-receptor agonistic monoclonal antibodies promote apoptosis in most cancer cells, and the differential expression of TRAIL-R2 between tumor and normal tissues allows its exploitation as a tumor-associated antigen. The use of these antibodies as anticancer agents has been extensively studied, but the results of clinical trials were disappointing. The observed lack of anticancer activity could be attributed to intrinsic or acquired resistance of tumor cells to this type of treatment. A possible strategy to circumvent drug resistance would be to strike tumor cells with a second modality based on a different mechanism of action. We therefore set out to generate and optimize a bispecific antibody targeting TRAIL-R2 and CD3. After the construction of different bispecific antibodies in tandem-scFv or single-chain diabody formats to reduce possible immunogenicity, we selected a humanized bispecific antibody with very low aggregates and long-term high stability and functionality. This antibody triggered TRAIL-R2 in an agonistic manner and its anticancer activity proved dramatically potentiated by the redirection of cytotoxic T cells against both sensitive and resistant melanoma cells. The results of our study show that combining the TRAIL-based antitumor strategy with an immunotherapeutic approach in a single molecule could be an effective addition to the anticancer armamentarium.

Introduction
The discovery of FasL, tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL), 1 which are natural cytokines that belong to the TNF superfamily, opened possibilities for the development of new cancer therapeutics thanks to their capability to induce apoptosis. 2 In contrast to TNF and FasL, which cause severe adverse effects in vivo, 3,4 TRAIL is a proapoptotic ligand that shows no toxicity because it seems to be specific to tumor cells, preserving normal cells. 5

TRAIL can trigger the extrinsic apoptotic pathway on tumor cells by binding TRAIL-R1 (or DR4) and TRAIL-R2 (or DR5), 6 inducing the trimerization of the receptors 7 and the activation of the caspase cascade. 8 TRAIL-R1 and TRAIL-R2 are up-regulated in many tumors, 9,10 and TRAIL-R2 seems to be an interesting target for cancer immunotherapy given its differential expression between tumor and normal tissues. Although good results had been obtained in preclinical models, different TRAIL receptor agonist compounds (a recombinant form of TRAIL and agonistic antibodies) failed in clinical trials. 11 The failure could be partly explained by the presence or development of intrinsic TRAIL resistance in human cancers. Tumor cells can acquire apoptosis escape mechanisms that preserve them from TRAIL-mediated killing. 12

In the past decade and with advances in antibody engineering, there has been an upsurge of therapeutic bispecific antibodies (bsAbs) research. BsAbs can simultaneously bind two different targets and could thus unite two mechanisms of action in one molecule. More than 50 different bsAb formats were engineered. 13 Good results were obtained in particular with bsAbs that were able to retarget T cells to lyse tumor cells.
in a TCR-independent way.\textsuperscript{14,15} The recently approved agent blinatumomab, which targets CD19 and CD3,\textsuperscript{16} and bispecific T-cell engagers (BiTEs) in general,\textsuperscript{17,18} have yielded excellent results.\textsuperscript{19,20} BiTEs are made up of two linked single-chain antibody variable fragments (scFv) in a tandem scFv (tascFv); the resulting compact structure allows the formation of the immunocytolytic synapsis between tumor and immune cells. Of note, only when both BiTE arms are engaged with their target antigens\textsuperscript{21} – even at subnanomolar concentrations – does the activation of T cells take place.

Building bsAbs composed only of variable domains can be a challenge because the final molecule may not attain proper folding and stability. In the antibody structure, the stabilizing interactions between constant domains of heavy and light chains are very important; the lack of these moieties could give the final construct poor thermal stability or lead to increased aggregation.\textsuperscript{22,23} Moreover, novel structural formats may create neoantigens or expose cryptic epitopes that may elicit immune responses. Overcoming immunogenicity during antibody development is essential to avoid neutralization of the biotherapeutic efficacy (i.e., anti-antibodies) and ensure the safety of exposed patients.\textsuperscript{24,25}

Here, we provide evidence that an anti-TRAIL-R2/anti-CD3 bsAb in single-chain diabody (scDb) format, rather than tascFv format, results in a more stable and functional reagent able to efficiently retarget T lymphocytes against TRAIL-R2\textsuperscript{+} tumor cells irrespective of their sensitivity to TRAIL treatment.

Results

Selection of anti-TRAIL-R2 scFvs from a phage display library

Three cycles of panning were performed on recombinant and natural human TRAIL-R2. In both cases there was an increment in colony number after each cycle. In particular, using PCR we observed an increase in colonies containing the insert at the right molecular weight (see supplementary Figure 1). Polyclonal phages isolated after the third cycle of selection against recombinant TRAIL-R2 were able to bind the recombinant protein in ELISA, but not the natural receptor expressed by cells (Suppl. Figure 2). On the other hand, polyclonal phages isolated after the third panning with native protein from cell lysate were able to bind the receptor expressed on TRAIL-R2\textsuperscript{+} cells. Single-clone ELISA, using phages isolated from selection on the natural receptor, was performed on Me15 cells and allowed us to isolate 13 clones; fluorescence-activated cell sorting (FACS) analysis demonstrated that the periplasm of six clones (7, 8, 44, 52, 56 and X) could specifically bind SU-DHL-4 cells without nonspecific binding on receptor-negative HDLM-2 cells (Suppl. Figure 3a). When these clones were sequenced (Eurofins Genomics), five different scFv genes were revealed.

Construction and production of an anti-TRAIL-R2/anti-CD3 tascFv bsAb

To construct a functional bsAb able to bind the two targets simultaneously, a first attempt was made to assemble the anti-TRAIL-R2 scFvs (isolated from the phage display library) with the TR66 anti-CD3 scFv\textsuperscript{26}; among the five assembled tascFvs only one clone, named 8/TR66, was able to bind both TRAIL-R2 and CD3 (Suppl. Figure 3b), but only when freshly produced. To isolate a functional bsAb we decided to build a bsAb library in a tascFv format. After transformation we obtained about $1.7 \times 10^6$ colonies containing the right size of the bsAb gene (validated by PCR). In fingerprint analysis, 9/11 clones proved to be cut in a different mode, indicating a variability of about 82% (Suppl. Figure 4). The variability was confirmed by sequencing analysis: of the 29 bsAb genes that were sequenced, 24 (82%) were found to be different (the CDR3 sequences of these different clones are presented in Suppl. Table 1). From this mini-library we isolated 15 bsAbs that were able to bind TRAIL-R2 in ELISA tests, but analysis by FACS indicated only three could bind both TRAIL-R2 and CD3. Two of these, E7/TR66 and F6/TR66, had the same sequence as the clone constructed with variable domains of 16E2 (patent no. US 8,409,570 B2); however, even though they were initially able to bind both targets simultaneously, they lost the TR66 arm functionality (Figure 1c). The third, F3/TR66, lost the ability to recognize both antigens.

To improve the bsAb performance, we decided to replace the murine TR66 variables with the variable domains of a different anti-CD3 antibody. In an attempt to reduce immunogenicity, which is crucial for the success of a biotherapeutic, we chose the humanized UCHT1 antibody variable domains. In this way we obtained a human/humanized tascFv bsAb. This tascFv was still specific and able to bind CD3, but binding to TRAIL-R2 was very poor (Figure 2a). The binding specificity of the anti-TRAIL-R2 moiety was confirmed by Biacore analysis on the recombinant protein (Figure 2b). Size exclusion chromatography (SEC) analysis revealed that about 52% of the bsAb was aggregated, with only 42.7% of the monomeric form (Figure 2c).

Remodeling of the anti-TRAIL-R2/anti-CD3 bsAb in a different format

To solve the stability problem of the tascFv, we changed the format and, using the variable domains of E7 (which corresponds to the 16E2 mAb described in the patent literature) and of UCHT1, we assembled the bsAb as a scDb. The scDb format consists of two single chains in which the C-terminus of a heavy-chain variable (VH) domain is connected to the N-terminus of a light-chain variable (VL) domain of another specificity using a short rigid linker to restrict intrachain pairing of VH and VL with the same specificity. The two chains pair, taking advantage of cognate variable domain natural pairing. In the scDb format, another longer linker between the two single chains stabilizes the structure (see cartoon of Figure 3a). FACS analysis demonstrated specific binding of the scDb on TRAIL-R2\textsuperscript{+} Me15 melanoma cells and CD3\textsuperscript{+} Jurkat cells. No binding was observed on MDA-MB-468 cells, which are completely negative for both specificities. The cell expression of the respective antigens was assessed using commercial control mAbs (Figure 3b). The mean fluorescence of the anti-TRAIL-R2 domain was higher than that of the tascFv (Figures
**Figure 1.** TascFv phage library construction and screening. 
(a) Cartoon illustrating the construction of the tascFv bsAb phage library. ScFv genes were used to construct the library derived from phages isolated after two cycles of selection of a scFv naïve library against TRAIL-R2. The genes were cloned in a pIT2 vector containing the anti-CD3 scFv (TR66 scFv). (b) Cartoon of the tascFv format: the anti-TRAIL-R2 scFv is connected by a GGGGS short linker to the anti-CD3 scFv. Moieties legend: VH\textsubscript{TRAIL-R2} orange; VL\textsubscript{TRAIL-R2} red; VL\textsubscript{CD3} blue; VH\textsubscript{CD3} grey. (c) Binding of isolated tascFv E7/TR66 is revealed by the anti-Myc-tag Ab (9E10) followed by Alexa Fluor 488 antimouse IgG Ab using FACS analysis. The same preparation was used immediately following production (upper panel) and after three days (bottom panel). Empty peak: negative control; grey peak: tascFv mAb control. Me15 cells were used to assess the TRAIL-R2 binding capacity and Jurkat cells were used to assess the CD3 specificity.
The increased binding capacity of the anti-TRAIL-R2 arm was confirmed by Biacore analysis (Figures 2b and 3c). In this format the bsAb was stable and maintained its binding capacity for up to two years (Suppl. Figure 5a). SEC analysis of the scDb with an elution time of 6.17 minutes (in agreement with the expected molecular weight) demonstrated that the scDb did not aggregate, with about 99.6% the monomeric form (Figure 3d), which was maintained at over 94% after storage for two years at 4°C (Suppl. Figure 5b). The scDb proved stable in human serum for 24 hours as demonstrated by FACS analysis (Suppl. Figure 5c).

Three-dimensional modeling

The three-dimensional model of the two antibodies shows that they have a different structural arrangement (Figure 4a). In both the scDb and tascFv antibodies, the anti-TRAIL-R2 VH and VL domains and the anti-CD3 VH and VL domains display the classical interaction typically observed in the scFv, whereas their relative orientation differs markedly in the two models. In fact, in the scDb, the anti-TRAIL-R2 VH/VL domains interact with the anti-CD3 VH/VL domains through several noncovalent interactions that confer a compact and globular structure on the protein, whilst in the tascFv no interactions at all are observed between the two domains, which behave as independent entities (Figure 4a).

The different three-dimensional structure arrangement confers different structural-dynamic properties, as monitored by molecular dynamics (MD) simulation. The plot of the root mean square deviation from the starting structure as a function of time indicated much larger values for the tascFv than the scDb (Figure 4b), showing that the tascFv structure fluctuates strongly and deviates markedly from the initial configuration, never reaching a stable conformation. The same information was obtained by analyzing the root mean square fluctuation (RMSF) of each residue belonging to the antibody, calculated on the Ca atom of every amino acid residue. The RMSF plot, reported in Figure 4c, indicated that the amino acid residues of the tascFv had four to five times larger fluctuation values than those of the scDb, again confirming that the tascFv antibody had a greater degree of flexibility and instability than the scDb.
Inspection of the simulation frames provides an explanation for the different behavior of the two antibodies. In fact, the presence of noncovalent interactions at the interface between the anti-TRAIL-R2 VH/VL domains and the anti-CD3 VH/VL domains allows the scDb to maintain a compact, globular shape throughout the simulation time. The most important interactions are by two salt bridges that are present over the entire trajectory: a salt bridge between ARG13 of the anti-TRAIL-R2 VH domain and ASP128 of the anti-CD3 VL domain, and a specular salt bridge between ASP384 of the anti-CD3 VH domain and ARG337 of the anti-TRAIL-R2 VL domain, both highlighted in Figure 4a. Moreover, in the scDb the interface between the anti-TRAIL-R2 VH/VL and the anti-CD3 VH/VL domains is characterized by the presence of several hydrogen bonds. Indeed, the plot of the number of the hydrogen bonds as a function of time indicates that on average five hydrogen bonds are detected along the entire trajectory of the scDb simulation (Suppl. Figure 6, black line), whilst an almost complete lack of hydrogen bonds is observed for the tascFv model (Suppl. Figure 6, red line), providing a further explanation for the different stability of the two models.

**scDb biochemical and functional characterization**

The standardized production yield, in laboratory flasks, from about 20 different production attempts of our scDb was in the...
The purity and integrity of E7/UCHT1 were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting; both assays revealed that the scDb migrated as a single band and had the expected molecular mass (Suppl. Fig. 7a), which was confirmed to be 54,415 kD by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) analysis (Suppl. Fig. 7b). To evaluate the binding specificity and kinetics of the anti-TRAIL-R2 scDb component, Biacore analysis was performed. It showed that anti-TRAIL-R2 scDb arm association and dissociation to and from the receptor occurred very fast ($K_a = 2.62 \times 10^5$ M$^{-1}$ sec$^{-1}$; $K_d = 3.88 \times 10^{-2}$ sec$^{-1}$), with an equilibrium dissociation constant of $1.48 \times 10^{-7}$ M (Suppl. Fig. 7c).

Figure 4. 3D model and RMSD and RMSF analyses of scDb and tascFv bispecific antibody simulations.
(a) Cartoon representation of the bispecific antibody in scDb (left) and tascFv format (right). Moieties legend: VH anti-TRAIL-R2: orange; VL anti-TRAIL-R2: red; VL anti-CD3: blue; VH anti-CD3: grey. The circles highlight the two stabilizing salt bridges, represented as sticks. (b) Time-dependent evolution of root mean square deviation (RMSD) values calculated for the scDb (black lines) and tascFv (red lines) antibodies. (c) Per-residue mean square fluctuation (RMSF) values calculated for the scDb (black lines) and tascFv (red lines) antibodies.
Figure 5. In vitro agonistic activity of scDb anti-TRAIL-R2 arm.

Cartoon illustrating the competition between sTRAIL and scDb (a) or no competition between sTRAIL and anti-TRAIL-R2 commercial mAb (b). Sensorgram of the binding competition of the scDb anti-TRAIL-R2 arm with sTRAIL for TRAIL-R2. In the first step sTRAIL was injected to saturate the receptor (c and d, black arrows). Once receptor saturation with sTRAIL was reached, 200 nM scDb (c, grey arrow) or 50 nM anti-TRAIL-R2 commercial mAb (d, grey arrow) was injected. Lack of binding of the scDb on the sTRAIL-saturated receptor indicated that the scDb competed with the ligand for binding to the receptor. By contrast, the anti-TRAIL-R2 commercial mAb that did not compete with the binding site of sTRAIL was able to improve the signal.(a) Cartoon showing the strategy used to increase scDb valence. To thoroughly characterize the potential agonistic activity of the anti-TRAIL-R2 arm, we used the scDb in its monomeric form or cross-linked with the anti-Myc-tag (9E10) mAb alone to artificially dimerize the scDb, or with the 9E10 mAb plus an antimouse antibody (recognizing 9E10) to artificially obtain the tetrameric form of the scDb.(f) sTRAIL-sensitive Me15 cells were treated with increasing concentrations of monovalent scDb or equal doses of dimerized or tetramerized scDb. Cell toxicity was assessed after 24 hours of treatment using the CellTiter-Glo assay. sTRAIL, at equal scDb concentrations, was used as positive control. The results were expressed as the percentage with respect to untreated cells. (g) 0.1, 1 or 10 µg/mL scDb was coated on a plate or added in soluble form to measure the difference in cytotoxic activity of monomeric scDb (S: soluble form) and multimerized scDb (C: coated) on sTRAIL-sensitive Me15 cells. Cell toxicity was assessed after 24 hours of treatment using the CellTiter-Glo assay. 0.1 ng/mL sTRAIL was used as positive control. The results were expressed as the percentage with respect to untreated cells. The graph represents mean ± SD, n = 3.
scDb antitumor activity in vitro

Evaluation of the agonistic activity of the anti-TRAIL-R2 scDb arm

sTRAIL showed affinity for TRAIL-R2 of $1.87 \times 10^{-9}$ M (Suppl. Fig. 8). To evaluate the binding competition of the scDb with sTRAIL, the recombinant receptor was immobilized on a chip and saturated with 1 µM of sTRAIL before the scDb or non-sTRAIL competing antibody was added (Figure 5a-b). No scDb binding on sTRAIL-saturated TRAIL-R2 was observed (Figure 5c), indicating competition for the same binding site, whereas additional binding was evident when a non-sTRAIL competing antibody was added (Figure 5d).

To demonstrate the potential agonistic activity of the anti-TRAIL-R2 arm, we used the scDb in its monomeric form or cross-linked with the anti-Myc-tag (9E10) mAb alone to artificially dimerize the scDb, or with the 9E10 mAb plus an antinouse antibody (recognizing 9E10) to artificially obtain the tetrameric form of the scDb (Figure 5e). The ability of the scDb to induce apoptosis by activating the death-receptor-mediated pathway was evaluated on the sTRAIL-sensitive cell line Me15 at different scDb concentrations (range 0.5–100 µg/mL). The activity of monomeric scDb was about 20% of the growth inhibition capacity at the maximum dose tested, whereas the use of cross-linked scDb, dimerized or tetramerized, led to improved inhibition, reaching a maximum of 65–80% of cell killing (Figure 5f). These levels of inhibition were obtained only with a dose of tetramerized scDb 20-fold higher than that of sTRAIL used as positive control. The agonistic effect increased substantially when the cells were incubated in 96-well plates coated with E7/UCHT1 scDb: 89%, 59% and 9% of Me15 cells were killed when 10, 1 and 0.1 µg/mL of scDb, respectively, was coated directly on plastic (Figure 5g).

Evaluation of the cytotoxicity capacity of scDb-redirected PBLs

To test the ability of scDb-redirected peripheral blood lymphocytes (PBLs) to inhibit tumor target growth, TRAIL-R2+ Me64 and Me15 cells were used. Three scDb concentrations (0.1, 0.5 and 1 µg/mL) and six different E:T (effector to target) ratios were used. The TRAIL-R2+ MDA-MB-468 cell line was used to exclude the possible off-target activity of scDb-retargeted lymphocytes; in this case the assay was performed using only the three higher E:T ratios. The test showed that after 96 hours of treatment the peak target growth inhibition was reached at an E:T ratio of 10:1. However, at this E:T ratio, the activity of PBLs alone was too high on both target cell lines. The best dose/response ratio (minimum dose to obtain the maximum effect with acceptable PBL allogeneic activity) was obtained with 0.5 µg/mL scDb and an E:T ratio of 5:1 (Figure 6a). Furthermore, the treatment result seemed
to correlate with the expression of the target, revealed by FACS analysis, on the surface of target cells (Figure 6b). No effect was observed when the MDA-MB-468 cell line was treated (Figure 6a).

**Discussion**

Here, we described a bsAb able to kill tumor cells by combining the possible induction of apoptosis due to TRAIL-R2 engagement with the ability to induce cytotoxicity by the CD3-mediated redirection of T cells.

To build an anti-TRAIL-R2/anti-CD3 bsAb, we selected a BITE-like conformation (tascFv) which, from the bivalent minibody experience, we thought could be a successful approach. This format is composed of two scFvs linked together by a nonflexible linker and is very compact and small; its compactness allows the formation of an immunological synapse between T cells and tumor cells.\(^{28}\) Due to the instability of the protein molecule, obtaining the bsAb in a tascFv format was not trivial. We tried combining many different anti-CD3 with anti-TRAIL-R2 scFvs isolated by the phage display technique, but the constructed molecules all proved unable to bind both antigens for very long. We therefore decided to construct a phage library expressing tascFvs and to perform the selection against TRAIL-R2 directly with bsAb-displaying phages. This molecular approach has the advantage that the clones, which were formerly in bispecific format, could be selected for their ability to bind the antigen, together with their intrinsic property to remain folded in tascFv format. Such a strategy allows the selection of tascFvs that have a working anti-TRAIL-R2 arm properly folded, and in our case proved superior to the strategy of combining working scFvs. However, in order to be functional bsAbs must co-bind the two specificities, and we found that the isolated clones either did not recognize CD3 at all or lost this ability over time. We also noticed that the well-folded structure of the anti-TRAIL-R2 arm could be influenced by changing the anti-CD3 moieties (humanized UCHT1 instead of TR66), resulting in poor functionality and stability. Computational analysis confirmed the instability of the bsAb in tascFv format, indicating that the short length of the linker region connecting the anti-TRAIL-R2 VL domain to the anti-CD3 VH domain hinders the establishment of noncovalent interactions and consequently the formation of a globular structure. Being composed of these variable regions, the tascFv is indeed characterized by a large degree of flexibility and the protein is unable to find a stable conformation, explaining its instability and propensity to aggregate.

As the tascFv was found to be unstable, we decided to change to a diabody-derived format. The diabody is a small bsAb similar in size to a tascFv, and for this reason has been widely used in T-cell retargeting strategies.\(^{29}\) However, since it is composed of two separate polypeptide chains, its use has been limited because the molecule proved unstable in vivo. It has been described that a stabilized diabody format, i.e., the dual-affinity retargeting molecule (DART; MacroGenics), solved this problem, showing both superior antitumor activity and improved stability with respect to the BITE constructed with the same variable moieties.\(^{30,31}\) The scDb we developed confirmed this tendency via another diabody-derived format that is stabilized by a linker between the two separate polypeptide chains,\(^{29}\) thereby providing another example of how the diabody format can be significantly better behaved. Since a molecule able to specifically bind both antigens and maintain stability over time was obtained merely by changing the anti-CD3 moieties and reshaping the bsAb into a scDb format, the selection strategy we describe here could be implemented in the future. For example it will be possible to prepare a bsAb phage library containing clones in both tascFv and scDb format. In addition, library clones may have different antibody moieties also for the second arm (in our case, the anti-CD3 antibody).

The scDb also showed promising anticancer activity during cytotoxicity experiments and, importantly, the cytotoxic activity was induced only when both arms were engaged with their target antigens.

Biacore experiments demonstrated that sTRAIL was a competitor of the TRAIL-R2/CD3 scDb binding site on TRAIL-R2, suggesting that the scDb could itself be an apoptosis inducer. However, as expected given its monomeric form, the scDb exerted only weak agonistic activity against TRAIL-sensitive cell lines compared to sTRAIL, which is consistent with the hypothesis that receptor trimerization is necessary for efficient death signaling.\(^5\) Indeed, by mimicking the trimerization ability of sTRAIL with an artificial method to multimerize the scDb, we showed that the agonistic activity of tetramerized scDb was superior to that of the dimeric and monomeric forms, albeit never reaching the level of sTRAIL activity. This result could be explained by the different binding strength of the two molecules to the receptor; in fact, literature data indicated that higher affinity influenced the triggering of death receptors, leading to increased apoptotic activity.\(^{32,33}\) Our measurements using Biacore defined the binding strength of trimeric sTRAIL to TRAIL-R2 in the nanomolar range (1.87 × 10\(^{-9}\) M), which is about 100 times stronger than that of monomeric scDb (1.48 × 10\(^{-7}\) M). We therefore speculate that the lower affinity of the scDb of the anti-TRAIL-R2 arm compared with that of sTRAIL could account for its lower agonistic activity even when it was used as an artificial tetramer.

When the scDb was multimerized directly on plastic, mimicking the T-cell-mediated multimerization process that we speculate would occur in vivo, its agonistic activity increased considerably. This result indicates that the bsAb is able to induce apoptosis when multimerization occurs, and hence demonstrates the dual mechanism of action of the reagent. In fact, in addition to this activity, it could efficiently retarget T cells, which provoked the killing of tumor cells expressing TRAIL-R2 on the plasma membrane. The bsAb-mediated retargeting of T cells resulted in growth inhibition of all treated TRAIL-R2\(^+\) tumor cell lines. This occurred also when the tumor cells were resistant to agonistic TRAIL-mediated apoptosis, as happened with Me64 melanoma cells.

The bsAb that we constructed, which retained its potency and absence of aggregates even after 30 months from production, holds promise as a novel therapeutic agent. We do, however, acknowledge the potential safety issues inherent to
an anti-TRAIL-R2/anti-CD3 bsAb and are therefore planning further studies to investigate and address these issues.

Materials and methods

Cell lines

Me41, Me15 and Me64 human melanoma cells were established at our institute by Dr Anichini’s group and cultured as described.34 MDA-MB-468 (triple-negative breast cancer) and Jurkat (non-Hodgkin lymphoma) cell lines were purchased from American Type Culture Collection (ATCC), and SU-DHL-4 and HDLM-2 non-Hodgkin lymphoma cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ); cells were grown in the media indicated by ATCC or DSMZ with 10% fetal bovine serum (FBS) and 2 mM L-glutamine in a humidified atmosphere with 5% CO₂ at 37°C. The hybridoma producing the anti-Myc-tag mAb 9E10 (CRL-1729) was purchased from ATCC; the hybridoma producing the anti-CD3 mAb TR66 was kindly provided by Prof. A. Lanzavecchia.26

PBLs were isolated from healthy-donoruffy coats kindly provided by the Immunohematology and Transfusional Medicine Unit of our institute. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat according to a standard Ficoll density gradient centrifugation protocol (Ficoll-Paque PLUS, GE Healthcare). Isolated PBMCs were resuspended in RPMI 1640 complete medium and the cell number was adjusted to 1 × 10⁶/mL. The flask was laid down for 30 minutes at 37°C in a 5% CO₂ humidified incubator to allow the monocytes to attach. PBLs present in the supernatant were removed and put in another flask. PBLs were cultured in RPMI 1640 with 5% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 mM non-essential amino acids, 0.1 mg/mL gentamycin and 1% vitamin complex and cultured at 37°C with CO₂.

Cells were lysed using RIPA buffer (50 mM TRIS-HCl PH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.2% SDS) plus a protease inhibitor cocktail (Roche).

Selection of anti-TRAIL-R2 scFvs from a phage display library

A premade human scFv phage display library35 was used and three rounds of selection (panning) were performed on immobilized antigen. The human recombinant TRAIL-R2 (R&D Systems) was coated (in phosphate-buffered saline (PBS)) on Immunotubes (Nalge Nunc International USA), whereas the human recombinant TRAIL-R2 derived from cell lysate of the Me15 cell line was immobilized on antiumous functionalized magnetic beads (Dynabeads, Dynal Invitrogen) previously coated with anti-TRAIL-R2 antibody (R&D Systems, Cat No: MAB6311). Pannings were performed as described.36 After each cycle, colonies were counted and screened for the presence of the correct insert by PCR. The PCR reaction was set up containing a single picked colony as the template, PCR nucleotide mix (10 mM each), 1x Green GoTaq® reaction buffer, 10 mM of LMB3 (CAGGAAACAGCTATGAC) and PHEN SEQ (CTATGCAGCCCGCATC) primers, 5 U/µL GoTaq® DNA polymerase (Promega), and Milli-Q water to 50 µL. Single-clone phage ELISA was performed to control the binding of the selected clones as described.36 Briefly, ELISA plates were coated overnight with 10 µg/mL TRAIL-R2 or with bovine serum albumin (unrelated protein) as negative control. Phage-containing supernatants were transferred to the ELISA plates and bound phages were detected by HRP/anti-M13 monoclonal conjugate (GE Healthcare) and TMB (Sigma). ELISA-positive phage clones were tested on SU-DHL-4 and HDLM-2 cells by FACS using a BD FACS Calibur flow cytometer (BD Biosciences). The genes of the isolated scFvs were sequenced and Kabat numbering was used to identify the three CDRs of the heavy-chain and light-chain variable regions (VH and VL).

Construction of a bsAb phage library in tascFv format

A tascFv gene cassette (VHTRAIL-R2-flexible linker-VLTRAIL-R2-flexible linker-VHCD3-flexible linker-VLCD3) – see cartoon in Figure 1a-b) was designed and synthesized by GeneArt (Thermo Fisher Scientific). The cassette was originally designed containing clone 8 from the above library selection and the variable domains of the anti-CD3 TR66. The sequences of the variable domains of the TR66 mAb were obtained from the TR66 hybridoma provided by Prof. Lanzavecchia. For construction of the library we kept the anti-CD3 TR66 variable domains fixed and cloned as anti-TRAIL-R2 the pool of VH and VL that we selected after the second panning against the native protein, amplified by PCR, using a set of primers specific for all VH and Jk/Jγ germ lines.37

The library also included the variable domains of other anti-TRAIL-R2 antibodies described in the literature: drozitumab,38 the clones 16E2 (patent no. US 8,409,570 B2) and 14A2,39 previously synthesized by GeneArt – Thermo Fisher Scientific) as well as the six clones isolated from the selection above.

The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega), cut with NcoI and NheI, and cloned in the gene cassette to generate the bsAbs in tascFv format. The presence of the right insert was checked using PCR as described above. To test the variability of the library, fingerprint analysis was performed: PCR products were purified and digested overnight with BstNI restriction enzyme and 29 colonies were sequenced (Metabion).

Randomly picked colonies derived from bacteria infected with the bsAb library phages were tested in single-clone phage ELISA as described.36 ELISA-positive phage clones were tested on Me15 and Jurkat cells by FACS and a clone called E7/TR66 was isolated.

bsAb reshaping and optimization

Human/humanized bsAbs (E7/UCHT1) were constructed using the variable domains of the E7 clone and the variable domain of the anti-CD3 humanized hybridoma UCHT1.40 The chosen/evaluated formats were single-chain diabody (scDb – VHTRAIL-R2-short linker-VLCD3-flexible linker-VHCD3-short linker-VLTRAIL-R2) and tandem single-chain antibody (tascFv – VHTRAIL-R2-flexible linker-VLTRAIL-R2-short linker-VHCD3-flexible linker-VLCD3): the genes were designed and then synthesized by GeneArt.

BsAb cassettes were cloned by unique restriction sites into a pIT2 vector that allows the insertion of a hexahistidine tag...
and a Myc tag at the bsAb C-terminus and the production of the bsAb in bacteria.

**Soluble bsAb expression**

The tascFvs and scDb were produced in the *E. coli* strain HB2151 [narl thi-1 ara lac-proAB [F' proAB+ lacIq lacZ (M15)]] and purified from periplasmic preparations using HiTrap Protein L or HisTrap FF crude chromatography columns (GE Healthcare) as described.41

**Three-dimensional modeling of the antibodies**

The three-dimensional structures of the dimers constituting the scDb and tascFv were modeled using the PIGSPro web server,42 a tool for the automatic modeling of immunoglobulin variable domains based on the canonical structure method. The obtained dimeric structures were docked using the High Ambiguity Driven protein-protein DOCKing program (HADDOCK),43 imposing interaction restraints to take into account the length of the linkers engineered to connect the monomers. HADDOCK carries out information-driven flexible docking of biomolecular complexes, using information from identified or predicted protein interfaces.43 The representative structures, obtained from the clustering of the docking results, were then subjected to a second modeling procedure aimed at shaping the linkers between the monomer chains, using the Modeller tool implemented in the UCSF Chimera program.44

**Classical molecular dynamics simulations**

The structural models were optimized through classical MD simulations. Hydrogen atoms were added using the Amber 16.0 tLeap tool45 and the systems were solvated with TIP3P water in a cubic box having 12 Å between the protein surface and the box boundary. Each system was electrostatically neutralized by addition of 5 chloride counterions. The GPU-enabled version of the PMEMD module of the Amber 16.0 package with the “ff14SB” force field was used for all MD simulations. Each system was energy-minimized for 2500 steps of the steepest descent algorithm followed by 500 steps of the conjugate gradient algorithm to eliminate close van der Waals contacts or solve structural issues induced by the modeling procedure. The systems were gradually heated from 0 to 300°K in 1 ns, followed by constant pressure equilibration at 300°K for 1 ns. Following this phase, 50-ns production MD runs were obtained with periodic boundary conditions in the NPT ensemble, at a constant temperature of 300°K using a Langevin thermostat and a constant pressure of 1 atm with isotropic molecule-based scaling. Bond lengths involving hydrogen were constrained using the SHAKE algorithm.46 Long-range electrostatic forces were calculated using the particle-mesh Ewald (PME) method.47 All analyses were performed using the Gromacs 2016.1 48 integrated tools.

**Flow cytometry experiments**

For detection of the bsAb recognition capacity, FACS analysis was performed as described.41 Briefly, 2 × 10^5 cells were incubated with primary antibody in PBS containing 1% of saturating FBS for 30 minutes at 4°C. TascFv or scDb was detected using 9E10 antibody and antimouse IgG (H + L specific) 488-labeled antibody (Thermo Fisher Scientific, Cat No: A-11,029). Anti-TRAIL-R2 antibody (R&D Systems – Cat No: MAB6311) and mouse anti-CD3 (TR66 mAb) plus antimouse IgG (H + L specific) 488-labeled (Thermo Fisher Scientific, Cat No: A-11,029) antibodies were used as positive controls. Fluorescence labeling was measured using a BD FACSCalibur system. Data were analyzed with the FlowJo software v. 10.1 (Tree Star Inc).

**Surface plasmon resonance (SPR) analysis**

SPR experiments were performed using Biacore 2000 (GE Healthcare). Standard N-ethyl-N-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide coupling was used to covalently immobilize recombinant TRAIL-R2 (R&D Systems) on a CM5 sensor chip (GE Healthcare). Spontaneous TRAIL-R2 dissociation was recorded for at least 600 seconds. A flow rate of 30 µL/min at 25°C was kept during all the experiments.

Different concentrations of tascFv/scDb/sTRAIL freshly diluted in HEPES-buffered saline, 3 mM EDTA and 0.05% surfactant P20 (GE Healthcare) were used to assay the binding and set up the system. Kinetic analyses were performed at concentrations ranging from 400 to 25 nM of scDb. The data obtained were analyzed with the BiAevaluation software 3.2 (global fitting) assuming a 1:1 Langmuir binding model. Competition assay for the receptor between sTRAIL and the scDb was performed using 1 µM of sTRAIL to saturate all receptors coated on the chip. Once saturation had occurred, 400 nM scDb was injected on the sTRAIL-saturated TRAIL-R2.

**Biochemical characterization and integrity of scDb**

The size and integrity of the bsAb were analyzed by SDS-PAGE and Western blotting. Samples containing 1 µg of purified scDb from two different batches of purification were used to test the integrity of the molecule as described.41 After the run, the protein bands on the gel were stained with Coomassie solution (0.1% Coomassie Brilliant Blue R-250, 30% methanol and 10% glacial acetic acid) or transferred onto a 0.45-µm nitrocellulose membrane (GE Healthcare). 9E10 antibody and antimouse IgG HRP (GE Healthcare, Cat No: GENA931-1ML) were incubated in two steps for 30 min-

**SEC was performed on a Superdex 200 5/150 GL (range separation: 600 kD-10 kD; GE Healthcare) as described.**41 The scDb size was analyzed using SELDI-TOF MS. SELEDI-TOF MS was performed following sample preparation, MS acquisition and data analysis as described.49 Assessment of the scDb stability in human serum was performed by incubating 20 µg/mL scDb in 80% human...
serum at 37°C. After 4, 8 and 24 hours the binding capacity of the scDb was assessed by FACS analysis.

**In vitro cytotoxicity studies**

Cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using PBLs as effectors and a panel of different cancer cell lines as targets. To avoid distortions due to the different cytotoxic power of PBLs, high-CD69-expressing PBL batches were considered just activated and excluded. Twelve thousand tumor cells/well were plated in 96-well flat-bottom plates with the appropriate medium and incubated overnight to allow their adherence. The assay was performed using a titration of the E:T ratio, ranging from 10:1 to 0.15:1, and the scDb concentration, ranging from 1 to 0.1 µg/mL. 100 ng/mL sTRAIL was used to assess the sensitivity of different cell lines. After 96 hours, the supernatant was removed and the wells were gently washed three times with PBS to remove non-adherent PBLs. In each well 100 µL fresh medium containing 0.5 mg/mL MTT was added. After three hours at 37°C, the supernatant and 150 µL of MTT solvent (isopropanol + 4 mM HCl + 0.1% NP40) was added. Absorbance at 590 nm (620-nm reference filter) was detected using a Bio-Rad 550 microplate reader. The percent cytotoxicity was calculated as the percentage with respect to untreated cells.

To test the agonistic activity of the scDb anti-TRAIL-R2 arm, 2 × 10⁴ Me15 cells were incubated with monomeric/dimerized/tetramerized or multimerized on plastic scDb at different concentrations (range 0.5–100 µg/mL). After 24 hours, tumor growth inhibition was assessed using the CellTiter-Glo viability assay (Promega) according to the manufacturer’s instructions.

For dimerization, the scDb was incubated with a half molar concentration of anti-Myc-tag for one hour at room temperature; for tetramerization, one-fourth molar concentration of anti-Myc-tag for one hour at room temperature; for tetramerization, one-fourth molar concentration (with respect to that of the scDb) of antimouse mAb (Sigma Aldrich) was added to the scDb/anti-Myc-tag mixture. For scDb multimerization on plastic, 0.1, 1 and 10 µg/mL of bsAb was added in a 96-well plate (Nunc) and incubated at 4°C overnight. The day after, the supernatant was discarded and cells were added.

**Statistical analysis**

The GraphPad Prism software was used to generate graphs and perform statistical analysis. One-way Anova followed by multiple comparison testing was carried out to determine the significance of differences between treatments.

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No potential conflict of interest was reported by the authors.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ATCC         | American Type Culture Collection |
| BiTE         | bispecific T-cell engager |
| bsAb         | bispecific antibody |
| DART         | dual-affinity retargeting |
| DMSZ         | German Collection of Microorganisms and Cell Cultures |
| E:T          | effector to target |
| FACS         | fluorescence-activated cell sorting |
| FBS          | fetal bovine serum |
| MD           | molecular dynamics |
| MTT          | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PBL          | peripheral blood lymphocyte |
| PBMC         | peripheral blood mononuclear cell |
| PBS          | phosphate-buffered saline |
| PME          | particle-mesh Ewald |
| RMSD         | root mean square deviation |
| RMSF         | root mean square fluctuation |
| scDb         | single-chain diabody |
| scFv         | single-chain antibody variable fragment |
| SDS-PAGE     | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SEC          | size exclusion chromatography |
| SELDI-TOF MS | surface-enhanced laser desorption/ionization time-of-flight mass spectrometry |
| SPR          | surface plasmon resonance |
| tascFv       | tandem scFv |
| TNF          | tumor necrosis factor |
| TRAIL        | TNF-related apoptosis-inducing ligand |
| VH           | heavy-chain variable |
| VL           | light-chain variable |

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