Advancing targeted co-stimulation with antibody-fusion proteins by introducing TNF superfamily members in a single-chain format

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ABSTRACT

Co-stimulation via receptors of the tumor necrosis factor superfamily (TNFSF) emerges as promising strategy to support antitumor immune responses. Targeted strategies with antibody-fusion proteins composed of a tumor-directed antibody part and the extracellular domain of a co-stimulatory ligand of the TNFSF constitute an attractive option to focus the co-stimulatory activity to the tumor site. Since TNFSF members intrinsically form functional units of non-covalently linked homotrimers, the protein engineering of suitable antibody-fusion proteins is challenging. Aiming for molecules of simple and stable configuration, we used TNFSF ligands in a single-chain format (scTNFSF), i.e., three units of the ectodomain connected by polypeptide linkers, folding into an intramolecular trimer. By fusing tumor-directed scFv antibody fragments directed against EpCAM or FAP to co-stimulatory scTNFSF molecules (sc4-1BLB, scOX40L, scGITRL or scLIGHT), a set of monomeric scFv-scTNFSF fusion proteins was generated. In comparison to the scFv-TNFSF format, defined by intermolecular homotrimerization via the TNFSF part, scFv-scTNFSF showed equal or enhanced co-stimulatory activity despite reduced avidity in antibody binding. In addition, enhanced serum stability and improved bioavailability in mice were observed. We show that the scFv-scTNFSF format can be applied to various members of the TNFSF, presenting targeting-dependent co-stimulatory activity. Hence, this format exhibits favorable properties that make it a promising choice for further therapeutic fusion protein development.

Abbreviations: EpCAM, epithelial cell adhesion molecule; ED-A, fibronectin extradomain A; FAP, fibroblast activation protein; GITRL, glucocorticoid-induced tumor necrosis factor receptor (GITR) ligand; LIGHT, homologous to lymphoid-tissue inducible proteins, shows inducible expression and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed by T lymphocytes; mAb, monoclonal antibody; scFv, single-chain fragment variable; TNF, tumor necrosis factor; TNFSF, tumor necrosis factor superfamily; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Introduction

Cancer immunotherapy has become a fast evolving field, with particular attention focusing on the modulation of the immune response. Thus, antagonistic antibodies directed against checkpoint inhibitors, e.g., CTLA-4 and PD-1, have been successfully introduced in the clinic, but also agonistic antibodies targeting co-stimulatory receptors have become of increasing interest for therapy.1 Co-stimulatory members of the TNF-receptor superfamily (TNFRSF) seem especially suitable, since those ligand–receptor interactions are defined by a spatio-temporal expression on different immune cell subtypes and play a crucial role in the complex network of immune regulation.2 Thus, co-stimulation via 4-1BB and OX40 was shown to enhance clonal expansion, survival, cytokine release and effector functions of immune cells, supporting mainly the shown to enhance clonal expansion, survival, cytokine release and play a crucial role in the complex network of immune regulation.2 Thus, co-stimulation via 4-1BB and OX40 was shown to enhance clonal expansion, survival, cytokine release and effector functions of immune cells, supporting mainly 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shedded form, is significantly reduced, but can be effectively restored either by oligomerization\textsuperscript{15,16} or antibody-mediated presentation on the tumor cell surface, mimicking the trans-membrane form of the ligand.\textsuperscript{16-18} From a structural point of view, TNFSF members are homotrimeric molecules formed by non-covalent assembly of three ectodomains.\textsuperscript{19} This property strongly influences the format adopted by the corresponding antibody-fusion proteins, resulting in rather bulky molecules. Thus, fusion of the extracellular domain of a TNFSF ligand to a scFv antibody leads to a homotrimeric molecule composed of the functional ligand trimer and three antibody units (scFv-TNFSF).\textsuperscript{17,18} In order to generate antibody-fusion proteins of smaller size and simpler configuration, we have introduced the co-stimulatory ligand in the single-chain format (scFv-scTNFSF), i.e., genetically connecting three extracellular domain units by linkers on the same polypeptide chain. We showed that the single-chain format is applicable to different co-stimulatory members of the TNFSF (4-1BB, OX40L, GITRL, LIGHT) and that the resulting scFv-scTNFSFs are functional. Furthermore, in comparison to the original scFv-TNFSF format, the scFv-scTNFSF appeared to be more stable, exhibiting also improved PK properties, thus presenting a promising option for further molecule development.

Results

Antibody-TNFSF ligand fusion proteins composed of a tumor-directed antibody moiety (scFv) and the extracellular domain of a co-stimulatory TNFSF member (ECD\textsubscript{TNFSF}) were generated in a homotrimeric and a monomeric format, respectively (Fig. 1A). Fusion of the scFv to a single ECD\textsubscript{TNFSF} led to ligand-mediated intermolecular trimerization and consequently to a homotrimeric molecule (scFv-TNFSF). On the other hand, fusion of the scFv with a single-chain ECD\textsubscript{TNFSF}, i.e., three consecutive ECD\textsubscript{TNFSF} in a row, led to intramolecular trimerization of the ligand, confining the functional ligand into a monomeric molecule (scFv-scTNFSF). Thus, fusion proteins with an antibody:ligand ratio of 3:1 (scFv-TNFSF) and 1:1 (scFv-scTNFSF) were designed. Furthermore, fusion proteins in both formats targeting the same co-stimulatory ligand (4-1BB) to different tumor associated antigens (FAP, EpCAM) and fusion proteins targeting different co-stimulatory ligands (4-1BB, OX40L) to the same tumor antigen (EpCAM) were generated. EpCAM-directed fusion proteins were designed with a Flag-tag at the N-terminus of the scFv, whereas FAP-directed fusion proteins were provided with a hexahistidyl-tag integrated in the linker connecting the antibody with the co-stimulatory ligand. The length of the glycine-serine-based linker connecting the antibody with the ligand was between 14 and 16 amino acids in all fusion proteins, whereas the glycine-serine-linker connecting the ECDs in the sc4-1BBL and scOX40L were of 20 and 7 amino acids, respectively. Here, the distance between N- and C-terminus of adjacent subunits in their trimeric structure was deduced from crystal structure data available.\textsuperscript{20,21} Fusion proteins were produced either in stably transfected HEK293 cells (1–5 mg/L) or transiently transfected HEK293-6E cells (5–10 mg/L), followed by affinity chromatography purification. SDS-PAGE analysis of the

![Figure 1](image-url)
purified proteins revealed single bands corresponding to the monomer of the scFv-TNFSF and scFv-scTNFSF, respectively (Fig. 1B). Size exclusion chromatography of the native fusion proteins in the scFv-TNFSF format showed for scFvFAP-4-1BBL and scFvEpCAM-OX40L a rather homogeneous peak consistent with a trimeric structure, while scFvEpCAM-4-1BBL presented as a mixture of trimers and dimers. All proteins in the scFv-scTNFSF format showed a dominant peak for the correctly assembled monomer and a small fraction of dimers (Fig. 1C). Thus, for the antibody-fusion proteins with 4-1BBL and OX40L in both formats correct expression and assembly without or only minor aggregation was demonstrated.

Next, the functionality of the antibody part of the fusion proteins was demonstrated by binding studies to tumor antigen-expressing cells by flow cytometry (Fig. 2A, Fig. S1). Here, antibody-mediated binding was shown for all fusion proteins. Nevertheless, fusion of the scFvEpCAM to the co-stimulatory ligands in the single-chain format considerably reduced the antibody binding capacity of scFvEpCAM-sc4-1BBL (EC50 92 ± 16 nM) and scFvEpCAM-scOX40L (EC50 344 ± 180 nM) in comparison to the scFvEpCAM (EC50 3.9 ± 0.7 nM) (Fig. 2A). For the fusion proteins in the homotrimeric format, i.e., endowed with three antibody moieties per molecule, an increased binding was observed for scFvEpCAM-4-1BBL (EC50 15 ± 2 nM) and scFvEpCAM-OX40L (EC50 4.2 ± 1.7 nM), however, not exceeding the binding capacity of the single scFvEpCAM molecule. This effect was more pronounced for the fusion proteins with OX40L than for those with 4-1BBL. In the case of the FAP-directed fusion proteins, the binding capacity of the antibody moiety was not impaired by fusion to the ligand. Here, scFvFAP (EC50 5.1 ± 0.5 nM) and scFvFAP-sc4-1BBL (EC50 7.7 ± 0.9 nM) showed similar EC50 values that were strongly exceeded by the binding capacity of the trimeric scFvFAP-4-1BBL (EC50 0.12 ± 0.09 nM).

Furthermore, we analyzed the binding properties of the ligand part of the fusion protein to corresponding receptors in ELISA (Fig. 2B). Similar binding capacity to recombinant 4-1BB-Fc was shown for scFvEpCAM-4-1BBL (EC50 2.1 ± 0.3 nM), scFvEpCAM-sc4-1BBL (EC50 2.7 ± 0.3 nM), 4-1BBL (EC50 2.2 ± 0.8 nM) and sc4-1BBL (EC50 1.7 ± 0.4 nM) (Fig. 2B). Likewise, in the case of OX40L (EC50 1.4 ± 0.4 nM) and scOX40L (EC50 1.3 ± 0.3 nM), conversion into the single-chain format did not affect the receptor binding properties. Also, fusion to scFvEpCAM had no influence on receptor binding for scFvEpCAM-

Figure 2. Binding properties of the fusion proteins. (A) Antibody-mediated binding was analyzed by flow cytometry. Bewo (EpCAM+) and HT1080-FAP were incubated with the respective fusion proteins. EpCAM-targeting fusion proteins and TNFSF ligands were detected via anti-Flag-PE mAb and FAP-targeting fusion proteins via anti-4-1BBL-PE or anti-His-PE mAb. (B) TNFSF ligand-mediated binding to recombinant TNFRSF-Fc was analyzed by ELISA. 4-1BB-Fc or OX40-Fc was coated and bound fusion protein detected via anti-Flag-HRP mAb. (C) TNFSF ligand-mediated receptor binding on cells. Stable TNFSF receptor expressing transfectants (HT1080-4-1BB, HT1080-OX40) were incubated with the corresponding fusion proteins and receptor activation measured via IL-8 release by ELISA. Graphics show mean ± SD, n = 3.
scOX40L (EC_{50} 1.1 \pm 0.3 \text{nM}), but showed a slight improvement in the case of scFvEpCAM-OX40L (EC_{50} 0.2 \pm 0.1 \text{nM}) (Fig. 2B). In addition, no significant difference in receptor binding capacity on HT1080-OX40 cells was detected comparing OX40L (1.5 \pm 1.2 \text{nM}) and scOX40L (1.5 \pm 0.4 \text{nM}) or scFvEpCAM-OX40L (0.9 \pm 0.3 \text{nM}) and scFvEpCAM-scOX40L (1.7 \pm 0.8 \text{nM}) (Fig. S2A). Thus, for 4-1BBL and OX40L fusion proteins, ligand–receptor binding capacity remained essentially conserved in the single-chain format and was not disturbed by the fusion to the antibody part.

In addition, we investigated if ligand binding leads to receptor activation. Therefore, fusion proteins were incubated on HT1080 cells stably transfected with 4-1BB or OX40, respectively, and receptor activation was measured via NF-κB induced IL-8 release (Fig. 2C). In solution, scFvEpCAM-4-1BBL (EC_{50} 3.6 \pm 0.7 \text{nM}) and scFvEpCAM-sc4-1BBL (EC_{50} 3.2 \pm 3.2 \text{nM}) were not able to induce IL-8 release. Likewise, scFvEpCAM-OX40L (EC_{50} 0.2 \pm 0.02 \text{nM}), scFvEpCAM-scOX40L (EC_{50} 0.6 \pm 0.05 \text{nM}) and scOX40L (EC_{50} 0.4 \pm 0.2 \text{nM}) did not induce receptor activation. Thus, the untargeted antibody-fusion proteins in both formats clearly showed receptor activation potential, while the corresponding recombinant co-stimulatory ligands were less stimulatory or not stimulatory at all. In particular, the antibody-fusion proteins with the ligand in the single-chain format were highly effective inducing IL-8 release.

Bi-functionality in terms of simultaneous binding capacity of the antibody-fusion proteins to target cells and the corresponding recombinant TNFSFR (4-1BB-Fc / OX40-Fc) was confirmed by flow cytometry (Fig. S2B). Next, we investigated how the antigen binding properties of the bi-functional antibody-fusion proteins would translate into targeting-mediated co-stimulatory activity. Therefore, co-culture assays with tumor target cells and human PBMCs in presence of suboptimal concentration of cross-linked anti-CD3 mAb and the corresponding scFv-TNFSF/scFv-scTNFSF in co-culture with PBMCs were performed. Co-stimulatory activity was measured in terms of enhanced IFNγ release and increased T cell proliferation (Fig. 3A, Fig. 3B). Antibody-fusion proteins of both formats (scFv-TNFSF and scFv-scTNFSF) targeting OX40L to EpCAM and 4-1BBL to EpCAM or FAP were analyzed. All of them showed to be co-stimulatory active, irrespective of their antibody specificity, the type and format of the ligand. In the proliferation assay, similar activity was observed for scFvEpCAM-4-1BBL and scFvEpCAM-sc4-1BBL as well as for scFvEpCAM-OX40L and scFvEpCAM-scOX40L, showing equivalent effects for both formats. In contrast, in the IFNγ release assay the single-chain format was more potent at lower concentrations, especially for the fusion protein with sc4-1BBL. This observation was confirmed by the FAP-targeting variants, where scFvFAP-sc4-1BBL was also co-stimulatory superior to scFvFAP-4-1BBL. In this case, the effect was not limited to the IFNγ release and manifested also in the proliferation assay. In accordance with the co-stimulatory nature of the ligands, in absence of TCR complex-stimulation with cross-linked anti-CD3 mAb, the antibody-fusion proteins did not induce T cell activation by themselves. Furthermore, it was demonstrated that the co-stimulatory activity was strictly ligand-dependent and could be blocked by the addition of the respective recombinant receptor (Fig. 4A). Additionally, we assessed the co-stimulatory potential of the untargeted antibody-fusion proteins (Fig. 4B). In absence of target cells, the effect of scFvEpCAM-OX40L and scFvEpCAM-scOX40L was severely diminished and stimulation of PBMCs only minimally enhanced. Similar effects were observed for scFvFAP-4-1BBL and to certain degree for scFvFAP-sc4-1BBL, but not for scFvEpCAM-4-1BBL and scFvEpCAM-sc4-1BBL.
rather similar for both formats under targeted and non-targeted conditions. Hence, antibody-mediated surface display of 4-1BBL and OX40L has a positive impact on the ligand activity in both formats. Nevertheless, target-dependent activity is strongly influenced by the particular fusion protein configuration.

Furthermore, we compared the serum stability of the antibody-fusion protein formats (Fig. 5A). Here, molecular integrity and ligand–receptor binding for scFvEpCAM-sc4-1BBL and scFvEpCAM-scOX40L was conserved up to 7 d in 50% serum at 37°C, while stability of scFvEpCAM-4-1BBL and scFvEpCAM-OX40L were approx. 25% decreased after 1 day. Thus, the single-chain format seems to confer higher stability to the fusion protein than the homotrimeric format. In addition, pharmacokinetic studies were performed in immunocompetent CD1 mice (Fig. 5B). Since there is no cross-reactivity between the human antibody/ligands and the corresponding mouse antigens/receptors, we focused here on aspects of size and stability of the homotrimeric scFvEpCAM-4-1BBL and scFvEpCAM-OX40L and monomeric single-chain scFvEpCAM-sc4-1BBL and scFvEpCAM-scOX40L molecules. Here, the initial half-life ($t_{1/2}$) was longer for scFvEpCAM-sc4-1BBL than for scFvEpCAM-4-1BBL and the AUC for scFvEpCAM-sc4-1BBL resulted in an approximately 3-fold enhancement in comparison to
scFvEpCAM-4-1BBL, pointing to a favorable bioavailability of the monomeric single-chain antibody-fusion protein format. In contrast, terminal half-life ($t_{1/2b}$) was considerably longer for scFvEpCAM-scOX40L than for scFvEpCAM-OX40L, while initial half-life was short in both cases and AUC values rather low (Table 1). In addition, thermal stability of the proteins was measured by dynamic light scattering. No difference in the melting point was observed for 4-1BBL, sc4-1BBL, OX40L and scOX40L (49°C). Similar results were obtained for scFvEpCAM-OX40L and scFvEpCAM-scOX40L (50°C) (Fig. S4).

Finally, the design of antibody-fusion proteins with co-stimulatory ligands of the TNFSF in the single-chain format was extended to the TNFSF members GITRL and LIGHT. Thus, in addition to the FAP-directed scFvFAP-sc4-1BBL described above and scFvFAP-scOX40L, also scFvFAP-scGITRL and scFvFAP-scLIGHT were generated. For the latter, a glycine-serine-linker of 10 amino acids and a single glycine were chosen to connect the ECDs in the scGITRL and scLIGHT, respectively. SDS-PAGE analysis of the purified proteins revealed the corresponding band of the respective monomer (Fig. 6A) and size exclusion chromatography confirmed the presence of the monomer in a main peak (Fig. 6B). Also here, a minor peak of a dimeric fraction was detected for scFvFAP-scOX40L and scFvFAP-scGITRL. Bi-functionality of the fusion proteins was shown by flow cytometry, where antibody-mediated binding of the fusion protein to target cells was detected via ligand–receptor interaction, using recombinant receptor-Fc followed by anti-Fc fluorescence-conjugated antibodies. Thus, simultaneous binding of the fusion protein to the tumor target on the one hand and recombinant co-stimulatory receptor on the other hand was demonstrated (Fig. 6C). Co-stimulatory activity of the fusion proteins was confirmed in co-culture assays of target cells and PBMCs in presence of suboptimal concentrations of cross-linked anti-CD3 monoclonal antibody (Fig. 6D).

| Fusion protein | MW (kDa) | Sr (nm) | $t_{1/2a}$ (h) | $t_{1/2b}$ (h) | AUC (h·μg/mL) |
|---------------|---------|--------|---------------|---------------|--------------|
| scFvEpCAM-4-1BBL | 145     | 4.81   | 1.28 ± 0.39   | 12.66 ± 0.44  | 66.27 ± 9.45 |
| scFvEpCAM-sc4-1BBL | 90      | 4.05   | 4.26 ± 2.58   | 13.34 ± 0.66  | 189.98 ± 23.23 |
| scFvEpCAM-OX40L | 132     | 5.45   | 0.52 ± 0.08   | 2.26 ± 1.45   | 8.15 ± 2.23  |
| scFvEpCAM-scOX40L | 76      | 4.8    | 0.68 ± 0.06   | 16.29 ± 0.61  | 10.20 ± 2.97 |

Figure 6. Characterization of further FAP-directed scFv-scTNFSF fusion proteins with the ligands OX40L, LIGHT and GITRL. (A) SDS-PAGE (12%) analysis under non-reducing (NR) conditions. (1) scFvFAP-scOX40L; (2) scFvFAP-scLIGHT; (3) scFvFAP-scGITRL. Coomassie staining. (B) Size exclusion chromatography on Yarra SEC-3000, mobile phase 0.1 M NaH2PO4/NaH2PO4, 0.1 M Na2SO4, pH 6.7 at 0.5 mL/min. (C) Bi-functional binding analysis by flow cytometry. Fusion proteins (40 nM) were incubated either on FAP+ (B16-FAP) or FAP– (B16) cells. Antibody-mediated binding was detected via co-stimulatory ligand–receptor interaction, using recombinant OX40-Fc, HVEM-Fc or GITR-Fc. Detection was performed by anti-human Fc-PE mAb. (D) Co-stimulatory activity of targeted and non-targeted antibody-fusion proteins. CFSE-labeled PBMCs were incubated with 4 ng/mL cross-linked anti-CD3 mAb and 90 nM of the respective fusion protein in presence or absence of target cells (HT1080-FAP). After 6 d, proliferation was measured by flow cytometry. Graphics (D) show mean ± SD, n = 3.
Enhanced proliferation was achieved by scFvFAP-scOX40L, scFvFAP-scGITRL and scFvFAP-scLIGHT in targeted form. In absence of target cells, no co-stimulatory activity was detected. Thus, these co-stimulatory members of the TNFSF can be successfully expressed in the single-chain format and introduced into the design of tumor-directed antibody-fusion proteins.

Discussion

Members of the TNF superfamily are type-II transmembrane proteins, characterized by the extracellular TNF homology domain (THD) that forms non-covalently associated trimers. Binding to the corresponding TNFSF-receptor occurs with 3:3 ligand:receptor stoichiometry, leading to receptor clustering and cell signaling. While the apoptotic members of the TNFSF, e.g., TNF, FasL and TRAIL, are classified as conventional family members presenting as tightly packed bell-shaped trimers, co-stimulatory members like 4-1BBL, OX40L and GITRL are assigned to the divergent family subtype, characterized by divergence in sequence and structure. Thus, 4-1BBL resembles a three-bladed propeller, while in the case of OX40L and GITRL, they are constituted by a shortened THD, presenting a more expanded, blooming flower-like organization. So far, generation of single-polypeptide chain variants had only been reported for apoptotic members of the conventional family, where improved stability was shown for TNF and TRAIL. Here, we report in the antibody-fusion protein context the generation of the single-chain format for TNFSF members of the divergent family, comparing binding and activation potential with corresponding recombinant wild-type proteins. 4-1BBL and OX40L showed comparable receptor binding capacity for both formats in ELISA that was not hampered by the fusion to the scFvEpCAM, indicating adequate linker length and ligand assembly in the single-chain format (Fig. 2B). As expected, the presence of only one antibody moiety in the scFv-scTNFSF format reduced the avidity and therefore the antigen binding capacity of scFv-sc4-1BBL and scFv-scOX40L (Fig. 2A). Nevertheless, the co-stimulatory effect achieved in our co-culture setting was not reduced. On the contrary, co-stimulation of PBMCs by scFv-sc4-1BBL and scFv-scOX40L measuring proliferation was similar and in terms of IFNγ release even enhanced in comparison to the corresponding scFv-4-1BBL and scFv-OX40L, indicating improved bioactivity (Fig. 3). In the single-chain format, the covalent linkage of the extracellular domains of the ligand is predicted to stabilize the natural occurring trimer formation. Current models of TNFSF-receptor activation propose an initial phase where the trimeric ligand interacts with the corresponding receptor, recruited and assembled into a trimeric structure as well, followed by a second phase of ligand-receptor complex clustering. The latter is efficiently supported either by a membrane-anchored, i.e., cell surface presentation of the ligand or its oligomerization in soluble form, depending on the activation requirement of the particular TNFSF-receptor. It is conceivable that stabilization of the trimeric ligand conformation by the single-chain format in the scFv-scTNFSF positively supports this process in both phases and targeting the fusion protein to the tumor cell via one scFv unit is sufficient for efficient cell surface presentation of the ligand. From a targeting point of view, it remains to be seen how differences in antibody binding capacity will reflect in the biodistribution pattern of the fusion proteins in vivo. On the other hand, it is important to keep in mind that targeting-independent effects can result from oligomerization. Size exclusion analysis revealed a small oligomeric fraction in the scFv-scTNFSF preparations indicating a certain degree of dimer formation. Partial dimer formation by antibodies in the scFv format has been described and could explain the oligomerization observed. In soluble form, 4-1BBL and OX40L have been described to be basically inactive and to require molecular cross-linking in order to regain their activity. We observed that our recombinant 4-1BBL and OX40L in both formats were indeed not capable of or very inefficient in activating the corresponding receptor-transfected HT1080 cell lines (Fig. 2C). In contrast, fusion to the scFvEpCAM clearly enhanced the receptor activation potential of the untargeted fusion proteins in both formats, especially for the OX40L constructs. Thus, trimer stabilization and oligomerization via the antibody part probably enhanced the receptor activation potential of the fusion protein. Interestingly, in the non-targeted setting, i.e., in absence of tumor target cells, co-stimulatory activity on PBMCs was almost absent for scFvEpCAM-OX40L and scFvEpCAM-scOX40L, but still conserved for scFvEpCAM-4-1BBL and scFvEpCAM-sc4-1BBL (Fig. 4B). For the latter, the particular scFv component seemed crucial, since the corresponding fusion proteins containing the scFvFAP showed also reduced activity in this condition, in accordance with former data. Thus, a careful selection of the antibody moiety is required to accomplish the concept of target-dependent co-stimulation. In addition, not only the structural properties, but also the position of the epitope on the target antigen and thereby the feasibility of ligand presentation needs to be considered, as shown from studies with bi-specific antibodies. Importantly, for all targeted fusion proteins no stimulatory effect was detected in absence of primary anti-CD3-stimulation, confirming their co-stimulatory nature and excluding any supra-agonistic activity.

The major advantage of the TNFSF single-chain format relies in the stabilization of the functional unit of the ligand. Besides the good functional performance, we could also observe improved serum stability and bioavailability for the scFv-scTNFSF format. Higher serum stability and blood retention has also been reported for scTNF in comparison to wild-type TNF. EGFR family-directed antibody fusion proteins have been produced with scTRAIL and antibodies in the scFv format. As well as the diabody (Db) format, the scFv-EHD format, generating either monovalent or bivalent molecules for each component, i.e., antibody:ligand ratios of 1:1 or 2:2, respectively. Although TRAIL is potentially active in soluble form, anti-body-mediated targeting showed to enhance the apoptotic activity in vitro and improve the antitumor effects in a xenograft tumor mouse model. Thus, it was demonstrated that defined ligand oligomerization can be combined with and still benefit from tumor targeting. Further linker engineering of scTRAIL led to improved thermal stability and solubility of the Db-scTRAIL molecule. Antibody fusion proteins in the homotrimeric scFv-TNF format have also been reported for several members of the mouse TNFSF, including CD40L, FasL, TRAIL, LIGHT, VEGI, lymphotoxin α, lymphotoxin β and lymphotoxin alpha1/beta2, in combination with an...
ED A-directed antibody moiety. Biodistribution studies in the F9-tumor mouse model revealed considerable differences in the performance of the scFv-TNFSF fusion proteins. Thus, apparently the homotrimeric format is not compatible with all members of the TNFSF and individual optimization by protein engineering will be necessary to meet the therapeutic requirements in each case. We have shown here that the single-chain format has the potential to improve the properties of TNFSF members of different classes. Therefore, this format appears as valuable starting point for further optimization processes in the antibody-TNFSF fusion protein development.

**Material and methods**

**Materials**

Antibodies were purchased from BioLegend (PE anti-human CD3, 317308; PE anti-human 4-1BB, 309804; PE anti-human 4-1BB, 311504; APC anti-human OX40, 350008), KPL (goat anti-mouse IgG H+L, 01-10-06), Miltenyi Biotec (PE anti-FLAG, 130-101-576; PE anti-His, 130-092-691), R&D Systems (anti-human CD3ε, MAB100) and Sigma-Aldrich (anti-FLAG® M2-HRP, A8592). CellTrace™ CFSE cell proliferation kit was obtained from Life Technologies (C34554). Human IFNγ DuoSet ELISA kit and human Interleukin-8 ELISA were purchased from R&D Systems (DY285) and ImmunoTools (31670089), respectively. FLAG peptide was obtained from peptides and elephants (EP01741) and Anti-FLAG metal ion afﬁnity chromatography was performed using a Yarra column from GeneArt (Thermo Fisher Scientiﬁc, 11875), 10% FBS (PAN Biotech, 3302-P121707). HT1080-4-1BB and HT1080-OX40 were kindly provided by Dr H. Wajant (University Hospital Würzburg, Germany) and were cultivated in RPMI 1640 (Thermo Fisher Scientiﬁc, 11875), 10% FBS (PAN Biotech, 3302-P121707). HT1080-FAP and B16-FAP (stable transfectants with human FAP) (Klaus Pﬁzermaier, IZI) were cultured in RPMI 1640, 5% FBS, supplemented with 200 μg/mL of G418 (Sigma, G8168) or zeocin (Thermo Fisher Scientiﬁc, R25001), respectively. The cell lines HT1080, HEK293 and Bewo were cultured in RPMI 1640, 10% FBS, RPMI 1640, 5% FBS or Ham’s F-12 Nutrient Mix (Thermo Fisher Scientiﬁc, 31765), 10% FBS, respectively. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of healthy donors (Klinikum Stuttgart, Germany) by Ficoll density gradi ent centrifugation (Lymphocyte Separation Medium 1077, Promocell, C-44010) and cultivated in RPMI 1640, 10% FBS. Human plasma was also obtained from healthy donors (Klinikum Stuttgart, Germany). CD1 mice were purchased from Charles River (Crl:CD1(ICR)). Animal care and the experiment carried out were in accordance with federal guidelines and had been approved by university and state authorities.

**Generation of antibody-fusion proteins**

Anti-EpCAM scFv323A35 was humanized in-house by complementarity-determining region grafting, generating scFv323A3hu3. Anti-FAP scFv36 had been generated and described previously. The genes encoding the extracellular domains (ECD) of 4-1BBL (amino acids 71-254), OX40L (amino acids 51-183), GITRL (amino acids 72-199) and LIGHT (amino acids 93-240) were codon-optimized for expression in human cells and synthesized by GeneArt (Thermo Fisher Scientiﬁc) either as single domain or as single-chain variant. For the latter, i.e., sc4-1BBL, scOX40L, scGITRL and scLIGHT, the linkers (GGGGS)4, GGGSGGG, GSGGGGGGSGG and G were used to connect the ECD, respectively.

The DNA sequences for the scFvEpCAM-TNFSF ligand fusion proteins were cloned into the mammalian expression vector pIRESpuro3 (Clontech, 631619). Recombinant protein was produced in stably transfected HEK293 cells and puriﬁed from the cell culture supernatant by FLAG afﬁnity chromatography according to the manufacturer’s instructions. Residual FLAG pepti de was removed by dialysis against PBS. The scFvFAP- TNFSF ligand fusion proteins were cloned into pSecTagA (Invitrogen, Thermo Fisher Scientiﬁc, V90020) and transiently produced in HEK-293-6E cells (NRC Biotechnology Research Institute, Canada) according to the standard protocol of the cell line provider. Purification was carried out by immobilized metal ion afﬁnity chromatography (IMAC) as described previously.

**Size exclusion chromatography (SEC)**

Size exclusion chromatography was performed using a Yarra SEC-3000 column (Phenomenex, 00H-4513-K0) and a Waters 2695 HPLC (Waters Corporation) in a 0.1 M Na2HPO4/NaH2PO4, 0.1 M NaSO4, pH 6.7 mobile phase at a flow rate of 0.5 mL/min. Thyroglobulin (669 kDa, Sr 8.5 nm), β-amylase (200 kDa, Sr 5.4 nm), bovine serum albumin (67 kDa, Sr 3.55 nm), carbonic anhydrase (29 kDa, Sr 2.35 nm) and FLAG peptide (1 kDa) served as standard proteins.

**Plasma stability**

Fusion proteins at 200 nM in 50% human plasma were incubated at 37°C for 1 d, 3 d and 7 d. Samples were frozen at −20°C immediately after preparation (0 d) or after the respective incubation period. The level of intact protein was determined via ELISA as described above. Pharmacokinetic parameters (initial half-life t1/2a, terminal half-life t1/2b, area under the curve (AUC)) were calculated using Excel.

**Pharmacokinetics**

Female CD1 mice received one i.v. injection of 25 μg fusion protein in 150 μL DPBS. Blood samples (50 μL) were taken from the tail vein after 3, 30, 60, 120 min, 6, 24 and 72 h. Serum was separated by centrifugation (30 min, 4°C) and samples were stored at −20°C. Serum concentration of fusion proteins was determined via ELISA as described above. Pharmacokinetic parameters (initial half-life t1/2α, terminal half-life t1/2β, area under the curve (AUC)) were calculated using Excel.
**Binding analysis by ELISA**

4-1BB-Fc or OX40-Fc (200 ng/well) were immobilized on ELISA plates overnight at 4°C followed by blocking and incubation with the fusion proteins at room temperature for 1 h. Bound proteins were detected with HRP-conjugated anti-FLAG antibody and absorbance was measured at 450 nm in an ELISA reader.

**Binding analysis by flow cytometry**

Antibody-mediated binding was assessed by flow cytometry. 2.5 × 10^5 target cells (Bewo (EpCAM^+^), HT1080-FAP) were incubated with serial dilutions of the fusion proteins for 1 h at 4°C. Cells were washed and bound fusion proteins were detected with a PE-conjugated anti-FLAG antibody (EpCAM-directed fusion proteins) or PE-conjugated anti-4-1BBL anti-body (FAP-directed fusion proteins).

Simultaneous binding of fusion proteins to FAP on tumor cells and recombinant TNFSF receptor was assessed by incubating 2.5 × 10^5 B16-FAP cells with 40 nM fusion protein for 1 h at 4°C. Bound fusion protein was detected via ligand–receptor interaction, incubating with recombinant receptor-Fc (5 μg/mL), followed by PE-conjugated anti-human Fc antibody. Cells were analyzed in a MACSQuant® Analyzer 10 (Miltenyi Biotec) and data was evaluated using MACSQuantify™ or FlowJo. Relative mean fluorescence intensities (MFI) were calculated as followed: relative MFI = (MFI_sample – (MFI_detection system – MFI_cells))/MFI_cells.

**Cytokine release assays**

Functionality of the TNFSF ligands was assessed in a cell-based reporter assay measuring interleukin-8 (IL-8) released by TNFSF–receptor transfected HT1080 cells upon TNFR-dependent NF-κB activation. 2 × 10^5 cells/well HT1080-4-1BB or HT1080-OX40 were seeded. The next day, supernatants were discarded to remove constitutively produced IL-8 and cells were incubated with serial dilutions of the fusion proteins for 18 h. Then, cell-free supernatants were analyzed directly by Sandwich ELISA using human Interleukin-8 ELISA kit according to the manufacturer’s instructions.

The co-stimulatory potential of the fusion proteins was evaluated by measuring IFNγ release from activated PBMC in a co-culture assay with target cells. 3 × 10^5 target cells/well (Bewo or HT1080-FAP) were seeded and the next day cells were incubated with serial dilutions of the co-stimulatory fusion proteins for 1 h at room temperature. 2 × 10^5 PBMC/well were added and T cells were activated with suboptimal concentration of anti-human CD3 mAb (50–100 ng/mL) cross-linked by previous incubation with an anti-mouse IgG mAb at a molar ratio of 1:3. After 48 h, IFNγ concentration in the supernatant was determined by Sandwich ELISA, using a human IFNγ DuoSet® ELISA kit according to the manufacturer’s instructions.

**T cell proliferation assays**

3 × 10^4 target cells/well (Bewo/HT1080-FAP) were seeded. The next day, cells were incubated with serial dilutions of the fusion proteins for 1 h at room temperature before adding 1.5 × 10^5 PBMC/well that were previously stained with carboxyfluorescein diacetate succinimydyl ester (CFSE) at a concentration of 625 nM per 1 × 10^6 cells/mL following the manufacturer’s instructions. T cells were activated with suboptimal concentrations of cross-linked anti-human CD3 mAb (approx. EC50) as indicated above. The effect of non-targeted scFv-ligand fusion proteins was analyzed in the absence of target cells. Furthermore, in some experiments the 4-1BBL- or OX40L-mediated effects of the fusion proteins (10 nM) were blocked by addition of 5-fold excess of recombinant 4-1BBL- or OX40-Fc (50 nM) before the addition of PBMC. After 6 d T cell proliferation was measured by flow cytometry in a MACSQuant® Analyzer 10.

**Statistical analysis**

Unless otherwise stated, all data are represented as mean ± SD of three independent experiments. Block shift correction was performed according to the formula: Xn = Xn – (Yn – Y) with Xn being the corrected value of X from the experiment n, Y the average of the X values from all experiments performed and Yn the average of the duplicate values of X from experiment n. Statistical significance was determined using one-way ANOVA followed by Tukey’s post-test (Graphpad Prism, Graphpad Software Inc.). p values below 0.05 were considered statistically significant (*p < 0.001, **p < 0.01, *p < 0.05).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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