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Fc-based Duokines: dual-acting costimulatory molecules comprising TNFSF ligands in the single-chain format fused to a heterodimerizing Fc (scDk-Fc)

Nadine Aschmoneit [iD][a], Katharina Kocher [iD][a], Martin Siegemund[a], Martina S. Lutz[a], Lennart Kühl [iD][a], Oliver Seifert [iD][a,b], and Roland E. Kontermann [iD][a,b]

aInstitute of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany; bStuttgart Research Center Systems Biology (SRCSB), University of Stuttgart, Stuttgart, Germany

ABSTRACT

Targeting costimulatory receptors of the tumor necrosis factor superfamily (TNFSF) to activate T-cells and promote anti-tumor T-cell function have emerged as a promising strategy in cancer immunotherapy. Previous studies have shown that combining two different members of the TNFSF resulted in dual-acting costimulatory molecules with the ability to activate two different receptors either on the same cell or on different cell types. To achieve prolonged plasma half-life and extended drug disposition, we have developed novel dual-acting molecules by fusing single-chain ligands of the TNFSF to heterodimerizing Fc chains (scDuokine-Fc, scDk-Fc). Incorporating costimulatory ligands of the TNF superfamily into a scDk-Fc molecule resulted in enhanced T-cell proliferation translating in an increased anti-tumor activity in combination with a primary T-cell-activating bispecific antibody. Our data show that the scDk-Fc molecules are potent immune-stimulatory molecules that are able to enhance T-cell mediated anti-tumor responses.

Introduction

Therapeutic concepts in the field of cancer immunotherapy aim to boost the patient's immune response against cancer cells that have evaded immune surveillance. One strategy is to interrupt this evasion and enhance the anti-tumor activity of T-cells using immune checkpoint inhibitors. Despite the success of already approved checkpoint inhibitors, i.e. antibodies targeting CTLA-4, PD-1 or PD-L1, more than 80% of patients do not respond to treatment or eventually experiencing resistance.[1] Hence, alternative strategies for promoting anti-tumor T-cell function, survival of T-cells and the formation of effector and memory phenotypes have gained increasing interest.

Multiple signals are mandatory for complete activation of a T-cell to induce clonal expansion and the acquisition of effector functions. Costimulatory and coinhibitory signaling represents a complex network of receptor ligand interactions qualitatively and quantitatively influencing the immune response. Upon activation, the expression of the respective molecules on the T-cell surface are induced whereby cell surface interaction and intracellular signaling vary in response to dynamic tissue environmental conditions.[2] In the initial phase of T-cell activation, ligand-receptor-interactions arise between T-cells and antigen-presenting cells (APCs) or B-cells, while in a later stage, activating ligands get upregulated on T-cells facilitating signaling between T-cells.[3,4]

Agonistic antibodies targeting costimulatory receptors from the TNF receptor superfamily (TNFRSF) expressed on the surface of T-cells such as 4–1BB,[5] OX40,[6,7] CD27[8] or CD40

on APCs[9] have been developed. Sufficient activation of these receptors requires receptor clustering, naturally occurring by binding of the cell membrane-displayed trimeric ligand to the respective receptor.[10,11] Therefore, monoclonal antibodies are, due to the bivalent binding mode, dependent on binding to Fcγ receptors on nearby cells to sufficiently cluster receptors and to induce signaling.

As an alternative to antibodies, soluble ligands can serve as activators of costimulatory signals. Conversion of the homotrimeric members of the TNFSF into a single-chain format further fused to an antigen-binding moiety of an antibody, e.g. a single-chain Fv (scFv) fragment, has been shown to successfully mimic cell surface-displayed activity for target-dependent T-cell activation.[12] Furthermore, we have recently developed a novel approach allowing simultaneous binding and activation of two different costimulatory receptors. This was achieved by combining two different members of the TNFSF into one molecule connecting either single subunits of two ligands (so-called Duokines, Dk) or single-chain derivatives of the ligand (single-chain Duokines, scDk) by flexible linkers.[13] We have demonstrated that these dual-acting costimulatory molecules can activate two different receptor types either on the same cell (i.e. acting in cis) or on different cell types (i.e. acting in trans). In combination with a bispecific T-cell engager, these molecules mediated an enhanced T-cell proliferation *in vitro* and showed promising anti-tumor effects *in vivo*.[13]

In the present study, we now developed novel dual-acting molecules by fusing single-chain ligands to heterodimerizing Fc chains (scDuokine-Fc, scDk-Fc) for a prolonged half-life

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and extended drug disposition. We analyzed receptor binding, enhancement of T-cell proliferation, pharmacokinetics and pharmacodynamic properties, demonstrating dual-acting immunostimulatory activity and improved anti-tumor activity in combination with a bispecific T-cell engager.

Material and methods

Materials

Antibodies were purchased from Biolegends (PerCP/Cy5.5 anti-human CD3, 317336; PE anti-human CCR7, 353204; APC anti-human CD45RA; 304112; PE anti-human CD69, 310906), Miltenyi Biotec (anti-human CD4-VioBlue, 130–097-333; anti-human CD8-PE/Vio770, 130–096-556; anti-His-PE, 130–092-691; KPL anti-mouse IgG (H + L), 01–10-06), Merck (anti-mouse IgG (whole molecule)-FITC, F9137; anti-mouse IgG (Fc specific)-HRP, A2554; anti-human Fc-HRP, A0170), Sigma-Aldrich (anti-FLAG M2-HRP, A8592) and R&D Systems (anti-human CD3ε, MAB100). CellTrace™ CFSE (C34554) and CellTrace™ Far Red Cell Proliferation Kit (C34564) were purchased from Thermo Fisher Scientific. CytoStim (130–092-172) was purchased from Miltenyi Biotec. Stable transfected HT1080-CD40, HT1080-CD27, HT1080-OX40 and HT1080-4-1BB 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, P30-3309). Mouse melanoma B16-FAP cells[13] were cultured in RPMI-1640, 5% FBS supplemented with 200 μg/mL zeocin (Thermo Fisher Scientific, R25001). Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors (Blood bank, Klinikum Stuttgart) by density gradient centrifugation (Lymphocyte Separation Medium 1077, Promocell, C-44010) and cultivated in RPMI-1640, 10% FBS.

Protein production and purification

Single-chain derivatives of the costimulatory ligands CD40L, CD27L OX40L and 4–1BBL were generated by connecting the ECD of the respective receptors with flexible linkers (scCD40L: GG; scCD27L: GG, scOX40L: $(GGGGS)_3$, sc4-1BBL: $(GGGGS)_4$) and fusion to either a human γ1 C_H2-C_H3_{hole} or to the complementary human γ1 C_H2-C_H3_{knob} chain. Genes encoding the different polypeptide chains were cloned into the pSecTagAL1 vector (a modified version of pSecTagA (Invitrogen, Thermo Fisher Scientific, V90020)). All proteins were produced in transiently transfected HEK293-6E cells (NRC Biotechnology Research Institute, Canada) using polyethylenimine (PEI; linear, 25 kDa, Sigma-Aldrich, 764604). Supernatants were harvested 96 hours post transfection, and human proteins were purified by protein A affinity chromatography followed by size-exclusion FPLC on a Superdex 200 10/300 GL column (PBS as mobile phase, 0.5 ml/min flow rate). For efficient heterodimer formation, mutations from the "knobs-into-holes" technology were introduced into a mouse IgG2a Fc-part.[14] LALA-PG mutations were introduced additionally to reduce Fc-dependent effector functions.[15] His-tagged mouse surrogates were produced as described for the human molecules, purified via immobilized metal ion affinity chromatography (IMAC) followed by size-exclusion chromatography. ScDks were produced and purified as previously described.[13]

Biochemical characterization

Purified proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie Brilliant Blue G-250. Integrity and purity of the proteins were further analyzed by size-exclusion chromatography using a Waters 2695 HPLC and a TSKgel SuperSW mAb HR column (Tosoh Bioscience) at a flow rate of 0.5 ml/min with 0.1 M Na_2HPO_4/NaH_2PO_4, 0.1 M Na_2SO_4, pH 6.7 as mobile phase. Thyroglobulin (669 kDa), β-Amylase (200 kDa), bovine serum albumin (67 kDa) and carbonic anhydrase (29 kDa) were used as reference proteins.

ELISA

96-well plates were coated with TNFRSF-Fc fusion proteins (200 ng/well in PBS) overnight at 4°C and residual binding sites were blocked with 2% (w/v) skim milk powder in PBS (MPBS, 200 μl/well). A serial dilution of purified proteins was incubated with immobilized receptors for 1 h at room temperature. Bound antibodies were detected with HRP-conjugated antibodies specific for human (for scDuokine-Fc), murine Fc (for TNFRSF-moFc) or FLAG-tag (for scDuokine and scDuokine-Fc). Detection antibodies were incubated for one additional hour at room temperature. 3,3′,5,5′-tetramethylbenzidine (TMB) (1 mg/ml; 0.006% (v/v H_2O_2) in 100 mM sodium-acetate buffer, pH 6 was used as substrate, reaction was terminated using 50 μl 1 M H_2SO_4 and absorption was measured at a wavelength of 450 nm.

Conjugate formation assay

To determine simultaneous binding of the scDuokine-Fc molecules to two different cell lines, TNFSFR-expressing HT1080 cells were stained with either carboxyfluorescein diacetate succinimidyl ester (CFSE) or Far Red at 625 nM/1x10^6 cells/ml following the manufacturer's instructions. 1×10^4 cells/well from two different TNFSFR-expressing cell lines either labeled with CFSE or Far Red were mixed in a 1:1 ration before 50 nM of respective protein was added. After incubation for 1 h at 37°C, 5% CO_2 formed conjugates were determined in flow cytometry.

T-cell proliferation

To analyze the proliferative effect on T cells, PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 625 nM/1x10^6 cells/ml. 2×10^5 CFSE-labeled PBMCs/well were incubated with different concentrations of fusion protein in presence of a suboptimal concentration of anti-CD3 mAb (crosslinked with goat anti-mouse IgG (H + L) in the ratio of 1:3) or CytoStim (diluted 1:4,000). After 6 days, immune cells of interest were labeled with fluorescence-conjugated antibodies directed against respective cell-surface

markers and proliferation was measured by multicolor flow cytometry analysis using MACSQuant Analyzer 10 (Miltenyi Biotec).

Animal experiments

All animal experiments were performed in accordance with federal guidelines and approved by the university and state authorities (registration number RPS35-9185-99/332). To determine the pharmacokinetic (PK) profile of the mscDuokine-mFc fusion proteins, mice received one intravenous (i.v.) injection (25 μg) and blood samples were taken after 3 min, 1 h, 6 h, 24 h, 72 h, and 168 h. After incubation on ice for 20 minutes, samples were centrifuged (16.000 x g, 4°C, 20 min) and stored at −20°C until analysis. Serum concentrations of mscDuokine-mFc fusion proteins were determined by ELISA using murine TNFSFR-Fc as immobilized antigen. Bound antibodies were detected with HRP-labeled anti-murine Fc secondary antibody. The PK data were calculated with Excel or PKSolver.

Anti-tumor activity was studied in a lung tumor model. B16-FAP cells (1×10^6 cells in 100 μl PBS) were injected intravenously into female C57BL/6 N mice (seven mice per group). Mice were treated with six intraperitoneal injections of 25 μg murine mscDuokine-mFc molecules (msc4-1BBL-mscOX40L-mFc or mscCD40L-mscCD27L-mFc) either alone or in combination with 0.2 μg bispecific antibody (scDbmo33x2C11) on days 1, 2, 3 and days 8, 9, 10 after tumor cell injection. Additionally, the mice were treated either with PBS or the bispecific antibody alone following the same treatment regimen. Mice were sacrificed on day 21, lungs were removed, fixed in Fekete solution and tumor foci counted.

Statistics

All data are represented as mean ± SD. Significances were calculated by GraphPad Prism 7.0 and results were compared by one-way ANOVA followed by Tukey's multiple comparison test (posttest) or t-test. $p < .05$ (*), $p < .01$ (**), $p < .001$ (***), $p < .0001$ (****), ns (not significant), n.d. (not determined).

Results

Generation of bifunctional costimulatory molecules comprising an Fc part

Previously, we have shown that bifunctional costimulatory molecules can be generated by fusing single-chain variants of two members of the TNFSF by a short flexible linker.[13] However, these molecules suffer from short half-lives due their small size and lack of an Fc portion. Therefore, we generated novel bifunctional costimulatory molecules called scDuokine-Fc comprising an Fc part (scDk-Fc). To generate the scDuokine-Fc, single-chain derivatives of the costimulatory ligands CD40L (aa 116–261), CD27L (aa 52–193), OX40L (aa 51–183) and 4–1BBL (aa 71–254) were fused to either a human γ1 C_H2-C_H3_{hole} or to the complementary human γ1 C_H2-C_H3_{knob} chain, further comprising mutations to silence Fc effector functions,[16] resulting in heterodimeric

bifunctional costimulatory molecules (Figure 1). Combining two of the ligands CD27L, OX40L or 4–1BBL into a scDuokine-Fc molecule resulted in cis-acting molecules targeting receptors expressed on T-cells. In contrast, scDuokine-Fc molecules combining CD40L with any other ligand (CD27L, OX40L or 4–1BBL) are trans-acting molecules that bind to receptors expressed on different cell types (CD40 on antigen presenting cells or B-cells and CD27, OX40 or 4–1BB on T-cells). In total, three cis-acting scDuokine-Fc and three trans-acting scDuokine molecules were generated.

All molecules were produced in transiently transfected HEK293-6E cell and purified via protein A affinity chromatography followed by a preparative size-exclusion chromatography step (SEC) with yields of 1.1 to 3.8 mg/L culture. SDS-PAGE analysis of the scDuokine-Fc molecules under non-reducing conditions revealed one single band for all analyzed molecules corresponding to disulfide-linked heterodimers. Under reducing conditions, all proteins revealed two bands, with the two bands for the scCD40L-Fc and sc4-1BBL-Fc chains running close together (Table 1). In analytical SEC, all analyzed molecules eluted with one major peak. All molecules comprising OX40L, predicted to be highly glycosylated (12 N-glycosylation sites per chain), migrated at higher apparent molecular masses than calculated from the sequence (Table 1). In summary, all six proteins could be produced as heterodimeric molecules.

scDuokine-Fc molecules are able to simultaneously bind two receptors

Binding to immobilized TNFSF receptor-Fc fusion proteins was determined in ELISA. All scDuokine-Fc molecules showed concentration-dependent binding to their respective receptors with EC_{50} values ranging from 0.2 to 0.7 nM (Figure 2a–f, left panel, Table 2). Additionally, simultaneous binding of scDuokine-Fc molecules to their respective receptors was confirmed in ELISA. Here, again a concentration-dependent binding was observed for all scDuokine-Fc molecules, with EC_{50} values in the sub-nanomolar range (0.1 to 0.4 nM) (Figure 2a–f middle panel, Table 2). As binding to receptors on two different cells is of vital importance for the trans-acting scDuokine-Fc molecules, simultaneous binding of two cell lines expressing different costimulatory receptors was analyzed in a conjugate formation assay. The cis-acting scDuokine-Fc molecules sc4-1BBL-scOX40L-Fc, scCD27L-scOX40L-Fc and the trans-acting scCD40L-scCD27L-Fc and scCD40L-scOX40L-Fc showed conjugate formation, while no significant formation of conjugates could be observed for sc4-1BBL-scCD27L-Fc and scCD40L-sc4-1BBL-Fc (Figure 2a–f right panel). In summary, all scDk-Fc molecules retained the receptor-binding activity of the fused ligands.

T-cell activation by scDuokine-Fc molecules

Two scDuokine-Fc molecules, one acting in cis and one acting in trans, were chosen for further analysis (The first scDk-Fc targets 4–1BBL and OX40L, which are both upregulated upon T-cell activation, the second scDk-Fc targets CD40L and CD27L constitutively expressed on APCs and T-cells, respectively). The costimulatory activity of the cis- and trans-
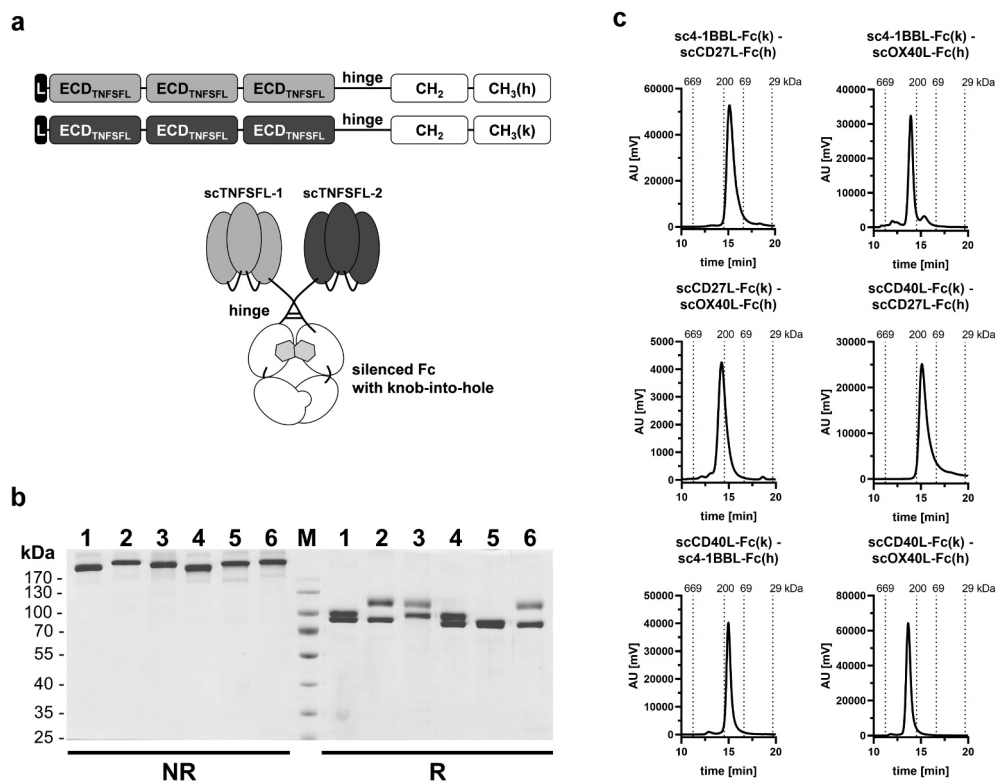
Figure 1. Biochemical characterization of scDuokine-Fc molecules. (a) Composition and schematic illustration of a scDuokine-Fc. L, Igκ chain leader sequence, ECD_TNFSFL, extracellular domain of the TNFSF ligand. (b) SDS-PAGE analysis (12% PAA, 3 μg/lane, Coomassie blue staining) of (1) sc4-1BBL-scCD27L-Fc; (2) sc4-1BBL-scOX40L-Fc, (3) scCD27L-scOX40L-Fc, (4) scCD40L-CD27L-Fc, (5) scCD40L-sc4-1BBL-Fc and (6) scCD40L-scOX40L-Fc under reducing (r) and non-reducing (NR) conditions. M, protein marker. (c) Size-exclusion chromatography by HPLC using a Tosoh TSKgel SuperSW mAb HR column.

Table 1. Biochemical characterization of scDuokine-Fc molecules. Calculation of the molecular mass (MW) based on the amino acid sequence.

	Calculated MW [kDa]	SDS-PAGE (R) [kDa]	SEC [kDa]
sc4–1BBL-Fc_knob	85	161	89	152
scCD27L-Fc_hole	76		83	
sc4–1BBL-Fc_knob	85	159	106	262
scOX40L-Fc_hole	74		83	
scCD27L-Fc_knob	77	151	103	242
scOX40L-Fc_hole	74		87	
scCD40L-Fc_knob	76	152	87	154
scCD27L-Fc_hole	76		79	
scCD40L-Fc_knob	76	161	79	174
sc4–1BBL-Fc_hole	85		79	
scCD40L-Fc_knob	76	150	100	270
scOX40L-Fc_hole	74		79	

acting scDuokine-Fc molecules was determined by measuring the enhancement of T-cell proliferation above the levels induced by a suboptimal concentration of a cross-linked anti-CD3 mAb providing the necessary first stimulus. Both scDuokine-Fc molecules were able to mediate statistically significant proliferative effects on CD4+ and CD8+ T-cells. On CD4+ T-cells, sc4-1BBL-scOX40L-Fc showed a 2-fold enhancement of T-cell proliferation compared to treatment with the anti-CD3 mAb, while scCD40L-scCD27L-Fc increased T-cell proliferation by a factor of 2.5 (Figure 3a left panel). Similar results were observed for CD8+ T-cells (Figure 3a right panel).

Regarding T-cell subpopulations, treatment with both scDuokine-Fc molecules mediated an increase in proliferation of naïve, effector and central memory CD4+ T-cells, while only a mild increase in proliferation of effector memory CD4+ T-cells was observed for scCD40L-scCD27L-Fc. Additionally, scCD40L-scCD27L-Fc was more potent in enhancing proliferation of central memory CD4+ T-cells compared to sc4-1BBL-scOX40L-Fc (Figure 3b left panel). For CD8+ T-cells, both scDuokine-Fc molecules were able to enhance the proliferation of all investigated T-cell subpopulations compared to treatment with the anti-CD3 mAb in a suboptimal concentration alone. While both investigated scDuokine-Fc molecules show a similar pattern regarding the composition of the CD4+ T-cell subpopulation, i.e. a slight shift toward CD4+ naïve T-cells compared to the anti-CD3 mAb alone (Figure 3c left panel), a tremendous proportional increase of CD8+ effector T-cells was observed for both scDuokine-Fc molecules compared to the treatment with the anti-CD3 mAb (Figure 3c right panel). Thus, both scDuokine-Fc fusion proteins were able to provide costimulatory signals to activated T-cells leading to increased proliferation of T-cells.

In further experiments, we compared binding and T-cell costimulatory activity of scDk-Fc targeting 4–1BBL and OX40L or CD40L and CD27L with the corresponding scDk molecules generated previously and lacking the Fc region.[13] ScDk-Fc CD40LxCD27L and scDk CD40LxCD27L showed similar binding with EC_50 values between 1.9 to 2.5 nM for binding to both receptors (CD40 and CD27). In contrast, a slightly increased binding to 4–1BB (4.9-fold) and OX40 (2.4-fold) was observed for scDk-Fc 4–1BBLxOX40L compared to its corresponding scDk (Figure 4a). In a T-cell proliferation assay identical
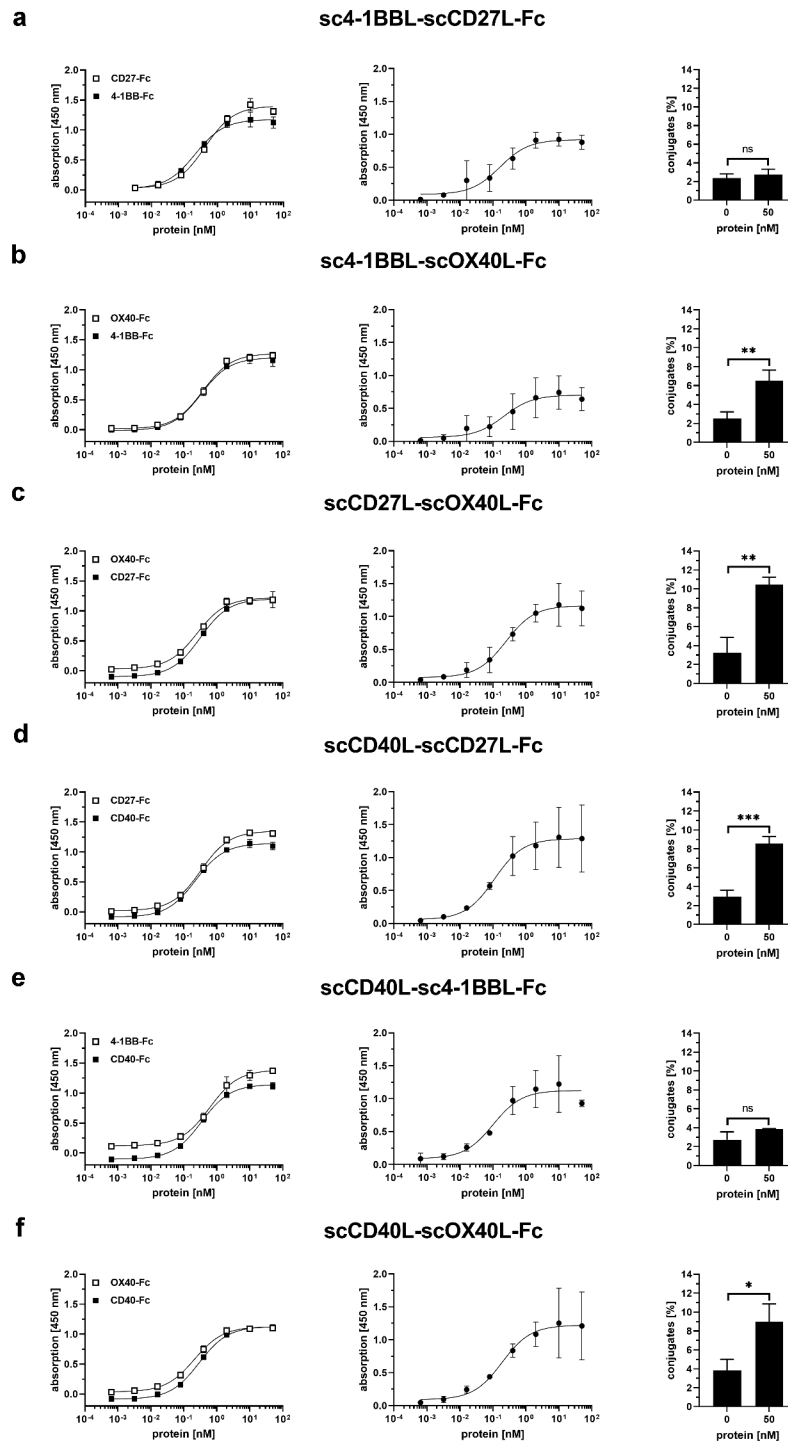
Figure 2. Binding properties of scDuokine-Fc molecules. Binding of (a) sc4-1BBL-scCD27L-Fc; (b) sc4-1BBL-scOX40L-Fc, (c) scCD27L-scOX40L-Fc, (d) scCD40L-scD27L-Fc, (e) scCD40L-sc4-1BBL-Fc and (f) scCD40L-scOX40L-Fc to immobilized receptors in ELISA (left panel). Binding of scDuokine-Fc molecules to immobilized TNFSFR-huFc and soluble TNFSFR-moFc sequentially (middle panel) and to two HT1080 cell lines expressing the respective receptors (right panel). For simultaneous binding to two HT1080 cell lines expressing two different TNFSFR, one cell line was dyed with CFSE and the other with Far Red. The double positive population (CFSE$^+$Far Red$^+$) was interpreted as formed conjugates. Mean ± SD, n = 3, statistics: t-test. *p < .05, **p < .01, ***p < .001, ****p < .0001, ns not significant.

activities were observed for scDk-Fc and scDk molecules titrated over a broad concentration range (Figure 4b). Thus, compared to the scDk molecules, where two single-chain ligands are connected by a flexible linker, the scDk-Fc fusion proteins retained their dual binding and costimulatory activity.
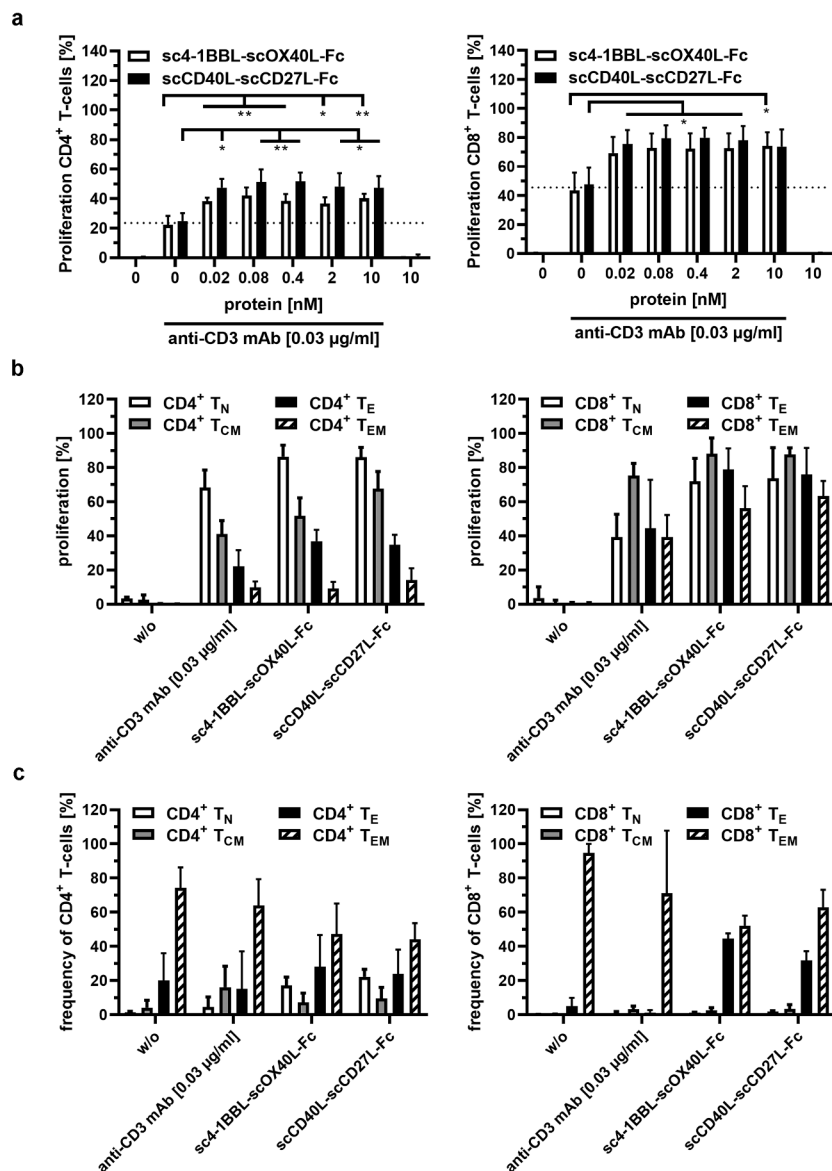
Figure 3. Activity of scDuokine-Fc molecules on T-cell proliferation. (a) Proliferation of CD4$^+$ and CD8$^+$ T-cells was measured by CFSE dilution in flow cytometry. Mean ± SD, n = 3. (b) Proliferation and (c) composition of naïve (T$_N$, CD45RA$^+$, CCR7$^+$), central memory (T$_{CM}$, CD45RA$^-$, CCR7$^+$), effector (T$_E$, CD45RA$^+$, CCR7$^-$) and effector memory (T$_{EM}$, CD45RA$^-$, CCR7$^-$) subpopulations of CD4$^+$ T-cells and CD8$^+$ T-cells was determined by CFSE dilution in flow cytometry. Mean ± SD, n = 3, statistics: one-way ANOVA followed by Tukey's posttest. *p < .05, **p < .01.

Pharmacokinetics of scDuokine-Fc molecules

In order to investigate the pharmacokinetic and pharmacodynamic properties of the scDuokine-Fc molecules in mice, we generated mouse surrogates composed of the respective single-chain mouse ligands and a heterodimerizing and silenced murine Fc. Pharmacokinetic properties of the scDuokine-Fc molecules were evaluated in the immunocompetent C57BL/6 N mouse strain. The murine homologs were produced in HEK293-6E cells and purified by IMAC via a His-tag added to the Fc knob chain introduced to prevent purification of Fc hole-hole homodimers. Characterization of the mouse surrogates was performed as described for the human scDuokine-Fc molecules. Thus, purity and correct assembly was confirmed by SDS-PAGE analysis (Figure 5a) and both molecules showed a concentration-dependent binding to their mouse receptors in ELISA (Figure 5b). Here, msc4-1BBL-mscOX40L-mFc bound to mOX40-Fc and m4-1BB-Fc with EC$_{50}$ values of

55.2 ± 15.1 nM and 9.2 ± 1.6 nM, respectively. MscCD40L-mscCD27L-mFc bound with EC$_{50}$ values of 0.4 ± 0.1 nM and 1.4 ± 0.3 nM to mCD27-Fc and mCD40-Fc, respectively.

Both scDuokine-Fc molecules showed pharmacokinetic profiles with a rapid decline in serum concentration during the initial phase, with half-lives of ~18 min (mscCD40L-mscCD27L-mFc) and ~84 min (msc4-1BBL-mscOX40L-mFc), respectively (Figure 5c, Table 3). The higher initial half-life of msc4-1BBL-mscOX40L-mFc resulted in a higher drug exposure (AUC 48.1 ± 12.5 h/μg/ml) compared to the mscCD40L-mscCD27L-mFc (AUC 14.0 ± 2.1 h/μg/ml).

Pharmacodynamics of scuokine-Fc molecules

The therapeutic anti-tumor potential of the murine scDuokine-Fc molecules was investigated in vivo in an established syngeneic lung tumor model in C7BL/6 N
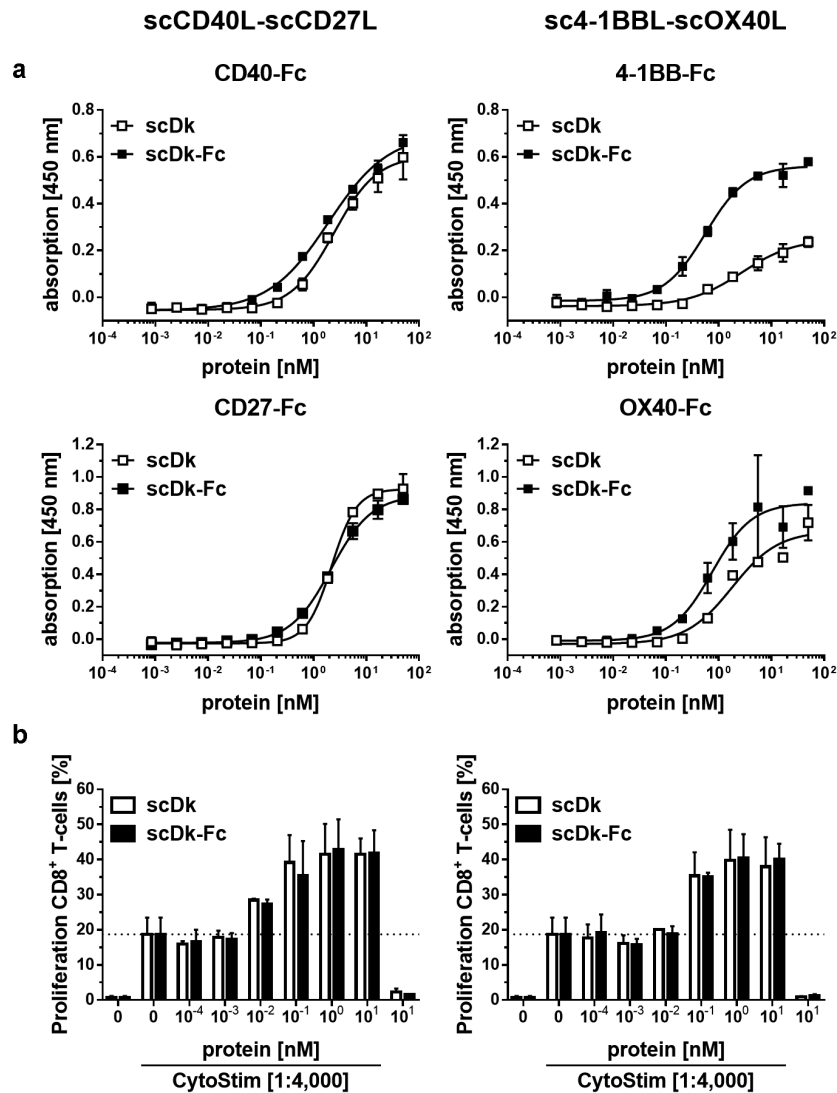
Figure 4. Binding properties and activity on T-cell proliferation for scDuokine (scDk) versus scDuokine-Fc (scDk-Fc) molecules. (a) Binding of scDk and scDk-Fc to immobilized receptors in ELISA (left panel: scCD40L-scCD27L/right panel: sc4-1BBL-scOX40L). (b) Proliferation of CD8^{+} T-cells for scDk and scDk-Fc was measured by CFSE dilution in flow cytometry (left panel: scCD40L-scCD27L/right panel: sc4-1BBL-scOX40L). Mean ± SD, n = 2.

Table 2. Binding analysis of the scDuokine-Fc molecules in ELISA. EC$_{50}$ [nM], Mean ± SD, n = 3.

	ELISA [nM]		
	4–1BB-moFc	CD27-moFc	4–1BB-huFc/CD27-moFc
sc4-1BBL-scCD27L-Fc	0.2 ± 0.04	0.4 ± 0.03	0.2 ± 0.2
	4-1BB-moFc	OX40-moFc	4-1BB-huFc/OX40-moFc
sc4-1BBL-scOX40L-Fc	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.4
	CD27-moFc	OX40-moFc	CD27-huFc/OX40-moFc
scCD27L-scOX40L Fc	0.3 ± 0.1	0.3 ± 0.02	0.3 ± 0.3
	CD40-moFc	CD27-moFc	CD40-huFc/CD27-moFc
scCD40L-scCD27L-Fc	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
	CD40-moFc	4-1BB-moFc	CD40-huFc/4-1BB-moFc
scCD40L-sc4-1BBL-Fc	0.4 ± 0.1	0.7 ± 0.3	0.1 ± 0.04
	CD40-moFc	OX40-moFc	CD40-huFc/OX40-moFc
scCD40L-scOX40L-Fc	0.3 ± 0.02	0.2 ± 0.03	0.5 ± 0.3

mice.[13,17–20] Human-FAP-expressing B16 cells were injected, i.v. into the mice, which received the murine scDuokine-Fc molecules either alone or in combination with a bispecific antibody in a suboptimal concentration providing the primary T-cell activation signal, or PBS as a control on day 1, 2, 3, 8, 9 and 10 post tumor cell inoculation.[13] On day 21, mice were sacrificed, lungs removed and tumors counted. Administration of the scDuokine-Fc molecules in combination with the scDb33x2C11 resulted in a stronger anti-tumor effect compared to the treatment with the murine scDuokine-Fc molecules without the scDb33x2C11 (~60-80% reduction of tumor lesions) or the PBS group (~60-70%) (Figure 5d). The scDb33x2C11 alone already reduced the tumor burden by ~40% compared to the treatment with PBS. Addition of scDk-Fc further reduced number of tumor lesions by ~30 and ~50%, respectively. All treatments were well-tolerated with no effects on body weight (Figure 5e). These observations confirm the potent anti-tumor effect of the scDuokine-Fc molecules as a costimulatory signal in combination with a primary MHC-independent T-cell activation signal.
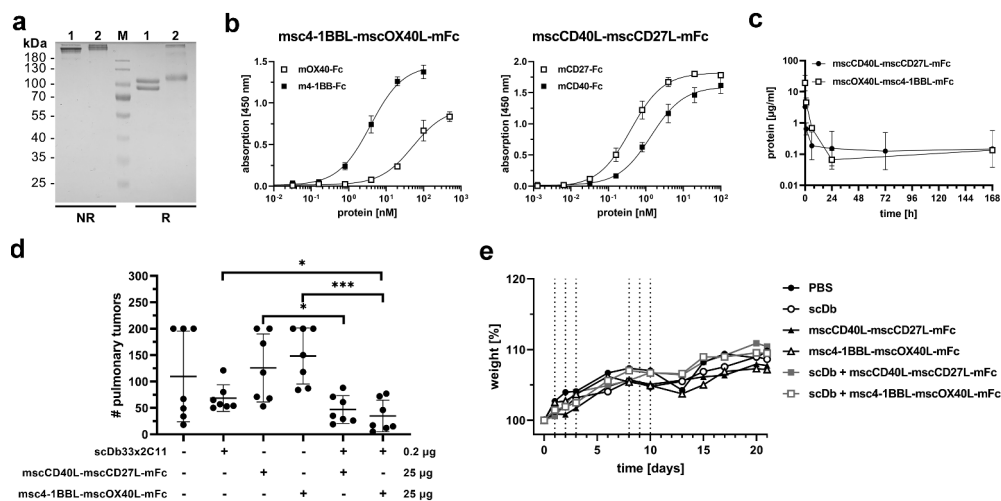
Figure 5. Pharmacokinetic and pharmacodynamics properties of scDuokine-Fc molecules. (a) SDS-PAGE analysis (12% PAA, 3 μg/lane, Coomassie blue staining) of murine surrogates (1) mscCD40L-mscCD27L-mFc and (2) msc4-1BBL-mscOX40L-mFc under non-reducing (NR) and reducing (r) conditions. M, protein marker. (b) Binding of msc4-1BBL-mscOX40L-mFc and mscCD40L-mscCD27L-mFc to immobilized murine receptors in ELISA. Mean ± SD, n = 3. (c) Female C57BL/6 N received 25 μg protein (i.v.) and blood samples were taken 3 min, 1 h, 6 h, 24 h, 72 h and 168 h after injection. Protein concentration in the serum was analyzed via ELISA with murine receptors. Mean ± SD, n = 3. (d) Murine scDk-Fc molecules were analyzed in a syngeneic lung tumor model in C57BL/6 N mice receiving FAP-expressing B16 tumor cells, i.v. at day 0 followed by treatment with i.p. injections of bispecific T-cell engaging single-chain diabody (scDb33x2C11) directed against human FAP and mouse CD3 either alone or in combination with murine scDk-Fc on day 1, 2, 3, 8, 9 and 10. As controls, murine scDk-Fc were injected without the scDb33x2C11. On day 21, lungs were removed and analyzed for tumor lesions. Mean ± SD, n = 7 mice/group, statistics: t-test. *p < .05, **p < .01, ***p < .001, ****p < .0001. (e) Changes of body weight in the different treatment groups.

Table 3. Pharmacokinetic properties of mouse surrogates of the scDuokine-Fc molecules. Initial half-life ($t_{1/2}\alpha$), terminal half-life ($t_{1/2}\beta$) and the areas under the curve (AUC) were determined over 168 hours. Mean ± SD, n = 3.

	$t_{1/2}\alpha$ [h]	$t_{1/2}\beta$ [h]	AUC [h*μg/ml]
msc4-1BBL-mscOX40L-mFc	1.4 ± 0.3	158.8 ± 3.1	48.1 ± 12.5
mscCD40L- mscCD27L-mFc	0.3 ± 0.1	307.4 ± 160.0	14.0 ± 2.1

Discussion

In this study, we demonstrated that dual-acting TNFSF ligand-based Fc fusion proteins either acting in cis or trans exhibit costimulatory activity. The scDuokine-Fc molecules can simultaneously bind to their two respective receptors, translating into enhancement of proliferation of CD4^{+} and CD8^{+} T-cells in presence of an anti-CD3 mAb in a suboptimal concentration. Furthermore, scDuokine-Fc molecules enhanced the anti-tumor activity of a bispecific antibody directed against FAP and CD3 in a B16-FAP mouse pulmonary tumor model.

Compared to the previously described Fc-less single-chain duokines (scDk),[13] we found that in ELISA binding of the scDk-Fc fusion proteins to their respective receptors was similar or slightly increased, indicating that fusion of the single-chain TNFSF members to separate Fc chains does not interfere with or even facilitate receptor binding. The ability to induce dual T-cell costimulation and proliferation was not affected by the new scDk-Fc format when compared to the Fc-less scDk format. However, drug exposure (AUC values) of the scDk-Fc fusion proteins after a single dose injection into mice were approximately 10-fold increased compared to Fc-less scDks previously described,[13] although both types of molecules were dominated by a short initial half-life.

Activating T-cells with agonistic antibodies against costimulatory receptors has the potential to unleash existing anti-tumor immune response, reduce tumor growth and increase survival in several tumor models.[8,21–23] However, some monoclonal antibodies have shown severe side effects like transaminitis resulting in hepatotoxicity-related deaths.[24] Presumably, FcγR interactions in the liver are responsible for the observed liver toxicity.[25–27] While monoclonal antibodies targeting costimulatory receptors need cross-presentation via Fcγ receptors,[28] activity of the scDk-Fc molecules in this study is not dependent on FcγR interaction. Therefore, mutations to abrogate interaction with FcγR avoid potential FcγR-mediated liver toxicity.[29]

The costimulation of T-cells with TNFSF ligands has been widely investigated for cancer therapy. 4–1BB stimulation has been shown to promote the formation of CD8^{+} memory T-cells,[30] restores the functionality of exhausted CD8^{+} T-cells, prolongs persistence of cytotoxic T-cells and enhances their function.[31,32] Additionally, the anti-tumor activity of OX40 mAbs is associated with the proliferation of effector T-cells at the tumor site[33] and stimulation of OX40 has been demonstrated to contribute to effector memory and central memory T-cell expansion.[34,35] In line with this, we found that dual costimulation with the sc4-1BBL-scOX40L-Fc scDuokine-Fc shifts the composition of CD8^{+} T-cells toward effector and effector memory T-cells compared to the stimulation with the CD3 mAb only. Interestingly, only small differences in the composition of CD4^{+} T-cell population were observed and proliferation of CD4^{+} T-cell was generally lower compared to CD8^{+} T-cells. In accordance, Lee et al. have shown that dual costimulation of OX40 and 4–1BB triggers CD8 effector functions to eliminate tumor cells even in the absence of CD4^{+} helper T-cells.[36]

Agonistic CD40-specific mAbs have been reported to induce cytotoxic T-lymphocyte-mediated anti-tumor effects by stimulating antigen-presenting cells.[37] Accordingly, treatment of MB49

(bladder carcinoma) tumors with an anti-CD40 mAb resulted in activation of intratumoral T-cells of the effector memory phenotype.[38] Furthermore, it has been shown that engagement of CD27 plays a critical role in CD8^{+} T-cell priming by CD40-licensed APCs[39] and that activation of CD27 is necessary for the expansion of memory CD8^{+} T-cells downstream of CD40.[40] Accordingly, our data show that dual costimulation with the scCD40L-scCD27L-Fc scDuokine-Fc resulted in proliferation of all CD8^{+} T-cell subtypes, shifting the composition of the population toward CD8^{+} effector and effector memory T-cells.

In this study, the murine surrogate molecules mscCD40L-mscCD27L-mFc and mscOX40L-msc4-1BBL-mFc exhibited a rather rapid initial clearance despite the presence of an Fc-region. This might be due to binding to immune cells resulting in target-mediated disposition. In line with this, it has been shown that the murine hexavalent CD40 agonist HERA-CD40L exhibited a 20-fold shorter half-life compared to the corresponding human molecule which is not able to bind to the murine receptor.[41] Regardless, the anti-tumor activity of both murine surrogate scDk-Fc molecules was demonstrated in a B16-FAP tumor model in combination with a bispecific antibody (scDb33x2C11), indicating that an immune response can efficiently be induced in vivo despite the short serum half-life. Recent studies suggest that the observed fast-in-fast-out pharmacokinetic profile of the scDk-Fc might be beneficial, as the over-stimulation and exhaustion of immune cells by prolonged exposure to a co-stimulatory ligand can be circumvented by a short serum half-life.[42–46]

The well-established melanoma B16-FAP lung tumor leson model[17,18] was used in the present study to demonstrate efficacy of scDk-Fc molecules. The available murine scDk-Fc surrogates allow testing in alternative syngeneic tumor models to further explore the potential of our cis- or trans-activating scDk-Fc. This includes subcutaneous and orthotopic tumor models, for example using mouse colon or breast carcinoma cell lines (e.g. CT26, MC38, 4T1).[47] However, cancer immunotherapy with our dual-acting costimulatory molecules requires a primary stimulus, e.g. through a bispecific T-cell engager as used in the present and previous studies.[13,18] Therefore, bispecific antibodies have to be developed targeting an antigen on the syngeneic tumor cell lines, e.g. transfected to express a respective target antigen. Further in vitro and in vivo studies may also include a direct comparison of the previously established Fc-less scDks with scDk-Fc molecules, to further evaluate effects of half-life, molecular composition and size on their costimulatory potentials as well as the contribution of CD4^{+} and CD8^{+} T-cell populations to the antitumor activity.

Importantly, single treatment with the human scDk-Fc molecules as well as the murine surrogates have not shown any induction of T-cell proliferation in vitro or in anti-tumor response in vivo. In line with previous studies investigating costimulatory antibody-ligand fusion proteins comprising the ligands 4–1BBL and OX40L,[18,48] T-cell costimulation with the scDk-Fc molecules requires a primary activation signal potentially lowering the risk of uncontrolled systemic T-cell activation. Thus, we have demonstrated that scDk-Fc comprising costimulatory ligands of the TNF superfamily are potent immune-stimulatory molecules enhancing T-cell proliferation resulting in an increased anti-tumor activity in combination with a bispecific antibody.

ORCID

Nadine Aschmoneit http://orcid.org/0000-0002-9385-9740
Katharina Kocher http://orcid.org/0000-0003-2331-3838
Lennart Kühl http://orcid.org/0000-0002-6887-8923
Oliver Seifert http://orcid.org/0000-0003-1876-4212
Roland E. Kontermann http://orcid.org/0000-0001-7139-1350

Author contributions

N.A., M.S., M.S.L., O.S., L.K. and K.K. performed cloning, protein expression and purification, biochemical analysis, binding studies, bioactivity assays and in vivo studies. N.A., M.S., L.K. and R.E.K. analyzed and interpreted the data. N.A., M.S., L.K. and R.E.K. were responsible for experimental design and supervised the work. N.A. and R.E.K. wrote the manuscript. All authors read and approved the final manuscript.

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