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ORIGINAL RESEARCH

Duokines: a novel class of dual-acting co-stimulatory molecules acting in cis or trans

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ABSTRACT

Co-stimulatory signals induced by ligands of the tumor necrosis factor superfamily (TNFSF) play a central role in T cell activation and have emerged as a promising strategy in cancer immunotherapy. Here, we established a novel class of bifunctional co-stimulatory fusion proteins with the aim to boost T cell activation at the level of T cell – antigen-presenting cell (APC) interaction. These novel dual-acting cytokine fusion proteins were created by connecting two different homotrimeric TNFSF ligands to form homotrimeric bifunctional molecules (Duokines) or by connecting single-chain derivatives of two different homotrimeric TNFSF with a single, flexible linker (single-chain Duokines, scDuokines). By linking the TNFSF ligands 4-1BBL, OX40L and CD27L in all possible combinations, cis-acting Duokines were generated that act on the same or adjacent T cells, while combining CD40L with 4-1BBL, OX40L and CD27L resulted in trans-acting Duokines acting simultaneously on APCs and T cells. *In vitro*, co-stimulation of T cells was seen for cis- and trans-acting Duokines and scDuokines in an antigen-independent as well as antigen-specific setting. Trans-acting molecules furthermore activated B cells, which represent a subclass of APCs. In a pilot experiment using the syngeneic B16-FAP mouse tumor model scDuokines displayed antitumoral activity in vivo in combination with a primary T cell-activating bispecific antibody, evident from reduced number of lung metastasis compared to the antibody-only treated group. Our data show that the bifunctional, co-stimulatory duokines are capable to enhance T cell-mediated anti-tumor immune responses, suggesting that they can serve as a new class of immuno-stimulatory molecules for use in cancer immunotherapy strategies.

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Introduction

Immunotherapy has emerged as a new modality for cancer treatment.[1] Therapeutic concepts to boost an anti-cancer immune response rely either on inhibiting immuno-suppressive activities or (co)-stimulating an immune response, or a combination of both, acting at different levels of the cancer-immunity cycle.[2,3] Several immune check-point inhibitors to "release the brake" on the immune response are meanwhile approved for cancer therapy, e.g. antibodies directed against CTLA-4, PD-1, or PD-L1.[4]

Besides co-inhibitory signals, co-stimulatory receptors and ligands are crucially contributing to the complex regulation network that controls the development of a functional immunity, including not only T cell activation, but also survival and the formation of effector and memory phenotypes.[4] During a primary T cell response, various signals are required at distinct stages of the immune response, controlled by an up – or down-regulation of receptors and ligands, depending on the dynamics of the inflammatory environment, cell subtypes and timing. This complex spatiotemporal expression of co-stimulatory modulators influences the progress of immune activation both quantitatively and qualitatively. Ligand and receptor interactions initially occur between T cells and APCs or B cells, but with ongoing activation ligands can get up-regulated on T cells and signaling between T cells arises.[5,6]

Various agonistic antibodies against co-stimulatory receptors are in development, including antibodies directed against CD40, CD27, OX40, 4-1BB and GITR, acting either on T cells or on antigen-presenting cells.[7–9] These receptors belong to the TNF receptor superfamily (TNFRSF), which are activated by binding of their cognate ligands of the TNF superfamily (TNFSF). However, efficient activation of the receptors often requires receptor clustering induced by binding of more than one ligand displayed on the cell membrane.[10,11] Here, antibodies are limited due to their bivalent binding mode and require binding to nearby Fcγ receptor-bearing cells for clustering and signal induction.

Fusion proteins comprising a soluble form of a TNFSF ligand fused to an antibody fragment directed against an antigen on a target cell have emerged as a possibility to mimic cell membrane-expressed ligands for target-dependent receptor clustering.[12–15] We and others have shown that this can result in an increased anti-tumor immune response, e.g. in combination with T-cell retargeting bispecific antibodies.-[16–20] However, although this approach incorporates a targeted delivery and accumulation in the tumor tissue, activation is restricted to a single receptor type.

Here, we now developed a novel strategy utilizing dual-acting molecules capable of binding and activating two different

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receptors either on the same cell type (i.e. acting in cis) or on different cell types (i.e. acting in trans) as co-stimulatory molecules. By combining important regulators in T cell co-stimulation and APC maturation, a novel tool exploiting the spatial proximity of T cells and antigen-presenting cells and differential up-regulation of TNFRSF receptors on primed T cells was generated. To this end, we fused two members of the TNFSF (CD40L, CD27L, OX40L, 4-1BBL) to produce either homotrimeric, bifunctional molecules or single-chain derivatives thereof, so-called Duokines and single-chain Duokines (scDuokines), collectively written as duokines. Functionality in terms of receptor binding and activation was analyzed *in vitro*, for example, with human PBMCs, demonstrating dual-acting immuno-stimulatory activity. Furthermore, induction of an increased anti-tumor activity was evaluated in a mouse tumor metastasis model in combination with a T-cell retargeting bispecific antibody as primary immune stimulus.

Results

General properties of duokines and scduokines

The tumor necrosis factor superfamily ligands CD40L, CD27L, 4-1BBL and OX40L were used for the generation of novel bifunctional co-stimulatory cytokine fusion proteins. These novel fusion proteins were named duokines, a neologism for dual-acting cytokines. Duokines were designed with a modular architecture simplifying the exchange and arrangement of the incorporated TNFSF ligands. The extracellular domains (ECD) of two different human TNFSF ligands (CD40L, aa 116 – 261; CD27L, aa 52 – 193; 4-1BBL, aa 71 – 254; OX40L, aa 51 – 183) were connected by a glycine-serine-rich linker (Figure 1A) and the intermolecular homotrimerization mediated by the ligands resulted in a bifunctional homotrimeric hexavalent molecule, the Duokine (Figure 1B). The length of the connecting linker was chosen with respect to the molecular shape of the incorporated ligands, i.e. $(GGGGS)_4$ if involving 4-1BBL as one partner and $(GGGGS)_3$ in the other cases. Furthermore, duokines were also generated in a single-chain format (scDuokines) by connecting the single-chain versions of two TNFSF ligands via a 15 amino acid $(GGGGS)_3$ linker (Figure 1A),[20] thereby creating a bifunctional hexavalent single-chain molecule (Figure 1B). By incorporating two different TNFSF ligands, two modes of action (cis-acting and trans-acting) were realized. Duokines combining two of the ligands CD27L, 4-1BBL and OX40L are targeting receptors (CD27, 4-1BB, OX40) expressed on T cells and were consequently specified to be cis-acting, irrelevant whether they were targeting two receptors on the same T cell or on two adjacent T cells. Duokines

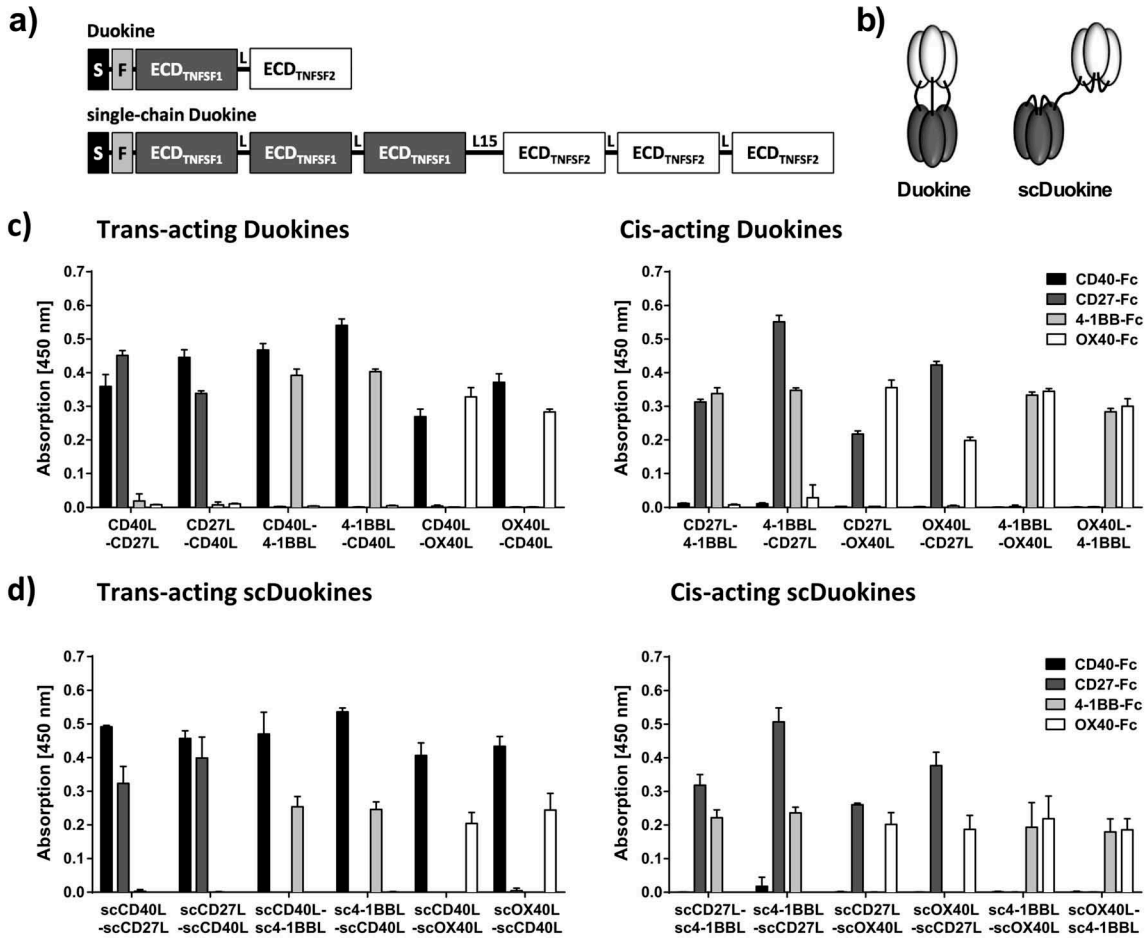

Figure 1. Dual-acting co-stimulatory molecules bind TNFRSF receptors. **(a)** Molecular composition and **(b)** schematic assembly of homotrimeric hexavalent Duokines and monomeric hexavalent scDuokines. S, signal sequence; F, FLAG tag; ECD, extracellular domain; L, linker. Both **(c)** Duokines (100 nM) and **(d)** scDuokines (5 µg/mL) bind to CD40-, CD27-, 4-1BB and OX40-Fc (200 ng) in ELISA. Mean ± SD, n = 3. Duokines with block-shift correction.

combining CD40L with one of the ligands CD27L, 4-1BBL and OX40L are targeting receptors expressed on T cells (CD27, 4-1BB, OX40) and CD40 expressed on e.g. antigen-presenting cells and B cells, thus are trans-acting between two different cell types. In summary, CD27L, 4-1BBL and OX40L were all combined in both molecular formats and with both possible arrangements of ligand order, resulting in six cis-acting Duokines and six cis-acting scDuokines. Likewise, CD40L was combined with each CD27L, 4-1BBL and OX40L in both possible orientations and both protein formats, resulting in six trans-acting Duokines and six trans-acting scDuokines. Overall, 12 different Duokines and 12 different scDuokines were generated (see also Supplementary Table 1).

All fusion proteins were designed with an N-terminal FLAG-tag for affinity chromatography-based purification from the supernatant of stably transfected HEK293T cells. Duokines were produced with average yields of 0.5 to 1.9 mg and scDuokines with average yields of 1.4 to 4.0 mg per liter culture supernatant. Protein purity was confirmed in SDS-PAGE analysis under non-reducing conditions, with all proteins appearing as major signals corresponding to the size of their monomers (Fig. S1a-b). Size exclusion chromatography under native conditions confirmed the correct assembly of Duokines into homotrimeric molecules and scDuokines into monomeric molecules with only minor fractions of higher-order complexes (Fig. S1c-d), indicating rather homogeneous protein configurations regardless of molecule format and ligand composition.

Monospecific binding of the Duokines and scDuokines to their respective TNFRSF receptors was analyzed in ELISA. All 24 recombinant fusion proteins showed specific ligand-receptor binding and no cross-reactivity with other TNFRSF receptors was observed (Figure 1c-d). Binding properties were in depth characterized by determining half-maximal effective concentrations in the low nanomolar range for the monospecific interaction of Duokines and scDuokines with their receptors (Table 1). The EC_{50} values ranged between 0.5 and 8.7 nM, with only three exceptions: CD40L-CD27L binding to CD40 (12.3 ± 3.8 nM), scCD27L-scOX40L binding to OX40 (14.3 ± 1.2 nM) and scOX40L-scCD27L binding to OX40 (60.2 ± 43.6 nM). Comparison with the EC_{50} values determined for binding of the soluble homotrimeric ligands and monomeric single-chain ligands confirmed that incorporation of TNFSF ligands into Duokines and scDuokines conserved or even strengthened their receptor binding capacities. Furthermore, simultaneous binding of the Duokines and scDuokines to both receptors was verified by applying an assay where the binding of one incorporated ligand to receptor-expressing target cells was detected via the interaction of the second incorporated ligand with its receptor presented as soluble Fc-fusion protein (Fig S2).

The *in vitro* stability of the novel Duokine and scDuokine protein formats was assessed by incubation in human serum at 37°C. Most of the Duokines retained 30% or more of their binding activity after 7 days, with the exception of three Duokines, all comprising CD27L, with remaining 10% activity after 7 days (Fig. S3a). In contrast, the plasma stability of scDuokines was more consistent with on average 24–58% intact protein remaining after 7 days (Fig. S3b). This finding indicated a stabilizing effect for some of the TNFSF members after conversion into a single-chain derivative.

Bioactivity of Duokines and single-chain Duokines (using the orientation with favorable integrity, stability and receptor binding, thereby reducing the total number of tested proteins to 6 Duokines and 6 scDuokines) was investigated using HT1080 cells stably transfected with CD40, CD27, 4-1BB or OX40 as reporter cell lines. Upon ligand binding, activated TNFRSF receptors induced NF-kB signaling, which resulted in measurable IL-8 release into the supernatant (Fig. S4).[21] In their soluble homotrimeric form, neither CD27L, 4-1BBL nor OX40L induced IL-8 release, but both CD40L and scCD40L as well as the other single-chain variants scCD27L, sc4-1BBL and scOX40L resulted in receptor activation. While the single-chain ligands predominantly required higher protein concentrations, the conversion of the ligands in both the Duokine and scDuokine format clearly enhanced receptor activation properties (Fig. S4). IL-8 release and therefore receptor activation was stronger for the single-chain Duokines (Fig. S4b), an effect especially prominent in case of targeting CD27 and 4-1BB, which were only weakly activated by Duokines. Bioactivity, as detected by induction of IL-8 release, was confirmed for all tested Duokines and scDuokines; sc4-1BBL-scCD40L induced strongest activation of both, CD40 and 4-1BB.

Immuno-stimulatory activity of scduokines

Because the single-chain Duokines appeared to be more stable and more bioactive, the immuno-stimulatory activity was analyzed *in vitro* for three trans-acting (scCD40L-scCD27L, sc4-1BBL-scCD40L, scOX40L-scCD40L) and two cis-acting (sc4-1BBL-scCD27L, scOX40L-scCD27L) scDuokines using freshly isolated PBMC. First, expression of the receptors CD40, CD27, 4-1BB and OX40 was measured on the various target cell types present in PBMC and the binding of scDuokines to these cell populations was identified. Regardless of pre-stimulation, CD40 and CD27 were constitutively expressed on all B cells and all T cells ($CD4^+$ and $CD8^+$), respectively. Moreover, about 30% B cells constitutively expressed CD27, too. In contrast, 4-1BB and OX40 were upregulated on both $CD4^+$ and $CD8^+$ T cells only upon CD3-mediated stimulation. Here, 4-1BB was predominantly upregulated on $CD8^+$ T cells, whereas OX40 was stronger induced on $CD4^+$ T cells (Figure 2A). In accordance with the observed receptor expression patterns, the three trans-acting scDuokines (scCD40L-scCD27L, sc4-1BBL-scCD40L and scOX40L-scCD40L) bound almost exclusively to B cells (Figure 2B). The trans-acting scCD40L-scCD27L targeting constitutively expressed receptors also bound to a minor fraction of T cells. In contrast, the cis-acting scDuokines were detected solely on T cells, with an increase in binding of scOX40L-scCD27L upon T cell activation, in accordance with the observed upregulation of OX40 under these conditions (Figure 2B). Generally, trans-acting scDuokines targeted B cells, while cis-acting scDuokines targeted activated $CD8^+$ and $CD4^+$ T cells.

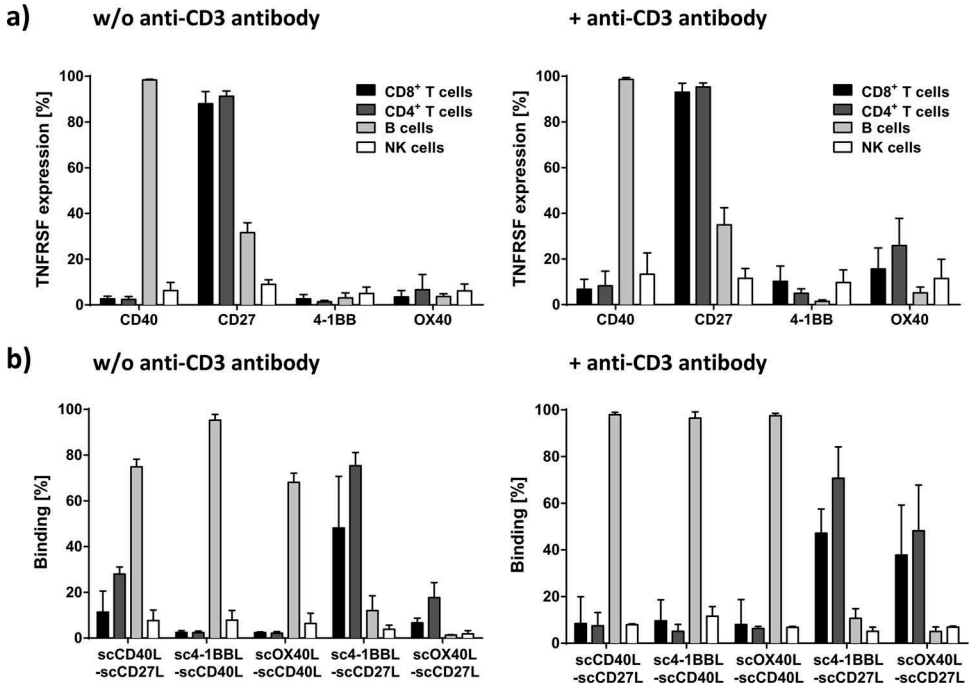

Figure 2. Selected scDuokines bind to human immune cells. **(a)** Subset populations of human bulk PBMCs were analyzed for expression of TNFRSF receptors with or without antigen-unspecific stimulation via an anti-human CD3 antibody (UCHT-1). **(b)** Binding of five different trans- and cis-acting scDuokines (10 nM) to the immune cell populations was analyzed by flow cytometry. Mean ± SD, n = 3 different PBMC donors.

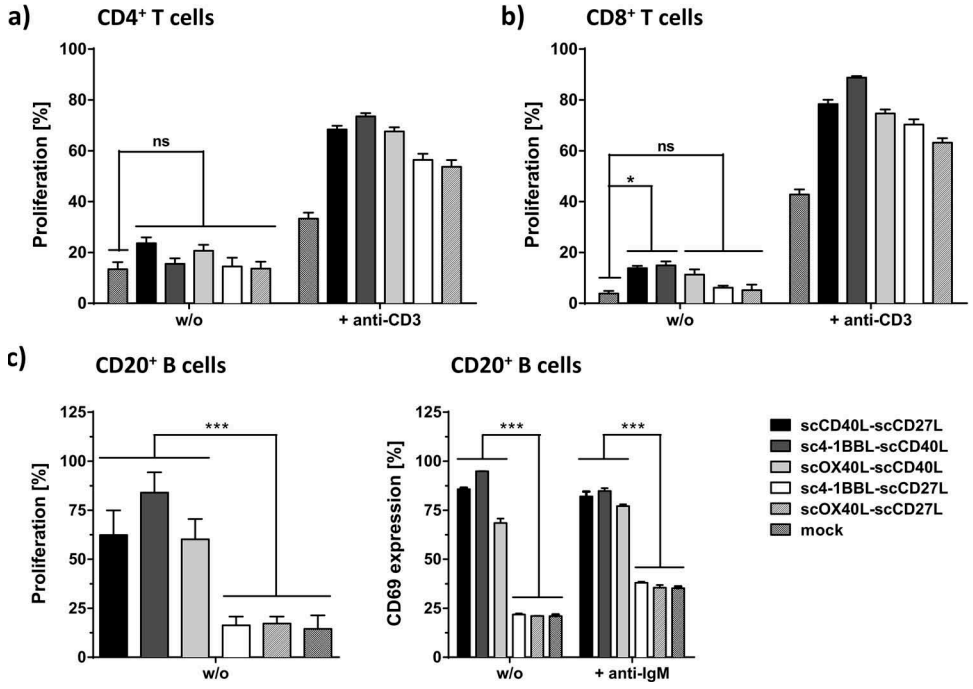

Figure 3. Selected scDuokines activate unstimulated and polyclonally prestimulated T cells and B cells. Bulk PBMC populations (3 donors) were incubated with trans- and cis-acting scDuokines (30 nM) in presence or absence of an anti-human CD3 antibody (UCHT-1) or coated anti-human IgM antibody. Proliferation of **(a)** $CD4^+$ T cells, **(b)** $CD8^+$ T cells and **(c)** $CD20^+$ B cells was analyzed after 6 days by flow cytometry. Expression of the activation marker CD69 on $CD20^+$ B cells was assessed after 24 h by flow cytometry. Mean ± SD, n = 3, block-shift correction, One-Way ANOVA.

All trans-acting scDuokines were able to activate B cells as determined by upregulation of the activation marker CD69 and proliferation induction. ScCD40L-scCD27L, sc4-1BBL-scCD40L and scOX40L-scCD40L increased the proliferation rate of $CD20^+$ B cells about 5-fold above the level of mock-treated cells, while no effects were observed for cis-acting scDuokines (Figure 3C). Strongest proliferation with 84% proliferating B cells was observed for sc4-1BBL-scCD40L. Likewise, upregulation of CD69 was promoted only by trans-acting but not cis-acting scDuokines (Figure 3C), in

this case, independent of the presence of an immobilized anti-IgM antibody, which is a known polyclonal activator of B cells (Figure 3C).

The immuno-stimulatory activity of scDuokines was assessed by measuring proliferation of $CD4^+$ and $CD8^+$ T cells induced by scDuokines alone or together with a primary CD3 stimulus. In combination with this antigen-independent stimulation, all analyzed trans-acting and cis-acting scDuokines enhanced proliferation of $CD8^+$ T cells up to 2-fold (Figure 3B). Strongest effects were achieved with the trans-acting sc4-1BBL-scCD40L leading to 89% proliferating $CD8^+$ T cells, while the cis-acting scOX40L-scCD27L resulted in a lower proliferation rate of only 63%. Without CD3-stimulation, the basal proliferation of $CD8^+$ T cells was neither enhanced by sc4-1BBL-scCD27L nor scOX40L-scCD27L, indicating that cis-acting scDuokines do not exert their co-stimulatory potential on resting $CD8^+$ T cells, although binding to T cells via CD27 was detected. In contrast, trans-acting scCD40L-scCD27L, sc4-1BBL-scCD40L and scOX40L-scCD40L activated resting $CD8^+$ T cells (Figure 3B). Despite resulting in only a minor portion of cells (11–14%) entering cell cycle upon trans-acting scDuokine treatment, this represents a significant ($p < 0.05$) 3-fold increase in proliferation compared to the basal unstimulated level. This indicated a trans-activation potential of trans-acting scDuokines for resting $CD8^+$ T cells via crosstalk with CD40-expressing B cells. Similar to $CD8^+$ T cells, CD3-stimulated $CD4^+$ T cells responded to all scDuokines, although the co-stimulatory effects were somewhat lower (Figure 3A).

Similar observations were made in an antigen-specific, MHC-dependent T cell proliferation assay, where $CD8^+$ T cells electroporated with IVT-RNA encoding a claudin-6 (CLD6) specific TCR have been co-cultured with donor-identical dendritic cells (DCs) electroporated with IVT-RNA encoding the corresponding antigen (CLD6). Here, Duokines represented by trans-acting 4-1BBL-CD40L and cis-acting 4-1BBL-CD27L further increased the T cell proliferation induced by specific antigen recognition on DCs, while the individual homotrimeric ligands or a mixture thereof did not (Figure 4). T cells electroporated with an irrelevant TCR were only weakly activated by the trans-acting 4-1BBL-CD40L, indicating a potential minor stimulatory activity of this Duokine for resting T cells (Figure 4B), similar to unstimulated T cells in the MHC-independent setting that were slightly activated by trans-acting scDuokines (Figure 3B).

In summary, both trans-acting and cis-acting scDuokines exerted co-stimulatory activity on $CD4^+$ and $CD8^+$ T cells, irrespective whether stimulated in an antigen-specific TCR-mediated or an antigen-unspecific CD3-mediated manner. Furthermore, *de novo* T cell-stimulatory activity was observed to some degree for two trans-acting scDuokines but not cis-acting scDuokines, which appeared to be linked to the fact that trans-acting scDuokines can bind to CD40-expressing APCs leading to crosslinking with T cells.

Duokines enhance the antitumor activity of a bispecific t-cell recruiting antibody

The TNFSF ligands used here for generation of duokines are only partially inter-species cross-reactive. Therefore, two mouse-specific scDuokines (trans-acting msc4-1BBL-mscCD40L and cis-acting msc4-1BBL-mscCD27L) were generated. The murine homologs were produced in HEK293T cells, purified via FLAG affinity chromatography and characterized as described for the human scDuokines. The therapeutic potential of the murine scDuokines was investigated *in vivo* using a syngeneic lung tumor model with human FAP-expressing B16 cells in C57BL/6N. The treatment protocol comprised a suboptimal dose of a bispecific single-chain diabody targeting human FAP and mouse CD3 (scDb332C11)[17,22,23] as primary MHC-independent T cell activation signal and the murine scDuokines as potential co-stimulatory signal. Groups of six C57BL/6N mice challenged

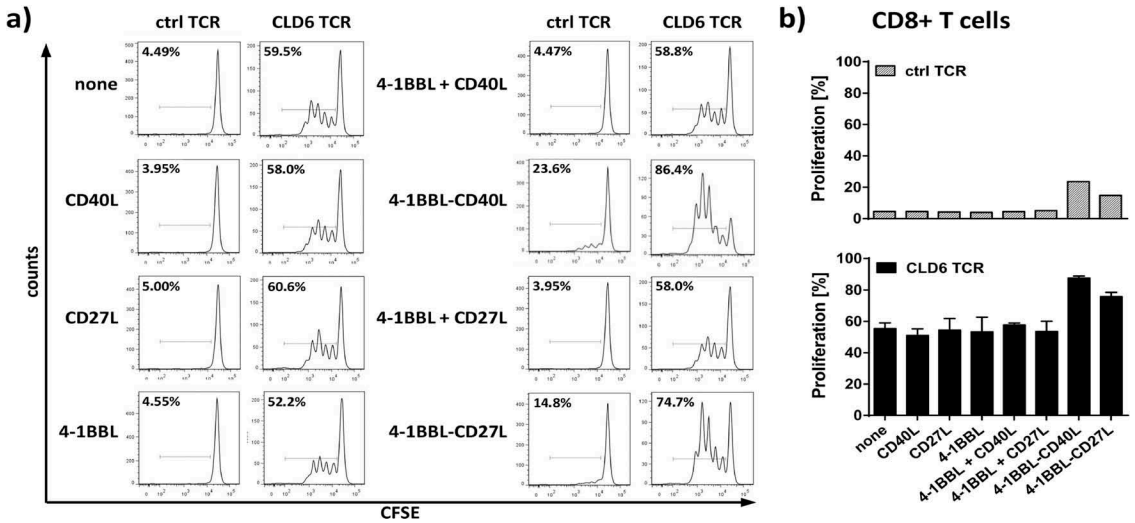

Figure 4. Selected Duokines enhance antigen-specific proliferation of $CD8^+$ T cells. Immature DCs (5,000/well) from HLA-$A2^+$ donors electroporated with CLD6 or irrelevant mRNA were co-cultured with CFSE-labeled $CD8^+$ T cells (50,000/well) from the same donor electroporated with CLD6-TCR or irrelevant TCR mRNA. 10 nM Duokines (4-1BBL-CD40L, 4-1BBL-CD27L) or corresponding control proteins were used for co-stimulatory treatment. After 4 days incubation at 37°C, 5% CO_2, proliferation was assessed by measuring CFSE dilution in flow cytometry. **(a)** Representative histograms and **(b)** Mean ± SD, n = 3.

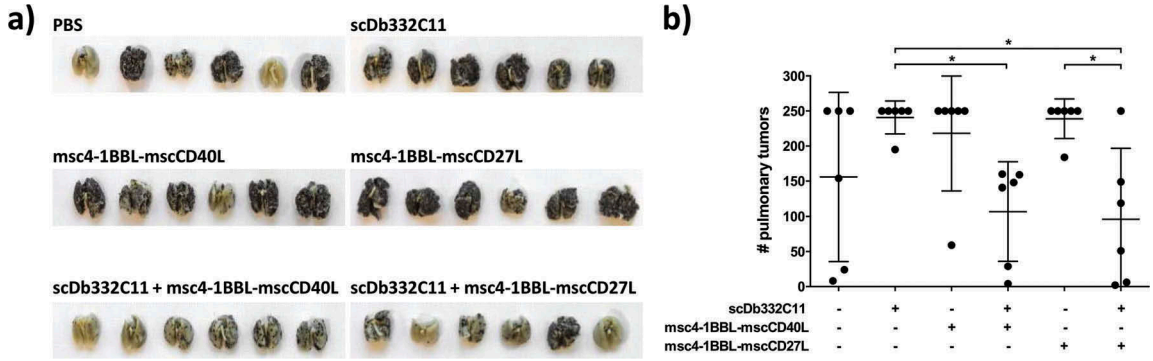

Figure 5. Selected scDuokines enhance the antitumoral activity of a T cell-recruiting antibody. One murine trans-acting scDuokine (msc4-1BBL-mscCD40L) and one murine cis-acting scDuokine (msc4-1BBL-mscCD27L) were analyzed in a syngeneic lung tumor model in C57BL/6 mice. Mice received human FAP-expressing B16 tumor cells i.v. at day 0 and were treated by i.p. injections of a bispecific T cell-recruiting single-chain Diabody (33C11) directed against human FAP and mouse CD3 either alone or together with the scDuokines. For control, scDuokines were injected without bispecific diabody at days 1, 2, 3, 8, 9 and 10. At day 21, **(a)** lungs were removed and **(b)** analyzed for tumor count. Lungs with more than 250 tumor lesions were considered uncountable and assigned with the fixed value of 250. Mean ± 95% CI, n = 6 mice/group, One-way ANOVA.

with B16-FAP (i.v.) were treated with combinations of scDb33x2C11 (4 pmol) and one mscDuokine each (0.2 nmol) at three early (day 1, 2, 3) and three late (day 8, 9, 10) time points, or PBS as negative control. 21 days after tumor inoculation, the mice treated with scDb33x2C11 only showed a very high tumor burden with lungs completely covered by black tumors (Figure 5A). In contrast, the combination treatment of either msc4-1BBL-mscCD40L or msc4-1BBL-mscCD27L together with the bispecific antibody clearly reduced tumor formation by 55–60%. No significant differences between the trans- and cis-acting mscDuokine were observed in this particular model (Figure 5B). Similar to the bispecific antibody, when applied as monotherapy, the mscDuokines exerted no therapeutic activity, as predicted from their action as co-factor in T cell activation. In summary, the *in vivo* study proved the ability of both trans- and cis-acting scDuokines to enhance anti-tumor activity of a bispecific T cell-recruiting antibody.

Discussion

Here we show that two members of the TNFSF can be fused by a short flexible linker to generate bifunctional immunostimulatory molecules. This arrangement is facilitated by the fact that the N- and C-termini of the subunits (protomers) forming the homotrimeric ligand are located on the same side of the molecule, which allows to link the C-terminus of the subunit of the first ligand to the N-terminus of the second ligand positioning the two ligands in an orientation with the receptor binding sites facing away from each other. Structurally, the TNFSF members employed in the present studies belong either to the conventional group (Type L) (CD40L) or the divergent group (Type V) (CD27L, OX40L, 4-1BBL).[24,25] While the conventional group members assemble into a very similar bell-shaped structure, the divergent group is not only characterized by a low sequence homology, but also by a structural diversity in the arrangement of the TNF homology domain (THD) in the homotrimeric structure, resulting in different positions of the N- and C-termini. For the construction of the Duokines, we have chosen linkers with a length of 15 or 20 residues (if 4-1BBL was part of the Duokine) for connecting the two THDs allowing sufficient flexibility to join the two ligands without introducing sterical constraints and loss of activity. Conversion of the homotrimeric ligands into single-chain derivatives further allowed generating scDuokines, with the single-chain ligands connected by a single, flexible linker. Compared to Duokines, this results in increased flexibility. Furthermore, conversion into single-chain derivatives can increase the stability of the individual ligands, as shown recently for TRAIL[26] by choosing appropriate linkers and the minimally required THD sequence. Comparing the plasma stability of the different Duokines and scDuokines, we did not observe a drastic improvement of stability for the scDuokines, except for some of the molecules, e.g. comprising CD27L and CD40L or CD27L and 4-1BBL. In some cases, the Duokines were more stable than the scDuokine derivatives, which might be due to the presence of three connecting linkers between the two ligands instead of one, potentially resulting in a more rigid structure. Further engineering of the linkers and THD domains can be applied to improve the stability of the scDuokines but also the Duokines.

All duokines retained the specificity for their respective receptors and exhibited dual activity as shown by receptor binding and activation. Using human PBMCs, we could also show that, in line with the mode of action, the trans-acting scDuokines were capable of stimulating T cells and B cells, while cis-acting scDuokines were co-stimulatory active only on T cells. Thus, Duokines and scDuokines act as soluble mediators between two different co-stimulatory receptors, mimicking the co-stimulatory signals between T cells and APCs, either requiring the presence of APCs or acting APC-independent. Using ligands against different receptors with either constitutive (CD40, CD27) or inducible (4-1BB, OX40) expression, different steps in the process of T-cell activation can be costimulated. Our *in vitro* studies with human PBMCs and the *in vivo* study in a mouse tumor model using duokines binding either to two constitutively expressed receptors (CD40 and CD27) or to one constitutively expressed (CD40 or CD27) and one inducible receptor (4-1BB or OX40)

revealed the co-stimulatory activity of both types of Duokines for CD3-stimulated T cells.

Further, enhancement of antigen-specific T cell proliferation was observed for a trans-acting Duokine (4-1BBL-CD40L) and a cis-acting Duokine (4-1BBL-CD27L) compared to application of single ligands, which failed to enhance proliferation. In this *in vitro* model, a slight stimulation was noted for the control-transfected cells, indicating that duokines may exhibit some TCR-independent co-stimulatory activity on T cells. However, *in vivo*, in a first pilot experiment using the B16-FAP tumor model, we showed that trans- and cis-acting scDuokines enhanced the antitumor activity only in the context of concomitant TCR triggering, i.e. via a bispecific T-cell recruiting antibody directed against FAP and CD3. In a previous study, using the same mouse tumor model, we showed that treatment with different formats of tumor-targeted co-stimulatory ligands (scFv-4-1BBL, B7.1-Db) only resulted in a marginal anti-tumor effect by themselves, but, as shown here for the duokines, strongest responses were observed in combination with a bispecific T-cell recruiting antibody.[17] Thus, both studies support the concept of an important role of co-stimulatory ligands for effective cancer immunotherapy. Certainly, further studies in this and other tumor models, investigating various duokines as co-stimulators, the role of dosage and combination with different primary stimuli, e.g. bispecific antibodies versus tumor vaccination, are necessary to confirm the presented results. Thereby, optimum conditions of inducing effective antitumoral responses and the full therapeutic benefit of the new class of co-stimulators described herein can be revealed. These studies should also resolve duokine pharmacokinetic profiles and the immuno-pharmacodynamic effects on different immune cell subsets.[27]

Compared to alternative strategies, such as antibodies against co-stimulatory members of the TNFRSF, duokines do not require a secondary cross-linking. In this context, trans-acting duokines will be cross-presented between two different cells, thus mimicking membrane-displayed ligands, generating a multivalent surface and acting bidirectional. Although we cannot exclude a trans-presentation of cis-acting duokines as well, e.g. between T cells or other cells expressing one of the relevant receptors, cis-acting duokines have the potential of multivalent receptor interaction on the same cell, which may favor a strong T cell activation. Indeed, because many of the trivalent TNFSF ligands themselves are poorly active, our finding with cis-acting duokines is in accordance with the view that not only signal cross-talk between the two involved receptors occurs, but their recruitment into larger heteromeric signal complexes may occur. Promiscuous binding of TRAFs (e.g. TRAF2, TRAF3) and activation of different pathways, such as PI3K, JNK and NF-KB, has been described for many of the co-stimulatory receptors, including CD27, OX40 and 4-1BB.[28,29] Thus although not naturally cross-linked by individual co-stimulatory ligands we propose that duokine-mediated clustering of different receptors results in potent activation of downstream signals of commonly used pathways.

Importantly, by addressing different receptors dual co-stimulation can be achieved. For example, dual co-stimulation of OX40 and 4-1BB with agonistic antibodies enhanced IL-2 signaling in $CD4^+$ T cells responding to cognate antigen. Besides eliciting a CD8 T cell-dependent tumor immunity, OX40 co-stimulation programmed CD4 T cells to express cytotoxic functions, while 4-1BB-costimulation maximized clonal expansion of the cytotoxic Th1 effector cells.[30] In line with this, simultaneous co-stimulation with agonistic anti-OX40 and anti-4-1BB antibodies resulted in synergistically induced CD8 T cell clonal expansion and induction of a massive burst of T cell effector functions.[31] Also in form of co-stimulatory ligands, co-application of tumor-directed antibody-fusion proteins with 4-1BBL and OX40L have shown to be effective in enhancing T cell stimulation in terms of proliferation and cytotoxic potential.[16]

Duokines might find various therapeutic applications by boosting naturally evolving or therapeutically induced immune responses. Various cancers, such as melanoma, are highly immunogenic and therapy might benefit from further stimulating this immune response. Here, one can also envisage a combination with immune check-point inhibitors thus allowing to block inhibitory signals and to boost the released immunological responses.[32] Alternatively, duokines can be combined with vaccination strategies or approaches of adoptive immune therapy, such as CAR-T cells or bispecific T cell-engaging molecules.[33] Duokines are not restricted to cancer therapy but might also be beneficially applied in other indications, such as viral infections, especially those being latent or chronic in nature.[34,35] Because of their modular structure, the duokine strategy described here allows combining different members of the co-stimulatory TNFSF family and thus, enables to specifically tailor the immunological responses to the therapeutic requirements.

Materials and methods

Materials

Antibodies were purchased from BioLegend (APC anti-human CD20, 302,309; PE anti-human CD69, 310,905; PE anti-human CD40, 334,307; PE anti-human CD27, 302,807; PE anti-human 4-1BB, 309,804; PE anti-human OX40, 350,003), KPL (goat anti-mouse IgG H + L, 01–10-06), Miltenyi Biotec (anti-CD3 FITC, 130–098-162; anti-CD4 VioBlue, 130–099-683; Anti-CD8 PE-Vio770, 130–098-060; Anti-CD56 PE-Vio770, 130–098-132; anti-FLAG PE, 130–101-576), R&D Systems (anti-human CD3ϵ UCHT1′ MAB100) and Sigma-Aldrich (anti-FLAG® M2-HRP, A8592; anti-human IgM HRP, A8786). CellTrace™ CFSE cell proliferation kit was purchased from Life Technologies (C34554) and human Interleukin-8 ELISA from ImmunoTools (31,670,089). FLAG peptide was obtained from peptides&elephants (EP01741) and Anti-FLAG® M2 affinity gel from Sigma-Aldrich (A2220). CD14 MicroBeads (130–050-201) for positive selection of human monocytes as well as hIL-4 (130–093-924) and hGM-CSF (130–093-868) for generation of immature DCs were purchased from Miltenyi Biotec. Human CD40-Fc, CD27-Fc, 4-1BB-Fc and OX40-Fc were generated in-house, as described in the following sections. Stable transfected HT1080-CD40, HT1080-CD27, 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, 11,875) with 10% FBS (PAN Biotech, 3302-P121707). B16-FAP (stable transfectants with human FAP, Klaus Pfizenmaier, University of Stuttgart) were cultured in RPMI 1640, 5% FBS supplemented with 200 µg/ml zeocin (Thermo Fisher Scientific, R25001). The production cell line HEK293T was cultured in RPMI 1640, 5% FBS. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of healthy donors (Klinikum Stuttgart or Universitätsklinikum Mainz, both Germany) by Ficoll density gradient centrifugation (Lymphocyte Separation Medium 1077, Promocell, C-44,010) and cultivated in RPMI 1640, 10% FBS. Human plasma was also obtained from healthy donors (Klinikum Stuttgart, Germany). C57BL/6NCrl mice were purchased from Charles River. Animal care, handling and experiments were in accordance with federal guidelines and approved by university and state authorities.

Cloning and production of recombinant proteins

The genes encoding the extracellular domains (ECD) of human CD40L (aa 116–261), human CD27L (aa 52–193), human 4-1BBL (aa 71–254) and human OX40L (aa 51–183) were codon-optimized for expression in human cells and synthesized by GeneArt (Thermo Fisher Scientific) either as single domain (TNFSF) or as single-chain variant (scTNFSF). Three identical ECDs were connected to single-chain variants using GGGSGGG linkers in the case of scCD40L, scCD27L and scOX40L, while $(GGGGS)_4$ linkers were used for sc4-1BBL. Duokines (TNFSF1-linker-TNFSF2) were generated by connecting two different TNFSF ligands with a $(GGGGS)_3$ or $(GGGGS)_4$ linker, with the longer linker being used in case of involvement of 4-1BBL. Similarly, single-chain Duokines (scDuokines, scTNFSF1-linker-scTNFSF2) were created by linking two single-chain TNFSF ligands via a GGGGSGGGGTGGGGS linker. For cloning of TNFRSF-Fc fusion proteins, the genes encoding the extracellular domains of human CD40 (aa 21–193), CD27 (aa 20–183), 4-1BB (aa 24–186) and OX40 (aa 29–214) were codon-optimized for expression in human cells and synthesized by GeneArt (Thermo Fisher Scientific). The ECDs were cloned N-terminally to the human Fcγ1 chain (hinge-CH2-CH3) via AgeI/NotI restriction sites. The DNA sequences encoding Duokines and scDuokines or TNFRSF-Fc fusion proteins were cloned into the mammalian expression vector pIRESpuro3 (Clontech, 631,619) or pSecTagA (Invitrogen™, Thermo Fisher Scientific, V90020), respectively. Recombinant proteins were produced in stably transfected HEK293T cells and purified from the cell culture supernatant via FLAG affinity chromatography (Duokines and scDuokines) or protein A affinity chromatography (TNFRSF-Fc fusion proteins) as described previously.[36]

Biochemical characterization of recombinant proteins

Purified proteins were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and stained with Coomassie Brilliant Blue G-250. Size exclusion chromatography (SEC) was performed as analytical gel filtration using a Waters 2695 HPLC equipped with a Yarra SEC-3000 column (Phenomenex, 00H-4513-E0). Proteins were analyzed in a 0.1 M Na_2HPO_4/NaH_2PO_4, 0.1 M Na_2SO_4, pH 6.7 mobile phase at a flow rate of 0.5 mL/min. Thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and FLAG peptide (1 kDa) served as standard proteins.

Elisa

TNFRSF-Fc fusion proteins (200 ng/well in PBS) were immobilized on 96-well ELISA plates overnight at 4°C followed by blocking with 2% (w/v) non-fat dry milk/PBS (MPBS). Purified proteins were serially diluted in MPBS and incubated with the coated receptors for 1 h at room temperature. Bound proteins were detected using HRP-conjugated anti-FLAG tag antibody and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Absorption was measured at 450 nm in a Tecan infinite M200 reader.

Flow cytometry

Generally, flow cytometry was performed using a MACSQuant Analyzer 10,MACSQuant VYB (both Miltenyi Biotec) or FACSCanto II (BD Biosciences) and in case of multi-color staining, spectral overlap was compensated prior to the experiment. For data analysis, MACSQuantify (Miltenyi Biotec) or FlowJo v7.6.5 (TreeStar) was used. If applicable, relative mean fluorescence intensities (MFI) were calculated as followed: relative MFI = $(MFI_{sample} - (MFI_{detection} - MFI_{cells}))/MFI_{cells}$.

For identification of leucocyte subpopulations ($CD3^+$, $CD4^+/CD8^+$ T cells; $CD3^-$, $CD20^+$ B cells; $CD3^-$, $CD56^+$ NK cells), detection of surface-expressed co-stimulatory receptors (CD40, CD27, 4-1BB, OX40) or activation markers (CD69), $2.5x10^4$ target cells (PBMCs) were incubated with fluorochrome-labeled monoclonal antibodies for 1 h at 4°C. Depending on the particular experiment, PBMCs were either kept untreated or pre-treated with cross-linked anti-human CD3 monoclonal antibody (T cell prestimulation) or plate-bound anti-human IgM antibody (B cell prestimulation).

Binding of Duokines or scDuokines to target cells (HT1080 transfectants or receptor-expressing leucocyte subpopulations) was determined by incubating $1.5–2.5x10^5$ cells with 10–100 nM Duokines or scDuokines for 1 h at 4°C. Cells were washed three times and bound fusion proteins were either detected directly with a PE-conjugated anti-FLAG antibody (monospecific binding) or indirectly via ligand-receptor interaction of the second Duokine/scDuokine binding site (bispecific binding). Therefore, the cells were incubated with 10 nM TNFRSF-Fc fusion proteins for 1 h at 4°C and detected with a PE-labeled anti-human Fc antibody.

Cytokine release assay

Bioactivity was evaluated in a reporter assay measuring the interleukin-8 (IL-8) release from TNFRSF-transfected HT1080

cells upon TNFRSF-dependent NF-KB activation. HT1080 ($2x10^{35}$ cells/100 µL) were grown in 96-well plates overnight at 37°C, 5% CO_2. The next day, culture medium was exchanged in order to remove constitutively produced IL-8 and cells were incubated with serial dilutions of Duokines, scDuokines and control proteins at 37°C, 5% CO_2. After 18 hours incubation, cell-free supernatants were collected and directly analyzed using a human IL-8 Sandwich ELISA kit (Immunotools) according to the manufacturer's instructions.

In vitro stimulatory activity: antigen-independent proliferation

Antigen-independent co-stimulatory activity was assessed by measuring proliferation of T cells and B cells in bulk PBMC populations. Therefore, PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 625 nM per 1×10^5 cells/mL according to the manufacturer's instructions and $1.5x10^4$ PBMCs per well were incubated with 30 nM scDuokines. Depending on the setting, T cells were activated with donor-specific suboptimal concentrations (3–11 ng/mL) of cross-linked anti-human CD3 antibody. After 6 days of stimulation, CFSE dilution was analyzed in flow cytometry. For identification of T and B cell populations antibody staining was performed as described above.

In vitro stimulatory activity: antigen-specific $CD8^+$ t cell proliferation

For analysis of antigen-specific $CD8^+$ T cell proliferation induced by Duokines, PBMCs were isolated from HLA-$A2^+$ buffy coats. $CD14^+$ monocytes were isolated from PBMCs using magnetic beads and immature dendritic cells (iDCs) were generated by stimulation with IL-4 and GM-CSF (each 1000 U/mL) over 4 days. Immature DCs were electroporated either with CLD6 or irrelevant mRNA as antigen and rested overnight. $CD8^+$ T cells were isolated from the same donor, electroporated either with CLD6-specific T cell receptor (TCR) mRNA or irrelevant TCR-mRNA and stained with CFSE. The next day, electroporated $CD8^+$ T cells (50,000/well) and iDCs (5,000/well) were co-cultured at an effector-to-target ratio of 10:1 and treated with 10 nM Duokines (4-1BBL-CD40L, 4-1BBL-CD27L) or control proteins. After 4 days of incubation at 37°C and 5% CO_2, $CD8^+$ T cell proliferation was assessed by measuring CFSE dilution in flow cytometry.

In vivo antitumor activity

Animal care and all experiments performed were in accordance with federal and European guidelines and have been approved by university and state authorities (Regierungspräsidum Stuttgart). B16-FAP cells ($1x10^5$ in 150 µL PBS) were injected intravenously into female C57BL/6NCrl mice (6 mice per group). As treatment, mice received six intraperitoneal injections of 200 pmol murine scDuokine (msc4-1BBL-mscCD40L or msc4-1BBL-mscCD27L) in combination with 4 pmol bispecific antibody (scDb332C11) on days 1, 2, 3 and days 8, 9, 10 after tumor cell engraftment. Additionally, mice were treated with the particular proteins alone or DPBS following the same treatment regimen. Mice were sacrificed 21 days after tumor cell inoculation. Lungs were removed, fixed in formaldehyde and pulmonary tumors were counted.

Plasma stability

200 nM Duokines or scDuokines were incubated in 50% human plasma at 37°C for 1, 3 or 7 days. Samples were frozen after preparation (0 d) or after the respective incubation time. Intact protein was determined in ELISA via binding to the TNFRSF-Fc fusion protein corresponding to the C-terminal ligand of the duokines and detection of the N-terminal FLAG-tag via HRP-conjugated anti-FLAG antibody. Protein concentrations were interpolated from a standard curve of purified protein and the average of detected intact protein at day 0 was fixed to 100%.

Statistical analysis

Unless stated otherwise, data are represented as mean ± SD of at least three independently performed experiments. If necessary, block shift correction was applied according to the formula $X'_n = X_n - (Y_n - Y)$ with X'_n being the corrected value of X from the experiment n, Y the average of the X values from all experiments performed and Y_n the average of the duplicate values of X from experiment n. One-way ANOVA followed by Tukey's post-test (Graphpad Prism 6) was used to calculate statistical significances from non-blockshifted data. p values below 0.05 were considered statistically significant (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

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Abbreviations

APC	antigen-presenting cell
ECD	extracellular domain
TCR	T-cell receptor
TNFSF	tumor necrosis factor superfamily
TNFRSF	tumor necrosis factor receptor superfamily (TNFRSF)

Disclosure statement

S.F., F.G., D.M., U.S., K.P. and R.E.K. are named inventor on a patent application covering the Duokine and scDuokine technology.

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