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The effects of affinity and valency of an albumin-binding domain (ABD) on the half-life of a single-chain diabody-ABD fusion protein

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Fusion of small recombinant antibody fragments to an albumin-binding domain (ABD) from streptococcal protein G strongly extends their plasma half-life. This ABD binds with nanomolar affinity to human (HSA) and mouse serum albumin (MSA). It was speculated that an increase in albumin-binding affinity should lead to a further increase in half-life. In the present study, we analyzed the effects of affinity and valency of the ABD on the pharmacokinetic properties of a bispecific single-chain diabody (scDb), applied previously to investigate various half-life extension strategies. The scDb is directed against carcinoembryonic antigen (CEA) and CD3 capable of mediating T cell retargeting to tumor cells. Two scDb derivatives with increased (scDb-ABD-H) and decreased (scDb-ABD-L) affinity as well as an scDb molecule fused to two ABD (scDb-ABD_2) were generated and produced in mammalian cells. The altered binding of these constructs to HSA and MSA was confirmed by ELISA and quartz crystal microbalance measurements. All constructs bound efficiently to CEA and CD3-positive cells and were able to activate T cells in a target cell-dependent manner, although T cell activation was reduced in the presence of serum albumin. All three derivatives showed a strongly increased half-life in mice as compared with scDb. Compared with the wild-type scDb-ABD, the half-life of scDb-ABD-H exhibited a prolonged half-life and scDb-ABD-L a reduced half-life, while the half-life scDb-ABD_2 was almost identical to that of scDb-ABD. However, these changes were only moderate, indicating that the half-life-extending property of the ABD in mice is only weakly influenced by affinity for serum albumin or valency of albumin binding.

Keywords: albumin-binding domain/bispecific antibody/carcinoembryonic antigen/retargeting/single-chain diabody

Introduction

Small recombinant antibody molecules, such as single-chain Fv (scFv) fragments, bispecific and bifunctional antibody derivatives but also the emerging class of antibody mimetics, find increasing applications in therapy (Enever *et al*., 2009; Kontermann, 2010; Müller and Kontermann, 2010). However, their small size, although facilitating tissue penetration, accounts for a rapid elimination from circulation (Batra *et al*., 2002; Jain *et al*., 2007). Consequently, various strategies have been established to extend the plasma half-life of these molecules (Kontermann, 2009). We have recently applied several of these strategies to extend the half-life of a bispecific single-chain diabody (scDb) molecule possessing a molecular mass of ~55 kDa. As a model, we used an scDb directed against carcinoembryonic antigen (CEA) and CD3 capable of retargeting effector T cells to tumor cells. Half-life extension strategies included PEGylation, *N*-glycosylation, fusion to human serum albumin (HSA), and an albumin-binding domain (ABD) from streptococcal protein G (Müller *et al*., 2007; Stork *et al*., 2007; Stork *et al*., 2008). A strongly prolonged half-life was observed for the PEGylated scDb, the scDb-HSA fusion protein and scDb-ABD, while *N*-glycosylation resulted only in a moderately increased half-life. We also showed that the long half-life, especially of scDb-ABD, translates into an increased accumulation in antigen-positive tumors (Stork *et al*., 2009).

The long half-life of scDb-ABD is mediated through recycling by the neonatal Fc receptor (FcRn) as shown by a comparative analysis of half-lives in the wild-type and FcRn heavy chain knockout mice (Stork *et al*., 2009). However, previous studies also revealed that the scDb-ABD fusion protein does not reach the long half-life of an IgG molecule (Stork *et al*., 2008). The ABD moiety of the scDb-ABD fusion protein binds with nanomolar affinity to HSA and mouse serum albumin (MSA) at neutral and acidic pH (Linhult *et al*., 2002; Stork *et al*, 2007; Jonsson *et al*., 2008; Stork *et al*., 2009). Based on the data from an Fab fused to an albumin-binding peptide, it was postulated that the fraction of unbound fusion protein has a profound effect on its clearance rate and, thus, that an increase in albumin-binding affinity should lead to a further increase in half-life (Nguyen *et al*., 2006). We therefore wondered whether the half-life of scDb-ABD could be further improved by increasing the affinity of the ABD moiety to albumin. Critical residues of the ABD involved in binding albumin were previously identified by a mutational study (Linhult *et al*., 2002) and, using phage display, ABD variants with femtomolar affinity for HSA were isolated from an ABD library with selected randomized positions (Jonsson *et al*., 2008).

In the present study, we generated two scDb-ABD derivatives with an increased and decreased affinity, respectively, for albumin. Furthermore, we fused a second ABD domain to the N-terminus of the original scDb-ABD molecule, thus generating a derivative that can bind two albumin molecules. These molecules were capable of binding to various extend

to HSA and MSA *in vitro*. The half-lives of these three scDb-ABD derivatives were, however, only slightly affected by affinity or valency of albumin binding. These data indicate that the affinity for albumin has only a moderate effect on half-life of albumin-binding fusion proteins.

Materials and methods

Materials

HRP-conjugated anti-His-tag antibody was purchased from Santa Cruz Biotechnology (CA, USA), and FITC-conjugated mouse anti-His-tag antibody from Dianova (Hamburg, Germany). CEA was obtained from Europa Bioproducts (Cambridge, UK). HSA and MSA were purchased from Sigma. The human colon adenocarcinoma cell line LS174T was purchased from ECACC (Wiltshire, UK) and cultured in RPMI, 5% FBS, 2 mM glutamine (Invitrogen, Karlsruhe, Germany). Jurkat cells were cultured in RPMI, 10% FBS. Buffy coat from healthy human donors were obtained from the blood bank (Ulm, Germany). The DuoSet IL-2 ELISA Development System kit was purchased from R&D Systems (Nordenstadt, Germany). CD1 mice were purchased from Elevage Janvier (Le Genest St. Isle, France).

Construction and production of an scDb-ABD fusion protein

DNA encoding the modified ABD domains was synthesized by GeneArt (Regensburg, Germany) adding an NotI and EcoRI restriction sites at the 5′ and 3′ end, respectively, for cloning into plasmid pAB1 scDbCEACD3. ABD-scDb-ABD was constructed by amplifying the ABD domain with primers SfiI-ABD-back (5′-GAC GCG GCC CAG CCG GCC CAG CAT GAT GAA GCG GTG GAT G-3′) and ABD-linker-NcoI-for (5′-CTG GCC GCG CTG CCG GGT GGT TCA GGA GGT GGT GGT TCA GGA GGT GCC ATG GCA TG-3′) and cloning as SfiI/NcoI fragment into pAB1 scDb CEACD3-ABD. All DNA fragments encoding scDb-ABD derivatives were then subcloned into mammalian expression vector pSecTagA (Invitrogen, Karlsruhe, Germany) as SfiI/EcoRI fragments. HEK293 cells were stably transfected and scDb-ABD derivatives were purified from cell culture supernatant by immobilized metal affinity chromatography (IMAC) essentially as described previously (Müller *et al*., 2007).

Production of a chimeric IgG and an scFv-Fc fusion protein

The chimeric anti-CEA IgG, possessing a human γ1 heavy chain was produced from stably transfected CHO-K1 cells as described previously (Stork *et al*., 2008). The scFv-Fc fusion protein was generated by fusing the anti-CEA scFv MFE-23 (Chester *et al*., 1994) to the human γ1 Fc region, including the hinge region and a hexahistidyl-tag at the C-terminus, essentially as described elsewhere (Müller *et al*., 2008). ScFv-Fc was purified from the cell culture supernatant of stably transfected HEK293 cells.

ELISA

CEA (300 ng/well) was coated overnight at 4°C and remaining binding sites were blocked with 2% (w/v) dry milk/PBS. Purified recombinant antibodies and serum samples were titrated in duplicates and incubated for 1 h at room temperature (RT). Detection was performed with mouse HRP-conjugated anti-His-tag antibody using TMB substrate (0.1 mg/ml TMB, 100 mM sodium acetate buffer pH 6.0, 0.006% H_2O_2). The reaction was stopped with 100 µl of 1 M H_2SO_4. Absorbance was measured at 450 nm in an ELISA reader.

Flow cytometry

Binding to CEA- and CD3-expressing cells was determined by flow cytometry (Benedict *et al*., 1997). LS174T or Jurkat-CD3 cells (2.5×10^5) were incubated with dilution series of antibodies for 2 h at 4°C. Cells were then washed with PBS, 2% FBS, 0.02% NaN_3 (PBA) and bound antibodies were detected using FITC-conjugated mouse anti-His-tag antibody. Data were fitted with GraphPrism software (La Jolla, USA) from three independent binding curves. From these three individual EC_{50} the mean and standard error was calculated.

Size exclusion chromatography

Apparent molecular weights and formation of scDb-ABD albumin complexes were analyzed by HPLC size exclusion chromatography using a BioSuite 450 (Waters Corporation, Milford, USA) and a flow rate of 0.5 ml/min. The following standard proteins were used: thyroglobulin, apoferritin, β-amylase, bovine serum albumin, carbonic anhydrase, cytochrome *c*. Complex formation of scDb-ABD with HSA or MSA was analyzed by incubating equimolar amounts of scDb-ABD and albumin (3 µM) in PBS at RT and subsequent analysis by size exclusion chromatography.

IL-2 release assay

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coat as described before (Müller *et al*., 2007). A total of 1×10^5 LS174T cells/100 µl/well were seeded in 96-well plates. The next day supernatant was removed and 150 µl of recombinant antibody added. After 1 h preincubation at RT, 2×10^5 PBMC/50 µl/well were added. PBMCs had been thawed the day before and seeded on a culture dish. Only cells that remained in suspension were used for the assay. After addition of PBMCs, the 96-well plate was incubated for 24 h at 37°C, 5% CO_2. Plates were centrifuged and cell-free supernatant collected. Concentration of human IL-2 in the supernatant was determined using the DuoSet IL-2 ELISA kit (R&D Systems) following the manufacturer's protocol.

Affinity measurements

Affinities of scDb-ABD for HSA and MSA at different pH were determined by quartz crystal microbalance measurements (Attana A-100 C-Fast system). Albumin was chemically immobilized on a carboxyl sensor chip according to the manufacturer's protocol at a density resulting in a signal increase of 70–80 Hz. Binding experiments were performed in PBS, pH 7.4 or 50 mM sodium phosphate buffer, 150 mM NaCl, pH 6.0 with at a flow rate of 25 µl/min. The chip was regenerated with 12.5 µl of 10 mM HCl. Before each measurement, a baseline was measured, which was subtracted from the binding curve. Data were collected by Attester 3.0 (Version 3.1.1.8, Attana, Stockholm, Sweden) and analyzed by ClampXP (Myszka and Morton, 1998). A mass transport model was fitted to the data (Myszka, 1997).

Pharmacokinetics

Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities. CD1 mice (8–16 weeks, weight between 30 and 40 g) received an intravenous (i.v.) injection of 25 μg scDb-ABD derivatives in a total volume of 150 μl. In time intervals of 3 min, 1, 2, 6 h and 1, 3, and 7 days blood samples (50 μl) were taken from the tail and incubated on ice. Clotted blood was centrifuged at 13 000 g for 10 min, 4°C and serum samples stored at −20°C. Serum concentrations of CEA-binding recombinant antibodies were determined by ELISA (as described above), interpolating the corresponding calibration curves. For comparison, the first value (3 min) was set to 100%. Half-lives ($t_{1/2}\alpha$, $t_{1/2}\beta$) and AUC were calculated with Excel. For statistics, student's *t*-test was applied.

Results

Single-chain diabody-ABD (scDb-ABD) derivatives

Three derivatives of single-chain diabody-ABD (scDb-ABD) fusion protein were generated using an scDb directed against CEA and CD3 (Stork *et al*., 2007). In scDb-ABD-H, the wild-type ABD domain was substituted by a high affinity ABD variant (Jonsson *et al*., 2008), whereas in scDb-ABD-L a low affinity ABD variant (Linhult *et al*., 2002) was used (Fig. 1). In addition, we generated an scDb-ABD derivative containing two wild-type ABD domains, one fused to the N-terminus and one fused to the C-terminus of the scDb molecule (Fig. 1a and b). All three fusion proteins were produced in stably transfected HEK293 cells and purified by IMAC. Yields varied between 2.3 and 4.1 mg/l culture. Purity was confirmed by SDS-PAGE (Fig. 1c) and

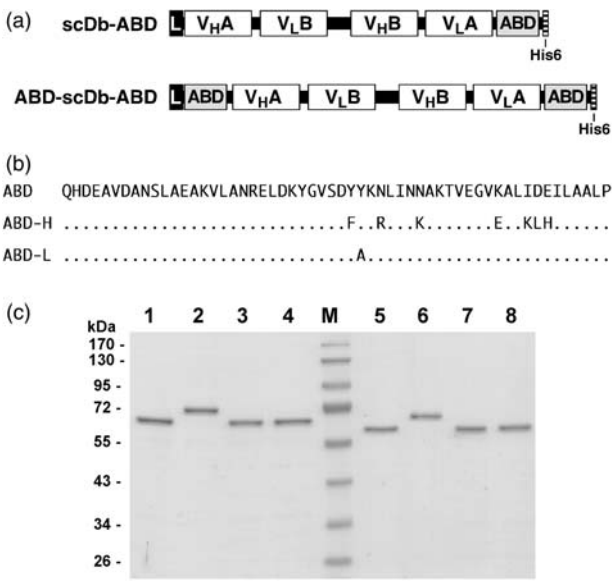

Fig. 1. ScDb-ABD derivatives. (**a**) Composition of scDb-ABD and ABD-scDb-ABD (scDb-ABD_2). (**b**) Sequences of the wild-type ABD (ABD) and two high (ABD-H) and low (ABD-L) affinity derivatives. (**c**) SDS-PAGE analysis of the wild-type scDb-ABD (1, 5), scDb-ABD_2 (2, 6), scDb-ABD-H (3, 7), and scDb-ABD-L (4, 8) under reducing (1–4) and non-reducing (5–8) conditions. Two micrograms of protein was analyzed per lane and gel was stained with Coomassie (M, molecular weight standards).

immunoblotting analysis (not shown). All molecules, including the original scDb-ABD, migrated as a single band under reducing and non-reducing conditions. Under non-reducing conditions, scDb-ABD, scDb-ABD-H and scDb-ABD-L exhibited an apparent molecular mass of 61 kDa, while that of scDb-ABD_2 was increased by ∼6 kDa in accordance with the molecular weight of the second ABD. All constructs recognized CEA in ELISA (not shown) and bound to CEA-expressing LS174T and CD3-expressing Jurkat cells (Fig. 2). EC_{50} values in the absence of HSA were in the range of 2.8–7.4 nM for binding to LS174T and 1.1–1.5 nM for binding to Jurkat, comparable to those determined for scDb CEACD3, i.e. without ABD fusion. Binding to LS174 and Jurkat cells was not or only marginally affected in the presence of 1 mg/ml HSA (Fig. 2; Table I). For scDb-ABD_2 a significant reduction of binding (approximately 2-fold for binding to LS174T and 4-fold for binding to Jurkat) was observed.

Interaction of scDb-ABD derivatives with albumin

In ELISA, all constructs showed a concentration-dependent binding to HSA and MSA, respectively (Fig. 3). Here, strongest binding was observed for scDb-ABD-H. Wild-type scDb-ABD and scDb-ABD_2 bound moderately to both albumins. ScDb-ABD-L showed the weakest binding of all constructs. The interaction of the scDb-ABD derivatives was further analyzed by size exclusion chromatography. Fusion proteins were incubated with HSA and MSA, respectively, at equimolar concentrations considering the number of ABD domains, i.e. scDb-ABD, scDb-ABD-H and scDb-ABD-L at a 1:1 molar ratio and scDb-ABD_2 at a 1:2 molar ratio. The scDb-ABD constructs eluted as a single major peak, except for scDb-ABD-H, which could not be resolved under the applied conditions. HSA and MSA eluted with a major peak corresponding to monomeric albumin and a minor peak corresponding to albumin dimers (Fig. 4). For both albumin species, the formation of scDb-ABD albumin complexes was observed after incubation with scDb-ABD, scDb-ABD-H and scDb-ABD_2, while no complexes were observed for scDb-ABD-L. ScDb-ABD and scDb-ABD-H showed an increased mobility with apparent molecular masses corresponding to one bound albumin. In contrast, the SEC analysis indicated that two albumin molecules were bound to one scDb-ABD_2 molecule (Fig. 4; Table II).

QCM measurements confirmed the altered affinity of the scDb-ABD derivatives for albumin (Fig. 5). At neutral pH, wild-type scDb-ABD had an affinity of 21.4 nM for MSA and 11.2 nM for HSA (Table III). The affinity of scDb-ABD-H was increased 12-fold for MSA and 80-fold for HSA, whereas the affinity of scDb-ABD-L was strongly reduced (approximately 30-fold for MSA). ScDb-ABD_2 exhibited a slightly increased binding to MSA (5.6 nM) and HSA (2.5 nM) (Table III).

IL-2 release

The influence of the ABD domain and binding to albumin on the T cell stimulating activity of the scDb moiety was analyzed in an IL-2 release assay using CEA-expressing LS174T cells as target cells and human PBMCs as effector cells. In the absence of HSA, all three scDb-ABD derivatives were capable of activating PBMCs to a similar extent as the wild-type construct and scDb without ABD (Fig. 6a). In the

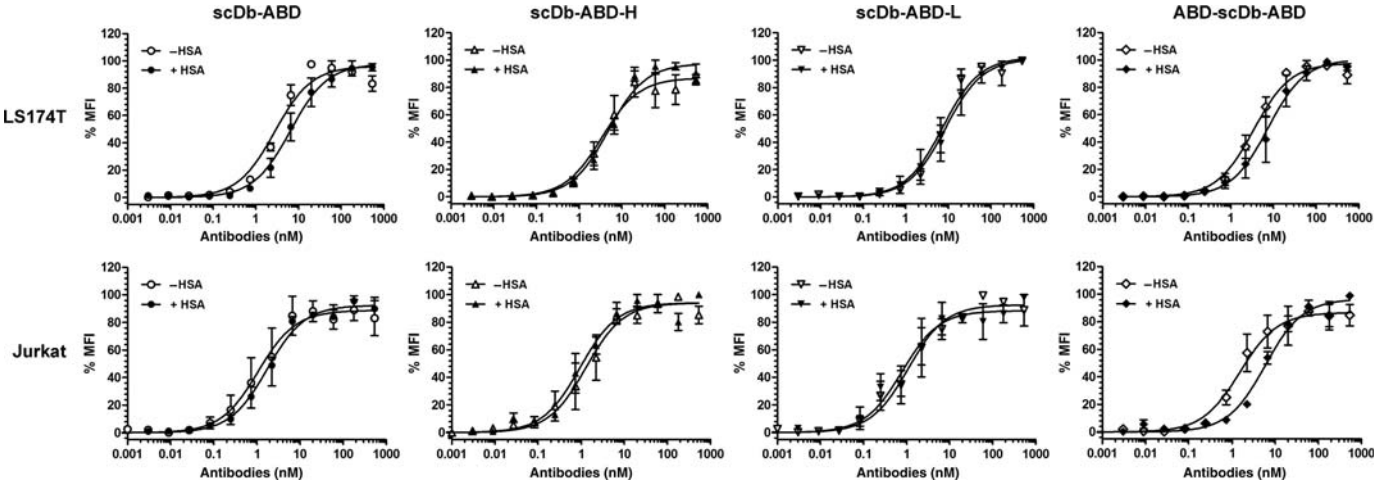

Fig. 2. Binding to CEA^+ and $CD3^+$ cells. Binding of the scDb-ABD derivatives to CEA-expressing LS174T cells and CD3-expressing Jurkat cells, respectively, was analyzed by flow cytometry in the presence (+HSA) or absence (−HSA) of HSA (1 mg/ml). Bound proteins were detected using an FITC-conjugated anti-His-tag antibody.

Table I. Binding to LS174T and Jurkat analyzed by flow cytometry

Construct	LS174T		Jurkat	
	−HSA	+HSA	−HSA	+HSA
scDb	2.2 ± 0.3	n.d.	0.7 ± 0.1	n.d.
scDb-ABD	2.8 ± 0.4	6.5 ± 1.0	1.1 ± 0.3	1.8 ± 0.3
scDb-ABD-H	3.4 ± 0.9	4.9 ± 0.6	1.3 ± 1.0	1.0 ± 0.2
scDb-ABD-L	7.4 ± 1.3	8.6 ± 1.8	1.1 ± 0.3	0.8 ± 0.2
scDb-ABD_2	3.4 ± 0.5	7.5 ± 1.5	1.5 ± 0.4	5.9 ± 0.9

n.d., not determined. EC_{50} values in nM ± SD.

presence of HSA (1 mg/ml), all scDb-ABD fusion proteins showed a reduction in the IL-2 secretion as compared with scDb. Here, scDb-ABD_2 exhibited almost no stimulatory activity.

Pharmacokinetics of scDb-ABD derivatives

Half-life of the scDb-ABD derivatives was analyzed after a single i.v. injection into CD1 mice. Serum concentrations were determined by ELISA, i.e. detecting molecules with CEA-binding activity. Compared with scDb, all three scDb-ABD derivatives showed a strongly prolonged circulation in the blood, similar to the wild-type scDb-ABD (Fig. 7). The longest terminal half-life was found for scDb-ABD-H (47.5 h) and the shortest for scDb-ABD-L (28.4 h). No significant differences between the different scDb-ABD constructs were observed comparing the $AUC_{0\text{-}7d}$ (Table IV). In this experiment we also included a chimeric anti-CEA IgG (Stork *et al.*, 2008) as well as an anti-CEA scFv-Fc fusion protein. The IgG molecule exhibited a terminal half-life of 6.8 days and a half-life of 2 days was measured for the scFv-Fc fusion protein.

Discussion

In this study, we compared the effects of altered binding of an ABD to albumin on the half-life of scDb-ABD fusion proteins in mice. All constructs were produced from stably

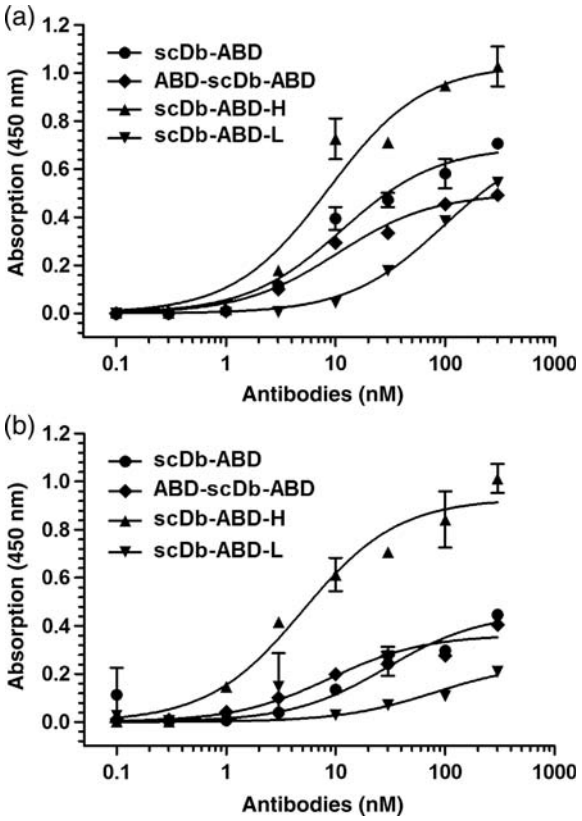

Fig. 3. Binding to albumin in ELISA. Binding of the scDb-ABD derivatives to HSA (**a**) and MSA (**b**) was analyzed in ELISA. Bound proteins were detected using an HRP-conjugated anti-His-tag antibody.

transfected HEK293 cells with similar yields as scDb and the wild-type scDb-ABD. The altered affinity of the ABD-H and ABD-L constructs for HSA and MSA was confirmed by ELISA and QCM measurements. The measured affinity of scDb-ABD for HSA and MSA is similar to that described in previous studies reporting an affinity of 4 nM for binding to HSA and 10 nM for MSA (Johansson *et al.*, 2002; Linhult *et al.*, 2002). The apparent affinity for albumin of the scDb-ABD_2 fusion protein, possessing two ABD wild-type

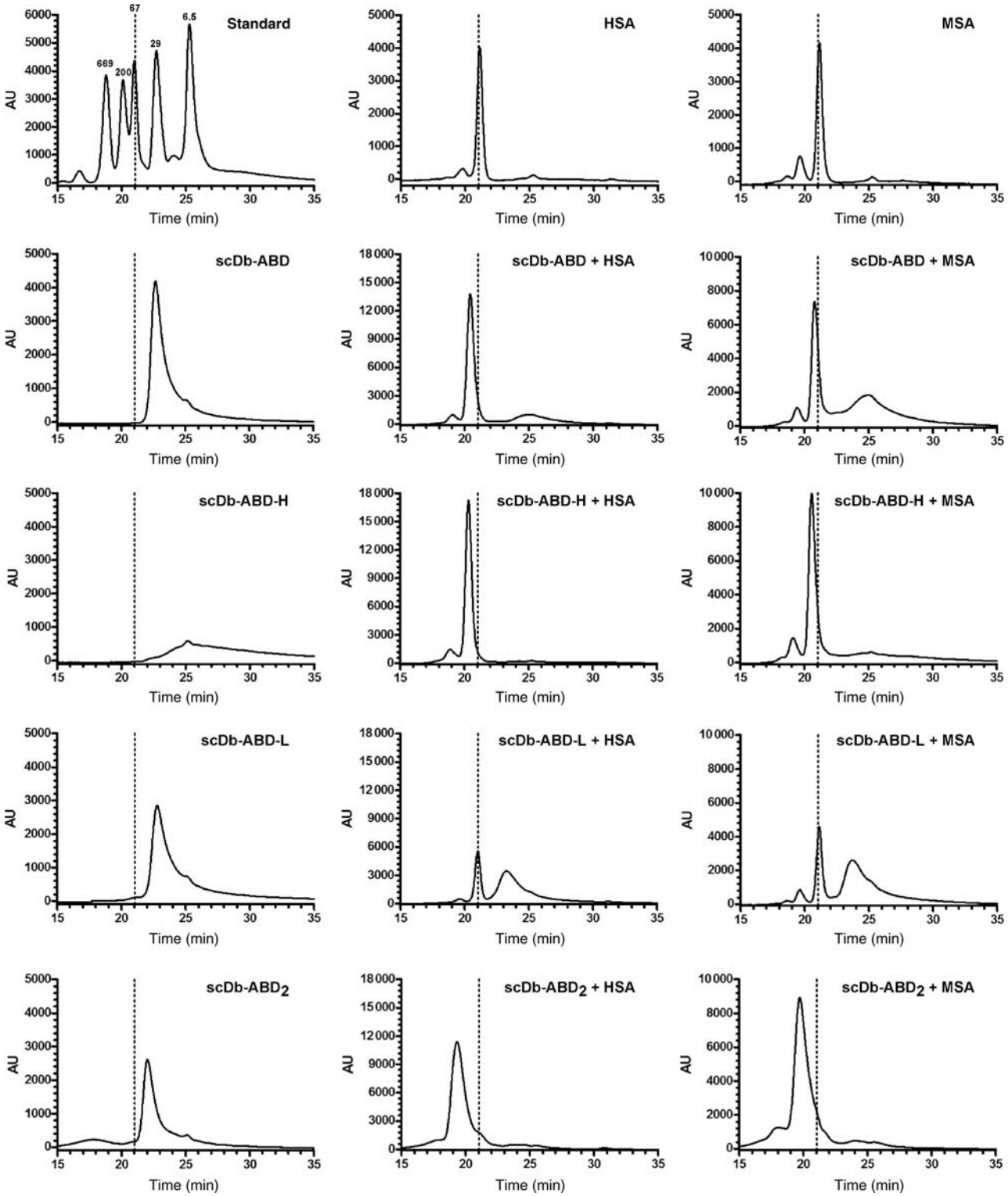

Fig. 4. Size exclusion chromatography analysis. ScDb-ABD molecules were incubated with HSA or MSA at equimolar concentrations and analyzed by HPLC size exclusion chromatography. Protein standards, the individual scDb-ABD molecules, as well as HSA and MSA alone were included for comparison. The dotted line indicates the position of albumin.

domains, was slightly increased, most likely reflecting avidity effects, i.e. binding of two albumin molecules as confirmed by the size exclusion chromatography experiments. The affinity of the scDb-ABD-H construct was increased 80-fold for HSA and 12-fold for MSA. A 200- to 2000-fold improved affinity over the wild-type ABD for binding to HSA was described in the original study for the ABD-H domain (ABD035), exhibiting an affinity in the range of 0.5–0.05 pM, which is much lower than the value determined in our study ($K_d = 140$ pM). This finding indicates that fusion of the ABD to the scDb has a negative influence on albumin binding. A similar effect was observed for an

Table II. Molecular mass and hydrodynamic radius

Construct	Calculated M_r (kDa)	S_r (nm)		
		−HSA	+HSA	+MSA
HSA	67.0	3.5	–	–
MSA	66.4	3.5	–	–
scDb-ABD	59.3	2.4	5.0	4.0
scDb-ABD-H	59.3	n.d.	5.0	4.4
scDb-ABD-L	59.2	2.3	–	–
scDb-ABD_2	65.7	2.7	5.9	5.7

anti-MSA single-domain antibody after fusion to IFN-α2b, which reduced affinity from 12 to 267 nM (Walker *et al.*, 2010). No data were reported for binding of ABD035 to MSA, although binding curves from Biacore experiments indicated a lower affinity for MSA (Jonsson *et al.*, 2008). An approximately 100-fold reduction in affinity for HSA was described for ABD-L (Linhult *et al.*, 2002). Binding of the scDb-ABD-L fusion protein to MSA in QCM was very weak in accordance with the low affinity for albumin and calculated to be around 600 nM. Weak binding of scDb-ABD-L was confirmed by size exclusion chromatography of antibody–albumin complexes allowed to form at equimolar concentrations. While in these experiments complexes were formed for the wild-type scDb-ABD, the scDb-ABD-H and the scDb-ABD_2 fusion proteins, none were observed for scDb-ABD-L.

All scDb-ABD derivatives showed a strongly improved half-life as compared with scDb lacking the ABD. Compared with wild-type scDb-ABD, the half-life of the scDb-ABD-H was slightly increased and that of scDb-ABD-L decreased. This finding demonstrates that the affinity for albumin influences the pharmacokinetic properties. However, differences were only marginal. Thus, even a low affinity for albumin caused a strong extension of the half-life. This finding is in agreement with half-lives determined for various other albumin-binding moieties, e.g. single-domain antibodies, peptides and small chemicals (Nguyen *et al.*, 2006; Trüssel *et al.*, 2009; Walker *et al.*, 2010). For example, a protein composed of interferon α2b (IFN-α2b) fused to an albumin-binding

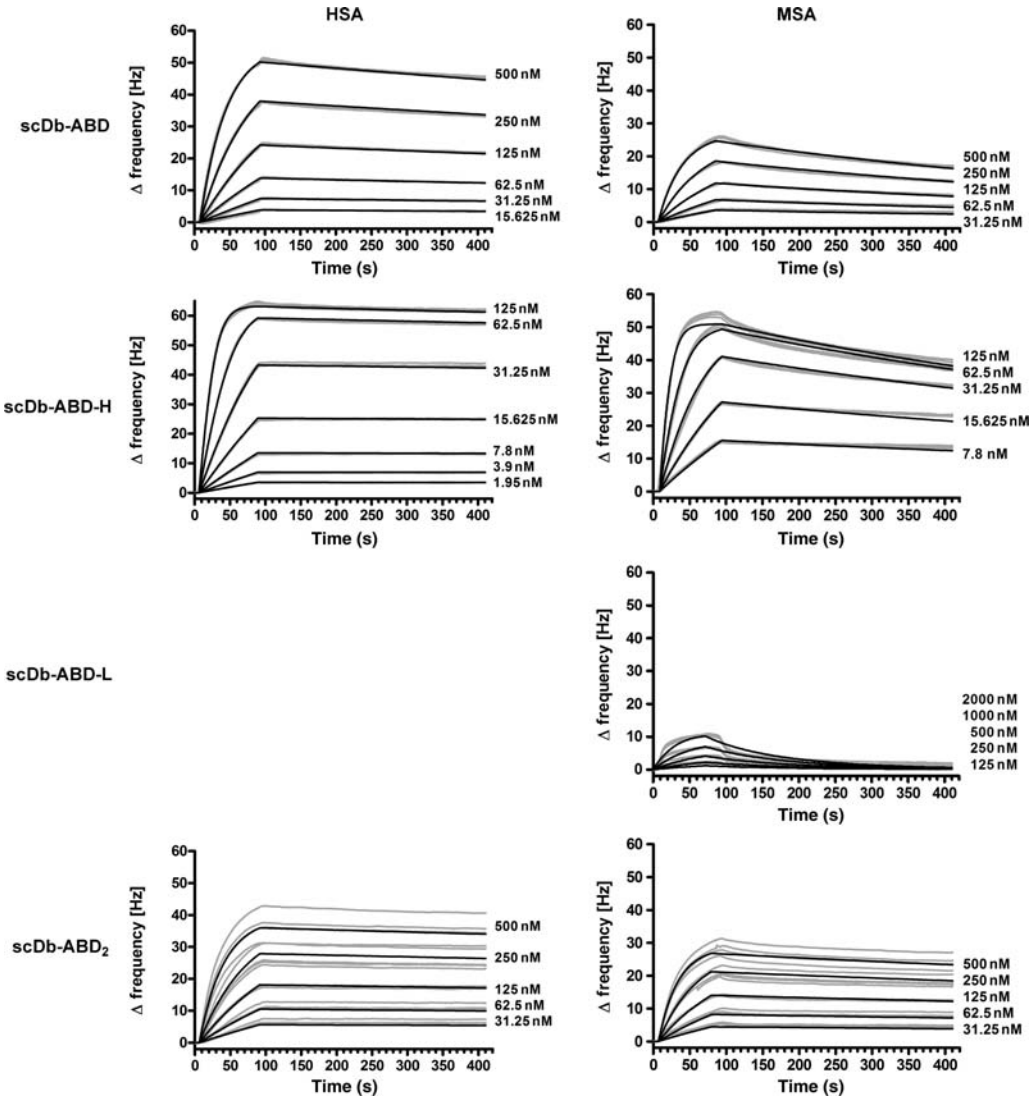

Fig. 5. Affinity measurements. Affinities of the scDb-ABD molecules were analyzed by quartz crystal microbalance measurements with immobilized MSA and HSA. Binding curves were fitted (black line) from four individual measurements (gray lines).

Table III. Affinities of scDb-ABD derivatives for MSA and HSA

Construct	K_d for HSA (nM)	K_d for MSA (nM)
scDb-ABD	11.2 ± 3.6	21.4 ± 2.2
scDb-ABD-H	0.14 ± 0.04	1.8 ± 0.5
scDb-ABD-L	n.d.	634 ± 30
scDb-ABD_2	2.5 ± 0.2	5.6 ± 0.2

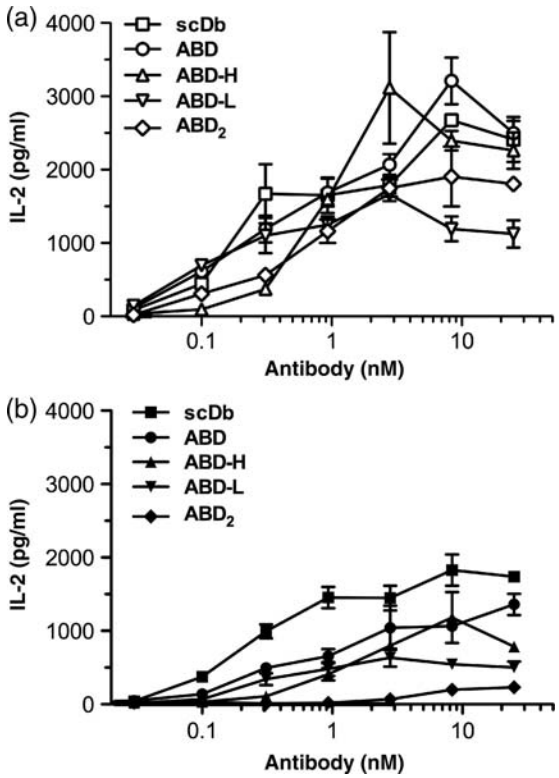

Fig. 6. scDb-ABD-mediated IL-2 secretion. LS174T were preincubated for 1 h with scDb-ABD derivatives at varying concentrations in the absence (**a**) or presence (**b**) of HSA (1 mg/ml) before adding human PBMCs. IL-2 release into the cell culture supernatant was measured after 24 h by ELISA. A representative experiment of three independently performed experiments using PBMCs from three different donors is shown.

single-domain antibody, which binds with a K_d of 267 nM to MSA increased half-life of IFN-α2b from 1.2 to 22.6 h in rats (Walker *et al*., 2010). Interestingly, the scDb-ABD_2 fusion protein, which exhibits a strongly increased hydrodynamic radius after binding of two albumin molecules (5.7 nM compared with 4 nM for scDb-ABD) had a terminal half-life similar to that of scDb-ABD. This finding indicates that a further increase in size does not lead to a further increase in half-life, which is supported by previous data with a PEGylated scDb possessing a hydrodynamic radius of 7.9 nM but nonetheless a similar half-life than scDb-ABD (Stork *et al*, 2008). The half-lives determined for the different scDb-ABD fusion proteins were also similar to that of an scFv-Fc fusion protein (2 days), while a chimeric IgG circulated very long with a half-life of 6.8 days. The shorter half-life of the scFv-Fc fusion protein compared with the full IgG is in accordance with other studies, which also reported half-lives of 1–2 days for similar scFv-Fc fusion proteins (Powers *et al*., 2001; Kenanova *et al*., 2007). This might be

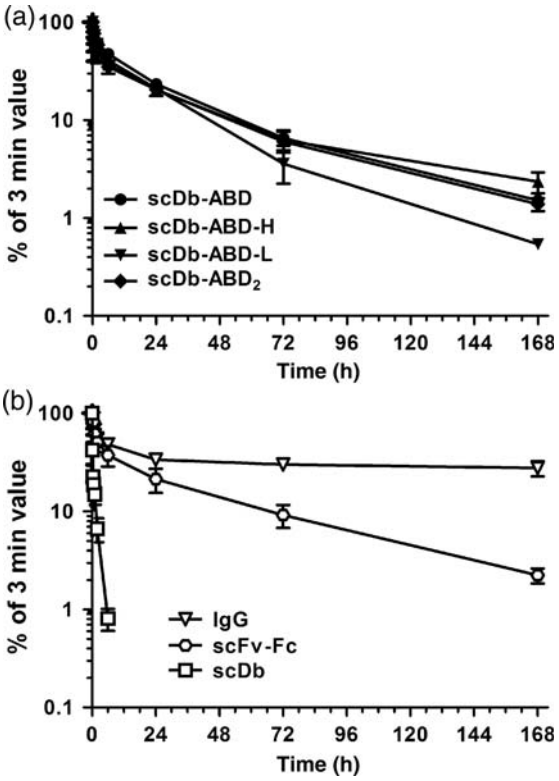

Fig. 7. Pharmacokinetics. ScDb-ABD derivatives (**a**) and anti-CEA chimeric IgG and scFv-Fc fusion protein (**b**). Proteins were i.v. injected into CD1 mice (25 μg/animal) and serum concentrations of the antibody molecules were determined at different time points by ELISA. Data were normalized considering maximal concentration at the first time point (3 min).

Table IV. Pharmacokinetic properties

Construct	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)	AUC_{0-7d} (%h)
scDb[a]	0.2 ± 0.1	5.6 ± 1.8	n.d.
scDb-ABD	1.8 ± 0.5	36.4 ± 4.8	2328 ± 462
scDb-ABD-H	1.5 ± 0.5	47.5 ± 14.8	2031 ± 153
scDb-ABD-L	2.6 ± 1.6	28.4 ± 1.9	1747 ± 444
scDb-ABD_2	1.2 ± 0.7	37.9 ± 1.1	1776 ± 366
scFv-Fc	1.2 ± 0.8	48.1 ± 8.4	2115 ± 1143
IgG	n.d.	163.1 ± 81.1	3586 ± 1196

[a]Data from Stork *et al*. (2007).

due to a lower affinity of Fc fusion proteins for the FcRn (Suzuki *et al*., 2010).

The long half-life of IgG in mice indicates that the scDb-ABD fusion proteins have not reached the maximal possible circulation time. The half-life of IgG and albumin is strongly influenced by FcRn-mediated recycling (Chaudhury *et al*., 2003; Chaudhury *et al*., 2006; Andersen and Sandlie, 2009). Binding studies showed that IgG binds at pH 6.0 with a much higher affinity to FcRn than albumin (Andersen *et al*., 2010). Hence, it becomes obvious that rather the affinity for FcRn than the affinity for albumin dictates the recycling and thus the half-life. Indeed, the half-life of IgG molecules has been further extended by increasing the affinity for FcRn at acidic pH (Hinton *et al*., 2005; Dall'Acqua *et al*., 2006). A recent study established a direct correlation between strength of FcRn binding and half-life (Zalevsky

et al., 2010). Furthermore, in this study it was shown that an extended half-life translates into improved antitumor activity of the engineered anti-VEGF and anti-EGFR IgGs.

An important aspect of half-life extension strategies relates to their influence on activity of the therapeutic protein. Thus, an 8-fold lower bioactivity was observed for IFN-α2b after fusion to an albumin-binding single-domain antibody, which was further reduced 3-fold in the presence of albumin (Walker *et al*., 2010). When analyzed for cell binding no or only a marginal reduction of the activity of the different scDb-ABD fusion proteins in comparison to scDb was observed. Also, the presence of HSA did not or only slightly affect binding. In contrast, the target cell-dependent stimulation of T cells as determined by IL-2 secretion was reduced in the presence of HSA, especially for the scDb-ABD_2 construct, which showed almost no stimulatory activity. This indicates that binding of albumin to the scDb-ABD molecules interferes with bringing target and effector cells into close contact required for efficient T cell triggering. This effect might, however, be antigen and epitope dependent, supported by data from a recent study of bispecific anti-melanoma chondroitin sulfate proteoglycan × anti-CD3 tandem scFvs recognizing different epitopes on the melanoma antigen, demonstrating that epitope distance and antigen size determine potency of T-cell mediated lysis (Bluemel *et al*., 2010).

In conclusion, we showed that altering the affinity or valency of albumin binding has only minor effects on the half-life of scDb-ABD fusion proteins in mice. For albumin-binding peptides, it was shown that the half-life of Fab-peptide fusion proteins, analyzed in mice, rats and rabbits, correlates with affinity of the peptide for albumin (Nguyen *et al*., 2006). In rats a 26-fold reduction in affinity for albumin (92 nM vs 2.4 μM) led to a 6-fold reduction of the terminal half-life. In the present study we measured an approximately 2-fold difference in half-life between the high and low affinity ABD fusion protein while the AUC was not significantly different. Currently, we have no data available whether a further reduction of affinity for albumin will lead to a more pronounced reduction of half-life. Most notably, our results demonstrate that an increased affinity of the ABD does not result in a significant improvement of half-life indicating a limit of half-life extension that can be reached through binding to albumin. Taken together, it becomes evident that even molecules with medium affinity for albumin are appropriate to prolong half-life of therapeutic proteins in accordance with various reports where low-to-medium affinity molecules such as albumin-binding chemicals, peptides and antibody domains were successfully employed to extend plasma half-lives (Nguyen *et al*., 2006; Trüssel *et al*., 2009; Walker *et al*., 2010).

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