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Supplementary Information for

An engineered 4-1BBL fusion protein with “activity-on-demand”
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Supplementary Information Text

Supplementary Methods

Cloning

A soluble single-chain trimer of murine 4-1BBL was designed by linking the TNF homology domain (amino acids 139 – 309) with a single glycine as a linker. The genetic sequence was ordered from Eurofins Genomics. The sequence was then introduced into a vector encoding the F8 in a diabody format by Gibson Isothermal Assembly. To clone the single-chain variable Fragment (scFv) linked to the 4-1BBL monomer, the genetic sequence encoding the diabody was replaced by the sequence encoding the scFv and two domains of 4-1BBL were removed by PCR followed by blunt-end ligation. Additional base pairs of 4-1BBL were added to the 4-1BBL sequence by PCR followed by blunt-end ligation. The IgG fusion proteins were cloned by fusing the 4-1BBL sequence to the sequence of the antibody in the IgG format by PCR before introducing it into an appropriate vector by restriction cloning. The protein sequences are provided in [**SI Appendix, Table S1**].

NF-κB response assay

CTLL-2_NF-κB reporter cells were starved by washing the cells twice with prewarmed HBSS (Gibco, #14175095) followed by growth in the absence of IL-2 for 6 - 9 h in RPMI 1640 (Gibco, # 21875034) medium supplemented with 10% FBS (Gibco, #10270106), 1 X antibiotic-antimycoticum (Gibco, # 15240062), 2 mM Ultraglutamine (Lonza, # BE17-605E/U1), 25 mM HEPES (Gibco, # 15630080) and 50 μM β-mercaptoethanol (Sigma Aldrich) in order to reduce the background signal. To coat the wells with antigen, 100 μL 100 nM 11-A-12 fibronectin in phosphate buffered saline (PBS) was added to each well and the plate was incubated at 37°C for 90 min. Cells were seeded in 96-well plates (50,000 cells/well) and growth medium containing varying concentrations of the antibody-cytokine conjugate was added. The cells were incubated at 37°C, 5% CO_2 for several hours. To assess luciferase production, 20 μL of the supernatant was transferred to an opaque 96-well plate (PerkinElmer, Optiplate-96, white, #6005290) and 80 μL 1 μg/mL Coelenterazine (Carl Roth AG, #4094.3) in phosphate buffered saline (PBS) was added. Luminescence at 595 nm was measured immediately. The relative luminescence was calculated by dividing the obtained results by the results obtained when no inducer was added. The data was fitted using the [Agonist] vs. response (three parameters) fit of the GraphPad Prism 7.0 a software to estimate the EC_{50}.

Immunofluorescence on tissue microarray

Immunofluorescence was performed onto Frozen Tumor and Normal Tissue Array (Biochain, #T6235700). The array was fixed by ice-cold aceton for 5 minutes. After fixation, sections were let

dry at room temperature for 10 minutes and then blocked for 45 min with 20% fetal bovine serum in PBS. FITC labeled IgG(F8) was added at 5 μg/ml in 2% BSA/PBS solution for 1h at room temperature. The tissue array was then washed twice with PBS and secondary rabbit anti-FITC antibody (Biorad, #4510-7804) was added to a final 1:1000 dilution in 2% BSA/PBS at room temperature for 1h. After washing the array twice with PBS, Goat Anti-Rabbit Alexa-488 (ThermoFisher, #A11032) was added to a final 1:500 dilution in 2% BSA/PBS. Dapi was used to counterstain nuclei. Slides were analyzed with Axioskop2 plus microscope (Zeiss).

Quantitative biodistribution studies

8 weeks old female 129/Sv mice were injected subcutaneously in the right flank with 10^7 F9 teratocarcinoma cells. The tumor size was measured daily with a caliper and the volume was calculated using the formula [volume = length x width x width x 0.5]. When the tumors reached a volume of 100 – 300 mm^3, 10 μg of radioiodinated protein was injected into the lateral tail vein. The mice were sacrificed 24 h after the injection and the organs were excised and weighed. The radioactivity of the different organs was measured (Packard Cobra II Gamma Counter) and expressed as percentage of injected dose per gram of tissue (%ID/g ± SD, *n* = 3).

***Ex vivo* detection of fluorescently labelled immunocytokines and serial scanning of the tumor section**

The tumor section was prepared and stained as described in the main text. Serial images of the tumor sections were acquired and electronically assembled using a Leica DMI6000B microscope equipped with a HC PL APO 20x/ 0.70 DRY objective (#11506166), a Leica K5-14400781 camera and a fast filter wheel. The acquisition settings were 100 ms exposure, 12.5 % intensity for the green channel (λ_{ex} = 490 nm, λ_{em} = 525 nm), 150 ms exposure, 25% intensity for the red channel (λ_{ex} = 552 nm, λ_{em} = 600 nm) and 50 ms, 6.25% intensity for the blue channel (λ_{ex} = 450 nm, λ_{em} = 422 nm). For image processing the LAS X and ImageJ software v1.52k were used. The thresholds for the red and green channels were set to 300 – 506 and for the blue channel to 250 – 6000.

Analysis of tumor-infiltrating lymphocytes by flow cytometry (detailed experimental protocol)

A single-cell suspension of the tumor was obtained by digesting it in RPMI 1640 supplemented with 1 mg/mL collagenase II and 100 µg/mL DNase I for 30 min at 37°C. After the digestion, the suspension was passed through a 70 μm cell strainer. If necessary, the red blood cells were removed using a red blood cell lysis buffer (Roche). The lymph nodes were smashed on a 70 μm cell strainer and washed with PBS. For cell surface staining, cells were incubated with a mix of suitable antibodies: αCD3-APC/Cy7 (Biolegend, #100222), αCD4-APC (Biolegend, #100412),

αCD8-FITC (Biolegend, #100706), αNK1.1-PE (Biolegend, #108708), αCD62L-BV421 (Biolegend, #104436), αCD44-APC/Cy7 (Biolegend, #103028), αMHCII(IA/IE)-BV421 (Biolegend, #107631), αPD-1-BV421 (Biolegend, #109121) and αCD39-APC (Biolegend, #143809). After staining of the cell surface markers, the cells were stained with 7-AAD (Biolegend) for live/dead discrimination. For intracellular staining, the cells were first stained with Zombie Red (SigmaAldrich) and then the cell surface stain was performed. The cells were fixed and permeabilized using the eBioscience™ FoxP3/Transcription Factor Staining Buffer Set (Thermofisher, #00-5523-00) according to the manufacturer's instructions. The fluorescence was measured using a Cytoflex Flow Cytometer and the data was evaluated using the FlowJo software. The gating strategy is depicted in [**SI Appendix, Fig. S8**]. Statistical evaluations were done using a regular two-way ANOVA followed by a Tukey's multiple comparison test or a regular one-way ANOVA followed by a Sidak's multiple comparison test in GraphPad Prism v8.4.1.

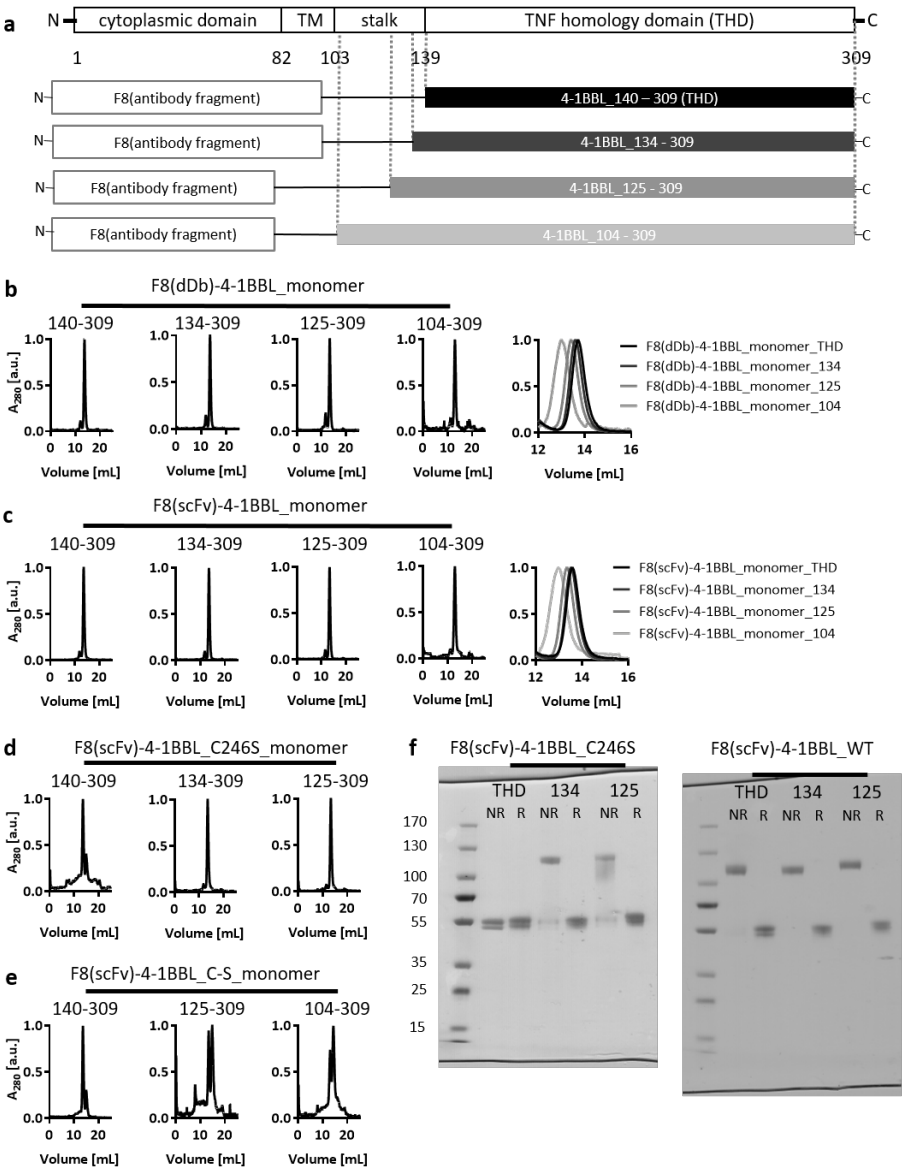


Fig. S1. Screening of the extracellular domain and C-S mutants of murine 4-1BBL to determine the optimal design of the 4-1BBL moiety in F8-4-1BBL (a) schematic depiction of the domain architecture of 4-1BBL and of the constructs that were screened containing different parts of the stalk region in addition to the TNF-homology domain (THD). The numbers correspond to the amino acid number in murine 4-1BBL. (b) Size exclusion profiles of F8 in the diabody (dDb) format linked to a single 4-1BBL subunit including different parts of the extracellular domain of 4-1BBL. Longer fragments of 4-1BBL were more prone to aggregation and also gave lower expression yields (data not shown) (c) size exclusion profiles F8 in the scFv format linked to a single 4-1BBL subunit (d) Size exclusion profiles of different F8-4-1BBL variants featuring a C246S mutation in the 4-1BBL subunit preventing the formation of the disulfide bond that links two THD of 4-1BBL (e) Size exclusion profiles of different variants of F8-4-1BBL where all cysteines in the 4-1BBL domain were mutated to serines demonstrating the role of the disulfide bond for the stability of the fusion protein (f) SDS PAGE of the different C246S mutants revealing the presence of a second disulfide-forming cysteine outside the THD (NR: non-reducing, R: reducing)

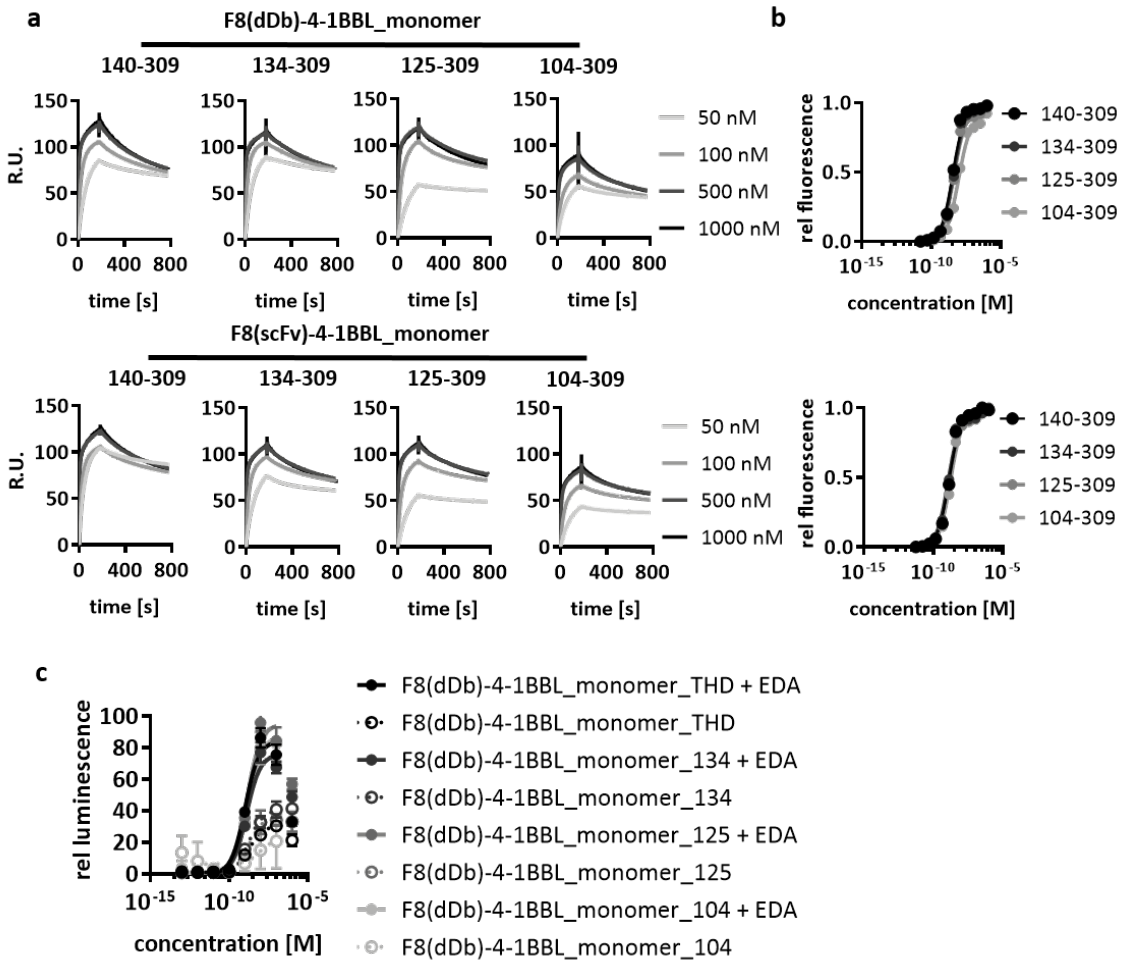
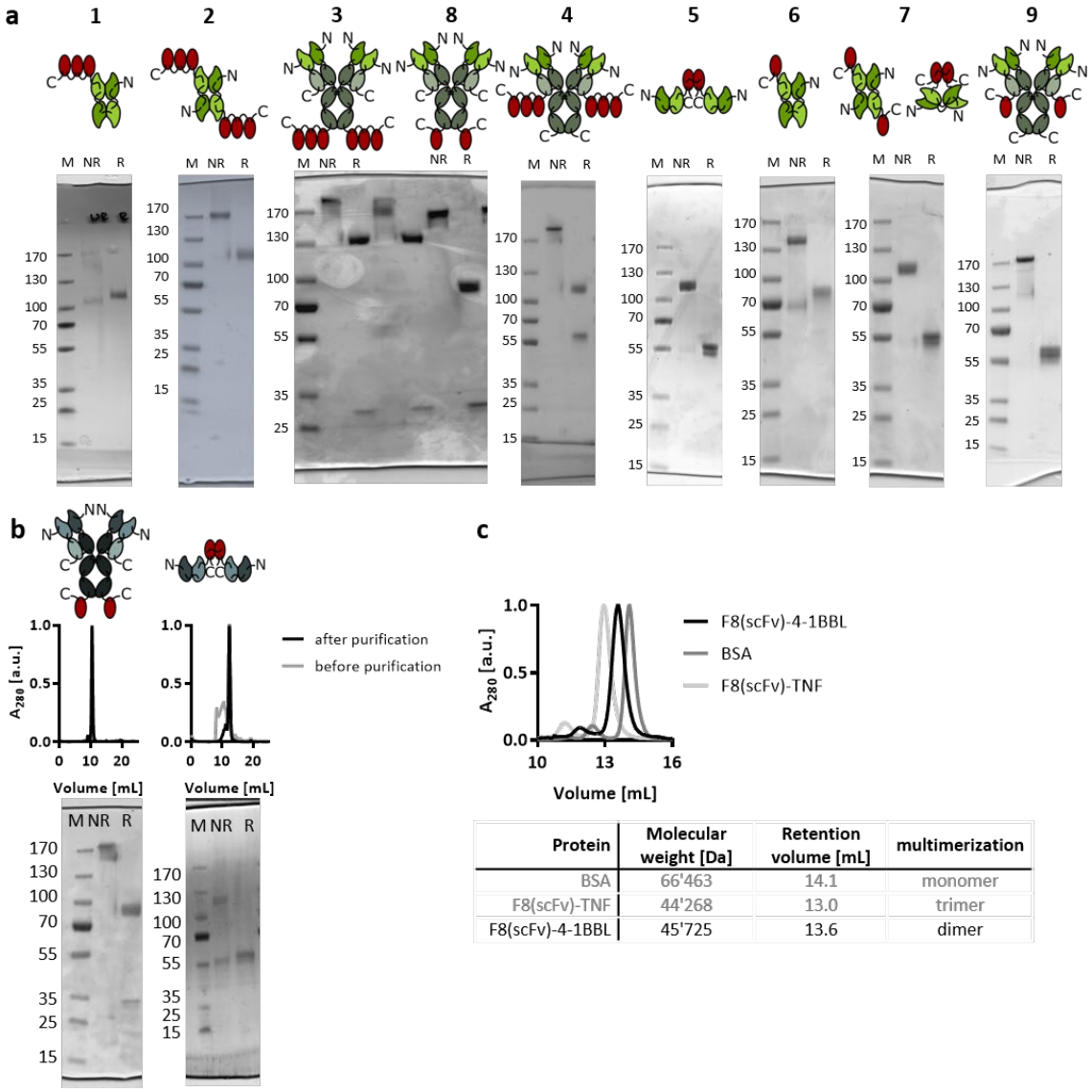


Fig. S2. The different F8-4-1BBL variants all retained binding to EDA and to 4-1BB as evidenced by (a) the Surface Plasmon Resonance measurements of binding to EDA and (b) the flow cytometry experiments with the 4-1BB expressing CTLL-2 cells (top: antibody in the diabody format, bottom: antibody in the scFv format) and (c) comparative bioactivity assays using the CTLL-2 NF-κB cell line revealed that the TNF homology domain is sufficient to achieve full signaling activity. The numbers refer to the amino acid numbers of murine 4-1BBL that were included in the F8-4-1BBL construct.



Protein	Molecular weight [Da]	Retention volume [mL]	multimerization
BSA	66'463	14.1	monomer
F8(scFv)-TNF	44'268	13.0	trimer
F8(scFv)-4-1BBL	45'725	13.6	dimer

Fig. S3. (a) SDS PAGE analysis of the nine F8-4-1BBL formats that were produced in this study (M: marker, NR: non-reducing sample buffer, R: reducing sample buffer) (b) Size exclusion chromatography profiles and SDS PAGE gels of the KSF constructs that were used for the biodistribution. The aggregates of the KSF(scFv)-4-1BBL could be efficiently removed by preparative size exclusion chromatography (c) comparative size exclusion chromatography showed that F8(scFv)-4-1BBL forms a dimer in solution since the retention volume was higher than for F8(scFv)-TNF that forms a non-covalent homotrimer(1)

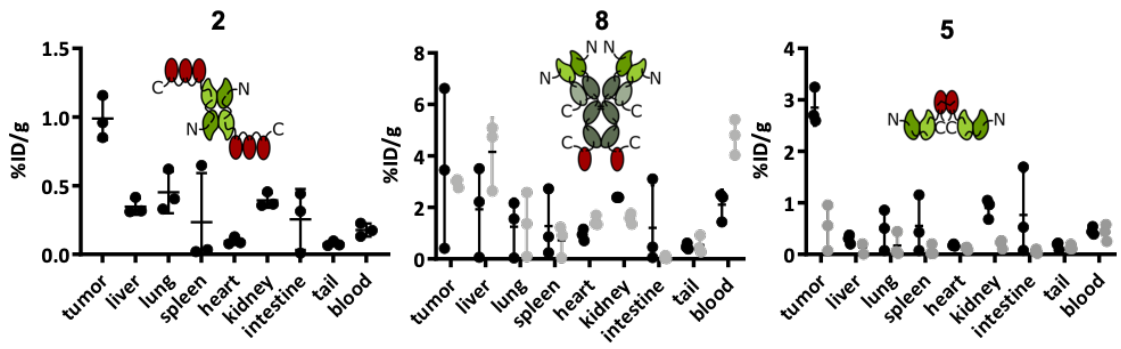


Fig. S4. *In vivo* biodistribution studies of three F8-4-1BBL formats. The mice were sacrificed 24 h after the injection of the radioiodinated proteins and the radioactivity of the excised organs was measured and expressed as percent injected dose per gram of tissue (%ID/g). The KSF antibody targeting hen egg lysozyme was used as untargeted control. Shown are individual measurements and mean ±SD (*n* = 3).

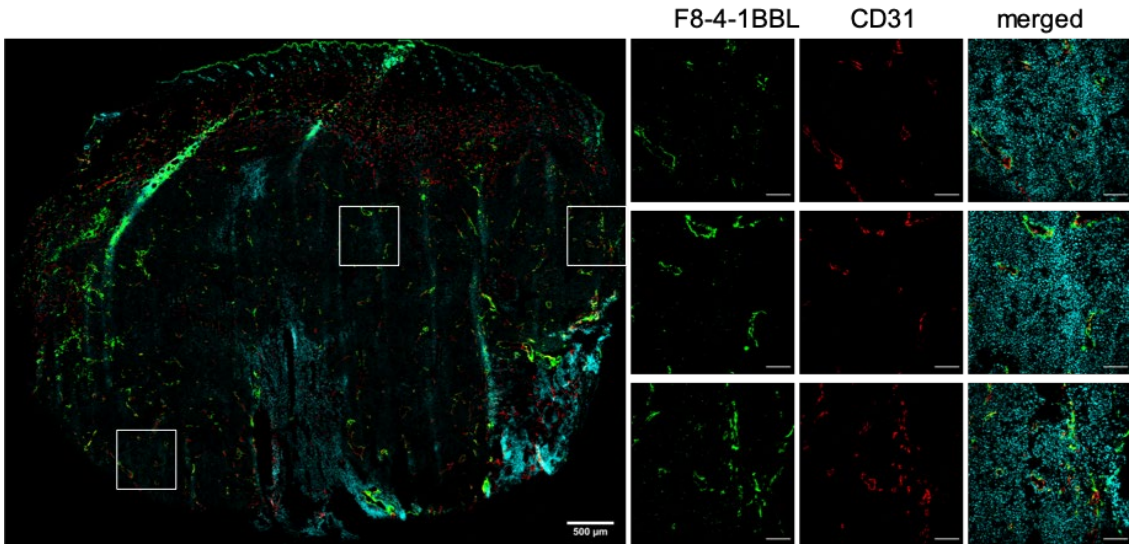


Fig S5. Serial images were taken of the tumor sections after *ex vivo* detection of FITC-labelled F8-4-1BBL and assembled to provide an overview of the entire tumor section. The channels showing CD31 and F8-4-1BBL are shown separately as well as the composite for relevant portions of the tumor sections (indicated by white squares) to show the colocalization of EDA-targeted antibody-cytokine conjugates and the tumor vasculature (green: αFITC, red: αCD31, cyan: nuclei; the scale bars in the single images represents 100 μm).

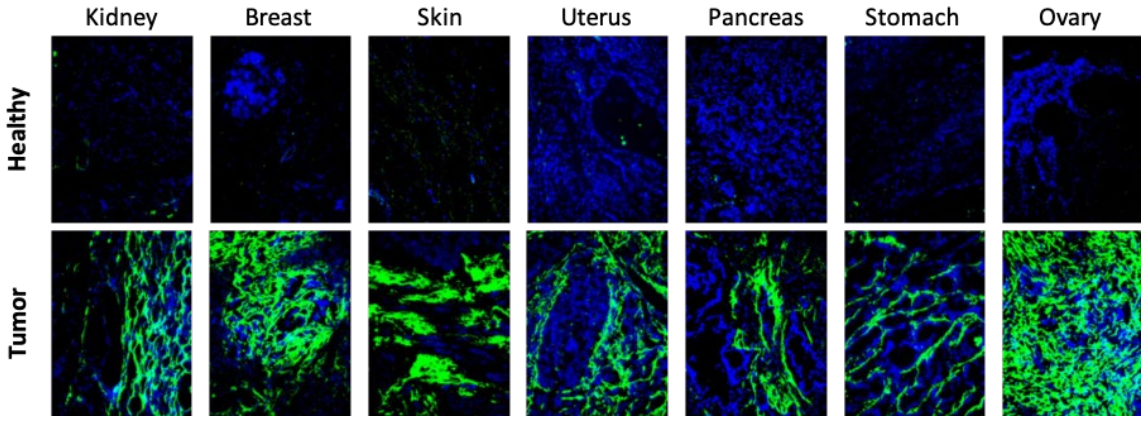


Fig S6. The expression of EDA was assessed on human tissue microarrays using FITC-labelled F8 in the IgG format. (green: EDA, blue: nuclei).

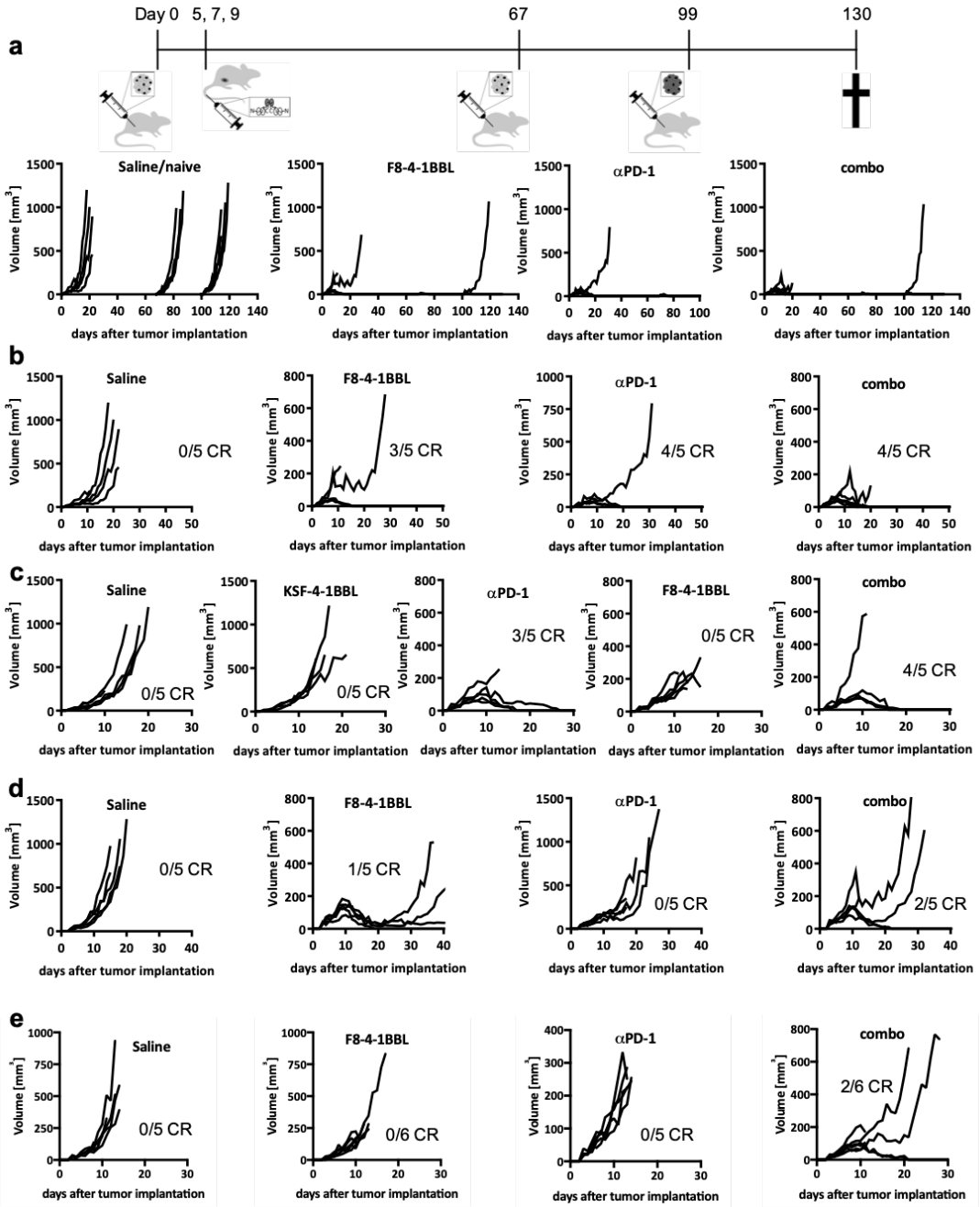


Fig. S7. Individual growth curves of the therapies (a) mice cured from WEHI-164 fibrosarcoma in the preventive setting were challenged with WEHI-164 fibrosarcoma cells on day 67 and mice from the F8-4-1BBL and the combo group also received a challenge with CT26 colon carcinoma cells on day 99. All the naïve mice developed tumors while the cured mice rejected subsequent challenges with WEHI-164 fibrosarcoma cells and most were also immune against CT26 colon carcinoma. (b) the individual growth curves of WEHI-164 fibrosarcoma bearing mice that received the treatment starting on day 5 after tumor implantation when the tumor volume was > 40 mm^3 (c) the individual growth curves of WEHI-164 fibrosarcoma-bearing mice that received the treatment starting on day 7 after tumor implantation when the tumor volume was > 80 mm^3 (d) individual growth curves of the CT26 colon-carcinoma-bearing mice that received the treatment starting on day 7 when the tumor volume was > 80 mm^3 (e) individual growth curves of the MC38 colon-

carcinoma-bearing mice that received the treatment starting on day 7 when the tumor volume was > 75 mm^3 (CR: complete response)

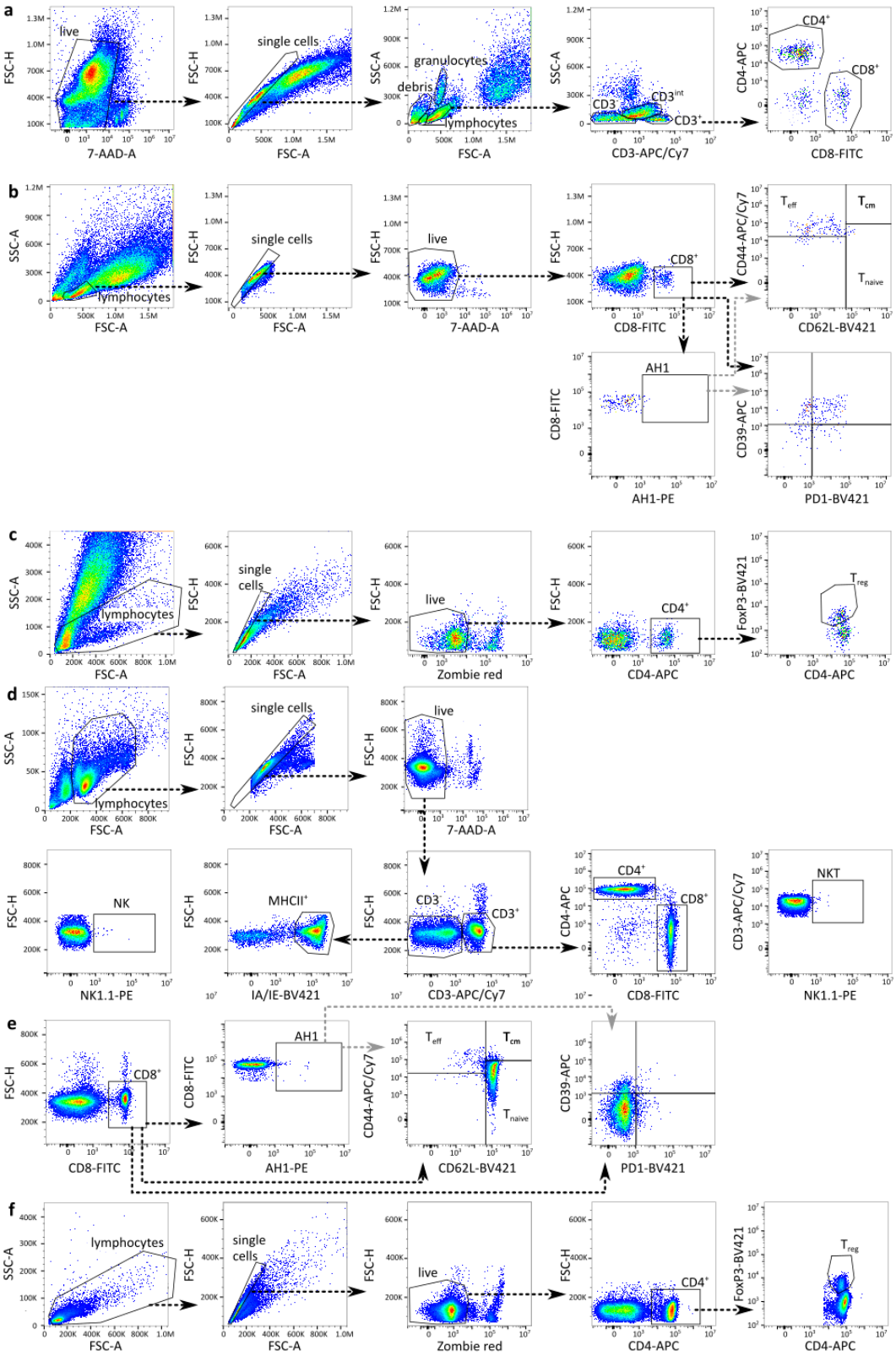


Fig. S8. Gating strategy for the flow cytometry analysis of the tumor-infiltrating lymphocytes (TILs) and the tumor-draining lymph nodes (TDLN) (a) in order to quantify the tumor infiltrates,

dead cells were excluded first by live/dead stain and then by scattering. Total living cells were calculated by subtracting dead cells and debris from the total number of events. Lymphocytes were further divided into the different subgroups (NKT, NK and MHCII+) as shown for the lymph nodes. (b) To assess the phenotype of tumor-infiltrating CD8+ cells, the events were gated by scattering and then live/dead, followed by CD8+ and AH1+. The same gates were applied to CD8+ and AH1+ cells for assessing the phenotypes. (c) To gate fixed tumor samples stained for intracellular FoxP3 the events were first gated by scattering and live/dead before the CD4+ cells were assessed for FoxP3 (T_{reg}). (d) To quantify the immune subsets in the tumor-draining lymph node (TDLN), the events were first gated by scattering and live/dead before the subsets were divided by CD3 expression and further markers. (e) The phenotype of CD8+ in the TDLN was assessed as described for the tumor. (f) Regulatory T cells in the TDLN were also assessed with the same gating strategy as described for the tumor (FSC: forward scattering, SSC: side scattering)

Table S1. Sequences of the fusion proteins that were developed in this study

F8(scDb)-(4-1BBL)$_3$: F8_VH-*linker*-F8_VL-*linker*- F8_VH-*linker*-F8_VL-*linker*-4-1BBL-*linker*-4-1BBL-*linker*-4-1BBL (Format **1**)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GGSG*
G-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*GGGGSGGGGSGGG*
GS-EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGST
YYADSVKGRFTISRDNSKNT LYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*G*
GSGG-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATG
IPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*SSSSGSSSSGSS*
SSG-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPG
LYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWS
QLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE-*G*-ATTQQGSP
VFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPT
FTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSV
GLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE-*G*-ATTQQGSPVFAKLLAKNQASL
CNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGW
VSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQD
AYRDWELSYPNTTSFGLFLVKPDNPWE

F8(dDb)-(4-1BBL)$_3$: F8_VH-*linker*-F8_VL-*linker*-4-1BBL-*linker*-4-1BBL-*linker*-4-1BBL (Format **2**)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GGSG*
G-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*SSSSGSSSSGSSSSG*
-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYY
VFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLL
LLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE-*G*-ATTQQGSPVFA
KLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTN
TGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLR
AYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE-*G*-ATTQQGSPVFAKLLAKNQASLCNTT
LNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVL
QAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRW
ELSYPNTTSFGLFLVKPDNPWE

F8(IgG)-(4-1BBL)$_3$_HC: VL-CL** VH-CH-*linker*-4-1BBL-*linker*-4-1BBL-*linker*-4-1BBL (Format **3**)

EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRF
SGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC**

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSASTKG
PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK-*SSSSGSSSSGSSSSG*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQD
GAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVD
DFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNT
TSFGLFLVKPDNPWE-*G*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLR
YEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFP
CSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNP
WE-*G*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSP

GLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSW
SQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE
F8(IgG)-(4-1BBL)$_3$_LC: VL-CL-*linker*-4-1BBL-*linker*-4-1BBL-*linker*-4-1BBL** VH-CH (Format **4**)
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KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC-*SSSSGSSSSGSSSSG*-ATTQQGSPVFAKLLAKNQ
ASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQ
GWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGA
QDAYRDWELSYPNTTSFGLFLVKPDNPWE-*G*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHS
QDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQ
VDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYP
NTTSFGLFLVKPDNPWE-*G*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQG
LRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVEL
FPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPD
NPWE**
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
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PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK
F8(scFv)-4-1BBL: F8_VH-*linker*-F8_VL-*linker*-4-1BBL (Format **5**)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GGGG
SGGGGSGGGG*-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYG
ASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*SSSSG
SSSSGSSSS*-GATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKE
LVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKL
VDRSWSQLLLLKAGHRLSVGLRALHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE
F8(scDb)-4-1BBL: F8_VH-*linker*-F8_VL-*linker*- F8_VH-*linker*-F8_VL-*linker*-4-1BBL (Format **6**)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GGSG
G*-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*GGGGSGGGGSGGG
GS*-EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTY
YADSVKGRFTISRDNSKNT LYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GG
SGG*-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGI
PDRFSGSGSGTDFTLTISRLEPEDFAVYYCQMRGRPPTFGQGTKVEIK-*SSSSGSSSSGSSSS
G*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLY
YVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQL
LLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE
F8(dDb)-4-1BBL: F8_VH-*linker*-F8_VL-*linker*-4-1BBL (Format **7**)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS-*GGSG
G*-EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK-*SSSSGSSSSGSSSSG*
-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYY
VFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLL
LLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE
F8(IgG)-4-1BBL_HC: VL-CL** VH-CH-*linker*-4-1BBL (Format **8**)
EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRF
SGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQL

KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC**
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSASTKG
PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK-*SSSSGSSSSGSSSSG*-ATTQQGSPVFAKLLAKNQASLCNTTLNWHSQD
GAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVD
DFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHGAQDAYRDWELSYPNT
TSFGLFLVKPDNPWE

F8(IgG)-4-1BBL_LC: VL-CL-*linker*-4-1BBL** VH-CH (Format **9**)
EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRF
SGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC-*SSSSGSSSSGSSSSG*-ATTQQGSPVFAKLLAKN
QASLCNTTLNWHSQDGAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKV
QGWVSLVLQAKPQVDDFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRAYLHG
AQDAYRDWELSYPNTTSFGLFLVKPDNPWE **
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSASTKG
PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK

KSF(IgG)-4-1BBL_HC: KSF_VL-KSF_CL** KSF_VH-KSF_CH-*linker*-4-1BBL
SELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSG
SSSGNTASLTITGAQAEDEADYYCNSSPLNRLAVVFGGGTKLTVLGCNSSPLNRLAVVFGGG
TKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTT
PSKQSNNKYAASSYLSLTPEQWKSHKSYSCQVTHEGSTVEKTVAPTECS**
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSPKVSLFDYWGQGTLVTVSSASTKG
PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK-*SSSSGSSSSGSSSS*-GATTQQGSPVFAKLLAKNQASLCNTTLNWHSQD
GAGSSYLSQGLRYEEDKKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVD
DFDNLALTVELFPCSMENKLVDRSWSQLLLLKAGHRLSVGLRALHGAQDAYRDWELSYPNTT
SFGLFLVKPDNPWE

KSF(scFv)-4-1BBL: KSF_VH-*linker*-KSF_VL-*linker*-4-1BBL
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSPKVSLFDYWGQGTLVTVSS-*GGGG
SGGGGSGGGGS*-SELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGK
NNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSSPLNRLAVVFGGGTKLTVLG -*SSS
SGSSSSGSSSS*-GATTQQGSPVFAKLLAKNQASLCNTTLNWHSQDGAGSSYLSQGLRYEED
KKELVVDSPGLYYVFLELKLSPTFTNTGHKVQGWVSLVLQAKPQVDDFDNLALTVELFPCSME
NKLVDRSWSQLLLLKAGHRLSVGLRALHGAQDAYRDWELSYPNTTSFGLFLVKPDNPWE

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