Supplementary Methods

Antibodies and reagents

Research antibodies

Antibodies were in general produced at Genmab in a human IgG1 backbone and included Fc-silencing mutations (L234F, L235E, D265A) and matched DuoBody mutations in the CH3 region for cFAE (F405L and K409R), unless otherwise specified.

Antibody	Description	Supplier (batch/cat. no.)
DuoBody-CD40x4-1BB	BsAb that contains a CD40-specific Fab arm, a 4-1BB-specific Fab arm and a human IgG1 backbone with Fc-silencing and the DuoBody mutations	Genmab (6306-06-08)
		Genmab (3898-197-EP)
		Genmab (6306-31-11-EP)
		Genmab (508332)
DuoBody-CD40x4-1BB surrogate	BsAb that contains a non-humanized variant of the CD40-specific Fab-arm of GEN1042, a non-humanized variant of the 4-1BB-specific Fab arm of GEN1042 and a human IgG1 backbone with Fc-silencing and the DuoBody mutations	Genmab (3593-060-EP)
mAb-CD40-FEAL	Bivalent CD40-specific mAb in a human IgG1 backbone with Fc-silencing and a DuoBody mutation; parental antibody for DuoBody-CD40x4-1BB	Genmab (4121-130-EP)
mAb-CD40 (active Fc)	Bivalent CD40-specific mAb that contains the CD40-specific Fab arm of DuoBody-CD40x4-1BB in an Fc-active human IgG1 backbone	Genmab (170802_PSM_0024#004)
mAb-4-1BB-FEAR	Bivalent 4-1BB-specific mAb in a human IgG1 backbone with Fc-silencing and a DuoBody mutation; parental antibody for DuoBody-CD40x4-1BB	Genmab (4121-131-EP)
bsAb-CD40xctrl	BsAb that contains a CD40-specific Fab arm, a non-binding control (ctrl) Fab arm based on IgG-b12, and a human IgG1 backbone with Fc-silencing and the DuoBody mutations	Genmab (170628_PSM_0016#001)
		Genmab (180202_PSM_0002#001)
		Genmab (180109_PSM_0050#001)
		Genmab (3727-032-EP)
bsAb-ctrlx4-1BB	BsAb that contains a 4-1BB-specific Fab arm, a non-binding control (ctrl) Fab arm based on IgG-b12, and a human IgG1 backbone with Fc-silencing and the DuoBody mutations	Genmab (170626_PSM_0016#001)
		Genmab (171205_PSM_0000#005)
		Genmab (180801_PSM_0031#002)
		Genmab (3593-072-EP)
mAb-ctrl-FEAL	Bivalent non-binding control (ctrl) mAb based on IgG-b12 in a human IgG1 backbone with Fc-silencing and a DuoBody mutation	Genmab (3666-078-EP)
		Genmab (3165-065-EP)
		Genmab (201029_PSM_0071#001)
		Genmab (3392-107-EP)
Urelumab analog	Bivalent urelumab analog in a human IgG1 backbone with Fc-silencing and DuoBody mutations	Genmab (181224_PSM_0026#002)
Mitazalimab analog	Bivalent Mitazalimab analog in a human IgG1 backbone with Fc-silencing and DuoBody mutations	Genmab (171205_PSM_0000#005)
Pembrolizumab	Research-grade pembrolizumab	Selleckchem (cat. no. A2005)
Pembrolizumab	Clinical-grade pembrolizumab	MSD, (article no. 10749880)

Muik A, *et al. J Immunother Cancer* 2022; 10:e004322. doi: 10.1136/jitc-2021-004322

Commercial antibodies

Target	Label[1]	Clone	Supplier	Cat. No.
CLDN6	DyLight650	IMAB 027	Ganymed	
TCRβ	BV421	H57-597	BD Biosciences	562839
IgG2,λ	BV421	Ha4/8	BD Biosciences	562629
CD20	FITC	2H7	BD Biosciences	556632
IgG, Fcγ Fragment specific	APC	-	Jackson ImmunoResearch	109-136-170
CD3	BV421	SP34-2	BD Biosciences	562877
IgG, Fcγ Fragment Specific	AF488	-	Jackson ImmunoResearch	109-546-098
CD137 (4-1BB)	APC	4B4-1	BD Biosciences	550890
AffiniPure F(ab') Fragment Goat Anti-human IgG, Fcγ fragment specific	R-PE	N/A	Jackson ImmunoResearch	109-116-098
LFA-1	unconjucated	HI111	eBioscience	14-0119-82
AffiniPure F(ab') Fragment Goat Anti-Mouse IgG (H+L)	AF488	polyclonal	Jackson ImmunoResearch	115-546-146
Phalloidin	AF546	N/A	Thermo Fisher	A22283
HLA-DR	BV421	G46-6	BD	562804
CD86	BUV737	Fun-1	BD	612784
CD45	AF700	H130	BD	560566
CD3	BUV496	UCHT1	BD	612940
CD40	FITC	LOB7/6	Invitrogen	MA5-16775
4-1BB	PE	4B4-1	Biolegend	309804
PD-L1	APC	29E.2A3	Biolegend	329708
CD27	BV786	L128	BD	563327
CD3	-	UCHT1	R&D systems	MAB100-500
CD4	PE	RPA-T4	TONBO Bioscience	50-0049
CD8	PE-Cy7	RPA-T8	TONBO Biosciences	60-0088
CD19	BV421	HIB19	Becton Dickinson	562440
CD8	PE	RPA-T8	TONBO	50-0088
GzmB	PE	GB11	BD	561142
CD107a	AF647	H4A3	Biolegend	328611
CD3	PE-Cy7	UCHT1	eBioscience	25-0038
CD4	FITC	M-T466	Miltenyi Biotec	130-080-501
CD56	APC	CMSSB	eBioscience	17-0567-042
CD20	APC	2H7	Becton Dickinson	559776
CD69	FITC	FN50	Becton Dickinson	557049
CD86	PE	2331/FUN-1	Becton Dickinson	555658
CD3	APC-H7	SK7	BD Biosciences	560176
CD4	PE	Sk3	BD Biosciences	345769
CD8	PerCP-Cy5.5	RPA-T8	BD Biosciences	560662
CD45RA	FITC	L48	BD Biosciences	335039
CCR7	BV510	3D12	BD Biosciences	563449
HLA-DR	APC	G46-6	BD Biosciences	559866
Ki67	BV421	B56	BD Biosciences	562899
CD45	BV605	HI30	BD Biosciences	564047
CD3	APC-H7	SK7	BD Biosciences	560176
CD16	PerCP-Cy5.5	3G8	BD Biosciences	338440
CD56	FITC	NCAM16.2	BD Biosciences	345811
CD19	PE-Cy7	Sj2SC1	BD Biosciences	341113
CD86	BV421	2331	BD Biosciences	562432
CD86	APC	2331	BD Biosciences	555660

[1] FITC: fluorescein isothiocyanate; PE: phycoerythin; BV: brilliant violet; APC: allophycocyanin; AF: AlexaFluor; Cy: Cyanine;

Cell lines

K562 cells (ATCC, CCL-243) were cultured in IMDM with 10% FBS. K562-h4-1BB cells were generated by retroviral transduction of human 4-1BB. K562-hCD40 cells were generated by stable transfection with human CD40.

Cell culture media and buffers

Name	Composition
X-Vivo15 medium	X-VIVO™ 15 chemically defined, serum-free hematopoietic cell medium (Lonza, BE02-060Q)
OptiPro SFM medium	OptiPro SFM medium (Thermo Fisher Scientific Europe B.V., 12309050) supplemented with 50 Units penicillin/50 Units streptomycin (Lonza, DE17-603E)
DC medium	RPMI 1640 medium supplemented with 5% plasma-derived human serum [PHS, One Lambda Inc., A25761], 1x minimum essential medium non-essential amino acids [MEM NEAA, Life Technologies GmbH, 11140-035], 1 mM sodium pyruvate [Life Technologies GmbH, 11360-039], 100 ng/mL [Miltenyi Biotec GmbH, 130-093-868] and 50 ng/mL IL-4 [Miltenyi Biotec GmbH, 130-093-924]
MLR medium	RPMI 1640 complete (ATCC modification formula, ThermoFisher, A1049101) supplemented with 10% fetal bovine serum (Gibco, 16140071)
T-cell assay medium	Iscove's Modified Dulbecco's Medium [IMDM, Life Technologies GmbH, 12440-053] supplemented with 5% PHS
Transport medium	HypoThermosol® FRS [BioLifeSolutions, 101104] supplemented with 3% Fungizone [Thermo Fisher, 15290-026] and 300 U/mL penicillin, 300 µg/mL streptomycin [pen/strep, Thermo Fisher, 15140-122]
Wash medium	X-Vivo15 medium [Lonza, BE02-060Q] supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin
TIL medium	X-Vivo15 medium supplemented with 10% human serum albumin [HSA, CSL Behring, PZN-00504775], 100 U/mL pen, 100 µg/mL strep, 1% Fungizone and 10 U/mL IL-2 [Proleukin®S, Novartis Pharma, PZN-02238131]
FACS buffer	DPBS supplemented with 2% FBS and 2 mM EDTA
FreeStyle 293 Expression Medium	FreeStyle™ 293 Expression Medium with 4 mM Glutamax (Invitrogen, 12338-018) supplemented with 50 Units penicillin/50 Units streptomycin (Lonza, DE17-603E)
B-cell medium	X-VIVO™ 15 chemically defined, serum-free hematopoietic cell medium (Lonza, cat. no. BE02-060Q) supplemented with 10% PHS (One Lambda Inc., cat. no. A25761), 1x Glutamax (Life Technologies GmbH, cat. no. 35050-061) and 20 mM HEPES (Life Technologies GmbH, cat. no. 15630-056)

Generation of parental antibodies mAb-CD40-FEAL and mAb-4-1BB-FEAR

The CD40-specific parental antibody for DuoBody-CD40x4-1BB (mAb-CD40-FEAL) was based on the murine anti-human CD40 antibody Lob 7/4. The 4-1BB-specific antibody for DuoBody-CD40x4-1BB (mAb-4-1BB-FEAR) was generated by immunization of rabbits and single B-cell cloning. Both antibodies were humanized using germline humanization (complementarity-determining region [CDR]-grafting) technology (Abzena). The selected V_L and V_H sequences were subsequently cloned in a human backbone that also contained human IgG1 constant heavy chain regions in which the one of the DuoBody mutation (F405L or K409R) and the Fc-silencing L234F, L235E and D265A (FEA) mutations had been introduced.

FcγR binding assays

Enzyme-linked immunosorbent assay (ELISA) plates (Greiner Bio-one, 655092) were coated with 1 µg/mL goat F(ab')2-anti-human (Jackson ImmunoResearch, 109-006-097), blocked with 0.2% bovine serum albumin (BSA) Fraction V (Roche, 10735086001), incubated with antibodies at RT for 1 h, followed by 1 µg/mL HIS- and biotin acceptor peptide (BAP-) tagged recombinant dimeric FcγR constructs (diFcγR HisBAP; previously described[1]) at RT for 1 h, Streptavidin-polyHRP (CLB, M2032) at RT for 30 min and finally 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Roche, 11112422001) at RT for 10-30 min. Absorption at OD405 was measured on an ELx808 Absorbance Microplate Reader (BioTek Instruments).

Biolayer interferometry (BLI)

Target binding affinity of DuoBody-CD40x4-1BB was determined by BLI on an Octet HTX instrument (FortéBio). Experiments were carried out while shaking at 1,000 RPM at 30°C.

Activated Amine Reactive 2nd Generation (AR2G) biosensors (FortéBio, 18-0026) were loaded with recombinant human CD40 ECD fused to a His-tag (CD40-His, 7.5 µg/mL; Sino Biological, 10774-H08H) or recombinant human 4-1BB ECD fused to a His-tag (41BBHis, 2.5 µg/mL; Sino Biological, 10041-H08H) diluted in acetate buffer (pH 6.0; FortéBio, 18-1070) and quenched with 1 M ethanolamine pH 8.5 (FortéBio, 18-1071). After a baseline measurement in Sample Diluent (FortéBio, 18-1048), association (200 s) and dissociation (2,000 s) of DuoBody-CD40x4-1BB was determined.

Data were acquired using Data Acquisition Software (FortéBio, v9.0.0.49d) and analyzed with Data Analysis Software (FortéBio, v9.0.0.14). Data were fitted with the 1:1 Global Full fit model.

Binding to target-transfected cells

CHO-S cells (ThermoFisher Scientific, R800-07) were transfected with full-length CD40 expression constructs (1.25 µg/mL) using Freestyle Max transfection reagent (Thermo Fisher Scientific, 16447100) and OptiPro SFM medium, according to the manufacturer's instructions. Binding of CD40-specific antibodies to CHO-S cells transiently transfected with human CD40 was analyzed by flow cytometry. HEK293F cells (ThermoFisher Scientific, R790-07) were transfected with full-length 4-1BB expression constructs (100 µg) using Freestyle Max transfection reagent and Freestyle 293 Expression medium, according to the manufacturer's instructions. Binding of 4-1BB-specific antibodies to HEK293F cells transiently transfected with human 4-1BB was analyzed by flow cytometry. Non-transfected CHO-S or HEK293F cells were used as a negative control.

Cell-based binding and blocking assays

Binding of CD40-specific antibodies to primary $CD20^+$ B cells was analyzed by flow cytometry using monocyte-depleted primary human PBMCs.

Binding of 4-1BB-specific antibodies to primary $CD3^+$4-$1BB^+$ T cells was analyzed by flow cytometry using the $CD14^-$ fraction of PBMCs that had been stimulated with Dynabeads™ Human T-Activator CD3/CD28 (Life Technologies GmbH, 11132D) for 48 h. Simultaneous binding of DuoBody-CD40x4-1BB to cells expressing CD40 or 4-1BB was analysed by flow cytometry assessment of doublet formation after co-incubation of K562-hCD40 cells labeled with CellTrace Violet (Thermo Fisher, C34557) and K562-h4-1BB cells labeled with CellTrace FarRed (Thermo Fisher, C34564), for 15 min at 37 °C.

Immunofluorescence and live-cell imaging

For immunofluorescence, DCs were seeded on poly-L-lysine (0.1 µg/mL; 2×10^6 cells/well) coated coverslips for 4 h. Subsequently, $CD8^+$ T cells, which had been activated by anti-CD3/anti-CD28 coated microbeads (Thermo Fisher, 11131D), were added (4×10^5 cells/well). The cells were co-cultured for 1 h in the presence of 0.5 µg/mL of DuoBody-CD40x4-1BB or control antibodies. The cells were fixed for 10 min at RT in 4% PFA, permeabilized with 0.25% Triton-X/PBS, blocked in 2% BSA/PBS and stained for cell surface antigens using commercial

antibodies and with Hoechst (Life Technologies, H3570). Coverslips were washed and mounted with Immomount media (Life Technologies, 9990402). Images were acquired at the confocal microscope Leica Sp8.

For live-cell imaging, claudin 6 (CLDN6)-electroporated iDC were seeded in a 96-well plate (1×10^4 cells/well). After O/N incubation CLDN6-TCR-electroporated $CD8^+$ T cells were added (2×10^4 cells/well), and the cells co-cultured in the presence of 0.125 µg/mL of DuoBody-CD40x4-1BB or control antibodies. Time lapse videos were performed at 37°C and 5% CO_2 at the Nikon Eclipse TiE microscope with a time frame of 15 min.

Image analysis was performed using the Fiji (ImageJ 1.52p) software. The intensity profile of the fluorescence at the synapse was generated selecting the single channels *Image* > *Color* > *Channel Tool*. At the interface between the DC and the $CD8^+$ T cell, a line was generated selecting the *segmented line* tool. By selecting *Analyze* > *Plot profile* an intensity profile was generated. For the live imaging, cells were manually followed and the number of contacts and contact duration was manually determined during the videos.

Flow cytometry

In preclinical studies, cells were stained for cell surface antigens using commercial antibodies for 15-30 min at 4°C. For intracellular staining, cells were fixed and permeabilized followed by intracellular staining for 30 min at 4°C. Viable cells were distinguished by staining with 7-aminoactinomycin D (7-AAD; Beckman Coulter, A07704), LIVE/DEAD™ Fixable Near-IR Dead cell stain kit (Thermo Scientific, L10119). Data were acquired on a BD FACSCanto™ or BD LSRFortessa™ X20 flow cytometer (BD Biosciences). For cell counting, negative control compensation beads (BD Biosciences, 51-90-9001291) were added to the cells prior to acquisition. Data was analyzed using FlowJo™ software V10.3. Proliferation analysis based on CFSE dilution was performed using the proliferation modeling tool from FlowJo, the generation peaks were automatically fitted and expansion index values were calculated.

Clinical study blood samples were collected just prior to DuoBody-CD40x4-1BB administration in Cycle 1 and 2, and on the following time points after administration in Cycle 1: Day 2, Day 3, Day 8 and Day 15. Cellular subsets in the blood were evaluated using antibody panels described in the Supplementary Methods and a Becton Dickinson FACSCanto™ flow cytometer equipped with FacsDiva software version 8.0.1.

Cytokine analysis

In preclinical studies, cell culture supernatants were analyzed using the V-PLEX Proinflammatory Panel 1 Human Kit (Meso Scale Diagnostics LLC, K15049D-2) on a MESO QuickPlex SQ 120 instrument (Meso Scale Diagnostics, LLC., R31QQ-3), according to the manufacturer's instructions. In the MLR assays, cytokines were analyzed by ELISA using the Alpha Lisa IFNγ kit (Perkin Elmer, AL217) or by Luminex using a custom Milliplex MAP Human cytokine/TH17 panel (Millipore Sigma, cat. no. SPR1526) on a Luminex FLEXMAP 3D instrument.

Clinical study blood samples were collected just prior to DuoBody-CD40x4-1BB administration in Cycle 1 and 2, and on the following time points after administration in Cycle 1: 2 h, 4-6 h, Day 2, Day 3, Day 8 and Day 15. Cytokines and chemokines were evaluated using V-PLEX Plus Human Biomarker 40-Plex kit (Meso Scale Diagnostics LLC, K15209G-1) on a Meso Sector S600 instrument (Meso Scale Diagnostics, LLC., IC0AA-0).

TCR repertoire analysis

For TCR repertoire sequencing, total RNA was isolated from tumor tissue or snap-frozen cultured TILs using the RNeasy Mini kit (Qiagen, 74104). Libraries were generated with the SMARTer human TCR-αβ profiling kit (Clontech, 635015) and were sequenced using the MiSeq system (Illumina). Data was analyzed using MiXCR[2] and VDJtools[3].

Muik A, *et al. J Immunother Cancer* 2022; 10:e004322. doi: 10.1136/jitc-2021-004322

Supplementary Table

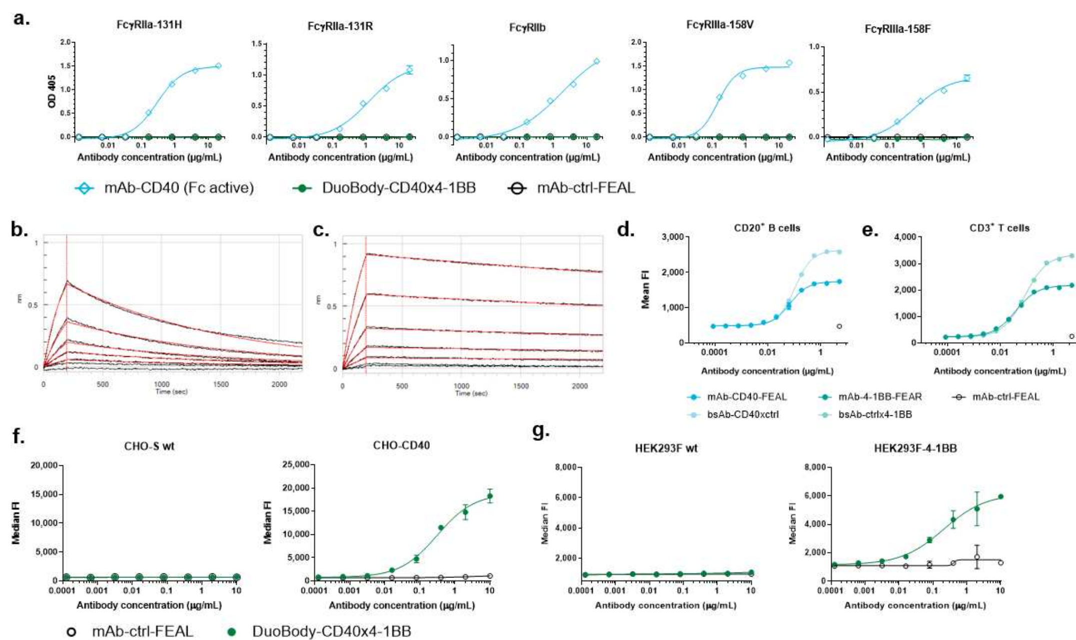
Supplementary Table 1 Binding affinity to recombinant human CD40 and 4-1BB

Binding of DuoBody-CD40x4-1BB to recombinant human CD40 and 4-1BB was analyzed using biolayer interferometry (BLI). Experiments were performed using the recombinant extracellular domains of CD40 or 4-1BB immobilized on the biosensors and an antibody concentration range of 0.16 – 10 nM. K_D (nM) refers to the equilibrium dissociation constant of the antibody-antigen interaction. k_d (s^{-1}) is the dissociation rate constant and k_a ($M^{-1}s^{-1}$) the association rate constant.

Antibody	**Antigen**	**n**	**k_a (1/Ms)**	**k_d (1/s)**	**K_D (nM)**
DuoBody-CD40x4-1BB	human CD40-His	**3**	$6.6×10^5 ± 1.5×10^5$	$6.7×10^{-4} ± 4.2 x 10^{-5}$	1.0 ± 0.19
DuoBody-CD40x4-1BB	human 4-1BB-His	**3**	$5.3×10^5 ± 1.1×10^5$	$8.9×10^{-5} ± 8.7×10^{-6}$	0.17 ± 0.02

Muik A, et al. J Immunother Cancer 2022; 10:e004322. doi: 10.1136/jitc-2021-004322

Supplementary Figures

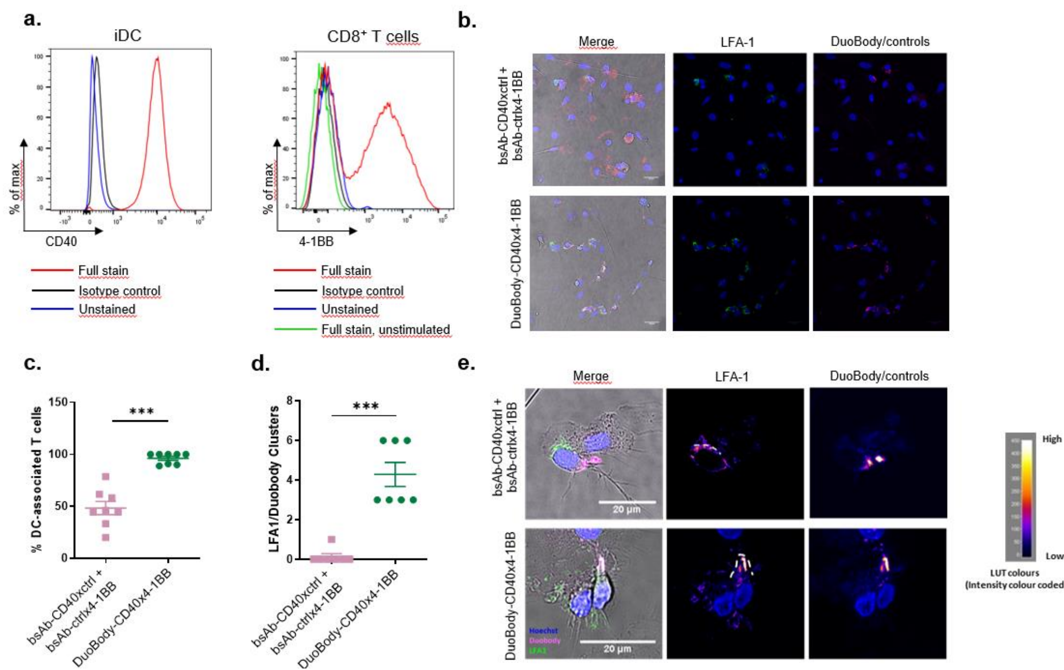


Supplementary Figure 1 Target binding characteristics of DuoBody-CD40x4-1BB (related to Figure 1)

A. Binding of FcγRIIa, -RIIb and -RIIIa to the indicated immobilized antibodies was determined by ELISA using the previously described HisBAP dimeric FcγR constructs[1]. Two common coding polymorphisms were tested for both FcγRIIa (131H and 131R) and –IIIa (158F and 158V)[4, 5, 6]. Data shown are mean optical densities at 405 nm (OD405) ± SD of duplicate measurements from one representative experiment (n=2). **B-C.** Binding of DuoBody-CD40x4-1BB to recombinant human CD40 (B) and 4-1BB (C) was analyzed by BLI. Experiments were performed using the recombinant ECD of CD40 or 4-1BB immobilized on the biosensors and an antibody concentration range of 0.023 – 1.5 µg/mL (0.16 - 10 nM). Association to and dissociation of DuoBody-CD40x4-1BB from CD40-His (B) or 4-1BB-His (C) is shown for one representative experiment (n=3). The black traces represent the association and dissociation of the antibody at different concentrations, the red traces represent the calculated global full fit using the 1:1 model. **D.** Binding of the CD40-specific Fab arm of DuoBody-CD40x4-1BB (bsAb-CD40xctrl) and control antibodies to human PBL *in vitro* was analyzed by flow cytometry. Data shown are MFI ± SD within the $CD20^+$ B-cell gate of triplicate measurements from one representative donor (n=2) **E.** Binding of the 4-1BB-specific Fab arm of DuoBody-CD40x4-1BB (bsAb-ctrlx4-1BB) and control antibodies to *in vitro* activated PBL was analyzed by flow cytometry. Data shown are MFI ± SD on $CD3^+4\text{-}1BB^+$ T cells of triplicate measurements from one representative donor (n=3). **F.** Binding of DuoBody-CD40x4-1BB to wild type or CD40-expressing CHO-S cells was determined by flow cytometry and compared with binding of the non-humanized variant bsIgG1-chCD40xch4-1BB. IgG1-ctrl-FEAL was taken along as negative control. Data shown are median fluorescence intensities (FI) ± SD from one representative experiment (n=3). **G.** Binding of DuoBody-CD40x4-1BB to wild type or 4-1BB-expressing HEK293F cells was determined by flow cytometry

Muik A, *et al. J Immunother Cancer* 2022; 10:e004322. doi: 10.1136/jitc-2021-004322

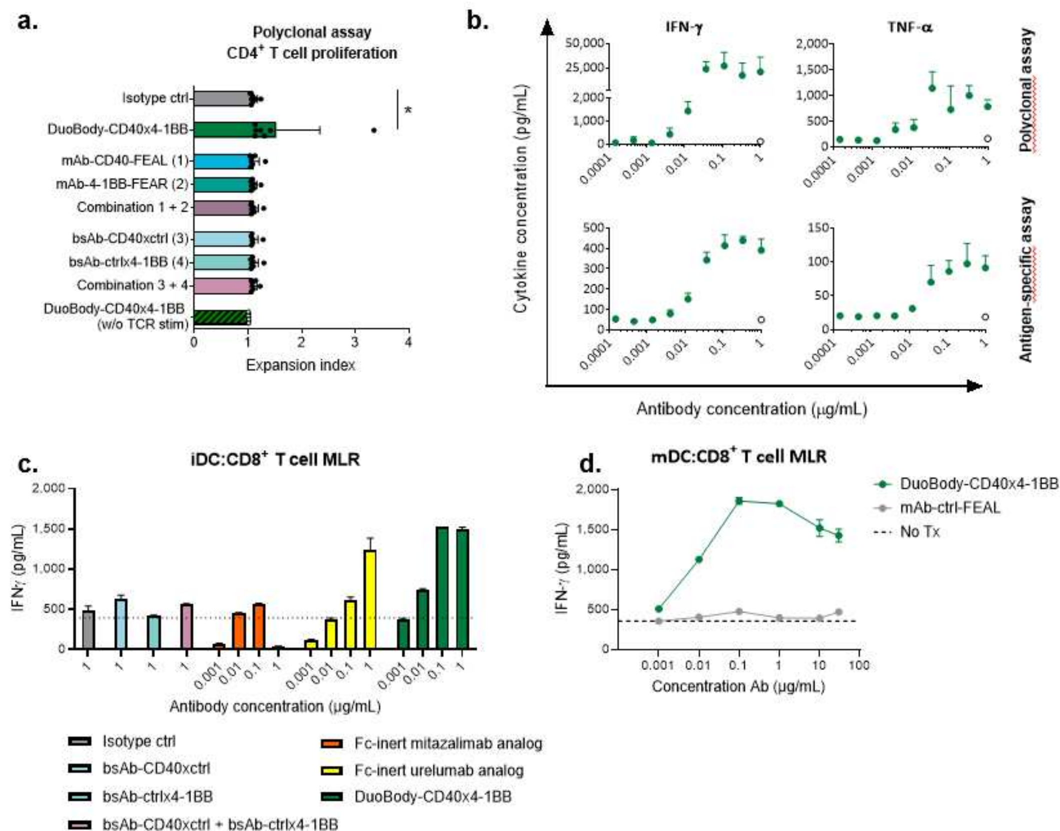
and compared with binding of bsIgG1-chCD40xch4-1BB. IgG1-ctrl-FEAL was taken along as negative control. Data shown are median FI ± SD from one representative experiment (n=3).



Supplementary Figure 2 DuoBody-CD40x4-1BB enhances formation of the immune synapse between APCs and T cells (related to Figure 2)

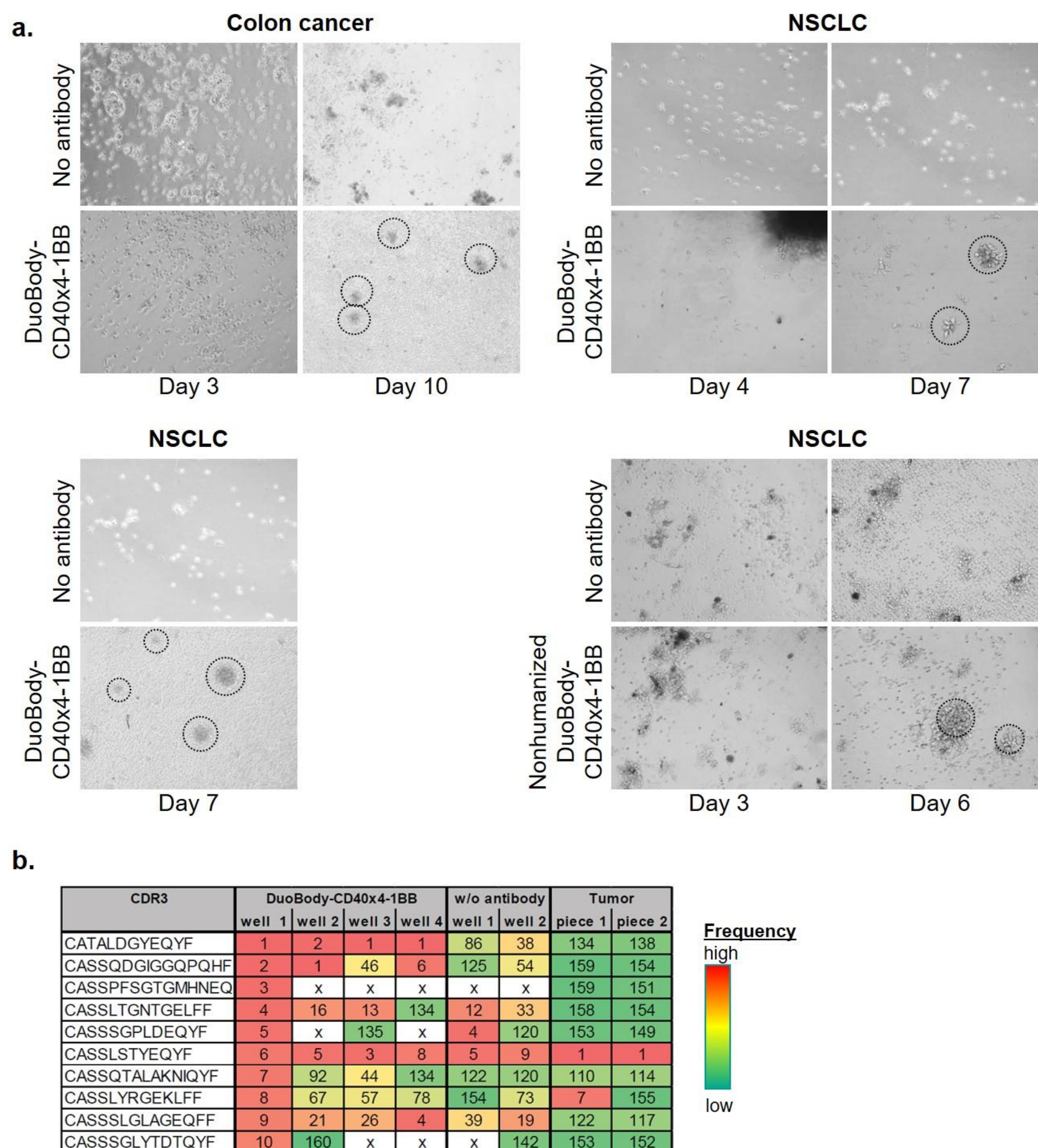
A. Flow cytometry analysis of CD40 expression on iDCs and 4-1BB expression on CD8+ T cells. **B.** Images of DC/T-cell co-cultures incubated with 0.125 µg/mL Alexa Fluor 647-conjugated DuoBody-CD40x4-1BB or a combination of bsAb-CD40xctrl and bsAb-ctrlx4-1BB (in magenta) and stained with an anti-human LFA-1 (in green) antibody. Nuclei were counterstained with Hoechst (in blue). Scale bars: 20 µm. **C.** The percentage of T cells that are associated with a DC. **D.** Clusters of LFA-1 with DuoBody-CD40x4-1BB or with control antibodies per field of view. ***, P<0.001; Mann-Whitney test. **E.** Higher magnification images of DC/T-cell co-cultures stained as in (B). Dashed line indicates the DC/T-cell interface.

Muik A, *et al. J Immunother Cancer* 2022; 10:e004322. doi: 10.1136/jitc-2021-004322



Supplementary Figure 3 DuoBody-CD40x4-1BB enhances T-cell activation *in vitro*

A. CFSE-labeled human PBMCs were stimulated with 0.03 µg/mL anti-CD3 and incubated with DuoBody-CD40x4-1BB or control antibodies (0.2 µg/mL) for 4 days. CFSE dilution in CD4⁺ T cells was analyzed by flow cytometry. Expansion index of individual donors and mean ± SD (n=7) is shown. *, P<0.05; Friedman test. **B.** Supernatant of the polyclonal and antigen-specific T-cell proliferation assays described in Figure 5A-B were collected after 48 h of culture in the presence of DuoBody-CD40x4-1BB or isotype ctrl antibodies. Cytokine secretion was analyzed using the V-PLEX Proinflammatory Panel 1 Human Kit. Data shown are mean concentration ± SD of triplicate wells from one representative donor. **C.** Purified CD8 T cells from healthy donors were co-cultured with immature allogeneic DCs in the presence of DuoBody-CD40x4-1BB, Fc-inert analogs of clinical mAbs mitazalimab and urelumab (0.001-1 µg/mL), or control antibodies (1 µg/mL; for combination of control antibodies 1 µg/mL of both antibodies was added) for 5 days. B. IFNγ concentrations in supernatant taken after 5 days from cultures (derived from the same donor as shown in Figure 3). Data shown are mean concentration ± SD of duplicate wells from one representative donor (n=2). Dotted line shows percentage of IFNγ in DC-T-cell cultures in the absence of treatment. **D.** Purified CD8⁺ T cells from healthy donors were co-cultured with LPS-matured allogeneic DCs in the presence of DuoBody-CD40x4-1BB or control antibodies for 4-5 days. The concentration of IFN-γ in the culture supernatant was analyzed by ELISA.

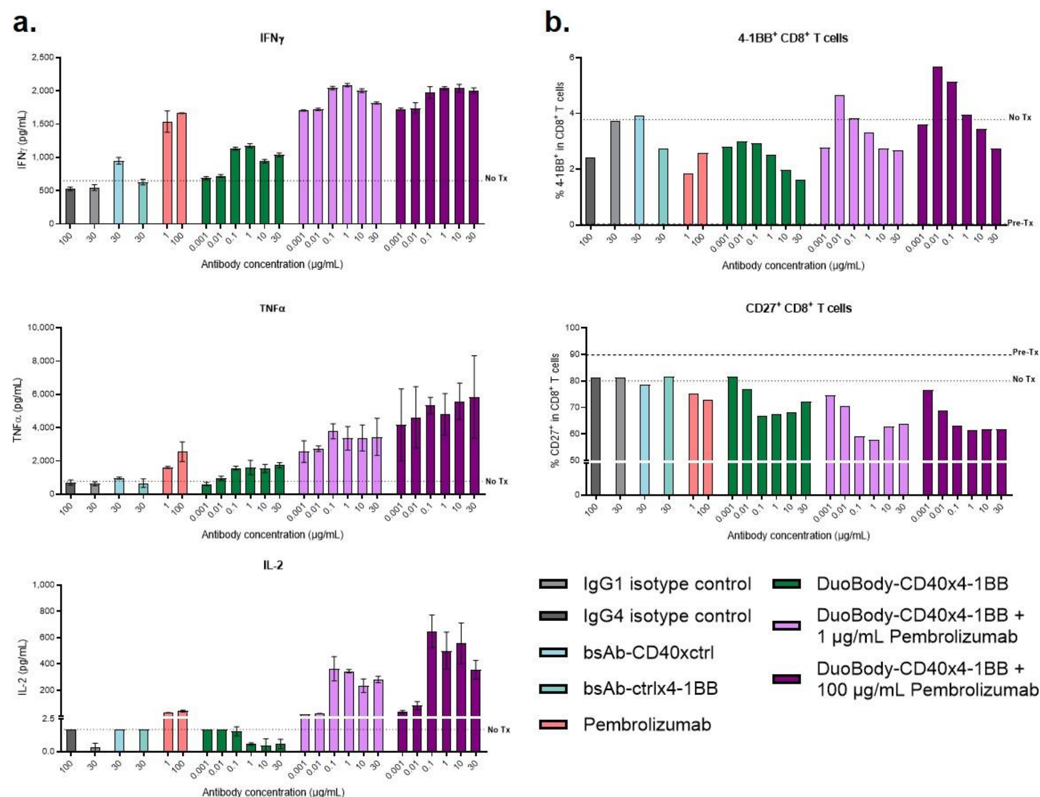


CDR3	DuoBody-CD40x4-1BB				w/o antibody		Tumor	
	well 1	well 2	well 3	well 4	well 1	well 2	piece 1	piece 2
CATALDGYEQYF	1	2	1	1	86	38	134	138
CASSQDGIGGQPQHF	2	1	46	6	125	54	159	154
CASSPFSGTGMHNEQ	3	x	x	x	x	x	159	151
CASSLTGNTGELFF	4	16	13	134	12	33	158	154
CASSSGPLDEQYF	5	x	135	x	4	120	153	149
CASSLSTYEQYF	6	5	3	8	5	9	1	1
CASSQTALAKNIQYF	7	92	44	134	122	120	110	114
CASSLYRGEKLFF	8	67	57	78	154	73	7	155
CASSSLGLAGEQFF	9	21	26	4	39	19	122	117
CASSSGLYTDTQYF	10	160	x	x	x	142	153	152

Supplementary Figure 4 DuoBody-CD40x4-1BB enhances TIL expansion

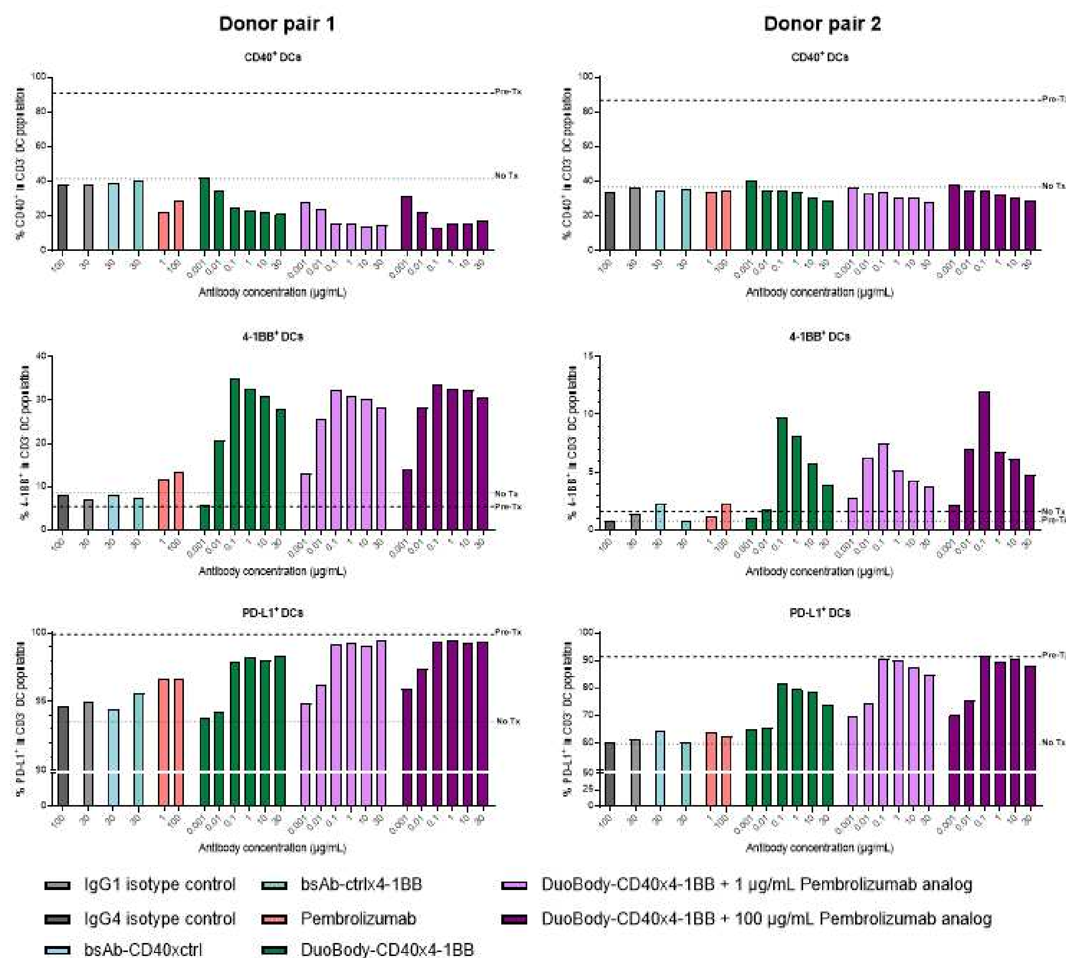
Tumor tissues resected from a colon cancer patient and three NSCLC patients were cut into pieces of 1-2 mm^3, and cultured in the presence of IL-2 (10-50 U/mL) with or without DuoBody-CD40x4-1BB (0.0008 – 0.2 µg/mL) or a non-humanized DuoBody-CD40x4-1BB surrogate (0.01 – 1 µg/mL). **A.** TIL microclusters are highlighted by the dashed circles. **B.** TCR repertoire analysis was performed by *TRB* RNA sequencing of the expanded TIL and the tumor fragments derived from the NSCLC specimen. The ten most frequent clones (1-10) for well 1 of DuoBody-CD40x4-1BB surrogate-treated cultures were used as reference and the frequency rank of these sequences in all other samples is shown in B (from high frequency [red] to low frequency [green]).

Muik A, *et al. J Immunother Cancer* 2022; 10:e004322. doi: 10.1136/jitc-2021-004322



Supplementary Figure 5 Combination of DuoBody-CD40x4-1BB with pembrolizumab amplifies the magnitude of the immune response

Purified CD8+ T cells from healthy donors were co-cultured with LPS-matured allogeneic DCs in the presence of DuoBody-CD40x4-1BB, research-grade pembrolizumab (either alone or in combination [concurrent treatment]) or control antibodies for 5 days. **A.** Cytokine concentrations in supernatant taken after 5 days of culture in the presence of indicated antibodies. Data shown are mean concentration ± SD of duplicate wells from one donor pair (DC: LS1166085; T: LS8843759B). Dashed line indicates co-cultures that were not treated with antibody (No Tx). **B.** The percentage of 4-1BB+ CD8+ T cells and CD27+ CD8+ T cells was measured by flow cytometry. Data shown are the percentage of positive cells within the total CD8+ T-cell population from one donor pair. Target expression (CD40, 4-1BB and PD-L1) on DCs is shown in Figure S6. Dashed line indicates co-cultures on Day 0 (before antibodies were added to the treated co-cultures) and co-cultures that were not treated with antibody at Day 5 of the MLR assay (No Tx).



Supplementary Figure 6 Target expression in co-cultures of mDCs with CD8+ T cells

Purified CD8 T cells from healthy donors were co-cultured with LPS-matured allogeneic DCs in the presence of DuoBody-CD40x4-1BB, research-grade pembrolizumab (either alone or in combination [concurrent treatment]) or control antibodies for 5 days. The percentage of CD40+, 4-1BB+ and PD-L1+ DCs was measured by flow cytometry. Data shown are the percentage of positive cells within the total CD3- DC population from two donor pairs (associated with Figure 7 and S5). PD-1 expression on T cells could not be evaluated due to the presence of pembrolizumab in the co-cultures, limiting binding of fluorescently-labeled PD-1 antibodies used for flow cytometry. Dashed line indicates co-cultures on Day 0 (before antibodies were added to the treated co-cultures) and co-cultures that were not treated with antibody at Day 5 of the MLR assay (No Tx).

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