BiAb redirect SAR T cell therapy in melanoma

1 **SUPPLEMENTARY METHODS**

2 **Processing and analysis of single-cell RNA-sequencing data**

3 All analyses from UMI count matrices were run with python 3 with the Scanpy API v.1.4.6[1] and
4 anndata v.0.7.1.[2] to obtain high quality cells, barcodes from GSE72056 were filtered based on
5 total UMI counts and total genes after visual inspection of distributions. Cells with over 20000
6 counts or 10000 genes as well as cells with under 6000 counts or 2000 genes were removed.
7 Genes with expression in less than 20 cells were excluded. Cells were normalised using the
8 SCRAN algorithm,[3] expression values were then log-transformed. The top 4000 highly variable
9 genes were selected based on normalised dispersion as described in.[4] To efficiently capture the
10 underlying data structure in two dimensions, a neighborhood graph was computed on the first
11 50 principal components using Scanpy's pp.neighbors with 15 neighbors. For 2D visualization,
12 embedding the neighborhood graph via UMAP was done by running Scanpy's tl.umap with
13 default parameters. Annotations of cells were provided by the authors.

14 **Analysis of TYRP1 and MCSP expression using flow cytometry**

15 Cells were detached using Accutase solution (Capricorn Scientific). Dead cells were stained
16 using the eFluor™ 780 fixable viability dye (1:1000, eBioscience, Thermo Fisher Scientific) for
17 15 minutes at room temperature, followed by staining of cell surface proteins using either 0.5
18 µg/mL of αMCSP/αE3 at 4°C or αTYRP1/αE3 at 37 °C for 30 minutes. Then, staining of primary
19 antibodies were conducted using a secondary goat polyclonal antibody against human IgG (2.5
20 µg/mL, Southern Biotech) for 15 minutes at room temperature. Median fluorescence intensity
21 (MFI) ratio was calculated based on ratio of MCSP or TYRP1 stain and secondary antibody only
22 stain.

23 Cells were analyzed on LSRFortessa (BD Biosciences) or CytoFLEX (Beckman Coulter Life
24 Sciences) flow cytometers, and data were analyzed with FlowJo software version 9.9.5 or
25 version 10.3.

26 **Analysis of TYRP1 and MCSP expression using confocal microscopy**

27 Cells were detached using Accutase solution (Capricorn Scientific), transferred to Poly-L-Lysine
28 coated SuperFrost Plus slides (Thermo Fisher Scientific) and incubated at 37 °C for 60 minutes
29 for cell attachment. Cells were first fixed with 1 % PFA solution (Carl Roth) for 10 minutes and
30 washed with PBS. For TYRP1 staining cells were permeabilized with Triton X100 (v/v 0,5%,
31 Carl Roth). Following permeabilization cells were stained with 1 μg/mL αTYRP1/αE3 for 60
32 minutes at room temperature. MCSP staining of non-permeabilized cells was conducted with 10
33 μg/mL αMCSP/αE3 for 60 minutes at room temperature. Then, secondary antibody against
34 human IgG (1 μg/mL, Southern Biotech) and DNA dye Hoechst 33342 (2 μM, Thermo Fisher
35 Scientific) were applied for 30 minutes at room temperature. After sealing cells with ProLong
36 Glass antifade mountant (Thermo Fisher Scientific), samples were analyzed using the laser-
37 scanning confocal microscope ZEISS LSM 800 (Carl Zeiss AG) and images were acquired
38 using Zen software (v2.3, Carl Zeiss AG).

39 **Preparation of single cell suspensions, antibody staining and flow cytometry**

40 Lymph nodes and spleens were passed through 30 μm cell strainers, followed by erythrocyte
41 lysis in the spleens. Tumors and lungs were digested with 1.5 mg/mL collagenase IV and
42 50 U/mL DNAse I for 30 minutes at 37 °C under agitation. Dead cells were stained using the
43 violet fixable viability dye (BioLegend) for 15 minutes at room temperature, followed by blocking
44 of Fc receptors with TruStain FcX (BioLegend) for 20 minutes at 4 °C. Following this, cell
45 surface proteins were stained for 20 minutes at 4 °C. For the analysis of human T cells
46 antibodies against CD45 (2D1), CD3 (OKT3), CD4 (OKT4) CD8a (RPA-T8), CD45RO (UCHL1),

47 CCR7 (G043H7), PD-1 (EH12.2H7), 4-1BB (4B4-1), CD69 (FN50) and EGFR (A-13) for
48 detection of SAR (all from BioLegend) were used. For the analysis of murine T cells antibodies
49 against CD45 (30-F11), CD3 (17a2), CD4 (GK1.5) CD8a (53-6.7) and EGFR (A-13) for
50 detection of SAR (all from BioLegend) were used. Additionally, tumor cells were detected using
51 the GFP expression of YUMM1.1 TYRP1-LUC-GFP cells.

52 Cells were analyzed on Canto or LSRFortessa flow cytometers (BD Biosciences), and data
53 were analyzed with FlowJo software version 9.9.5 or version 10.3.

54 **Construction of 2 + 1 bispecific antibodies**

55 The construction of expression vectors for BiAb was performed by standard recombinant DNA
56 technologies. All antibody chain genes were separately inserted into expression vectors under
57 control of a MPSV or a SV40E hCMV promoter. The plasmids were cotransfected and
58 transiently expressed in HEK293 or CHO cells. The 2 + 1 antibody contained two Fabs for
59 hMCSP or TYRP1 and one Fab for EGFRvIII which was N-terminally fused to one arm of the
60 hMCSP or TYRP1 IgG. In order to obtain high yields of correctly paired molecules the "knobs-
61 into-holes" technology was used for heterodimerization. P329G, L234A, and L235A (PG LALA)
62 mutations were inserted in CH3 and CH2 domains to prevent binding to Fcγ Rs and C1q.[5 6] To
63 ensure correct pairing of the different chains, the CrossMAb^{VH-VL} technology (αEGFRvIII) and
64 charged residues (αMCSP) were used.[7]

65 **Purification and quality control**

66 All antibodies were transiently produced in HEK293 or CHO cells, purified and analyzed for
67 integrity and monomer content, as previously described.[5]

68 **Bispecific antibody binding assays**

Apparent dissociation constants (K_D) were measured by calibrated flow cytometry on a Fortessa II instrument (BD Biosciences) with 3.0 to 3.4 µm Rainbow Calibration particles (BioLegend) as calibration control.[8] After normalization, data points were fitted to a one-site specific binding model.

Supplementary Table 1: Patient characteristics

Name / cells	Biopsy	Patient age at biopsy time	Gender	Clinical stage at biopsy time	Site of biopsy	Tx before biopsy	Tx after biopsy	Primary tumor subtype	Primary tumor location	Breslow's index of primary tumor	Genotype
Patient 1	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 2	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 3	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 4	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 5	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 6	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 7	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 8	Metastasis (skin)	61 yo	Female	III	Skin (leg)	Dabrafenib (BRAFi) and trametinib (MEKi)	Ipilimumab; Encorafenib (BRAFi) + Binimetinib (MEKi) + Infigratinib (FGFRi); Pembrolizumab; radiation therapy (30Gy), multiple excisions; electro-chemotherapy (bleomycin); vemurafenib; TVEC	Nodular melanoma	Leg	4 mm	BRAF V600E, NRASwt
Patient 9	Metastasis (skin)	n. a.	n. a.	n. a.	Skin	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 10	Metastasis (skin)	n. a.	n. a.	n. a.	Skin	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Patient 11	Primary melanoma	73 yo	Male	IV	Skin (abdomen)	None	Transarterial chemoembolization (patient was also diagnosed with hepatocellular carcinoma 2 months later)	Nodular melanoma with ulceration mitotic index $5/mm^2$ T3b	Abdomen	4 mm	n. a.
Patient 12	Primary melanoma	86 yo	Female	III	Skin (neck)	None	n. a.	Nodular melanoma	Neck	n. a.	n. a.
Patient 13	Metastasis (brain)	36 yo	Female	IV	Brain	None	None	n. a.	Forehead	3 mm	BRAF V600

REFERENCES

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82 1. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis.
83 *Genome Biology* 2018;19(1):15. doi: 10.1186/s13059-017-1382-0
84 2. Virshup I, Rybakov S, Theis FJ, et al. anndata: Annotated data. *bioRxiv*
85 2021:2021.12.16.473007. doi: 10.1101/2021.12.16.473007
86 3. L. Lun AT, Bach K, Marioni JC. Pooling across cells to normalize single-cell RNA sequencing
87 data with many zero counts. *Genome Biology* 2016;17(1):75. doi: 10.1186/s13059-016-
88 0947-7
89 4. Zheng GX, Terry JM, Belgrader P, et al. Massively parallel digital transcriptional profiling of
90 single cells. *Nat Commun* 2017;8:14049. doi: 10.1038/ncomms14049 [published Online
91 First: 20170116]
92 5. Karches CH, Benmebarek M-R, Schmidbauer ML, et al. Bispecific Antibodies Enable Synthetic
93 Agonistic Receptor-Transduced T Cells for Tumor Immunotherapy. *Clinical Cancer*
94 *Research* 2019;25(19):5890-900. doi: 10.1158/1078-0432.Ccr-18-3927
95 6. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is
96 important in antibody protection against HIV. *Nature* 2007;449(7158):101-4. doi:
97 10.1038/nature06106
98 7. Schaefer W, Regula JT, Bähner M, et al. Immunoglobulin domain crossover as a generic
99 approach for the production of bispecific IgG antibodies. *Proceedings of the National*
100 *Academy of Sciences* 2011;108(27):11187-92. doi: 10.1073/pnas.1019002108
101 8. Benedict CA, MacKrell AJ, Anderson WF. Determination of the binding affinity of an anti-
102 CD34 single-chain antibody using a novel, flow cytometry based assay. *Journal of*
103 *Immunological Methods* 1997;201(2):223-31. doi: https://doi.org/10.1016/S0022-
104 1759(96)00227-X
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