1 **Supplementary material**

2

3 **Supplementary Methods**

4 *Flow cytometry*

5 Fluorescently labeled antibodies were used to detect cell surface markers and intracellular proteins

6 (supplementary table 2) . Cells were stained as previously described[1]. In short: cells were harvested and

7 stained on ice for 20 minutes with fluorescently labeled antibodies in PBA; a solution of 0.5% bovine serum

8 albumin and 0.02% sodium azide in PBS. Cells were then washed in PBA, resuspended in an appropriate

9 volume of PBA and acquired on an LSR Fortessa (BD Biosciences).

10

11 *Cell culture*

12 PBMCs from CLL patients and HD were thawed and either directly analyzed or cultured for 1-29 days as

13 indicated. PBMCs, CAR-T cells, the mantle cell lymphoma (MCL) cell line JeKo-1 (CRL-3006; ATCC), and the

14 NIH3T3 fibroblast (3T3) and CD40L-transfected NIH3T3 fibroblast (3T40) cell lines were cultured in RPMI

15 1640 (#22400089; Gibco) supplemented with 10% FCS by volume and supplemented with Penicillin and

16 Streptomycin (#15140-122; ThermoFisher Scientific) at 37°C and 5% CO_2. All final PBMC concentrations

17 were adjusted to $3*10^6$ cells per ml. T cells were stimulated using soluble anti-CD3 IgM (91 ng/µl, clone

18 1XE; Sanquin) and anti-CD28 IgG (3 µg/ml, clone 15E8; Sanquin). CLL cells were co-cultured with 3T3 or

19 3T40 cells as previously described[23,24]. After 2 days of culturing CLL cells with either 3T3 or 3T40 cells with

20 or without ibrutinib (S2680, Selleckchem; 0.1 µM, or 1 µM), imatinib (13139, Cayman Chemical) (1 µM),

21 dasatinib (S1021, Selleckchem) (1 µM), and Bay 11-7082 (196870, Calbiochem) (250 nM), CLL cells were

22 detached by gently resuspending the cell culture medium. The kinase inhibitors were washed away

23 thoroughly before proceeding an autologous T cell co-culture. The treated or untreated CLL cells were

24 then co-cultured in a 1:1 ratio with autologous PBMCs.

25

26 *CAR T-cell production*

27 To generate CAR-T cells, 293T cells (CRL-3216, ATCC) were transfected at 80% confluency with 15 ug of

28 the lentiviral vector encoding the anti-CD19 4-1BB CAR (CAR19, Tisagenlecleucel; Novartis)[7], and

29 packaging plasmids pRSV REV (18 ug, Rev expression plasmid; plasmid #12253, Addgene), pMDLg/p RRE

30 (18 ug, Gag/Pol expression plasmid; plasmid #12251, Addgene), and pVSV-G (7 ug, VSV glycoprotein

31 expression plasmid; plasmid #138479, Addgene) using Lipofectamine 20000 (11668019, Thermofisher).

32 The lentivirus was harvested after 24 hours, and concentrated by ultracentrifugation. Prior to transduction

33 with the CAR19 encoding lentivirus, T cells were purified from PBMCs from HD or CLL patients using a

34 negative T-cell selection kit following manufacturer's instructions (17951; StemCell). T cells were

35 stimulated using CD3/CD28 coated beads according to manufacturer's instructions (11131D;

36 ThermoFisher Scientific), and supplemented with 100 U/ml recombinant human IL-2 (200-02; Peprotech).

37 24h post activation, the T cells were transduced with the lentiviral particles encoding CAR19. The CAR-T

38 cells were subsequently expanded for 2 weeks to obtain sufficient CAR-T cells while the cell culture media

39 was refreshed every 3-4 days. Purified CAR-T cells were obtained by staining CAR-T cells with biotinylated
40 protein L (29997, ThermoFisher Scientific), and Streptavidin (554061, BD Biosciences), and subsequently
41 cell sorting using a BD FACSAria™ II.
42
43 *RNA sequencing*
44 FACS sorted CLL cells (based on CD19 positivity and cell viability using the SH800 Cell Sorter (Sony)) were
45 cultured for 48 hours on 3T3 fibroblasts, 3T40 fibroblasts or 3T40 fibroblasts with Dasatinib (100 nM). The
46 cells were pelleted and total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the
47 manufacturer's protocol. RNA quality was assessed using the High Sensitivity RNA ScreenTape (Agilent
48 Technologies). Sequencing libraries were prepared with the KAPA mRNA HyperPrep kit (Roche), all
49 samples were amplified for 14 cycles. Barcoded samples were pooled based on Qubit DNA HS
50 (ThermoFisher Scientific) measurement and sequenced on Illumina HiSeq 4000 (Illumina) using a single-
51 read 50 bp standard protocol. Sequencing depth was approximately 20M reads per sample. Raw FASTQ
52 files were subjected to quality control using FastQC and trimmed using Trimmomatic (v0.32). Reads were
53 aligned to the human reference genome (GRCh38/hg38) using HISAT2(v2.1.0)[2]. Gene level counts were
54 obtained using HTSeq (v0.11.0) and the human GTF (gene transfer format) file from Ensembl (release 94).
55 X and Y chromosomal genes were removed prior to differential expression analysis due to a large sex-
56 specific effect. Differential expression was assessed with DESeq2[3], using an FDR cut-off of 0.05.
57 Differentially expressed genes (DEGs) between the different conditions were used for retrieving the

58 inversely regulated genes, which were defined as genes that changed in opposite directions comparing

59 the 3T40 vs 3T3 DEGs with the 3T40 + dasatinib vs 3T40 DEGs. From this set of genes, those involved in

60 contact-dependent cell-cell interactions were extracted using the publicly available ligand-receptor pair

61 repository curated by Armingol et al. 2021 (https://github.com/LewisLabUCSD/Ligand-Receptor-Pairs)[4,5].

62 Plots were generated with ggplot2 (Bioconductor)[6] and gplots (CRAN) using R[7], Bioconductor and

63 Rstudio[8,9].

64 **Supplementary table 1** Characteristics of treatment-naive CLL patients included in this study

ID	Sex	Age	Rai Stage	Mutation Status	Leuco $*10^9$/L	% CD5+ CD19+	% CD3+	CMV Status
1	Male	72	0	Mutated	71.4	96.7	1.7	+
2	Female	74	0	Mutated	39.6	96.9	7.1	+
3	Male	66	I	Mutated	36.2	89.9	6.7	+
4	Female	69	I	Mutated	30.1	85.7	6.9	N.D.
5	Male	65	0	N.D.	127	55.7	1.6	N.D.
6	Male	68	I	Mutated	39	19.5	0.4	N.D.
7	Male	27	N.D.	Unmutated	286	65.4	0.6	+
8	Female	55	N.D.	Mutated	92.6	91.9	5.4	N.D.
9	Male	73	0	N.D.	129.7	91.7	0.3	+
10	Female	60	0	Mutated	152	91.8	5.2	+
11	Male	56	0	Mutated	123.33	92.9	2.4	+
12	Male	74	II	Mutated	69.67	96.8	2.3	+
13	Female	74	0	Mutated	59.6	91	3.1	+
14	Male	72	0	Unmutated	77.8	93.08	4.55	+
15	Female	62	I	Mutated	139	91.7	9.1	N.D.
16	Male	66	I	N.D.	90.92	96.14	2.94	N.D.
17	Male	66	IV	Mutated	41.06	82.7	15.86	N.D.
18	Male	66	0	N.D.	51.1	88.45	9.67	N.D.
19	Female	70	N.D.	N.D.	24.87	90.68	7.59	N.D.
20	Male	55	0	N.D.	69.64	92.33	6.05	N.D.
21	Female	57	0	Mutated	89.9	95.89	3.55	N.D.
22	Male	58	II	Mutated	116.93	94.81	4.73	+
23	Female	79	0	N.D.	50.39	90.91	7.64	N.D.
24	Male	61	III	Mutated	200.68	94.82	4.43	-

25	Female	75	N.D.	Unmutated	85.54	87.45	10.21	N.D.
26	Male	64	0	Mutated	52.26	94	4.69	+
27	Female	69	I	Mutated	76.82	90.29	7.49	N.D.
29	Female	63	II	Mutated	60.37	86.44	12.02	N.D.
30	Female	70	N.D.	Mutated	223.94	94.98	4.34	N.D.
31	Male	66	N.D.	Mutated	221.75	95.62	3.66	+
32	Female	62	0	Mutated	91.81	91.67	7.34	+
33	Female	69	I	Mutated	91.17	93.59	5.29	N.D.
34	Male	67	0	Mutated	118.03	98.09	1.77	N.D.
35	Male	51	N.D.	Mutated	167.31	96.05	3.06	+
36	Female	60	N.D.	N.D.	124.82	96.16	3.19	N.D.
37	Male	71	0	N.D.	19.89	89.18	8.64	N.D.
38	Female	68	0	Mutated	24.5	90.1	6.8	-
39	Male	73	N.D.	Mutated	29.5	89.3	3.8	N.D.
40	Female	83	I	Mutated	25.05	85.25	11.75	+
41	Male	79	I	Mutated	44.9	74	2.4	-
42	Male	59	III	Mutated	58.7	93	4.4	-
43	Male	75	II	Mutated	58.4	92.7	1.7	+
44	Female	77	0	Unmutated	55.87	88.68	3.65	N.D.
45	Male	81	I	Mutated	183.48	78	1.2	-
46	Male	60	III	Mutated	85.2	93.7	5.7	-
47	Male	81	N.D.	Mutated	85.2	98.23	1.42	+

65 N.D.: Not determined, +: Positive, -: Negative

66

67 **Supplementary table 2**

Marker	Manufacturer	Product ID
CD3	eBioscience	56-0038-82
CD4	BD Biosciences	555349
CD4	Biolegend	317442
CD5	eBioscience	12-0059-42
CD8	BD Biosciences	580347
CD8	BD Biosciences	563823
CD19	BD Biosciences	555415
CD19	Beckman	IM3628
CD24	Biolegend	311120
CD25	BD Biosciences	340907
CD25	BD Biosciences	563701
CD27	BD Biosciences	563816
CD45RA	BD Biosciences	563953
CD45RA	Biolegend	304135
CD52	Biolegend	316004
CD54	Biolegend	322720

CD58	Biolegend	330916
CD70	Biolegend	355111
CD71	BD Biosciences	563768
CD71	eBioscience	11-0719-41
CD80	eBioscience	11-0809-41
CD86	BD Biosciences	555660
CD95	BD Biosciences	564710
CD107a	Biolegend	561348
PD-1	BD Biosciences	561272
PD-L1	Biolegend	329731
4-1BBL	Biolegend	311507
OX-40L	Biolegend	326307
MHC-I	Biolegend	311438
MHC-II	Biolegend	361716
Siglec-10	BD Biosciences	566588
Siglec-10	Biolegend	347603
IFNγ	BD Biosciences	563563
IL-2	BD Biosciences	340450
TNFα	BD Biosciences	557996
Granzyme B	BD Biosciences	562462
Perforin	Antibodychain	2140540

Supplementary references

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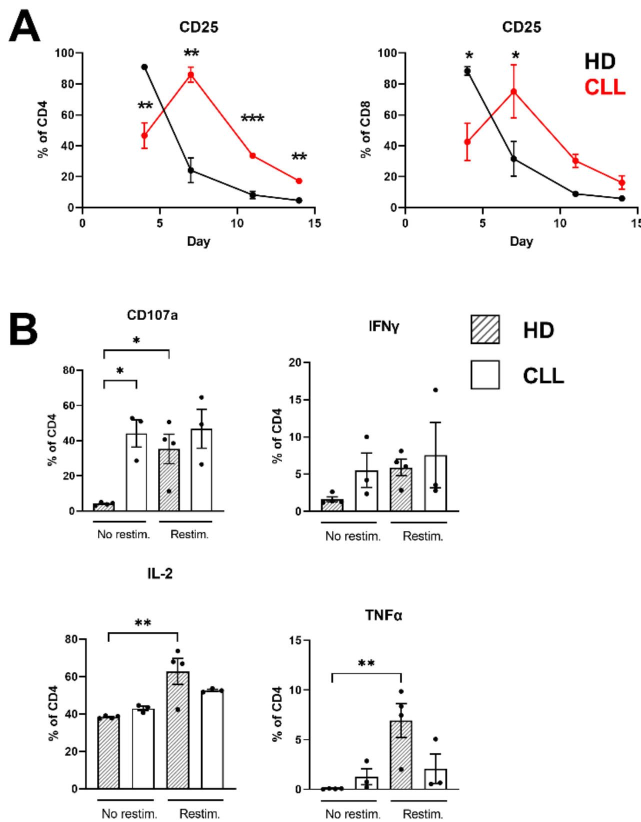
90 **Supplemental figures**

91



92

93 **Supplemental figure 1:**

94 (**A**) PBMCs from HD and CLL patients were stimulated with a single dose of soluble CD3/CD28 antibodies

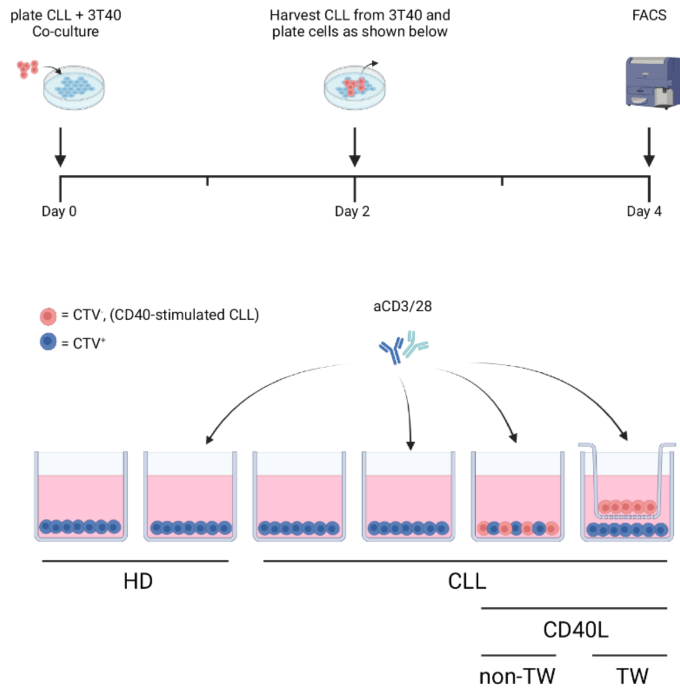
95 and kept in culture for 14 days. Expression of CD25 was measured during T-cell activation over a period

96 of 14 days (n=3-5). (**B**) Additionally, after an initial 14 days of stimulation, the T cells were re-stimulated

97 for 2 days and degranulation (CD107a), IFNγ, IL-2, and TNFα were measured on CD4 T cells (HD, n=4; CLL,

98 n=3). *P* values were calculated by a 1-way ANOVA, followed by a Šídák's test for multiple comparisons (B).

99 Data are presented as mean ± SEM, *p≤0.05, **p≤0.01, ***p≤0.001.



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101 **Supplemental figure 2:**

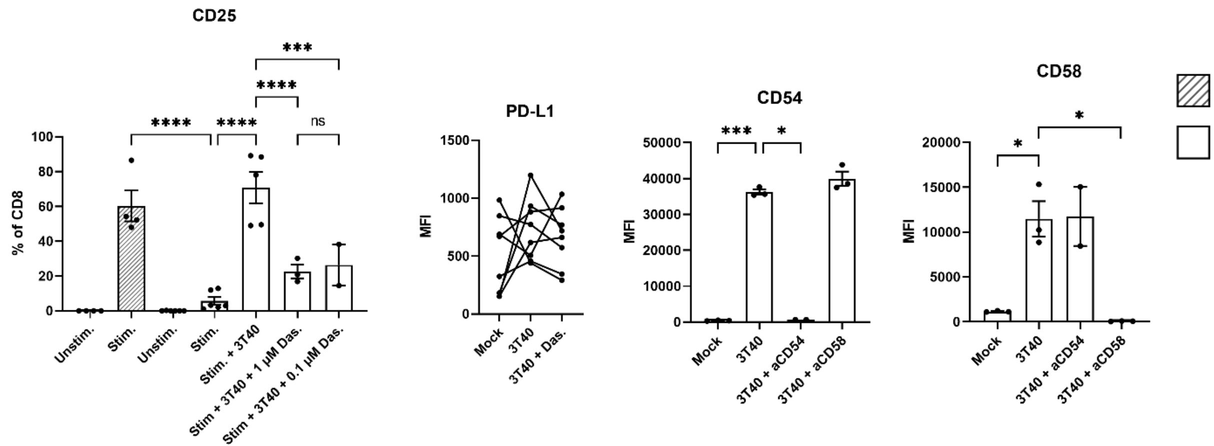
102 Schematic overview of a transwell experiment to determine the relevance of cell-cell contact of CD40-

103 stimulated cells on T-cell activation. Red cells indicate CD40-primed CLL cells and blue cells indicate fresh

104 CLL PBMCs labelled with CTV, these cells are mixed either allowing cell-cell contact or preventing cell-cell

105 contact due to the transwell insert.

106



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108 **Supplemental figure 3:**

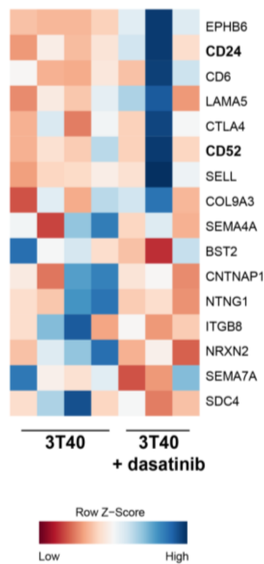
109 (**A**) PBMCs of CLL patients were cultured on a layer of mock or CD40L expressing fibroblasts with or
110 without 1 uM or 0.1 uM dasatinib and harvested after 2 days. These CLL cells were co-cultured with
111 autologous PBMCs for 2 days with (stim) or without (unstim) CD3/28 antibodies and CD25 expression was
112 measured using FACS analysis afterwards (HD, n=4; CLL, n=2-6). (**B**) Expression of PD-L1 on CLL cells after
113 a 2 day culture with mock or CD40L-expressing fibroblasts, and with or without dasatinib. (**C**) Detection of
114 CD54 and CD58 expression on CLL cells after a 2-day culture with or without CD40L-expressing fibroblasts,
115 and with or without CD54 or CD58 blocking antibodies. *P* values were calculated by a 1-way ANOVA,
116 followed by a Šídák's test for multiple comparisons (A-C). Data are presented as mean ± SEM, *p≤0.05,
117 ***p≤0.001, ****p≤0.0001.

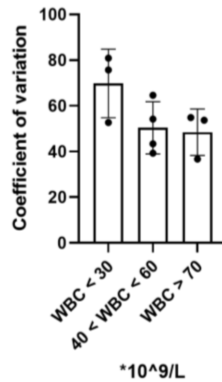
118

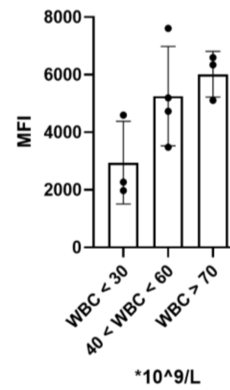
A

B

C





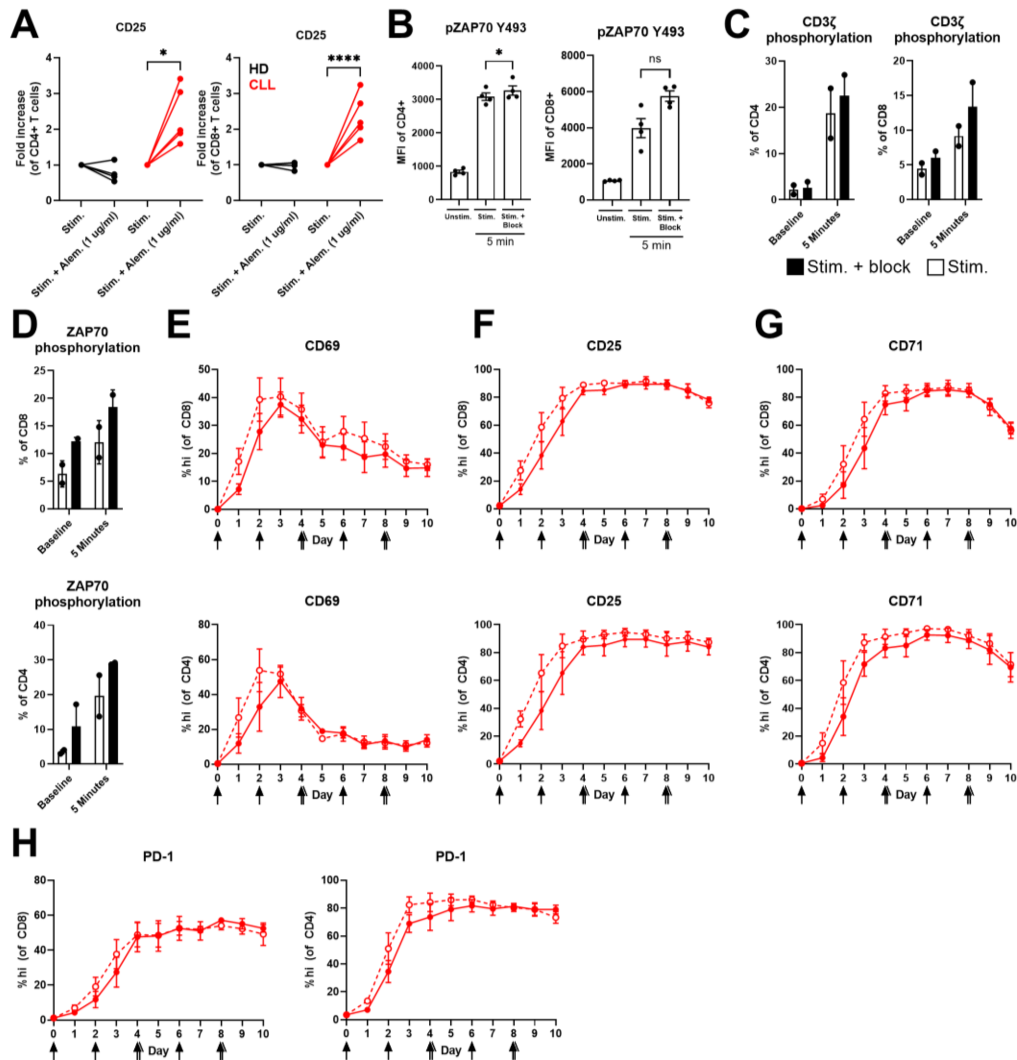


119

120 **Supplemental figure 4:**

121 (**A**) PBMCs from 4 CLL patients were thawed and cultured on a layer of CD40L expressing fibroblasts for 2
122 days with or without 1 uM dasatinib. Heat map showing DESeq2's normalized counts for each patient
123 (column) of genes (rows) that are inversely correlated between the 3T40 vs 3T3 comparison and the 3T40
124 + DAS vs 3T40 comparison. Genes known to be involved in contact-dependent cell-cell interaction were
125 selected. *CD24* and *CD52* were identified as potential targets of interest. (**B**) Variation in CD24 expression
126 on CLL cells from patients in different disease stages. PBMCs from patients in different disease stages
127 indicated by the WBC/leucocyte count in the blood, were thawed and CD24 expression on CLL cells
128 (CD5+CD19+) was measured using flow cytometry. (**C**) Geometric Mean fluorescence intensity of CD24 on
129 CLL cells from patients in different disease stages

130



131

132 **Supplemental figure 5:**

133 (**A**) PBMCs derived from CLL patients and HD were incubated with 1 µg/ml alemtuzumab for 1 hour after

134 which T cells were stimulated. T cells were measured for expression of CD25 (HD, n=4; CLL, n=5). (**B**)

135 PBMCs from CLL patients were stimulated using soluble CD3/CD28 antibodies in presence or absence of

136 CD24 and CD52 blocking antibodies. Five minutes after activation, the T cells were stained and analyzed

137 for phosphorylation of tyrosine residue 493 in ZAP70 (n=4). (**C** and **D**) PBMCs from CLL patients were pre-

138 incubated for 1 hour with CD24 and CD52 blocking antibodies or without. Next, T-cell stimulation was

139 performed using soluble CD3/CD28 antibodies (baseline and 5 minutes) and CD3ζ (**C**) and ZAP70 (**D**)

140 phosphorylation was measured (n=2). (**E-H**) PBMCs from CLL patients were cultured for 16 days in

141 presence of a single dose of soluble CD3/CD28 antibodies with or without CD24 and CD52 blocking

142 antibodies. During the culturing period, T-cell activation (**E**: CD69, **F**: CD52, and **G**: CD71), as well as

143 expression of PD-1 (**H**) was measured. Single arrows denote days on which CD24 and CD52 blocking

144 antibodies were re-added, and double arrows indicate days on which both cell culture medium was

145 refreshed as well as the addition of fresh CD24 and CD52 blocking antibodies. P values were calculated by

146 an unpaired or paired t-test (A and B). Data are presented as mean ± SEM, *$p \leq 0.05$, ****$p \leq 0.0001$.

147