

Supplementary Data

Materials

Media and Buffers

RPMI 1640 medium (PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% FCS + 1% penicillin/streptomycin/L-glutamine + 1% HEPES—hereafter referred to as culture medium—was used for cell lines, co-cultures of T cells, and all functional assays unless otherwise indicated. hCD19-Ba/F3 cells were cultured in culture medium supplemented with 10% WEHI supernatant containing IL-3. Antibody staining for flow cytometry was performed in PBS + 0.1% BSA + 2 mM EDTA.

Bispecific antibody construct

The half-life-extended CD19xCD3 bispecific antibody construct AMG 562 was produced and provided by AMGEN Research Munich (Munich, Germany). It consists of a human anti-CD3 and anti-CD19 single-chain variable fragment and an engineered Fc region. A control construct (cBiTE) was provided in which the anti-CD19 binding domain is exchanged for a single-chain variable fragment specific for an irrelevant antigen.

Cell lines

Human ALL and diffuse large B-cell lymphoma (DLBCL) cell lines and murine Ba/F3 cells were kindly provided by Oliver Weigert and Karsten Spiekermann (LMU Munich). Ba/F3 cells were transduced in-house with human CD19 (hCD19-Ba/F3).

26 **Antibodies for flow cytometry**

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Antibody (anti-human)	Clone	Isotype	Manufacturer	Cat. #
AquaLiveDead	-	-	Thermo Scientific Fisher	L34957
CD2-PerCP/Cy5.5	RPA-2.10	mIgG1,k	Biolegend	300216
CD2-BV421	TS1/8	mIgG1,k	Biolegend	309218
CD2-APC	RPA-2.10	mIgG1,k	Biolegend	300214
CD3-PerCP/Cy5.5	HIT3a	IgG2a,k	Biolegend	300328
CD4-FITC	OKT4	mIgG2b,k	Biolegend	317408
CD4-PE-Cy7	RPA-T4	mIgG1,k	Biolegend	300512
CD4-APCVio770	REA623	IgG1 (REA)	Miltenyi Biotec	130-113-223
CD8-APC-Cy7	SK1	mIgG1,k	Biolegend	344714
CD8-VioBlue	REA734	IgG1, REA	Miltenyi Biotec	130-110-683
CD19-PE-Cy7	SJ25C1	mIgG1,k	Biolegend	363012
CD19-PE	HIB19	mIgG1,k	Biolegend	302208
CD19-FITC	HIB19	mIgG1,k	Biolegend	302206
CD45-AF700	HI30	mIgG1,k	Biolegend	304024
CD80-PE	2D10	mIgG1,k	Biolegend	305208
CD86-PE	IT2.2	mIgG2b,k	Biolegend	305406
CD274-PE (PD-L1)	29E.2A3	mIgG2b,k	Biolegend	329706
CD223-APC (LAG-3)	REA351	IgG1 (REA)	Miltenyi Biotec	130-119-567
CD279-FITC (PD-1)	EH12.2H7	IgG1,k	Biolegend	329904
CD279-PE-Cy7 (PD-1)	EH12.2H7	mIgG1,k	Biolegend	329918
CD366-BV421 (Tim-3)	F38-2E2	mIgG1,k	Biolegend	345008
Granzyme B-PE	QA16A02	mIgG1,k	Biolegend	372208
IFN- γ -PE	B27	mIgG1,k	Biolegend	506507
IL-2-PE-Cy7	MQ1-17H12	rat IgG2a,k	eBioscience	25-7029-41
TCR $\alpha\beta$ -PE	IP26	mIgG1,k	Biolegend	306708
TNF- α -APC	MAb11	mIgG1,k	Biolegend	502912
TOX-PE	REA473	IgG1, REA	Miltenyi Biotec	130-120-785
Antibody (anti-mouse)	Clone	Isotype	Manufacturer	Cat. #
CD45-BV510	30-F11	Rat IgG2b,k	Biolegend	103138

28 m=murine

29 Reagents

Name	Manufacturer	Cat. #
Brefeldin A	Sigma–Aldrich	B7651-5MG
Dasatinib	Selleckchem	S1021
DPBS	PanBiotech	P04-36500
Dynabeads™ Human T-Activator CD3/CD28	Thermo Fisher Scientific	11131D
FcR blocking reagent	Miltenyi Biotec	130-059-901
Fetal bovine serum	Thermo Fisher Scientific	10270106
Ficoll Histopaque®-1077 Hybri-Max	Sigma–Aldrich	H8889-500ML
HEPES buffer solution (1 M)	Gibco Life Technologies	15630-056
Hoechst 33342 (20 mM)	Thermo Fisher Scientific	62249
Ionomycin, calcium salt	Sigma–Aldrich	I0634
MACS BSA stock solution	Miltenyi Biotec	130-091-376
Monensin, sodium salt	Sigma–Aldrich	M5273-1G
Opdivo (nivolumab, 40 mg/4 ml)	Bristol Myers Squibb	
Penicillin/streptomycin/L-glutamine, 100X	Thermo Fisher Scientific	10378016
Phorbol 12-myristate 13-acetate (PMA)	Sigma–Aldrich	P1585
Poly-D-lysine	Santa Cruz Biotechnology	sc-136156
RPMI 1640	PanBiotech	P04-16500
Seahorse XF RPMI, pH 7.4	Agilent	103576-100
Seahorse XF, 200 mM glutamine solution	Agilent	103579-100
Sodium pyruvate (100 mM), 100X	Gibco Life Technologies	11360-70
UltraPure™ 0.5 M EDTA	Invitrogen	15575-038

31 Kits

Name	Manufacturer	Cat. #
Cytofix/Cytoperm™ Fixation/Permeablization Kit	BD Biosciences	554714
EasySep™ Human T Cell Isolation Kit	STEMCELL Technologies	17951
Foxp3/Transcription Factor Staining Buffer Set	eBioscience	00-5523-00
Human Granzyme B Flex Set	BD Biosciences	560304
Human Th1/Th2 Cytokine Kit II	BD Biosciences	551809
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina	New England BioLabs	E7760
Pan T Cell Isolation Kit, human	Miltenyi Biotec	130-096-535
RNA Clean and Concentrator-25 Kit	Zymo Research	R1017
Seahorse XF Cell Mito Stress Test Kit (oligomycin, FCCP, Rotenone + Antimycine A)	Agilent	103015-100
Seahorse XF Glycolysis Stress Test Kit (glucose, oligomycin, 2-DG)	Agilent	103020-100
Seahorse XFe96 FluxPak mini	Agilent	102601-100

32

Methods

Sample collection from r/r BCP-ALL patients and healthy donors

Peripheral blood (PB) of healthy donors (HDs) and ALL patients prior to and during blinatumomab therapy was collected in accordance with the Declaration of Helsinki and with approval from the Institutional Review Board of the Ludwig-Maximilians-Universität (Munich, Germany). PB mononuclear cells were isolated by Ficoll density gradient centrifugation and stored at -150°C .

Flow cytometry and cytokine quantification

Immunophenotyping and functional T-cell assays, with the exception of metabolic studies, were assessed by flow cytometry on Cytoflex S or LX instruments (Beckman Coulter) at the iFlow Core facility, LMU University Hospital, Munich. Flow-cytometric data was analyzed using FlowJo version 10.5.2 (Tree Star Inc., Ashland, OR, USA). Cytokine concentrations in cell culture supernatants were quantified by cytometric bead array (CBA) using the Th1/Th2 kit and the Granzyme B Flex Set (both BD Biosciences, Franklin Lakes, NJ, USA). Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm Kit (BD Biosciences). For staining of transcription factors, the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) was used. All kits were used according to the manufacturers' instructions. Corresponding isotype controls were used for quantifying expression of intracellular cytokines and other proteins.

Metabolic stress assays

The protocol was adapted from Van der Windt et al.¹. For the mitochondrial stress test, T cells were incubated for 1 h in Seahorse XF RPMI medium + 1 mM sodium pyruvate + 2 mM L-glutamine + 10 mM glucose at 37°C without CO_2 . The oxygen consumption rate (OCR, pmol/min) was measured at baseline and after injection of oligomycin (1 μM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 1 μM) and antimycin A/rotenone (1 μM ; all Agilent, Santa Clara, CA, USA), respectively. Maximal OCR and spare respiratory capacity (SRC) were calculated as follows:

$$(1) \text{ Maximal OCR} = (\text{OCR after FCCP}) - (\text{non-mitochondrial respiration after rotenone})$$

(2) $\text{SRC} = (\text{maximal OCR}) - (\text{basal OCR})$

For the glycolysis stress test, T cells were starved for 1 h in glucose-free Seahorse XF RPMI medium + 2 mM L-glutamine at 37°C without CO₂. The extracellular acidification rate (ECAR, mpH/min) was measured after injection of glucose (10 mM), oligomycin (1 µM) and 2-deoxy-D-glucose (2-DG, 50 mM), respectively. Glycolysis, glycolytic capacity and glycolytic reserve were calculated as follows:

(3) $\text{Glycolysis} = (\text{ECAR before oligomycin}) - (\text{ECAR before glucose})$

(4) $\text{Glycolytic capacity} = (\text{maximal ECAR after oligomycin}) - (\text{ECAR before glucose})$

(5) $\text{Glycolytic reserve} = (\text{glycolytic capacity}) - (\text{glycolysis})$

Bioinformatic analysis of RNA Sequencing

Sequencing reads were aligned to release 101 of Ensembl GRCh38 prior to counting reads per gene with Star (version 2.7.6a) using default parameters. Values for expression (in transcripts per million, TPM) were calculated with RSEM (1.3.3) using default parameters. Differential expression was estimated with DSeq2 (1.28.1). Pathway enrichment analysis was performed on ranked test statistics with the Bioconductor package "fgsea" (version 1.14.0) using the human MSigDB Collection v5p2. Principal component analyses and heatmap visualizations were based on log₂-transformed TPM values.

In vivo studies

All animal trials were performed in accordance with the current ethical standards of the official committee on animal experimentation (written approval granted by Regierung von Oberbayern, tierversuche@reg-ob.bayern.de; ROB-55.2Vet-2532.Vet_02-16-7 and ROB-55.2-2532.Vet_02-20-159). NSG mice (The Jackson Laboratory, Bar Harbor, ME, USA) were injected intravenously (i.v.) with 1.2×10^6 patient-derived xenograft (PDX) ALL-265 cells expressing enhanced firefly luciferase and GFP (<https://www.addgene.org/104834/>). Tumor burden was monitored repeatedly by *in vivo* bioluminescence imaging (BLI). Following engraftment, mice were transplanted i.v. with $0.8\text{--}1.3 \times 10^6$ T cells from four HDs stimulated *in vitro* with AMG 562 for 14

days continuously (CONT) or with a treatment-free interval (TFI) from day 7 to 14. Mice were treated with AMG 562 (0.2 mg/kg in PBS, i.v.) or control construct on day 1 and day 7 post-T-cell injection. Leukemia burden was monitored twice per week by BLI. Peripheral blood (PB) was collected on days 2 and 7 post T-cell injection, and analyzed for human cytokines by CBA, and for CD3⁺ T-cell expansion and the presence of PDX cells using flow cytometry. Mice were monitored daily for clinical signs of illness (weight loss, poor posture, rough fur, reduced mobility). On day 15 after T-cell injection, mice were sacrificed and PDX cell burden in the bone marrow was quantified by flow cytometry. One mouse intended to receive TFI T cells from donor 2 died in inhalation anesthesia and was therefore excluded from the study.

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Table S1: Characteristics of ALL and lymphoma cell lines

Cell line	Species	Origin
OCI-Ly1	human	Large B cell lymphoma
OCI-Ly8	human	Diffuse large B cell lymphoma
Nalm6	human	B cell precursor acute lymphoblastic leukemia in relapse
REH	human	B cell precursor acute lymphoblastic leukemia in relapse
SU-DHL-5	human	Diffuse large B cell lymphoma
SEM	human	B cell precursor leukemia
Ba/F3	Murine (C3H background)	IL-3 dependent pro B cell line

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118 **References**

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- 120 1. van der Windt GJ, Chang CH, Pearce EL. Measuring Bioenergetics in T Cells
121 Using a Seahorse Extracellular Flux Analyzer. *Current protocols in immunology*.
122 2016;113:3.16b.11-13.16b.14.

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Supplementary Figures

Figure S1: Differences in AMG 562-mediated lysis between CD19⁺ cell lines. A)

Representative histograms of AMG 562-mediated upregulation of PD-L1 in OCI-Ly1 cells after 3 days in co-culture with HD T cells (AMG 562 = 5 ng/ml; E:T = 1:1). **B)** CD2⁺ fold change and cytotoxicity of HD T cells against OCI-Ly1, Nalm6, or hTim-3-Ba/F3 cells (CD19⁻) after 3 days (AMG 562 = 5 ng/ml); *n* = 3. **C)** Percentage of CD69⁺ and PD-1⁺ cells among CD4⁺ and CD8⁺ T cells after 3 days of assaying for cytotoxicity against OCI-Ly1 and Nalm6 cells (AMG 562 or cBiTE = 5 ng/ml); *n* = 3. Line plots represent the mean \pm SEM.

Figure S2: Gating Strategy, T-cell exhaustion in cocultures using other BsAbs and cell lines. **A)** Gating strategy for quantifying co-expression of PD-1, Tim-3, and LAG-3 in CD4⁺ and CD8⁺ T cells. **B)** Percentage of CD4⁺ T cells co-expressing PD-1, Tim-3, and LAG-3, and the median fluorescence intensity (MFI) ratio of TOX during continuous AMG 562 stimulation; $n = 6$. Bars represent the mean \pm SEM. **C)** Correlation between TOX expression on CD8⁺ T cells and cytotoxic capacity assessed in cytotoxicity assays upon continuous BsAb stimulation; $n = 6$, mean values are shown for day 0, 7, 14, 21, 28. **D)** Expression of individual IRs on CD8⁺ and CD4⁺ T cells and **E)** Granzyme B expression of CD8⁺ T cells analyzed by intracellular cytokine staining upon continuous AMG 562 stimulation. **F)** T-cell proliferation, cytotoxic capacity and secretion of IFN- γ and IL-2 upon continuous AMG 562 stimulation in coculture with the OCI-Ly1 or Nalm6 cell line. **G)** T-cell proliferation, cytotoxic capacity and secretion of IFN- γ and IL-2 upon continuous AMG 562 or blinatumomab stimulation in coculture with the OCI-Ly1 cell line. Statistical analysis: Kruskal–Wallis and Dunn's multiple comparison test (panels B+E); ns $p > .05$; * $p < .05$; ** $p < .01$; *** $p < .001$.

Figure S3: T-cell function and metabolic capacity after 21–28 days of CONT vs TFI AMG 562 stimulation. **A)** Percentage of CD4⁺ T cells co-expressing PD-1, Tim-3, and LAG-3; *n* = 6. **B)** AMG 562-mediated CD2⁺ fold change (*n* = 3), cytotoxic capacity against hCD19-Ba/F3 cells (*n* = 6), and granzyme B expression (*n* = 6) after 21 days of co-culture. **C)** Kinetic graph of normalized OCR and corresponding bar graphs obtained during mitochondrial stress test of T cells after 21 days of continuous vs TFI AMG 562 stimulation; *n* = 3. **D)** Kinetic graph of normalized ECAR and corresponding bar graphs obtained during glycolysis stress test of T cells after 21 days of continuous vs TFI AMG 562 stimulation; *n* = 3. **E)** and **F)** Kinetic graphs of OCR (E) and ECAR (F) obtained during mitochondrial and glycolysis stress testing of T cells after 28 days of continuous vs TFI AMG 562 stimulation; *n* = 3. **G)** CD33xCD3-mediated cytotoxic capacity against hCD33-Ba/F3 cells (E:T = 1:1, CD33xCD3/cBiTE = 5 ng/ml, 72h) after 0, 7 and 14 days of co-culture with the AML cell line Molm-13 and CD33xCD3 bispecific antibody (5 ng/ml) or a TFI (day 7–14); *n* = 3. Boxplot (panel B) whiskers indicate minima and maxima, and boxes represent the lower quartile, the median, and the upper quartile. All other plots present mean ± SEM values. Statistical analysis: two-way ANOVA and Sidak's multiple comparison test (panels A–D, G); ns *p* > .05; ** *p* < .01; **** *p* < .0001.

167 **Figure S4: TFI-mediated T-cell reinvigoration can be mimicked by combinatorial**
168 **treatment with AMG 562 and dasatinib. A)** Timeline of AMG 562+dasatinib-
169 mediated T-cell stimulation in comparison to continuous or TFI stimulation over
170 17 days. Dasatinib = 100 nM. **B)** Percentage of CD4⁺ T cells co-expressing PD-1, Tim-
171 3, and LAG-3. **C)** Cytokine levels in co-culture supernatants determined by CBA; *n* =
172 3. **D)** Representative plots of AMG 562-mediated cytotoxic capacity against hCD19-
173 Ba/F3 cells of differentially treated T cells on day 14. All graphs (panels B+C) present
174 mean ± SEM values; statistical analysis: two-way ANOVA and Sidak's multiple
175 comparison test; ns *p* > .05; * *p* < .05.

Figure S5: PD-1 blockage does not prevent AMG 562-mediated T-cell exhaustion.

A) Timeline of continuous AMG 562 stimulation with or without 10 µg/ml nivolumab over 28 days. **B)** Percentage of Tim-3⁺LAG-3⁺CD8⁺ and Tim-3⁺LAG-3⁺CD4⁺ T cells. **C)** Cytokine levels in co-culture supernatants determined by CBA. **D)** AMG 562-mediated T-cell proliferation as fold change and cytotoxic capacity against hCD19-Ba/F3 cells of T cells stimulated with AMG 562 with or without nivolumab; *n* = 3. All graphs present mean ± SEM values.

Figure S6: Transcriptome analysis of day 21 TFI vs CONT and day 21 TFI vs day 7 T cells. **A)** Volcano plot of day 21 TFI vs CONT T cells; $p_{\text{adj}} < .05$. Selected genes are highlighted as significantly downregulated (blue) or significantly upregulated (red) in TFI cells. **B)** Unsupervised clustering of the top 100 differentially expressed genes in day 21 TFI vs CONT T cells; $p_{\text{adj}} < .05$. Selected genes are indicated. **C)** $\text{Log}_2(\text{TPM})$ expression of the genes *BTLA* and *BMF* at timepoints 0, 7, 14, and 21 days in TFI vs CONT T cells. **D)** Pathway enrichment analysis of day 14 TFI vs CONT (red) and day 21 TFI vs CONT (red-grey stripes); $p_{\text{adj}} < .05$. **E)** Gene set enrichment analysis of day 21 TFI vs CONT T cells using MSigDB and the gene set GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_UP. **F)** Volcano plot of day 21 TFI vs day 7 T cells; $p_{\text{adj}} < .05$. Selected genes are highlighted as significantly downregulated (blue) or significantly upregulated (red) in day 21 TFI vs day 7 T cells. **G)** Unsupervised clustering of the top 100 differentially expressed genes in day 21 TFI vs day 7 T cells; $p_{\text{adj}} < .05$. Selected genes are indicated. NES, normalized enrichment score. **H)** $\text{Log}_2(\text{TPM})$ expression of NK receptor genes at timepoints 0, 7, 14, 21 days in TFI vs CONT T cells. Line plots present the mean \pm SEM values. **I)** PCA colored by sample batch.

Figure S7: *In vivo* tumor burden and T-cell quantification. **A)** Gating strategy to quantify ALL burden and T-cell expansion. **B)** 1.2×10^6 PDX ALL-265 cells expressing enhanced firefly luciferase and GFP were injected i.v. and engraftment was monitored twice per week using BLI. One mouse, intended to receive TFI T cells from donor 2, died in inhalation anesthesia and was therefore excluded from the study.

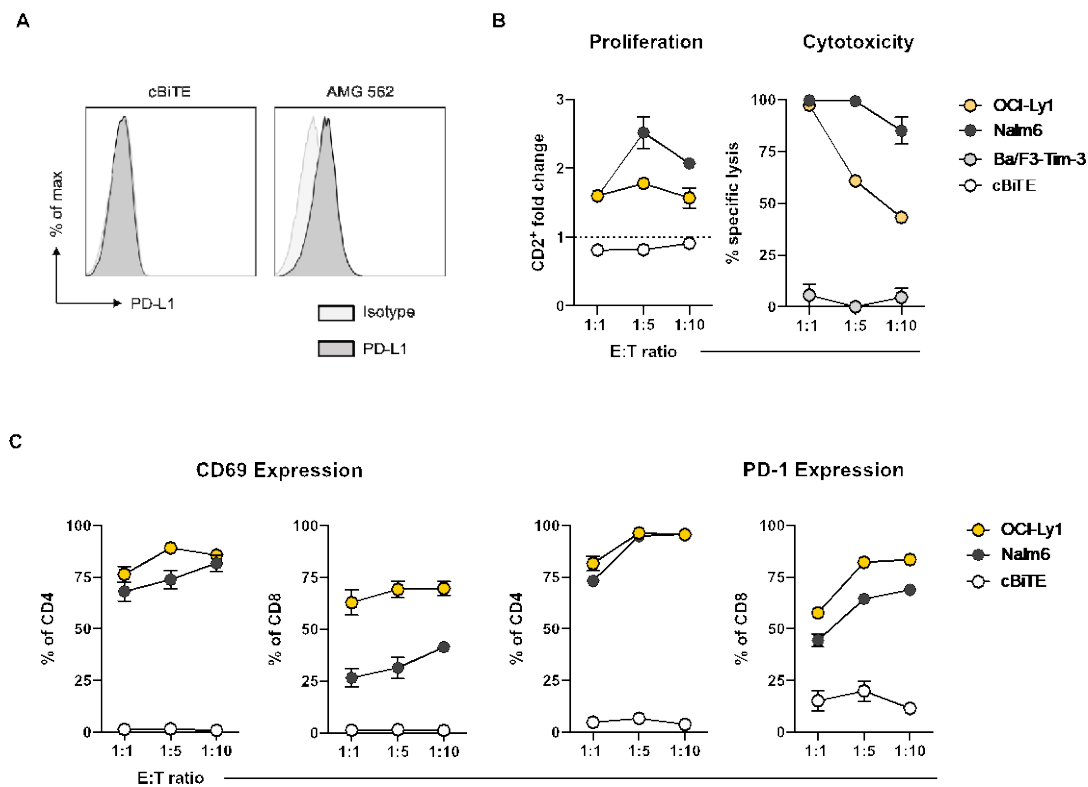
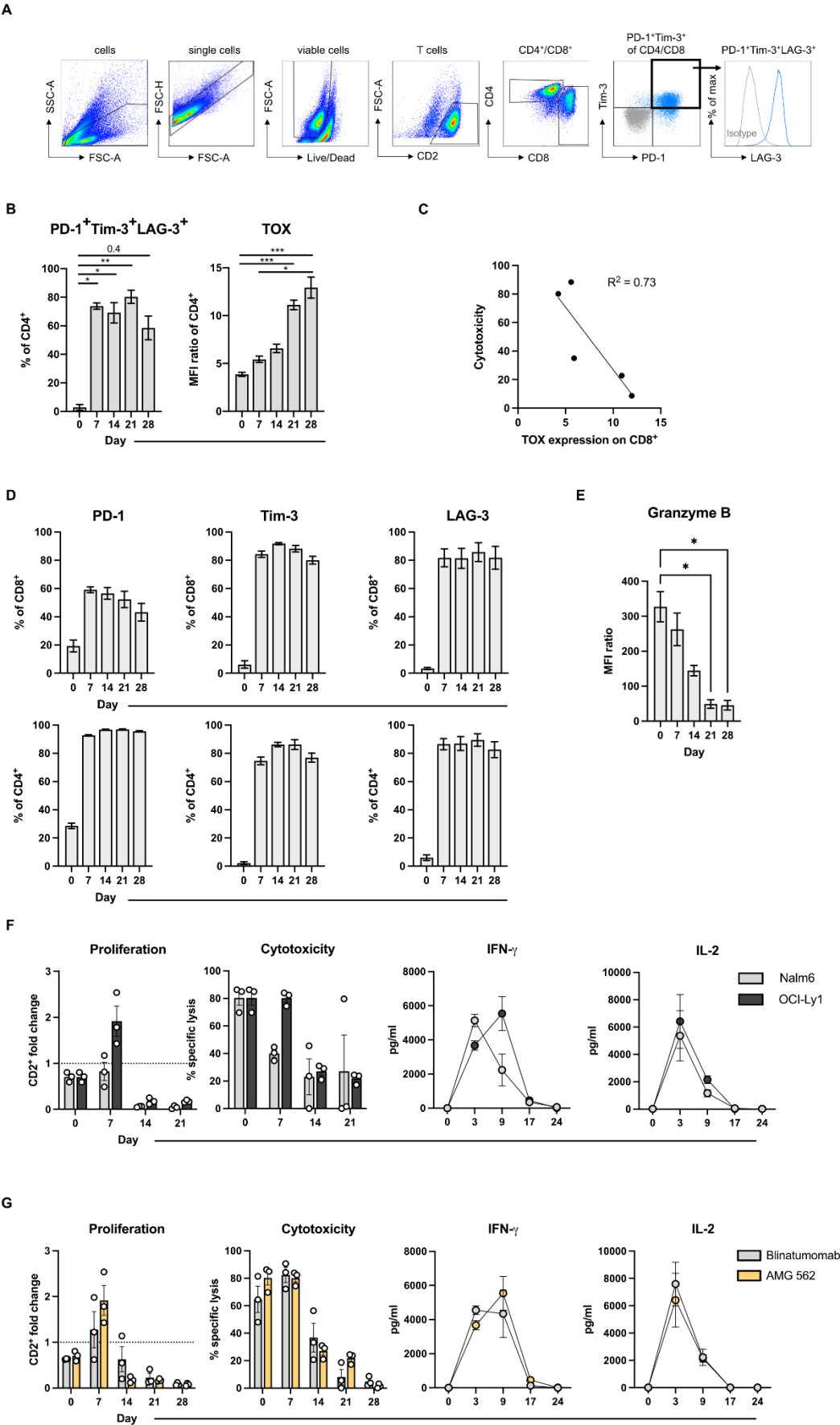


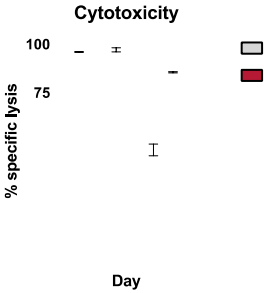
Figure S2



A

Day 21

Day 21



B

