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Supplemental Information

ARI0003: Co-transduced CD19/BCMA dual-targeting CAR-T cells for the treatment of non-Hodgkin lymphoma

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Supplemental Figures

Figure S1

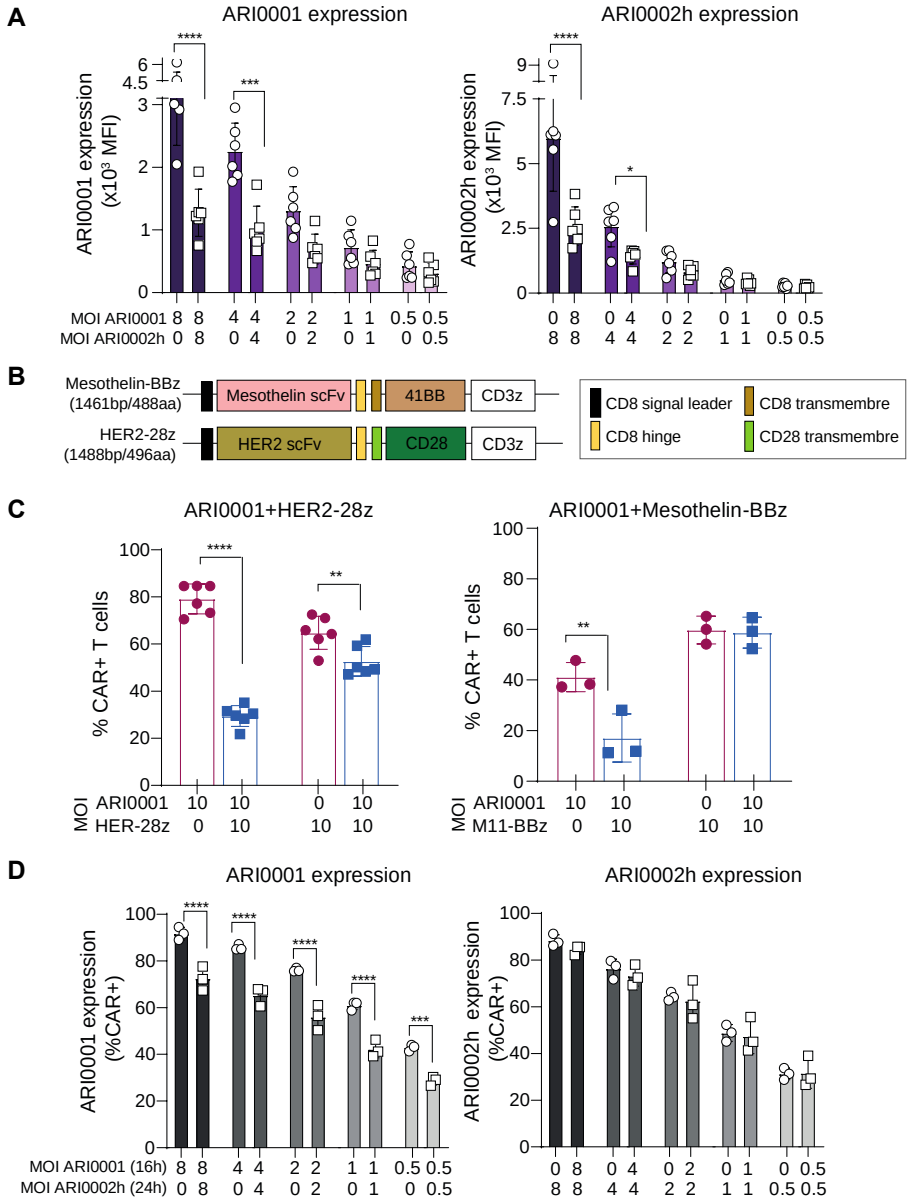
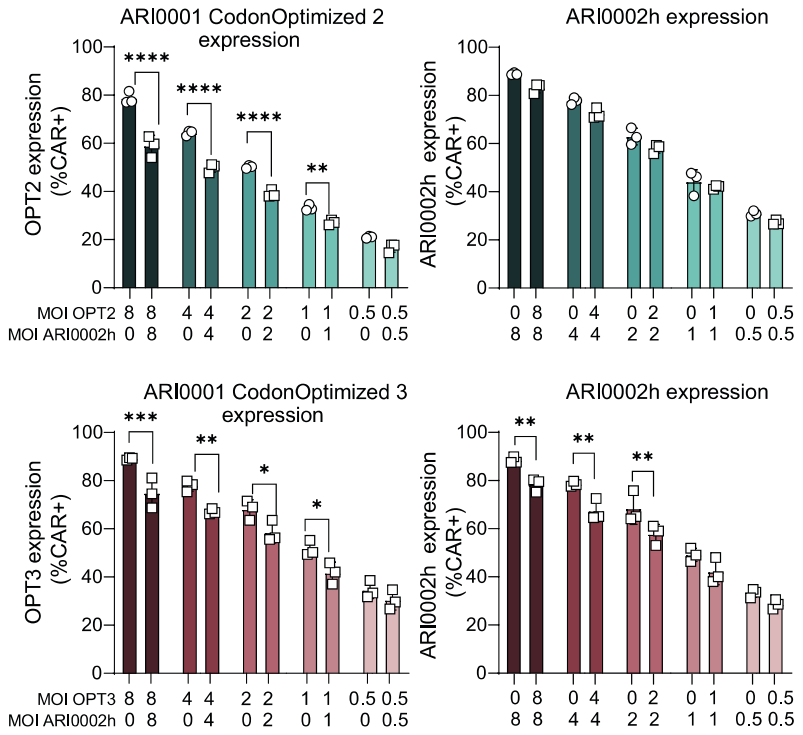


Figure S1. Characterization of ARI0001 and ARI0002h co-transduction. T cells were activated using CD3/CD28 beads and 24 hours later (unless otherwise indicated) transduced with the indicated MOI of the different lentiviral vectors (as single vectors or in co-transduction). Analysis of CAR expression was performed at day 6 of CAR-T cell expansion using recombinant proteins in a minimum of three different healthy donors. Each dot/square represents an individual donor. **A)** Co-transduction of lentiviral vectors expressing ARI0001 or ARI0002h at equivalent MOIs resulted in decreased mean fluorescent intensity (MFI) for both CARs compared to single transductions. **B)** Schematic representation of CAR constructs, showing base pair lengths and amino acid

sequences. Both CARs feature the CD8α signal peptide and extracellular spacer. Mesothelin-BBz incorporates the CD8α transmembrane domain with the 4-1BB co-stimulatory domain, while HER2-28z includes the CD28 transmembrane domain with CD28 co-stimulatory domain. **C)** Percentage of CAR expression on the T cell membrane in single compared to co-transduced CAR-T cells, assessed by flow cytometry (n=3-6 healthy donors). **D)** Transduction of T cells with ARI0002h-expressing lentiviral vectors 8 hours after the addition of ARI0001. ARI0001 expression is shown in the left panel and ARI0002h in the right panel. Statistical significance in A-D was determined using two-way ANOVA. *P*-values: ****<0.0001, ***<0.0005, **<0.005, *<0.05.

Figure S2

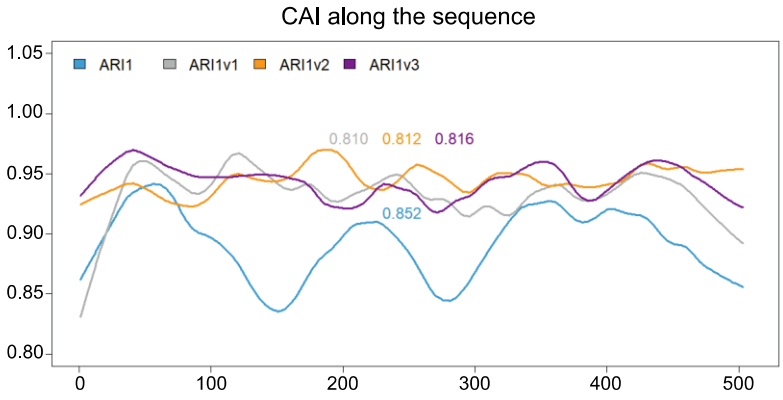
A



B

Name	Length	CAI	%GC	%GC1	%GC2	%GC3
ARI0001 (no CodonOptimized)	1509	0.852	56.7	59.4	50.9	59.8
ARI0001 CodonOptimized 1	1509	0.810	61.6	59.8	51.1	73.8
ARI0001 CodonOptimized 2	1509	0.812	61.6	60.2	51.1	73.6
ARI0001 CodonOptimized 3	1509	0.816	61.3	59.8	51.1	73.0

C



D

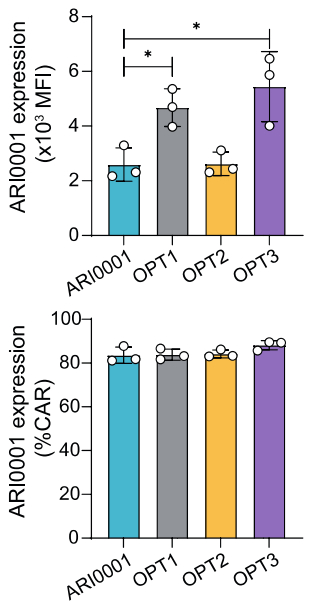


Figure S2. ARI0001 codon optimization did not improve its expression in co-transduction with ARI0002h. **A)** T cells were activated with CD3/CD28 beads and 24 hours later transduced with the indicated MOI of the different lentiviral vectors (as single vectors or in co-transduction). Analysis of CAR expression was performed at day 6 of CAR-T cell expansion using recombinant proteins (CD19-Fc and BCMA-His) followed by a secondary antibody (against Fc or His) in a minimum of three different healthy donors. When co-transducing two lentiviral vectors, one or both CARs were expressed at lower levels on the T cell membrane than when transduced individually. Impaired CAR expression in dual CAR-T cells was exacerbated when increasing multiplicities of infection (MOI) were used. Co-transduction of codon-optimized versions of ARI0001 with ARI0002h did not improve ARI0001 expression in co-transduction. **B)** Table of codon usage analysis of CD19 sequences corresponding to ARI0001 versions. Global CAI, average GC content and the % of GC at positions 1, 2 and 3 of the different codons are indicated. **C)** Representation of the CAI along the non-optimized and optimized DNA sequences encoding CD19 protein from the different ARI0001 versions. **D)** Quantification of CAR expression presented in the upper panel as MFI and in the lower panel as the percentage of expression for ARI0001 and its codon-optimized versions in single transductions. Statistical analyses were performed using Two-way ANOVA tests, with p-values denoted as ****<0.0001, ***<0.0005, **<0.005, *<0.05. The data represents the Mean ± SD from n=3 healthy donors.

Figure S3

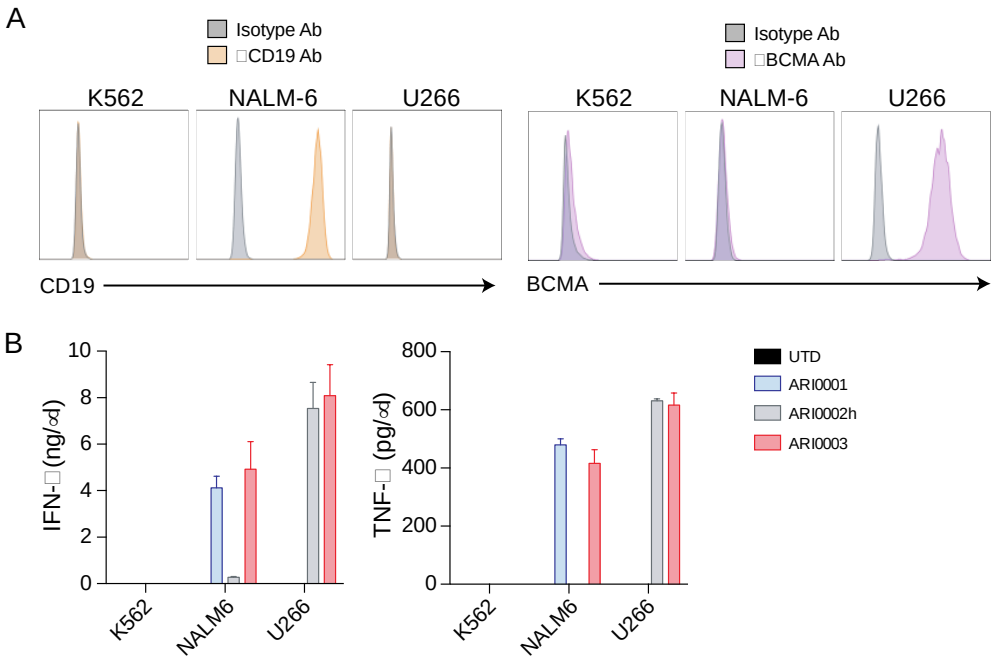


Figure S3. ARI0003 activation in the absence of target antigens or in the presence of a single target antigen. A) Analysis of the expression of CD19 and BCMA in control cell lines K562 ($CD19^{-}BCMA^{-}$), NALM-6 ($CD19^{+}BCMA^{-}$) and U266 ($CD19^{-}BCMA^{+}$) by flow cytometry. **B)** Monospecific CAR-T cells (ARI0001 and ARI0002h) and dual CAR-T cells (ARI0003) were cultured with the indicated target cells at an E:T=3:1. IFN-γ and TNF-α cytokine release in supernatants was analyzed by ELISA 24h after co-culture. The mean ± SD of triplicates from a healthy donor is shown. These experiments have been repeated twice, with two different healthy donors, with similar results.

Figure S4

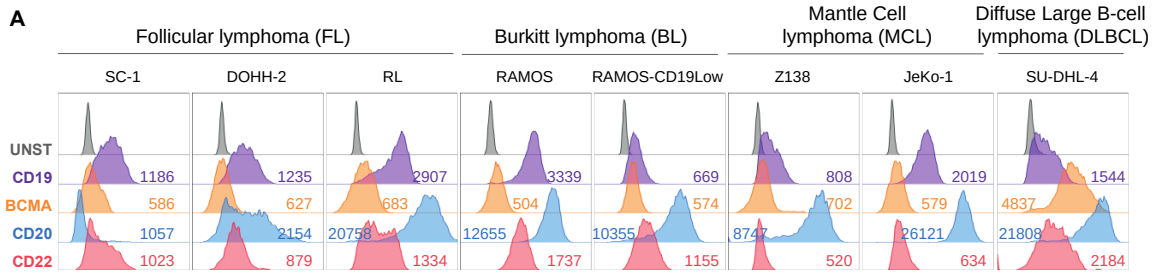


Figure S4. Expression of CD19, BCMA, CD20 and CD22 in a panel of non-Hodgkin's lymphoma tumor lines. A) Antigen expression was analyzed by flow cytometry. The panel includes cell lines from follicular lymphoma (SC-1, DOHH-2 and RL), Burkitt lymphoma (Ramos WT and Ramos CD19 Low), mantle cell lymphoma (Z138 and JeKo-1) and diffuse large B-cell lymphoma (SU-DHL-4).

Figure S5

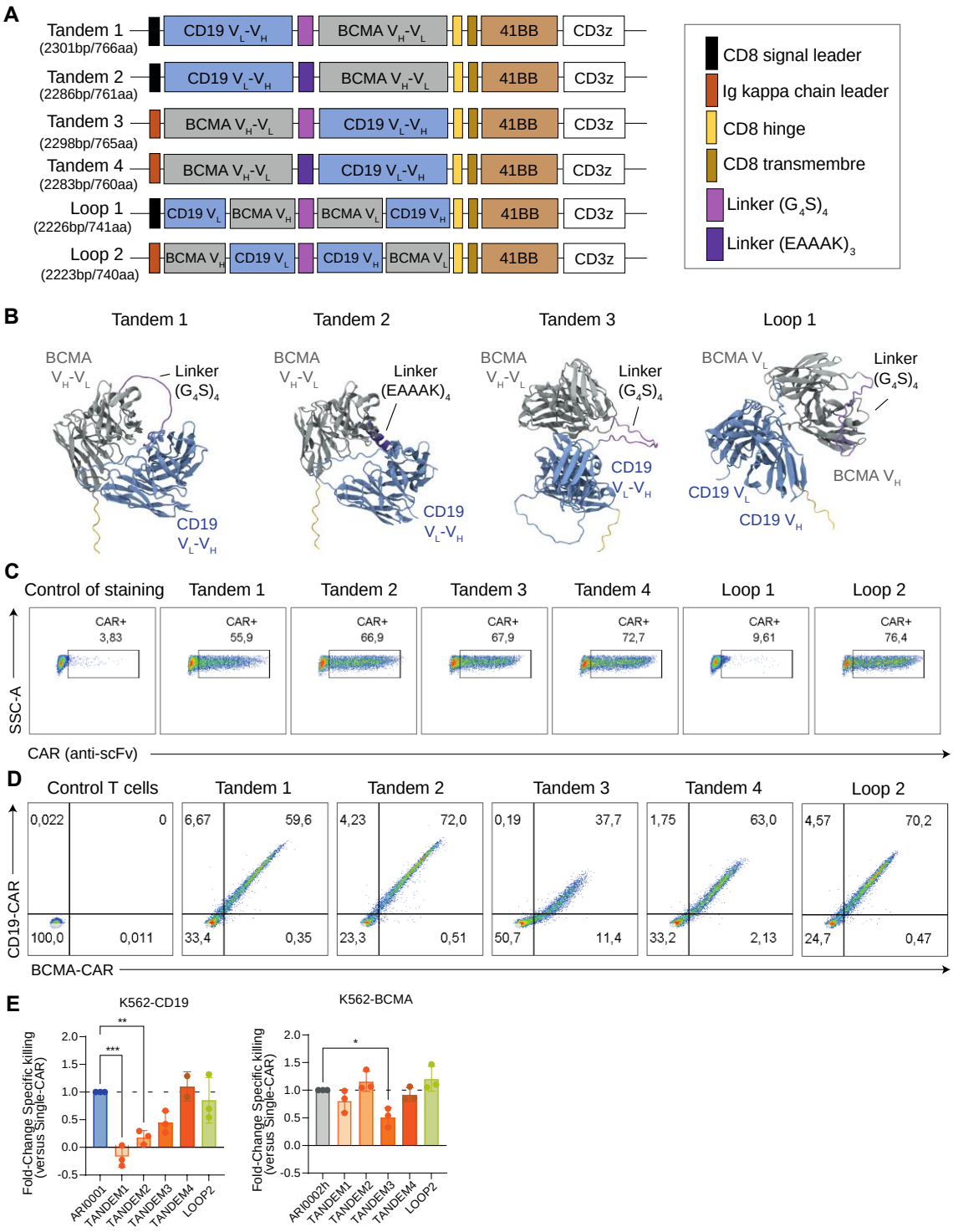


Figure S5. Selection of tandem and loop constructs based on CAR surface expression and specific lysis against targeted antigens. A) Schematic representation of the different tandem and loop CAR constructs. **B)** Structural models of the Tandem (Tandem1-3) and Loop (Loop1-2) constructs were generated using AlphaFold3. The models were based on the input sequences of their extracellular domains, specifically the CD8a hinge and scFv. The BCMA-targeting regions are

represented in grey, the CD19-targeting regions in blue, and the linkers connecting both regions are shown in purple. Structure of single CARs, ARI0001 and ARI0002h and selected Tandem4 and Loop2 are shown in Figure 3B. **C-D)** Representative flow cytometry plots displaying CAR expression on T Cells using anti-mouse IgG antibody (C) or recombinant proteins (D). **E)** Indicated CAR-T cells and target cells were mixed at an E:T=1:1 and cytotoxicity was evaluated 24 hours later in a luciferase-based assay. All data are means ± SD from triplicate wells of a representative healthy donor. Dotted lines indicate the percentage of lysis of ARI0001 in the K562-CD19 cell line and ARI0002h in the K562-BCMA cell line. Fold-change in specific lysis for each CAR-T cell group is shown in n=3 healthy donors.

Figure S6

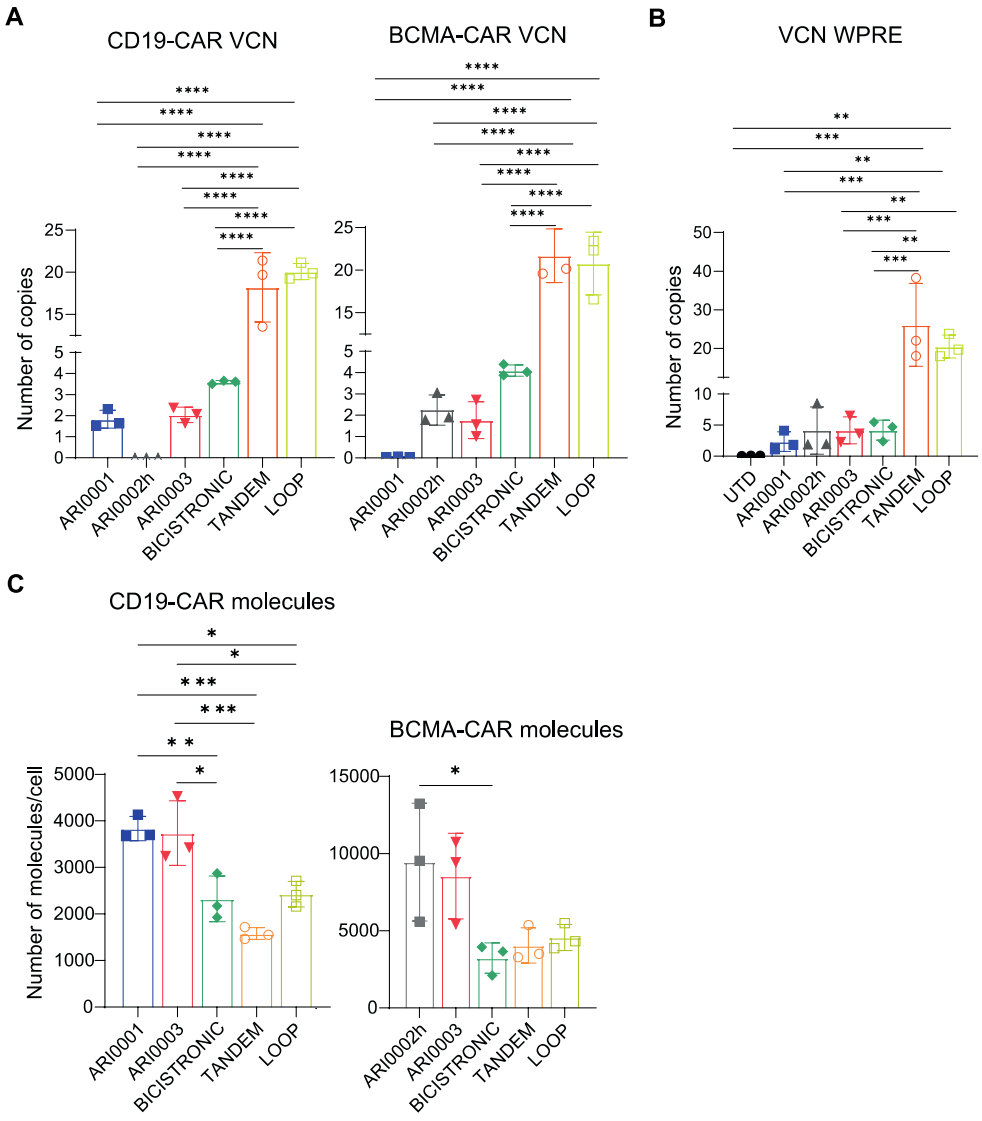


Figure S6. Expression profile of various dual CAR-T Cell Modalities. A-B) Single and dual-targeting strategies were studied at the level of DNA. The absolute number of lentiviral DNA copies integrated per genome for CD19-CARs and BCMA-CARs was assessed using qPCR using specific primers for CD19 scFv and BCMA scFv (A) or using specific primers targeting WPRE (B). Each dot is a healthy donor (n=3). Statistical significance was assessed using a one-way ANOVA test, with p-values defined as follows: ****<0.0001, ***<0.0005, **<0.005. **C)** The absolute number of CAR molecules for both single and dual-targeting strategies was evaluated using Quantibrite technology. Each dot represents a healthy donor (n=3). Statistical significance was determined through a one-way ANOVA test, with p-values indicated as: ****<0.0001, ***<0.0005, **<0.005, *<0.05.

Figure S7

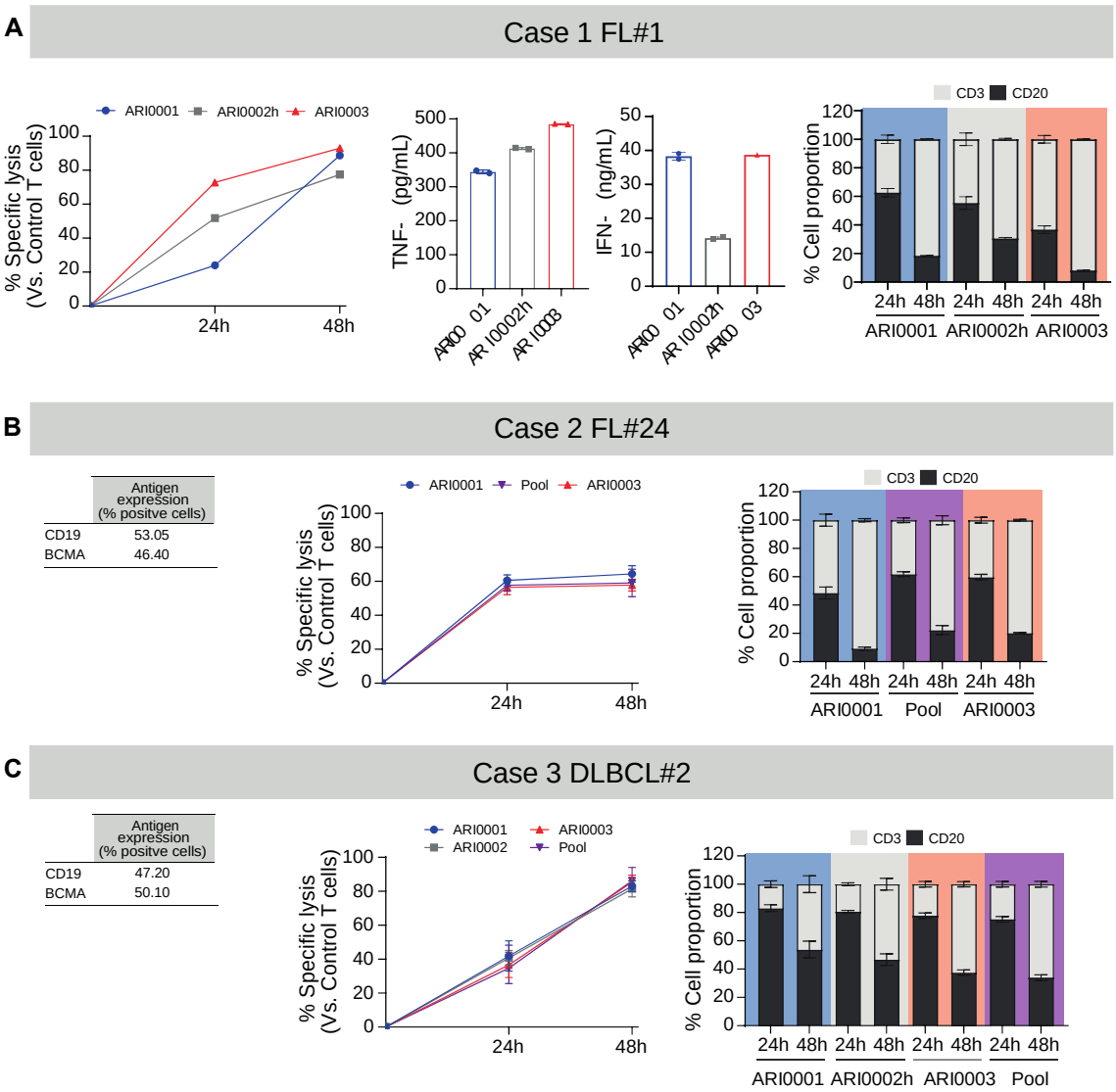


Figure S7. PDLS as pre-clinical model for CAR-T cells. Patient derived NHL spheroids (PDSL) from FL (A, B) and DLBCL (C) were generated. Specific lysis of remaining tumor cells was analyzed by flow cytometry after co-culture at E:T=1:2 ratio at indicated time-points. Cytokine production by ELISA (TNF-α and IFN-γ) after 24h co-culture at E:T=1:2 was assessed. Each dot in the ELISA represents a spheroid (n=1-2). CD19 and BCMA antigen expression was analyzed before CAR-T cell addition for co-culture. Percentage cell proportion was analyzed after 24h and 48h co-culture by flow cytometry. Results represent the mean ± SD for triplicates.

Figure S8

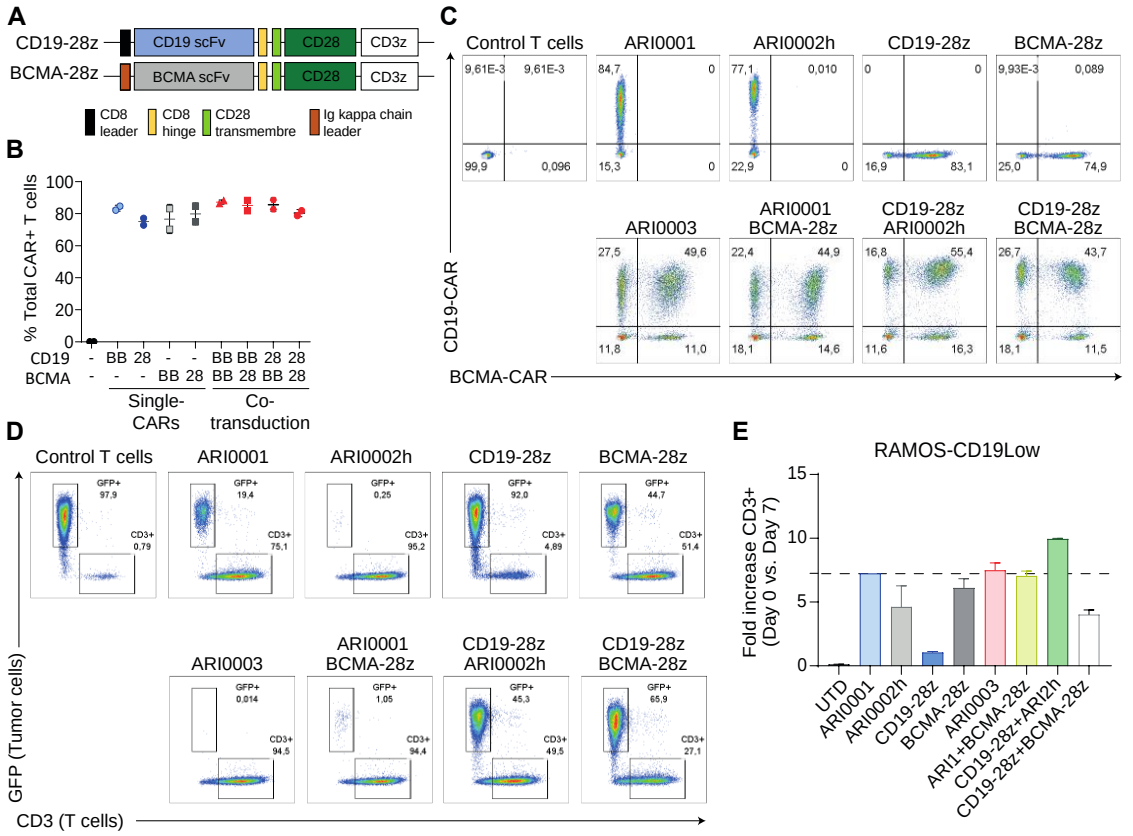


Figure S8. Generation and evaluation of dual CAR-T cells containing different intracellular domains. A) Schematic representation of CD28-based CAR constructs. **B)** Percentage of T cells expressing CARs at the cell surface as analyzed by flow cytometry. Each dot represents a healthy donor (n=2). **C)** Flow cytometry plots illustrating CD19-CAR and/or BCMA-CAR expression on the T cell membrane. **D-E)** Long-term cytotoxicity and proliferation of dual CAR-T cells in co-culture with Ramos –CD19 Low. The different groups of single- and co-transduced CAR-T cells or untransduced control T cells (UTD) were co-cultured with Burkitt lymphoma cells modified to express low levels of CD19 (Ramos –CD19 Low) at E:T=1:4. 7 days after co-culture, cells were harvested and stained with specific antibodies to be analyzed by flow cytometry. Cells were selected for live/dead, CD3 (for T cells) or GFP (for tumor cells). **D)** CD3+ (T lymphocytes) and GFP+ (tumor) populations after 7 days of co-culture. **E)** The proliferation of T lymphocytes after 7 days of co-culture with target cells was analyzed by flow cytometry using quantification beads. Dotted line indicates ARI0001 proliferation. All data are means ± SD from duplicate wells of a healthy donor.

Figure S9

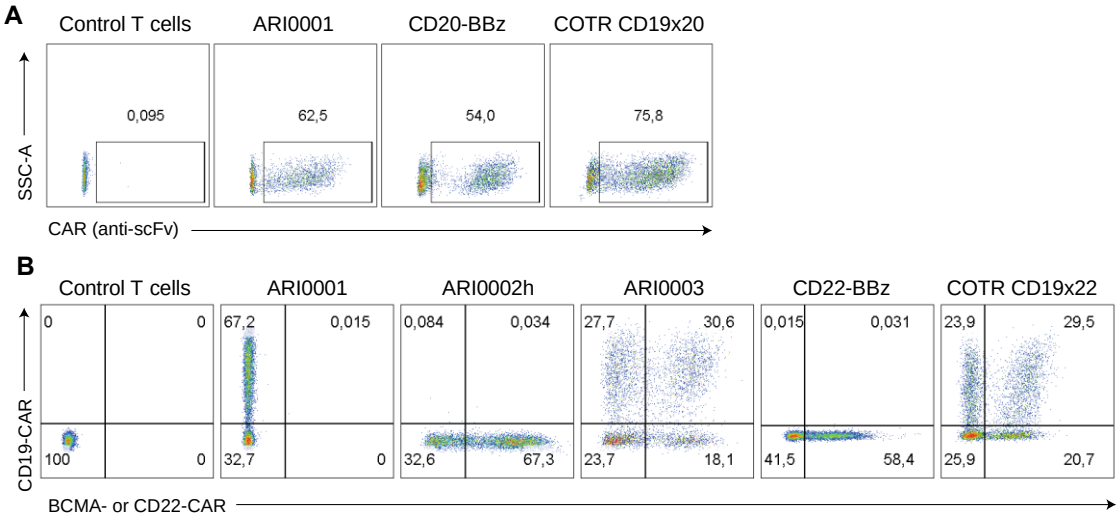


Figure S9. CAR expression frequencies in transduced T cell populations. CAR expression on the T cell membrane was analyzed by flow cytometry using (A) anti-mouse IgG antibody for CD20-CAR or (B) recombinant proteins for CD22-CAR. Flow plots are representative of one healthy donor.

Figure S10

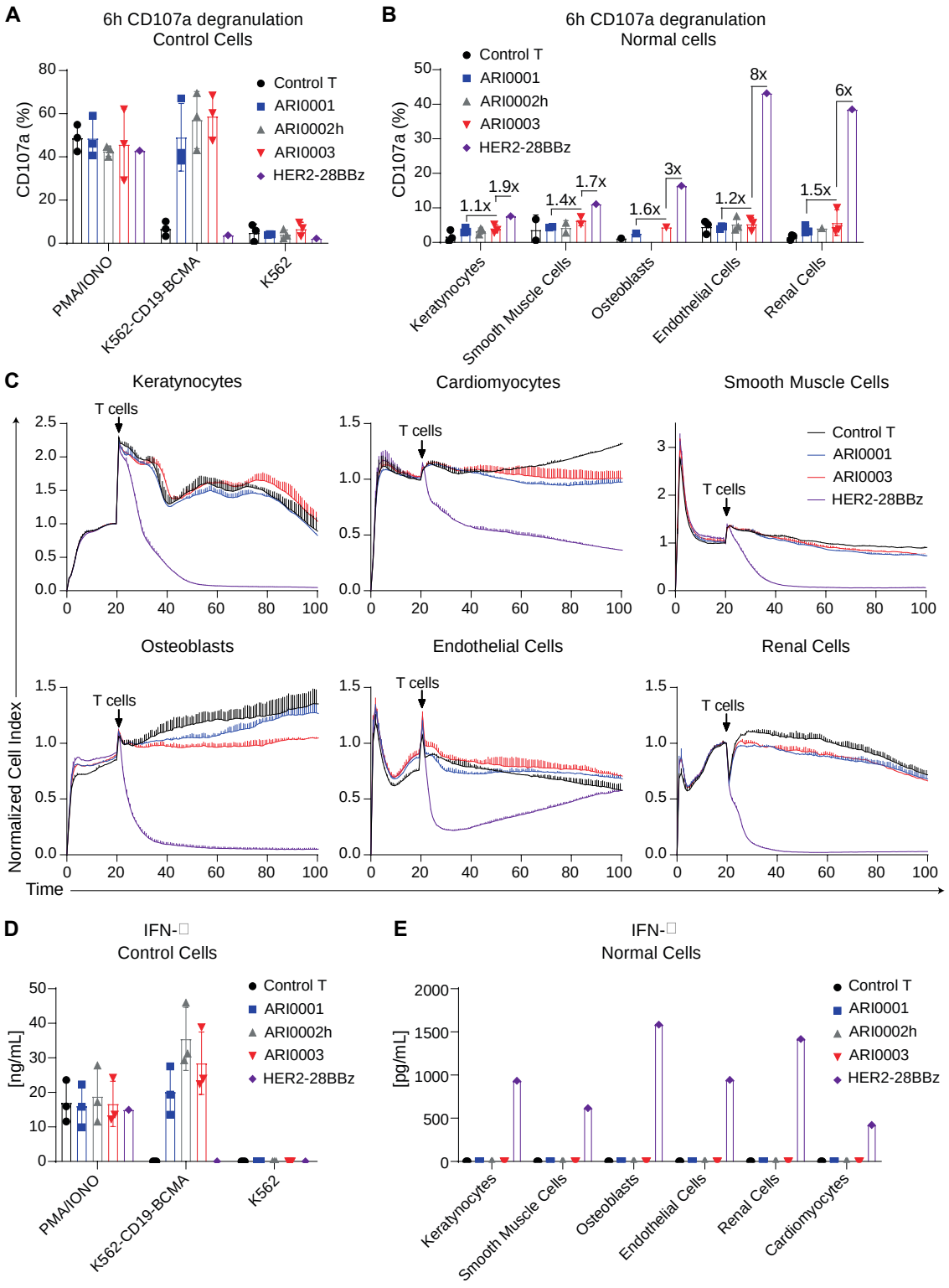

A
6h CD107a degranulation
Control Cells
CD107a (%)
Control T
ARI0001
ARI0002h
ARI0003
HER2-28BBz
PMA/IONO
K562-CD19-BCMA
K562
B
6h CD107a degranulation
Normal cells
1.1x
1.9x
1.4x
1.7x
1.6x
3x
1.2x
8x
1.5x
6x
Keratynocytes
Smooth Muscle Cells
Osteoblasts
Endothelial Cells
Renal Cells
C
Keratynocytes
Cardiomyocytes
Smooth Muscle Cells
Osteoblasts
Endothelial Cells
Renal Cells
T cells
Normalized Cell Index
Time
D
IFN-γ
Control Cells
[ng/mL]
E
IFN-γ
Normal Cells
[pg/mL]

Figure S10. Dual CD19/BCMA targeting by ARI0003 cells does not induce degranulation, killing and cytokine production when co-cultured with normal cells. A-B) Indicated CAR-T cells were stimulated with stimulation cocktail (PMA/ionomycin), co-cultured with K562-CD19-BCMA or K562 tumor cells or normal cells at an E:T=1:1. The percentage of T cells expressing CD107a was analyzed by flow cytometry 6 hours after co-culture. The fold-change in CD107a expression in ARI0003 CAR-T cells versus ARI0001 or high affinity HER2-28BBz CAR-T cells is shown for each cell line. **C)** A real-time cytotoxicity assay (xCelligence) was used to evaluate the lysis of normal cells when treated with control T cells or CAR-T cells at an E:T=3:1 over a 100-hour period. **D)** Indicated CAR-T cells were stimulated with stimulation cocktail or co-cultured with K562-CD19-BCMA (positive control), K562 (negative control) and normal cells at an E:T=3:1. Supernatants were obtained 24 hours after co-culture and IFN-γ production was analyzed by ELISA. Each dot represents a healthy donor.

Table S1. FL patients characteristics.

[1]F: Female, M: Male; [2] PB: Peripheral Blood, LN: Lymph Node, BM: Bone marrow. [3] Samples were obtained at D: diagnosis, R: relapse, Pt: Pretreatment, NA: Not Available; [4]Evaluated by two independent pathologists; [5]Ann Arbor stage; [6]FLIPI: Follicular Lymphoma International Prognostic Index (High (H):≧3; Medium (M):2; Low (L):0-1); [7]All treatments; R: Rituximab; R-mnt: Maintenance Rituximab; RTx: Radiotherapy; GA: Obinutuzumab; Benda: Bendamustine; CHOP: Chemotherapy combination of Cyclophosphamide Hydroxydaunorubicin, Oncovin and Prednisone; ASCT: Autologous Stem Cell Transplantation;DA-EPOCH: dose adjusted combination of etoposide phosphate, prednisone, vincristine sulfate (Oncovin), cyclophosphamide, and hydroxydaunorubicin; ICE: ifosfamide, carboplatin, and etoposide; GEMOX: gemcitabine-oxaliplatin: IT therapy: inthratecal therapy. CR: Complete Response; PR: Partial Response; SD: Stable Disease; [9]POD24: Progression of disease within 2 years; [10] from B-cell panel (CD19, C20, CD22 and CD79b) in routine diagnosis. Y: Yes; N: No; N/A: Not available.

Study label	**Sex /Age[1]**	**Sample type[2]**	**Disease status at sampling[3]**	**Histological grade[4]**	**Stage[5]**	**FLIPI6**	**Treatments[7]**	**Response to 1st tt[8]**	**POD24[9]**	**Cell count Lympho B (10^9/L)**	**% Lympho B[10]**
FL1	F/75	PB	D	2	IVA	H	R-COP/R-mnt	PR	N	74.46	78
FL2	F/52	LN	D	1	IV	H	R-CHOP/P-mnt R-ESHAP ASCT R-GEMOX Ifosfamide Rx CD19 CART (axi-cel)	CR	N	3.13	85
FL24	F/51	PB	D	2	IV	H	R-CHOP/P-mnt	CR	N	9.64	70

Table S2. DLBCL patients characteristics.

[1]F: Female, M: Male; [2] PB: Peripheral Blood, LN: Lymph Node, BM: Bone marrow. [3] Samples were obtained at D: diagnosis, R: relapse, Pt: Pretreatment, NA: Not Available; [4]Evaluated by two independent pathologists; [5]Ann Arbor stage; [6]FLIPI: Follicular Lymphoma International Prognostic Index (High (H):≧3; Medium (M):2; Low (L):0-1); [7]All treatments; R: Rituximab; R-mnt: Maintenance Rituximab; RTx: Radiotherapy; GA: Obinutuzumab; Benda: Bendamustine; CHOP: Chemotherapy combination of Cyclophosphamide Hydroxydaunorubicin, Oncovin and Prednisone; ASCT: Autologous Stem Cell Transplantation;DA-EPOCH: dose adjusted combination of etoposide phosphate, prednisone, vincristine sulfate (Oncovin), cyclophosphamide, and hydroxydaunorubicin; ICE: ifosfamide, carboplatin, and etoposide; GEMOX: gemcitabine-oxaliplatin: IT therapy: inthratecal therapy. CR: Complete Response; PR: Partial Response; SD: Stable Disease; [9]POD24: Progression of disease within 2 years; [10] from B-cell panel(CD19, C20, CD22 and CD79b) in routine diagnosis. Y: Yes; N: No; N/A: Not available. *progression after CD19 CAR-T therapy

Study label	**Sex /Age[1]**	**Sample type[2]**	**Disease status at sampling[3]**	**Subtype**	**Stage[5]**	**Genetic alterations**	**Treatments[7]**	**Response to 1st tt[8]**
DLBCL2	M/37	LN	D	DLBCL	IE	del1p36, BCL6 gains	R-CHOP +RTx	CR
DLBCL4	M/54	PB	R*	GC-DLBCL (from tFL)	IVB	N/A	RTx +R-DA-EPOCH+IT therapy RTx ASCT R-ICE R-GEMOX CD19 CART (aci-cel)	PR
DLBCL5	M/30	BM	R*	HGBCL, NOS	IVB	delTP53, t(8;14)(q24;q32)	BURKIMAB-14 R-HyperCVAD CD19 CART (ARI0001)	CR

Supplemental Methods

Codon usage analysis

Homo sapiens codon usage was extracted from the Codon Usage Database available at http://www.kazusa.or.jp/codon/. Codon Adaptation Index (CAI) along the sequence was represented using R v3.2.3 software. The Codon Adaptation Index (CAI) of each sequence, the percentage of GC and the percentage of codons with G or C at the first %GC1, second %GC2 or third position %GC3 were calculated using the CAIcal server from http://ppuigbo.me/programs/CAIcal/[19].