This appendix has been provided by the authors to give readers additional information about their work.

SUPPLEMENTARY MARETIALS TO:

Bendamustine Lymphodepletion Before Axicabtagene Ciloleucel Is Safe and Associates with Reduced Inflammatory Cytokines

Content:

SUPPLEMENTARY MATERIAL: PATIENTS AND METHODS

DNA isolation and qPCR analysis.

Peripheral blood mononuclear cells from LBCL patients were collected on day 7 post-treatment with axi-cel. The cells were separated from EDTA-preserved whole blood using FICOLL (Sigma-Aldrich; Cat #26873-85-8) gradient purification, followed by direct isolation of genomic DNA. In a subset of 15 patients (Flu/Cy: 6, Benda: 9), sufficient material allowed for DNA isolation and subsequent quantitative PCR to assess axi-cel CAR levels per microgram of genomic DNA. For the qPCR analysis, transgene-specific primers targeting the integrated CD19 CAR transgene sequence (FMC63 scFv; amplicon: GCCATTTACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACTACTGGGGC CAAGG) were used. The qPCR was performed using TaqMan technology (Applied Biosystems), with each reaction including triplicates of 200 ng genomic DNA per time point sourced from peripheral blood samples [1, 2]. To ensure the quality of the assay, a parallel amplification was conducted with 20 ng genomic DNA using a primer/probe combination specific for a non-transcribed sequence upstream of the cyclin-dependent kinase inhibitor 1A (p21) gene. This parallel amplification generated a correction factor, which was applied to adjust for the differences between the calculated and actual DNA input. The lower limit of quantification for this method was established at 25 copies per microgram of genomic DNA.

T cell memory phenotype analysis by flow cytometry

Cells were resuspended in FACS staining buffer (phosphate buffered saline (PBS) + 2% FBS) using the following antibodies: CD45RA (FITC-2H4), CCR7 (APC-G043H7), and CD3 (BUV421-OKT3). Cell viability was established using ViaKrome 808 Fixable Viability Dye (Beckman; Cat# C36628). Cells were processed for flow cytometry using the CURIOX AUTO1000 automation platform with a laminar washer paired with a direct reading grid for reading on the cytometer without perturbation of the samples. Data were acquired on a CytoFlex LX Flow Cytometer (Beckman). All data analysis was performed using FlowJo 10.8.0 software (FlowJo, LLC).

Cytokine assay and Liquid chromatography/Mass spectrometry.

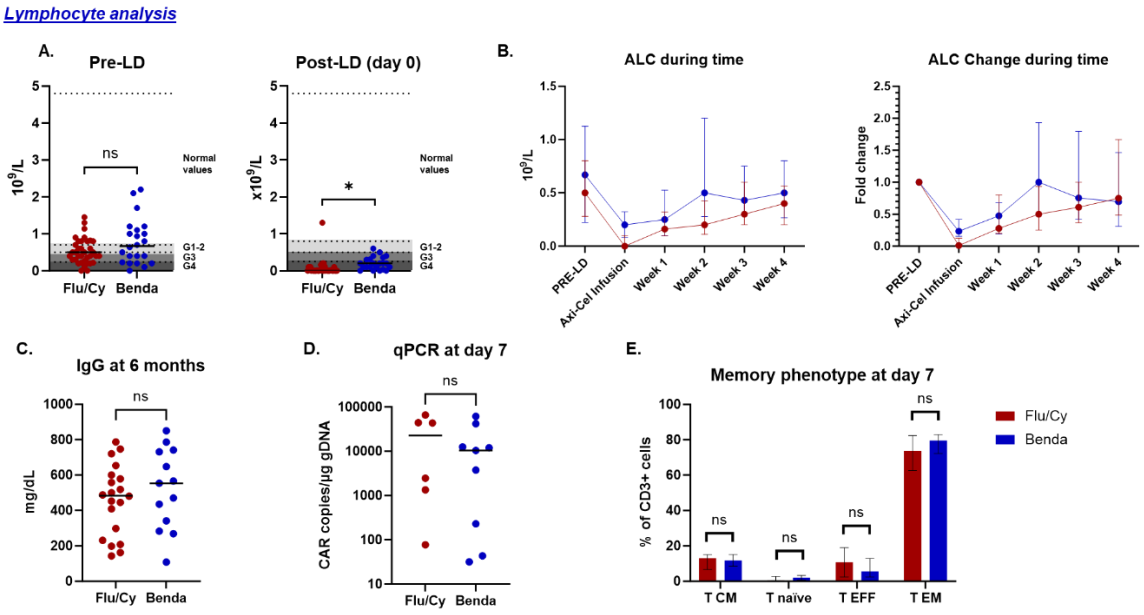
Due to sample availability, a separate cohort of patients receiving either Benda (n=25) or Flu/Cy (n=7) LD was analyzed. We selected serum samples before lymphodepletion start (Flu/Cy: day -5; Benda: day -4) and after lymphodepletion (day 0) (**Fig. 4A**). For the cytokine evaluation, we performed *Luminex* immunoassay to detect cytokines levels (n=32) on serum samples of 32 NHL

patients collected before lymphodepletion administration and before CART19 infusion. In this cohort, patients received either Bendmustine 90 mg/m^2 days over 2 days or Flu/Cy over 3 days before CART19 infusion. For cytokines analysis, we performed Luminex immunoassay (Life Technologies) according to the manufacturer's instructions. For the metabolomic analysis, we performed liquid chromatography/mass spectrometry of the same matched serum samples of NHL patients (n=32) used for cytokine analysis. Specifically, metabolites were extracted by adding 300 µL of 0ºC cold methanol to 100 µL of serum samples, vortexed, rested on ice 10 minutes, and centrifuged at 21000 G for 15 minutes at 0ºC. The collected supernatant underwent solid phase extraction using Agilent Positive Pressure Monifold 48 Processor equipped with Agilent Captiva EMR-Lipid cartridges. The extracted supernatants were transferred to a new microcentrifuge tube and centrifuged at 21000 G for 15 minutes. A 100 µL aliquot was transferred to autosampler Liquid Chromatography (LC) vial with 250 µL polyethylene inserts. Patient samples were analyzed by hydrophilic interaction chromatography (HILIC) coupled with negative-mode electrospray-ionization high-resolution Mass Spectrometer (MS) on a stand-alone 6546 Q-TOF (Agilent Technologies, CA, USA). An aliquot of 2 µL of the blood serum extract was applied to an InfinityLab Poroshell 120, HILIC-Z, 2.1 × 150 mm, 2.7-micron LC column (Agilent Technologies, CA, USA). The metabolites were eluted with an 85 to 10% nonlinear gradient of LC-MS hypergrade acetonitrile (Supelco LiChrosolv, mobile phase B) and 10mM ammonia acetate (LC-MS grade) in high-performance liquid chromatography (HPLC) water with added 5 µM medronic acid (Agilent) and LC-MS grade ammonia hydroxide (phase A, pH=9.30) at a flow rate of 400 µL/min with an Agilent 1290 Infinity II Series LC system. The negative-ion MS data for a mass range of 50 to 1100 Da were acquired at one scans/s. Data were collected in both centroid and profile modes in 4-GHz high-resolution mode. Negative-ion reference masses of 112.985587 and 1033.988109 m/z solution were analyzed to ensure mass accuracy. To monitor instrument performance, quality control samples consisting of a metabolite extract of pooled serum samples were analyzed at the beginning of each analysis day and every six samples during the analysis. The labeled U-13C metabolite standard mixes and compounds from yeast extracts (Cambridge Isotope Laboratories, MA, USA) were used as internal standards for the identification of serum metabolites. Data were analyzed using MassHunter Profinder version 10.0 software (Agilent Technologies, CA, USA).

General Statistics. For statistical analysis, continuous variables were summarized as the median with inter-quartile range (IQR, unless otherwise specified) and categorical variables as percentages. Patient demographics, response rates, and adverse events were compared using

chi-square and Student's t-tests. Survival analyses were estimated using the Kaplan–Meier method and compared using the log-rank test. All statistical tests were two-sided, and statistical significance was defined as p-value <0.05. Analyses were performed with the Statistical Package for the Social Sciences software v.22.0 (Chicago, IL, USA). For metabolite analysis, log2 fold change was calculated by R package tools (v.3.9.4) and visualized using pheatmap (v1.0.12) and ComplexHeatmap (v2.15.3). Two-sided Student's t-test was performed on the log2 fold change between Benda and Flu/Cy. As for the heatmap of log2 fold change for two groups, metabolites were selected as the top ones with the highest absolute values of log2 fold change difference between the two groups and plotted with the median value of each group. The heatmap of Pre-LD per patient was visualized in log scale by default natural logarithms using the same R package. For cytokine analysis, the heatmap of log2 fold change per patient was also visualized using the same R package pheatmap (v1.0.12) and ComplexHeatmap (v2.15.3). Figures were generated using GraphPad Prism software 9.5.0 (730).

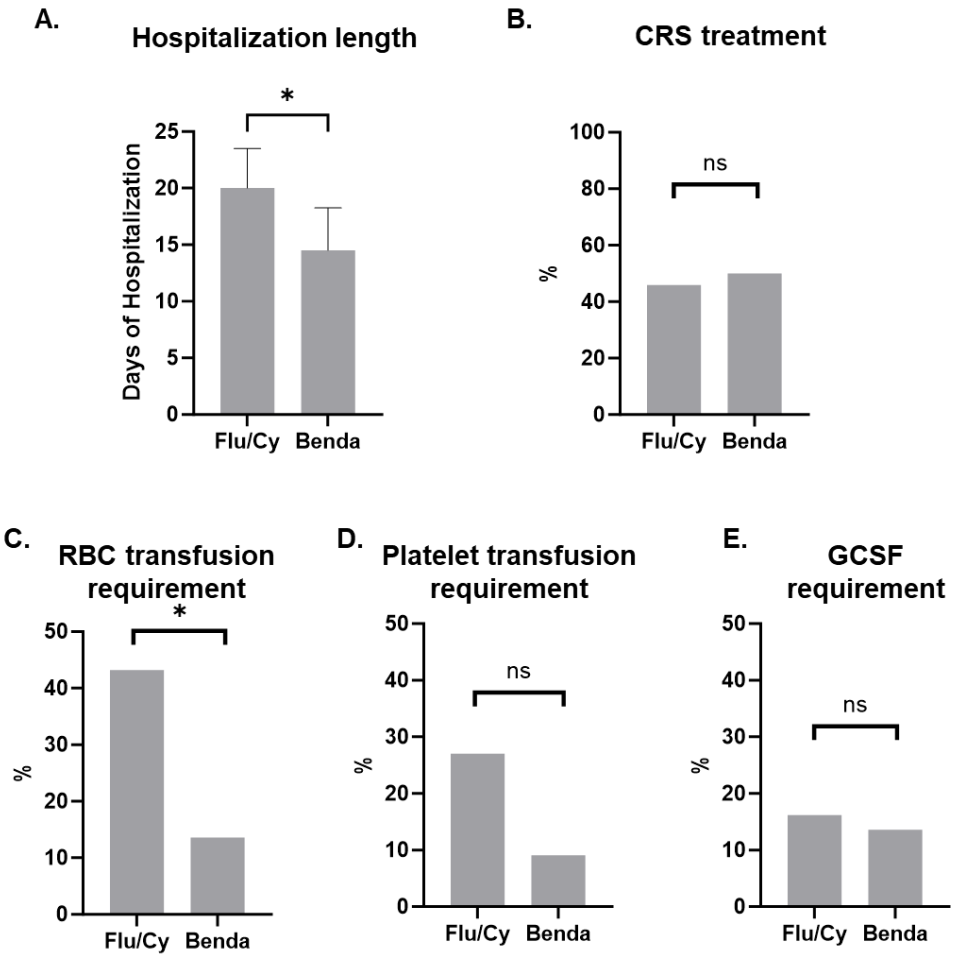
SUPPLEMENTARY FIGURES



Supp. Figure 1. Lymphocyte analysis after CD28 costimulated CART19 infusion according to lymphodepletion regimen administered. A. Dot plots show differences between the two lymphodepletion regimens for lymphocyte counts before lymphodepletion (Pre-LD) and on the day of axi-cel infusion (Post-LD, day 0). Shadows of gray background highlight the range of specific abnormal values. B. Median values of lymphocyte counts over time and change in lymphocyte counts from baseline (Pre-LD values) according to lymphodepletion regimen. Red lines represent Flu/Cy-treated patients, while blue line represents bendamustine-treated patients. C. Dot plot shows differences between the two lymphodepletion regimens for serum immunoglobulin G levels at 6 months after axi-cel infusion in patients in persistent remission. D. Dot plot shows differences between the two lymphodepletion regimens for axi-cel CAR copies measured at day 7 after infusion. E. Histograms show differences between T cell memory phenotype populations measured at day 7 after axi-cel infusion.

*Abbreviations: Benda: Bendamustine lymphodepletion regimen; Flu/Cy: fludarabine/cyclophosphamide lymphodepletion regimen; T CM: central memory T cells; T EFF: Effector T cells; T EM: Effector memory T cells; T naïve: Naïve T cells; p: p-value. * = p<0.050; ns = Not statistically significant.*

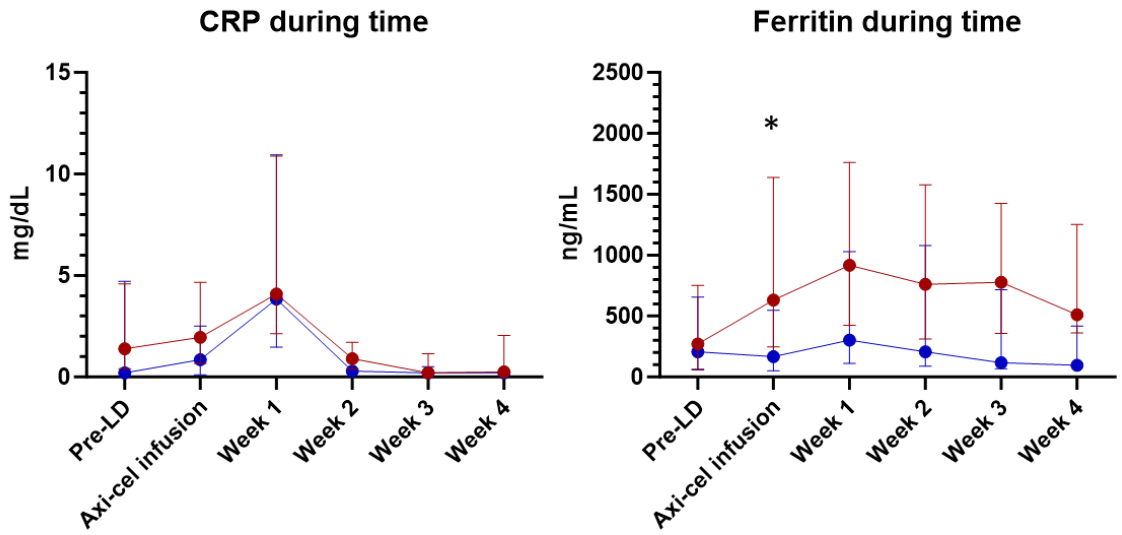
Supp. Fig. 2



Supp. Figure 2. Toxicity management analysis after CD28 costimulated CART19 infusion according to lymphodepletion regimen administered. A. Histograms show hospitalization duration according to the lymphodepletion regimen received. B. Histograms show the percentage of axi-cel-treated patients requiring specific treatment for CRS and ICANS according to lymphodepletion. C. Histograms show the percentage of axi-cel-treated patients requiring red blood cell transfusion over the 30 days after axi-cel infusion; D. Histograms show the percentage of axi-cel-treated patients requiring platelets transfusion over the 30 days after axi-cel infusion. D. Histograms show the percentage of axi-cel treated patients requiring G-CFS administration over the 30 days after axi-cel infusion.

*Abbreviations: Axi-cel: axicabtacene cilolecel; Benda: Bendamustine lymphodepleting regimen; CRS: cytokines releasing syndrome; Flu/Cy: fludarabine/cyclophosphamide lymphodepleting regimen; p: p-value; * = p<0.050; ns = Not statistically significant.*

Supp. Fig. 3

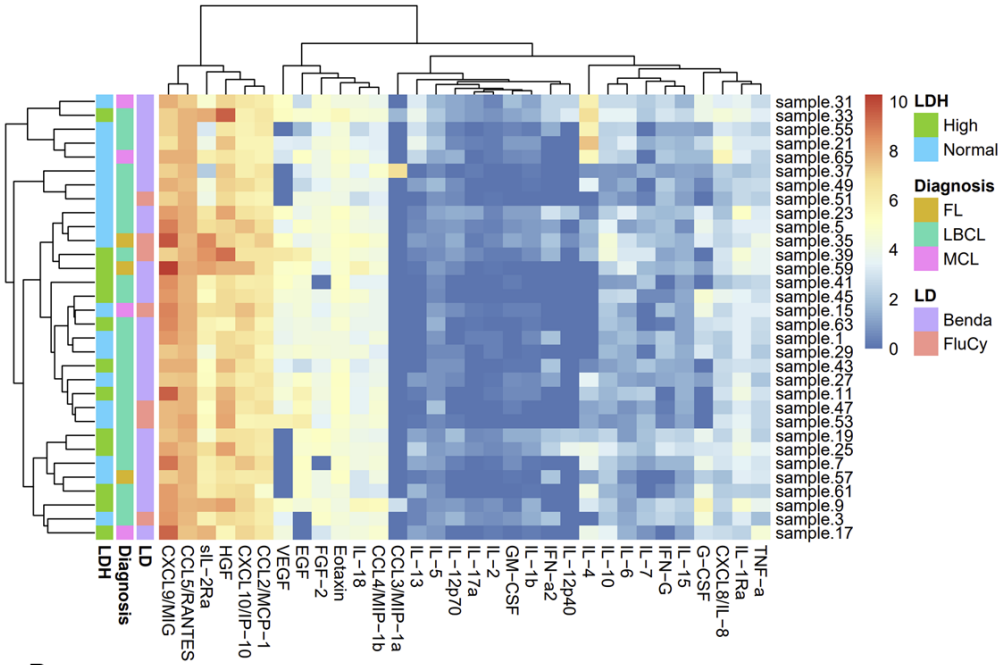


Supp. Figure 3. CRP and ferritin levels over time after CD28 costimulated CART19 infusion according to the lymphodepleting regimen administered. Red lines represent Flu/Cy-treated patients, while blue line represents bendamustine-treated patients.

*Abbreviations: Benda: Bendamustine lymphodepleting regimen; CRP: C reactive protein; Flu/Cy: fludarabine/cyclophosphamide lymphodepleting regimen; * = p<0.050*

Supp. Fig. 4

A. Pre-LD cytokine levels



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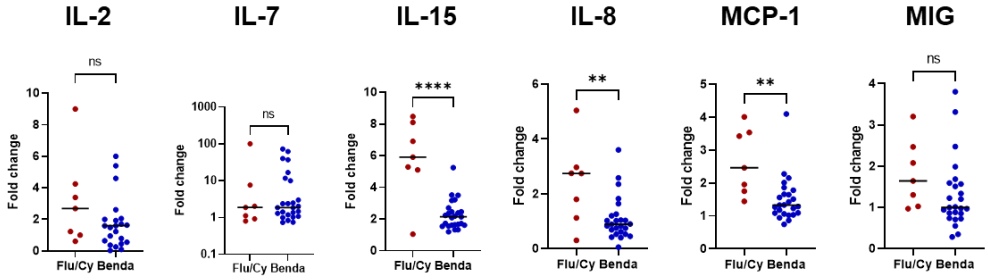

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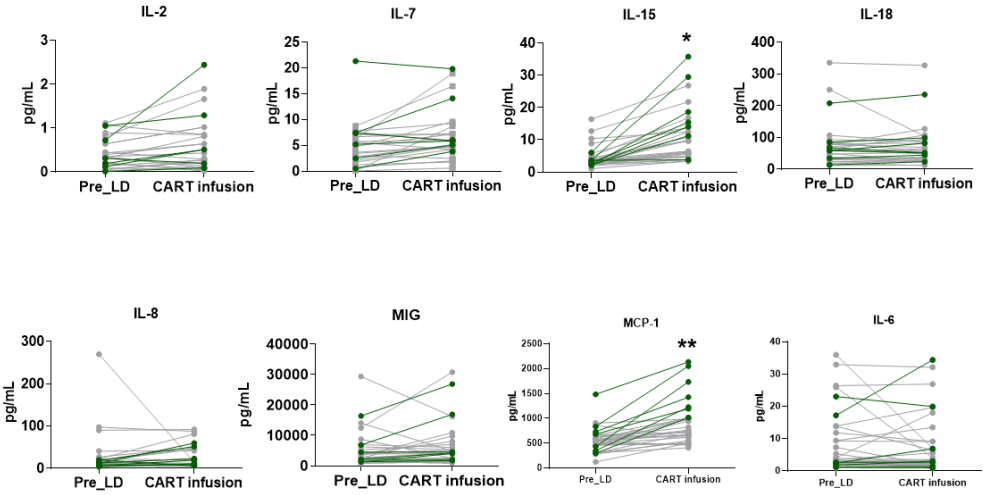
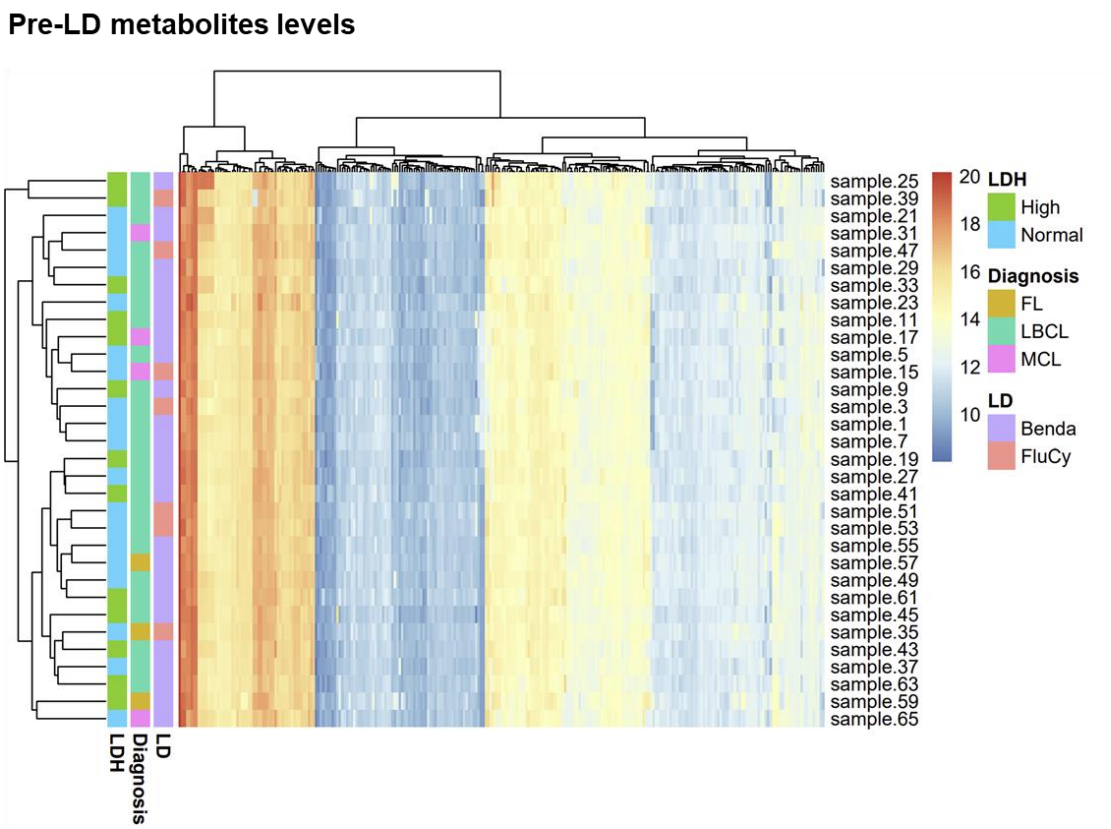


Suppl. Figure 4. Cytokine level changes of cytokine involved in T cell expansion and the emergence of toxicities. A. Heatmap of Pre-LD cytokine values distribution. Each row represents a patient, while each column represents a metabolite. None pre-LD clustering based on LDH, diagnosis, and lymphodepletion was identified by hierarchical clustering. B. Cytokines affecting lymphocyte engraftment and expansion, and cytokines modulating myeloid cells changes according to lymphodepletion. Dot plots show differences between the two lymphodepletion regimens for IL-2, IL-7, IL-15, IL-8, MCP-1, and MIG changes related to lymphodepletion. C. Cytokine absolute levels change from pre-lymphodepletion to CART19 infusion according to lymphodepletion administered. Each line represents a specific patient. Green lines represent Flu/Cy-treated patients, while gray lines represent Benda-treated patients.

*Abbreviations: Benda: Bendamustine lymphodepletion regimen; FL: Follicular lymphoma; Flu/Cy: fludarabine/cyclophosphamide lymphodepletion regimen; LBCL: large B cell lymphomas; LDH: Lactate dehydrogenase; MCL: mantle cell lymphoma * = p<0.050; ** = P<0.005; ns = Not statistically significant.*

Supp. Fig. 5



Suppl. Figure 5. Heatmap of metabolites pre-lymphodepletion. Each row represents a patient while each column represents a metabolite. None pre-LD clustering based on LDH, diagnosis, and Lymphodepletion was identified by hierarchical clustering.

Abbreviations: Benda: Bendamustine lymphodepletion regimen; FL: Follicular lymphoma; Flu/Cy: fludarabine/cyclophosphamide lymphodepletion regimen; LBCL: large B cell lymphomas; LDH: Lactate dehydrogenase; MCL: mantle cell lymphoma

SUPPLEMENTARY TABLES

Supp. Table 1 Hematological toxicities

Characteristic	Flu/Cy 37 (62.7%)	Benda 22 (37.3%)	p
Pre-LD Lymphocytes (x10^9/L)	0.50 [0.30-0.80]	0.67 [0.23-1.10]	0.969
Pre-LD Neutrophils (x10^9/L)	2.90 [1.55-3.96]	3.40 [2.46-4.23]	0.464
Pre-LD Platelets (x10^9/L)	172 [108-199]	196 [131-230]	0.237
Pre-LD Hemoglobin (g/dL)	11.2 [8.9-12.4]	11.8 [10.3-12.5]	0.431
Post-LD Lymphocytes (x10^9/L)	0.00 [0.00-0.10]	0.20 [0.10-0.31]	0.022
Post-LD Neutrophils (x10^9/L)	1.20 [0.60-2.00]	2.70 [1.80-3.20]	<0.001
Post-LD Platelets (x10^9/L)	139 [108-163]	166 [124-193]	0.270
Post-LD Hemoglobin (g/dL)	10.4 [8.3-11.9]	11.4 [8.6-11.9]	0.290
Neutrophils nadir (x10^9/L)	0.02 [0.00-0.20]	0.90 [0.66-1.89]	<0.001
Platelets nadir (x10^9/L)	61 [23-100]	89 [57-143]	0.097
Hemoglobin nadir (g/dL)	7.6 [7.2-8.8]	9.8 [7.9-10.9]	0.004
4-weeks Lymphocytes (x10^9/L)	0.40 [0.20-0.55]	0.50 [0.30-0.70]	0.279
4-weeks Neutrophils (x10^9/L)	1.50 [1.00-2.60]	1.10 [0.80-2.50]	0.873
4-weeks Platelets (x10^9/L)	112 [34-202]	105 [57-146]	0.585
4-weeks Hemoglobin (g/dL)	11.2 [9.1 -12.2]	11.6 [9.8-12.3]	0.593

Abbreviations: Pre-LD: Before lymphodepletion start: Post-LD: Post lymphodepletion, the same day of axi-cel infusion. Benda: Bendamustine lymphodepletion regimen; Flu/Cy: fludarabine/cyclophosphamide lymphodepletion regimen.

Supp. Table 2. Characteristics of patients evaluated for cytokines and metabolite levels

Characteristic		Total 32 (100%)	Flu/Cy 7 (21.9%)	Benda 25 (78.1%)	p
Age at infusion	≤65 years	20 (62.5%)	6 (85.7%)	14 (56.0%)	0.151
	> 65 years	12 (37.5%)	1 (14.3%)	11 (44.0%)	
Diagnosis	LBCL	25 (78.1%)	5 (71.4%)	20 (80.0%)	0.859
	FL	3 (9.4%)	1 (14.3%)	2 (8.0%)	
	MCL	4 (12.5%)	1 (14.3%)	3 (12.0%)	
Sex	Female	3 (9.4%)	0 (0.0%)	3 (12.0%)	0.336
	Male	29 (90.6%)	7 (100%)	22 (88.0%)	
Number of previous therapies	Median [IQR]	3 [2-4]	2 [2-3]	3 [2-4]	0.110
ECOG grade (n=28)	≤1	27 (96.4%)	6 (100%)	21 (95.5%)	0.595
	>1	1 (3.6%)	0 (0.0%)	1 (4.5%)	
LDH levels pre-LD	Normal	19 (59.4%)	6 (85.7%)	13 (52.0%)	0.108
	Elevated	13 (40.6%)	1 (14.3%)	12 (48.0%)	

Abbreviations: Benda: Bendamustine lymphodepletion regimen; FL: follicular lymphoma; Flu/Cy: fludarabine/cyclophosphamide lymphodepletion regimen; LBCL: large B-cell lymphomas; ECOG PS: Performance status according to Eastern Cooperative Oncology Group; IQR: interquartile range; LDH: Lactate dehydrogenase; MCL: mantle cell lymphoma; n: number; p: p-value; Pre-LD: pre-lymphodepletion

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