# CD19 Chimeric Antigen Receptor T-cell Shows Activity in Patients with B-cell Lymphoma Without CD19 Expression by Immunohistochemistry

**Tao Wang**
Changhai Hospital Department of Hematology https://orcid.org/0000-0002-1789-9767
**Lili Xu**
Changhai Hospital Department of Hematology
**Yang Wang**
Changhai Hospital Department of Neurosurgery
**Gusheng Tang**
Changhai Hospital Department of Hematology
**Lei Gao**
Changhai Hospital Department of Hematology
**Li Chen**
Changhai Hospital Department of Nephrology
**Jie Chen**
Changhai Hospital Department of Hematology
**Wenqin Yue**
Changhai Hospital Department of Nephrology
**Weijia Fu**
Changhai Hospital Department of Nephrology
**Miaoxia He**
Changhai Hospital Department of Nephrology
**Jianmin Yang** ( chyangjianmin@163.com )
Department of Hematology, Changhai Hospital

**Research**

**Keywords:** Diffuse large B-cell lymphoma, Chimeric antigen receptor T-cell, CD19 expression, Immunohistochemistry

**Posted Date:** February 5th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-191492/v1

# Abstract

**Background:** Some clinical trials have reported that CD19-negative B-cell lymphoma patients responded to CD19 CART therapy. The mechanism of CD19 CART activating in patients with B-cell lymphoma that do not express CD19 by immunohistochemistry (IHC) remains unknown.

**Methods:** 8 CART treated diffuse large B-cell lymphoma (DLBCL) patients and other 90 DLBCL tissues were tested by IHC using 7 anti-CD19 antibodies. Besides, CD19 mRNA and protein of 6 human DLBCL cell lines were assessed using Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western blotting analysis, and CD19 expression was evaluated by IHC as well as flow cytometry (FCM).

**Results:** 6 out of 8 (75%) DLBCL patients who received CD19 CART therapy were identified as CD19-negative by at least one antibody. In the 6 patients, 5 objective responses were seen (response rate, 83.3%). For the 90 DLBCL samples, 68 samples were involved in analysis, and 57 out of 68 (83.8%) samples were assessed as CD19 negative by at least one antibody. IHC assay for 6 DLBCL cell lines showed that 3 cell lines CD19 expression by IHC had inconsistent results with CD19 mRNA and Western blotting assays, while FCM assay for the 6 DLBCL cell lines showed high sensitivity and good concordance to CD19 expression. Antibody HIB19 mean fluorescence intensity showed a positive correlation with CD19 expression ($R^2$=0.8125, P=0.0137).

**Conclusion:** These results demonstrate the mechanism that CD19-negaive B-cell lymphoma patients respond to CD19 CART therapy, suggesting that inclusion or exclusion of lymphoma patients for CD19 CART therapy based on current CD19 IHC seems unwarranted. FCM assay is recommended as a complementary diagnostic method to CD19 IHC.


# Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), accounting for 30% of newly diagnosed NHL[1]. Diagnosis of DLBCL mainly relies on immunohistochemistry (IHC) assay for CD20, CD3, CD5, CD10, CD45, BCL2, Ki-67, IRF4/MUM1 and MYC[2]. Rituximab combined with CHOP immunochemotherapy (R-CHOP) is the standard-of-care treatment for DLBCL, with remission rates of 58–75% [3–6]. However, 20–50% of the patients are primary refractory to or relapsed after R-CHOP immunochemotherapy[7]. For refractory or relapsed DLBCL (r/r DLBCL), the outcomes are very poor even with salvage therapy.

Chimeric antigen receptor (CAR) T-cell immunotherapy has achieved remarkable success in relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL)[8–13] and B-cell NHL[14–17]. Three CART products have been approved by Food and Drug Administration, including Tisagenlecleucel (Kymariah) for r/r DLBCL and B-ALL, Axicabtagene Ciloleucel (Yescarta) for relapsed or refractory large B-cell lymphoma and KTE-X19 (Tecartus) for relapsed or refractory mantle cell lymphoma [18–20]. Interestingly, recent studies have reported that CD19 CART is active in B-cell lymphoma that do not express CD19 by IHC. In a

clinical trial of Axicabtagene Ciloleucel, 8 CD19-negative DLBCL patients were observed to have similar response rates as CD19-positive patients[15]. In a recent reported clinical trial results of KTE-X19, 3 CD19-negative mantle cell lymphoma patients were shown to have a 100% response rate to CD19 CAR-T cell therapy[21]. By far, IHC has been the most commonly used method in lymphoma diagnosis and CD19 analysis before CART therapy. The observations in above studies seem to support the hypothesis that CD19 IHC negative lymphoma patients would be able to respond to CD19 CART. CD19 IHC positivity as an indicator for CD19 CART therapy is limited, however, how to determine B-cell lymphoma patients whether to use CD19 CART therapy is not well defined.

Here, we reviewed 8 DLBCL patients treated with CD19 CART and 90 DLBCL samples by IHC with 7 different CD19 antibodies. Both mRNA and protein CD19 expression were evaluated in 6 human DLBCL cell lines, followed by IHC with 7 different CD19 antibodies and Flow cytometry (FCM) with 3 different CD19 antibodies to determine heterogeneity between CD19 detection methods and develop a reliable method for CD19 assessment before CART therapy.

## Methods And Materials

# Patients and lymphoma samples

A total of 98 pretreatment tumor biopsy samples were collected from newly diagnosed with DLBCL in the Naval Military Medical University affiliated Changhai Hospital from January 2012 to January 2019. Among them, 8 patients have received CD19 CART therapy. Histologic specimens were examined by two pathologists in line with the 2016 updated World Health Organization Classification of lymphoid neoplasias [2]. Informed consents were obtained from all patients in accordance with requirements of the Declaration of Helsinki.

# Micro-immunohistochemistry (uIHC)

The sample were transferred into IHC tissue chip (9×10 array) using a sample needle and then cut into 4–6 μm sections. To conduct the uIHC test, antibody solutions were localized on surfaces of micrometre-sized tissue sections using a vertical microfluidic probe for primary antibodies incubation. Secondary antibody incubation was performed following conventional IHC procedure as described in detail previously[22]. Seven commercially available CD19 antibodies were used which were extracellular-epitope monoclonal antibodies including LE-CD19 (Novus Biologicals, USA, NB100-65672), OTI1F2 (LifeSpan BioSciences, USA, LS-C338081), D4V4B (Cell signaling technology, USA, 90176), OTI1F9 (LifeSpan BioSciences, USA, LS-C338089), OTI3B10 (LifeSpan BioSciences, USA, LSC174739), EPR5906 (Abcam, USA, ab134114) and SP291 (Abcam, USA, ab227688). The results of CD19 were scored using a semi-quantitative scoring system (Allred score)[22]. The final immunostaining scores were defined as 0, 1, 2, 3, 4. 0 represents tissue loss during manufacture, 1 stands for CD19 negative, 2 and 3 represent CD19 < 30% but > 0 (low positive) and > 30% but < 100% (high positive), respectively. Samples were excluded for IHC

score analysis if tissues lost more than 2 out of the 7 antibodies assays and the data of percentage of positive cells included only samples without tissue loss.

# Cell lines and cell culture

The human DLBCL cell lines TMD-8, OCI-LY1, HBL-1 and SUDHL-2 were donated by Department of Urology, Changhai Hospital, Naval Medical University, Shanghai, China. The human lymphoma cell lines SUDHL-4 and DB were donated by School of life sciences and technology, Tongji University, Shanghai, China. TMD-8, HBL-1, SUDHL-2, SUDHL-4 and DB cell line were cultured in RPMI-1640 Medium containing 20% fetal bovine serum (Gibco, USA) and OCI-LY1 was cultured in IMDM medium containing 20% fetal bovine serum. All of the cell lines were cultured in incubator containing 5% CO2 at 37℃. Fresh medium was supplied every 3 days.

# FCM assay

A patient's pleural effusion and 6 DLBCL cell lines including TMD8, OCI-LY1, HBL-1, SU-DHL-4, SU-DHL-2 and DB were analyzed by FCM. Cell density was adjusted to $5\times10^5$/ml before staining. 3 CD19-APC antibodies applied were used including SJ25-C1 (clone Invitrogen, USA, MHCD1905), HIB19 (BD Pharmingen, USA, 555415) and 4G7 (Novus Biologicals, USA, NBP2-52664). FCM was performed using Beckman Coulter Flow Cytometer (Navios, California, USA) and the results were analyzed using Flowjo v10 software.

# Western Blotting

Cells were collected and washed twice with PBS. Cell pellets were lysed in RIPA and PMSF (1:100) buffer (Thermo Scientific 89900, 36978), and protein quantification by BCA assay (Thermo Scientific, 23227). Proteins were separated by 4–20% SDSPAGE at 180V for 30 minutes and transferred to PVDF membranes for 1.5 hours. PVDF membranes were blocked with 5% BSA for 1 hour and washed with TBST. The membranes were incubated with primary antibodies D4V4B (Cell signaling technology, USA, 90176) and β-Actin (Cell signaling technology, USA, 4970T) overnight at 4˚C, and further with a secondary antibody (Cell signaling technology, USA, 7074S) for 1 hour at room temperature. The membranes were washed three times and visualized with a chemiluminescence system. Proteins were quantified using Image J software.

# Reverse Transcription Polymerase Chain Reaction (RT-PCR)

$5\times10^6$ cells were collected and washed twice with PBS. RNA was extracted using TRIzol reagent (Introvigen, 15596-026) and eluted in 50ul nuclease-free water. Reverse-transcription reaction was performed with PrimeScript™ RT Master Mix (Takara, RR036A), following the manufacturer's instructions. The primers sequences of CD19 were: Forward: GATAACGCTGTGCTGCAGTG and Reverse: ACACATACAGCTTGGGGCTC. The assay was carried out in a 20 μl reaction mixture containing 10 μl of 2× TB Green Premix Ex Taq (Takara, RR820A), 1ul of 10 μM of each primer, 0.4 μl of 50×ROX Reference Dye, 2 μl of Template and 5.6 ul sterile purified water. The optimized thermal cycling conditions were as follows: 1 cycle of 95°C for 30 sec and 40 cycles of PCR amplification at 95°C for 3 sec and 60°C for 30

sec, followed by a melt curve stage. Real-time RT-PCR reactions were performed on the Biosystems 7500 real-time PCR System (Biosystems, CA, USA).

# Statistical analysis

Continuous data were expressed as median and standard deviation. For analysis of DLBCL, IHC percentage of positive cells among different antibodies and comparisons among cell lines RNA, protein levels, MFIs and IHC intensity, the general linear model and Student-Newman-Keuls test were used. To determine the associations between the protein level in different cell lines and MFIs or IHC intensity, Spearman's rank correlation coefficients were calculated. All statistical analyses were performed using SPSS (version 23.00) software. P value < 0.05 was considered to be statistically significant.

# Results

### 1. CD19 expression in DLBCL patients treated with CART.

In this study, one DLBCL patient who received CD19 CART therapy and obtained CR, was observed to be CD19 negative in her pleural effusion lymphoma cells at initial diagnostic IHC assay (Fig. 1A). To further investigate these results, the sample was evaluated using 7 IHC antibodies targeting different clones, and found that the sample was positive in antibodies OTI1F9 and 3B10 detection, low positive in antibodies LE-CD19 and D4V4B detection, and negative in antibodies OTI1F2, EPR5906 and SP291 detection (Fig. 1B). To determine the results, we evaluated CD19 expression in the pleural effusion cells by FCM, which identified that CD19 expression in the lymphoma cells was positive (Fig. 1C). To further investigate the relevance of CD19 expression and patients' response to CART therapy, IHC assays of CD19 expression in other 7 DLBCL patients treated with CD19 CART therapy were performed. The patients' characteristics were showed in Table 1. 6 out of 8 DLBCL patients who received CD19 CART therapy obtained complete or partial remission (response rate, 75%). It is observed that 6 DLBCL tumors were identified as CD19-negative by at least one antibody. Among the 6 patients, 5 objective responses were seen (response rate, 83.3%). 2-year progression-free survival rate 38% (95% CI 4.68%-71.32%), 2-year overall survival 50% (95% CI 4.68%-71.325). As the data were too small to analyze IHC staining bias, additional 90 DLBCL sample were reviewed by IHC with 7 antibodies.

### 2. CD19 expression in DLBCL tissues varies with different IHC antibodies.

To compare the performance of different CD19 IHC antibodies, we tested 90 formalin-fixed and paraffin-embedded (FFPE) DLBCL samples with 7 CD19 IHC antibodies by uIHC (Fig. 2A). 68 DLBCL qualified samples were involved in the analysis, samples showed different CD19 expression status among different antibodies. 57 out of 68 (83.8%) samples were assessed as CD19 negative by at least one antibody. The results are displayed by a heat map (Fig. 2B) The percentage of positive cells were found to differ when detected by different antibodies. Antibody LE-CD19 had highest percent of CD19 positive cells, 1F2, D4V4B, 1F9 and 3B10 had moderate percent of CD19 positive cells, and EPR5906 and SP291

had the lowest percent of CD19 positive cells ($P < 0.05$) (Fig. 2C). CD19 IHC showed a poor consistency among different antibodies.

### 3. RT-PCR, Western blotting and IHC analysis of CD19 mRNA and CD19 protein expression in human DLBCL cell lines.

As CD19 IHC showed low concordance among antibodies, to find out a method with good consistency, we compared IHC and FCM in 6 DLBCL cell lines, including TMD-8, OCI-LY1, HBL-1, SUDHL-2, SUDHL-4 and DB. CD19 mRNA levels in the 6 cell lines was determined by RT-PCR, and all of these cell lines were detected CD19 mRNA. TMD-8 (0.201%±0.019%) and SUDHL-4 (0.246%±0.016%) showed high levels of CD19 mRNA, OCI-LY1(0.142%±0.012%), HBL-1(0.0605 ± 0.004%) and SUDHL-2 (0.121%±0.002%) showed moderate levels of CD19 mRNA, while DB ($3.65\times10^{-5}$%±$1.195\times10^{-5}$%) cell line showed the lowest level of CD19 mRNA (Fig. 3A). As the protein level, CD19 expression was detected by Western Blotting analysis(Fig. 3B). TMD-8 (41.6235%±2.2582%), OCI-LY1 (42.1468%±0.0683%) and SUDHL-2 (38.1858% ±2.4609%) showed high CD19 protein expression, HBL-1 (30.2584%±3.4691%) and SUDHL-4 (28.6253% ±2.5132%) showed moderate CD19 protein expression, and DB cell line showed undetectable CD19 expression（Fig. 3C）. IHC assay with 7 different CD19 antibodies was performed in the 6 cell lines and results were displayed by a heat map (Fig. 3D). CD19 expression in cell lines TMD-8, SUDHL-2 and SUDHL-4 were identically strong positive (Fig. 3E,H-I). Cell line OCI-LY1 showed CD19 positive in antibodies OTI1F9, 3B10, EPR5906 and SP291, while showed CD19-negative in antibodies LE-CD19 and D4V4B (Fig. 3F). Cell line HBL-1 showed low positive in antibodies LE-CD19 and OTI1F2, and strong positive in remain antibodies (Fig. 3G). Cell line DB showed strong positive in antibodies 3B10 and low positive in antibodies LE-CD19 while negative in the remaining antibodies (Fig. 3J). The discrepancy of CD19 expression in cell lines OCI-LY1, HBL-1 and DB indicated that CD19 expression could be false negative due to single assay with one antibody, emphasizing the importance of developing a complementary method to assess the CD19 expression level following CART therapy.

### 4. FCM had high sensitivity and good concordance for CD19 detection.

Unlike IHC assay which was used FFPE samples, FCM was applied in living cells. CD19 expression was detected using FCM with 3 antibodies targeting different CD19 epitopes of SJ25-C1, HIB19 and 4G7. All the 6 cell lines tested CD19 positive expression. For the SJ25-C1 and HIB19 antibodies, the TMD-8, SUDHL-2 and SUDHL-4 cell lines were observed high CD19-Mean fluorescence intensity (MFI), OCI-LY1 and HBL-1 cell lines showed moderate CD19-MFI and cell line DB showed low CD19-MFI (Fig. 4A-B). For the 4G7 antibody, TMD-8 cell line was observed high CD19-MFI, while OCI-LY1 and HBL-1, SUDHL-2 and SUDHL-4 cell lines showed moderate CD19-MFI and DB cell line showed low CD19-MFI (Fig. 4C). A positive correlation was observed between CD19 expression level determined by Western blotting assay and that of CD19-MFI measured by FCM with the HIB19 antibody ($R^2 = 0.8125$, $P = 0.0137$) (Fig. 4D). FCM and Western blotting showed good concordance in measuring the expression of CD19 expression.

## Discussion

To explore the reason of CD19 CART actives in CD19 negative lymphoma patients, 98 DLBCL samples were retested for CD19 expression with 7 different antibodies. 6 DLBCL cell lines was detected by RT-PCR and Western blotting followed by IHC and FCM. This study revealed the potential mechanism of the presumably CD19-negative lymphoma patients responding to CD19 CART therapy, since these patients were actually false CD19-negative due to current IHC limitation. FCM with sensitivity and good concordance to CD19 expression was recommended as a complementary method for IHC.

False CD19 negative in IHC detection may be attributed to two mechanisms: First, formalin fixation and paraffin embedding altered the three-dimensional structure of antigen protein[23]. The reaction between antibodies and antigens depends on the antigen conformation. Mason and O'Leary reported that the secondary structure of proteins was locked in the process of formalin fixation[24], while the tertiary and quaternary antigen conformation decreased the final antigen-antibody reaction intensity, and consequently, leading to a false-negative result[25]. Second, single CD19 antibody cannot cover all kinds of CD19 isoforms. A gene can generate various protein isoforms due to alternative splicing of the primary mRNA and posttranslational modification[26]. Different CD19 antibodies have distinct epitopes, so one CD19 antibody cannot identify all the epitopes in the CD19 isoforms repertoire[27]. Most pathological laboratory performing IHC use only one CD19 antibody, and samples are likely to be judge as "negative" if the CD19 molecule dose not express the isoforms that the CD19 antibody can identify.

To our knowledge, no theoretical basis and data support the mechanism of CD19 CART mediating CD19-negative killing. In preclinical research, there are reports that CART could mediate non-target cell lysis via bystander killing effect, a mechanism which is cytokine-dependent that CART were activated by target antigen-positive cells and release cytokines such as interferon-γ and tumor necrosis factor α[28]. Bystander killing effect occurs only when CART are co-cultured with target antigen positive and negative cell lines, and target antigen negative cell lines are not killed by CART without target antigen positive cell lines[29,30]. The antigen recognition domain of CAR-T cells is single-chain variable Fragment (scFv), a molecule from both the heavy and light chain of the antigen-binding domain of a monoclonal antibody[31]. Based on the antigen-antibody reaction (Ag-Ab reaction) mechanism, the ScFv-based CART recognizes protein structure other than epitopes. Therefore, the test for both the target antigen structure and epitopes are equally important in prior treatment evaluations.

Since IHC preserves the tissue architecture, it is used for routine clinical practice in lymphoma diagnosis while FCM is restricted to NHL with peripheral blood or bone marrow involvement or in chronic lymphocytic leukemia[32].Over the years, multiple analytical techniques have been developed for clinical diagnosis, classification and monitoring of hematopoietic disease. FCM is a sensitive, accurate and real-time detection that was widely used in the evaluation of hematopoietic diseases [33]. FCM is a laser-based technology, which measures living cells immunophenotype based on Ag-Ab reaction without structural alteration. FCM has some advantages compared with IHC: (1) FCM provides faster and accurate quantitative results; (2) FCM evaluates millions of cells labeled with different markers simultaneously and can easily define clonal populations; (3) FCM directly examines living cells and immunophenotypes

in physiological state[32,34]. What's more, CART immunotherapy is effective for CD19-dim B-cell ALL, according to research in vitro, CART was able to recognize and lyse cells with CD19 expression in very low levels [35]. It is important to evaluate CD19 expression in patients by a method with high sensitivity. FCM can give timely and reliable information, with detection limit of $10^{-5}$ [36]. These suggest that FCM be a reliable method in the assessment of CD19 expression in DLBCL. Therefore, for prior CD19 CART infusion evaluation, FCM can be used as a complimentary detection method.

Collectively, these fundamental insight of "CD19-negative" enhance the understating of CD19-negative DLBCL patients responding to CD19 CART therapy. Based on the findings, we suggest that lymphoma patients perform both IHC and FCM for accurate CD19 expression measurement before CD19 CART therapy.

## Abbreviations

DLBCL: Diffuse large B-cell lymphoma; NHL: non-Hodgkin lymphoma; IHC: immunohistochemistry; r/r DLBCL: refractory or relapsed DLBCL; CAR: Chimeric antigen receptor; FCM: Flow cytometry; RT-PCR: Reverse Transcription Polymerase Chain Reaction; uIHC: Micro-immunohistochemistry; FFPE: formalin-fixed and paraffin-embedded; scFv: single-chain variable Fragment.

## Declarations

# Ethics approval and consent to participate

The use of remnants of archived diagnostic tissues for manufacturing of IHC and FCM was approved by the institutional review boards of Naval Military Medical University affiliated Changhai Hospital. All work has been carried out in compliance with the Helsinki Declaration. Informed consents were obtained from all patients in accordance with requirements of the Declaration of Helsinki.

# Consent for publication

Not applicable.

# Conflict of interest statement

The authors have declared that no conflict of interest exists.

# Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

# Funding

This work was support by National Natural Science Foundation of China (NSFC 81770209) and National Science and Technology Major Project of China (No. SQ2017ZX090304007002).

# Authors' contributions

Jianmin Yang and Miaoxia He designed the study. Tao Wang conducted the research and prepared the manuscript. Lili Xu acquired and analyzed the data. Yang Wang and Gusheng Tang provided reagents and analyzed data. Lei Gao, Li Chen, Jie Chen, Wenqin Yue and Weijia Fu recruited the patients for the study.

# Acknowledgements

We are grateful to Yujie Wang for excellent samples preparation.

## References

1. Zelenetz AD, Gordon LI, Abramson JS, Advani RH, Bartlett NL, Caimi PF, et al. NCCN Guidelines Insights: B-Cell Lymphomas, Version 3.2019. *J Natl Compr Canc Netw*. 2019;17(6):650-661.
2. Quintanilla-Martinez L. The 2016 updated WHO classification of lymphoid neoplasias. *Hematol Oncol*. 2017;35 Suppl 1(37-45.
3. Pfreundschuh M, Schubert J, Ziepert M, Schmits R, Mohren M, Lengfelder E, et al. Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60). *Lancet Oncol*. 2008;9(2):105-116.
4. Cunningham D, Hawkes EA, Jack A, Qian W, Smith P, Mouncey P, et al. Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisolone in patients with newly diagnosed diffuse large B-cell non-Hodgkin lymphoma: a phase 3 comparison of dose intensification with 14-day versus 21-day cycles. *Lancet*. 2013;381(9880):1817-1826.
5. Coiffier B, Thieblemont C, Van Den Neste E, Lepeu G, Plantier I, Castaigne S, et al. Long-term outcome of patients in the LNH-98.5 trial, the first randomized study comparing rituximab-CHOP to standard CHOP chemotherapy in DLBCL patients: a study by the Groupe d'Etudes des Lymphomes de l'Adulte. *Blood*. 2010;116(12):2040-2045.
6. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346(4):235-242.
7. Crump M, Neelapu SS, Farooq U, Van Den Neste E, Kuruvilla J, Westin J, et al. Outcomes in refractory diffuse large B-cell lymphoma: results from the international SCHOLAR-1 study. *Blood*. 2017;130(16):1800-1808.

8. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med*. 2013;368(16):1509-1518.
9. Laetsch TW, Myers GD, Baruchel A, Dietz AC, Pulsipher MA, Bittencourt H, et al. Patient-reported quality of life after tisagenlecleucel infusion in children and young adults with relapsed or refractory B-cell acute lymphoblastic leukaemia: a global, single-arm, phase 2 trial. *Lancet Oncol*. 2019;20(12):1710-1718.
10. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet*. 2015;385(9967):517-528.
11. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371(16):1507-1517.
12. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med*. 2018;378(5):439-448.
13. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med*. 2018;378(5):449-459.
14. Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol*. 2019;20(1):31-42.
15. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med*. 2017;377(26):2531-2544.
16. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. *N Engl J Med*. 2019;380(1):45-56.
17. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak Ö, et al. Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas. *N Engl J Med*. 2017;377(26):2545-2554.
18. Bouchkouj N, Kasamon YL, de Claro RA, George B, Lin X, Lee S, et al. FDA Approval Summary: Axicabtagene Ciloleucel for Relapsed or Refractory Large B-cell Lymphoma. *Clin Cancer Res*. 2019;25(6):1702-1708.
19. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-174.
20. Jacobson CA. CD19 Chimeric Antigen Receptor Therapy for Refractory Aggressive B-Cell Lymphoma. *J Clin Oncol*. 2019;37(4):328-335.
21. Wang M, Munoz J, Goy A, Locke FL, Jacobson CA, Hill BT, et al. KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma. *N Engl J Med*. 2020;382(14):1331-1342.
22. He M, Chen K, Li S, Zhang S, Zheng J, Hu X, et al. Clinical Significance of "Double-hit" and "Double-protein" expression in Primary Gastric B-cell Lymphomas. *J Cancer*. 2016;7(10):1215-1225.
23. Ramos-Vara JA. Technical aspects of immunohistochemistry. *Vet Pathol*. 2005;42(4):405-426.

24. Mason JT, O'Leary TJ. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J Histochem Cytochem*. 1991;39(2):225-229.
25. Montero C. The antigen-antibody reaction in immunohistochemistry. *J Histochem Cytochem*. 2003;51(1):1-4.
26. Sharp PA. Split genes and RNA splicing. *Cell*. 1994;77(6):805-815.
27. Klesmith JR, Wu L, Lobb RR, Rennert PD, Hackel BJ. Fine Epitope Mapping of the CD19 Extracellular Domain Promotes Design. *Biochemistry*. 2019;58(48):4869-4881.
28. Zhang B, Karrison T, Rowley DA, Schreiber H. IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J Clin Invest*. 2008;118(4):1398-1404.
29. Ross SL, Sherman M, McElroy PL, Lofgren JA, Moody G, Baeuerle PA, et al. Bispecific T cell engager (BiTE®) antibody constructs can mediate bystander tumor cell killing. 2017;12(8):e0183390.
30. Lanitis E, Poussin M, Hagemann IS, Coukos G, Sandaltzopoulos R, Scholler N, et al. Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor. *Mol Ther*. 2012;20(3):633-643.
31. Dotti G, Gottschalk S, Savoldo B, Brenner MK. Design and development of therapies using chimeric antigen receptor-expressing T cells. *Immunol Rev*. 2014;257(1):107-126.
32. Debliquis A, Baseggio L, Bouyer S, Guy J, Garnache-Ottou F, Genevieve F, et al. Multicentric MFI30 study: Standardization of flow cytometry analysis of CD30 expression in non-Hodgkin lymphoma. 2020.
33. Orfao A, Matarraz S, Pérez-Andrés M, Almeida J, Teodosio C, Berkowska MA, et al. Immunophenotypic dissection of normal hematopoiesis. *J Immunol Methods*. 2019;475(112684.
34. Gaidano V, Tenace V, Santoro N, Varvello S, Cignetti A. A Clinically Applicable Approach to the Classification of B-Cell Non-Hodgkin Lymphomas with Flow Cytometry and Machine Learning. 2020;12(6).
35. Pillai V, Muralidharan K, Meng W, Bagashev A, Oldridge DA, Rosenthal J, et al. CAR T-cell therapy is effective for CD19-dim B-lymphoblastic leukemia but is impacted by prior blinatumomab therapy. *Blood Adv*. 2019;3(22):3539-3549.
36. Cheminant M, Derrieux C, Touzart A, Schmit S, Grenier A, Trinquand A, et al. Minimal residual disease monitoring by 8-color flow cytometry in mantle cell lymphoma: an EU-MCL and LYSA study. *Haematologica*. 2016;101(3):336-345.

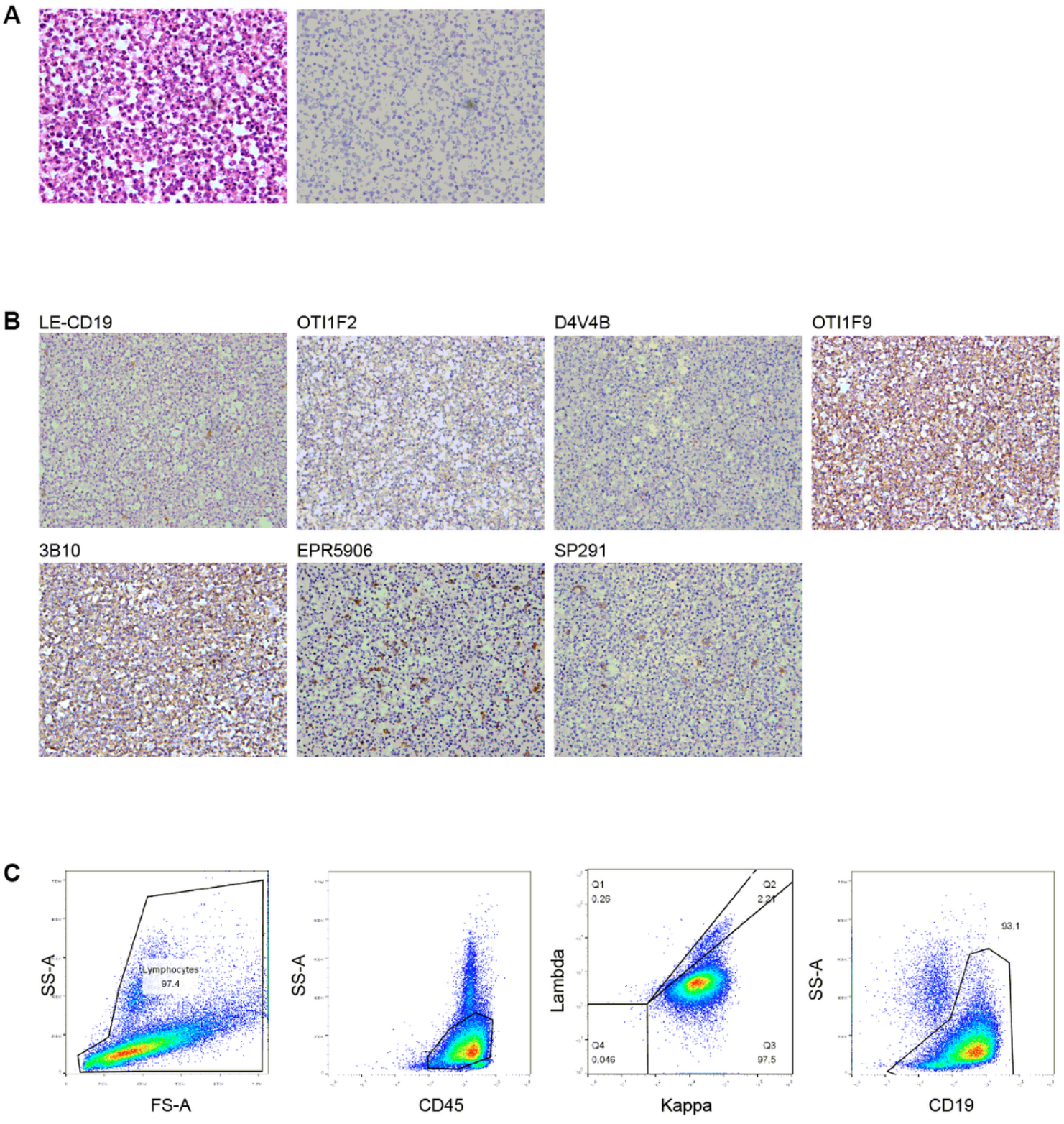
# Table

**Table 1**. DLBCL Patients CD19 IHC and response to CART therapy.

| No. | Age | Gender | Prior treatments, No. | Status prior CART | CART cell dose (× $10^6$/Kg) | CD19 IHC with 7 antibodies | Best response | Duration of response (months) | Survival (months) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 69 | M | 9 | PD | 6.3 |  | PD | 1 | 1 |
| 2 | 58 | M | 4 | PD | 4.8 |  | PD | 1 | 12 |
| 3 | 27 | M | 5 | PD | 1.72 |  | PR | 29 | 29 |
| 4 | 64 | M | 12 | R | 0.8 |  | PR | 7 | 10 |
| 5 | 68 | M | 6 | SD | 2.86 |  | CR | 38 | 38 |
| 6 | 56 | F | 8 | PD | 2.76 |  | PR | 33 | 33 |
| 7 | 44 | M | 5 | PD | 6.32 |  | PR | 4 | 12 |
| 8 | 19 | M | 10 | PD | 6.32 |  | CR | 19 | 25 |

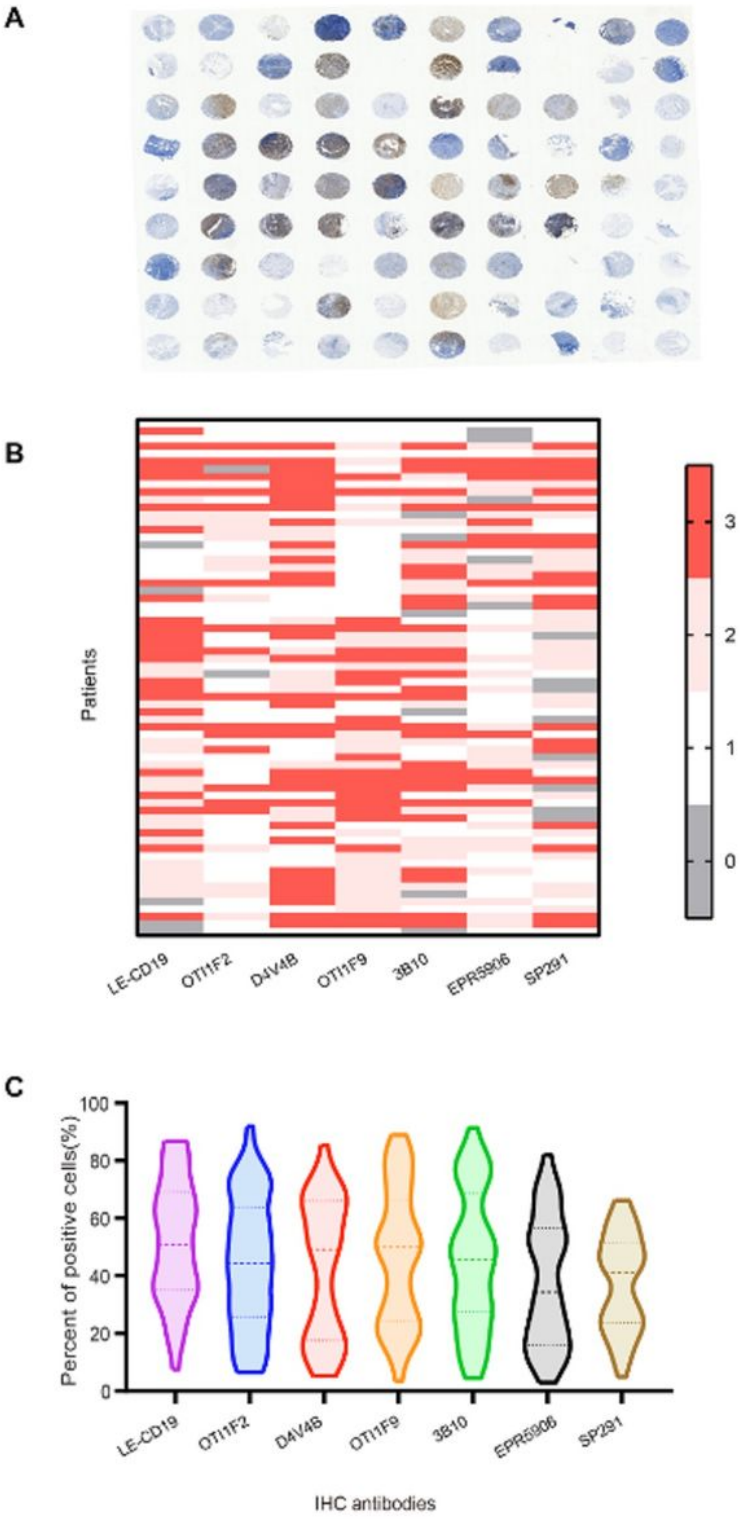
Abbreviations: PD, progressive disease; R, relapse; SD, stable disease; CR, complete remission.

# Figures




## Figure 1

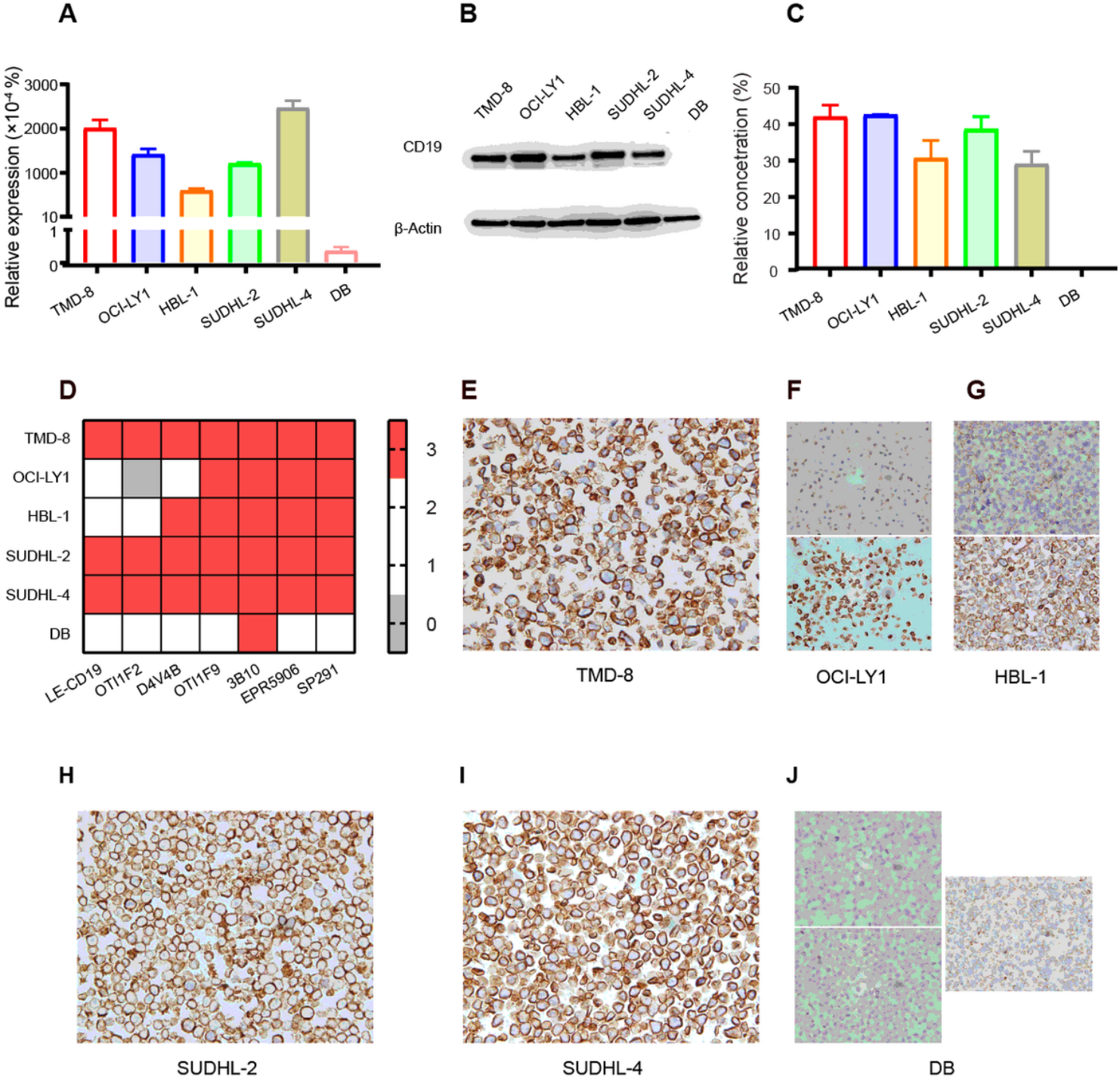
CD19 expression and CART response in diffuse large B-cell lymphoma (DLBCL) sample. A. One DLBCL patient showed CD19 negative at initial diagnosis by immunohistochemistry (IHC) but obtained complete remission in CART treatment. B. IHC with antibodies targeting different clones in the DLBCL sample. C. Flow cytometry (FCM) assay presented this patient was CD19 positive.




## Figure 2

Evaluation of CD19 expression by micro-IHC (uIHC) using 7 different antibodies in DLBCL samples. A. Tissue microarray stained by uIHC. B. Heat map of CD19 expression was detected by uIHC (n=68). Score 0 represents tissue loss, 1 represented CD19 negative, 2 and 3represeted CD19 low and high positive CD19 expression, respectively. C. Percentage of positive cells in each sample. The percentages of positive cells samples were detected by different antibodies (P<0.0001). Antibody LE-CD19 showed the highest

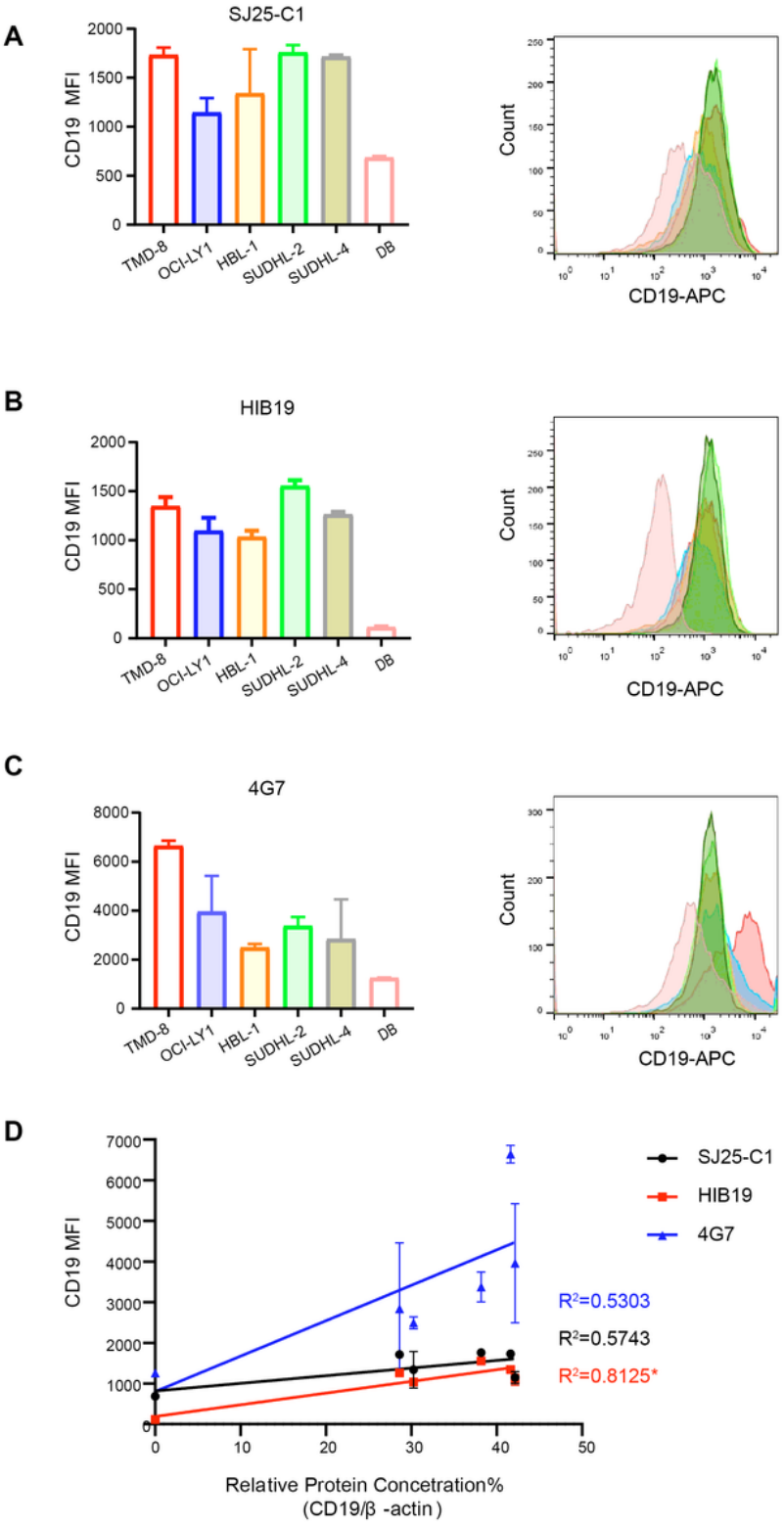
percent of CD19 positive cells, OTI1F2, D4V4B, OTI1F9 and 3B10 showed the moderate percent of CD19 positive cells and antibodies EPR5906 and SP291 presented the lowest percent of CD19 positive cells (P<0.05). The data was tested by the general linear model and Student-Newman-Keuls test.




## Figure 3

CD19 mRNA and CD19 expression level by RT-PCR, Western blotting and IHC analysis in cell lines of TMD-8, OCI-LY1, HBL-1, SUDHL-2, SUDHL-4 and DB. A. CD19 mRNA levels in TMD-8 (0.201%±0.019%), OCI-LY1 (0.142%±0.012%), HBL-1 (0.0605±0.004%), SUDHL-2 (0.121%±0.002%), SUDHL-4 (0.246% ±0.016%) and DB (3.65×10-5%±1.195×10-5%). Data were normalized to GAPDH mRNA levels. B. The

CD19 protein band was revealed by Western Blotting analysis. C. Quantification of CD19 protein level in TMD-8 (41.6235%±2.2582%), OCI-LY1 (42.1468%±0.0683%), HBL-1 (30.2584%±3.4691%), SUDHL-2 (38.1858%±2.4609%), SUDHL-4 (28.6253%±2.5132%) and DB (0). D. Heat map of CD19 expression in 6 cell lines using IHC with 7 antibodies targeting different clones. Score 0 represents tissue losing, 1 represents CD19 negative, 2 and 3 implied CD19 low-and high-positive CD19 expression. D. TMD-8 shows high-positive CD19 expression with 7 different antibodies. This image was stained by antibody 3B10. E. OCI-LY1 shows CD19 negative expression when detected by antibody LE-CD19 (up panel) and D4V4B, but showed a high-positive CD19 expression with detection of 3B10 (down panel) and OTI1F9, EPR5906 and SP291. F. HBL-1 shows CD19 low-positive expression of CD19 detected by antibodies OTI1F2 and LE-CD19 (up panel), high-positive expression of CD19 detected by antibodies OTI1F9, EPR5906, SP291 and D4V4B, 3B10 (down panel). G. SUDHL-2 shows high-positive CD19 expression with 7 different antibodies. The image was stained by antibody 3B10. H. SUDHL-4 displays high-positive CD19 expression with 7 different antibodies. The image was stained by antibody 3B10. I. DB shows negative CD19 expression detected by antibodies OTI1F2 D4V4B, EPR5906 and SP291, OTI1F9 (left up panel), a low-positive CD19 expression detected by antibodies LE-CD19 (left down panel) and a high-positive CD19 expression detected by antibodies 3B10 (right panel).




## Figure 4

Flow cytometry analysis of 6 cell lines CD19 expression. A. CD19 mean fluorescence intensity of 6 cell lines detected by antibody Sj25-C1 (left panel) and a histogram of the 6 cell lines detected by Flow cytometry (right panel). B. CD19 mean fluorescence intensity of 6 cell lines detected by antibody HIB19 (left panel) and a histogram of 6 cell lines detected by Flow cytometry (right panel). C. CD19 mean fluorescence intensity of 6 cell lines detected by antibody 4G7 (left panel) and a histogram of 6 cell lines

detected by Flow cytometry (right panel). D. Correlations between the CD19-MFI of 3 antibodies and CD19 protein level of 6 cell lines. Antibody HIB shows a strong correlation with the CD19 protein level (P=0.0137).Spearman’s rank correlation coefficients were used in the data analysis.