LETTER TO THE EDITOR

Homogeneously high expression of CD32b makes it a potential target for CAR-T therapy for chronic lymphocytic leukemia

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Abstract

CD19 chimeric antigen receptor (CAR)-T cells have been used to treat patients with refractory chronic lymphocytic leukemia (CLL). However, approximately 50% of patients do not respond to this therapy. To improve the clinical outcome of these patients, it is necessary to develop strategies with other optimal targets to enable secondary or combinational CAR-T cell therapy. By screening a panel of surface antigens, we found that CD32b (FcγRIIb) was homogeneously expressed at high site density on tumor cells from CLL patients. We then developed a second-generation CAR construct targeting CD32b, and T cells transduced with the CD32 CAR efficiently eliminated the CD32b+ Raji leukemic cell line in vitro and in a mouse xenograft model. Furthermore, CD32b CAR-T cells showed cytotoxicity against primary human CLL cells that were cultured in vitro or transplanted into immunodeficient mice. The efficacy of CD32b CAR T cells correlated with the CD32b density on CLL cells. CD32b is not significantly expressed by non-B hematopoietic cells. Our study thus identifies CD32b as a potential target of CAR-T cell therapy for CLL, although further modification of the CAR construct with a safety mechanism may be required to minimize off-target toxicity.

Keywords: Chronic lymphocytic leukemia, CD32b, Chimeric antigen receptor, Antigen site density

To the editor:

Chronic lymphocytic leukemia (CLL) is a hematological neoplasm mostly diagnosed in the elderly. Refractory and relapsed (r/r) CLL patients have a poor prognosis with limited therapeutic options [1, 2]. Chimeric antigen receptor (CAR)-T cells targeting CD19 have shown activity in CLL, but can only induce complete remission in about 30%-60% of the patients [3, 4]. It is essential to develop alternative targets for secondary or combinational CAR-T cell therapies for CLL.

Since target antigen site density and expression percentage on tumor cells are critical determinants of CAR-T cell efficacy [5], we aimed to identify a target antigen that was expressed at high levels on all CLL cells. The expression levels of B cell-associated antigens (CD19/CD20/CD22/CD32) and 3 previously suggested targets (CD23/ROR1/FcμR) were examined on leukemic cells from CLL patients (Additional file 1: Table S1). CD32 (FCGR2) was expressed on 100% CD5+CD19+ CLL cells from all patients, similar to CD19 (Fig. 1a, Additional
CD32b was not significantly expressed on hematopoietic stem/progenitor cells and most mature blood cells, but was expressed in a small proportion of dendritic cells (Fig. 1g–h).

Second-generation CAR constructs with scFv derived from the CD32b-specific antibodies 2B6 and NOV2108 were developed (Fig. 2a, b, Additional file 2). Since the CLL cell line MEC1 only partially expressed CD32, we used the Raji cell line, which had homogeneous CD32b expression, to evaluate the activity of CD32b CAR-T cells (Additional file 3: Fig. S1a). CD32b was not significantly expressed on hematopoietic stem/progenitor cells and most mature blood cells, but was expressed in a small proportion of dendritic cells (Fig. 1g–h).

In vitro cytotoxicity of 2B6bbz to primary CLL cells was higher than that of 2108bbz (Additional file 3: Fig. S3b). 2B6bbz T cells displayed similar anti-CLL cytotoxicity with CD19 CAR-T cells when the expression of CD19 and CD32 in leukemia was similar, and 2B6bbz was superior to CD19 CAR-T cells when the expression of CD32 in leukemia was higher than CD19 (Fig. 2j). Moreover, cytotoxicity of 2B6bbz T cells positively correlated with CD32 density across different samples (Fig. 2k).

The in vivo anti-CLL activity of 2B6bbz T cells was assessed in NSG mice transplanted with patient samples (Fig. 2l). 2B6bbz T cells were as potent as CD19 CAR-T cells: they achieved complete clearance of CLL in 80% (8/10) of mice and showed robust proliferation in most mice (Fig. 2m-n, Additional file 3: Fig. S4b, c). Loss of CD32b expression was not observed (Additional file 3: Fig. S4d). Due to the limited persistence of CLL in mice, we could not evaluate whether 2B6bbz T cells could provide a long-term cure effect. Since this model has been widely used to evaluate the in vivo efficacy of new drugs in CLL [7], our results indicate that CD32b CAR-T cells have potent cytotoxicity against CLL cells in vivo.

CD32b CAR-T cells may cause B cell aplasia, which can be managed with immunoglobulin infusion. Previous reports have shown the expression of CD32b in some normal tissues and cells, including airway smooth muscle cells, liver sinusoidal endothelial cells, Kupffer cells and placenta [8, 9], which may cause potential off-target toxicities of CD32b CAR-T cell therapy. However, CD32b may still be an applicable target, since the potential off-target toxicity could be alleviated by decreasing CAR affinity for antigen or adopting a syn-Notch or zipper safety gate, which has been validated in various CAR-T cell studies [10–12]. Therefore, it would be feasible to improve the safety of CD32b CAR-T cells based on these modifications.

In summary, our study identifies CD32b as an antigen that is homogeneously expressed at high levels on CLL cells. CD32b CAR-T cells showed killing efficacy against primary CLL cells in vitro and in vivo. CD32b is therefore a promising target for CAR therapy in CLL, although further evaluation of off-target toxicities and optimization with safety modifications are needed before conducting clinical trials.

(See figure on next page.)

**Fig. 1** CD32b is homogeneously expressed at high level on primary CLL cells, but not significantly expressed on non-B hematopoietic cells. a Expression (% positive) of CD32 (n = 41), CD19 (n = 41), CD20 (n = 33), CD22 (n = 29), CD23 (n = 29), ROR1 (n = 22) and FcμR (n = 22) in CLL samples from CLL patients (Additional file 1: Table S1). b Evaluation for site density of CD32 and other antigens in CLL patients (sample size was the same as a) using Quantibrite-PE beads. c Transcriptional profile of FcγR2a, FcγR2b and FcγR2c from 2 CLL samples and Raji cell line by RNA sequencing. d Flow cytometric analysis of surface expression of CD32, CD32b and CD19 in 7 CLL patients. e Expression (% positive) of CD32, CD32b and CD19 in CLL patients (n = 7). f Site density comparison among CD32b, CD32 and CD19 in CLL patients (n = 7). g–h Data in e–f belong to Pt 42–48, and the expression of CD32 and CD19 on samples from Pt 42–48 is not included in a–b. h Flow cytometric analysis of surface expression of CD32 on peripheral blood cells and HSPCs (CD34* CD38− HSCs and CD34+ CD38+ HPCs) from a healthy donor. i Flow cytometric analysis of CD32b expression on normal peripheral blood cells and HSPCs from a healthy donor. FPKM: expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced. HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; NK, natural killer; DC, dendritic cells. Data were representative of two independent experiments. Unpaired two-tailed Student’s t test was used for statistical analyses in a, b, paired two-tailed Student’s t test was used in e and f (**P < 0.01, ***P < 0.001)
Fig. 1 (See legend on previous page.)
### Fig. 2 (See legend on next page.)

#### a

| 5'LTR | VL | VH | CD6aTM | 4-1BB | CD3ζ | 3'LTR |
|-------|----|----|--------|-------|------|-------|
| 2B6 scFv | NOV2108 | scFv | 2B6bbz | 2B6bbz | M1-CD4+ CAR-T | M2-CD4+ CAR-T |
|       |       |      |        |       | M1-CD8+ CAR-T | M2-CD8+ CAR-T |
|       |       |      |        |       | M3-CD4+ CAR-T | M3-CD8+ CAR-T |
|       |       |      |        |       | PCDH M3-CD4+ CAR-T | PCDH M3-CD8+ CAR-T |

#### b

| E:T | % specific lysis |
|-----|------------------|
| 1:15 | 20 |
| 1:11 | 40 |
| 1:2.5 | 60 |
| 1:5 | 80 |

#### c

- **2B6bbz**
  - **CD32**: 50617.68
  - **CD19**: 11425.91

- **PCDH**
  - **CD32**: 26585.37
  - **CD19**: 24202.55

#### d

- **day-5 Raji cells IV**
- **day0 BLI**
- **day0 CAR-T cells IV**
- **Weekly BLI**
- **Monitor Survival**
- **day 7**
- **day 14**
- **day 42**

#### e

- **Luminescence**
  - **Color Scale**: Min = 1.00e5, Max = 5.00e6
  - **Radiance (p/sec/cm²/sr)**: Min = 1.30e3, Max = 1.00e4

#### f

- **Day 0**
- **CD32b**
- **Ig-like C2-type1**
- **NOV2108**
- **ITIM**

#### g

- **Fig. 2 (continued)**

#### h

- **% percent suvival**
  - **CD32b**
  - **Ig-like C2-type2**

#### i

- **Radiance**
  - **Color Scale**: Min = 1.00e5, Max = 5.00e6

#### j

- **P<0.001**

#### k

- **% specific lysis**
  - **Site density (×10⁴)**

#### l

- **Day 0**
- **Analysis of Leukemia and CAR-T in PB**

#### m

- **Number of mice**
- **CC**
- **NC**

#### n

- **Recepto**
- **CD19 CAR-T**
- **2B6bbz**

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**Legend:**
- **BM**: Bone Marrow
- **Spleen**
- **Liver**
- **PB**
**Fig. 2.** CD32b CAR-T efficacy against Raji cells and primary CLL cells. a Diagram indicating constructions of two CD32b CAR sequences (scFvs from clone 2B6 or NOV2108). b 2018 Nov scFv binds Ig-like C2-type 1 domain of CD32b, whereas 2B6 binds binding domain of CD32b. c Cytotoxicity of CD32b CAR-T targeting Raji cells after incubation for 36 h at the indicated effector-to-target (E:T) ratios; control T cells were used as negative controls. d Schematic of the Raji xenograft model. NSG mice were injected with tail vein with 3 x 10^6 luciferase^+ Raji cells on day-5. Bioluminescent imaging was performed on day 0 to quantify engraftment and then weekly measured. Control T cells or 2B6bbz T cells (1 x 10^6) were injected IV on day 0. e Representative bioluminescent imaging at day 0, 7, 14 and 42 after injection of Raji cells. f Flow cytometric analysis of Raji cells in peripheral blood from Raji-NSG mice (from e). g Bioluminescent signal for each treatment group over time. Data represent mean values of each group ±SD. h Log-rank survival curve was used for survival analysis of Raji xenograft mice treated by 2B6bbz or control T cells. Data of g and h were summarized from 4 independent experiments. (Control, n = 12; 2B6bbz, n = 14). i Flow cytometric analysis of CAR-T cells in peripheral blood from Raji-NSG mice (from e). j Specific cytotoxicity targeting of CLL by 2B6bbz and CD19 CAR-T cells after incubation with primary CLL cells for 36 h at the indicated E:T ratios; Three representative CLL patient examples are shown. k Correlation between 2B6bbz T cytotoxicity and CD32 density across different patient CLL samples. l Schematic of the primary CLL xenograft model. NSG mice were sublethally irradiated (150 cGy) on day -3 and injected with 2-4 x 10^7 CLL PBMCs via the tail vein on day -3. Engraftment was confirmed by flow cytometry in PB around day 0. Mice were then injected with 5 x 10^6 2B6bbz T, CD19 CAR-T cells or control T cells via the tail vein and bled weekly to quantify CLL burden. m Response of primary CLL-NSG mice treated with 2B6bbz T (CC, n = 8; NC, n = 2), CD19 CAR-T (CC, n = 4; NC, n = 6) or control T cells (NC, n = 10). n Number of CAR-T tumoricidal responses in BM, BM, liver, spleen and lung from CD32b-NSG mice; Data from CLL-NSG mice. Numbers indicate tumor cells (n = 10). o Log-rank (Mantel–Cox) test was used for statistical analysis in m. p Pearson correlation analysis was used in k. (*P < 0.05, **P < 0.01, ***P < 0.001)

**Supplementary Information**

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**Abbreviations**

CR: Chronic lymphocytic leukemia; CR: Complete response; r/r: Refractory and relapsed; FcγRIIb: Low affinity immune receptor for CD32; NC: Not clearance; HSC: Hematopoietic stem cell; HPC: Hematopoietic progenitor cell; BM: Bone marrow; NK: Natural killer; DC: Dendritic cells.

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**Authors’ contributions**

GW, XS and XF designed the study; GW and XS performed most of the experiments and analyzed the data; SZ, CL, YM and ML helped with the plasmid construction and animal experiments; QN, ZF, XY, YJ and DZ helped with the basic lentivirus and plasmid and CAR-T generation system; YX, SW, CH, HH and EJ helped with the collection of patient samples; JP and QD helped with the study design and provided clinical consultation; XS, GW and XF wrote the manuscript; QD, JP and XF jointly directed and supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials
All data needed to evaluate the conclusions in the paper are present in the paper or the additional files.

Declarations

Ethics approval and consent to participate
The studies were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College (approval number: KT2020005-EC-3).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Barr PM, Munir T, Brown JR, O’Brien SM, Barrientos JC, Reddy NM, et al. Final analysis from RESONATE: six-year follow-up in patients (pts) with previously treated chronic lymphocytic leukemia or small lymphocytic lymphoma (CLL/SLL) on ibrutinib. J Clin Oncol. 2019; 37(15).
2. Stilgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Dohner K, et al. Gene mutations and treatment outcome in chronic lymphocytic leuke- mia: results from the CLL8 trial. Blood. 2014;123(21):3247–54.
3. Siddiqi T, Soumerai JD, Dorrinie KA, Stephens DM, Riedell PA, Arnason JE, et al. Updated follow-up of patients with relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma treated with lisoctabtagene maraleucel in the phase 1 monotherapy cohort of transcen CLL 004, including high-risk and ibrutinib-treated patients. Blood. 2020;136(Supplement 1):40–1.
4. Frey NV, Gill S, Heeney EO, Schuster S, Nasta L, Loren A, et al. Long-term outcomes from a randomized dose optimization study of chimeric anti- gen receptor modified T cells in relapsed chronic lymphocytic leukemia. J Clin Oncol. 2020;38(25):2862–71.
5. Ramakrishna S, Highfill SL, Walsh Z, Nguyen SM, Lei H, Sherr JF, et al. Modulation of target antigen density improves CAR T-cell functionality and persistence. Clin Cancer Res. 2019;25(17):5329–41.
6. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fc receptor and their polymor- phic variants for human IgG subclasses. Blood. 2009;113(16):3716–25.
7. Hermann SEM, Montraveta A, Niemann CU, Mora-Jensen H, Gulrajani M, Kranz F, et al. The Bruton Tyrosine Kinase (BTK) inhibitor acalabrutinib demonstrates potent on-target effects and efficacy in two mouse models of chronic lymphocytic leukemia. Clin Cancer Res. 2017;23(11):2831–41.
8. Xia YC, Schuliga M, Shepherd M, Powell M, Harris T, Langenbach SY, et al. Functional expression of IgG-Fc receptors in human airway smooth muscle cells. Am J Respir Cell Mol Biol. 2011;44(5):665–72.
9. Bruggerman CW, Houtzager J, Dierdorf B, Kers J, Pals ST, Lutter R, et al. Tissue-specific expression of IgG receptors by human macrophages ex vivo. PLoS ONE. 2019;14(10):e0223264.
10. Drent E, Thelemi M, Poels R, de Jong-Korlaar R, Yuan H, de Buijn J, et al. A rational strategy for reducing on-target off-tumor effects of CD38-chimeric antigen receptors by affinity optimization. Mol Ther. 2017;25(8):1946–58.
11. Morsut L, Roybal KT, Xiong X, Girdley RA, Coyle SM, Thomson M, et al. Engineering customized cell sensing and response behaviors using synthetic notch receptors. Cell. 2016;164(4):780–91.
12. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell. 2018;173(6):1426-1438.e11.