



Immunotherapy

CD133-directed CAR T-cells for MLL leukemia: on-target, off-tumor myeloablative toxicity

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To the Editor:

Chimeric antigen receptors (CARs) have undoubtedly revolutionized immunotherapy, especially in the B-cell acute lymphoblastic leukemia (ALL) arena where over 80% of complete remissions are observed in refractory/relapsed (R/R) B-cell ALL patients treated with CD19-directed CAR T-cells (CARTs) [1]. However, despite holding an unprecedented promise, several issues still have to be resolved before CARTs can be expanded to novel targets and/or malignancies or even provided as first-line treatment in B-cell ALL [2]. For instance, toxicities such as cytokine release syndrome and immune escape mechanisms including loss of the antigen under CART-mediated pressure remain major concerns, urging further research on the mechanisms underlying CARTs cytotoxicity.

In this sense, loss of CD19 antigen is frequently observed after CD19-directed CARTs therapy in B-cell ALL [3, 4], but is particularly common in MLL-rearranged (MLLr) B-cell ALL, an aggressive subtype of B-cell ALL (dismal in MLL-AF4+ infants) associated with lymphoid-to-myeloid lineage switch [3, 5, 6]. We read with interest the work recently published in *Leukemia* by Li et al. reporting a novel CAR targeting both CD19 and CD133 [7]. This study proposes to use a bi-specific CAR targeting both CD19 and CD133 antigens in a Boolean OR-gate approach for MLLr B-cell ALL as a strategy to avoid and treat CD19- relapses. The authors reasoned that CD133, encoded by *PROM1* gene, is a specific marker for MLLr leukemia because *PROM1* is an MLL target, especially in MLL-AF4 B-cell ALL [8–10]. They went on and performed in vitro assays showing that CD19/CD133 bi-specific CAR triggers robust cytotoxicity against CD19 + CD133 + and CD19-CD133+ B-cell lines [7], thus suggesting it may help in reducing subsequent lineage switch in MLLr B-cell ALL.

A major drawback for CD133 as target in immunotherapy is its expression in hematopoietic stem and progenitor cells (HSPCs), which would likely exert “on-target off-tumor” myeloablative, life-threatening toxicity [11, 12]. Because B-cell ALL is molecularly heterogeneous and can be diagnosed during infancy, childhood and adulthood, we have characterized *PROM1*/CD133 expression in a large cohort of cytogenetically distinct B-cell ALL subgroups ($n = 212$ patients) as well as in different subpopulations of normal CD34+ HSPCs obtained across hematopoietic ontogeny from 22-weeks old human fetal liver (FL, prenatal), cord blood (CB, perinatal), and adult G-CSF-mobilized peripheral blood/bone marrow (PB/BM, postnatal). An initial analysis of publicly available RNA-seq data [13] from 170 diagnostic B-cell ALL patients confirmed that *PROM1* is overexpressed in patients with MLLr B-cell ALL, although its expression is not significantly higher than in other cytogenetic

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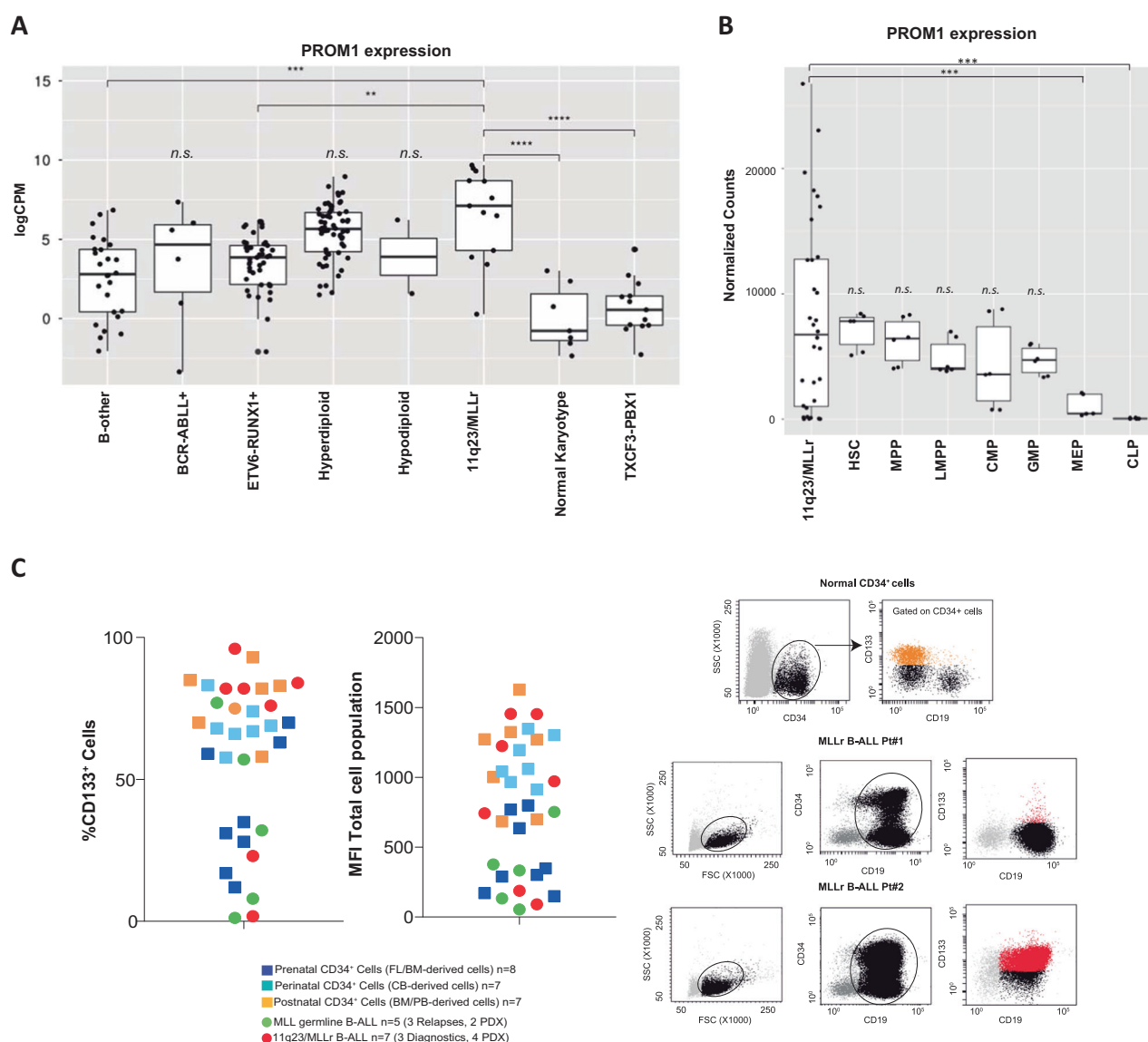


Fig. 1 Characterization of CD133/*PROM1* expression in B-cell ALL and normal HSPCs. **a** Expression level of *PROM1* in the indicated cytogenetic subgroups of B-cell ALL ($n = 170$ patients at diagnosis) determined by RNA-seq represented in log₂(CPM) scale, with CPM = counts per million [13]. **b** RNA-seq analysis comparing the expression of *PROM1* in 11q23/MLLr B-cell ALL ($n = 29$ patients) with that in distinct fractions of Lin-CD34⁺ + CD38-CD19- non-lymphoid normal HSPCs (HSC hematopoietic stem cells, MPP multipotent progenitors, LMPP lymphoid-primed multipotent progenitors, CMP common myeloid progenitors, GMP granulocyte-monocyte progenitor, MEP megakaryocyte-erythroid progenitors) and in

common lymphoid progenitors (CLP) [14]. Data shown as normalized counts. The boxes define the first and third quartiles. The horizontal line within the box represents the median. **c** Frequency (left) and mean fluorescence intensity (MFI, middle) of CD133⁺ BM blasts/cells in MLLr ($n = 7$) and non-MLL B-cell ALLs ($n = 5$) primary diagnostic/relapse samples or primografts (PDXs), and normal CD34⁺ HSPCs derived from FL ($n = 8$), CB ($n = 7$) and adult PB/BM ($n = 7$). Representative FACS dot plots for CD133 in normal CD34⁺ HSPCs (upper right) and BM samples from two independent MLLr B-cell ALL patients (bottom right)

subgroups (Fig. 1a). We then analyzed *PROM1* during HSPC development and observed that *PROM1* is highly expressed in early normal hematopoietic stem cells (HSC) and multipotent progenitors (MPP) with its expression decreasing from the lymphoid-primed multipotent progenitors (LMPP) onwards with its expression being marginal at later stages of myeloid differentiation (megakaryocyte-erythroid progenitors, MEP) and

common lymphoid progenitors (CLP) [14] (Fig. 1b). Importantly, 70% (22/32) of 11q23/MLLr B-cell patients (both MLL-AF4 and MLL-AF9) express equal (9/32) or lower (13/32) *PROM1* levels that HSCs and MPPs, which raises doubts about the suitability of *PROM1* as a target for B-cell ALL immunotherapy [15].

FACS clinical immunophenotyping provides a priori a more rapid and feasible clinically relevant diagnostic

information than RNA-seq during the decision-making process. Thus, we next FACS-analyzed the expression of CD133 (*PROM1* gene product) in the cell surface of BM-derived primary blasts and primografts (PDXs) obtained from 11q23/MLLr ($n = 7$) and non-MLL ($n = 5$) B-cell ALL patients, and in comparison with healthy prenatal (22 weeks old FL), perinatal (CB) and adult (PB/BM) CD34 + HSPCs (Fig. 1c). Consistent with the RNA-seq data, the expression of CD133 in 11q23/MLLr blasts is intermingled with that observed in CD34+ HSPCs across hematopoietic ontogeny (Fig. 1c).

Our data demonstrates that *PROM1*/CD133 is similarly expressed between MLLr B-cell ALL primary blasts and normal non-lymphoid HSPCs across ontogeny, thus indicating that “on-target, off-tumor” toxic/myeloablative effects are likely to occur if used in a bi-specific CAR approach where CD133 antigen will be constantly targeted regardless of the co-expression of CD19 in the same cell. Our data therefore raises concerns about using CD133 as a target for MLLr B-cell ALL immunotherapy. An alternative to circumvent HSPC toxicity would be to engineer dual CAR T-cells with one CAR engaging an antigen (i.e., CD19) mediating T-cell activation and another CAR engaging a second antigen (i.e., CD133) mediating T-cell co-stimulation [16]. Unfortunately, although such a CD19/CD133 dual CAR might be likely safe due to its cytotoxicity being restrained only to cells co-expressing CD19 and CD133, its specific cytotoxic performance will be poor since not the entire MLLr B-cell ALL blast population is CD19 + CD133+ (Fig. 1c). Another alternative approach to prevent HSPC toxicity would be to have in place a potent molecular switch (i.e., iCas9) to eliminate CAR133-expressing T-cells as necessary [17]. Further long-term in vivo studies using both primary B-cell ALL cells and normal HSCPs remain to be conducted to elucidate the efficacy versus the myeloablative toxicity of a CAR CD133 [18, 19].

Data availability

All genomic data is already publicly available. A full data availability will be provided.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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