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A novel chimeric antigen receptor T-cell therapy targeting CD84 for the treatment of acute myeloid and T-cell lymphoblastic leukemias

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Despite the remarkable clinical successes of chimeric antigen receptor (CAR) T-cell therapies in treating B-cell malignancies and multiple myeloma, similar outcomes have not been achieved in other indications. For patients with relapsed or refractory (R/R) acute myeloid leukemia (AML) or T-cell acute lymphoblastic leukemia (T-ALL), treatment options are limited, yet CART-cell therapies offer significant potential to address this unmet need. Here, we introduce a first-in-class CART-cell therapy targeting CD84, a novel antigen, for the treatment of R/R AML and T-ALL. CD84 is highly expressed on leukemic blasts, with limited expression on hematopoietic stem progenitor cells (HSPC), and is largely absent in healthy human tissues. Our second-generation CARTs targeting CD84 (CART84) demonstrate potent cytotoxicity against AML and T-ALL cells both in vitro and in vivo in patient-derived xenograft (PDX) models. Furthermore, CART84 eliminated primary leukemic blasts while exhibiting low cytotoxicity against CD34+ HSPC in vitro and in humanized mouse models in vivo, suggesting a low risk of myelotoxicity. These results support CD84 as a promising target for AML and T-ALL and provide the foundation for our upcoming first-in-human phase I/II clinical trial using CD84-directed CAR T cell therapy for patients with R/R AML and T-ALL (EudraCT 2024-519966-31-00).

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INTRODUCTION

Chimeric antigen receptor (CAR) T therapy involves genetically modifying T cells to express synthetic receptors that target antigens on malignant cells, enabling them to exert a cytotoxic effect in an HLA-independent manner [1]. CART therapies have demonstrated outstanding clinical outcomes in relapsed or refractory (R/R) B-cell malignancies and R/R multiple myeloma, leading to the approval of several products [2–7]. However, no CART therapy has yet been approved for acute myeloid leukemia (AML) or T-cell acute lymphoblastic leukemia (T-ALL).

Major challenges in AML treatment are resistance, which is observed in 10–40% of cases, and relapse after an initial response, which affects 75% of patients [8]. Similarly, ~20% of pediatric and 40% of adult T-ALL patients experience relapse, with poor long-term survival rates in R/R T-ALL cases [9]. This underscores a significant medical need where CART cells hold substantial potential. The ideal target for CART therapy should be expressed exclusively on malignant cells [10], but many antigens explored for AML, such as CD33 [11–13], CD123 [14], and CLL-1 [15, 16], are also expressed to some extent on healthy hematopoietic stem cells [17] or other tissues [18]. Clinical trials so far have reported limited efficacy and significant on-target/off-tumor toxicities [18, 19]. In T-ALL, CART therapies predominantly target lineage-specific antigens like CD1a [20, 21], CD5 [22], and CD7 [23, 24]. These approaches face significant challenges, including fratricide during CART production and T-cell aplasia as an on-target/off-tumor side effect, further complicating their clinical application.

CD84 (SLAMF5) is a homophilic cell surface glycoprotein in the SLAMF family, with two extracellular immunoglobulin-like

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domains: a membrane-distal V-set and a membrane-proximal C2-set [25, 26]. It is widely expressed on immune cells, especially monocytes, memory B and T lymphocytes, and platelets, where it plays a role in monocyte activation, T-B cell interactions, and B-cell tolerance at the germinal center checkpoint [27].

In chronic lymphocytic leukemia (CLL) [28], CD84 is overexpressed and promotes leukemic cell survival within the tumor microenvironment [29], with a similar role observed in multiple myeloma [30]. In AML, CD84 supports leukemogenesis; its silencing reduces cell viability and induces apoptosis [31]. Additionally, transcriptome and proteome studies have identified elevated CD84 levels in AML and T-ALL cell lines and patient samples [32–34].

Here, we demonstrate CD84 as a tumor antigen in AML and other hematological malignancies, identifying it as a potential pan-hematologic target. We also present promising preclinical data on a first-in-class CART-cell therapy targeting CD84, supporting its potential as a treatment for AML and T-ALL, and our planned first-in-human phase 1/2 clinical trial in these indications.

MATERIALS AND METHODS

CD84 expression assessment by flow cytometry

CD84 expression was analyzed by staining 1×10^6 cells with anti-human CD84-PE (clone CD84.1.21, Biolegend) or an isotype control, as detailed in Supplementary Methods. Data were acquired on a BD LSR Fortessa 4L or BD Canto II and analyzed with FlowJo v10.8.1 or Infinicyt (Cytognos).

CART expansion

T cells were isolated by density gradient from either PBMCs of healthy donors or from patients diagnosed with AML. Following CD3/CD28 Dynabead activation and IL-2 (50 IU/mL) stimulation, cells were transduced at an MOI of 5. CARTs were expanded in X-VIVO^{TM}15 medium supplemented with 5% human serum and 1% penicillin-streptomycin for 8–10 days.

Cytotoxicity assays

Target cells were incubated with CARTs at the indicated ratios for 24–48 h, with CARTs normalized to 40% CAR expression. Cytotoxicity was calculated as: % of live cells = 100 × (number of tumor cells with effector cells at time *X*/number of tumor cells alone at time *X*).

Xenograft mouse models

Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Barcelona and the Generalitat de Catalunya. Immunodeficient NSG mice (NOD-Cg-Prkdc^{scid}-Il2rg^{tm1Wjl}/SzJ) were purchased from Charles River and housed under pathogen-free conditions at the University of Barcelona's Animal Facility. Mice were euthanized upon meeting endpoint criteria.

AML and T-ALL xenograft model

On day zero, 1×10^5 MOLM-13 (AML) or 7×10^5 MOLT-4 (T-ALL) cells expressing GFP-ffLuc reporter were injected intravenously (i.v.) into mice. Mice were allocated into experimental groups based on tumor burden to ensure similar tumor loads. Subsequently, 3×10^6 to 5×10^6 CART84 were injected i.v. 2–13 days later (figure legends). Tumor progression was monitored by bioluminescence using the Xenogen IVIS 50 Imaging System, with imaging data analyzed using Living Image version 4.74 (PerkinElmer).

AML and T-ALL patient-derived xenograft (PDX) model

Animals were irradiated (2 Gy), and 1×10^5 AML or 7×10^5 T-ALL PDX cells were injected i.v. after 6 h. One day before the CART-cell injection, bone marrow (BM) was aspirated to equally allocate animals to experimental groups. On day eleven, 5×10^6 CART84 cells were injected i.v. Tumor progression was monitored weekly through peripheral blood (PB) and monthly through BM aspirates. Animals were examined for extramedullary tumors. AML PDX cells were derived from a case with t(11;19)(q23;p13.3) KMT2A/MLLT1 (ELN 2022 adverse), and TALL-PDX cells from a cortical (CD1a+) T-ALL case.

CD34+ humanized mouse model

CD34+ cells (1×10^5) were intratibially injected into previously irradiated (2 Gy) mice. On day 39, after confirming a >60% human CD45+ engraftment, mice were allocated to experimental groups. On day 48, 1×10^6 autologous CART84, CART19 (ARI0001/varnimcabtagene autoleucel) or untransduced (UT) T cells were i.v. injected. Myelotoxicity was monitored weekly through PB and monthly through BM aspirates.

Statistical analysis

The exact sample size and the statistical analyses (performed using GraphPad Prism 9.1.2) are detailed in each figure legend. A *T*-test was used to compare two groups, while ANOVA with Dunnett's post-test was applied for multiple comparisons involving more than two groups. Kaplan–Meier analysis was used for animal survival curves, with log-rank (Mantel-Cox) tests for group comparisons, with a corrected *p* value for 3 comparisons. A *p* value of <0.05 was considered statistically significant.

RESULTS

CD84 receptor: a novel target in hematological malignancies

Using GEPIA [33] to analyze the TCGA dataset, we found that CD84 mRNA expression is 4.2 times higher in diffuse large B-cell lymphoma (DLBCL) and 10 times higher in AML than in healthy counterparts (Fig. S1A). Additionally, CCLE data revealed high CD84 mRNA levels across various hematological cancer cell lines (Fig. S1B). On BloodSpot [35], CD84 expression is notably high in ELN 2022 [36] favorable-risk AML with inv (16)/t(16;16) and adverse-risk AML with t(v;11q23.3)/KMT2A rearrangement (Fig. S1C).

We analyzed CD84 surface expression in hematologic cell lines and primary patient samples. High CD84 expression (85.3–95.4%) was found in Burkitt lymphoma (Ramos), B-ALL (NALM-6), AML (MOLM-13 and HL-60/S4), and T-ALL (MOLT-4) cell lines, while low expression (21.7%) was observed in the AML line U937 (Fig. S2A).

We evaluated CD84 expression on primary AML blasts from 51 adult AML patients, including cases of de novo and R/R AML with various genetic abnormalities and risk profiles, notably AML with NPM1 mutation, AML-MR, and high-risk AML with TP53 (Supplementary Table 1). In TP53-mutated AML, CD84 expression was consistently high across samples, with a median of 95%. Compared with CD123 and CD33—the primary CART targets in AML—CD84 expression closely resembled that of CD33 (Fig. 1A). Notably, high CD84 expression was observed in 5 of 7 AML with TP53 cases, and both cases with MECOM rearrangement showed >95% expression. The mean normalized mean fluorescence intensity (nMFI) for CD84 was 26.30 across all AML samples (Fig. 1B). In addition, CD84 expression was confirmed in a formalin-fixed, paraffin-embedded (FFPE) biopsy from an AML patient with MECOM rearrangement (Fig. S2B).

Next, we studied CD84 expression on primary T-ALL blasts from eight adult patients and found that 6 out of 8 displayed over 95% expression (Fig. S2C). Finally, CD84 was overexpressed on leukemic cells from 17 CLL patients, corroborating previous reports [28, 29, 37] (Fig. S2D).

CD84 expression in the hematopoietic system

To study CD84 expression in the hematopoietic system, we quantified the percentage (Figs. S2E and S3A, B) and nMFI (Fig. 1C) of CD84 in various cell populations from healthy donor BM samples and PB. High CD84 expression was observed across multiple hematopoietic cell types (Fig. S2E), although nMFI levels varied significantly (Fig. 1C). While AML cells displayed moderate CD84 nMFI of 26.3 (Fig. 1B), stem cells exhibited a lower CD84 nMFI of 14.63 (Fig. 1C). Common myeloid progenitors displayed high intensity, and lymphoid progenitors showed moderate intensity, consistent with recent findings [38] (Fig. 1C).

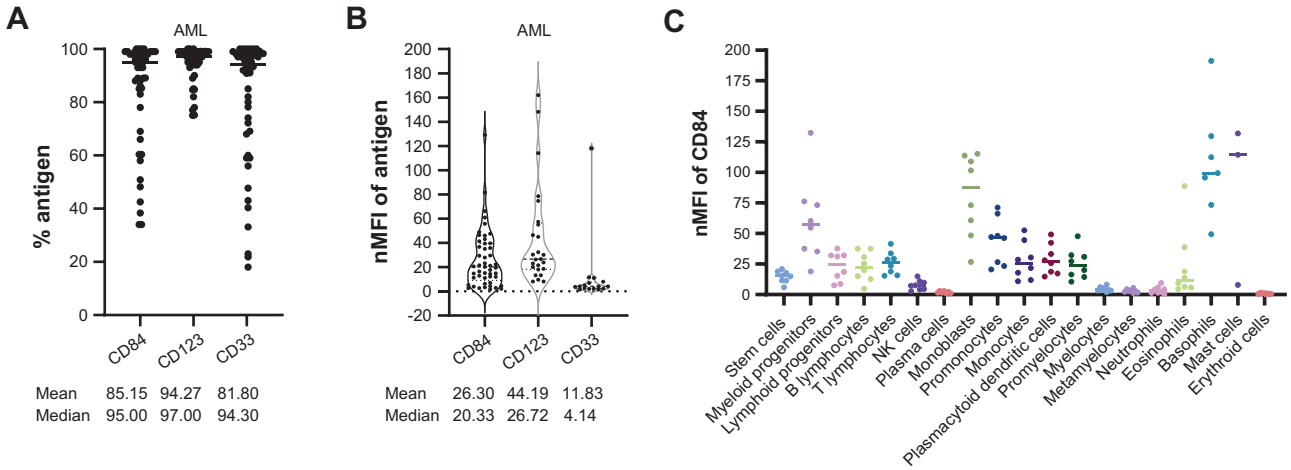

Fig. 1 CD84 expression on leukemic blasts and healthy bone marrow cells. A Percentage of CD84, CD123, and CD33-positive cells in primary AML patient samples, with the median indicated ($n = 51$ AML patient samples). **B** The normalized mean fluorescence intensity (nMFI) of CD84, CD123, and CD33 in primary AML patient samples, calculated as the ratio of the MFI of cells stained with specific antibodies to the MFI of cells stained with isotype controls (median is shown; $n = 51$ AML patient samples). See Supplementary Table 1 for AML diagnoses. **C** The nMFI of CD84 expression was analyzed as the ratio of the MFI of cells stained with the specific anti-CD84 antibody to the MFI of cells stained with the isotype control. Eight samples were analyzed ($n = 8$: 5 healthy donor bone marrow samples and 3 samples from individuals with monoclonal gammopathy of undetermined significance) (median is represented).

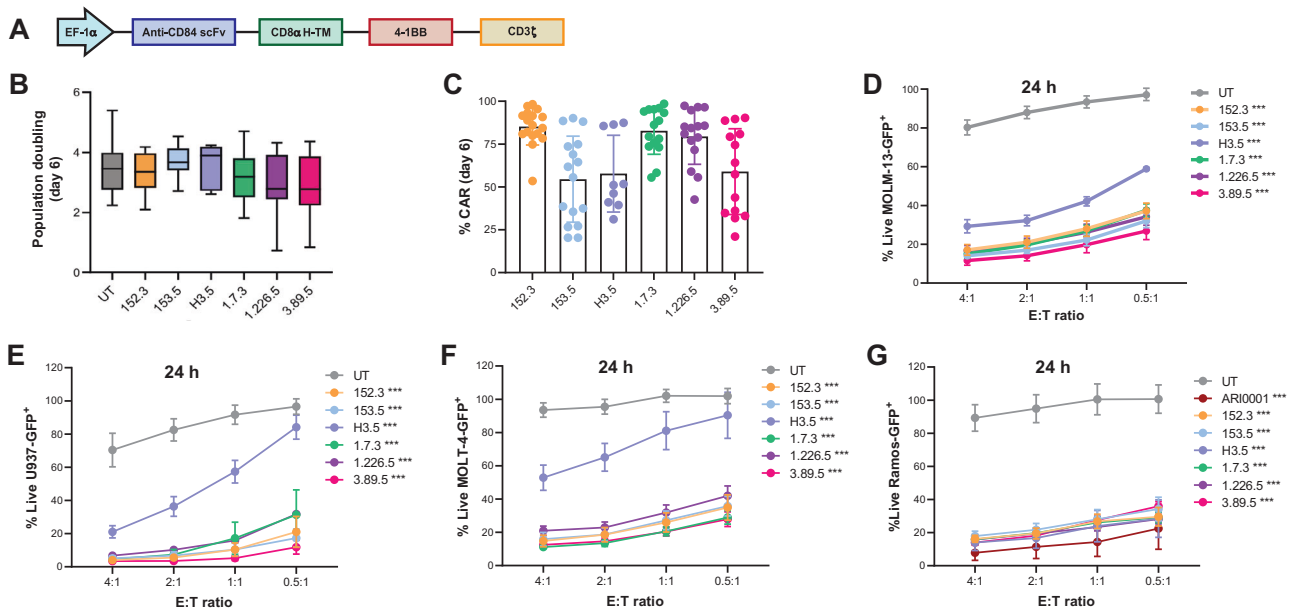

Fig. 2 CD84-targeting CART cells are successfully generated and expanded. A Scheme of second-generation CAR vector with EF1-α promoter, anti-human CD84 single-chain variable fragment (scFv), CD8α as hinge (H), and transmembrane (TM) domain, 4-1BB as intracellular costimulatory domain, and CD3ζ as signaling domain. **B** Population doubling of CART84 cells in comparison to UT cells at day 6 of expansion (Min to Max) $n = 5$–13. **C** Percentage of CAR-positive cells in CART84 cells at day 6 of expansion (Mean ± SD) $n = 9$–17. Cytotoxicity of CART84 towards different cell lines: CD84^{high} MOLM-13-GFP+ (AML) (**D**); CD84^{low} U937-GFP+ (AML) (**E**); CD84^{high} MOLT-4-GFP+ (T-ALL) (**F**); CD84^{high} Ramos-GFP+ (Burkitt lymphoma) (**G**). The percentage of surviving target cells relative to untreated cells (target cells alone) is shown at different effector-to-target (E:T) ratios at 24 h. Mean of at least 5 independent experiments ± SEM. UT untransduced T cells. Statistical significance was determined with a two-way ANOVA test (multiple comparisons to UT): ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

In terms of myeloid differentiation, we observed high CD84 intensity in common myeloid progenitors, as well as in promyelocytes. This intensity decreased progressively along the differentiation pathway, becoming nearly negative in myelocytes, metamyelocytes, and neutrophils. However, CD84 expression reappeared at moderate levels in mature eosinophils and at high levels in mature basophils and mast cells (Fig. 1C).

Regarding monocytic differentiation, CD84 intensity was high in monoblasts and promonocytes, and moderate in monocytes. CD84 expression was moderate in plasmacytoid dendritic cells, T and B cells, low in NK cells, and almost absent in plasma cells. As previously described [27], erythrocytes were negative for CD84 (Fig. 1C).

CD84-targeting CARs are successfully generated and expanded

We engineered multiple CD84-targeting CAR-T cells (CART84) using murine and fully human single-chain variable fragments (scFvs) included in a second-generation lentiviral CAR construct (anti-CD84scFv-CD8αH-TM-4-1BB-CD3ζ) (Supplementary M&M; Figs. 2A and S4A). CART84 constructs 152.3, 153.5, 1.226.5, 1.7.3,

3.89.5, and H3.5 exhibited higher expansion rates and over 40% CAR positivity, making them suitable for further evaluation (Fig. 2B, C).

Given that T cells express CD84, we anticipated potential fratricide among CART84. To investigate this, we examined CD84 expression in both CAR-negative and CAR-positive fractions. A decrease in CD84 was observed in CART84 variants 152.3, 1.226.5, and 3.89.5 by the end of expansion. In contrast, CART84 variants 153.5 and H3.5 maintained CD84 levels comparable to untransduced (UT) cells and CD19-directed CARTs (varnimcabtagene autoleucel/ARI0001), which serve as negative controls for fratricide as they lack CD19 (Fig. S4B, C). Although the decrease in CD84 could suggest fratricide, it appears time-limited, as CART84 cells expanded significantly and achieved high CAR expression by day 6 (Fig. 2B, C). Additionally, CART84 exhibited a higher CD4/CD8 ratio compared to UT, likely due to the removal of CD8 central and effector memory cells, which express elevated levels of CD84 (Fig. S4D).

To evaluate the impact of CAR84 expression on the CART immunophenotype, we analyzed exhaustion markers LAG-3, Tim-3, CTLA-4, and PD-1 and T-cell subsets at the end of expansion. CART84 showed a non-significant increase in exhaustion markers compared to UT in most instances. Notably, CART84 3.89.5 exhibited the highest expression of exhaustion markers in both CD4+ and CD8+ populations (Fig. S5A). Regarding T-cell subsets, there were no significant differences between CART84 and UT at the end of expansion (Fig. S5B). Most CART84 cells displayed a central memory phenotype, which is associated with enhanced in vivo persistence [39].

CART84 demonstrates high efficacy against hematological malignancies in vitro

To evaluate the cytotoxic potential of CART84 in vitro, we cocultured CART84 with CD84-positive tumor cell lines derived from AML ($CD84^{high}$ MOLM-13 and $CD84^{low}$ U937), T-ALL (MOLT-4), and aggressive B-cell lymphoma (Ramos). ARI0001 served as a positive control of cytotoxicity towards Ramos cells. All CART84 constructs displayed strong cytotoxicity in a dose-dependent manner, from high to low effector-to-target (E:T) ratios at 24 h, with enhanced effects observed at 48 h (Figs. 2D–G and S6A–D). Although H3.5 was notably cytotoxic, it displayed lower cytotoxicity towards U937 (Fig. 2E) and MOLT-4 compared to other CART84 constructs (Fig. 2F).

We then assessed the cytokine secretion by CART84 cocultured with MOLM-13, MOLT-4, and Ramos. All CART84 secreted higher cytokine levels than UT when cocultured with target cell lines (Fig. S6E–G). Notably, H3.5 produced the highest cytokine levels with Ramos. On the other hand, 1.7.3 secreted higher cytokine levels with MOLM-13 and MOLT-4 compared to other CART84 constructs, despite not exhibiting higher cytotoxicity towards these cells.

We also assessed CART84 proliferation when exposed to CD84 antigen, either with CD84-positive 300.19 or CD84-positive MOLM-13 cells (Fig. S6H, I). All CART84 constructs proliferated more in the presence of CD84-positive than CD84-negative 300.19 cells (Fig. S6H), except for H3.5. CART84 1.226.5 and 3.89.5 cells showed enhanced proliferation when exposed to CD84-positive 300.19 cells, though they also proliferated with CD84-negative cells. Conversely, H3.5, 1.7.3, and 3.89.5 exhibited similar proliferation in response to both specific and nonspecific stimuli (IL-2), suggesting possible tonic signaling (Fig. S6I). CART84 152.3 and 153.5 exhibited the most specific proliferation when cocultured with CD84-positive 300.19 or MOLM-13 cells (Fig. S6H, I).

In summary, CART84 152.3 and 153.5 demonstrated high cytotoxic activity, robust cytokine secretion, and specific proliferation in response to their target antigen, making them ideal candidates for further studies. To confirm their specificity, CART84 152.3 and 153.5 were cocultured with CD84-negative HEK-293 and HEK-293 engineered to express CD84. Both CART84 selectively eliminated CD84-positive HEK-293 without affecting parent cells (Fig. 3A).

We also assessed the cytotoxicity of CART84 against primary AML patient blasts. CART84 exerted cytotoxic effects towards these cells at 24 h, which became more pronounced at 48 h (Figs. 3B and S7A). Next, we successfully expanded CART84 from AML patients p#52 and p#53 (Fig. S7B). CART84 expansion was

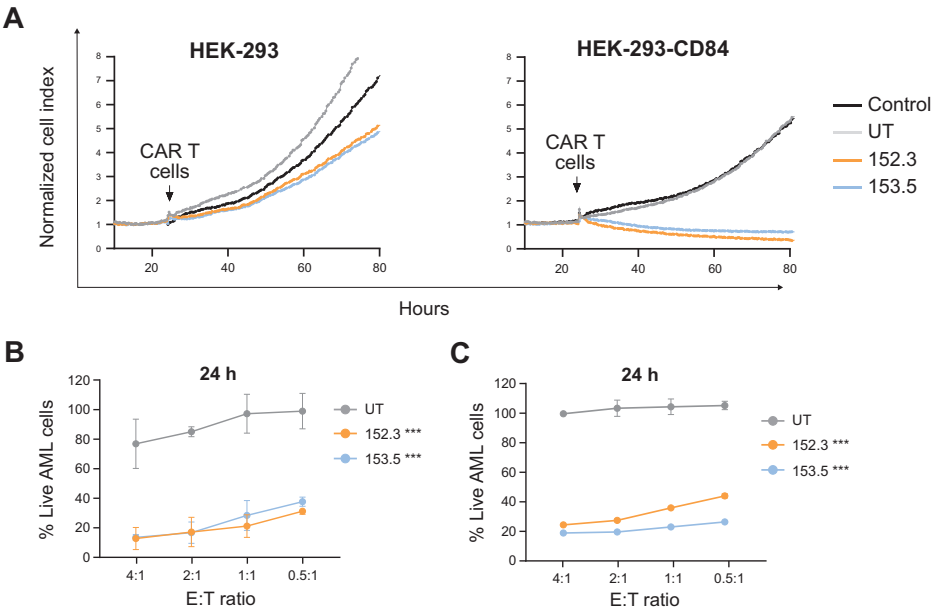

Fig. 3 CART84 selectively kills CD84-positive cells and targets primary AML blasts in vitro. A Cytotoxicity of CART84 cells towards CD84-negative (left) and CD84-positive (right) HEK-293 cells, at an E:T ratio of 4:1, assessed with the xCELLigence. **B** Cytotoxicity of CART84 cells against primary AML cells from patient sample #13 (see Suppl. Table 1). Data represent the mean of two CART84 productions derived from T cells of two healthy donors (BC#215 and BC#216), tested against AML cells from patient #13. One representative example out of three is shown. Statistical significance was determined with a two-way ANOVA test (multiple comparisons to UT). **C** Cytotoxicity of CART84 cells derived from AML patient #52 against autologous AML blasts. Details of AML diagnoses are provided in Supplementary Table 1. Statistical significance was determined with a two-way ANOVA test (multiple comparisons to UT). UT untransduced T cells. ***$p < 0.001$.

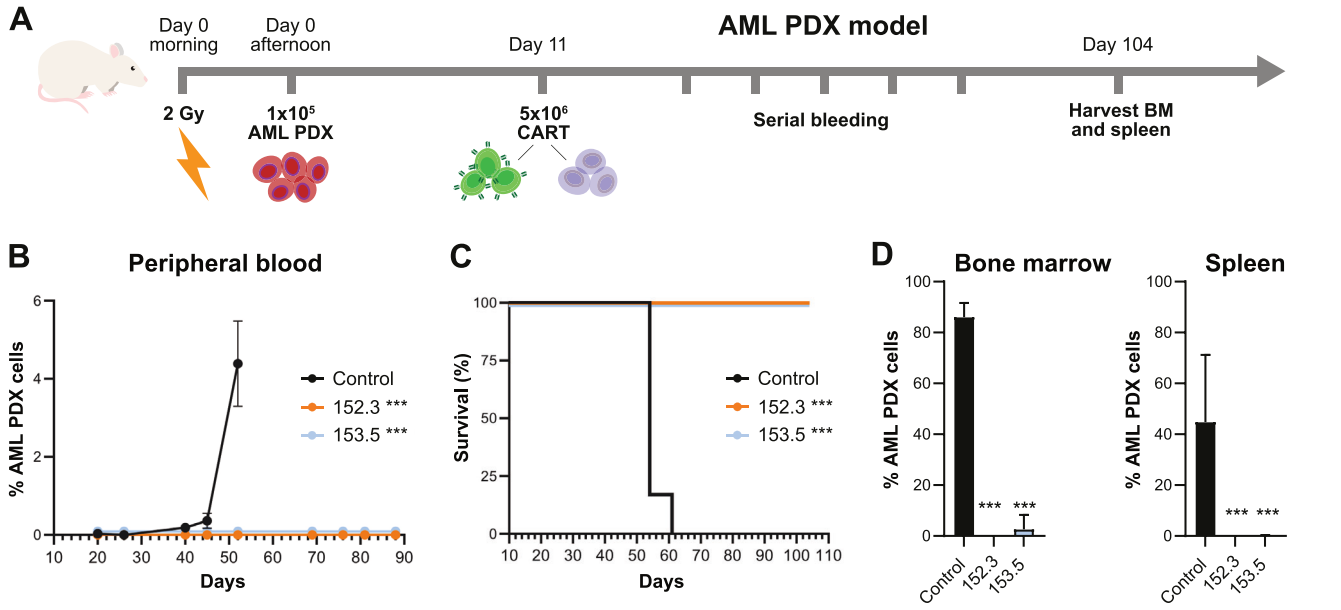

Fig. 4 In vivo efficacy of CART84 in an AML PDX model. A Experimental design. **B** Percentage of AML PDX cells in the peripheral blood of mice ($n = 6$ mice per group). Statistical analysis was performed with a two-way ANOVA with Dunnett's multiple comparisons vs. control mice (Mean ± SEM). **C** Kaplan–Meier survival curves for each experimental group. Statistical significance was determined with a log-rank test, with a corrected p value for 3 comparisons (control vs.152.3 / 153.5; 152.3 vs. 153.5). **D** Percentage of AML PDX cells in the bone marrow or spleen of mice at the end of the experiment (Mean ± SD). Statistical analysis was performed with a one-way ANOVA with Dunnett's multiple comparisons vs. the control group. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

similar to that observed in healthy donors (Fig. 2B). Moreover, CART84 demonstrated a specific antileukemia effect when cocultured with autologous AML blasts (Fig. 3C).

CART84 eliminates AML cells in a xenograft mouse model

We assessed the in vivo efficacy of CART84 in an AML xenograft model using the MOLM-13 cell line. On day zero, 1×10^5 MOLM-13-GFP-ffLuc cells were i.v. injected into NSG mice. Tumor engraftment was confirmed by bioluminescence the following day, and on day 2, mice were assigned to receive 5×10^6 CART84 152.3/153.5 or UT (Fig. S8A). Although CART84-treated mice showed reduced disease progression, a second dose was administered on day 13 due to continued disease advancement. Following this dose, a marked reduction in disease was observed (Fig. S8B, C). CART84-treated mice demonstrated increased survival when compared with the UT-treated group (Fig. S8D). Except for two animals in the 153.5-treated group that developed extramedullary tumors (data not shown), all CART84-treated mice were leukemia-free at the end of the experiment (Fig. S8E).

We also evaluated CART84 in an AML PDX model (Fig. S9A). On day zero, 1×10^5 AML PDX cells were i.v. injected into previously irradiated mice. Following leukemia engraftment on day eleven, 5×10^6 CART84 were administered i.v. (Fig. 4A). CART84 152.3 and 153.5 effectively eliminated the disease, as evidenced in both PB (Fig. 4B) and BM of CART84-treated mice (Fig. S9B), significantly prolonging their survival compared to controls (Fig. 4C). At the study endpoint, CART84 eliminated the disease in the BM or spleen of treated animals (Fig. 4D).

CART84 controls disease progression in a T-ALL xenograft model

We evaluated the in vivo efficacy of CART84 in a T-ALL xenograft model using the MOLT-4 cell line. On day zero, 7×10^5 MOLT-4-GFP-ffLuc cells were injected i.v. into mice, followed by 3×10^6 CART84/UT on day 5 (Fig. S10A). Animals in the control and UT groups were euthanized between days 26–32, whereas CART84-treated mice exhibited disease control and extended survival by an additional 2–3 weeks (Fig. S10B–D).

We also tested CART84 in a T-ALL PDX model (Fig. S10E). On day zero, 7×10^5 T-ALL PDX cells were injected i.v. into previously irradiated mice. On day 11, mice received 5×10^6 CART84 (Fig. 5A). CART84 effectively eradicated leukemia, as evidenced in PB (Fig. 5B), and significantly increased survival compared to the control group (Fig. 5C). At the end of the experiment, leukemic cells were undetectable in the BM and spleen of nearly all CART84-treated mice, with a single exception observed in the 153.5-treated group (Fig. 5D).

CD84 expression in healthy tissues: addressing potential on-target/off-tumor toxicities

CD84 expression was evaluated in FFPE human healthy tissues using an anti-CD84 antibody. No expression was detected in a wide-tissue microarray of healthy human parenchymal tissues (Figs. S11 and S12), except in lymphoid organs (Figs. S13 and S14). CD84 was found in the lymph nodes and in megakaryocytes and promyelocytes in the BM (Figs. S13 and S14).

Additionally, CD84 expression was assessed in fresh-frozen tissues using the antibodies 152-1D5 and 153-4D9, from which the scFv of 152.3 and 153.5 are derived, to evaluate the safety of our candidates. Neither 152-1D5 nor 153-4D9 bound to the parenchyma of healthy human tissues, including the heart, lung, liver, kidney, or brain, except for lymph nodes (Fig. S15). Importantly, microglial cells in the brain stained positively for 152-1D5 but were negative for the 153-4D9 mAb. Staining of lymph nodes and other lymphoid-associated tissues showed no significant differences between the antibodies (data not shown). Consequently, we selected CART84 153.5 as our candidate for the first-in-human clinical trial using CD84-directed CART cells for R/R AML and T-ALL patients.

In light of CD84 expression on immune cells (Fig. S3A, B), we conducted cytotoxicity assays of CART84 towards autologous T cells to study potential T-cell toxicity. Although CART84 exhibited an overall low cytotoxic effect (Fig. S16A), the highest cytotoxicity was observed against central memory T cells (Fig. S16B). Nonetheless, all T-cell subsets remained present at the end of the experiment (Fig. S16B).

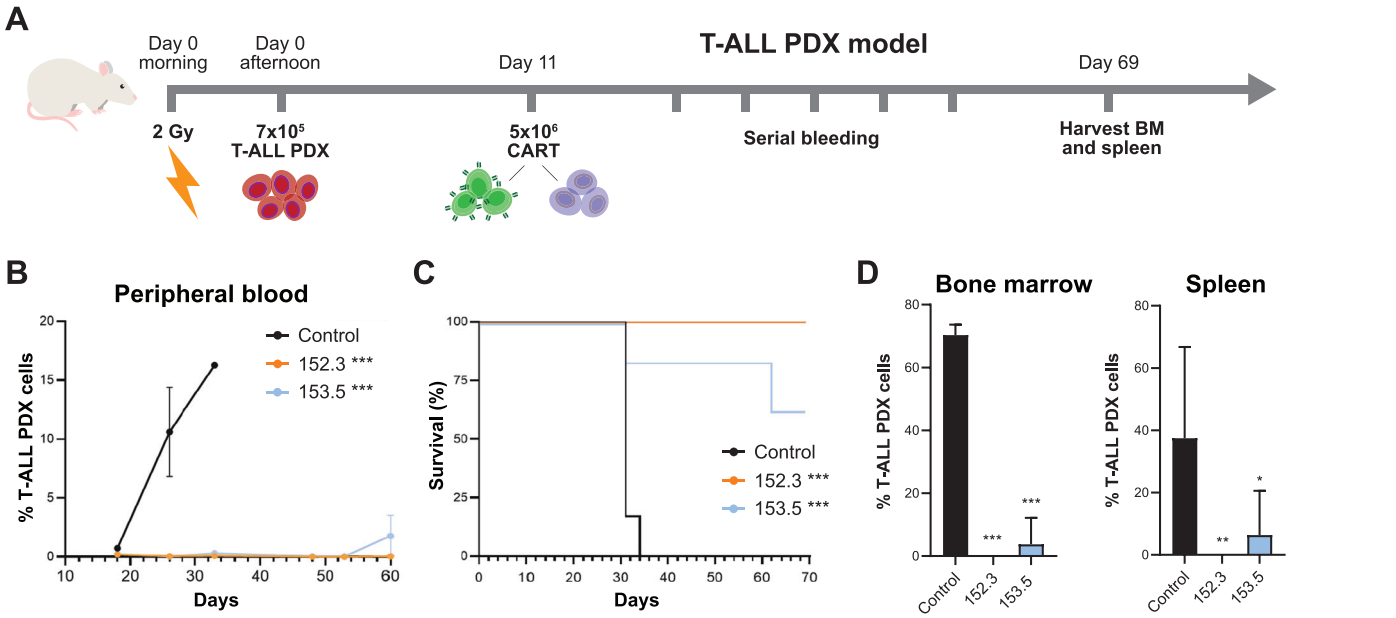

Fig. 5 In vivo efficacy of CART84 in a T-ALL PDX model. A Experimental design. **B** Percentage of T-ALL PDX cells in the peripheral blood of mice ($n = 5$–6 mice per group). Statistical analysis was performed with a two-way ANOVA model with Dunnett's multiple comparisons vs. control mice (Mean ± SEM). **C** Kaplan–Meier survival curves for each experimental group. Statistical significance was determined with a log-rank test, with a corrected p value for three comparisons (control vs.152.3 / 153.5; 152.3 vs. 153.5). **D** Percentage of T-ALL PDX cells in the bone marrow or spleen of mice at the end of the experiment (Mean ± SD). Statistical analysis was performed with a one-way ANOVA with Dunnett's multiple comparisons vs. the control group. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

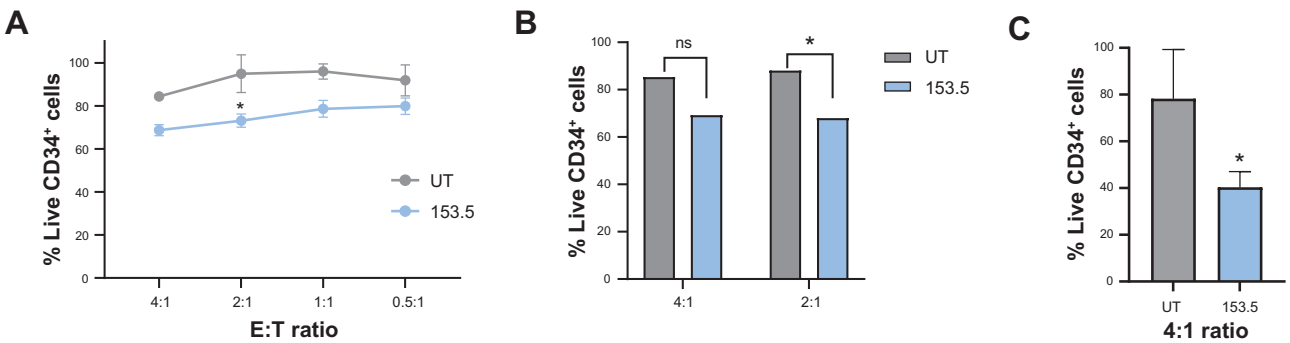

Fig. 6 Myelotoxicity of CART84 cells in vitro. A Cytotoxicity assay of CART84 cells against G-CSF mobilized peripheral blood CD34$^+$ hematopoietic stem/progenitor cells (HSPC) after 24 h (mean of technical duplicates; one representative example out of three is shown). Statistical analysis was performed with a two-way ANOVA with Sidak's multiple comparisons. **B** Cytotoxicity of CART84 against CD34+ HSPC from cord blood donors at 24 h (mean of 5 assays using CD34+ cells from five different cord blood units ± SEM). Statistical significance was determined using a two-way ANOVA model with Sidak's multiple comparisons. **C** Cytotoxicity of CART84 against donor bone marrow CD34+ HSPC (Mean of 4 assays using CD34+ cells from four different bone marrow samples ± SEM). Statistical significance was determined using an unpaired t-test. *$p < 0.05$.

We evaluated whether soluble CD84 (sCD84) shed from platelets during infection [40] could affect CART84 efficacy or induce on-target off-tumor toxicity when present. CART84 remained effective against MOLM-13 in the presence of sCD84, though efficacy slightly declined in a dose-dependent manner. Additionally, CD84-negative HEK-293 cells were not targeted by CART84, even by adding sCD84, suggesting that sCD84 might not induce on-target off-tumor toxicity (Fig. S17A, B).

CART84 demonstrates hematologic toxicity in vitro and in vivo in a humanized mouse model

Like other CARTs targeting AML [17], CD84 is expressed on CD34+ hematopoietic stem/progenitor cells (HSPC), though at lower levels than on other immune cells (Figs. 1C and S2E). We evaluated the cytotoxicity of our candidate CART84 153.5 against healthy donor CD34+ HSPC from three sources: G-CSF-mobilized PB, cord blood, and BM. Cytotoxicity assays revealed that CART84 exhibited low cytotoxic activity against CD34+ HSPC from G-CSF-mobilized PB and cord blood, but moderate activity against those from BM after 24 h, with statistical significance at some E:T ratios (Fig. 6A–C).

To further study hematological toxicity, we assessed the impact of CART84 on the viability and clonogenic capacity of CD34+ HSPCs. CART84 slightly reduced CD34$^+$ colony forming units (CFU) compared to UT, but without statistical significance (Fig. S18A, B).

Finally, we assessed the potential hematologic toxicity of CART84 in vivo using a humanized NSG mouse model (Fig. 7A±G). By day 48, 60% of CD45+ cells in the BM of mice were human, confirming successful engraftment (Fig. S18C, D). We then administered 1×10^6 UT, CART84 153.5, or ARI0001 (a non-myelotoxic control). By day 64, PB analysis revealed an increase in human CD45+ and CD3+ cells in UT- and ARI0001-treated mice,

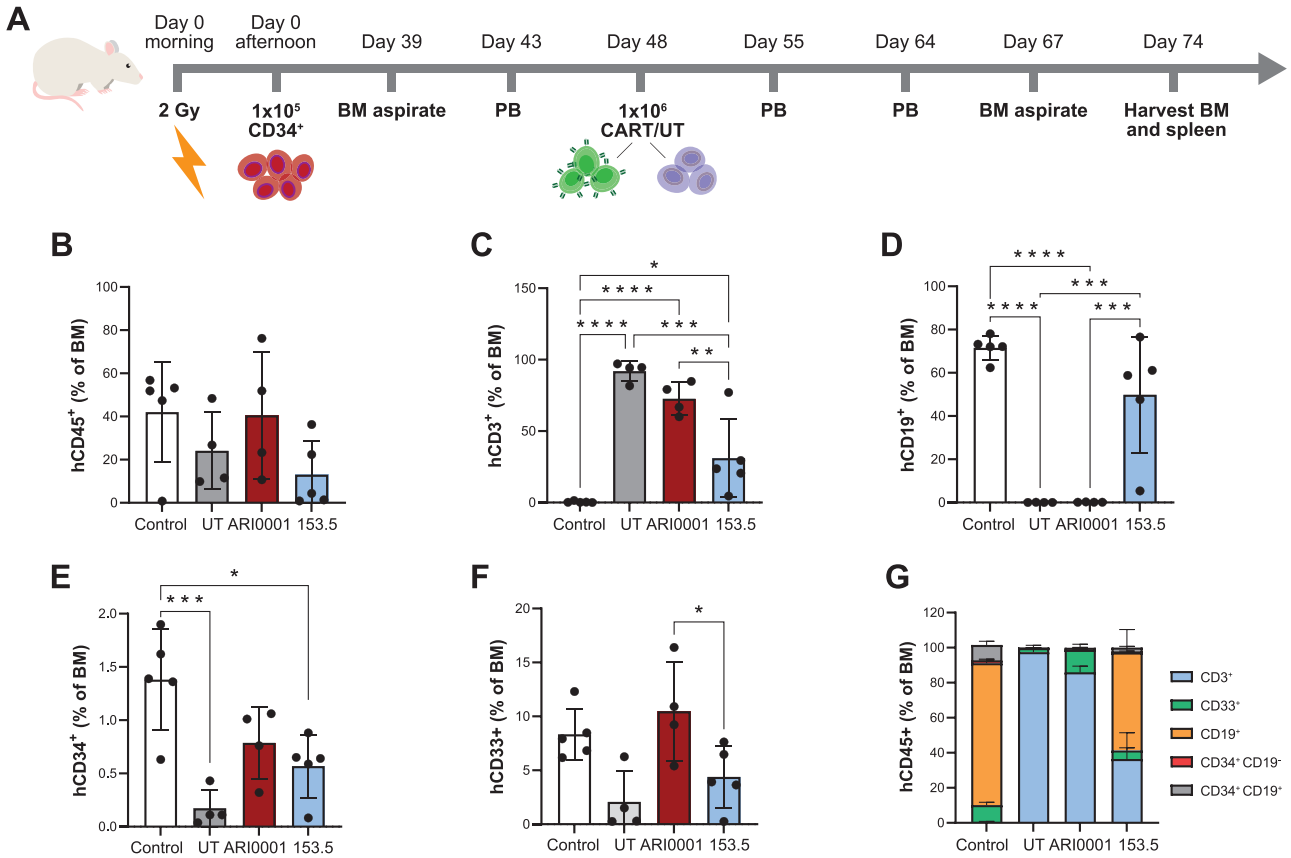

Fig. 7 Myelotoxicity of CART84 cells in vivo. A Experimental design of the humanized NSG mouse model ($n = 5–6$ mice per group). **B–F** Percentage of different human subsets in the bone marrow (gated from the human CD45+ population) at day 67 of the experiment. **B** Percentage of CD45+ human cells gated from the entire bone marrow. **C** Percentage of human T lymphocytes (hCD3+). **D** Percentage of human B lymphocytes (hCD19+). **E** Percentage of human HSPC (hCD34+). **F** Percentage of human myeloid cells (hCD33+) **G** Human cell populations per mouse group. Statistical analysis of bone marrow cell populations was performed using a one-way ANOVA with multiple comparisons (all vs. all). UT: untransduced T cells. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

attributed to xeno-GvHD and CD19+ B-cell targeting, respectively (Fig. S18E, F). In contrast, CART84-treated mice showed no significant changes in CD45+, CD3+, or CD14+ cells, but exhibited a reduction in CD19+ B cells in PB when compared to the control group (Fig. S18G, H).

By day 67, BM analysis revealed B-cell depletion in ARI0001-treated mice, validating the experimental setup. In CART84-treated mice, CD34⁺ cell numbers were reduced compared to controls. A decrease in CD19⁺ B cells and CD33⁺ myeloid cells was also observed, though not statistically significant (Fig. 7D–F). Notably, by the end of the experiment, all cell populations remained detectable in CART84-treated mice (Fig. 7G).

DISCUSSION

CART therapy for AML and T-ALL remains a significant challenge due to the absence of a specific antigen equivalent to CD19 for B-ALL or B-cell lymphoma. In this study, we demonstrate that CD84 is expressed across various hematological malignancies, positioning it as a promising pan-hematologic target. We developed an anti-CD84 CART therapy for AML and T-ALL, addressing the critical unmet need in these diseases, which currently lack an approved CART therapy.

Designing CART therapy for AML presents a major challenge due to the risk of on-target/off-tumor toxicity, particularly myelotoxicity. The most studied antigens—CD123, CD33, and CLL-1—are also present on healthy cells, leading to varying degrees of hematologic toxicity. Although CD123-directed CART cells have shown efficacy against AML [17], cases of myeloablation have been reported [41]. CD33-targeting CARTs have demonstrated limited clinical efficacy, despite promising preclinical results [42, 43]. CLL-1 has emerged as a promising CART target, with early trials showing complete responses in children [44] and adults, without myelotoxic effects, though some adult patients experienced high-grade cytokine release syndrome and severe infections due to CLL-1 expression on granulocytes [45]. Here, we observed robust efficacy of CART84 against AML in vitro and in a PDX model derived from an AML with t(11;19)(q23;p13.3) KMT2A/MLLT1 in vivo (ELN 2022 adverse). In the 51 de novo or R/R AML samples analyzed, CD84 expression (nMFI) was higher in malignant cells than in HSPCs, and it was comparable to those of CD123 and CD33. Moreover, high CD84 expression was observed in AML cases with TP53 mutations and MECOM rearrangements, presenting a potential therapeutic option for these high-risk patients. Importantly, the anti-CD84 antibody 153-4D9—from which the scFv of our clinical trial candidate is derived—did not bind to the parenchyma of healthy human tissues, such as heart, lung, liver, kidney, or brain. Based on our in vitro and in vivo data, we believe that CART84 therapy may provide an additional therapeutic option for AML patients.

For T-ALL, CD7 [46–48] and CD5 [49, 50] are common antigens, with CD1a exclusive to cortical T-ALL [20]. Clinical trials using CD7-directed CART therapy for T-ALL have shown promising results [46, 47]. However, challenges like T-cell fratricide and limited CART expansion remain unsolved. To prevent fratricide in T-ALL, strategies include antigen knockout [48, 51], intracellular retention

of the antigen [52], pharmacological inhibition [53], or using naturally selected [54] or naturally occurring T cells [55]. An additional challenge in CART therapy for T-ALL is T-cell aplasia, as the target antigens are also expressed on healthy T cells. While CD84 is also expressed on T cells, our results demonstrate that CART84 expanded successfully without requiring gene modifications, unlike what is common for CD7 or CD5. CART84 expansion exhibited a higher CD4/CD8 ratio compared to UT, likely due to the removal of $CD84^{high}$ CD8+ memory and CD8+ effector memory cells. When we evaluated T-cell toxicity in vitro to investigate potential T-cell aplasia, CART84 exhibited low cytotoxic effects. It is important to note that all T-cell subsets were still present after 24 h of co-culture, suggesting that complete T-cell depletion is unlikely.

To our knowledge, this is the first report introducing CD84 as a novel target for CART therapy in acute leukemias. Previous studies have highlighted the role of CD84 in various malignancies [32, 34]. Binsky-Ehrenreich et al. demonstrated that CD84 supports leukemic cell survival in CLL [28, 29] and plays a similar role in the tumor microenvironment of multiple myeloma by suppressing T-cell activity [30]. Additionally, CD84 has been identified as a driver of leukemogenesis in AML [31], and Pigazzi and collaborators [56] reported high CD84 expression in pediatric AML. Given its role in leukemic cell survival, antigen loss is unlikely to serve as an escape mechanism; however, clinical trials are needed to determine whether CD84 downregulation could occur under the selective pressure of CAR T cell therapy [57].

The expression of CD84 in BM and lymphoid tissues, particularly in HSPCs, remains controversial [38, 58, 59]. Recently, Kim and collaborators reported that $CD84^{low}$ HPSCs proliferate significantly more and differentiate into all lymphoid progeny, while $CD84^{high}$ HPSCs primarily differentiate into myeloid cells [38]. In our study, while the in vitro CFU assays suggest low myelotoxicity of CART84, cytotoxicity assays against $CD34^{+}$ HSCs indicate low to moderate myelotoxicity, depending on the cell source. In the humanized mouse model, CART84 reduced $CD34^{+}$ cells in the BM compared to controls and exhibited cytotoxicity against B cells in PB. Moreover, although not statistically significant, some degree of CART84-mediated cytotoxicity against $CD33^{+}$ myeloid cells was observed. Taken together, while our preclinical findings suggest that CART84 may not induce severe hematologic toxicity, the limitations of current preclinical models must be acknowledged. Moreover, targeting myeloid antigens can potentially lead to myeloablation, as demonstrated in clinical trials with other targets, such as CD33 and CD123 [12, 60]. Given the CD84 expression profile, patients could experience profound aplasia after CART84 treatment, requiring careful management and appropriate infectious prophylaxis. To overcome the potential myelotoxicity and B/T cell depletion associated with CART84 exposure, we plan to proceed with allogeneic hematopoietic stem cell transplantation after CART84-induced leukemia clearance, aiming to both eliminate CART cells and restore the patient's hematopoietic and immune system.

In conclusion, our results establish CD84 as a promising therapeutic target in hematological malignancies, particularly acute leukemias. Given the demonstrated efficacy of CART84 in our preclinical studies, we plan to advance it to a first-in-human clinical trial as a bridge therapy to allo-HSCT in patients with R/R AML and T-ALL (EudraCT 2024-519966-31-00).

DATA AVAILABILITY

The datasets analyzed during the current study were obtained from publicly available databases, including GEPIA (http://gepia.cancer-pku.cn/), the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle), and BloodSpot (https://servers.binf.ku.dk/bloodspot/). All datasets are accessible online and were used in accordance with their terms of use. Additional datasets generated during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

LPA, NKG wrote the manuscript. LPA, MAU, MVC, BMH, JA, SP, GF, AA, and BCA carried out the experiments. LPA, MAU, MVC, BMH, JA, SP, GF, AA, BCA, and NKG analyzed the data and prepared the figures for the article. FG, AM, AB, IN, MG, DSM, NT, TV, PMe, MR, DC, and JE contributed to data collection and provided patient samples. CS, MUH, DSM, NT, TV, PMe, AM, EAGN, JE, AUI, PMo, and JD collaborated in data analysis and supervised the manuscript. MJ and NKG designed the study and supervised the project. All authors contributed to manuscript revisions and approved the final version.

COMPETING INTERESTS

PM is a cofounder of OneChain ImmunoTx, a spin-off company unrelated to this work. LP, MAU, SP, and NKG are employees of Gyala Therapeutics S.L., NKG is partially employed by Gyala Therapeutics S.L., a spin-off company of the Hospital Clínic of Barcelona related to this work. CS is the CEO of Gyala Therapeutics S.L., a spin-off company related to this work. LP, CS, MJ, and NKG declare to be co-inventors in the patent of PCT/EP2023/050194. MJ declares Research Funding from Gyala Therapeutics S.L.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods were performed in accordance with the relevant guidelines and regulations, including the Declaration of Helsinki. Approval for the use of human samples was obtained from the Clinical Research Ethics Committee of Hospital Clínic of Barcelona, under protocol numbers HCB/2021/0977 and HCB/2016/0045. Informed consent was obtained from all participants or their legal guardians prior to sample collection (reference code R121004-094). Approval for experiments involving animals was obtained from the Animal Experimentation Ethics Committee of Generalitat de Catalunya, under protocol number 11577.

ADDITIONAL INFORMATION

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