Nature Communications

https://doi.org/10.1038/s41467-026-72361-4

Article in Press

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Received: 12 April 2025

Accepted: 1 April 2026

Cite this article as: Pigazzi, M., Merlini, S., Da Ros, A. *et al.* CD84 is a specific target for acute myeloid leukemia CAR-T cell therapy. *Nat Commun* (2026). https://doi.org/10.1038/s41467-026-72361-4

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CD84 is a specific target for Acute Myeloid Leukemia CAR-T cell therapy

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ABSTRACT

Chimeric antigen receptor (CAR)-T cell therapy has transformed the treatment of hematologic malignancies, yet its application to acute myeloid leukemia (AML) remains challenging due to the scarcity of disease-specific antigens. The identification of a highly selective target is crucial to enhance efficacy while minimizing off-tumor toxicity. Here, we identify CD84 as a promising target for AML immunotherapy, displaying a unique expression profile: it is robustly and stably expressed by blasts, particularly in relapsed disease, and negligible on normal hematopoietic stem/progenitor cells. This profile renders CD84 an ideal target, with potential for improved therapeutic precision and potency, and with reduced risk of off-target effects and toxicity. To assess its potential, we generate CD84-directed CAR-T cells and teste them *in vitro* and *in vivo* on clinically relevant models. The engineered cells exhibit potent cytotoxicity against CD84-expressing AML cell lines and patient-derived xenograft (PDX) cells, eliminating leukemic blasts even with low CD84 expression. In AML-PDX models, CAR-T treatment leads to sustained reduction of leukemia burden, doubling the survival of the treated animals compared to controls. No downregulation of CD84 expression on the blasts in the treated models is seen. Importantly, CD84 CAR-T cells spare normal hematopoietic stem/progenitor cells that after treatment retain their repopulation potential in humanized models. These findings establish CD84 as a target for AML immunotherapy and provide a compelling rationale for clinical development of CD84-directed approaches that may address an urgent need for treatment in aggressive and refractory AML.

INTRODUCTION

Acute leukemias constitute about one-third of all pediatric malignancies and are the leading cause of cancer-related mortality in children [1]. Acute lymphoblastic leukemia (ALL) is the most diagnosed entity overall, whereas acute myeloid leukemia (AML), though less prevalent in children, is the most frequent form of leukemia in adolescents. Generally, AML is less curable than ALL [2]. Although the prognosis for children with AML has improved significantly over the past three decades, reaching up to 80% survival at 5 years in the most recent clinical trials, more than 25% of pediatric patients still experience disease recurrence [3–6]. Nowadays, despite chimeric antigen receptor (CAR)-T cell therapies have revolutionized the treatment of B-cell neoplasms [7,8] AML still awaits a broadly successful CAR-T cell approach. Indeed, translating the success of CAR-T cell therapies targeting CD19 in B-cell malignancies [9,10] to AML has proven difficult due to the distinct biology of the disease and the lack of universally expressed and specific target antigens [11,12]. In fact, AML is a heterogeneous malignancy, both genetically and phenotypically, with an increasing number of AML subgroups being identified, and with distinct biological and prognostic characteristics that affect the development of targeted therapies [13–15]. Furthermore, AML sub-clonal dynamics evolve over time and in response to treatment [13,16,17]. This intra-tumoral dynamic heterogeneity has significantly affected the identification of candidate target antigens for CAR-T cell therapies, which should be expressed along treatment and relapse by most if not all AML cells, including leukemia stem cells, but not by healthy hematopoietic cells to spare normal hematopoiesis from off-tumor toxicity. In the continuous effort to identify such antigens, different targets have been tested. Robust data exists on CD33 and CD123 that are highly expressed on AML

blasts [18,19], but also on normal hematopoietic stem/progenitor cells (HSCs), with consequent significant challenges in balancing efficacy with safety [20,21]. More selective targets have been recently identified such as CD7, C-type lectin-like molecule 1, CD38, TIM3, CD7, FLT3, CD70, and CD47, which are currently tested in early phase clinical trials, frequently as bridge-to-transplant [22–28]. Early evidence from these studies indicate that more research is needed to identify either new and specific targets and/or additional signaling, i.e. enforcing the lysis efficacy and/or limiting unwanted signaling from the niche, that could render CAR-T cell therapy a viable option also in AML. To address these challenges, we conducted *in silico* analyses for target antigen discovery on a large dataset. CD84 (SLAMF5) resulted highly and stably expressed in a large cohort pediatric of AML cases at diagnosis and at relapse, confirmed also in adult AML[29,30], but not in immature healthy hematopoietic cell compartments, making it a potentially ideal candidate as tumor-associated antigen (TAA). We here describe the target validation process results, as well as the proof-of-concept of efficacy and feasibility of a CAR-T cell approach targeting CD84.

RESULTS

CD84 is robustly expressed by AML blasts but not by early hematopoietic precursors

With the goal of identifying new antigens associated to AML blasts of pediatric patients, we analysed the transcriptome of 85 childhood AML cases at diagnosis by gene expression arrays [31]. Exploiting this dataset, we selected 6 TAAs for being hyper-expressed in all AML cases and overexpressed as compared to 3 reference age-matched

healthy bone marrow samples (Figure 1A). Among them, we prioritized CD84 based on its expression profile in AML cell lines (Figure 1B-C, Table 1S). Interestingly, high CD84 expression was documented in several sources, including: an independent large cohort of pediatric AML cases (n=1491) from the TARGET-COG database that also included 68 bone marrow samples from healthy donors (Figure 1D, Supplemental Figure 1A); several datasets comprising adult AML cases of the Cancer Genome Atlas (TCGA, n=179), the BeatAML collaborative research program (n=223) and the SWOG Cancer Research Network (n=174); and the public bloodspot tool (https://www.fobinf.com/)[32]. Interestingly, CD84 was also recently identified as a leukemia stem cell (LSC) target, over-expressed *versus* healthy BM cells, in a single cell multimodal analysis applied to 15 pediatric AML cases [33]. Based on these early findings, we conducted a prospective study in which CD84 protein expression was evaluated by flow cytometry on a cohort of *de novo* AML cases at diagnosis (n=99) and at relapse (n=32) (Table 1-2), as well as on samples collected during treatment or follow-up when minimal residual disease (MRD) was detected (n=9, blasts range 0.02-4%, average 1.3%). Moreover, we evaluated CD84 expression on a series of cases classified as secondary AML, myelodysplastic syndromes or pre-treated/refractory diseases with AML blasts >40% (n=28). CD84 was expressed in 85 of the 99 AML cases at diagnosis (86%), in 29 of 32 samples collected at relapse (91%), in 100% of the follow-up samples with detectable MRD (Figure 1E-F), and in 27 of other 28 myeloid diseases (96%) (Supplemental Figure 1B). No clear association between the expression of CD84 and a specific AML molecular lesion was found (Figure 1F), with a trend towards higher expression in *KMT2A* and *NUP98* rearranged cases, supported also by the COG database (Supplemental Figure 1A).

The expression pattern of CD84 in non-AML cells and tissues was then evaluated by multiple approaches. Publicly available gene expression and protein datasets (Bloodspot, The Human Protein Atlas, GTEX) and our flow cytometry analyses demonstrated limited to no expression of CD84 in most of human tissues, as well as primary human cells (Supplemental Figure 1C and D). Importantly, CD84 expression projected on healthy BM specimens analyzed by single cell sequencing was low in hematopoietic cells, especially on prospectively identified HSCs (Figure 2A and B). These data were confirmed by a prospective flow cytometry analysis that revealed CD84 protein expression to be significantly lower than that on CD84+ blasts in HSCs, including the long-term HSC compartment (Supplemental Figure 3), and all other non-leukemic cells identified within healthy-regenerating BM samples (n=15), showing CD84+ expression levels comparable to those of the blasts classified as negative according to WHO criteria (Figure 2C). Only monocytes and myeloid cells showed a trend towards higher expression levels, which anyhow remained significantly lower than those of the positive blasts. MFI data were consistent with the number of CD84 molecules (Supplemental Figure 2).

T lymphocytes expressing anti-CD84 CARs efficiently lyse AML cell lines *in vitro*

To assess whether CD84 targeting may constitute a valuable strategy to eliminate leukemia with limited or no impact on normal hematopoiesis, we generated two candidate anti-CD84 ScFV sequences (B8 and F12) by phage display (Supplemental Figure 4S) that were then cloned into a second-generation CAR construct containing the CD28 H/TM and 4-1BB costimulatory domains in combination with the zeta (CD3zeta) signaling domain (Figure 3A). The two CAR sequences were then inserted into a third-generation lentiviral transfer plasmid under the control of the human phosphoglycerate kinase

promoter (hPGK) for high-titer LV production. The corresponding LVs were then employed for generating F12 and B8 CAR-T cells (Supplemental Figure 5S). Briefly, healthy donor T cells (n=5 different donors) were isolated from total PBMCs based on $CD4^+$ and $CD8^+$ magnetic separation, followed by cell activation via IL-7 and IL-15, transfer of the CAR construct via LV transduction at multiplicity of infection (MOI) into the activated T cells and expansion for 14 days. Control cells were produced employing a reporter gene (mCherry)-encoding equivalent LV. At the end of the 17-day process, the T cells were efficiently transduced (Figure 3B) and expressed the G4S linker (Supplemental Figure 6A, B), showed good expansion rates with high viability (Supplemental Figure 7A-B) and a preserved CD4:CD8 ratio (Supplemental Figure 7C), were enriched in central memory (T_{cm}, $C197^+$ $CD45R0^+$) cells (Supplemental Figure 7D), activated as *per* CD69 and CD25 expression and with cytotoxic potentialities, as documented by an increased expression of CD107a, CD137 and CD154 as compared to the beginning of the manufacturing process (Supplemental Figure 7E). The CAR-T cells did not show an exhausted phenotype as they expressed low levels of CD366, CD233, and CD279 (Supplemental Figure 7F). Importantly, the CAR cassette did not affect these parameters at comparison with the reporter cassette.

To assess their potency, the CAR-T cells were first tested on HL-60 and SHI-1 as target $CD84^+$ AML cell lines expressing LUC. B8 and F12 CAR-T cells maintained in co-culture for 48 hours at a 1:1 E:T ratio with either of the two AML cell lines exhibited a potent lytic activity (ranging from 60 to 95%) (Figure 3C-D). After co-culture, the CAR-T cells also showed a cytokine production and release pattern consistent with activation and the cytotoxicity they exerted (Figure 3E). ELISA quantitative assay confirmed the presence

of abundant cytotoxic secreted factors in the co-culture medium (Figure 3F). The cytolytic ability of B8 and F12 CAR-T cells was also evaluated in a dose-response test employing the MKPL1 and the SHI-1 cell lines with high and low CD84 density, respectively (Figure 3G). The T cells engineered with either F12 or B8 chains showed a strong and comparable cytolytic activity on MKPL1 cells at all the tested E: T ratios, while they exhibited a strong lytic capacity (>40%) on SHI-1 only up to a 1:8 E: T ratio (Figure 3H), with some possible advantage in terms of induced lysis of B8 over F12.

To assess if the B8 and F12 chains have a different antigen affinity, we compared the ScFVs sequences and discovered that they were 94% identical with only 16 different aminoacidic residues, 9 of which with preserved chemical properties (Supplemental Figure 8A). The complementarity-determining regions (CDRs) of the V_H and V_L were the same for both chains and contained the residues (n=12 for B8 and n=11 for F12) responsible for the contact with CD84. A model prediction of the structure of the binding between the scFVs and the extracellular domain (ED) of CD84 identified specific amino acids involved in the binding. Results indicated that despite 3 different contact residues, both ScFVs bind to the CD84 ED *via* the same epitopes (Supplemental Figure 8B-C). Consistently, plasmon resonance confirmed that both ScFVs exhibit a strong affinity for the target with similar dissociation constant values in the nanomolar range (Figure 3I).

T lymphocytes expressing anti-CD84 CARs recognize and potently kill AML cell lines *in vivo*

To study CAR-T cell cytotoxic activity and killing potency *in vivo*, we injected B8 and F12 CAR-T cells or mCherry control T cells (day 3) in NSG mice previously engrafted with the LUC-expressing SHI-1 AML cell line (day 1) (E:T 3:1). Starting from day 24 days post

AML induction, CAR-T cell treated mice showed a significantly lower leukemic burden, measured as luciferase signal, as compared to controls (Figure 4A). At the end of the observation time (day 42), the BLI captured in the mice treated with the B8 or F12 CAR-T cells was 93% and 84% lower than the control BLI, respectively (Figure 4A). Treatment with B8 or F12 CAR-T cells resulted also in a significantly extended median survival of the recipient mice as compared to controls (Figure 4B). Upon sacrifice, 60 to 90 days after AML induction, we observed the presence of abundant T lymphocytes ($CD3^+$, $CD45^+$ cells) in the peripheral blood, BM, and spleen of the CAR-T cell treated xenografts, but not of the mice treated with the control cells, consistent with a T-cell expansion consequent to antigen recognition (Figure 4C).

To test B8 and F12 CAR-T cell specificity we generated CD84 knock-out SHI-1 AML cells (SHI-1 $CD84^{KO}$) and injected these cells, as well as the two CD84 negative hematopoietic cell lines U937 and K562 (Figure 4D), in NSG mice followed by CAR-T or mCherry T cells. Neither B8 nor F12 CAR-T cells limited the growth of SHI-1 $CD84^{KO}$ cells, nor extended the survival of the engrafted mice that died similarly to mice treated with control T cells. Similarly, B8 was inefficient in reducing non-target AML U937 and K562 cell line expansion *in vivo* (Figure 4E-F). Notably, at mice sacrifice, we documented AML cell spread in BM, spleen, and tumor masses of the SHI-1 $CD84^{KO}$ xenografts treated with the CAR-T cells. At these sites, AML cells were negative for CD84 expression, confirming CAR specificity (Supplemental Figure 9A-B).

T lymphocytes expressing anti-CD84 CARs display robust anti-leukemia activity on AML PDX models

To evaluate the anti-leukemia activity of anti-CD84 CAR-T cells against primary AML, we employed pediatric AML-PDX models characterized by heterogeneous CD84 expression levels (Figure 5A). We first tested B8 and F12 CAR-T cells *in vitro* on primary cells and observed a heterogeneous, but consistently cell lysis potency (Figure 5B). The specificity of CD84 binding and activation was supported by the measurement of relevant cytokines release (Figure 5C), including TNFα, IFNγ, IL-2, and Granzyme B in the co-culture medium (Figure 5D). We then conducted a feasibility *in vivo* study to determine the optimal timing and dosing for CAR-T cell infusion in our PDXs (Supplemental Figures 10A-D). Thus, we inoculated 0.25 x10^6 LUC AML cells/mouse derived from three different pediatric PDXs characterized by low, medium and high CD84 expression (MFI: PDX #1= 371, considered barely negative for CD84; PDX #3 = 861; PDX # 8= 2147), and then either control mCherry T cells, or B8 or F12 CAR-T cells on day +3 at a 3:1 E: T ratio (Supplemental Figure 11A). We then monitored AML disease burden in treated and control mice as bioluminescence signal starting at week 4 after inoculation. The disease progressed rapidly in all the animals that received control T cells (Figure 5E and Supplemental Figure 11B). On the contrary, treatment with B8 or F12 CAR-T cells resulted in leukemia clearance and/or remarkable delay of its progression in the animals engrafted with the PDXs characterized by medium (PDX#3) and high (PDX#8) CD84 expression (Figure 5E-F and Supplemental Figure 8C). The treatment effect was striking in PDX#1 likely because of the substantially lower CD84 density of this model as compared to the other PDXs (Supplemental Figure 11B-C). Importantly, treatment with B8 or F12 CAR-T cells significantly extended the median survival of AML-PDXs (Figure 5F, n=19 vs n=6 control group, ****p<0.0001; Supplemental Figure 11D). Leukemia

clearance, documented by luciferase and by CD33 staining at flow cytometry, was associated with an early expansion (from week 3-4) and persistence of T lymphocytes in the peripheral blood of the CAR-T cell treated PDXs, and not in the controls (Figure 5GH). Interestingly, whenever residual myeloid blasts were detected at mice sacrifice of the treated PDX-bearing mice at the end of the study, CD84 was also detected on the tumor cells at levels comparable to the originally injected PDX cells, excluding the occurrence of antigen escape mechanisms also in this clinically relevant experimental setting (Supplemental Figure 11E).

T lymphocytes expressing anti-CD84 CARs do not target *bone fide* hematopoietic stem/progenitor cells

To evaluate the off-tumor activity of the anti-CD84 CAR-T cells, we co-cultured commercial $CD34^+$ HSCs derived from the BM of healthy donors with either B8 or F12 CAR-T cells or control T cells at 1:1 E:T ratio for 48 hours. $CD34^+$ viability was not affected by the incubation with the B8 or F12 CAR-T cells (Figure 6A). Moreover, no significant reduction in the number of colony-forming units (CFUs) generated by the $CD34^+$ cells exposed to B8 or F12 CAR-T cells *versus* control T cells *versus* untreated cells, nor variations in their differentiation pattern were observed (Figure 6B). To further assess any possible cytotoxic effect of the anti-CD84 CAR-T cells on the early human hematopoietic compartment, we established a humanized mouse model (Figure 6C). We intravenously injected $0.7x10^6$ BM-derived $CD34^+$ HSCs in NSG mice pre-treated with a busulfan myeloablative regimen and 3 days later, we infused the recipient mice with $3x10^6$ unmanipulated total BM cells from NSG donors to rescue murine hematopoiesis. Twelve weeks after, when human cell engraftment was robust, we inoculated the animals with

$2.1x10^6$ F12 or B8 CAR-T or control T cells (Figure 6C). Seven days later, a robust human cell engraftment was observed in the peripheral blood, BM, and spleen of all the study animals independently from the T cells received (control or B8 or F12 CAR-T) (Figure 6D and Supplemental Figure 12A-B). Notably, the content and clonogenic capacity of the $CD34^+$ HSCs retrieved from the CAR-T recipients were not different from those of animals injected with control cells (Figure 6E-F and Supplemental Figure 12C-D). Of note, we documented expression of CD84 at low levels (as compared to AML blasts) in freshly isolated T cells and in cells at the end of the transduction and expansion processes (Supplemental Figure 13A-B). CD84 was expressed at low levels also in PBMC subsets, and the expression was substantially lower than in blasts (Supplemental Figure 13C). To exclude that this expression could account for any off-target effects exerted by the anti-CD84 CAR-T cells on other hematopoietic cells, we tested the lysis potency of B8 CAR-T cells on unmanipulated T cells or whole PBMCs from healthy donors that resulted negligible (Supplemental Figure 13D). To assess the possible occurrence of fratricide activity by the anti-CD84 CAR-T cells we performed an *in vivo* kinetic study monitoring the presence of human $CD3^+$ cells. Importantly, this experiment showed that: i) in mock treated animals, the injected mCherry control T cells were no longer detectable while AML expanded, as expected; ii) in treated animals, the B8 CAR-T cells persisted in the long term and expanded at the expenses of the AML that was not detected; iii) although the human T cells continued to express CD84 as they did *ex vivo* and *in vitro*, CD84 levels remained substantially lower than those of the target AML CD33+ cells; iv) the fraction of G4S+ cells within human CD3+ cells in the treated mice remained consistent with the *in vitro* data along the entire *in vivo* monitoring, thus excluding a fratricide effect exerted by the effectors (Supplemental Figure 13E,F,G). The frequency of G4S+ in B8-CAR-T cells

is reported (Supplemental Figure 13F). Finally, as low CD84 protein expression was reported for respiratory tissue in protein-atlases, we tested B8 and F12 CAR-T cell lytic activity against the NL20 cell line, which is a not tumorigenic epithelial cell line isolated from normal human bronchi. Importantly, despite we confirmed a low but detectable CD84 expression in NL20 cells by flow cytometry (Supplemental Figure 14A), exposure of these cells to F12 and B8 CAR-T cells resulted in no or very minimal lysis as compared to the mCherry controls (Supplemental Figure 14B).

DISCUSSION

AML presents significant challenges in the development of targeted immunotherapies, largely due to its heterogeneous nature. A critical hurdle is the lack of TAAs specific to leukemic cells, expressed across the diverse genetic patients' subgroups and the disease stages including recurrence, while absent or minimally expressed on early hematopoietic progenitors [27,34,35]. Despite intense efforts and studies, none of the AML TAAs currently explored for clinical immunotherapy applications completely fulfills these requirements. One of the most explored TAAs in AML is CD123 as preclinical studies demonstrate robust anti-leukemia effects of CD123-directed CAR-T cells. However, being CD123 expressed on healthy myeloid cells [36] and because of conflicting data on the potential toxicity of CD123-targeted CAR-T cell approaches on the hematopoietic stem/progenitor cell compartment, this strategy in most trials is employed only as a bridge to allogeneic hematopoietic cell transplant. To address this lack of specificity, novel immunotherapies under investigation target CD123 or other TAAs in a combinatorial design with CD3-engagers [37–40]. Moreover, intense studies are ongoing to identify novel univocally and

reliably expressed AML target antigens, with PRAME, CLEC2A, TIM-3, CLL-1, and NKG2A being advanced in pre-clinical testing [39,41–45].

To contribute to these efforts, we prospectively interrogated a vast gene expression dataset from a cohort of >1500 pediatric AML patients and validated initial results by flow cytometry on an additional prospective cohort of >150 pediatric AML cases. This multistep process allowed us identifying in CD84/SLAMF5 the most promising candidate potentially fulfilling the AML TAA desired characteristics. In our AML cohorts, CD84 resulted expressed at diagnosis, relapse, and along treatment course, whenever myeloid blasts were detected. Importantly, CD84 was found expressed at disease recurrence also in the samples characterized by very low levels of MRD, and in refractory and secondary diseases, findings of outmost importance as these forms, which are often driven by the survival of residual LSCs evading initial therapy and acquiring additional mutations, remain the greatest challenge in AML treatment [46]. Notably, high CD84 expression was associated by recent literature to the most primitive LSC compartment in prospectively enrolled AML pediatric cases analyzed at the single cell level [33]. Importantly, our prospective study also revealed that CD84 expression is broad across AML subtypes and is not limited nor specifically associated to any genetic variants of AML, making it a promising target for therapies designed to treat the disease in a heterogeneous patient setting [47]. These findings are supported by the increasingly recognized role of SLAM-family receptors in cancer and cancer immunity [48], and their recent association to AML [29,33,49]. Importantly, we also documented a differential expression pattern of CD84 in AML *versus* healthy BM samples. CD84 expression in hematopoietic stem/progenitor cells was negligible if not completely absent at the protein and transcriptomic levels, respectively.

Similarly, other hematopoietic cells did not express CD84 at significant levels, except for myelo-monocytic cells that showed an expression detectable over the negative samples, but significantly lower than those measured in the AML blasts, with no documented functional effects in our *in vitro* and *in vivo* studies. In fact, our anti-CD84 CAR-T cells demonstrated neither direct toxicity nor lytic ability on the HSC/early progenitor compartment *in vitro*, nor hematologic toxicity *in vivo* in chimeras. Rather, clonogenic potential of the hematopoietic progenitors and their ability to sustain hematopoiesis *in vivo* upon treatment with anti-CD84 CAR-T cells were preserved. Overall, these data stand for a high targeting specificity for CD84, even at the LSC level, its expression persists at every stage of leukemogenesis, and at disease recurrence, and low risk of off-target effects, making it a uniquely viable target in the current AML landscape [50].

Likely because of these CD84 characteristics, the two ScFvs generated for CD84 targeting exerted a potent and specific antitumor activity against AML cell lines with different CD84 membrane densities, both *in vitro* and *in vivo*. This activity was confirmed on pediatric AML-PDX models with different CD84 expression levels. The anti-CD84 CAR-T cells showed lytic activity on the patients' cells also *in vivo* with significant amelioration of the survival of the treated *versus* control tumor-bearing animals. Notably, these findings were paralleled by a specific and coherent expansion of the anti-CD84 CAR-T cells in the peripheral blood of the treated mice, and not of the animals that had received the control T cells in which blasts were expanding. Efficacy was limited in the case of PDX#1 characterized by the lowest, barely absent, expression of CD84, thus confirming further the specificity and potential safety of the approach. Importantly, the persistence of CD84 expression on residual AML cells after CAR-T cell treatment,

provides further support for its role as 5TAA for AML and for the approach to be relevant in translational perspective. This latter finding could confer to CD84 targeting improved therapeutic precision and potency compared to more recent optimized approaches, such as the CD70-targeted [27] or CD7 CAR-T cells [35].

Overall, the expression of CD84 across multiple AML subtypes and genotypes, within the LSC subset and in relapsed samples, and after targeted treatment, in pediatric and adult patients, combined with its negligible expression in normal immature/precursor hematopoietic cells, render this antigen an ideal candidate for AML immunotherapy. Our data of efficacy and lack of off-target toxicities obtained with anti-CD84 CAR-T cells further supports this concept. Importantly, the *in vivo* long-term persistence of a mixed population of T cells (80% of which stably expresses the B8 CAR, as per G4S-linker staining) dismisses the potential occurrence of a direct lysis of the T cells by the B8 CAR T cells, albeit the former displays some basal CD84 expression. This may suggest that a certain CD84 expression threshold is needed to trigger a cytotoxic effect by T cells expressing B8. Thus, moving forward, research efforts should concentrate on translating these findings into early phase clinical trials. Indeed, although great expectations for AML treatment may reside in dual-targeting approaches or adoptive cell therapies based on CAR-NK, TCR-T cells or CRISPR-Cas9 engineered HSCs, and the choice between autologous and allogeneic cell therapy strategies is still unsolved [51,52], our findings on CD84 may bear the potential to boost a treatment opportunity for patients with relapsed and chemotherapy-recalcitrant AML forms, overcoming current limitations of AML TAAs in terms of efficiency of targeting and specificity. The recently highlighted role of CD84 in autoimmune diseases suggests that CD84 blockade may help controlling pathological

autoantibody production, allowing to hypothesize also a future broader interest for this immunotherapy approach [53,54].

METHODS

Ethics approval and consent to participate. This study included patients enrolled in the AIEOP-AML trials (AIEOP AML 2002/01 trial, EudraCT:2014-000976-25; AIEOP AML 2013/01 trial, EudraCT: 2014-000652-28). A signed informed consent was obtained, and the study was performed in accordance with the Declaration of Helsinki and approval by our local institutional review board. All clinical trials were approved by institutional review boards or ethics committees. All experiments and procedures involving animals were performed in compliance with all relevant ethical regulations and were explicitly approved by the Italian Ministry of Health and by the ethical committee of Institutional Animal Care and Use Committee (OPBA of University of Padova) (authorization n. 131/2022PR).

Human Cells and Cell lines. All AML cell lines were purchased by DSMZ. HL-60, Kasumi-1, and MOLM-13 were cultured in RPMI (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 1% Penicillin-Streptomycin (Gibco), 1% L-Glutamine (Gibco) and 10 % Fetal Bovine Serum (Gibco). MV4-11 and SHI-1 were cultured in Dulbecco's Modified Medium (GIBCO), supplemented with 1% Penicillin-Streptomycin, 1% L-Glutamine, and 10 % Fetal Bovine Serum. SHI-1-CD84^{KO} AML cell line was used as control being the CD84 gene knock-out (Synthego Corporation, Redwood City, CA). All cell lines were cultured in a humidified incubator with 5% CO2. Primary AML *ex vivo* cells were cultured at 37°C in RPMI Medium 1640 with 10% FBS, 2mM glutamine (Gibco), 100U/mL streptomycin/penicillin (Gibco), and supplemented with 50 ng/mL thrombopoietin (TPO), 50 ng/mL stem cell factor (SCF), 50 ng/mL FMS-like tyrosine

kinase 3 ligand (Flt3L), 20 ng/mL interleukin-3 (IL 3) and 20 ng/mL interleukin-6 (IL 6); all these cytokines purchased from Miltenyi Biotec (Miltenyi Biotec, Bergisch Gladbach, DE). Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors by Ficoll density separation after obtaining written, informed consent. Human bone marrow (BM) $CD34^+$ cells for flow cytometric evaluation (n=5) (StemExpress/ CGT global, Folsom, CA, USA) and for in vitro experimentation were cultured in Serum-free StemSpan SFEM II medium (StemCell Technologies, Vancouver, Canada), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 ng/ml hSCF (Miltenyi Biotec), 100 ng/ml hFlt3-L (Miltenyi Biotec), hTPO 100 ng/mL (Miltenyi Biotec), hIL-6 (Miltenyi Biotec) and 0.75 µM StemRegenin1 (SR1) (Cellagen Technology, San Diego, CA, USA). CD34+ cells were cultured in a 5% CO2 humidified atmosphere at 37 °C.

RNA-seq analysis. We used deposited transcriptome (GSE75461) data of 85-AML cases[31] and we generated a list of genes being hyperexpressed (of >2.4 fold), as compared to healthy controls, predicted by cellular surface protein antigens tool (CSPAs, http://wlab.ethz.ch/cspa/). These results were further refined with data deposited at National Cancer Institute's Genomic Data Commons (https://gdc.cancer.gov/) under the TARGET-AML project. The initial list included 76 genes that were hyperexpressed in AML cells, with 26 out of 76 genes found to be over-expressed with respect to healthy bone marrow (BM). We refined our selection process by analyzing public databases via GeneCards. This analysis included examining TAA functional descriptions, disease associations, mRNA and protein expression patterns, subcellular locations, and relevant pathways. We included only genes not previously identified as cancer-related TAAs, particularly those unassociated with AML or unused in prior CAR-T development projects.

Then, we conducted flow cytometry tests on at least 4 AML cell lines and healthy PBMC to finally select 6 novel AML TAAs with CD84 being the best candidate for immunotherapy.

Flow cytometry. BM samples from AML patients and healthy controls were stained for 20 minutes with CD84 (APC, Biolegend; 326010) in combination with antibodies specific for detecting blasts or BM other residual populations [55]. Samples were resuspended with 5% BSA in PBS, acquired using a FACSCanto II (Beckton Dickinson) and analysed by Kaluza software (version 2.2, Beckman Coulter). CD84 expression was rated comparing the fluorescence shift and distribution pattern of blasts compared to appropriate negative controls.

Immunofluorescence. CD84 antibody (Novus Biological, Centennial CO, USA) and a goat-anti mouse IgG Alexa488 antibody (Life Technologies) as secondary antibody were used. Cells (3×10^5) were seeded to the bottom of culture chamber slides (FALCON, Big Flats, NY, USA) pre-coated with fibronectin (40 ug/ml) (Corning, NY, USA) for 2 hours at 37°C. After this incubation time, cells were stained with MemBrite Fix dye (Biotium), a specific membrane dye, for 5 minutes at 37°C according to guidelines. Cells were fixed with 4% formaldehyde in PBS for 15 minutes and, after washing, blocked with 3% BSA in PBS for 30 minutes. After saturation, cells were stained with CD84 together with Fc Receptor Blocking (Milteny Biotec) overnight at 4°C, and then secondary antibody for 1 hour at room temperature. Cells were imaged with a Zeiss 2.6 laser scanning microscope. Images were analyzed using Image J win 32 software.

***In vitro* phage display biopanning single-chain variable fragment (scFV) generation and cloning.** *In vitro* phage display biopanning (4 rounds) was performed using a human naïve LiAb-SFMax library (high diversity of $5.37x10^{10}$ variants) with CD84 Antigen purified by SDS-PAGE. Phage clones were then isolated through bacterial infection. Screening and validation of at least 96 single phage binders was performed by polyclonal ELISA; then, monoclonal phage ELISA (single phage binders screening) was used to select clones and peptides to be sequenced (ProteoGenix, France). Two different unique sequences (namely B8 and F12) were identified and re-tested by monoclonal ELISA with CD84 antigen to confirm binding. Then, two CAR cassettes (1506 bp) were synthesized and cloned from pUC57 vectors into a 3rd generation LV transfer plasmid under the control of the human phosphoglycerate kinase promoter. One Shot TOP10 chemically competent E.coli (ThermoFisher, Waltham, MA, USA) were transformed, and DNA was extracted with Plasmid DNA Maxiprep Kit (ThermoFisher). The procedure was controlled by digestion with BamHI and SalI enzymes and agarose gel electrophoresis. Sanger Sequencing of the plasmids was performed to verify the correct insertion of the cassette in the backbone.

Lentiviral vector production and transduction of human T cells. Lentiviral vectors (LVs) were produced via transient transfection of the HEK293T packaging cell line. Briefly, 70% of confluent cells were co-transfected with Gag/Pol (III gen), Env and Rev packaging plasmids, pAdvantage plasmid (pADV), and the transfer vector plasmid. After 48 hours from the transfection, the lentiviral supernatant was collected, ultracentrifuged, aliquoted, and stocked at -80°C. T cells in TransACT activation-expansion were LV transduced as described above. To evaluate transduction efficiency, the vector copy

number (VCN) per cell was measured. Total genomic DNA from transduced T cells was extracted after 14 days from the transduction with the Dneasy Blood & Tissue Kit (Qiagen). To determine the VCN per cell, digital droplet PCR with the reaction mixture containing ddPCR Supermix (Bio-Rad) and the primer-probe sets for target and reference genome (ddPCRTM CNV Assay (FAM); ddPCRTM CNV Assay (HEX)) was used. The droplets were made by Automated Droplet Generator (Bio-Rad) and read with a QX200 droplet reader (Bio-Rad). The VCN was analyzed with QuantaSoft droplet reader software and determined by the ratio of the target-gene concentration to the reference-gene concentration, multiplied by the number of copies of the reference gene in the reference genome.

$CD4^+$ and $CD8^+$ cell isolation from peripheral blood mononuclear cells and T-cell expansion. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density gradient centrifugation using Lymphoprep (StemCell technologies). From PBMCs, $CD4^+$/$CD8^+$ T cells were magnetically isolated using autoMACS cell separator (Miltenyi Biotec) with CD4 and CD8 MicroBeads (Miltenyi Biotec) according to manufacturer's instructions. Isolated T cells were then activated in vitro for three days in TexMACS Medium (Miltenyi Biotec) additioned with TransACT (Miltenyi Biotec), 1% P/S and recombinant human IL-7 (500 IU/ml) and of IL-15 (84 IU/ml) (Miltenyi Biotec). Transduction was performed on day 3 by supplementing the media with the selected LV supernatant at a multiplicity of infection (MOI) of 5 in the presence of 0.01 mg/mL Vectofusin-1 (Miltenyi Biotec), as per optimized conditions, for a total incubation time of 16 hours. On day 4, cells were washed from TransACT and lentivirus and then maintained in TexMACS with IL-7 and IL-15 at $1x10^6$ cells per mL for 14 days of expansion. The state

of % T cell transduction was monitored by mCherry expression in control T cells and by G4S linker antibody (Cell Signaling Technology, The Netherland) in B(-CAR T cells. T cell activation and exhaustion were regularly tested at the end of manufacturing.

Activation, exhaustion, and differentiation markers by Flow Cytometry. Cells were stained with fluorochrome-conjugated primary antibodies and isotype control for 15 minutes at RT. Stained cells were washed and immediately analyzed with FACSCelestaTM Cell Analyzer (BD Biosciences). The following antibodies were used (by Miltenyi Biotec): CD3 (PE-Vio615), CD4 (VioGreen), CD8 (APC), CD45 (VioBlue), CD56 (PE), CD16 (PE), CD14 (APC), CD34 (PE), CD38 (APC), CD197 (VioBlue), CD95 (PE), CD62L (PE), CD45RO (APC), CD223 (VioBlue), CD279 (PE), CD366 (APC), CD154 (VioBlue), CD25 (PE), CD137 (APC), CD69 (PE), CD84 (APC) CD107a (PE), CD33 (FITC). Analyses were performed by using FlowJo software (BD Biosciences, v10).

***In Vitro* Cytotoxicity Assay.** Cytotoxic assays were conducted by co-culturing AML target cell lines (positive for CD84) or *ex vivo* AML cells from PDX models or PBMCs or T cell with CAR-T cells or untransduced T cells for 48 hours at an E:T ratio of 1:1 at the end of T cell expansion (day 17). Co-cultured cells were stained with Annexin V-PE (Miltenyi Biotec) and 7-AAD Staining Solution (Miltenyi Biotec) and analyzed by flow cytometry with FACSCelesta^{TM} Cell Analyzer and FlowJo software. The percentage of cell lysis was calculated using the following formula:

% of lysis = [100 - (N of alive AML cells in coculture with CAR T cells)/(N of alive AML cells cultured with empty CAR or alone)] * 100

Alternatively, the bioluminescence of GFP-Luciferase (LUC) transduced AML cells was analyzed after 48 hours from co-culture with CAR-T cells. Briefly, cells were centrifuged and resuspended in 50uL of PBS 1X and then incubated with XenoLight D-luciferin firefly (15mg/mL in PBS; Perkin Elmer, Waltham, MA) for 10 min. LUC activity was measured by a Spark–Tecan multi-well plate reader (Tecan Group Ltd., Männedorf, Switzerland), and data were reported as a percentage of AML cell lysis (BLI).

Cytokine intracellular staining. Following 6, 24 and 48 hours of co-culture between anti-CD84 B8, F12 and $CD84^+$ targets (SHI-1, HL-60 and *ex vivo* AML cells from AML-PDXs) at 1:1 E:T ratio, the cytokine production capacity of anti-CD84 B8, F12 ScFv(s) was evaluated by flow cytometry. Briefly, $0.4x10^6$ cells were initially labeled for cell surface antigen expression (CD3-APC (Beckam Coulter, Marseille, France), CD33-PC5 (Beckam Coulter) for 20 minutes, to discriminate between effector CAR-T cells and target AML cells. Then, by using the Inside Stain Kit (Miltenyi Biotec) and according to the manufacturer's instructions, the cells were fixed, permeabilized, and stained with anti-IFNγ and anti-TNFα antibodies. Eventually, percentages of IFNγ and TNFα positive-expressing cells were estimated with respect to total lymphocytes.

CAR-T cytokine release assay. BioRad Bio-Plex200 was used to analyze CAR-T cytokine release alone or in coculture with AML cell lines. After 48 h coculture of CAR-T cells (B8, F12, or mCherry) and AML cell lines at 1:1 E:T ratio, cell culture supernatants were collected, clarified by centrifugation, and stored at -80°C until analysis. Cytokine levels were quantified by an external service provider using a multiplex immunoassay platform BioRad Bio-Plex200, adhering to standard protocols.

Colony forming cell (CFC) assay. Commercially available $CD34^+$ cells from healthy donors (Hu BM CD34+, StemExpress) were co-cultured either with media alone, mCherry or anti-CD84 B8 or F12, and CAR T cells at E:T ratio of 1:1 for 4 hours. Following incubation, the cell suspension was added to the semisolid methylcellulose-based medium Methocult H4534 Classic without EPO (StemCell Technologies Inc, Vancouver, British Columbia, Canada) and plated into 3-cm tissue culture dishes. After 12 days, colonies derived from granulocyte-macrophage (G, M, and GM) and multipotential granulocyte, erythroid, macrophage, megakaryocyte (GEMM) progenitors were scored and enumerated according to manufacturer's instructions and total number of colonies was assessed.

***In vivo* experiments in NSG mice.** Procedures involving animals and their care were carried out in accordance with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December 1987) and with "ARRIVE" guidelines (Animals in Research Reporting *In Vivo* Experiments). Ministry authorization approved: 131/2022-PR. NSG mice (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ female or male of 4-5 weeks old, 20-25 g/mouse, maximum 5 animals/cage) were injected intravenously (tail vein) with either 0.5×10^6 when using SHI-1-LUC, or HL60-LUC cell lines, and at day+3 from AML injection treated by intravenous injection of 1.5×10^6 target or empty CAR-T cells as control at a E:T ratio of 3:1. For AML-PDX experiments, NSG mice were tail-injected intravenously with 0,25 or 0,5 or $1x10^6$ AML *ex vivo* from AML-PDX cells transduced with the luciferase gene. Then, at day +3 or day +28 from AML cells injection mice were tail-injected with CAR-T cells. The final E:T 3:1 dose starting from $0,25x10^6$ AML injection was used. Bioluminescence was

monitored to verify tumor growth, by intra-peritoneal injections with XenoLight D-luciferin firefly (15mg/ml in PBS; Perkin Elmer, Waltham, MA) 10 min before measurement (Xenogen IVIS Spectrum bioluminescence/optical imaging system, Xenogen Corporation, Alameda, CA). To monitor tumor burden and T cell persistence we used human CD33 (hCD33) and CD3 (hCD3) in peripheral blood (PB) by flow cytometry every week until mice sacrifice. At sacrifice (performed at the humane endpoint, without exceeding maximal tumor burden as defined by ethics committee; ministry authorization approved: 131/2022-PR), organs were recovered and flushed or mechanically dissociated to harvest and analyze human cells.

Off-tumor effect model in NSG mice. NSG (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ of 4-5 weeks old, 20-25 g/mouse, maximum 5 animals/cage) were conditioned with busulfan (16.25 mg/kg per 4 days). Cells that were administered i.v. (0.7×10^6/mouse) were CD34+ HSCs (StemExpress) derived from the human BM. Mice were then provided with syngeneic BM for hematopoietic rescue 4 days post-transplant. At week +12 post-transplantation, mice were tail-vein injected with $2.1x10^6$ (3:1 E:T) control T or anti-CD84 B8 ScFv CAR T cells. After 1 week animals were sacrificed and cells from femour and spleen were collected and analyzed for hCD45 and CD34+ expression by flow cytometry; 6000 cells were seeded in semisolid media to test the clonogenic potential.

Statistical analyses. The Mann Whitney or T-test was adopted for detecting significance between differences in means of 2 groups/conditions.One-way ANOVA or Kruskall Wallis for multiple comparisons was used when more than 2 groups/conditions were compare; when indicated, pairwise comparisons between specific groups were performed using Mann–Whitney test. Log-Rank MantelCox test was used for survival analyses. Details

on the exact statistical test used as well as the number of subjects and samples are included in the respective figure legends. P-values are reported in the respective figure legends whenever possible. No statistical method was used to predetermine sample size. Graphs and associated statistical analyses were generated using GraphPad Prism 8.

DATA AVAILABILITY

RNA-data are deposited in public repositories: NCBI's Gene Expression Omnibus Accession number, GEO GSE75461 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75461); National Cancer Institute's Genomic Data Commons (https://gdc.cancer.gov/) under the TARGET AML (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000465.v18.p7). Source data relative to all figures are provided as a Source Data excel file.

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ACKNOWLEDGMENTS. This manuscript is *in memoriam* of Professor Giuseppe Basso, a mentor and a guide, for his legacy at the Division of Pediatric Hematology, Oncology and Stem Cell Transplant in Padova. The authors thank the staff of Division of Pediatric Hematology, Oncology and Stem Cell Transplant, Women's and Children's Health Department for diagnostic activity and biobanking (Pediatric Oncology BioBank-BBOP) for the biological samples management, in particular Katia Polato for the genetic screening, to Drs Pamela Scarparo, Barbara Michielotto and Chiara Frasson for flow cytometry. We are grateful to Drs Barbara Montini and Elena Porcù for the preliminary data support. We are grateful to all AIEOP centers.

This work was supported by grants from Istituto di Ricerca Pediatrica-Fondazione Città della Speranza (to AB and MP); Fondazione Associazione Italiana Ricerca sul Cancro (AIRC, IG grant 20562 to MP); by Italian PNRR CN3 "National Center for Gene Therapy and Drugs Based on RNA Technology" (to FL and AB); by sponsored research Altheia science (to AB).

AUTHOR CONTRIBUTIONS STATEMENT. S.Mer., A.D.R., O.M, N.F performed *in vitro* and *in vivo* experiments; G.F., R.M. and B.B. contributed to flow cytometry and immunophenotipic analysis; S.Mes and F.L. contributed to sample collection, data curation, manuscript review and editing; G.B: who passed away during the course of this work, initiated the project, conceptualized the study, and secured key laboratory resources; P.R., M.P. and A.B. contributed to conceptualization, resources, supervision, investigation, methodology, project management, manuscript writing–review and editing.

COMPETING INTERESTS STATEMENT

AB and MP are inventors on the following patent applications: PCT/EP2022/059031 and PCT/EP2023/059054. Altheia Science owns the rights for their exploitation. PR is an employee of Altheia Science. AB and MP are scientific advisors for Altheia Science; AB is a shareholder of the company. The remaining authors declare no competing interests.

TABLES

Table 1. AML patients' characteristics at diagnosis.

Patients (n)	99
Age (years, average)	8,2
Gender	
Male	54
Female	45
Blasts, bone marrow (%, average)	65
Genetics (n)	
t(8;21)*RUNX1::RUNX1T1*	8
inv(16)*CBFB::MYH11*	10
NPM1	4
NPM1 and FLT3-ITD	7
KMT2A-r	24
FLT3-mut	12
t(16;16)*CBFA2T3::GLIS2*	2
NUP98-r	3
No marker	28
Others (rare)	1

Table 2. AML patients' characteristics at relapse.

Patients (n)	32
Age (years, average)	10,6
Gender	
Male	16
Female	16
Blasts, bone marrow (%, average)	38
Genetics (n)	
t(8;21)*RUNX1::RUNX1T1*	5
inv(16)*CBFB::MYH11*	2
KMT2A-r	9
FLT3-mut	2
t(16;16)CBFA2T3::GLIS2	3
NUP98-r	1
Others (rare)	1
No marker	9

FIGURE LEGENDS

Figure 1. Transcriptomic profiling of primary patient samples identifies CD84 as highly expressed in pediatric AML. A) Experimental workflow to candidate TAA identification. Created in *BioRender. Smith, J. (2025). BioRender.com/c248457*. **B**) Immunofluorescence staining showing localization of CD84 (green dots) on the cell

membrane (orange-colored dots represent Membrite membrane-specific distribution) of the HL-60 AML cell line. Representative of n=3 biological replicates. **C**) Expression level of CD84 evaluated by flow cytometry on different AML cell lines. The expression peaks are scaled as percentage of the maximum count. Representative of n=3 biological replicates. **D**) TARGET dataset-derived CD84 expression levels in 1491 AML patients' samples. **E**) Dot plots from 2 representative pediatric AML BM samples processed by flow cytometry for CD84 staining; blasts are identified according to CD45 expression and physical parameters. **F**) CD84 Mean fluoresce intensity (MFI) measured on the blasts identified according to the approach shown in (E). A total of 99 samples from patients at diagnosis, 32 at relapse and 11 at follow up with MRD (each dot corresponds to an individual patient sample) are represented. 85 cases at diagnosis, 29 at relapse and 11 samples with MRD were positive for CD84 expression with respect to 14 at diagnosis and 3 at relapse that were negative as per WHO classification significance Kruskal-Wallis test, ****p<0.0001; CD84 expression of the same positive AML samples is shown on the right panel subdivided based on genetic markers.

Figure 2. CD84 expression profiling in not-leukemic hematopoietic cells. (**A**) Unsupervised clustering by uniform manifold and projection (UMAP) analysis of single cell RNA sequencing profile from a healthy BM sample. **B**) Normalized CD84 expression on same samples showing low/no expression in HSC and CMPs. The arrows in (A) and (B) indicated the HSC compartment. **C**) Quantification of CD84 expression (MFI) in the reference AML blast population at diagnosis (n=99, CD84 positive blasts in red and CD84 negative blasts in white) and the normal counterparts from healthy BM. HSCs (n=13) are

shown in grey. Individual values are shown; significance Kruskal-Wallis, **p=0.0012, ****p<0,0001.

Figure 3. Anti-CD84 CAR-T cells recognize and kill AML cell lines *in vitro*. A) Diagram of the anti-CD84 CAR constructs, depicting the ScFVs (B8 or F12), the hinge, the transmembrane domain (TMD), the costimulatory domain (CD) and the signaling tail (ST). Control LV encodes mCherry. Created in *BioRender. Smith, J. (2025). BioRender.com/c248457*. **B)** Transduction efficiency of T lymphocytes by the LVs shown in (A) was determined at end of in vitro culture (day 17) by measurement of the number of integrated viral copies per cell (vector copy number, VCN) by ddPCR, Data obtained from CAR T-cell products generated from 5 independent donors across 10 independent productions (n=12 mCherry, n=10 B8, n=3 F12 measurements). **C**, **D**) Anti-CD84 CAR-T cell ability to lysate target LUC-transduced AML cell lines C) measured by bioluminescence (BLI). Two-sided Mann-Whitney test, ****p<0,0001, ***p=0,0003, **p=0,0061and D) by Annexin V and 7AAD staining. Two-sided Mann-Whitney test, ****p<0,0001, ***p=0,0001, ***p=0,0003, **p=0,002 respectively. Percentage of cell lysis exerted by a different CAR-T product was normalized respect to the mCherry. Data are represented as mean±SD of 3 to 11 different experiments. **E**) TNFα (left panel) and IFNγ (right panel) expression on mCherry or B8 or F12 LV transduced and expanded T cells after 48 hours of co-colture with target SHI-1 or HL-60 AML cell lines at 1:1 E:T ratio, measured by flow cytometry. Data are normalize to mCherry and represented as mean±SD of 3 to 6 independent experiments. Two-sided Mann-Whitney test, left panel ***p=0,0002, **p=0,0045, ***p=0,0005, **p=0,0045 respectively, right panel **p=0,0030, **p=0,0083, **p=0,0030, **p=0,0083 respectively. **F**) Cytokine concentrations of secreted

TNFα, IFNγ, IL-2 and Granzyme B in the supernatant from the experiments shown in (E, SHI-1). **G**) CD84 molecules per cell in AML cell lines (MKPL-1 and SHI-1); data are represented as mean±SD, n=3 biological replicates. **H**) Dose response *in vitro* cytotoxicity of B8 or F12 CAR-T at increasing E:T ratios normalized to mCherry in a 48-hour co-colture assay, evaluated by AV/7AAD expression at flow cytometry. Data are represented as mean±SD of 5 to 10 biological replicates. **I)** Surface plasmon resonance (SPR) sensorgrams of binding kinetics between CD84 and B8 (D-upper) and F12 (D-lower) ScFvs.

Figure 4. *In vivo* efficacy and specificity of anti-CD84 B8 and F12 CAR-T cells on AML cell lines. A) NSG mice were injected with $0.5x10^6$ SHI-1 AML-LUC positive cells and, 24 hours later, with $1.5x10^6$ B8 or F12 or CAR-T cells or control mCherry T cells (3:1 effector to target ratio). AML engraftment was monitored weekly by LUC bioluminescence signal reduction (represented as total flux). n=4–12 mice per group at each time point. Two-sided Mann-Whitney test, *p=0.0117 **p=0.0078. **B**) Survival rates of NSG mice injected with SHI-1 AML cell line and F12/B8 or mCherry CAR-T cells; n=5 animals/group. Log-Rank MantelCox test, *p=0.01 **p=0.0038. **C**) B8/F12 CAR-T and mCherry T cell distribution in peripheral blood (PB) and organs (bone marrow, BM, and spleen, SPL) at sacrifice measured by flow cytometry (CD3+ cells on total human CD45 alive cells). n=4–12 mice per group per organ. Two-sided Mann-Whitney, **p=0.0016, ***p=0.0007, **p=0.0031 respectively. **D**) CD84 cell membrane expression on not-target AML cell lines analyzed by flow cytometry. The expression peaks are scaled as a percentage of the maximum count. **E**) SHI-1^{CD84KO} vs K562 vs U937 cell lines engraftment following treatment with F12 or B8 or mCherry CAR-T cells was monitored by LUC. Data are

represented as mean±SD of n=3 to 10 mice per group. **F**) Survival rates of NSG mice injected with the SHI-1^{CD84KO} AML cell line and F12/B8 or mCherry T cells. n=4 to 5 animals group.

Figure 5. *In vivo* efficacy of B8 and F12 CAR-T cells on pediatric AML patients' derived xenograft models. A) CD84 expression by flow cytometry in different *ex vivo* cell samples from pediatric AML-PDXs. The expression peaks are scaled as a percentage of the maximum count. **B**) B8 and F12 CAR-T cell (and mCherry T cell) killing potency on *ex vivo* cells from AML-PDXs co-coltured for 48 hours at 1 to 1 E:T ratio and analyzed by flow cytometry (% of dead cells 7AAD+ and/or AV+; n=14 B8 and n=7 F12 technical replicates derived from independent *ex vivo* AML-PDX models (7 models for B8 and 6 models for F12). Data are represented as mean±SD. Two-sided Mann-Whitney, ****p=0.0001.**C**) TNFα and IFNγ expressing T cells following 48 hours of co-colture in experiments in (B) measured by flow cytometry. Data are represented as mean±SD. One-way ANOVA, **p=0.0032, n=3 biological replicates. **D**) Cytokine concentrations of secreted TNFα, IFNγ IL-2 and Granzyme B in the supernatant from the experiments in (B) measured by multiplex bead assay using a BioRad Bio-Plex 200 system. **E**) AML-PDX #3 and #8 engraftment and spread monitored weekly by LUC bioluminescence analysis (LUC signal reduction is shown represented as total flux), after administration of the B8 or F12 CAR (or mCherry control) T cells. Representative images (left) and total flux quantification (right); graph shows pooled data from AML-PDX #3 and #8 (n=8 mCherry, n=7 B8, n=10 F12). Data are represented as mean±SD. One-way ANOVA performed at week 10 comparing all groups to mCherry,****p<0.0001. (**F**) Survival rates of treated mice is reported (n=19 B8/F12 treated AML-PDX, n=6 mCherry treated AML-

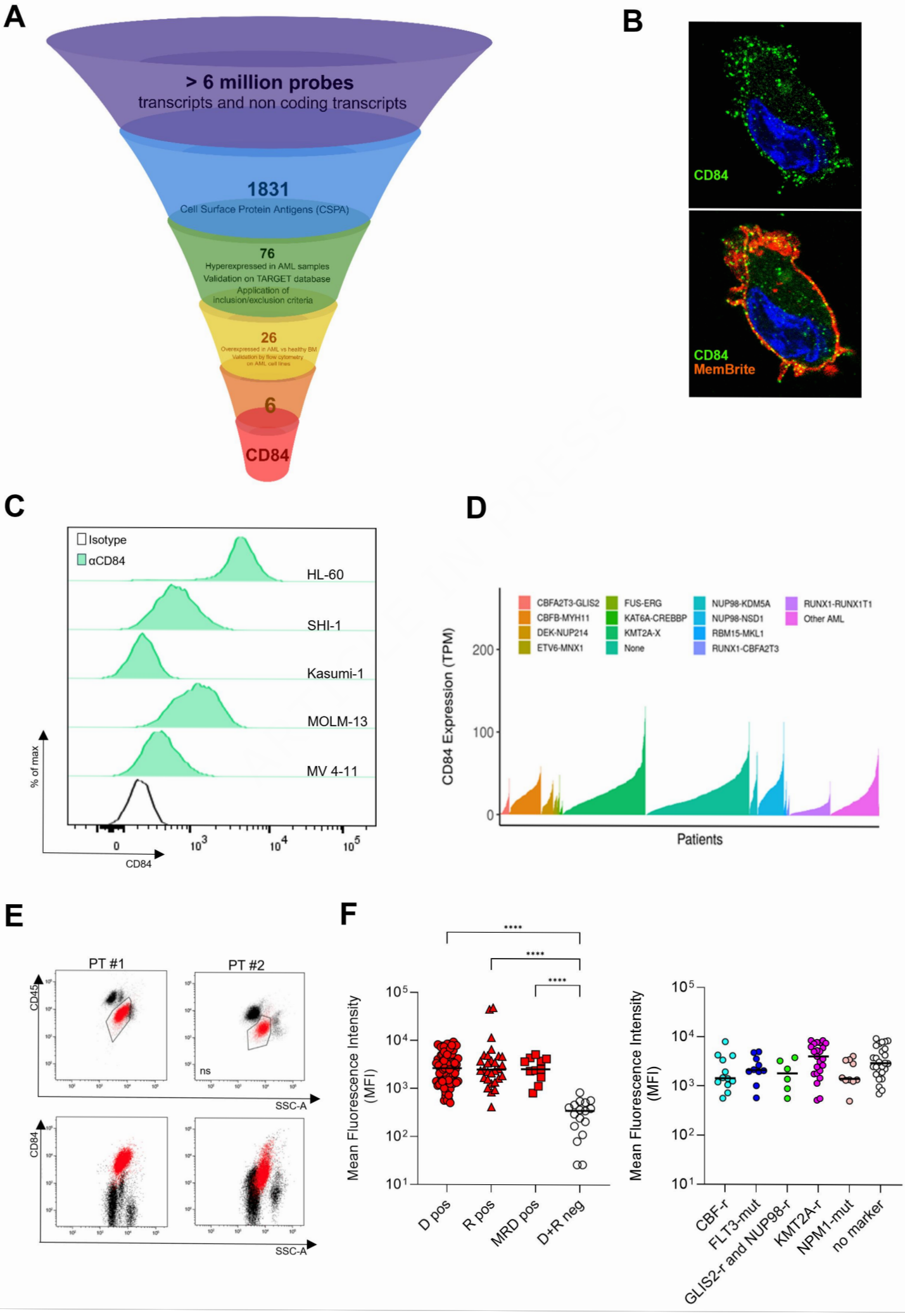
PDX). Log-Rank MantelCox test, ****p<0.0001. **G**) % T cells and AML cells were monitored weekly in the peripheral circulation by flow cytometry via anti-hCD3 and anti-hCD33 antibodies. Data are represented as mean±SD. n=9 mCherry, n=10 B8, n=15 F12 mice.

Figure 6. Off-target cytotoxicity of B8 and F12 scFv chains evaluation on *bona fide* hematopoietic stem/progenitor cells. A) Viability (7AAD+ and/or AV+ dead cells) of CD34+ HSCs after exposure to effector CAR-T (or mCherry) T cells *in vitro* in a 48-hour co-culture assay, measured by flow cytometry. Data are represented as mean±SD. n=11 mCherry, n=6 B8, n=5 F12, across n=3 biological replicates. **B**) Absolute total number (left panel) and morphology (right panel) of colony forming cells (CFCs) generated by $CD34^+$ HSCs untreated, or treated with CAR-T or control mCherry T cells in an E:T ratio of 1:1 for 6 hours and then plated in MethoCult medium for 12 days (multipotential granulocyte, erythroid, macrophage, and megakaryocyte colonies, granulocyte-macrophage colonies, granulocyte and macrophage colonies: CFU-GEMM, CFU-GM, CFU-G and CFU-M, respectively). Data are represented as mean±SD. n=4 untreated, n=9 mCherry, n=6 B8, n=6 F12, across 3 biological replicates. **C**) Schematic representation of the *in vivo* study. Created in *BioRender. Smith, J. (2025). BioRender.com/c248457*. **D**) Human cell engraftment quantified by flow cytometry (hCD45 expression) in the peripheral blood (PB), spleen and bone marrow (BM) of mice injected with control or B8 or F12 CAR-T cells upon sacrifice. Data are normalized on mCherry and represented as mean±SD. n=8 mCherry, n=9 B8, n=3 F12 mice across n=2 independent experiments. Two-sided Mann-Whitney, *p=0.0121. **E**) Frequency of $CD34^+$ human cells in the BM of mice at sacrifice. Data are represented as mean±SD. n=8

mCherry, n=9 B8, n=3 F12. **F**) CFCs generated by plating the $CD34^+$ cells isolated from the BM of the mice. Data are normalized on mCherry and represented as mean±SD. n=8 mCherry, n=9 B8, n=3 F12 across n=2 independent experiments.

Chimeric antigen receptor (CAR)-T cell therapy has been shown successful in a range of malignancies but due to an absence of notable antigenic targets has limited its application to acute myeloid leukaemia (AML). Here the authors identify CD84 as a potential target for AML and show that the application of CD84 CAR-T in models of AML results in reduced tumour burden and enhanced survival.

Peer review information: *Nature Communications* thanks David Sedloev, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. A peer review file is available.


A
> 6 million probes
transcripts and non coding transcripts
1831
Cell Surface Protein Antigens (CSPA)
76
Hyperexpressed in AML samples
Validation on TARGET database
Application of
inclusion/exclusion criteria
26
Overexpressed in AML vs healthy BM
Validation by flow cytometry
on AML cell lines
6
CD84
B
CD84
CD84
MemBrite
C
Isotype
αCD84
HL-60
SHI-1
Kasumi-1
MOLM-13
MV 4-11
% of max
0
10^3
10^4
10^5
CD84
D
CBFA2T3-GLIS2
CBFB-MYH11
DEK-NUP214
ETV6-MNX1
FUS-ERG
KAT6A-CREBBP
KMT2A-X
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NUP98-KDM5A
NUP98-NSD1
RBM15-MKL1
RUNX1-CBFA2T3
RUNX1-RUNX1T1
Other AML
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100
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Patients
E
PT #1
PT #2
CD45
ns
SSC-A
CD84
SSC-A
F
****
****
****
Mean Fluorescence Intensity (MFI)
10^5
10^4
10^3
10^2
10^1
D pos
R pos
MRD pos
D+R neg
Mean Fluorescence Intensity (MFI)
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FLT3-mut
GLIS2-r and NUP98-r
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NPM1-mut
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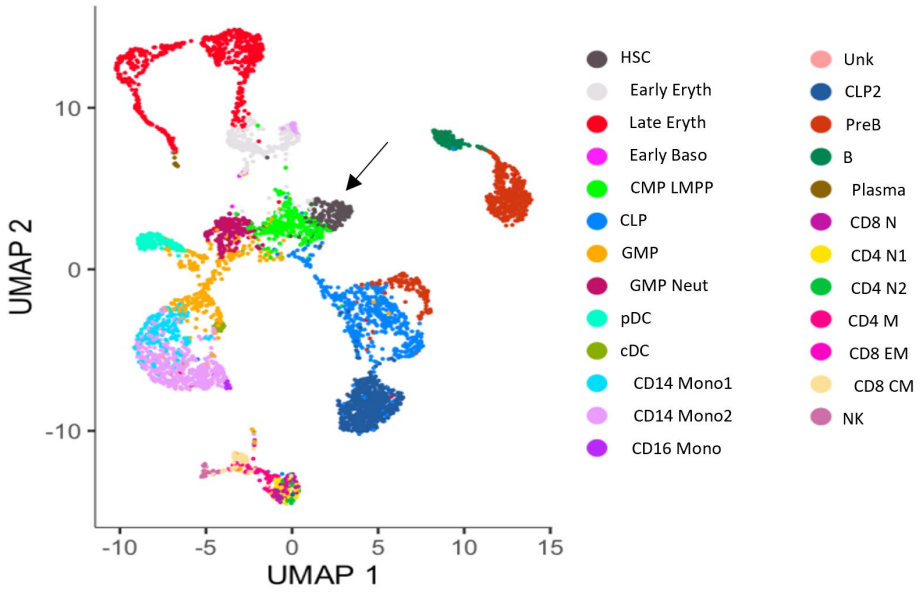
A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UMAP 1
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-10
-10
-5
0
5
10
15
HSC
Early Eryth
Late Eryth
Early Baso
CMP LMPP
CLP
GMP
GMP Neut
pDC
cDC
CD14 Mono1
CD14 Mono2
CD16 Mono
Unk
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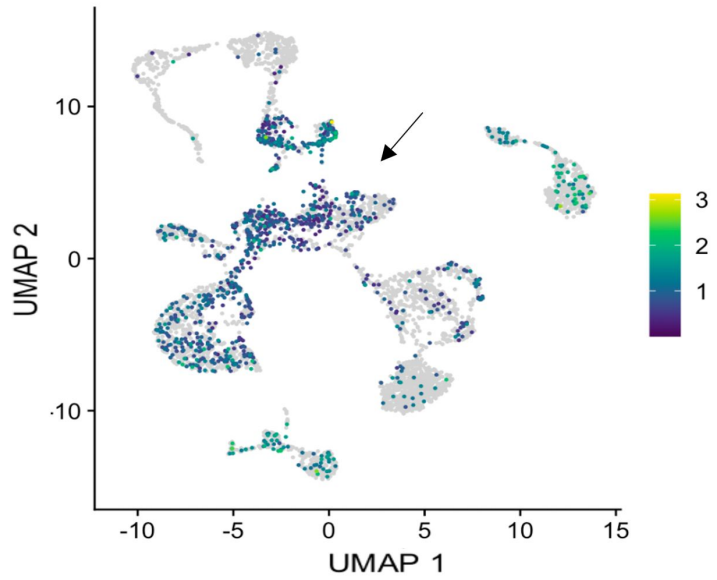
B
Plasma
CD8 N
CD4 N1
CD4 N2
CD4 M
CD8 EM
CD8 CM
NK

B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15
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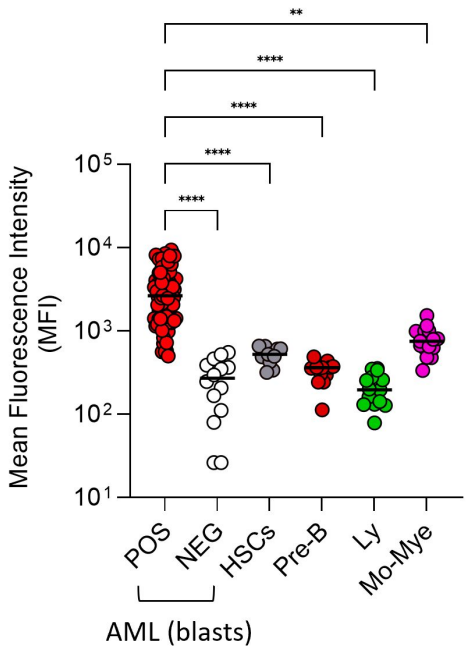
2
1

C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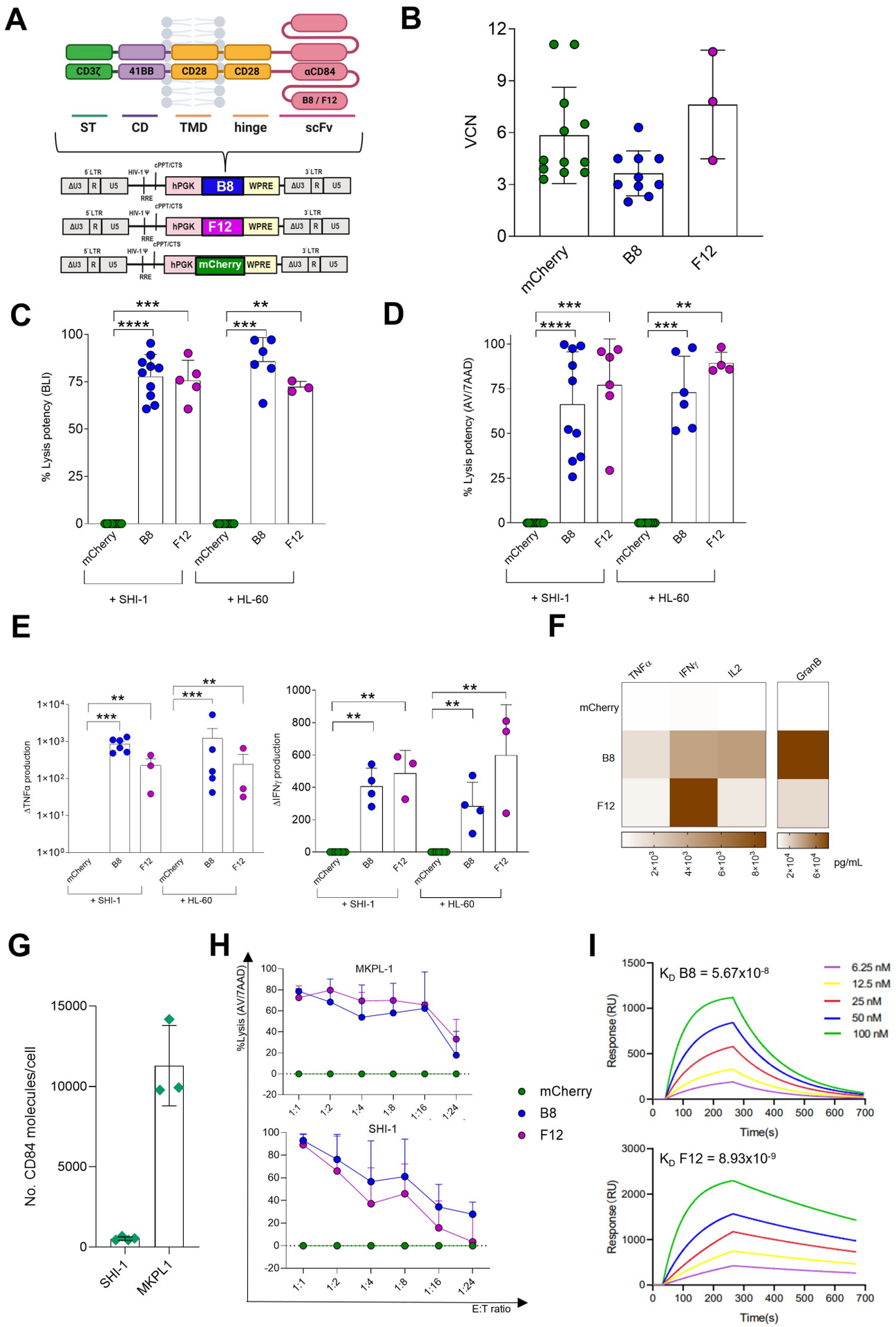
10^4
10^3
10^2
10^1
**
****
****
****
****
POS
NEG
HSCs
Pre-B
Ly
Mo-Mye
AML (blasts)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CD3ζ
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CD28
CD28
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B8 / F12
ST
CD
TMD
hinge
scFv
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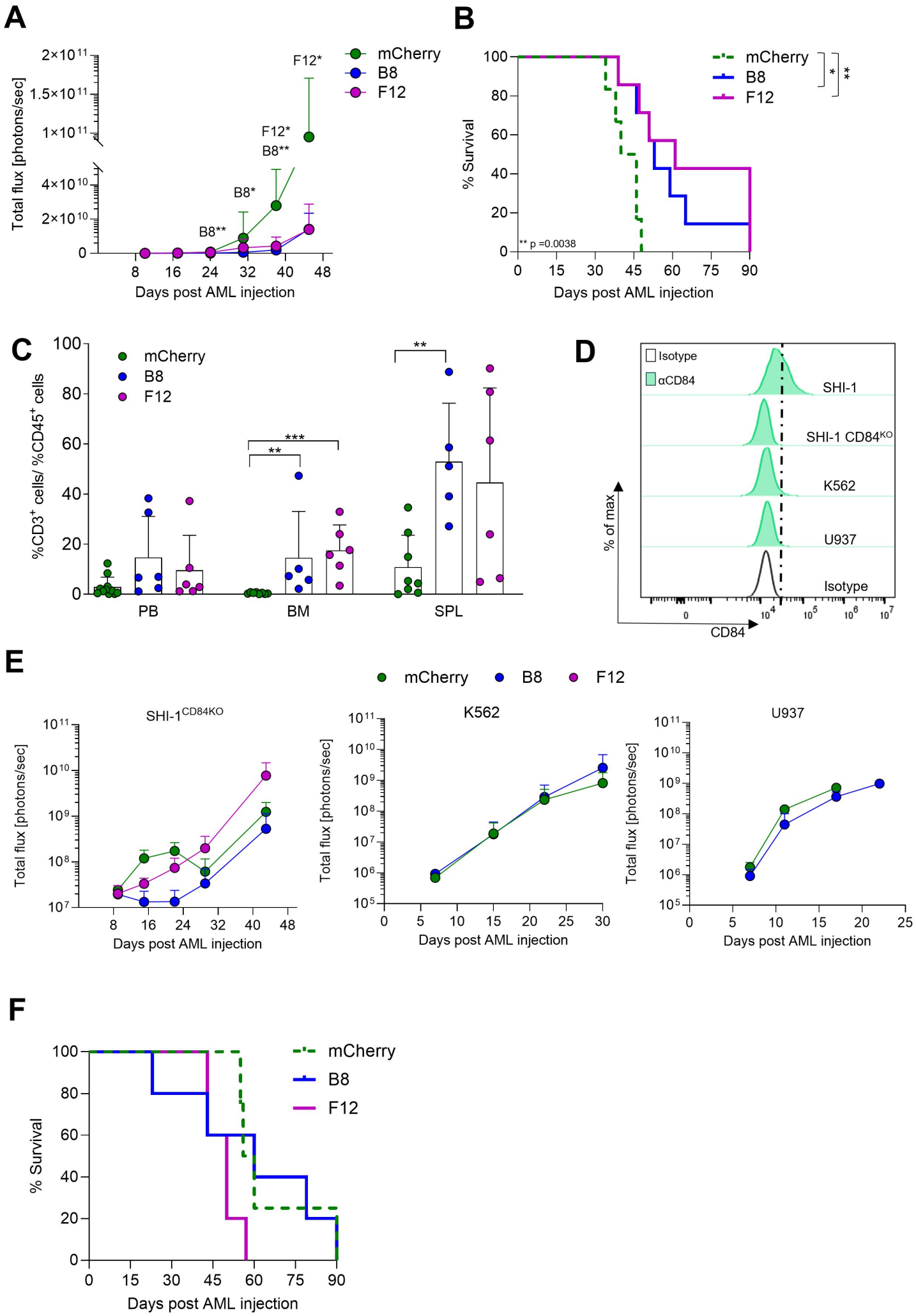
HIV-1 Ψ
cPPT/CTS
RRE
hPGK
B8
F12
mCherry
WPRE
3´LTR
ΔU3
R
U5
B
VCN
mCherry
B8
F12
C
% Lysis potency (BLI)
D
% Lysis potency (AV/7AAD)
+ SHI-1
+ HL-60
E
ΔTNFα production
ΔIFNγ production
F
TNFα
IFNγ
IL2
GranB
pg/mL
G
No. CD84 molecules/cell
SHI-1
MKPL-1
H
%Lysis (AV/7AAD)
E:T ratio
1:1
1:2
1:4
1:8
1:16
1:24
I
K_D B8 = 5.67x10^-8
K_D F12 = 8.93x10^-9
6.25 nM
12.5 nM
25 nM
50 nM
100 nM
Response (RU)
Time(s)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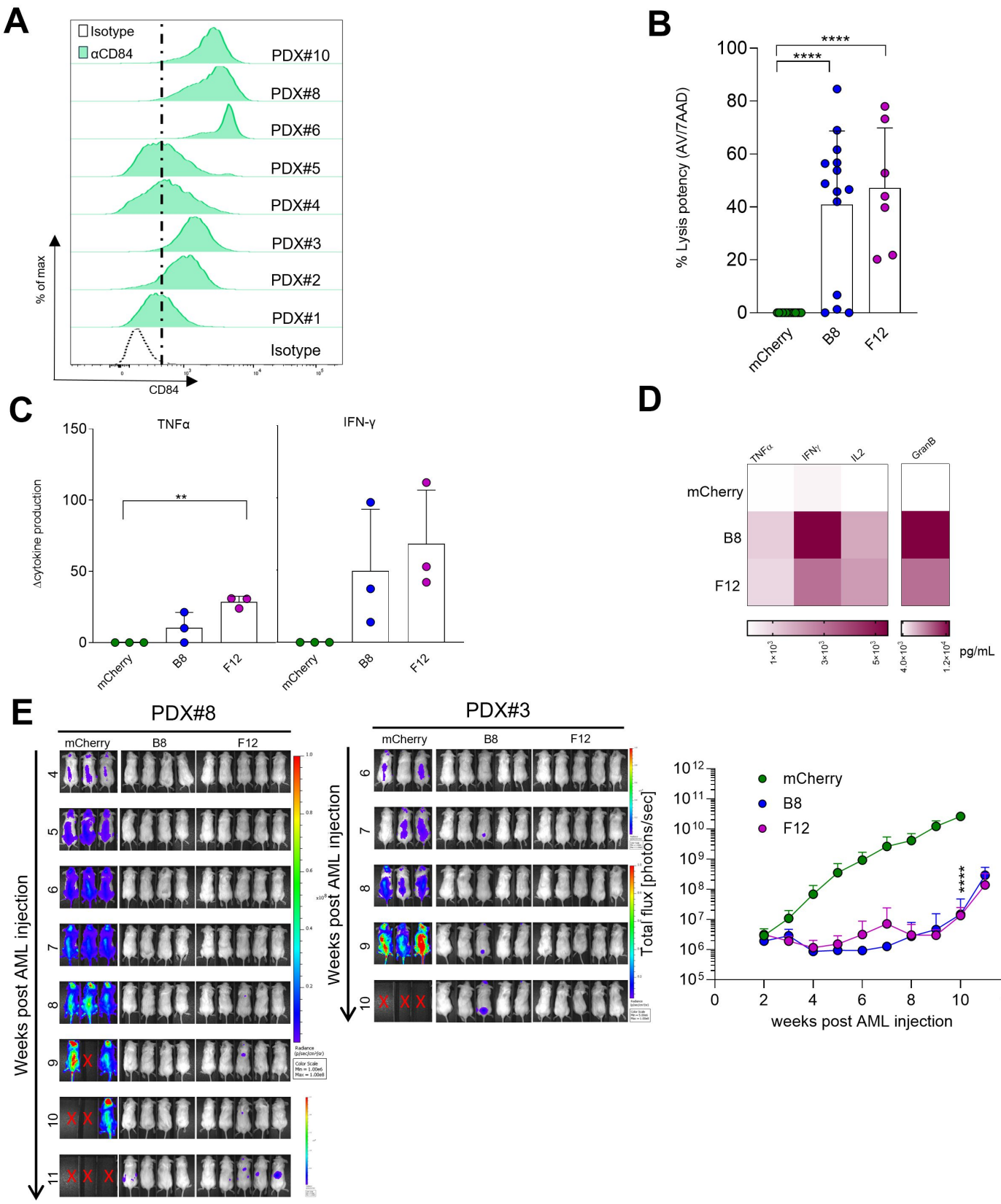
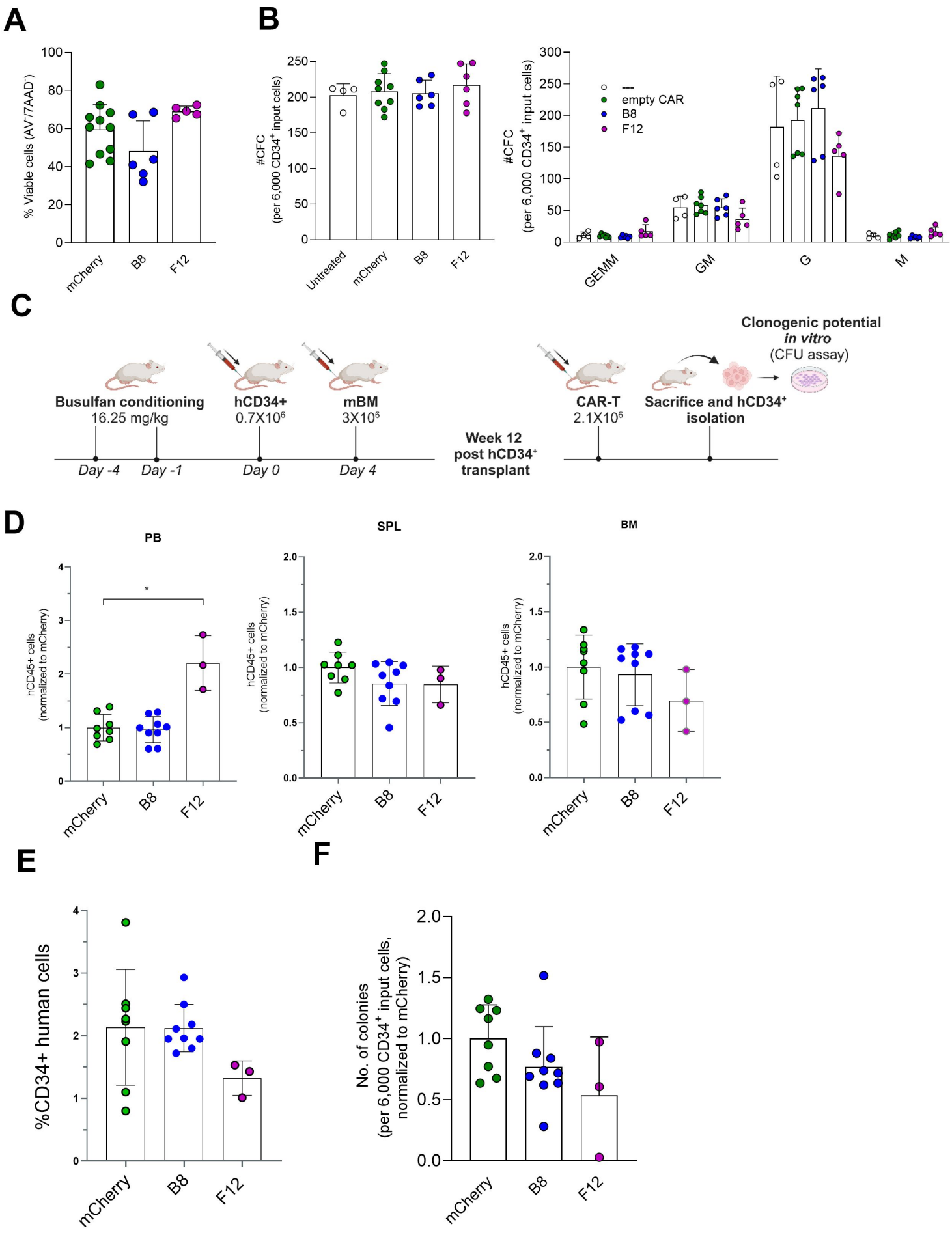
Total flux [photons/sec]
Days post AML injection
mCherry
B8
F12
F12*
B8**
B8*
B8**
B
% Survival
Days post AML injection
mCherry
B8
F12
** p =0.0038
C
%CD3+ cells/ %CD45+ cells
PB
BM
SPL
D
Isotype
αCD84
SHI-1
SHI-1 CD84KO
K562
U937
Isotype
% of max
CD84
E
mCherry
B8
F12
SHI-1CD84KO
K562
U937
Total flux [photons/sec]
Days post AML injection
F
% Survival
Days post AML injection
mCherry
B8
F12


A
Isotype
αCD84
PDX#10
PDX#8
PDX#6
PDX#5
PDX#4
PDX#3
PDX#2
PDX#1
Isotype
% of max
CD84
B
% Lysis potency (AV/7AAD)
****
****
mCherry
B8
F12
C
TNFα
IFN-γ
Δcytokine production
**
mCherry
B8
F12
D
TNFα
IFNγ
IL2
GranB
mCherry
B8
F12
pg/mL
E
PDX#8
PDX#3
mCherry
B8
F12
Weeks post AML injection
Total flux [photons/sec]
mCherry
B8
F12
****
weeks post AML injection
F
% Survival
mCherry
B8
F12
****
****
****p<0.0001
Weeks post AML injection
G
% AML cells
% Lymphocytes
mCherry
B8
F12


A
% Viable cells (AV-/7AAD-)
mCherry
B8
F12
B
#CFC (per 6,000 CD34+ input cells)
Untreated
mCherry
B8
F12
#CFC (per 6,000 CD34+ input cells)
---
empty CAR
B8
F12
GEMM
GM
G
M
C
Busulfan conditioning
16.25 mg/kg
Day -4
Day -1
hCD34+
0.7X10⁶
Day 0
mBM
3X10⁶
Day 4
Week 12
post hCD34+
transplant
CAR-T
2.1X10⁶
Sacrifice and hCD34+
isolation
Clonogenic potential
in vitro
(CFU assay)
D
PB
SPL
BM
hCD45+ cells
(normalized to mCherry)
mCherry
B8
F12
E
%CD34+ human cells
F
No. of colonies
(per 6,000 CD34+ input cells,
normalized to mCherry)