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Supplemental Information

A Novel Anti-LILRB4 CAR-T Cell for the Treatment of Monocytic AML

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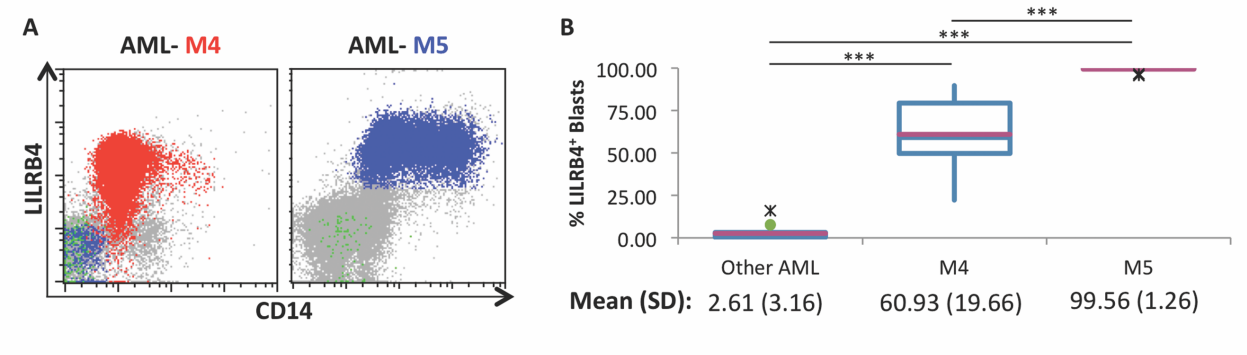
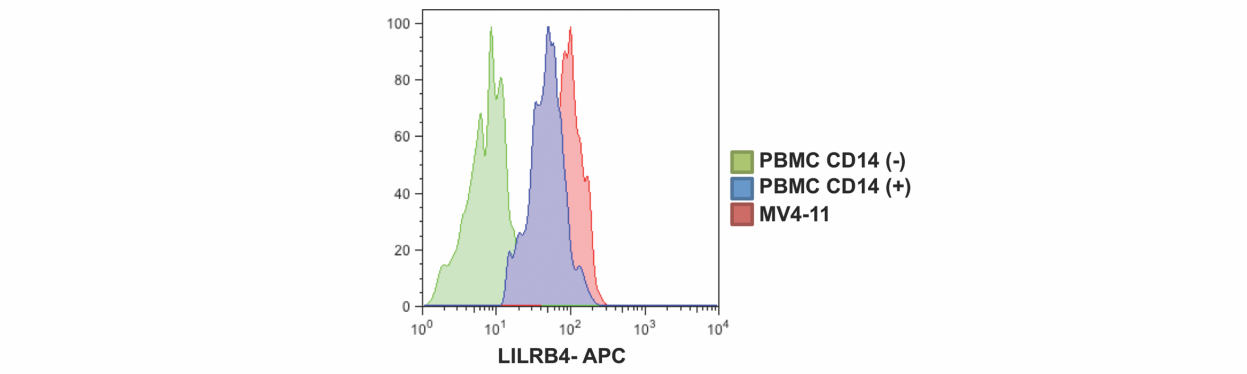
Supplementary Figures



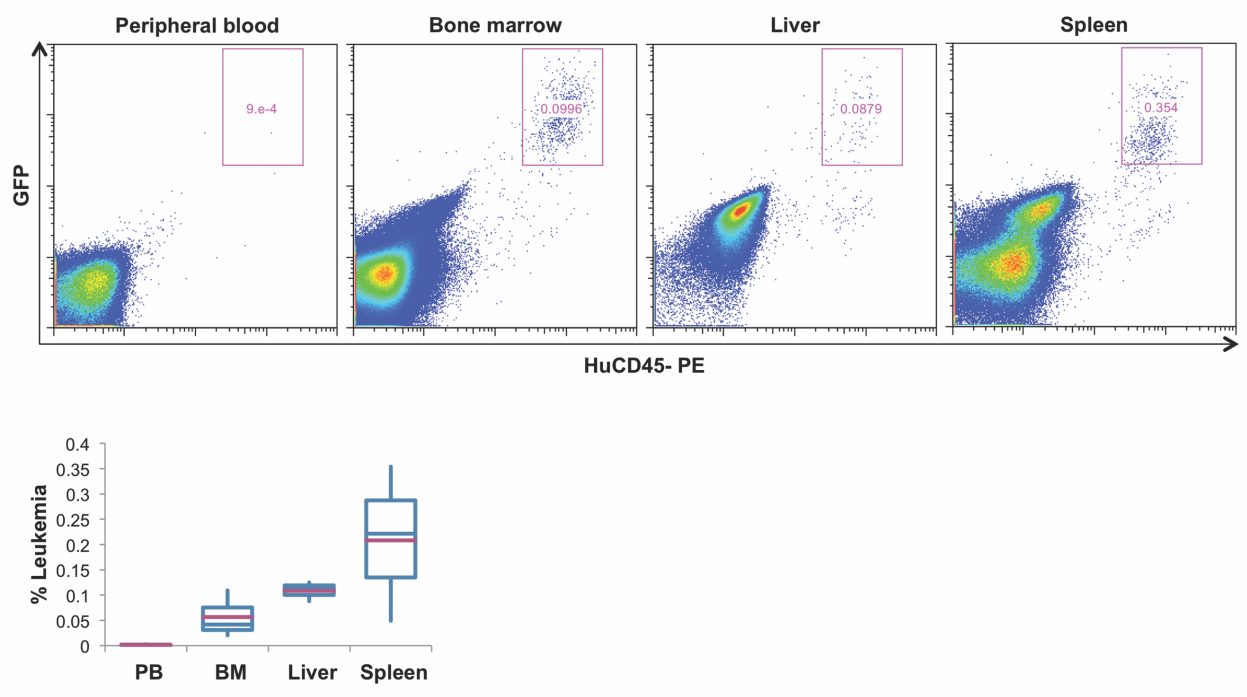
Supplementary Figure 1: LILRB4 is expressed only on M4 and M5 AML

A) Flow cytometry plot of representative patient sample for myelomonocytic AML (M4) and monocytic AML (M5), gated from mid to large FSC, low to mid SSC, CD45- dim, demonstrating that LILRB4 is expressed on the partial population of M4 blasts and the full population of M5 blasts. (B) Quantification of LILRB4 expression in patients with AML demonstrating that LILRB4 is not expressed on AML M0- M2 (M3 excluded from analysis), and displays partial expression on AML M4, however is expressed on greater than 99% (SD= 1.25) of leukemia cells in all patients with monocytic AML.



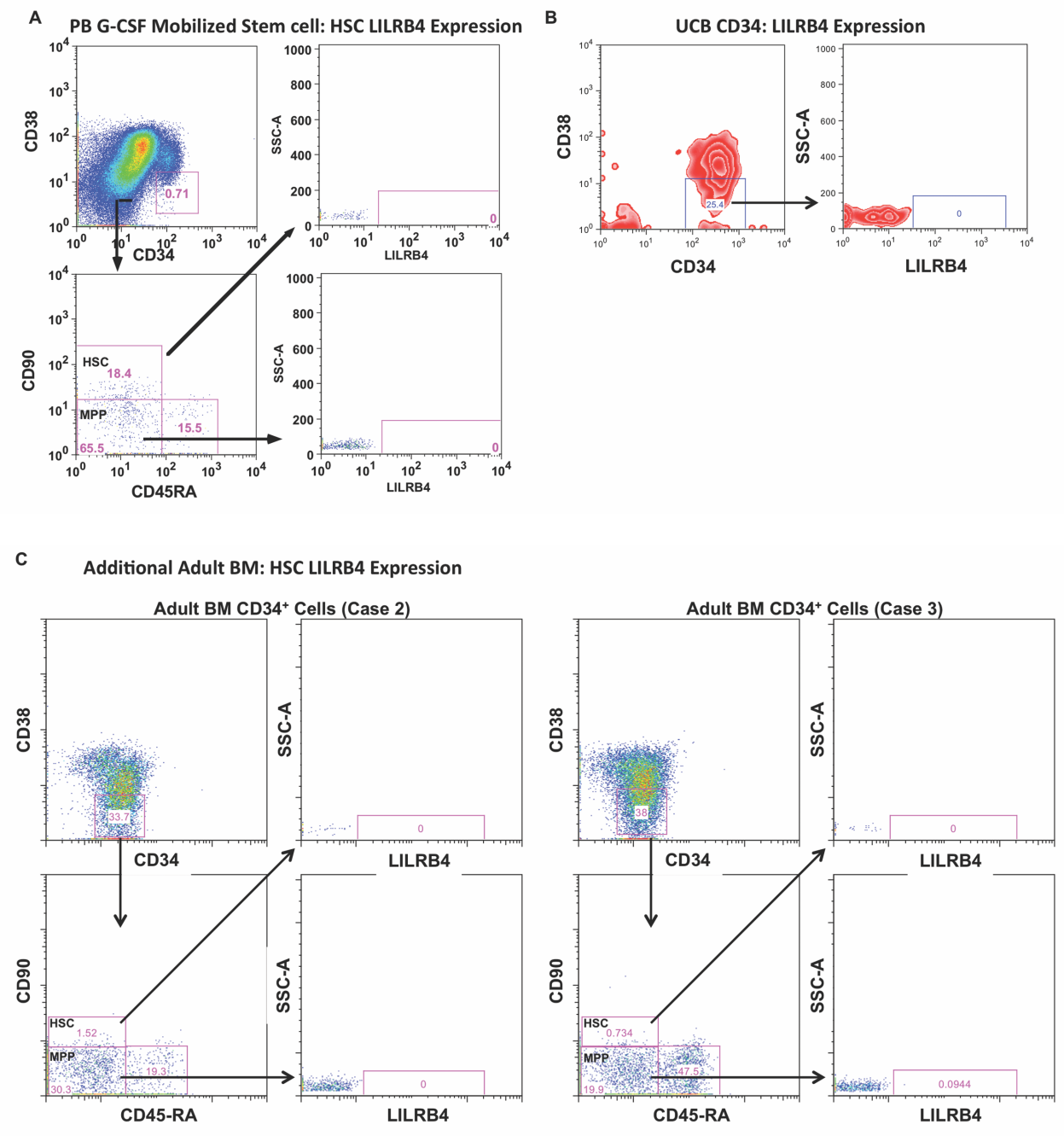
Supplementary Figure 2: LILRB4 is expressed on normal monocytes

LILRB4 expression analyzed by flow cytometry on MV4-11 AML cells (Red), monocytes (Blue, $CD14^+$) and non- monocytes (Green, $CD14^-$) from normal healthy donor peripheral blood mononuclear cells (PBMCs).



Supplementary Figure 3: MV4-11 engraftment in NSG mice

NSG mice were irradiated on day -1, injected with MV4-11 AML cells on day 0 and sacrificed on day 5. Peripheral blood (PB), bone marrow (BM), liver and spleen was harvested to determine AML cell engraftment. MV4-11 cells were identified as GFP^+/Human $CD45^+$. (Top) Flow cytometry of representative mice shows no circulating MV4-11 cells are seen in PB, however can be identified within BM, liver and spleen. (Bottom) Quantified flow cytometry data (n=3).



Supplementary Figure 4: LILRB4 is not expressed on human HSCs obtained from multiple sources

LILRB4 expression analyzed on human HSCs and MPPs obtained from (A) normal- healthy adult G-CSF peripheral blood mobilized stem cells, (B) normal- healthy umbilical cord blood

cells and (C) normal healthy adult bone marrow (2 additional cases). Cells were gated from Low SSC/Low FSC/CD45-Dim.

Supplementary Methods

Cell lines and cell culture methods

Cell lines were purchased from ATCC (Rockville, MD). MV4-11, THP-1 and MOLM-13 cell lines were maintained in RPMI-1640 media supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Sigma- Aldrich). 293-T cells were maintained in DMEM supplemented with 10% heat- inactivated FBS and 1% penicillin/streptomycin. Healthy donor primary human T-cells ($CD3^+$, frozen) were purchased from All Cells (Alameda, CA). T-cells were maintained in Immunocult-XF T-cell expansion media (Stemcell, Cambridge, MA) supplemented with 100 units/ml of recombinant human-IL2 (Peprotech, Rocky Hill, NJ). Human umbilical cord blood $CD34^+$ cells were purchased from Stemcell and used in humanized mice experiments or colony forming unit (CFU) assay as described below. Primary human AML samples were obtained from the tissue bank at UTSW and analyzed by flow cytometry as described below. Patient specimens were banked after informed consent was obtained under a protocol reviewed and approved by the Institutional Review Board at UTSW. The UTSW cohort included 69 AML patients representative of AML subtypes by the French-American-British (FAB) classification: acute myelomonocytic leukemia (M4, n=19), acute monocytic leukemia (M5, n=17), and other subtypes of AML (n=33).

Flow cytometry

For flow cytometry analyses of human cells and AML cell lines, cells were stained with mouse anti-human ILT3-APC (ZM4.1- eBioscience, Grand Island, NY), mouse anti human- CD3-APC (OKT3- Biolegend, San Diego, CA), goat anti-human IgG- FcGamma fragment specific-APC (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse anti-human CD14-FITC

(61D3- eBioscience), mouse anti human-CD19-APC (HIB19- Biolegend), mouse anti-human CD41-APC (HIP8- Biolegend) mouse anti-human CD38-FITC (HIT2- BD Pharmingen, San Diego, CA), mouse anti-human CD34-eFluor® 450 (4H11- eBioscience), mouse anti-human CD90-APC/Cy7 (5E10- eBioscience), mouse anti-human CD45RA-PE/Cy7 (HI100- Biolegend), monoclonal antibodies at 1:100 dilution. For analysis of cytotoxicity, AML cells were stained with DDAO-SE and propidium iodide as detailed in *Cytotoxicity assay* below. For analysis of AML cell and human hematopoietic cell engraftment in NSG mice, a previously published protocol was followed (23, 27, 28). Briefly, we collected peripheral blood from mice by retro-orbital bleeding, lysed red blood cells (RBC) in RBC lysis buffer, blocked with anti- mouse CD16/32 (93- Biolegend) and stained cells with mouse- anti-human CD45-PE (HI30, 1:100 dilution- BD Pharmingen) and anti-human CD3-APC (OKT3- Biolegend), to quantify total human AML cells ($GFP^+/CD45^+/CD3^-$). Cells were run on either FACSCalibur (BD Biosciences, San Jose, CA) for analysis or FACSAria (BD Biosciences) for analysis and sorting. Flow data were analysed by Flowjo software (Ashland, OR).

Generating anti-LILRB4 humanized scFv

The anti-LILRB4 antibody used in the CAR construct was a humanized rabbit antibody. The original rabbit antibody was isolated from a memory B cell of a New Zealand white rabbit immunized with the human LILRB4 ECD protein (SinoBiological, Beijing, China). Two rounds of PCR were performed by incorporating overlapping sequences at the 3′ and 5′ ends allowing infusion cloning of the antibody variable regions into rabbit IgG heavy- and light-chain vectors, based on protocols described previously[1]. The anti-LILRB4 antibody was expressed transiently in HEK 293 cells and purified by protein A/G for testing, based on protocols described

previously[1]. Humanization of the anti-LILRB4 antibody was based a CDR-grafting strategy as described previously[2]. Briefly, CDRs in the heavy and light chains of the rabbit antibody were defined by combination of three methods: Kabat, IMGT and Paratome[3-5]. The humanized VK and VH fragments were synthesized and cloned into human IgG1 CK and CH vectors separately, followed by expression in HEK 293 cells to confirm the biological activities compared with parental rabbit antibody. After confirmation of binding affinity of the humanized antibody, we performed PCR to link the VH and VL genes into scFv with a $(G_4S)_3$ linker[6]. The constructed scFv DNA product was then cloned into lentivirus expression plasmid for generation of anti-LILBR4-CAR.

Lentivirus production

The lentivirus expression plasmid encoding the anti-LILBR4-CAR and GFP genes was packaged in VSV-G pseudotyped lentiviral particles in 293T cells and concentrated by polymer precipitation followed by ultracentrifugation over sucrose cushion by Signagen (Signagen Laboratories, Rockville, MD). Lentiviral particles were endotoxin free and replication incompetent, and titer was determined by p24 ELISA as tested by Signagen. The concentrated lentivirus was thawed and resuspended in T cell medium one hour prior to use in T-cell transduction.

ELISA binding assay

Corning 96-well EIA/RIA plates were coated overnight at 4°C with LILRA or LILRB recombinant proteins (1 μg/mL) and blocked for 2 hours at 37°C with 5% non-fat milk. After washing with PBS-T (Tween 20, 0.05%) 3 times, 100 μL of serial dilution of anti-LILRB4 antibodies were added and incubated for 30 min at 37°C. Subsequently, the plates were washed

with PBS-T and incubated for 30 min with anti-human $F(ab')_2$ HRP-conjugated antibody (Jackson ImmunoResearch Laboratories). The immunoreactions were developed with TMB substrates (Sigma-Aldrich) and stopped by the addition of 2 M sulfuric acid before plate reading at 450 nm.

Flow cytometry binding assay

Control (untransduced) T-cells or anti-LILRB4 CAR-T cells (5×10^4 cells in total volume 100 µl) were incubated with 0.5 µg of LILRB4-Fc fusion protein, on ice for 90 minutes. Human IgG was used as control. Cells were then washed in ice-cold PBS + 2% FBS, followed by secondary staining with APC-Goat-anti Human IgG-FcGamma (Jackson-ImmunoResearch). Cells were washed in ice-cold PBS twice and re-suspended in PBS + 2% FBS for flow cytometry analysis on FACS-Calibur (BD Biosciences).

Cytotoxicity assay

AML cells were labeled with far-red DDAO-SE (Life Technologies, Grand Island, NY) per manufacturer's directions. Control- (untransduced) T cells or anti-LILRB4 CAR-T cells (effectors) were co-cultured with AML cells (targets) for 4 hours in RPMI in 96 well U-bottom plates at the indicated effector to target (E:T) ratios. Following this, each sample was resuspended in PBS with 0.1% propidium iodide (by volume) with 1×10^4 flow cytometry counting beads per sample. Live AML target cells were defined as the DDAO-SE^+/PI^- cell population. Experiments were performed in triplicates, and cytotoxicity was calculated as:

AML = AML cell count/# of beads (Co-culture WITHOUT T cells)

X = Living target cell count/# of Beads (Co-culture WITH T cells)

*% Cytotoxicity= (AML - X)/AML*100.*

Cytokine release

Control (untransduced) or anti-LILRB4 CAR-T cells (2-5 x 10^4) were co-cultured with AML cells (1:1) in 96-well U-bottom plates for 24 hours. Human IFN-γ and TNF-α release into culture supernatants was quantified by ELISA (Biolegend) following the manufacturer's protocol.

Colony-forming unit (CFU) assay

Human umbilical cord blood $CD34^+$ cells (1 x 10^3) were co-cultured with 1 x 10^4 control (untransduced)- T cells or anti-LILRB4 CAR-T cells for 4 hours, resuspended in MethoCult Classic (Stemcell), plated, and incubated in a humidified chamber per manufacturer's directions. Colonies were classified and counted after 14 days.

Gene and protein expression analysis

Expression profile of LILRB4 in normal tissue at the mRNA and protein level was assessed by utilizing publically available databases for gene expression analysis (Human body index - transcriptional profiling, *GSE7307: 210152_at*) and mass- spectrometry proteomic analysis (Human Proteome Map: *LILRB4- http://www.humanproteomemap.org/query.php*), respectively.

Immunohistochemistry

Hematoxylin staining and immunostaining were performed on normal human tissue microarrays (FDA-808j-1, BN1002b) purchased from US Biomax (Rockville, MD), and paraffin embedded sections of THP-1 wild-type and LILRB4-knock out tumors. Antibodies used were against LILRB4 (1:100). The images were visualized using the Hamamatsu NanoZoomer 2.0-HT

(Meyer instruments Inc., Houston, TX) and viewed in NPDview2 software (Hamamatsu, Japan).

CD34$^+$ Humanized mouse xenograft

4-6 week-old NOD.*Cg-Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ (NSG) mice (Jackson Laboratory) were sublethally irradiated (200 cGy) on day 0. Following this, 4-6 hours later, each mouse was injected via tail-vein, with 8 x 10^4 human umbilical cord blood CD34$^+$ cells resuspended in 200 µl PBS. Mice were determined to be engrafted when greater than 1% human CD45$^+$ cells was detected in peripheral blood by flow cytometry (approximately 4 weeks). Following engraftment, 1 x 10^6 anti-LILRB4 CAR-T cells resuspended in 200 µl PBS were injected into each mouse via tail-vein. Control mice were treated with PBS. Peripheral blood was analyzed weekly and mice were sacrificed on day 28 following anti-LILRB4 CAR-T cell treatment. Peripheral blood, bone marrow, liver and spleen was harvested from each mouse, and analyzed by flow cytometry for specific human hematopoietic cell populations (CD19, CD33, CD14, CD34, CD38, LILRB4).

Statistical analyses

Data are expressed as mean ± SE. Data comparing effect between Control- and anti-LILRB4 CAR-T treatment groups were analyzed by Student's t-test and considered statistically significant if $p < 0.05$. Kaplan- Meier survival curves of control- and anti-LILRB4 CAR-T treatment groups were analyzed using a log-rank test and were considered statistically significant if $p < 0.05$.

References

1. Freed DC, Tang Q, Tang A, Li F, He X, Huang Z, et al. Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine. Proc Natl Acad Sci U S A 2013; 110:E4997-5005.
2. Yu YL, Lee P, Ke YH, Zhang YK, Yu Q, Lee J, et al. A Humanized Anti-VEGF Rabbit Monoclonal Antibody Inhibits Angiogenesis and Blocks Tumor Growth in Xenograft Models. PLoS ONE 2010; 5:e9072.
3. Retter I, Althaus HH, Munch R, Muller W. VBASE2, an integrative V gene database. Nucleic Acids Res 2005; 33:D671-4.
4. Lefranc MP, Ehrenmann F, Ginestoux C, Giudicelli V, Duroux P. Use of IMGT((R)) databases and tools for antibody engineering and humanization. Methods in molecular biology 2012; 907:3-37.
5. Kunik V, Ashkenazi S, Ofran Y. Paratome: an online tool for systematic identification of antigen-binding regions in antibodies based on sequence or structure. Nucleic Acids Res 2012; 40:W521-4.
6. Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, et al. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol 1996; 14:309-14.