Supplemental information

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Supplementary information

RESOURCE

Engineering chimeric antigen receptor neutrophils from human pluripotent stem cells for targeted cancer immunotherapy

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Fig. S1 Generation of aorta-like CD34+SOX17+ hemogenic endothelium (HE) and hematopoietic stem and progenitor cells (HSPCs), related to Figure 1. (A) Schematic of HE and HSPC generation from hPSCs. (B-C) hPSC-derived day 5 cultures were subjected to flow cytometry analysis for CD34/SOX17 and quantified in (C). (D) Representative images of immunostaining for SOX17 and VEcad on day 5. Scale bars, 50 μm. (E-H) hPSCs were differentiated as illustrated in (A). Representative flow plots of CD45/CD43 expression in H9 hPSCs at different time points were shown in (E) and quantified in (F). Representative flow plots of CD44/CD43 in H9 (G) cultures and CD34/RUNC1c (H) in H9 RUNX1c-GFP (Ng et al., 2016) cultures at the indicated days were shown. (I-J) Cell viability before and after froze was assessed by flow cytometry with Calcein-AM stain.
Fig. S2 Multipotent evaluation of hPSC-derived myeloid progenitors, related to Figure 1. (A-C) Schematic of colony forming unit-macrophage (CFU-M) and -granulocyte-macrophage (CFU-GM) analysis at indicated days during differentiation was shown in (A). Representative images of G and GM colonies formed by hPSC-derived myeloid progenitors collected at indicated days were shown in (B) and quantified in (C). Data are represented as
mean ± s.d. of three independent replicates. Scale bars, 100 μm. (D-E) Monocyte/Macrophage differentiation potential of hPSC-derived myeloid progenitor cells was assessed by GM-CSF treatment. Representative flow plots of CD14 and CD45 under different culture conditions were shown in (D) and quantified in (E). (F-H) Schematic of neutrophil differentiation potential evaluation of hPSC-derived myeloid progenitors at indicated days with G-CSF and AM580 treatment was shown in (F). Expressions of CD11b and CD16 in cell cultures collected at indicated days were assessed by flow cytometry (G) and quantified in (H). (I-J) Representative flow cytometry images of CD16 on neutrophils before and after sorting were shown in (I). (J) Sorted CD16- cells were subjected for flow cytometry analysis of other markers, including CD10, CD14, FcεRIα and EPX. Representative Wright-Giemsa staining (K) and fluorescent staining of DAPI and CD16 (L) of hPSC-derived and primary peripheral blood (PB) neutrophils were shown. Scale bars, 5 or 10 μm. Data are represented as mean ± s.d. of three independent replicates.
Fig. S3 Characterization of CAR knockin hPSCs and hPSC-derived neutrophils, related to Figure 2. (A) Representative RT-PCR analysis of IL-13 and CLTX-IgG4 expression on CLTX-T-CAR, CLTX-NK-CAR, and IL13-T-CAR knockin hPSCs during neutrophil differentiation is shown. Representative fluorescent images (B) and flow plots (C) of OCT4 and SSEA4 expression on CAR hPSCs were shown. Scale bars, 50 μm. (D) Absolute counts and neutrophil differentiation efficiency (% CD11b+CD16+) of wildtype and CAR-expressing hPSCs were quantified. Data are represented as mean ± s.d. of five independent replicates. (E-H) Bulk RNA sequencing (RNA-seq) analysis was performed on hPSC-derived and primary neutrophils. (E) 3D score plot of first three principal components (PCs) from principal component analysis (PCA) is shown. Each data point corresponds to different biological samples and black arrows show the development transition from hPSCs to hPSC-neutrophils, then to
peripheral blood mature neutrophils. (F) Heatmaps show selected antitumor N1 and protumor N2 markers. (G) Venn diagram shows the number of gene sets that were enriched in different cell types (relative to hPSCs). Top 150 significantly enriched gene ontology (GO) (p<0.05), ranked by normalized enrichment score (NES) for each neutrophil group, were used for analysis. (H) Heatmaps show 29 GO only enriched in CLTX and IL13-CAR neutrophils. (I-J) Wildtype hPSCs were differentiated into three germ layer lineages: mesoderm, endoderm, and ectoderm. Representative immunostaining images of cTnT, HNF4A and β-III tubulin were shown in (I). Scale bars, 100 μm. (J) CAR-neutrophils were derived from CLTX-T-CAR hPSCs and incubated with normal SVG p12 glial cells, H9 hPSCs, hPSC-derived mesoderm, endoderm and ectoderm at indicated neutrophil-to-target ratios. The numbers of viable cells were quantified. Data are represented as mean ± s.d. of three independent replicates.
Fig. S4 MMP2 mediates CLTX-T-CAR hPSC-neutrophil interaction with glioblastoma cells, related to Figure 4. (A) Schematic of Cas13d PiggyBac Transposon platform for inducible knockdown of targeted genes in tumor cells. Glioblastoma U87MG cells were transfected with indicated Cas13d gRNA constructs along with the hyPBase plasmid. Representative brightfield and fluorescent images (B), and flow plots (C) of eGFP signal after doxycycline (DOX) treatment were shown. Scale bars, 100 μm. Tumor cells transfected with CLCN3- (D), ANXA2- (E), and MMP2- (F) targeting Cas13d gRNAs were subjected to RT-PCR analysis with or without doxycycline (DOX) treatment. Antitumor cytotoxicity of CLTX-T-CAR hPSC-neutrophils against these modified tumor cells were quantified at a neutrophil-to-target ratio of 10:1 with or without DOX treatment. Wildtype tumor cells were used as a positive control. Data are represented as mean ± s.d. of three independent replicates. (G) Off-target effects of Cas13d-targeted gene knockdown in the other two genes were assessed in MMP2, CLCN3 and ANXA2 knockdown tumor cells via RT-PCR analysis. (H-K) RT-PCR (H) and linear regression analysis of ANXA2 (I), CLCN3 (J), and
MMP2 (K) expression in different tumor cells and their correlation to corresponding tumor lysis efficiency were performed. (L) Expression of MMP2 in normal cells was assessed by RT-PCR analysis and U87MG cells were used as a positive control. (M) Representative brightfield image of endothelial cells in the implemented in vitro blood-brain-barrier model is shown. Scale bar, 100 μm. (N-O) Tumor infiltration (N) and tumor lysis (O) of CLTX-T-CAR neutrophils with or without 50 ng/mL MMP2 and 5 μM cytochalasin D (CytoD) were evaluated. Scale bars, 200 μm.
Fig. S5 *In situ* antitumor activities of CLTX-T-CAR neutrophils and CLTX-NK-CAR natural killer (NK) cells were evaluated via intratumoral injection, related to Figure 6. (A-E) Schematic of stage-specific NK cell differentiation from hPSCs. (B) Representative histogram plots of indicated markers and corresponding isotype controls are shown. (C) Anti-glioblastoma cytotoxicity of indicated hPSC-derived NK cells were performed at different effector-to-target ratios under normoxia or hypoxia. Data are presented as mean ± s.d. of three independent replicates, *p<0.05. (D) Cytotoxicity of CLTX-NK-CAR hPSC-NK cells against indicated tumor and healthy cells at a ratio of 10:1 is shown. Data are presented as mean ± s.d. of three independent replicates, *p<0.05. (E) *In vitro* cytotoxicity of mixed CAR-neutrophils and CAR-NK cells with indicated mixing ratios was evaluated. (F-J) Schematic of intratumoral injection of indicated hPSC-neutrophils or NK cells for *in vivo* antitumor cytotoxicity study is shown in (F). 5×10^5 luciferase (Luci)-expressing U87MG cells were stereotactically implanted into the right forebrain of NRG mice. After 3 hr, PBS, 5×10^6 wildtype or CLTX-T-CAR hPSC-neutrophils, 5×10^6 wildtype or CLTX-NK-CAR hPSC-NK cells were injected to the same position. Time-dependent tumor burden was determined (G) and quantified (H) by bioluminescent imaging (BLI) at indicated days. Data are mean ± s.d. for the mice in (G). n=3. (I) Body weights of indicated experimental mice were measured and recorded weekly. (J) Kaplan-Meier curve demonstrating survival of experimental mice is shown.
Fig. S6 In vivo antitumor activities of CLTX-T-CAR neutrophils and CLTX-NK-CAR NK cells were assessed via intravenous injection, related to Figure 6. (A) Schematic of intravenous injection of Cy5-labeled hPSC-neutrophils for in vivo cell tracking study. 5×10^5 luciferase (Luci)-expressing U87MG cells were stereotactically implanted into the right forebrain of NRG mice. After 4 days, mice were intravenously treated with PBS, 5×10^6 Cy5-labeled wildtype or CLTX-T-CAR hPSC-neutrophils. Time-dependent biodistributions of Cy5+ neutrophils in whole body (B), brain (C) and other organs (D) were determined and quantified by fluorescence imaging at indicated hours. (E) Body weights of experimental mice were measured and recorded weekly. (F-G) Representative brightfield and H&E staining images of glioblastoma xenografts isolated from indicated mice at day 28 were shown in (F) and quantified in (G). Data are represented as mean ± s.d. of three independent replicates. *p<0.05. (H) Wildtype and CAR-neutrophils, isolated from mouse blood 24 hours after systemic injection of neutrophils at indicated days in Fig. 6A, were subjected for RT-PCR analysis of antitumor N1 and protumor N2 markers and quantified. (I-J) Representative immunostaining images of glioblastoma xenografts isolated from indicated mice at day 26 were shown in (I), and expression of Arginase and iNOS were quantified in (J). Data are represented as mean ± s.d. of three independent replicates. *p<0.05.
Table S1. Off-target analysis of CLTX-CAR hPSCs

| NAME             | LOCUS     | SEQUENCE                        | MISMATCH   | INDEL |
|------------------|-----------|---------------------------------|------------|-------|
| sgRNAT2 target   | AAVSI     | GGGGCCACTAGGGACAG\ GATTGG      |            | ND    |
| Off-target 1     | chr10:+121198679 | GGAGACATTAGGGACAG\ GATAAG      | 3MMs [3:5:8] | ND    |
| Off-target 2     | chr7:+122484177  | GGCCTTACGGGACAG\ GACGAG        | 3MMs [3:4:20] | ND    |
| Off-target 3     | chr14:+93436934    | CAGGGCCTGGGGACAG\ GATCAG       | 4MMs [1:2:5:10] | ND    |
| Off-target 4     | chr15:-45827888    | GGGGTCACTGGGGACAA\ GATTGG      | 3MMs [5:10:17] | ND    |
| Off-target 5     | chr15:-25467890    | GGGACCCTGGGCACAG\ GATCGGG      | 3MMs [4:10:13] | ND    |

*ND: not detected.