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Supplemental Information

Human pluripotent stem cell-derived eosinophils reveal potent cytotoxicity against solid tumors

Weifeng Lai, Huangfan Xie, Yuting Liu, Feng Zheng, Yingfeng Zhang, Qi Lei, Lejun Lv, Jiebin Dong, Jian Song, Xue Gao, Ming Yin, Chengyan Wang, and Hongkui Deng

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Supplemental Experimental Procedures

Figure S1

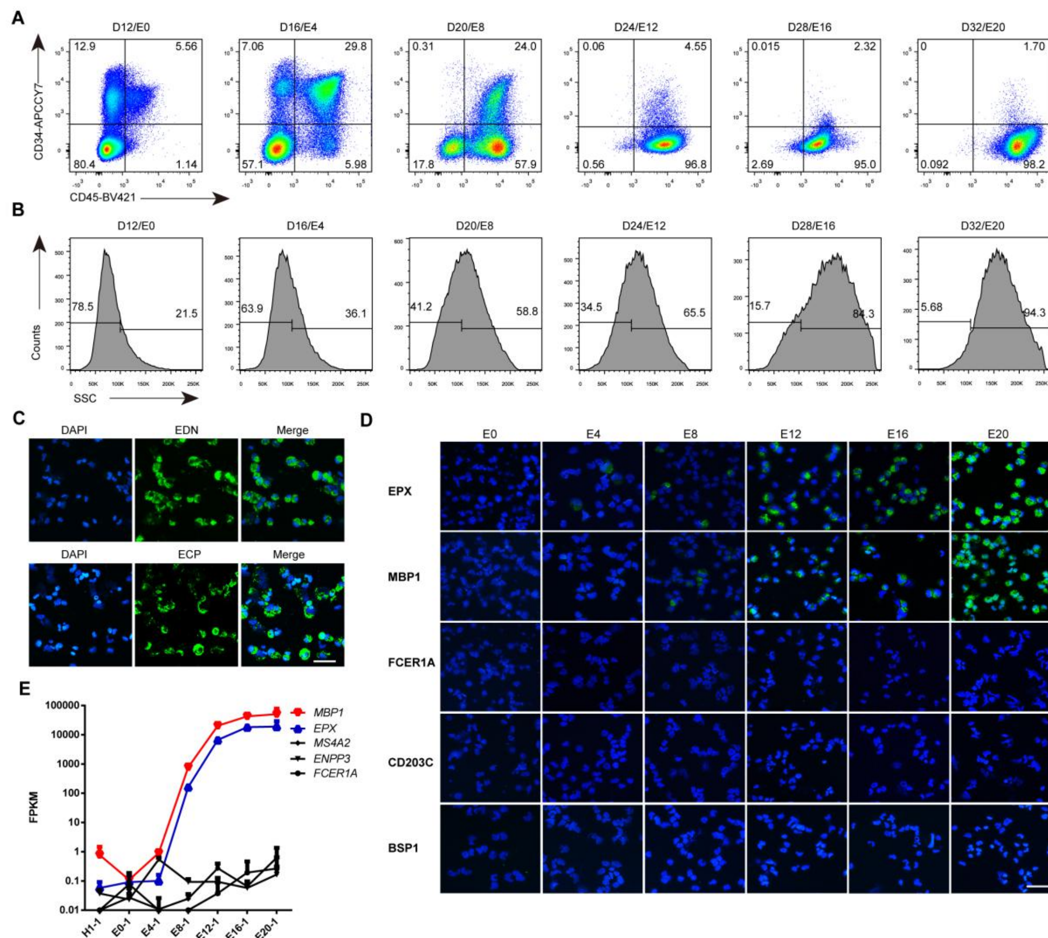

Figure S1. Generation of eosinophils from hESCs, related to Figure 1*.*

(A) Representative flow plots from 3 independent experiments showing the kinetics of the percentage of $CD34^+CD45^+$ cells during eosinophil induction from hESCs (H1).

(B) Representative flow histogram from 3 independent experiments showing the kinetics of the percentage of the SSC^{high} cells in the population during eosinophil induction from hESCs (H1).

(C) Representative immunostaining data from 3 independent experiments showing the EDN and ECP expression of E20 eosinophil induced from hESCs (H1), scale bar, 50 µm.

(D) Representative immunostaining data from 3 independent experiments showing the gene expression of induced cells harvested on E0, E4, E8, E12, E16 and E20, scale bar, 50 µm.

(E) Gene expression of H1 cells and induced cells harvested on E0, E4, E8, E12, E16 and E20 from RNA-seq data (n=2 replicate).

Figure S2

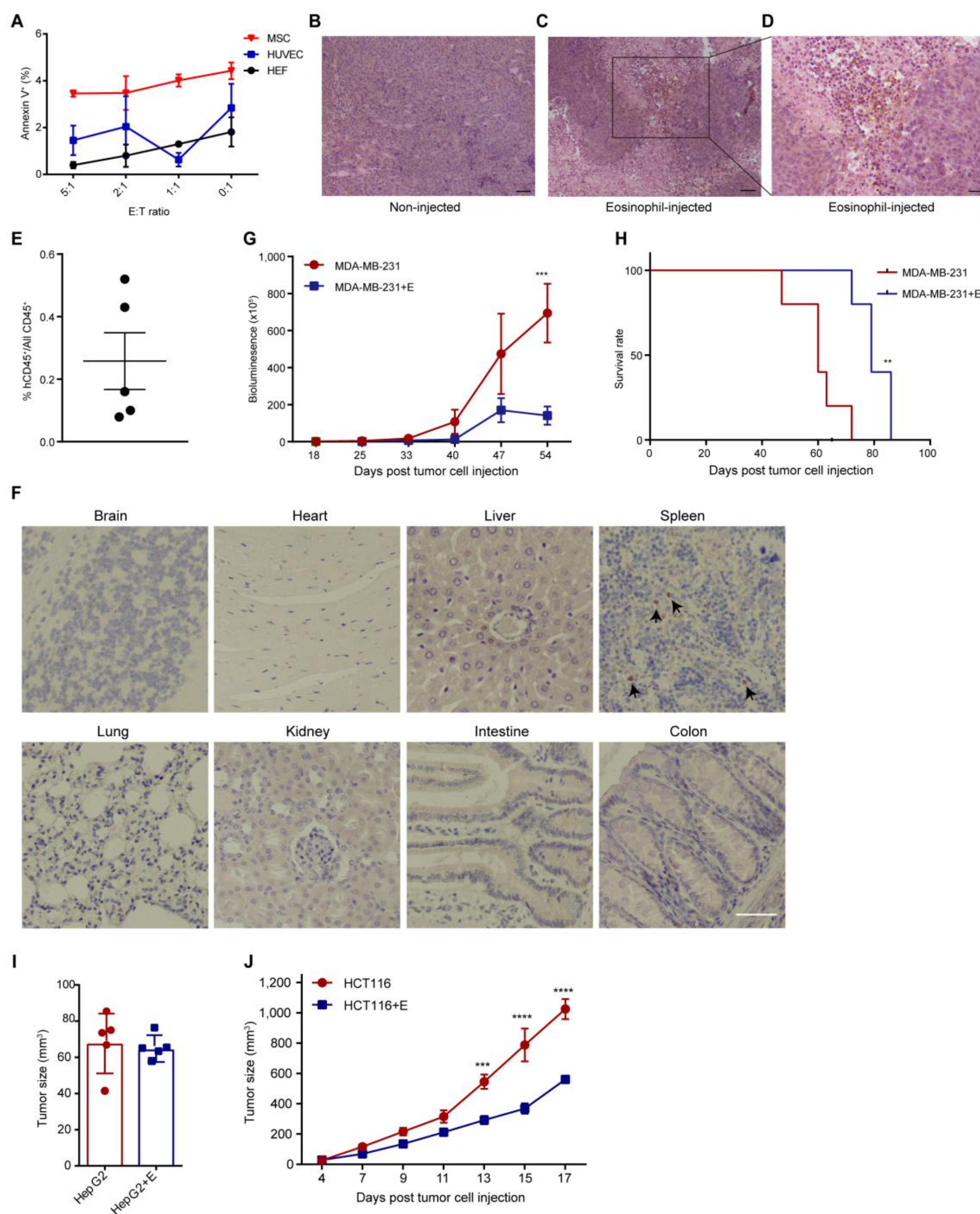

Figure S2. The infiltration of eosinophils into tumors and the anti-tumor activity of hESC-derived eosinophils, related to Figure 3, Figure 4.

(A) H1-derived eosinophils were cocultured with primary human umbilical vein endothelial cells (HUEVC), human embryonic fibroblasts (HEF) and human mesenchymal stroma cells (MSC) as target cells respectively. Annexin V^+ cells were shown as the mean percentage value ± SD (n=3), representative data from 3 independent experiments.

(B-D) Immunohistochemical analysis of H1-derived eosinophils infiltrated in HCT116 tumors, as detected by EPX antibody, in (B) control tumor, (C and D) H1-derived eosinophils injected tumor. For (B) and (C), scale bar, 50μm; for (D), scale bar, 20μm.

(E) Statistics showing the percentage of human $CD45^+$ cells in tumor tissues of MDA-MB-231

cell-burdened mice at 48 h after injection of H1-derived eosinophils (n = 5 mice); data shown as the mean values ± SEM.

(F) Immunohistochemical analysis of H1-derived eosinophils infiltrated in main organs, as detected by EPX antibody (arrowhead in the spleen indicate human EPX^+ eosinophils), scale bar, 50 μm.

(G) The MDA-MB-231 tumor cell burden of each group was measured on the indicated days after tumor cells injection by bioluminescent imaging (n = 5 mice for each group). Statistical significance was assessed using two-tailed ANOVA, where ***p < 0.001; data shown as the mean values ± SEM.

(H) Kaplan-Meier curve representing the survival rate of the experimental groups in the MDA-MB-231 xenograft mouse models (n = 5 mice for each group). Statistical analysis was calculated using log-rank (Mantel-Cox) test, where **p < 0.01.

(I) Tumor size of HepG2 on day 7 after tumor cell injection (n = 5 mice for each group), data shown as the mean values ± SD.

(J) HCT116 tumor size of each group determined on the indicated days after tumor cell injection (n = 5 mice for each group). Statistical significance was assessed using two-tailed ANOVA, where ***p < 0.001 and ****p < 0.0001; data shown as the mean values ± SEM.

Figure S3

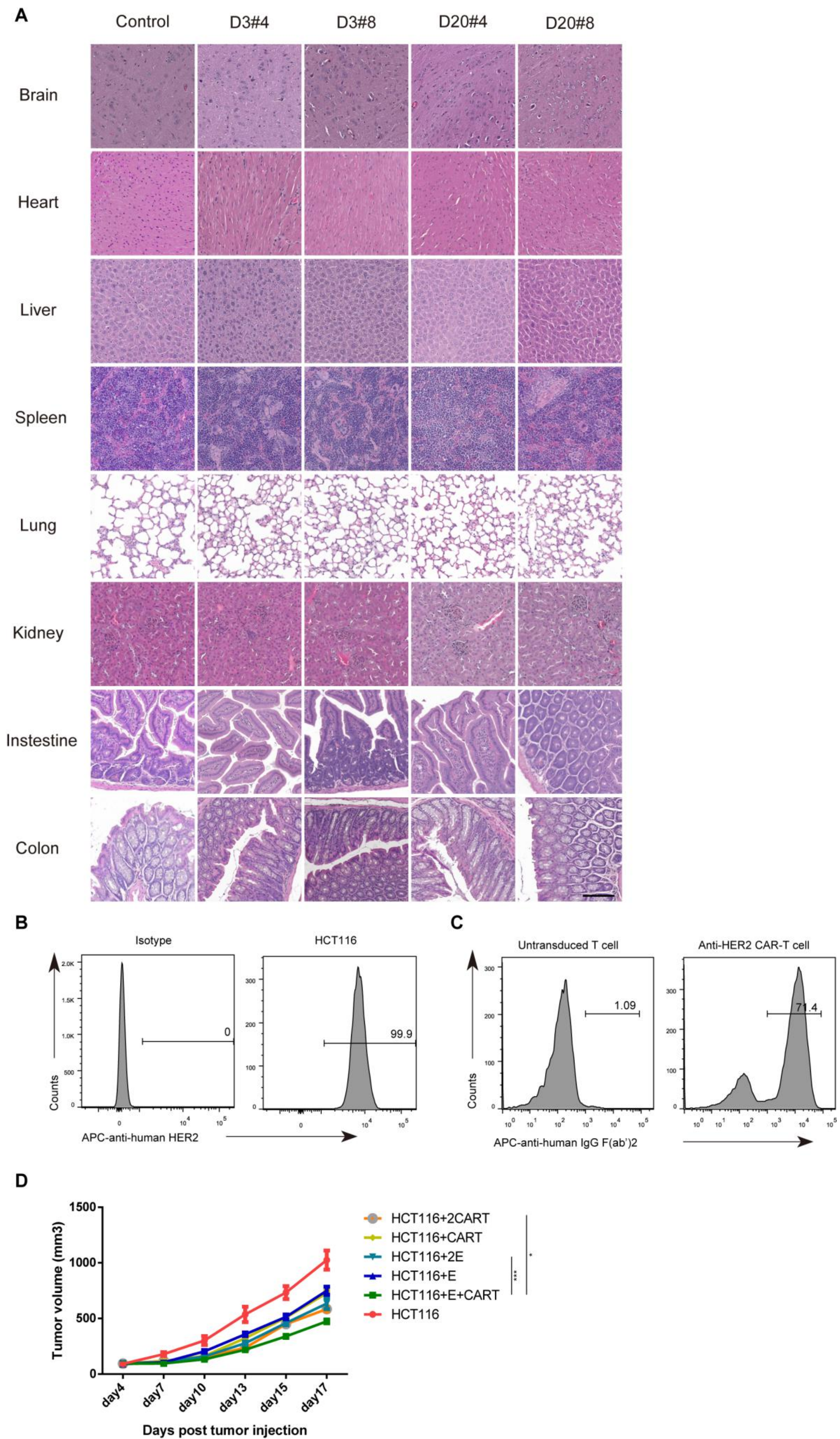

Figure S3. Histological analysis of major organs of mice injected with H1-derived eosinophils and the generation of CAR-HER2 T cells, related to Figure 4.

(A) Sections of brain, heart, liver, spleen, lung, kidney, intestine and colon were collected after the injection of H1-derived eosinophils at 4×10^6 cells and 8×10^6 cells per mouse at day 3 and day 20 respectively and stained with Hematoxylin and Eosin for the detection of changes in tissue morphology (D3#4: day 3, 4×10^6 cells; D3#8, day 3, 8×10^6 cells; D20#4, day 20, 4×10^6 cells; D20#8: day 20, 8×10^6 cells), scale bar, 100 μm.

(B) Representative flow histogram showing HER2 expression of HCT116 cells.

(C) Representative flow histogram showing anti-HER2 CAR expression of T cells.

(D) HCT116 tumor size of each group measured on the indicated days after tumor cell injection (n=5 mice for each group). Statistical significance was assessed using two-tailed ANOVA, where *$p < 0.05$, and ***$p < 0.001$; data are shown as the mean values ± SEM.

Figure S4

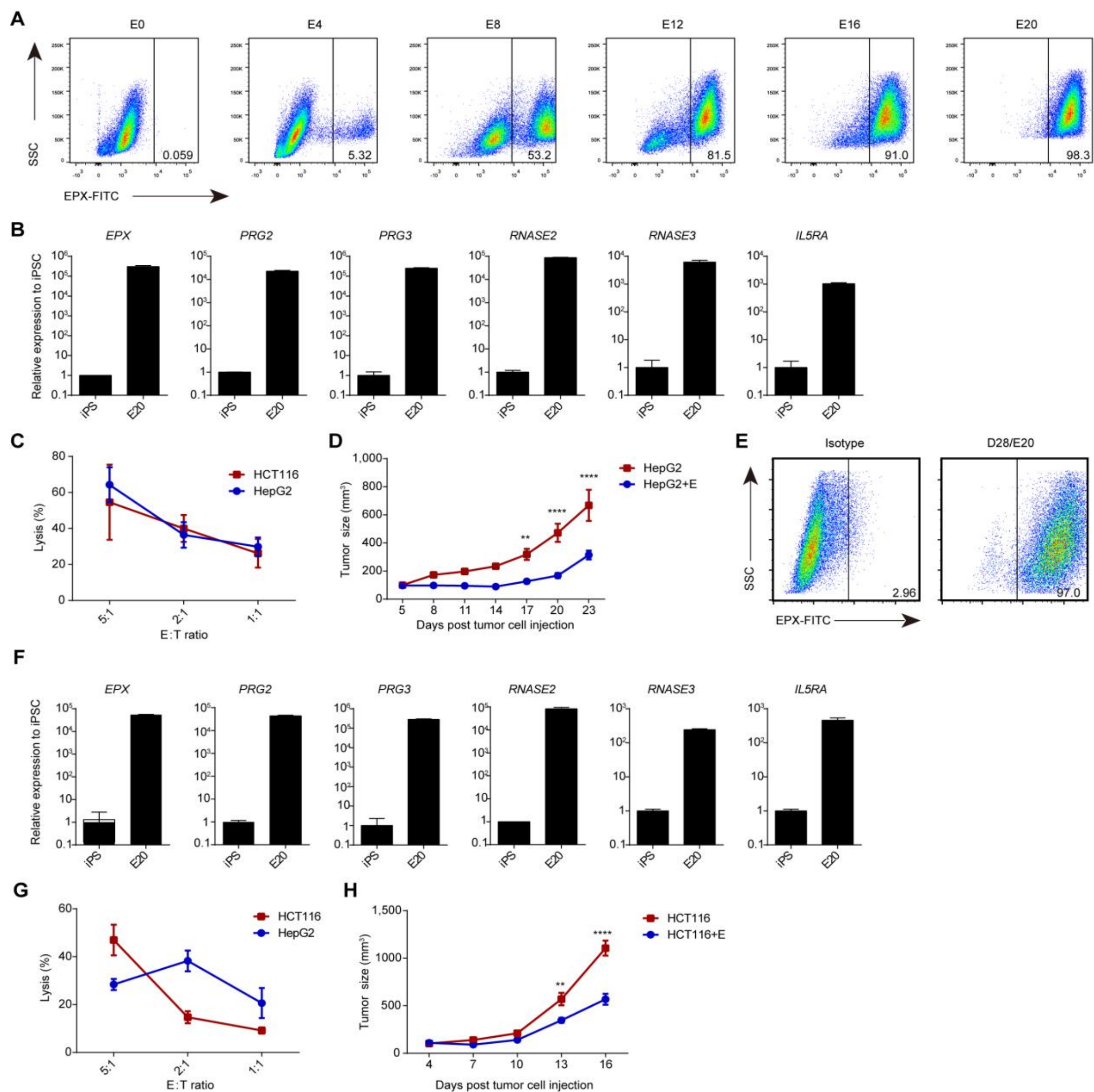

Figure S4. Efficient generation of eosinophils from human iPSCs.

(A) Representative flow plots from 3 independent experiments showing the kinetics of EPX^+ cell numbers during eosinophil differentiation from iPS-#7 cells.

(B) qPCR analysis of key gene expression in iPS-#7 cells and iPS-#7-derived eosinophils recovered on E20 (n = 3). Data shown as the mean percentage value ± SD. This is a representative data from 3 independent experiments.

(C) Cytotoxicity of the iPS-#7-derived eosinophils toward HCT116 and HepG2 target cells at the indicated effector-to-target ratios, respectively (n = 3). The lysis rate of target cells was shown as the mean percentage value ± SD. This is a representative data from 3 independent experiments.

(D) Tumor size determined on the indicated days after HepG2 tumor cells injection (n = 5 mice for each group). Statistical significance was assessed using two-tailed ANOVA, where **p < 0.01 and ****p < 0.0001; data shown as the mean values ± SEM.

(E) Representative flow plots from 3 independent experiments showing EPX^+ E20 eosinophil differentiation from iPS-#8 cells.

(F) qPCR analysis of key gene expression in iPS-#8 cells and iPS-#8-derived eosinophils

recovered on E20 (n = 3). Data shown as the mean percentage value ± SD. This is a representative data from 3 independent experiments.

(G) Cytotoxicity of the iPS-#8-derived eosinophils toward HCT116 and HepG2 target cells at the indicated effector-to-target ratios, respectively (n = 3). The lysis rate of target cells was shown as the mean percentage value ± SD. This is a representative data from 3 independent experiments.

(H) Tumor size determined on the indicated days after HCT116 tumor cells injection (n = 5 mice for each group). Statistical significance was assessed using two-tailed ANOVA, where $^{**}p < 0.01$ and $^{****}p < 0.0001$; data shown as the mean values ± SEM.

SUPPLEMENTAL TABLES

Table S1. List for qPCR primers, related to Figure 2, Figure S4 .

*β-Actin*_ Forward	GACAGCAGTCGGTTGGAGCG
*β-Actin*_ Reverse	GGGACTTCCTGTAACAACGCATC
*EPX*_ Forward	GTCCTGCGAGACTGCATAGC
*EPX*_ Reverse	TATAATCTGCGGCCCGAACAA
*PRG2*_ Forward	AAACTCCCCTTACTTCTGGCT
*PRG2*_ Reverse	GCAGCGTCTTAGCACCCAA
*PRG3*_ Forward	TCTGGAGAGCCTAGAGACACA
*PRG3*_ Reverse	CCTCCGTCAGAGCCAAGTC
*RNASE2*_ Forward	TTTACCTGGGCTCAATGGTTTG
*RNASE2*_ Reverse	TGCATCGCCGTTGATAATTGT
*RNASE3*_ Forward	CCCACAGTTTACGAGGGCTC
*RNASE3*_ Reverse	ACCCGGAATCTACTCCGATGA
*IL5RA*_ Forward	ATCATCGTGGCGCATGTATTAC
*IL5RA*_ Reverse	AAAGAACTTGAGCCAAACCAGT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Mouse experiments were conducted according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University. All of the mice were NOD-Prkdc^{scid} Il2rgtm1/Vst (NPG) mice (Stock Number: VS-AM-001) purchased from Beijing Vitalstar Biotechnology, and they ranged from 8 to 12 weeks of age.

Cord blood

This study was approved by the Institute of Review Board in Peking University (IRB 00001052-15087) and conducted according to the approved protocol. Samples were collected from consenting donors according to ethically approved procedures at China-Japanese Friendship Hospital and 307 Hospital of People's Liberation Army of China.

Cell culture

Human embryonic stem cells (H1) were obtained from Wicell (NIH: hESC-10-0043), and their usage was annually approved. H1 cells were cultured in Matrigel (BD Biosciences, Cat: 354230)-coated plates with pluripotent stem cell culture medium (PSCM, PSCeasy, Beijing CELLAPY Biotechnology) under 20% O_2 and 5% CO_2 at 37 °C. H1 cells were passaged by treatment with 0.5 μM EDTA (Gibco, Cat: 25300-062) for 5-6 min at 37 °C, and cells were collected and split at 1 to 6 - 1 to 10 ratios with pluripotent stem cell culture medium containing 5 μM Y-27632 (Selleck, Cat# S1049).

iPS cells (iPS-#7, iPS-#8) were purchased from Cauliscell Biotechnology and were cultured in Matrigel-coated plates with pluripotent stem cell culture medium (PSCeasy, Beijing CELLAPY Biotechnology). iPS cells were passaged by splitting at ratios of nearly 1 to 6 following treatment with 0.5 μM EDTA for 5-6 min at 37 °C, and then the cells were collected and plated with pluripotent stem cell culture medium containing 5 μM Y-27632.

HCT116 and MDA-MB-231 cell lines were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). The HepG2 cell line was a gift from Kuanhui Xiang (Peking University Health Science Center). All these cell lines were transduced with lentiviral GFP-luc vector, were sorted to isolate GFP$^+$ populations, and then were cultured in DMEM (Thermo Fisher Scientific) plus 10% FBS (HyClone), 1% GlutaMAX (Gibco), 1% PS (Gibco) and 1% NEAA (Gibco) under 5% CO_2 at 37 °C conditions. Trypsin-EDTA (0.25%) was used for passage.

Human embryonic fibroblasts (HEFs) were isolated and approved by IRB of Clinical Research Ethics Committee of China-Japan Friendship Hospital (Ethical approval No: 2009–50) and Stem Cell Research Oversight of Peking University (SCRO201103-03). They were cultured in DMEM (Thermo Fisher Scientific) plus 10% FBS (HyClone), 1% GlutaMAX (Gibco), 1% PS (Gibco) and 1% NEAA (Gibco) under 5% CO2 at 37 ℃ conditions. Human umbilical vascular and endothelial cells (HUVECs) were purchased from Lonza and cultured according to the manufacturer's manual. Trypsin-EDTA (0.25%) was used for the passage of HEFs and HUVECs.

Eosinophil differentiation from human ESCs and iPSCs

Pluripotent stem cells were cultured in Matrigel-coated plate with low density from 1 × 10^4~5 × 10^4/well in 6-well plate in pluripotent stem cell culture medium at day 1 before the differentiation. At differentiation day 0, Activin A (20ng/ml), BMP4 (20ng/ml) (StemImmune LLC, Cat: HST-B4-0100) and CHIR-99021 (3-5 μM) were administrated in the medium RPMI 1640 supplemented with B27 (without vitamin A) and 50 μg/ml ascorbic acid. From day 2 to day 6, 5 ng/ml BMP4, 50 ng/ml human vascular endothelial growth factor (VEGF, StemImmune LLC, Cat: HVG-VF5-1000), 50 ng/ml human basic fibroblast growth factor (bFGF, Origene, Cat: TP750002) and 10 μM SB-431542 (Selleck, Cat: S1067) were added. From day 6 to day 12, 5 ng/ml BMP4, 10 ng/ml VEGF, 20 ng/ml recombinant human stem cell factor (SCF, StemImmune LLC, Cat: HHM-SF-1000), 30 μM NAC (Sigma, Cat: A7250-5G), and 2 μM minocycline hydrochloride (Selleck, Cat: 3268) were supplemented in IMDM (Thermo Fisher Scientific) containing B27 without vitamin A and 50 μg/ml ascorbic acid. After day 12, eosinophil induction medium was used, which consisted of IMDM supplemented with B27 (without vitamin A), 30 μM NAC, 2 μM minocycline hydrochloride, 10 ng/ml human recombinant interleukin-3 (IL3, StemImmune LLC, Cat: HCT-I3-1000) and 10 ng/ml human recombinant interleukin-5 (IL5, Novoprotein, Cat: CI59).

Isolation of human primary naïve eosinophils from cord blood

Human primary naïve eosinophils were isolated based on anti-CD16-negative selection protocol via certain modifications (Wacht et al., 2018). Briefly, cord blood unit was diluted by sterile PBS, aliquoted gently to human lymphocyte separation medium (DRKEWE, Cat: DKW-KLSH-0100), and centrifuged at 1,500 rpm for 20 min according to the manufacturer' s protocol to obtain blood cells separated in different layers. Cell pellets in the bottom layer, which contained mainly granulocytes and red blood cells, were collected and suspended in 1 × RBC lysis buffer (Biolegend, Cat: 420301) to lyse the red blood cells according to the product manual. After lysis of red blood cells, the remaining cells were mainly granulocytes. They were washed twice with PBS, centrifuged at 1,500 rpm for 5 min. Cell pellets were then collected and stained with BV421 anti-human CD45 (Biolegend, Cat: 304032) and PE-Cy7 anti-human CD16 (Biolegend, Cat: 302016). Then the $CD45^+CD16^-$ cell population were isolated by the flow cytometer (MoFlo XDP) and collected in sterile PBS. These $CD45^+CD16^-$ cells were regarded as primary naïve eosinophils.

Isolation of human mesenchymal stem cells from cord blood

For mesenchymal stromal cells, monocyte cells after lymphocyte separation were cultured in DMEM (Gibco) containing 10% FBS (Hyclone) for 48 hours and the suspending cells were discarded. Adherent mesenchymal stromal cells were further cultured to 80% confluence.

Flow cytometry analysis

For surface marker detection, cultured cells were collected at the indicated times, digested with accutase (Millipore, Cat: SCR005) at 37 °C for 5 min, diluted with an equal volume of PBS (Corning, Cat: 21-040-CV), centrifuged at 1,800 rpm for 3 min to obtain cell pellets, and resuspended with PBS containing 0.5% BSA (Sigma, Cat: A1470–100G) to form a single-cell suspension. Next, the indicated antibodies were added, and they were incubated with the cells

for 15 min in the dark at room temperature. Then, the cells were washed three times with PBS, were suspended in 300 μl of PBS, and then were filtered through a 40 μm nylon cell strainer for analysis. Each antibody (0.2 μl) was added to each sample. The antibodies used were as follows: 7-AAD (BD Pharmingen, 559925), BV421 anti-human CD45 (Biolegend, Cat: 304032), PE anti-human CD69 (Biolegend, Cat: 310906), PE anti-human CD11b (Biolegend, Cat: 301306), PE-Cy7 anti-human Siglec-8 (Biolegend, Cat: 347112), and APC-Cy7 anti-human CD34 (Biolegend, Cat: 343614), and the Isotype Ctrl Antibody are: Brilliant Violet 421™ Mouse IgG1, κ Isotype Ctrl Antibody (Biolegend, Cat: 400157), PE/Cyanine7 Mouse IgG1, κ Isotype Ctrl Antibody (Biolegend, Cat: 400125), PE Mouse IgG1, κ Isotype Ctrl Antibody (Biolegend, Cat: 400111), APC/Cyanine7 Mouse IgG2a, κ Isotype Ctrl Antibody (Biolegend, Cat: 400229).

For intracellular staining of EPX, the cultured cells were collected at the indicated times and were digested into single-cell suspensions as described above. Then they were stained with Fixable Viability Stain 575V (BD Horizon™, Cat: 565694) according to the product manual. Next, the cells were fixed and permeabilized using a BD Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD, Cat: 554714), and then they were stained with an anti-EPX antibody (Abcam, Cat: ab190715) according to the manual; a proportion of the cells were stained with mouse clonal IgG1 (BD Pharmingen™, Cat: 555751) as an isotype control. The cells were then washed twice with 1 × BD Perm/Wash buffer before being incubated with Alexa Fluor 488-AffiniPure donkey anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch, Cat: 715-545-150) at 37 °C for 15 min. Then, the cells were washed twice and were filtered through 40 μm nylon cell strainer for analysis.

To analyze the infiltration of human ESC-derived eosinophils in solid tumors, mice were euthanized, and tumors were isolated. Isolated tumors were cut into 1 mm pieces with scissors and then were digested by incubation with 1 ug/ml Collagenase IV (Sigma, Cat: 17104019) and 1mg/ml Dnase (Sigma, Cat: DN25-1G) at 37 °C and 5% CO_2 for 30 min. Single cells were pipetted to a tube after digestion and were collected by centrifugation at 1800 rpm for 5 min. Tumor-derived cells were stained with PE anti-mouse CD45 (Biolegend, Cat: 103106) and BV421 anti-human CD45 (Biolegend, Cat: 328114) antibodies, were washed three times with PBS, and were filtered through a 40 μm nylon cell strainer for flow cytometry.

Flow cytometry analysis was conducted using LSRFortessa (BD). The data were analyzed using FlowJo-V10 (BD).

Giemsa staining

Differentiated cells were collected, counted and then centrifuged at 1,800 rpm for 3 min. Cell pellets were resuspended in PBS at a density of 5 × 10^6/ml, and 20- 30 μl of cells were added to adhesive microscope slides (CITOTEST, Jiangsu, Cat: 188105) and were centrifuged in StatSpin CytoFuge 2 (CYTOCENTRIFMGE) according to the protocol. Cells on the slides were fixed by incubation with 100% methanol (Beijing Chemical Work) in a glass bottle for 5 min. After air-drying, cells were stained by adding 200 μl of Eosinophil-specific staining solution A (Carbol 2R) (Baso Zhuhai, Cat: DA0164) for 5 minutes, which was followed by ten times of distilled water wash. Then, 200 ul of Eosinophil-specific staining solution B (Baso

Zhuhai, Cat: DA0164) were added and treated for 5 minutes. The solutions were discarded, and the slides were washed 4-5 times with distilled water. Stained cells were observed under a microscope (Olympus, BX-43), and pictures were taken with Cellsens software (Olympus Life Science).

Electron microscopy

Eosinophils differentiated on day 28 (D28/E16) were fixed with 2% paraformaldehyde / 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 min at 37 ℃, and then they were incubated for another 30 min at room temperature and overnight at 4 ℃. After rinsing several times in phosphate buffer, cells were postfixed in 2% OsO_4 with 1.5% potassium ferrocyanide for 2 h at room temperature. Following several washes in distilled water, samples were stained with 2% aqueous uranyl acetate overnight at 4 °C. After washing several times in distilled water, the cultures were dehydrated in a graded alcohol series and subsequently were embedded in Spurr's resin (SPI supplies, PA, USA). Ultrathin sections (70 nm) were cut with a diamond knife on an ultramicrotome (UC7, Leica Microsystem) and were collected on copper grids with a single slot. Sections were stained with uranyl acetate and lead citrate and then were observed under an electron microscope (Tecnai G2 Spirit, FEI) at 120 kV.

Immunofluorescence

The cells were fixed in 4% paraformaldehyde (DingGuo, AR-0211) at room temperature for 15 min and blocked with PBS that contained 0.2% Triton X-100 (Sigma-Aldrich, T8787) and 3% normal donkey serum (Jackson Immuno Research, 017-000-121) at room temperature for 45 min. The cells were incubated with primary antibodies at 4°C overnight. Secondary antibodies (Jackson ImmunoResearch) were incubated at room temperature for 1 hr. The nuclei were stained with DAPI (Roche Life Science, 10236276001). Antibody details were provided below.
anti-EPX (1:200, Abcam, Cat: ab190715); anti-Bsp-1 (1:200, BD Pharmingen, Cat: 552754); anti-MBP1 (1:200, invitrogen, Cat: PA5-112670); anti-RceR1 alpha (1:200, eBioscience, Cat: 11589942); anti-CD203c (1:200, biolegend, Cat: 324610); anti-EDN (1:200, CUSABIO, Cat: P10153); anti-ECP (1:200, CUSABIO, Cat: P12724).

RNA sequencing and bioinformatics analysis

During H1 differentiation, total RNA was isolated from cultured cells on day 0 (H1), day 12 (E0), day 16 (E4), day 20 (E8), day 24 (E12), day 28 (E16), and day 32 (E20) using RNeasy Plus Micro kit (Qiagen, 74034). Total RNA of cord blood primary naïve eosinophils was isolated using the same kit. RNA sequencing libraries were constructed using an NEB Next, Ultra RNA Library Prep kit for Illumina (NEB England BioLabs, E7530L). The fragmented paired-end libraries were sequenced using an Illumina HiSeq-PE150. All sequencing was performed at Novogene.

For bioinformatics analysis of the RNA-seq data, Fastq reads were aligned to the human reference genome (hg19) or mouse reference genome (mm10) using TopHat. Counting and FPKM values were calculated with cuffquant and cuffnorm, respectively. Clustering analysis and gene expression heatmaps in Figures were based on FPKM values. DESeq2 was used with default parameters to identify differentially expressed genes between samples. We used

log2 (fold change) >1 or < -1 and FDR < 0.01 as the cutoff.

Quantitative real-time PCR

Total RNA was isolated from the indicated cells with an RNA isolation kit (QIAGEN, Cat: 74034) according to the manufacturer's protocol. The Easy transcriptase kit (Transgene, Cat: AT311–03) was used for synthesizing cDNA from total RNA. Quantitative real-time PCR was performed in triplicate from at least three biological samples with a BIO-RAD CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Cat: 1855201). Quantitative PCR was carried out in a volume of 20 μl using FastStart Essential DNA Green Master (Roche, Cat: 06924204001). The PCR protocol was as follows: first, 95 °C for 10 min to activate the polymerase, followed by 40 cycles at 95 °C for 10 s (for denaturation), 60 °C for 10 s (for annealing), and 72 °C for 10 s (for extension). Values for mRNA expression were normalized to the expression of H1 or iPSC. The primer sets used to detect single genes are listed in Table S1.

Lentiviral vectors and transduction

The lentiviral vector (Plenti3) EF1a-GFP-2A-Luc2-SV40-puro (GFP-luc for short), which encodes separate GFP protein and luciferase, was packaged, and the titre was determined according to the protocol previously described (Xiao et al., 2019). HCT116, MDA-MB-231 and HepG2 cell lines were transduced with the lentiviral vectors with 8 μg/ml polybrene (Yeasen, Cat: 40804ES86), and then the GFP^{+} cells were flow sorted and propagated, and the luciferase activity of each was confirmed before further use.

Isolation, activation and infection of human T cells

All Peripheral Blood Mononuclear Cells (PBMCs) used in our study were obtained from healthy donors who provided informed consent (Blood Center of Beijing Red Cross Society). Human T lymphocytes were cultured in RMPI-1640 medium (Gibco, USA) supplemented with 10% FBS and 300 U/ml interleukin-2 (Peprotech, USA). After 48 hours of activation with anti-human CD3/CD28 Dynabeads (Gibco, USA), 8 μg/ml polybrene (Millipore, USA) and 300 U/ml IL-2 were added to each well and T cells were transduced twice over the next 48 h with meso-CAR lentivirus by spinoculation for 1 h. Transduction efficiency was determined by flow cytometry 7 days later.

Immunohistochemistry and histological analysis

To analyze the infiltration of human ESC-derived eosinophils in solid tumors and mouse main organs, tumor tissues and mouse main organs were fixed with formalin (10% and 20% respectively 24 h) at room temperature. After paraffinization, 5 μm slices were cut and affixed on slides for immunohistochemical staining. We used the anti-EPX antibody (Abcam, Cat: ab19075) to identify eosinophils according to the manual (ZSGB-BIO, SP-9000). Main organs of mouse were collected after the injection of hPSC-derived eosinophils at 4 × 106 and 8 × 106 per mouse at day 3 and day 20 respectively, Hematoxylin and Eosin(H&E) staining was used for tissue and cell identification.

***In vitro* cytotoxicity assays**

Tumor cells bearing the GFP-luc transgene were seeded at a density of 1×10^{4}cells/well in

96-well plates in 100 ul DMEM containing 10% FBS. Six to ten hours later, the eosinophils were added to the target cells at E (effector): T (target) = 5: 1, 2: 1, 1: 1, and 0: 1, and the medium volume of eosinophil suspension was 100 μl per well. After incubation for 20 h, the apoptotic cells of the tumor target cells were quantified by a standard bioluminescence assay based on luciferase using a multimode plate reader (PerkinElmer). The percentage of lysed cells was calculated using the following equation: % lysis = 100 × (spontaneous death RLU - test RLU) / (spontaneous death RLU). RLU: relative light units.

***In vivo* tumor assay**

For forming inoculated tumors, luciferase-marked target cells were injected subcutaneously into the recipient NPG mice at a dose of 5×10^4 cells per mouse, which was followed by two intravenous injections of eosinophils; each mouse received 2×10^6 eosinophils suspended in 100 μl of culture medium on day 3 and day 6. The control groups were injected with only an equal volume of culture medium to enable comparison with the experimental groups. The tumor burden of each mouse was monitored by *in vivo* bioluminescence imaging using Xenogen IVIS (Caliper Life Sciences). Mice were injected intraperitoneally with 150 mg/kg D-luciferin (GoldBio, Cat: LUCK-100) and were imaged 10 min later;

For forming established tumors, 1.5×10^6 HepG2 tumor cell were injected subcutaneously and 2×10^6 eosinophils (pre-activated 20 hour with 10 ng/ml IFNγ and 10 ng/ml TNFα) were intravenously injected on day 7 and day 10; 1×10^6 A375 tumor cell were injected subcutaneously and 2×10^6 eosinophils (pre-activated 20 h with 10 ng/ml IFNγ and 10 ng/ml TNFα) were intravenously injected on day 6 and day 9; 1×10^6 HCT116 tumor cell were injected subcutaneously and 2×10^6 eosinophils (pre-activated 20 h with 10 ng/ml IFNγ and 10 ng/ml TNFα) were intravenously injected on day 4 and day 7;

For evaluating the combination effects of CAR-T and hESC-derived eosinophils, we designed CAR-T or hESC-derived eosinophils alone as controls. Tumor cells were injected subcutaneously at a dosage of 1×10^6 HCT116 tumor cells per mouse. Two intravenous injections of eosinophils, CAR-T cells or their combination were performed. Each mouse received 2×10^6 eosinophils, which were pre-activated 20 h with 10 ng/ml IFNγ and 10 ng/ml TNFα on day 4 and day 7. In the following day, 1×10^6 CAR-T cells were respectively injected. To increase the dose of CAR-T or hESC-derived eosinophils, each mouse received 4×10^6 eosinophils on day 4 and day 7, or each mouse received 2×10^6 CAR-T. The control groups were injected with only an equal volume of culture medium to enable comparison with the experimental groups. The tumor size was measured at the indicated time points using the following formula: V = 1/2 × (length × width × width). Tumor-bearing mice in this study were randomized to different groups. Mice with large tumor masses more than 15 mm in length were euthanized.

SUPPLEMENTAL REFERENCES

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