Supplementary materials and methods

NSG and Humice

All manipulations and procedures with mice were approved by Agency for Science, Technology and Research (A*STAR) Institutional Animal Care and Use Committee (IACUC). The diet provided was irradiated TEKLAD GLOBAL 18% Protein Rodent Diet (2918). Mice were housed in a sterile environment and only accessed under a BSL2 hood. Mice were fed, given water and monitored daily for health, and cages were changed weekly.

NSG mice were purchased from The Jackson Laboratory and bred in a specific pathogen free facility at the Biological Resource Centre (BRC) in A*STAR, Singapore. One to three days old NSG pups were irradiated with a 55 s exposure equaling 1.1 Gy and transplanted with 1×10^5 $CD34^+$ human fetal liver cells by intra-hepatic injections. The mice were bled at 8 weeks post-transplantation to determine the fraction of human immune cell reconstitution. Reconstitution was calculated by [$\%hCD45^+$ / ($\%hCD45^+$ + $\%mCD45^+$)]. 8-10 weeks old humanized mice reconstituted with 20–50% of human $CD45^+$ cells were used for engraftment.

HCC-PDX tumor maintenance and xenografts

For *in vivo* HCC-PDX subcutaneous humanized model establishment, patient HCC tumors were collected from HCC surgical specimens. Before surgery, all patients gave written informed consent for their HCC samples to be used for research. After appropriate clinical tissue is taken, the remainder of the HCC is transferred on ice with media consisting of DMEM containing 10% FCS, 1% penicillin/streptavidin to where the PDX is to be established. Within 4 hours, HCC fragments were cut into pieces of ca. $3 \times 3 \times 3$mm using sterile surgical instruments. Once the mice are anaesthetized, and shaved, for subcutaneous placement, using forceps to lift up the skin to ensure no peritoneal violation a small 1cm incision is made in the skin with scissors. The subcutaneous is probed to create a pocket, the tissue is placed inside the pocket and the skin is closed with adhesive, suture or clips.

To maintain HCC-PDX tumors in NSG mice, HCC obtained from the first generation of mice (P1) were serially transplanted to the next cohorts of mice (P2 and P3). HCCs were harvested from established PDXs were cut into pieces of ca. $3\times3\times3$ mm^3 using sterile surgical instruments in a laminar flow cabinet. Pieces were transferred into sterile cryotubes containing 1.5 mL 95%FCS/5% DMSO. Cryotubes were put in CoolCell container

(Biocision), placed in a −80°C freezer overnight and transferred to liquid nitrogen storage the next day. For thawing, cryotubes were held in a water bath (37°C) until melted.

Determination of tumor size

Tumor volume was measured in two dimensions (length and width) using calipers and the tumor volume was calculated using the formula: Tumor size= (length ^{2}x width) x $\pi/6$.

Isolation of leukocytes from blood, spleen, bone marrow and HCC-PDX tumor

150-200 µl blood was collected in potassium EDTA MiniCollect® tubes (Greiner bio-one, 450475) via cheek bleeding. 30 µl of blood mixed with 20µl CountBright™ Absolute Counting Beads (ThermoFisher) were plated in 96-well V-bottom plates at room temperature before processing and data acquisition. Fresh spleen was excised from mouse and placed in PBS on ice. Crush spleen through a 100µm cell strainer (Falcon) using a syringe plunger until only connective tissue is left. For bone marrow cells isolation, tibias, femurs, hip and spine were dissected from mice. The clean bones are crushed with mortar and pestle in medium (PBS+2%FCS+2mM EDTA). The cell mixture obtained from each mouse is kept separate and filtered through a 100µm cell strainer (Falcon). All samples were processed within one hour of collection. To isolate the TILs from HCC, tumor was cut up into 1-2 mm^2 fragments after trimming away fat and connective tissue and disaggregated with human tumor dissociation Kit (Miltenyi Biotec) using gentleMACS™ Dissociator (Miltenyi Biotec). The cell suspension was filtered through a 100µm cell strainer (Falcon). Cell suspension layered over a discontinuous 40% followed by a 80% Percoll® Density Gradient Media (GE Healthcare). Leukocytes are located at the interface between 40% and 80% Percoll. The enriched TILs were then washed in D-PBS, 1% BSA and then processed as the described.

Flow cytometry

Cell mixture from blood, spleen, bone marrow and tumor was suspended in ammonium-chloride-potassium (ACK) lysing buffer (Life Technologies) and incubated for 10 minutes at room temperature with gentle mixing to lyse contaminating red blood cells (RBC). For surface staining, leukocytes were washed twice in Fluorescence-activated cell sorting (FACS)

buffer [Phosphate-buffered saline (PBS) + 2% BSA + 1 mM EDTA + 0.1% sodium azide], incubated with Fc blocking reagent (Miltenyi Biotec) and stained with directly conjugated antibodies. For intracellular staining, blood leukocytes were labeled with surface markers as previous described and then fixed and permed with Transcription Factor Buffer Set (BD Pharmingen™). Five antibodies panel were used for this study. Human T cell panel (15 colors): hCD4- BUV395, hCD8-BUV373, hCD183-BV421, hCD197-BV510, hCD25-BV605, hCD196-BV650, hCD38-BV711, hCD45RO-BV785, hCD45RA-FITC, hCD127-PE, hCD194-PE-CF594, hCD3-PERCP5.5, hCD185-PE-CY7, hCCR10-APC and hHLA-DR-APC-CY7. Human Non T cell panel (15 colors): hCD45- BUV395, hCD19-BUV373, hCD56-BV421, hIgD-BV510, hCD11c-BV605, hCD27-BV650, hCD38-BV711, hCD16-BV785, hCD123-FITC, hCD20-PE, hCD24-PE-CF594, hCD66b-PERCP5.5, hCD3-PE-CY7, hCD14-APC and hHLA-DR-APC-CY7. Human Tex cell panel (15 colors): hCD4- BUV395, hCD8-BUV373, hCD272-BV421, hCD197-BV510, hKLRG-1-BV605, hCD28-BV650, hCD279-BV711, hCD366-BV785, hCD45RA-FITC, hCD57-PE, hCD152-PE-CF594, hCD160-PERCP5.5, hTIGIT-PE-CY7, hCD223-APC and hCD244-APC-CY7. Human Tc cell panel (11 color): hCD4- BUV395, hCD8-BUV373, Granzyme B-BV421, hCD197-BV510, hIFN-γ-BV605, hTNF-α-BV650, hCD3-BV785, hCD45RA-FITC, Granulysin-PE, Granzyme A-PERCP5.5, Perforin-APC and hIL-2-APC-CY7.TAM and MDSC panel(13 color): hCD45- BUV395, hCD11b-BV421, hCD86-BV605, hCD15-BV650, hCD204-BV711, hCD16-BV785, hCD33-FITC,Lineage (hCD3, hCD19 and hCD56) -PE, hCD68-PE-CF594, hCD163-PERCP5.5, hCD124-PE-CY7, hCD14-APC and hHLA-DR-APC-CY7. Dead cell exclusion was performed with the addition of DAPI (Life Technologies).

Human multiplex cytokine analysis

Plasma cytokines were analyzed using the LEGENDplex™ human Th Cytokine Panel (13-plex) array kit, human cytokine Panel 2 (13-plex) array kit and human CD8/NK panel assay kit (13-plex) from Biolegend according to the manufacturer's protocol. The data were collected on a LSR II flow cytometer and analyzed using the LEGENDplex™ software version 7.0 (Biolegend).

Isolation human $CD3^+$ cells and activation *in vitro*

Human $CD3^+$ cells were isolated from blood, spleen and TILs cells using dead cell removal kit (Miltenyi Biotec), mouse cell depletion kit (Miltenyi Biotec) and human pan T cell isolation kit (Miltenyi Biotec). $2x10^5$ human T cells mixed with anti-human CD2/3/28

MACSiBead particles from human T cell activation/ expansion Kit (Miltenyi Biotec) were seeding into TexMACS medium (Miltenyi Biotec). For the drug testing in vitro, Pembrolizumab and Ipilimumab were added to final concentration 2.5µg/ml. At third days, the supernatant was used for IFN-γ measurement.

IFN-γ ELISA

The supernatant levels of human IFN-γ were determined using a sandwich ELISA kit from Biolegend; Human IFN-γ ELISA MAX ™ Deluxe, exactly as described by the manufacturer.

Histology

For histology analyses, HCC-PDX tumors were formalin-fixed, paraffin-embedded (FFPE), sliced into 5-µm sections, and subjected to standard hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC). Paraffin sections (5 µm) were stained with hematoxylin and eosin (H&E) for initial histopathological evaluation. IHC was performed using rabbit specific IHC polymer detection kit HRP/DAB (ab209101) and mouse on mouse polymer IHC Kit (ab127055) according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. Primary antibodies from Abcam used for IHC were: Mouse anti-human CD45 (ab781), Mouse anti-human CD3 (ab17143), Rabbit anti-human CD4 (ab133616), Rabbit anti-human CD8 (ab93278), Rabbit-anti-human PD-1 (ab137132), Mouse anti-human NCAM (ab200698), Mouse anti-human CD68 (ab955) and Rabbit-anti-human PDL1 (ab58810).

Hep3B cell culture and IFN-γ treatment

The human HCC cell line Hep3B was purchased from ATCC and cultured in minimal essential medium (MEM) containing 10% fetal bovine serum. IFN-γ (Biolegend) was added to final concentration (0.1 ng/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 for 24 h.

RNA isolation and quantitative real-time PCR

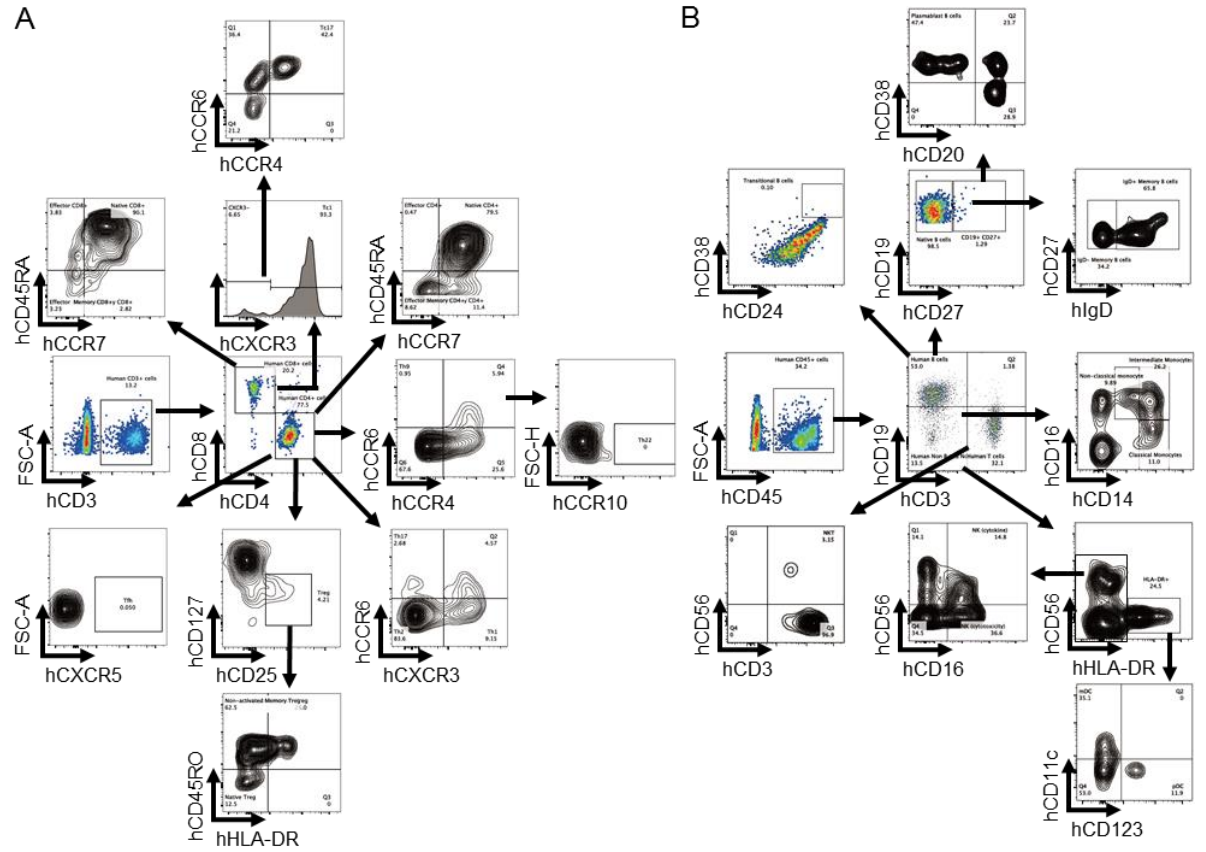
Total RNA was extracted from Hep3B cell line and HCC-PDX tumor tissue using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA was prepared using QuantiTect Reverse Transcription Kit (QIAGEN). qRT-PCR was performed in triplicates using iQ SYBR Green Supermix on an iCycler Real-Time Detection System (BioRad). All values were normalized with glyceraldehydes 3-phosphate dehydrogenase

(GAPDH) as an endogenous control. Data was analyzed using the comparative cycle threshold (Ct) method in which gene expression is calculated as $2^{-\Delta\Delta CT}$, where Delta Ct = (Ct gene of interest – Ct GADPH internal control). The primers used for the qRT-PCR analysis are listed in Supplementary table S2.

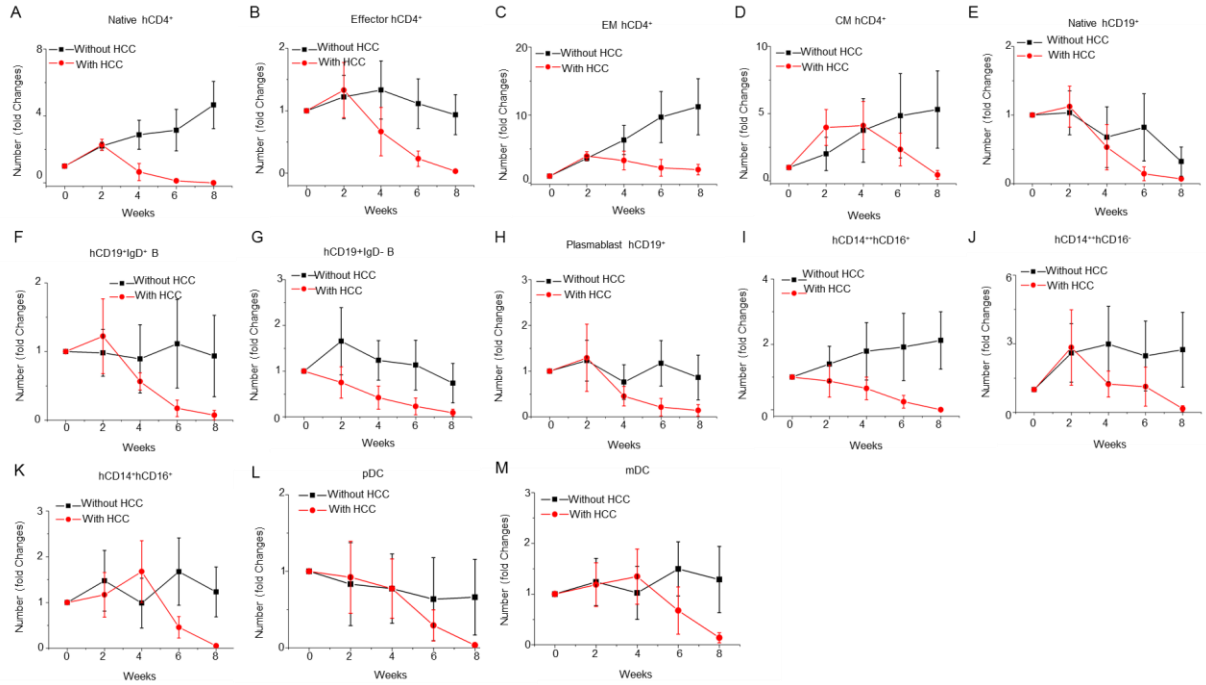
Statistical analysis

Statistical analysis was performed using JMP 13 (SAS Institute Inc. Cary, NC, USA) or Origin 9 (Origin Lab Corp., Northampton, MA, USA). All data are represented as mean ± standard error of the mean (SEM) and were tested for statistical significance using Mann-Whitney U test or analysis of variance (ANOVA). *P*-values of <0.05 were interpreted as statistically significant.

Supplementary figures

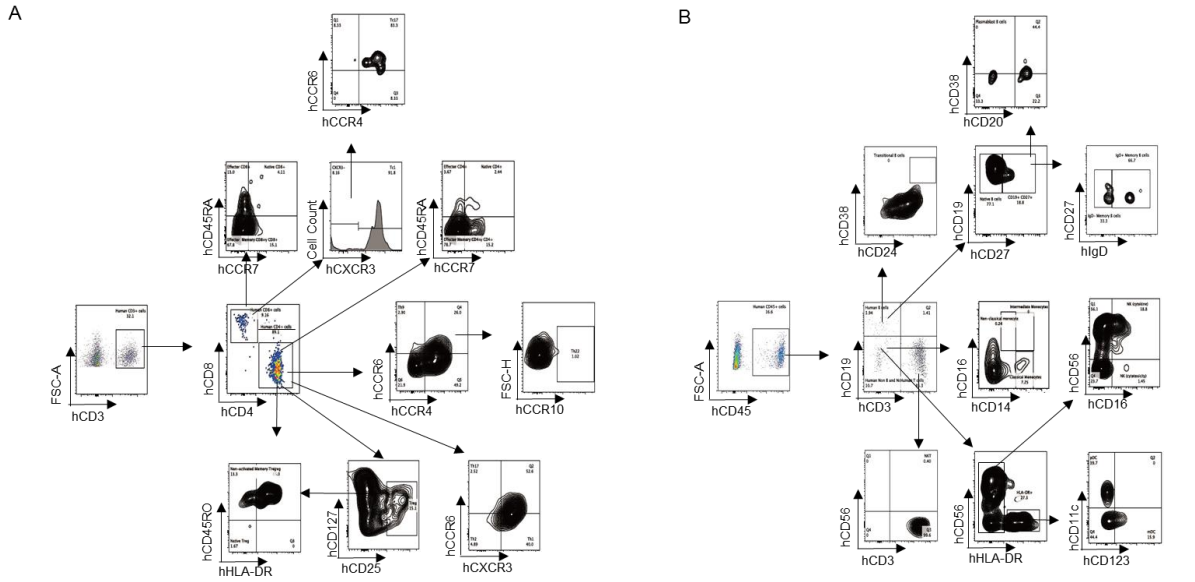


Supplementary figure 1. Human blood immune cell profiling in humanized mice. The gating scheme for human blood T cell (A) and non-T cell (B) populations in humanized mice.

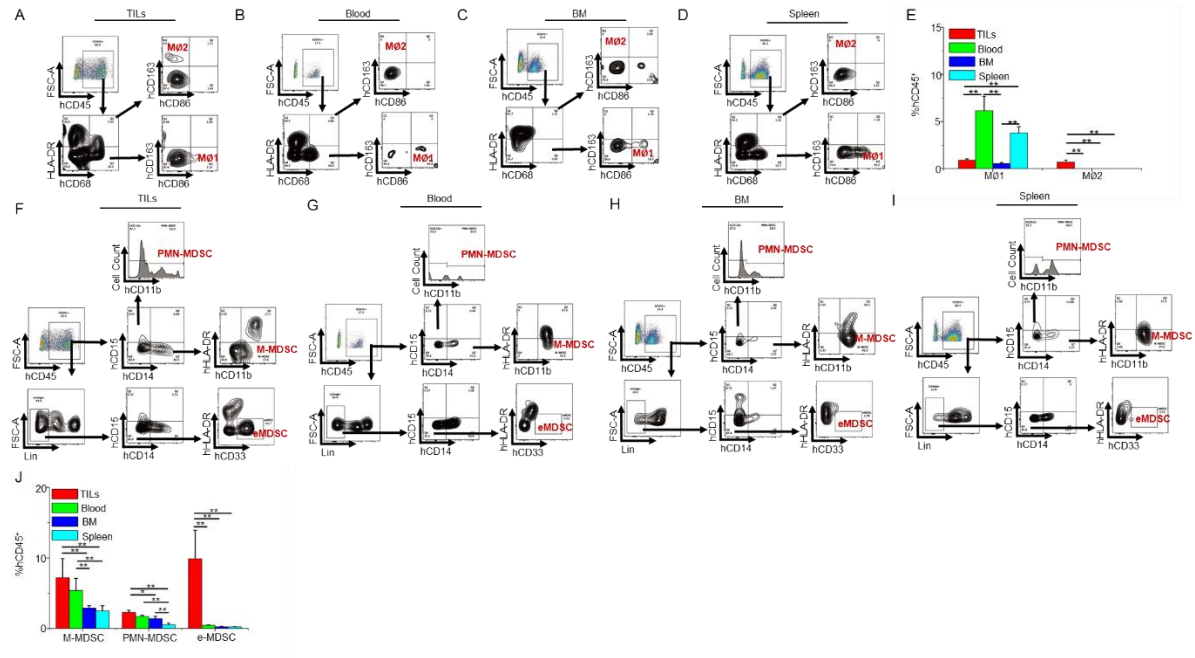


Supplementary figure 2. The kinetics of blood immune cell number count changes after tumor inoculation. PDX tumors were transplanted s.c. to 8-10 weeks old humice (n = 5). Blood immune cell frequencies and absolute numbers were analyzed biweekly by flow

cytometry. Data are presented as fold changes normalized to the cell numbers of specific cell types before PDX transplantation (week 0). (A) Native hCD4^{+} T cells, (B) Effector hCD4^{+} T cells, (C) Effector memory hCD4^{+} T cells, (D) Central memory hCD4^{+} T cells, (E) Native hCD19^{+} B cells, (F) IgD^{+}CD19^{+} B cells, (G) CD19^{+}IgD^{-} B cells, (H) Plasmablast CD19^{+} B cells, (I) CD14^{++}CD16^{+} monocytes, (J) CD14^{++}CD16^{-} monocytes, (K) CD14^{+}CD16^{+} monocytes, (L) pDC, (M) mDC.

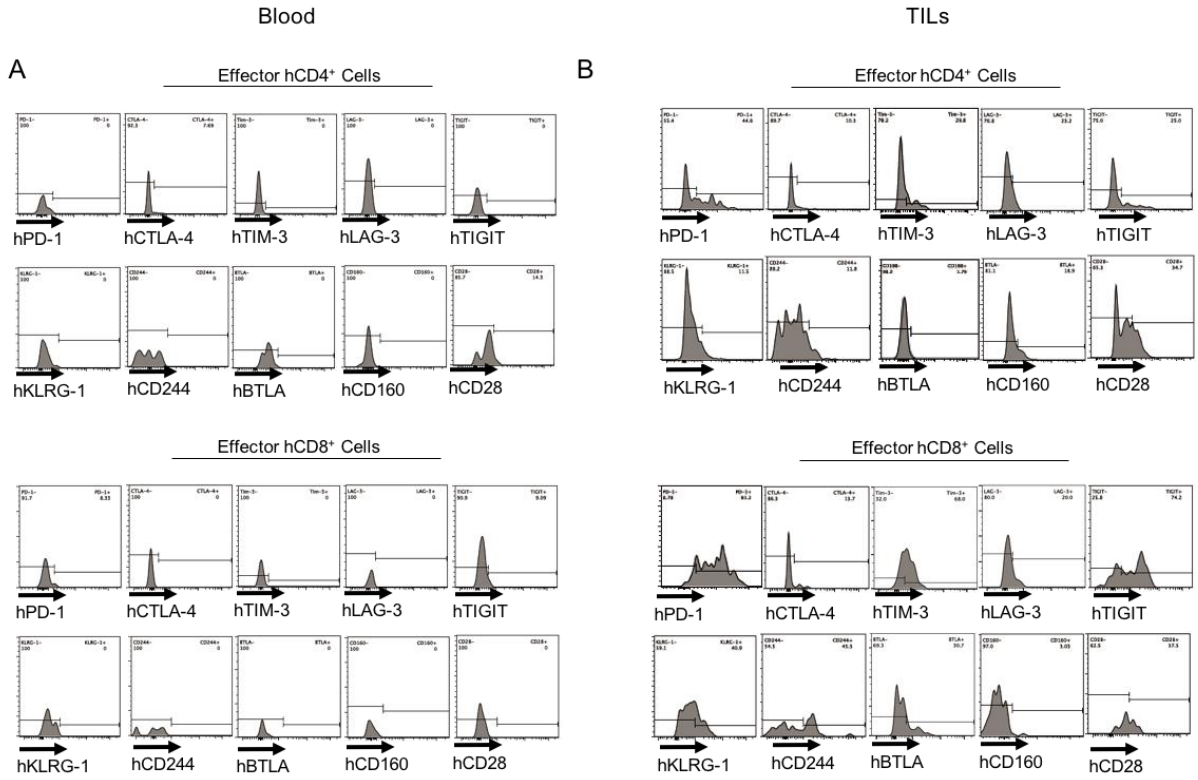


Supplementary figure 3. The gating scheme for human tumor infiltrated T cell (B) and non-T cell (C) populations in humanized mice.

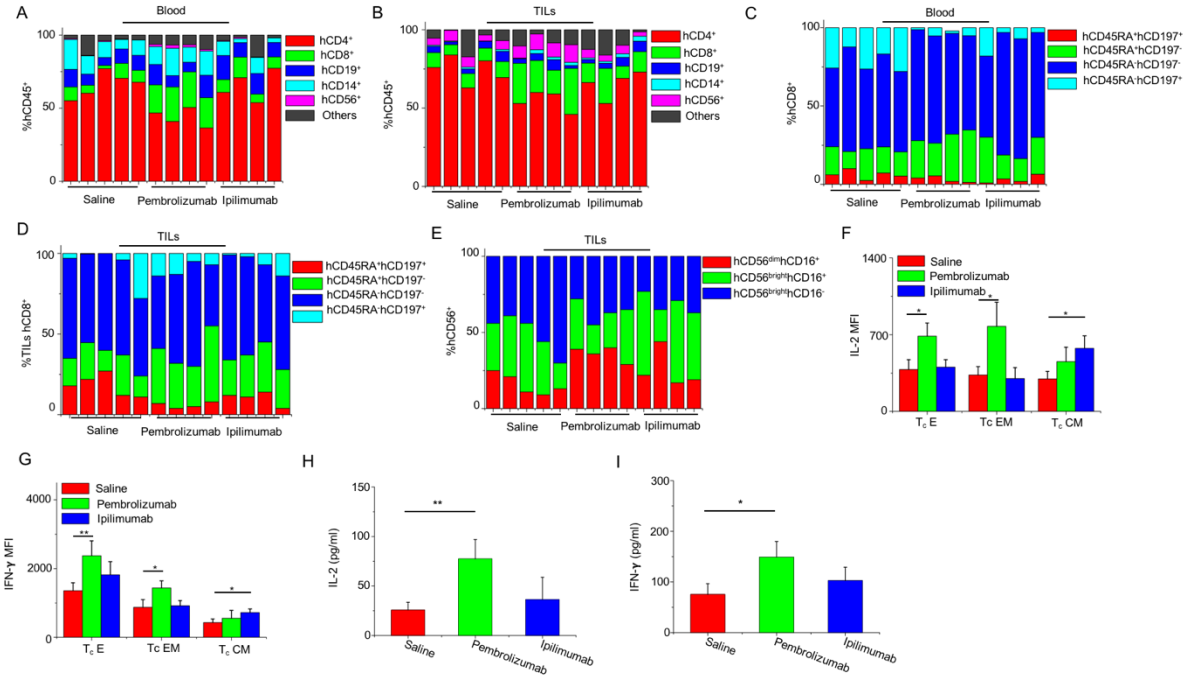


Supplementary figure 4. The profiling of HCC-tumor associated macrophage (TAM) in TILs (A), Blood (B), BM (C) and Spleen (D). (E) The statistical analysis of MØ1 and MØ2 in

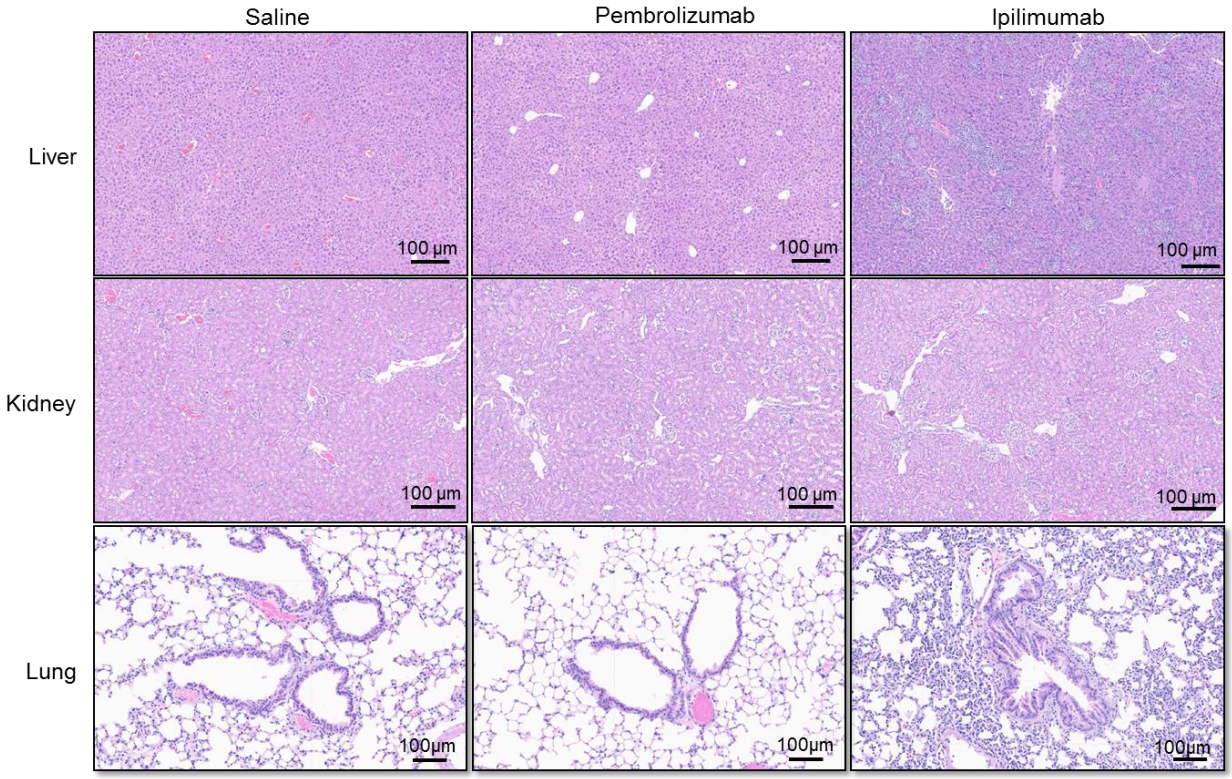
different organs. The profiling of MDSC and in TILs (F), blood (G), BM (H) and spleen (I). (J) The statistical analysis of MDSC subsets in different organs.



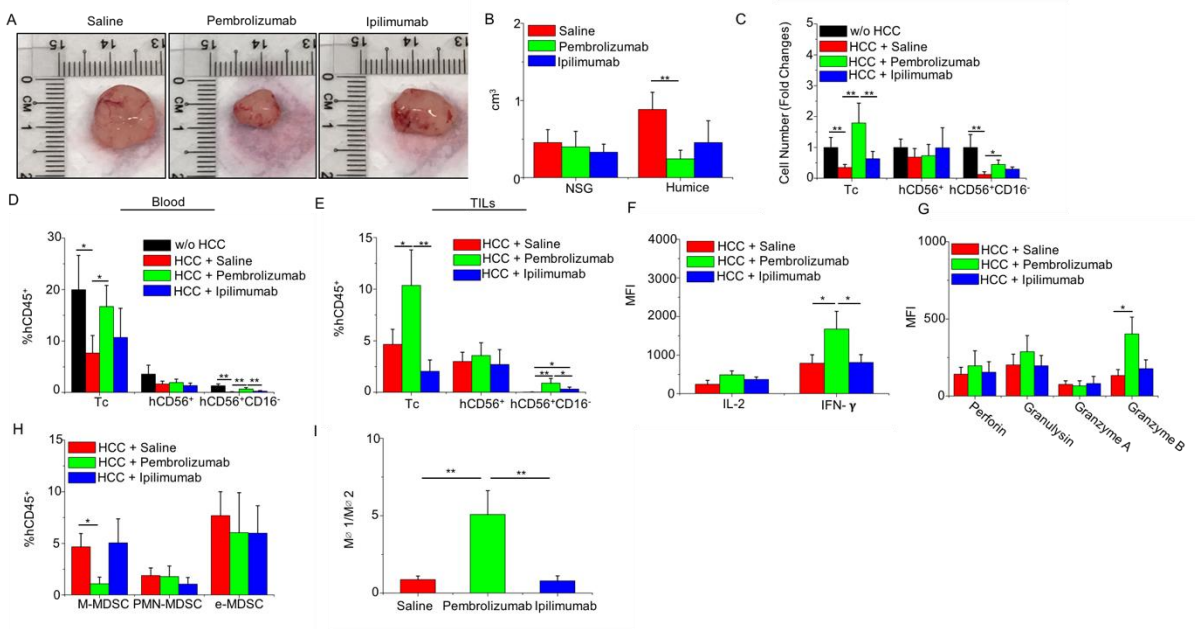
Supplementary figure 5. PDX tumors were transplanted s.c. to 8-10 weeks old humice. Eight weeks post transplantation, blood and tumor leukocytes were isolated and analyzed by flow cytometry for the expression of immune checkpoint receptors. Shown are the representative plots of immune checkpoint receptor stains in effector $hCD4^+$ and $hCD8^+$ T cells from blood (A) and TILs (B).



Supplementary figure 6. Drug treatment effects on immune cells and cytokine production. Four weeks after tumor inoculations, humice and NSG were treated with saline, Pembrolizumab or Ipilimumab for another 4 weeks (n = 4). (A and B) The proportion changes of main human immune cell subtypes in blood (A) and TILs (B) after drug treatments. Each bar presents one mouse. (C and D) The proportion changes of human Tc subtypes in blood (C) and TILs (D). (E) The proportion changes of human NK subtypes in TILs. (F and G) Intercellular staining of human IL-2 (F), IFN-γ (G) in Tc subsets. (H and I) Plasma levels of human IL-2 (H) and IFN-γ (I) after drug treatment. *: $p < 0.05$. **: $p < 0.01$.

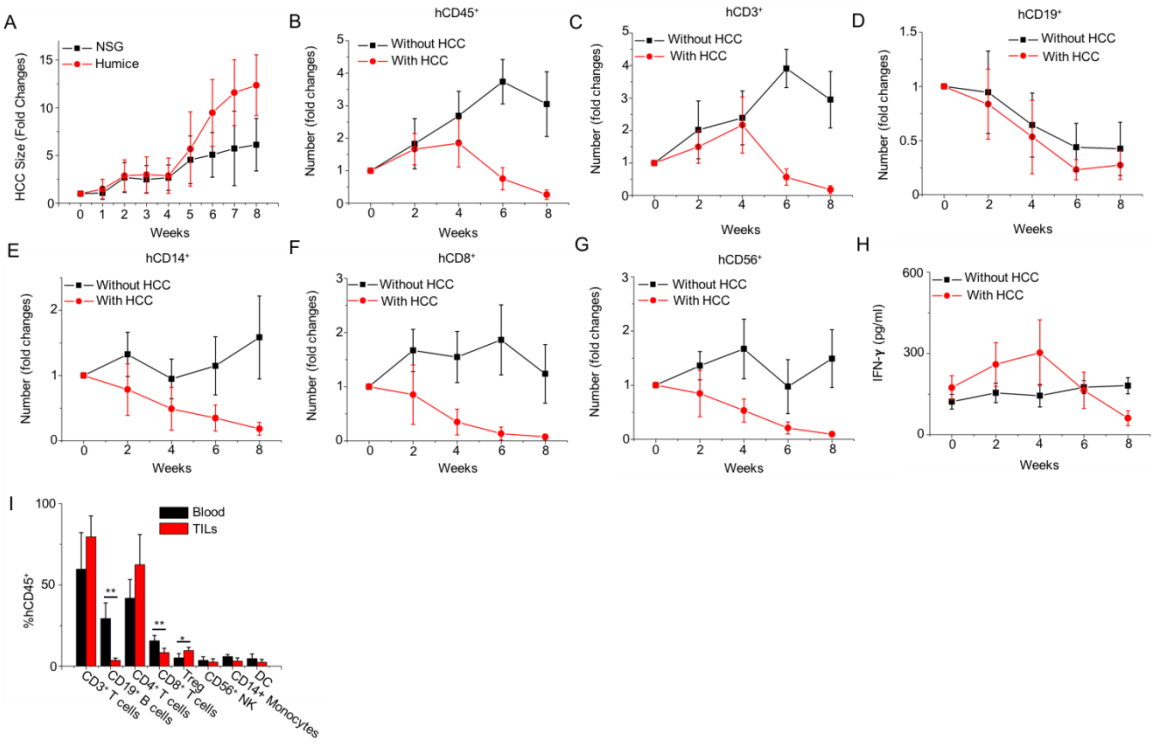


Supplementary figure 7. Four weeks after tumor inoculations, humice and NSG were treated with saline, Pembrolizumab or Ipilimumab for another 4 weeks. Liver, kidney and lung were collected for histological analysis. Shown are representative images of H&E stain in liver, kidney and lung.

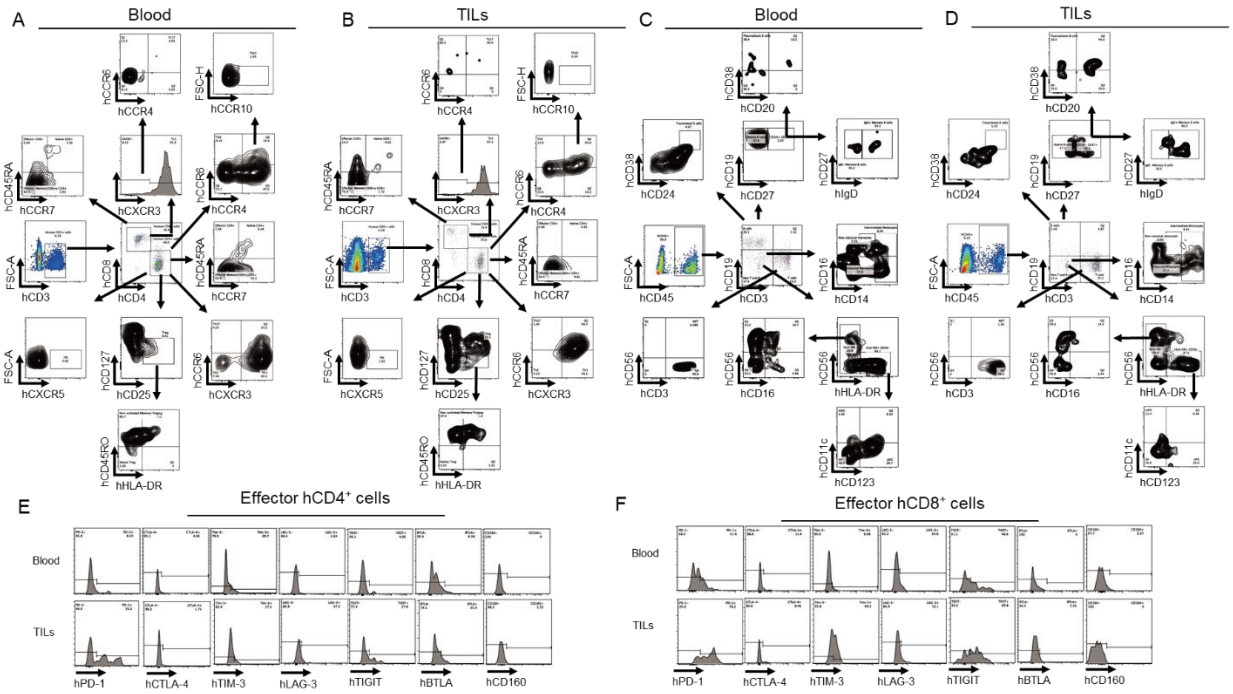


Supplementary figure 8. The effects of immune checkpoint inhibitors (Pembrolizumab and Ipilimumab) treatment in HCC#4 PDX humice. Four weeks after tumor inoculations, humice

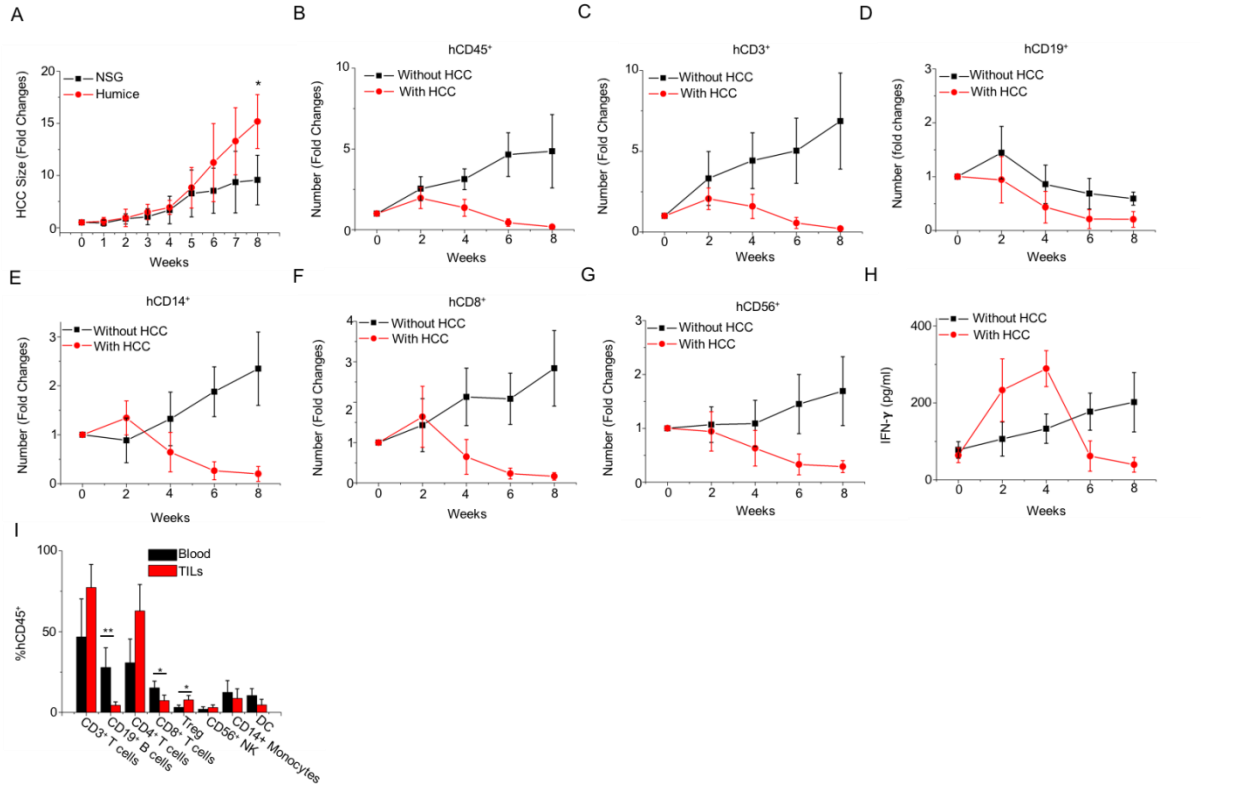
and NSG were treated with saline, Pembrolizumab or Ipilimumab for another 4 weeks before tumor samples and plasma were collected for analysis (n = 8). (A) Shown are the representative images of HCC-PDX tumors. (B) The statistical analysis of HCC-PDX tumor size in NSG and humice with different treatments. (C) The absolute number counts of blood cytotoxic cells after treatments. The data are presented as fold changes normalized to counts from humice without HCC. (D) The proportion changes of major immune cell types in blood after drug treatments. (E) The proportion changes of major immune cell types in TILs after drug treatments. Intercellular staining to analyze the expression of human IL-2 and IFN-γ (F) and cytolytic proteins (G) in Tc. The proportion changes of MDSC (H) and ratio changes of MØ1/MØ2 (I) in TILs after drug treatments. *: $p < 0.05$. **: $p < 0.01$.



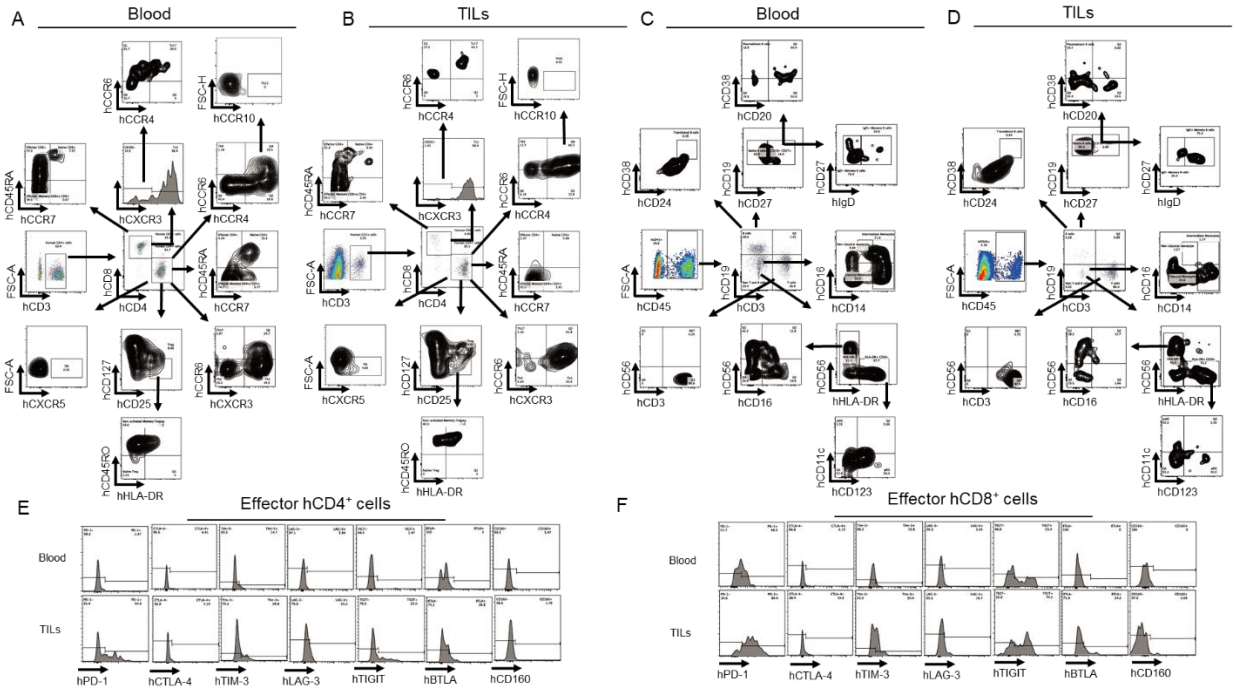
Supplementary figure 9. The growth and immune kinetics changes of HCC#2. (A) HCC#2 growth curve in NSG and humanized mice (n = 7). (B-G) The fold changes of human main immune cells in blood: Human $hCD45^+$ cells (B), $hCD3^+$ (C), $hCD19^+$ (D), $hCD14^+$ (E), $hCD8^+$ (F), $hCD56^+$ (G) cells. (H) The kinetics of plasma human IFN-γ after HCC#2 engraftment in humice. (I) The proportion of major human immune cells from blood and HCC#2 PDX tumor. *: $p < 0.05$. **: $p < 0.01$.



Supplementary figure 10. The immune profiling and expression of immune checkpoint receptors of HCC#2. The T cells subsets from blood (A) and TILs (B). The other subsets of human immune cells from blood (C) and TILs (D). The plots of immune checkpoint receptors in effector human $CD4^+$ (E) and effector human $CD8^+$ (F) T cells from blood and TILs.



Supplementary figure 11. The growth and immune kinetics changes of HCC#3. (A) HCC#3 growth curve in NSG and humanized mice (n = 7). (B-G) The fold changes of human main immune cells in blood: Human hCD45$^+$ cells (B), hCD3$^+$ (C), hCD19$^+$ (D), hCD14$^+$ (E), hCD8$^+$ (F), hCD56$^+$ (G) cells. (H) The kinetics of plasma human IFN-γ after HCC#3 engraftment in humice. (I) The proportion of major human immune cells from blood and HCC#3 PDX tumor. *: $p < 0.05$. **: $p < 0.01$.



Supplementary figure 12. The immune profiling and expression of immune checkpoint receptors of HCC#3. The T cells subsets from blood (A) and TILs (B). The other subsets of human immune cells from blood (C) and TILs (D). The plots of immune checkpoint receptors in effector human CD4$^+$ (E) and effector human CD8$^+$ (F) T cells from blood and TILs.