Cell Reports, Volume *20*

Supplemental Information

Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18

Biliang Hu, Jiangtao Ren, Yanping Luo, Brian Keith, Regina M. Young, John Scholler, Yangbing Zhao, and Carl H. June

Experimental Procedures

Mice and cell lines

NOD-SCID-γ chain-/- (NSG) and C57BL/6 (CD45.1 donor and CD45.2 recipient) mice were purchased from Jackson Laboratories, and all animal procedures were performed in animal facility at the University of Pennsylvania in accordance with Federal and Institutional Animal Care and Use Committee requirements. Wild-type parental AsPC1, K562 cells and Nalm6 cells (purchased from American Type Culture Collection) were engineered to express CBG-T2A-GFP as a fluorescent reporter gene and tracer of luciferase activity *in vitro* and *in vivo*. K562 with CBG-T2A-GFP cells were further engineered to express human CD19 (K562-CD19) or human mesothelin (K562-Meso). B16F10 (ATCC) was engineered to stably express murine CD19 (GeneCopoeia).

Molecular cloning

The parental vector encoding mesothelin CAR pTRPE SS1BBz contains scFV recognizing human mesothelin, 4-1BB and CD3z intracellular signaling domains. The sequence encoding mature human IL18 (Uniprot Q14116) was synthesized from IDT and cloned into pTRPE SS1BBz along with a T2A (Kim et al., 2011) linker (pTRPE SS1BBz-T2A-hIL18). GFP and humanized CAR CD19BBz were cloned to replace SS1BBz to generate pTRPE CD19BBz-T2A-hIL18 and pTRPE GFP-T2A-hIL18. GFP was cloned to replace SS1BBz to generate pTRPE GFP. GFP was also cloned to replace hIL18 to generate pTRPE SS1BBz-T2A-GFP and pTRPE CD19BBz-T2A-GFP. The parental vector encoding anti-murine CD19 CAR MSGV muCD19BBz contains the 1D3 scFV recognizing murine CD19 (gift from Dr. Taku Kambayashi, UPENN Pathology and Laboratory Medicine) was originally obtained from ATCC (HB-305), murine 4-1BB and CD3z intracellular signaling domains. MSGV muCD19BBz vector was further inserted with a T2A linked GFP or murine IL18 sequence downstream of the CAR.

Primary human T cell expansion

Primary donor T cells were transduced with lentivector encoding CAR or GFP with or without hIL18 and expanded as previously reported (Milone et al., 2009). In brief, primary donor T cells were activated with anti-CD3/CD28 Dynabeads (ThermoFisher) at D0, and transduced with lentivector at D1. Fresh medium was added at D3 and the activated T cells were de-beaded at D5 and maintained at 7e5/ml until rested. T cells were frozen in liquid nitrogen.

Mouse syngeneic T cell expansion

Spleens were harvested from CD45.1 donor mice to isolate mouse T cells via anti-CD3 beads (Stemcell Technologies). Purified mouse T cells were activated with Dynabeads mouse CD3x28 beads (ThermoFisher) at a 2:1 ratio of bead:cell. Activated mouse T-cells were transduced with retroviral vector MSGV for CAR expression. Resting murine CART cells following ex vivo expansion were confirmed to have CAR expression and mIL18 secretion, and then washed and injected to CD45.2 recipient mice intravenously.

CRISPR/Cas9 gene knockout

Primary donor T cells were transduced with lentivector encoding CAR or GFP with or without hIL18 and engineered with CRISPR/Cas9 system as previously reported (Ren et al., 2016). In brief, primary donor T cells were activated with anti-CD3/CD28 Dynabeads (ThermoFisher) at D0, and transduced with lentivector at D1. The activated T cells were de-beaded at D3 and electroporated with sgRNA and Cas9 RNA. Then the T cells were maintained at 7e5/ml until rested. T cells were frozen in liquid nitrogen.

TCR-deficient T cells were made with sgRNA targeting GGAGAATGACGAGTGGACCC within the TCRB region. For the IL18R knockout we designed 7 sgRNA targeting IL18RA (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013), and utilized RNA electroporation to induce CRISPR/Cas9 mediated gene editing following the procedure of generating TCR KO universal CART (Ren et al., 2016). The screening of IL18RA KO showed that the A4 sgRNA produced the most efficient gene knockout, and sequencing of the PCR product spanning the gene edited site confirmed mutations in the DNA sequence (GTACAAAAGCAGTGGATCAC) targeted by the A4 sgRNA (data not shown).

Supplemental References

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A.*, et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819-823.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*, 816-821.

Kim, J.H., Lee, S.R., Li, L.H., Park, H.J., Park, J.H., Lee, K.Y., Kim, M.K., Shin, B.A., and Choi, S.Y. (2011). High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One *6*, e18556.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.

Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D., Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G.*, et al.* (2009). Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther *17*, 1453-1464.

Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H., and Zhao, Y. (2016). Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. Clin Cancer Res *23*, 2255-2266.

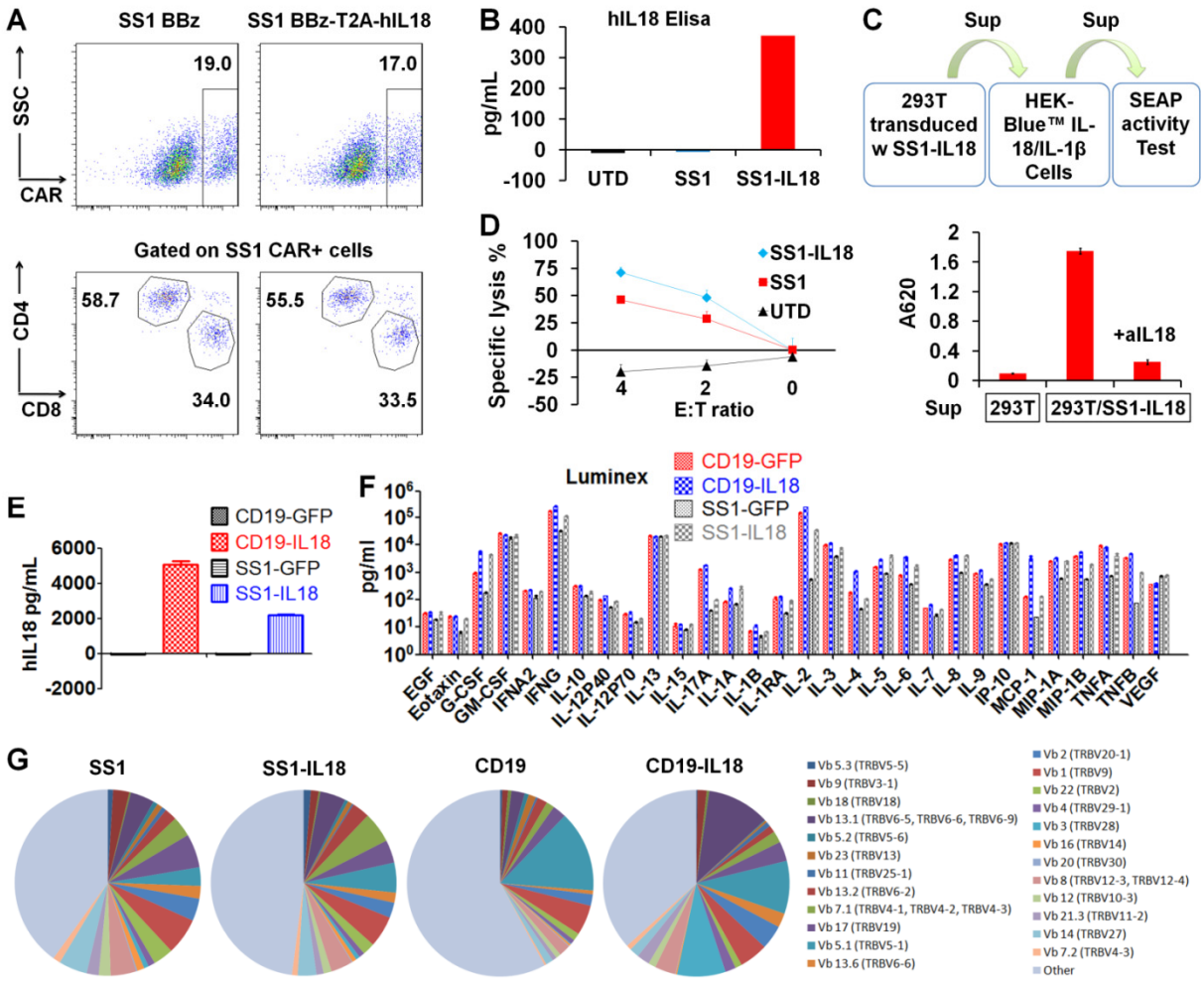

Figure S1. SS1-IL18 CART cells displayed enhanced cytotoxicity and cytokine production. Related to Figure 1. (A-D) Normal donor primary T cells were activated and transduced with lentivector SS1-IL18. SS1 CAR expression and the CD8 and CD4 distribution was assessed by flow cytometry **(A)**, and IL18 secretion determined by ELISA **(B)**. A commercial IL18 reporter cell line (Invitrogen) was used to test the bioactivity of IL18 **(C)**. 293T cells were transduced with lentivector SS1-IL18. Culture supernatant was transferred to IL18 reporter cells to activate IL18R mediated downstream production of SEAP, which was analyzed by a SEAP detection medium. SEAP activity was determined by A620 (duplicate), and anti-IL18 antibody was included as a control to confirm the specificity of IL18 induced SEAP production in the reporter cells. **(D)** SS1-IL18 CAR T or SS1 CAR T cells were co-cultured with BxPC3 (a mesothelin+ pancreatic tumor cell line) cells expressing CBG-GFP for 3 days at the ratios indicated. Remaining viable BxPC3 cells were lysed, and luciferase activity was determined by bioluminescence (triplicate). Specific lysis=(Sample-Max luciferase activity)/(Minimum luciferase activity- Max luciferase activity)*100%. Maximum luciferase activity was derived from BxPC3 cells only, and Minimum luciferase activity was derived from BxPC3 cells treated with triton X-100. **(E)** The concentration of IL18 was assessed by ELISA (duplicate) at D3 of 1st restimulation without exogenous IL2. **(F)** Luminex analysis (duplicate) was performed to determine cytokine production in the D3 culture supernatant of GFP- or IL18-CAR T cells following 1st aAPC restimulation without exogenous IL2. **(G)** IL18 expanded T cells are polyclonal through phenotyping of TCRB variants. Spleens of the mice displaying vigorous expansion of IL18-CAR T cells were harvested and processed to stain TCRB variants by IOTest® Beta Mark TCR V beta Repertoire Kit (Beckman Coulter). CD45+ cells were gated to estimate the frequency of each TCRB variant. Splenic SS1 or CD19 CAR T cells were included as controls.

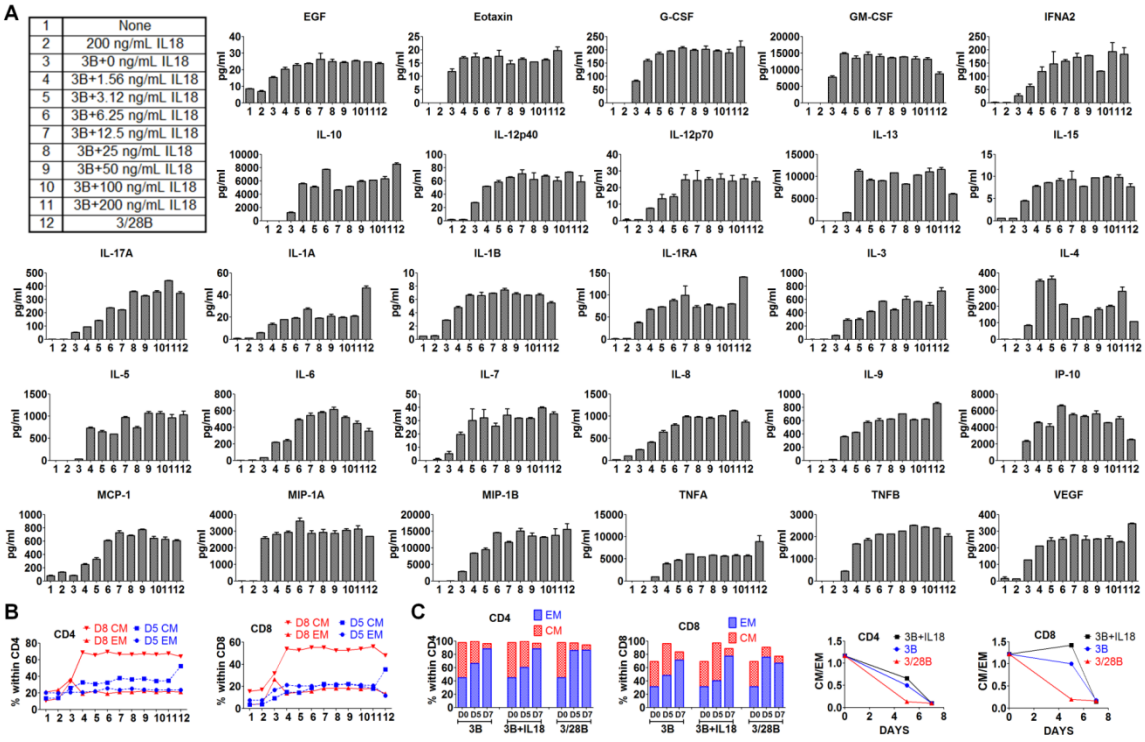

Figure S2. Cytokine profile and memory phenotype of IL18 and anti-CD3 bead activated T cells. Related to Figure 2. (A-B) Primary T cells from normal donors were activated with anti-CD3 beads and increasing concentrations of recombinant IL18, ranging from 1.56 to 200 ng/ml, added at D0 and D3. **(A)** Luminex analysis (duplicate) was conducted to assess the concentration of 30 cytokines in the D5 supernatant of T cells stimulated as indicated. (B) Memory phenotype at D5 and D8 was evaluated by staining for CCR7 and CD45RO. Central memory (CM) was defined as CCR7+CD45RO+, and effector memory (EM) as CCR7-CD45RO+. **(C)** Anti-CD3/CD28 beads were used to expand T cells from a normal human donor and the expanded T cells were then re-activated with anti-CD3 beads and 100ng/ml IL18, added at D0 and D3. Memory phenotype was analyzed by flow cytometry at D0 before restimulation, and D5 and D7 following restimulation.

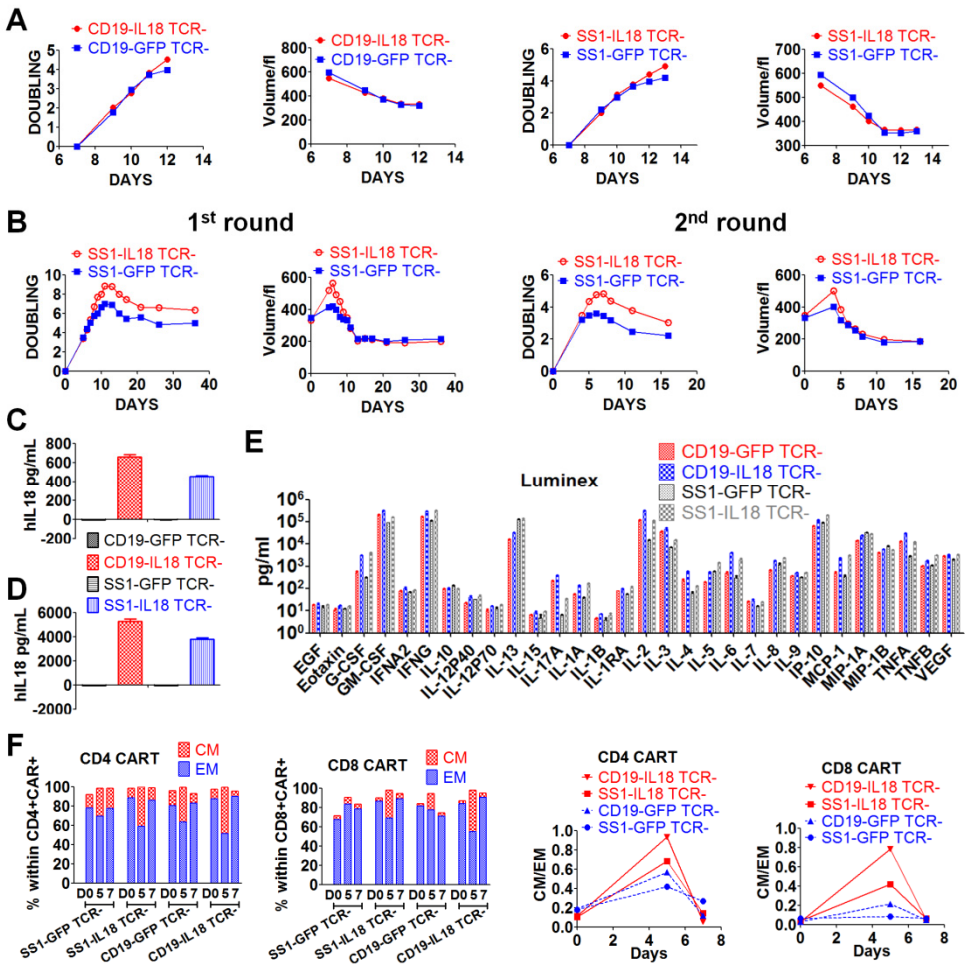

Figure S3. Characterization of TCR-deficient CAR T cells during *ex vivo* expansion and aAPC restimulation. Related to Figure 4. (A) Expansion kinetics and size of TCR-deficient (TCR-) GFP-CAR T or IL18-CAR T cells following stimulation with anti-CD3/CD28 beads and RNA electroporation. **(B)** Expansion kinetics and size of TCR-deficient SS1-GFP CAR T or SS1-IL18 CAR T cells following 1st (with exogenous IL2) and 2nd (without exogenous IL2) round of restimulation with irradiated mesothelin-expressing K562 cells. The concentration of IL18 was assessed by ELISA (duplicate) from TCR-deficient T cells following expansion with anti-CD3/CD28 beads and RNA electroporation of gRNA and Cas9. **(C)**, or at D3 of 1st restimulation without exogenous IL2 for TCR- deficient CART cells **(D)**. **(E)** Luminex analysis (duplicate) was performed to determine cytokine production in the D3 culture supernatant of TCR-deficient GFP-CAR T or IL18-CAR T cells following 1st aAPC restimulation without exogenous IL2**. (F)** IL18-CAR T was expanded by standard anti-CD3/28 beads protocol plus CRISPR/Cas9 targeted knockout of TCR. The IL18-CAR T/TCR KO cells were restimulated with irradiated APC initially with exogenous IL2 and subsequently further restimulated without exogenous IL2. T cell memory phenotype was analyzed by flow cytometry at D0 before 2nd restimulation, and D5 and D7 following the 2nd restimulation.

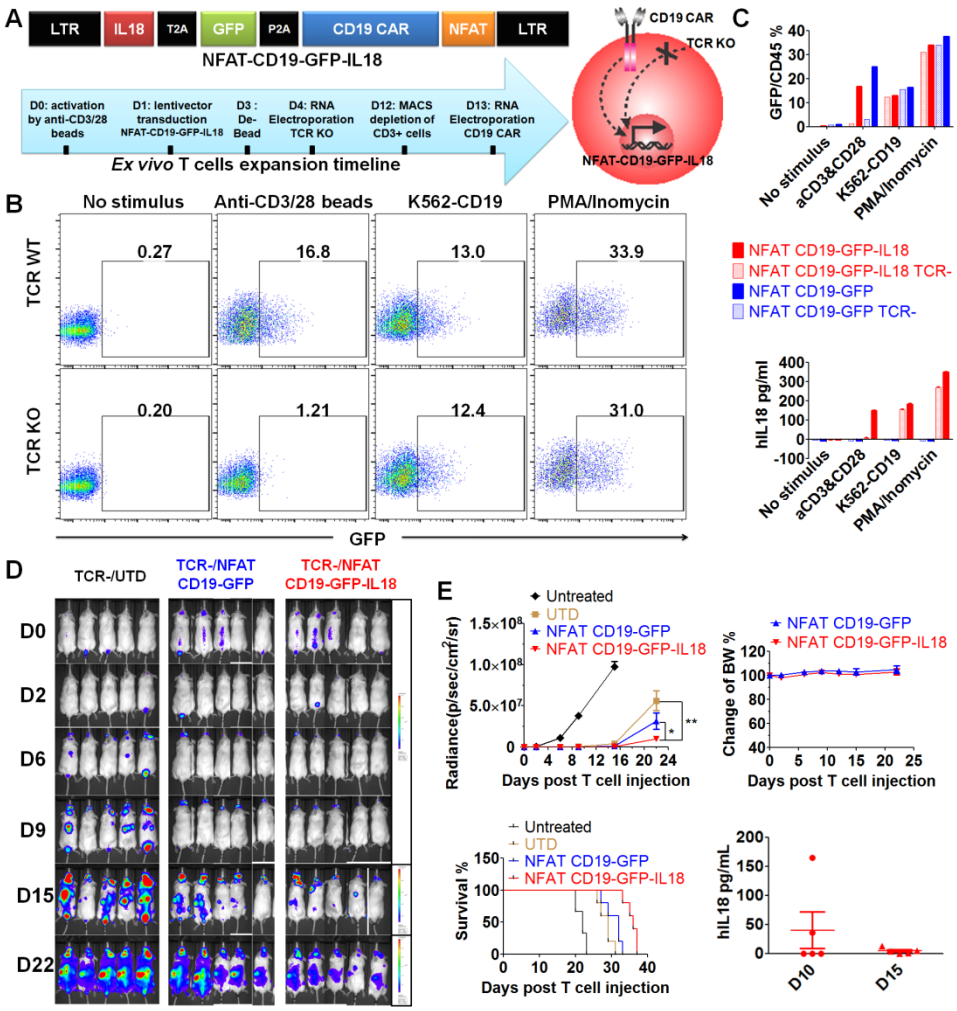

Figure S4. A novel inducible system for IL18 expression in CART cells. Related to Figure 4. **(A)** Upper panel: schematic of lentiviral construct in which the NFAT promoter drives expression of CD19-GFP-IL18. Lower panel: the ex vivo timeline to generate TCR-deficient T cells transduced with NFAT-CD19-GFP-IL18 and electroporated with mRNA CD19 CAR, designated as TCR-/NFAT-CD19-GFP-IL18. **(B)** TCR-/NFAT-CD19-GFP-IL18 CAR T cells were stimulated with anti-CD3/28 beads, K562-CD19, or PMA/ionomycin, after which CD45GFP+ cells were quantified by flow cytometry. **(C)** Summary of GFP+/CD45+ percentages (upper panel) and IL18 concentrations (lower panel) under different conditions as indicated. **(D)** NSG mice (n=5) were inoculated by tail vein injection with 1x10e6 Nalm6-CBG/GFP cells, and treated 1 week later with TCR-deficient untransduced, NFAT-CD19-GFP, or NFAT-CD19-GFP-IL18 CAR T, all bearing mRNA encoded CD19 CAR. Tumor progression was monitored by live animal imaging. **(E)** Tumor growth kinetics (Student's t-test: **p=0.0057, *p=0.0359), survival rate after T cell infusion, change of body weight, and IL18 concentrations at D10 and D15 following T cell injection. All data with error bars are presented as mean±SEM.