Supplementary Table.

The sequences of CARs

CD28-based CD19-targeted CAR (1928z):

MLLLVTSLLLCELPHPAFLLIPIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITGSTSGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQ
PPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
YYGGSYAMDYWGQGTSVTVSTGAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFP
GPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHY
QPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM
GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

4-1BB-based CD19-targeted CAR (19BBz):

MLLLVTSLLLCELPHPAFLLIPIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITGSTSGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQ
PPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
YYGGSYAMDYWGQGTSVTVSTGAAATTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV
HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDG
CSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE
MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD
ALHMQALPPR

HER2-targeted CAR:

MLLLVTSLLLCELPHPAFLLIPDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKP
GKAPKLLIYSASFLESGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT
KVEIKRTGSTSGSGKPGSGEGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR
QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSR
WGGDGFYAMDVWGQGTLVTVSSGSTGAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPS
PLFPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTR
KHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRD
PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT
YDALHMQALPPR

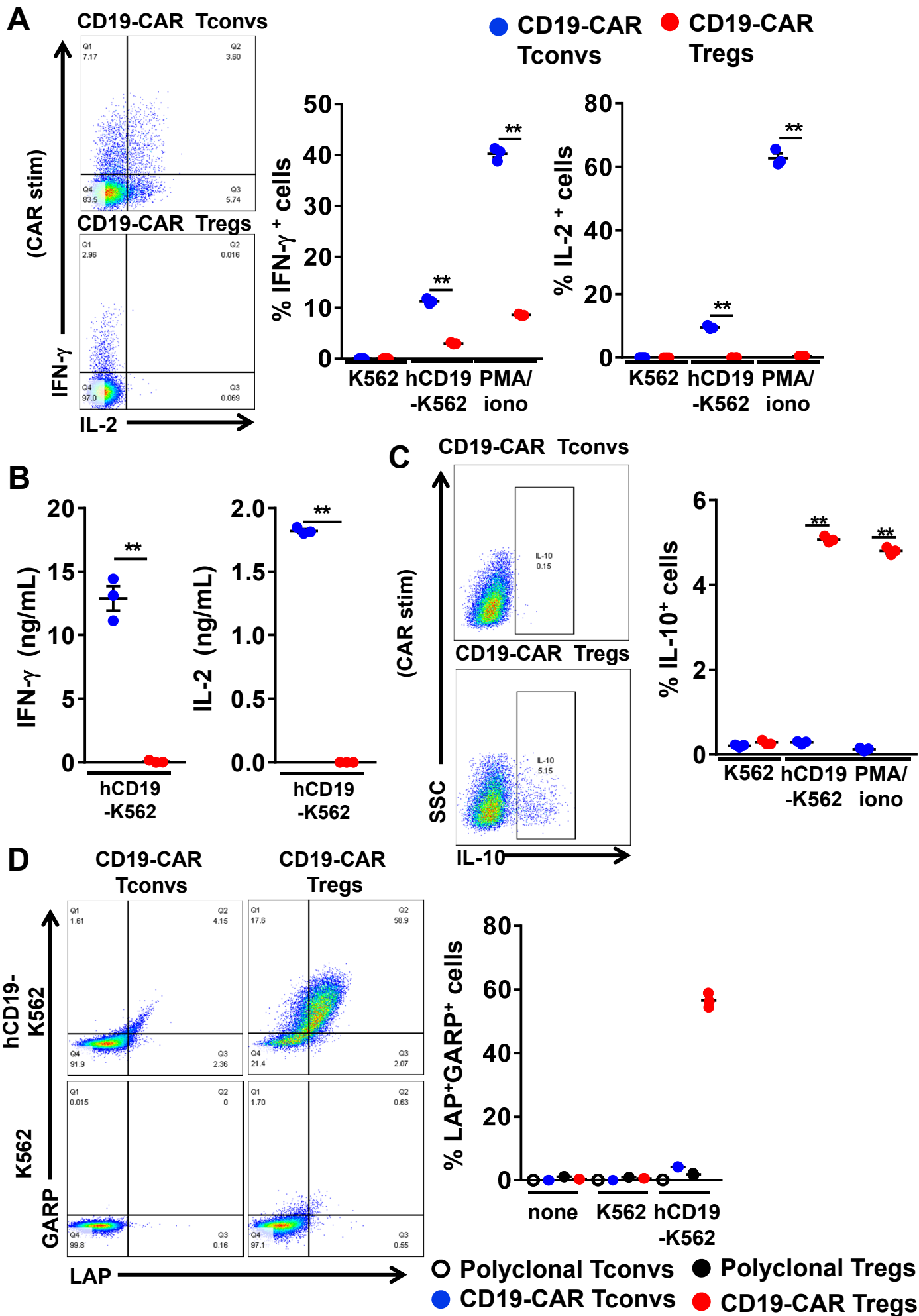1 **Supplementary Figure and legends**

2

A

B

C

D
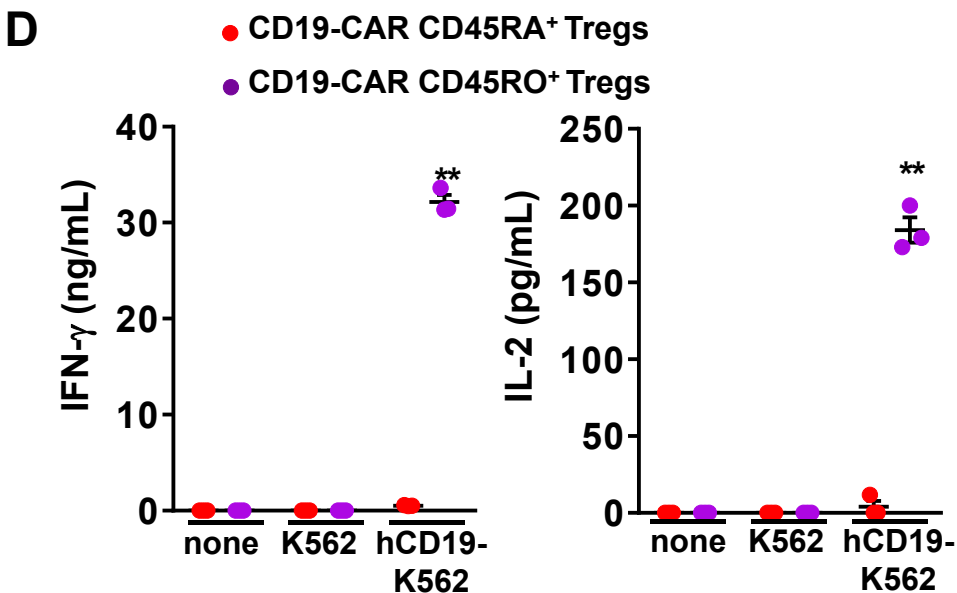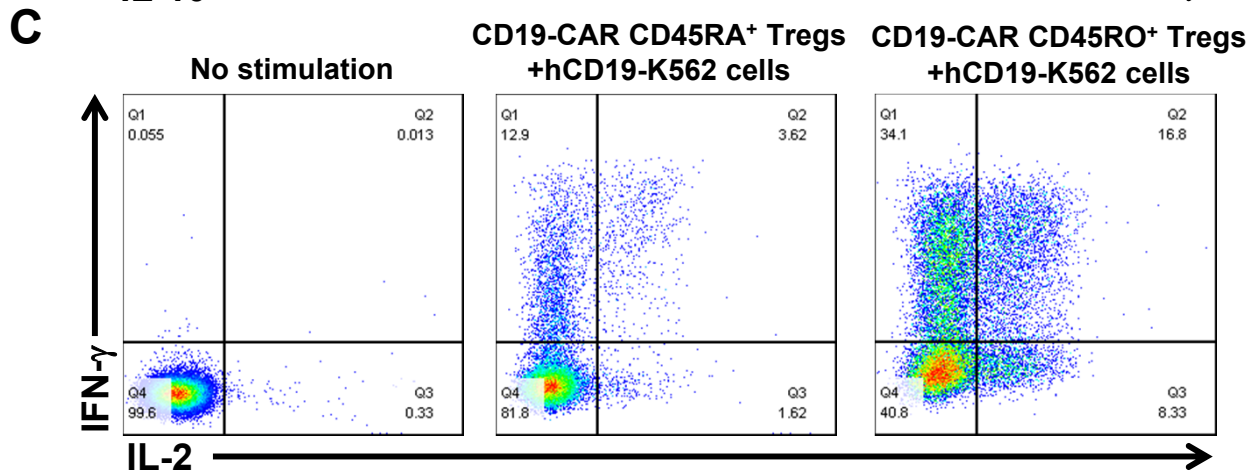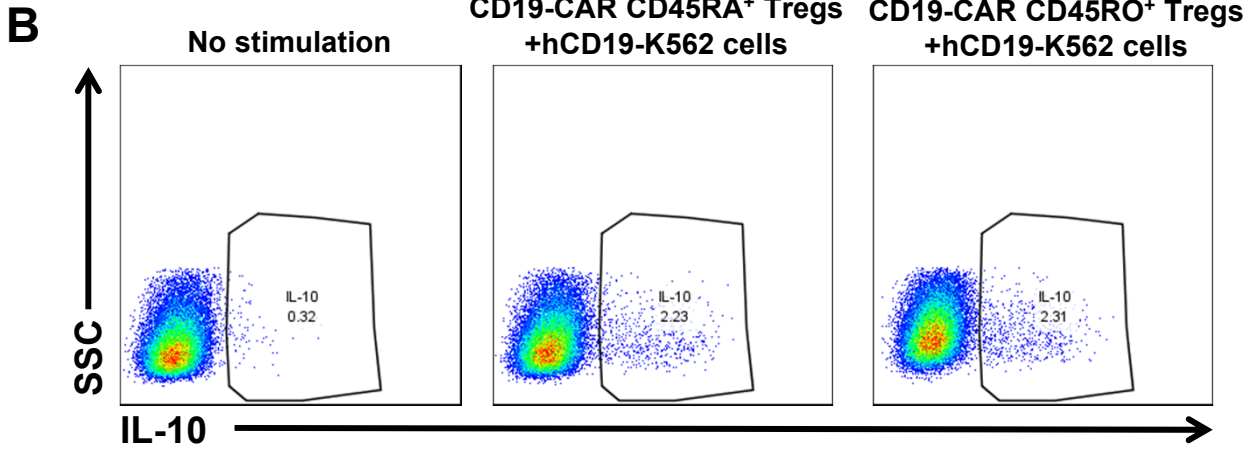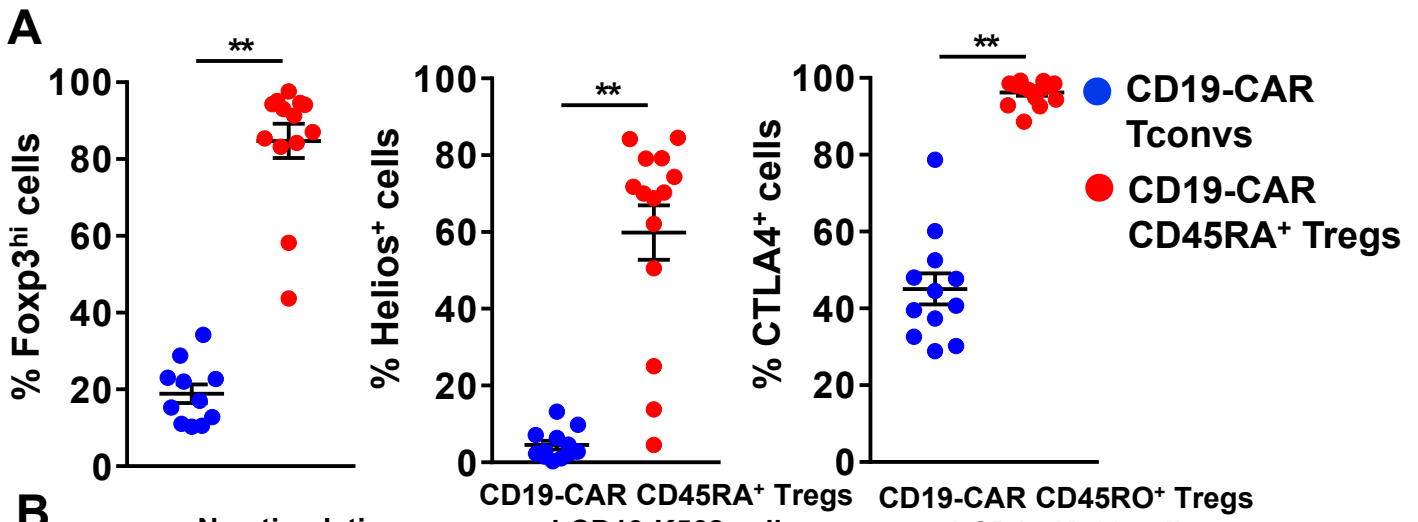
1   **Supplementary Figure 1.**

2   **Phenotype of CD19-targeted CAR Tregs from CD45RA$^+$ Tregs and CD45RO$^+$**

3   **Tregs**

4   (A) Frequency of Foxp3^{hi}, Helios$^+$, and CTLA4$^+$ cells in CD19-CAR Tconvs and

5   CD19-CAR CD45RA$^+$ Tregs after expansion measured by flow cytometric analysis (n =

6   12 or 14). (B and C) Flow cytometric analysis of (B) IL-10, (C) IFN-γ and IL-2 in the

7   indicated cells 4 h after stimulation with hCD19-K562 cells. (D) The amount of IFN-γ

8   or IL-2 produced by CD19-CAR CD45RA$^+$ Tregs and CD19-CAR CD45RO$^+$ Tregs 1

9   day after co-culture with hCD19-K562 cells or K562 cells (n = 3*). (A) Data are

10   representative of fourteen independent experiments using human samples provided by

11   five healthy donors. (D) Data are collected using human samples that were provided by

12   one healthy donor. (A and D) *p* values were determined using a two-tailed Student's

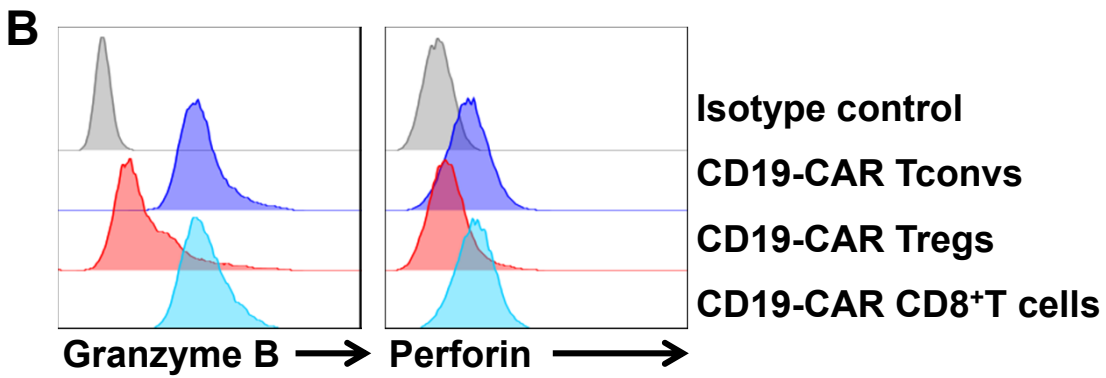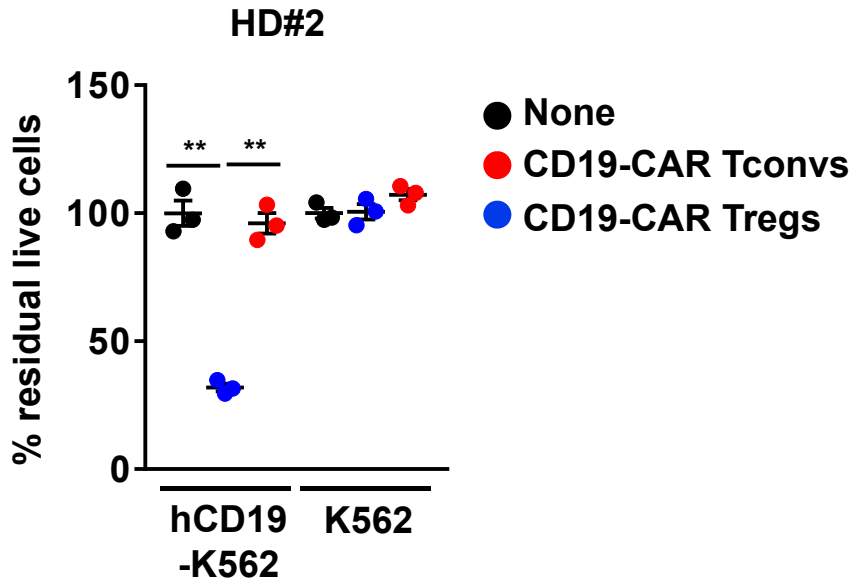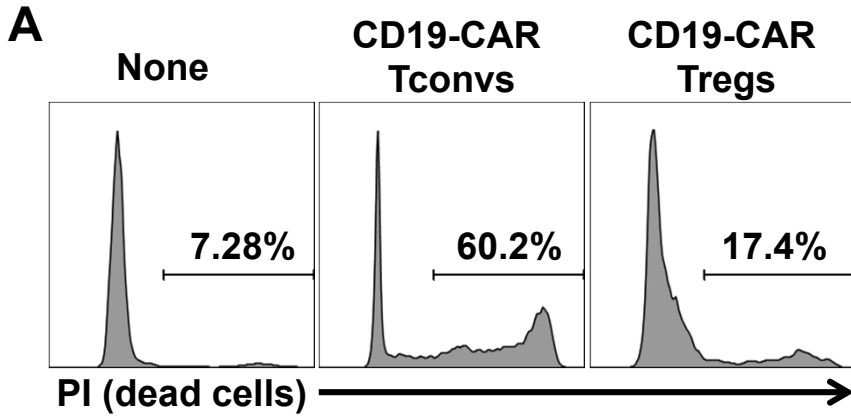13   t-test (**p* < 0.01). Data are mean ± SEM.

14

1 **Supplementary Figure 2.**

2 **Phenotype of CD19-targeted CAR Tregs and CD19-targeted CAR Tconvs**

3 (A) Flow cytometric analysis of IFN-γ and IL-2 in the indicated cells 4 h after

4 stimulation with hCD19-K562 cells, K562 cells, or PMA/ionomycin (PMA/iono) (n =

5 3) (B) The amount of IFN-γ or IL-2 produced by indicated cells 1 day after co-culture

6 with hCD19-K562 cells (n = 3). (C) Flow cytometric analysis of IL-10 in the indicated

7 cells 4 h after stimulation with hCD19-K562 cells, K562 cells, or PMA/ionomycin

8 (PMA/iono) (n = 3) (D) Flow cytometric analysis of LAP and GARP in the indicated

9 cells 1 day after co-culture with hCD19-K562 cells or K562 cells (n = 3*). Data are

10 collected using human samples that were provided by one healthy donor. (A-C) *p* values

11 were determined using a two-tailed Student's *t*-test (**p < 0.01). Data are mean ± SEM.

12

A

None   CD19-CAR Tconvs   CD19-CAR Tregs

7.28%   60.2%   17.4%

PI (dead cells)

HD#2

% residual live cells

- None
- CD19-CAR Tconvs
- CD19-CAR Tregs

hCD19-K562   K562

B

Isotype control
CD19-CAR Tconvs
CD19-CAR Tregs
CD19-CAR CD8$^+$T cells

Granzyme B   Perforin
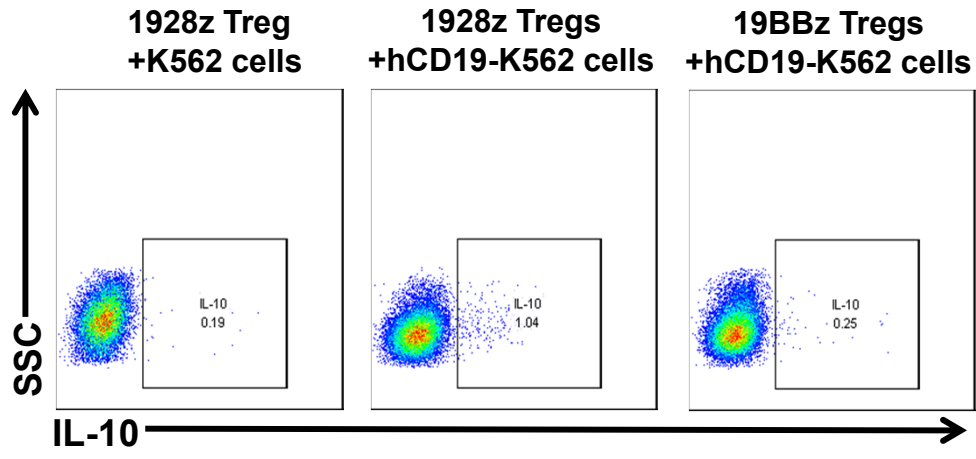
1   **Supplementary Figure 3.**

2   **Effector functions of CD19-targeted CAR Tregs**

3   (A) The cytotoxicity of CD19-targeted CAR Tregs (CD19-CAR Tregs) and

4   CD19-targeted CAR Tconvs (CD19-CAR Tconvs) 1 day after co-culture with

5   hCD19-K562 cells or K562 cells. The upper panels show the representative FACS

6   profiles and the lower panels show residual live cells (the healthy donor is different

7   from the one used in **Figure 2E**) (n = 3). (B) Flow cytometric analysis of granzyme B

8   and perforin in the indicated cells after expansion. (A) p values were determined using a

9   one-way analysis of variance (ANOVA) (**$p < 0.01$). Data are presented as the mean $\pm$

10   SEM.

11

12

(Gated on CD4+ cells)

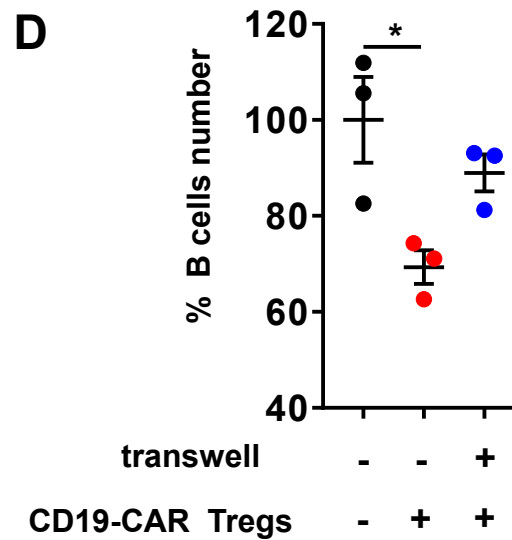| 1928z Treg +K562 cells	1928z Tregs +hCD19-K562 cells	19BBz Tregs +hCD19-K562 cells

IL-10
0.19
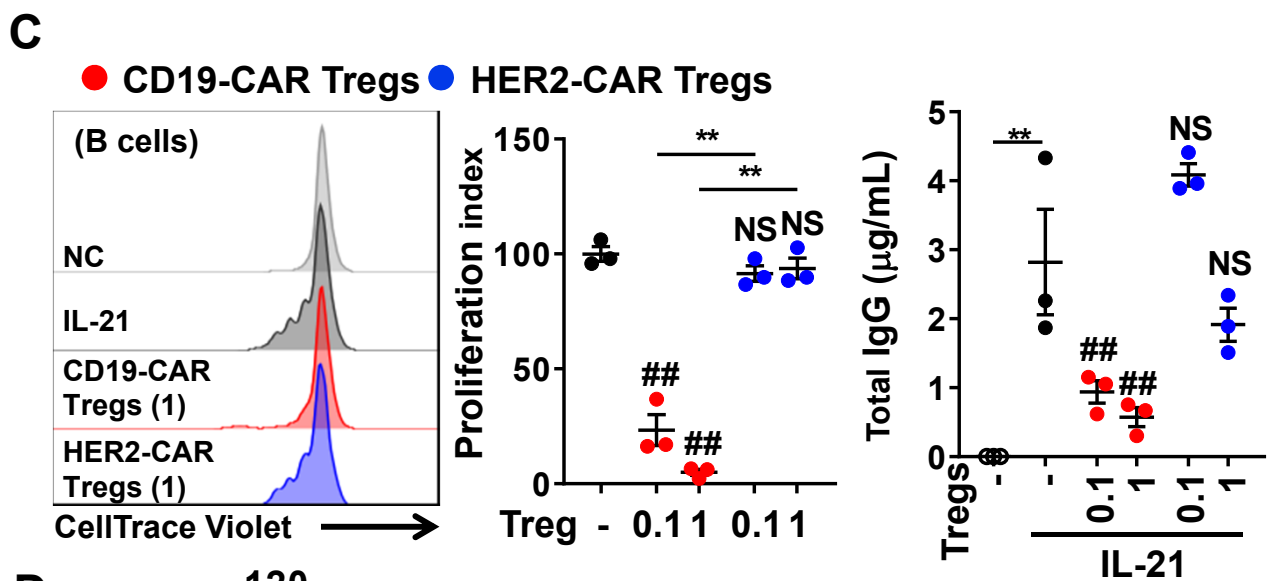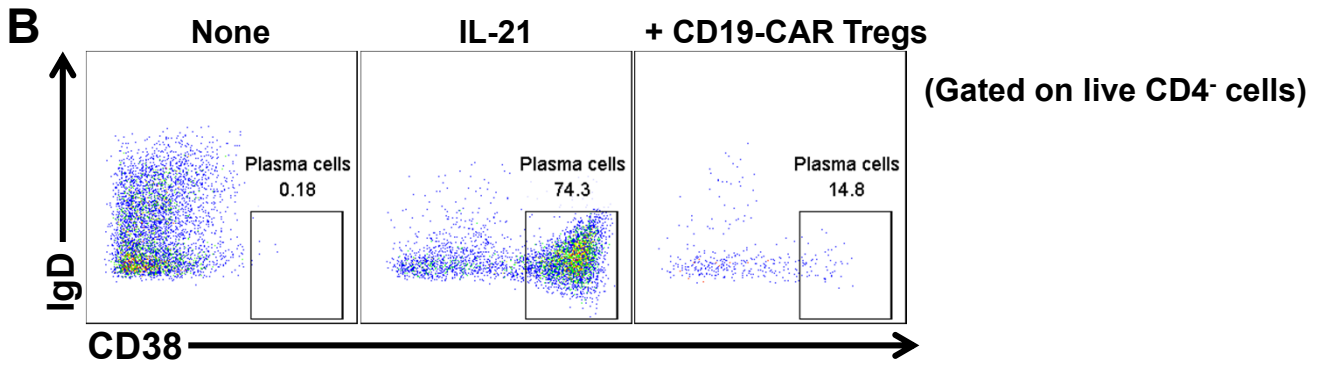
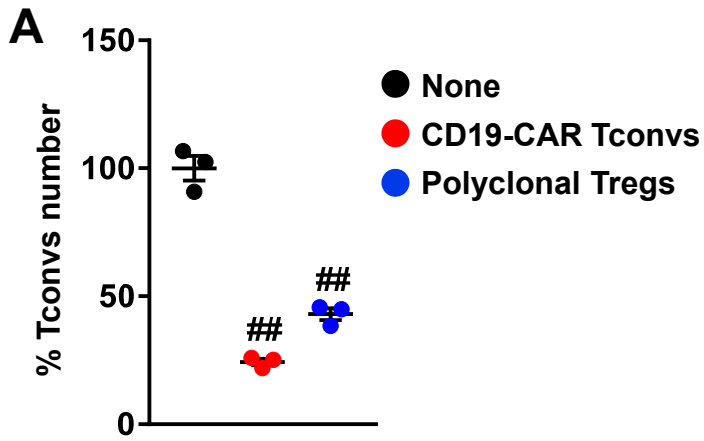IL-10
1.04

IL-10
0.25

SSC

IL-10

1   **Supplementary Figure 4.**

2   **Flow cytometric analysis of IL-10 in 1928z Tregs and 19BBz Tregs**

3   Flow cytometric analysis of IL-10 in the indicated cells 4 h after stimulation with

4   hCD19-K562 cells or K562 cells.

5

6

1   **Supplementary Figure 5.**

2   **Suppression on primary human B cells by CD19-targeted CAR Tregs**

3   (A) Flow cytometric analysis of the number of TCR-stimulated CD19-CAR Tconvs 2

4   days after co-culture with CD19-CAR Tregs or polyclonal Tregs at ratios of 1:1

5   (Tconvs: Tregs) (n = 3). The number of CD19-CAR Tconvs in the absence of Tregs is

6   shown as 100%. (B) Flow cytometric analysis of differentiated B cells

7   (CD4$^-$FVD$^-$IgD$^-$CD38$^+$) 7 days after co-culture with CD19-CAR Tregs. (C and D)

8   Primary human B cells were stimulated with anti-IgM and anti-CD40 antibodies in the

9   presence of IL-21. (C) Flow cytometric analysis of 1) CellTrace violet dilution of

10   CellTrace violet-labeled primary human B cells 3 days and 2) the amount of total IgG 7

11   days after co-culture with CD19-CAR Tregs or HER2-CAR Tregs at ratios of 1:0.1 and

12   1:1 (B cells : Tregs) (n = 3) The fraction of CellTrace violet^{low} B cells in the absence of

13   CD19-CAR Tregs is shown as 100% in the middle panel. (D) Flow cytometric analysis

14   of the number of primary human B cells 3 days after co-culture with CD19-CAR Tregs

15   in Transwell assay (n = 3). The B cells number in the absence of CD19-CAR Tregs is

16   shown as 100%. Data are collected using human samples that were provided by one

17   healthy donor. (A, C and D) p values were determined using a one-way analysis of

18   variance (ANOVA) (*$p < 0.05$, **$p < 0.01$ compared with indicated two columns, $^\#p <$

19   0.05 and $^{\#\#}p < 0.01$; NS, not significant, compared with each black circle). Data are

20   presented as the mean ± SEM.

21

22

A   (Gated on live hCD45$^+$ cells)

B   (Gated on live hCD45$^+$hCD4$^+$ cells)

C

(Gated on live hCD45$^+$hCD4$^-$hCD8$^-$ cells)
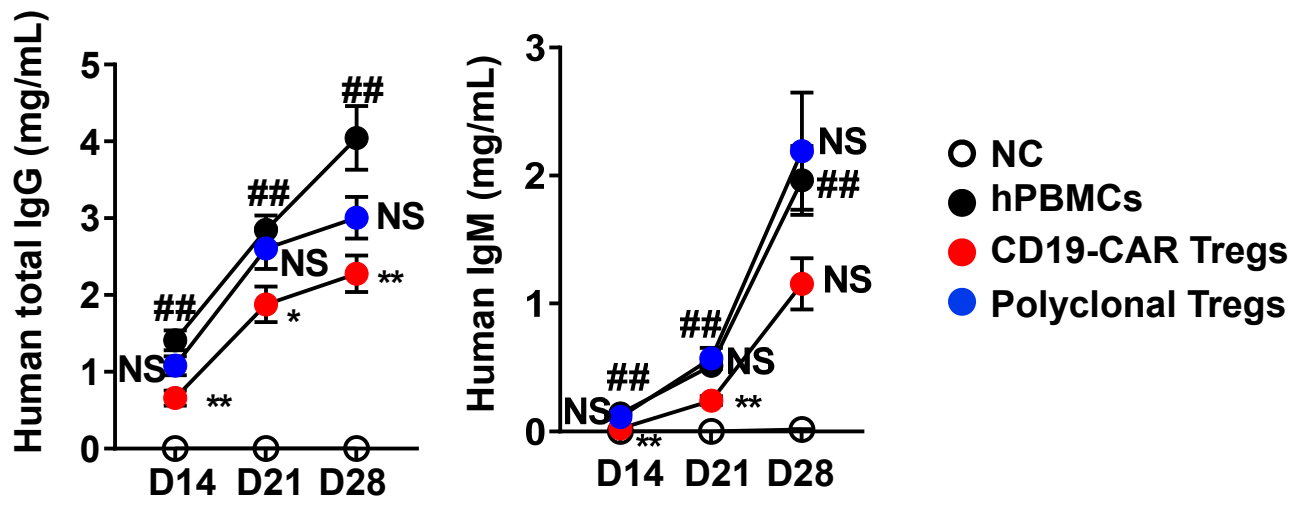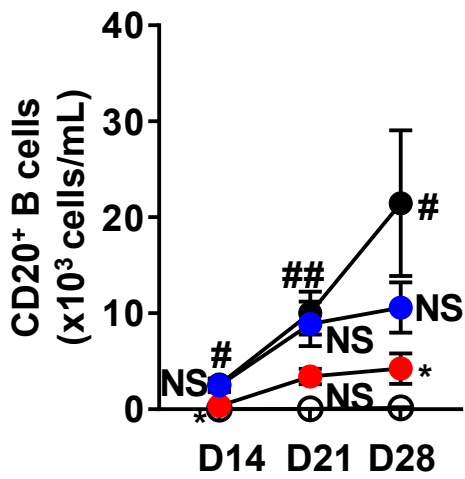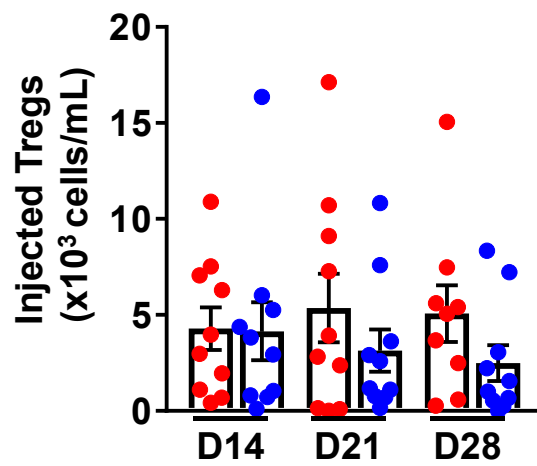
1 **Supplementary Figure 6.**

2 **Flow cytometric analysis of therapeutic adoptive therapy of CD19-targeted CAR**

3 **Tregs in vivo**

4 Severely immunodeficient (NOD.Cg-PrkDC cidIl2rgtm1Wjl/Szj, NSG) mice were

5 intravenously (IV) injected with 5×10^6 human PBMCs. Autologous CD19-CAR Tregs

6 and polyclonal Tregs (2×10^6) were adoptively transferred 7 days after PBMC injection.

7 (A and B) Flow cytometric analysis of (A) B cells (FVD⁻hCD45⁺hCD20⁺), (B) injected

8 Tregs (FVD⁻hCD45⁺hCD4⁺Venus⁺) in the peripheral blood on day 21. (C) Flow

9 cytometric analysis of plasma cells (FVD⁻hCD45⁺hCD4⁻hCD8⁻hCD20⁻CD138⁺) in the

10 spleen on day 28 (n = 5*). (C) *p* values were determined using a two-tailed Student's

11 *t*-test *(*p* < 0.05)*. Data are presented as the mean ± SEM.

12

13

A

Human total IgG (mg/mL)

##
NS   ## ## **
NS   NS *
**

NC
hPBMCs
CD19-CAR Tregs
Polyclonal Tregs

D14   D21   D28

Human IgM (mg/mL)

NS   ## NS
NS
NS **

D14   D21   D28

B

CD20⁺ B cells (x10³ cells/mL)

#
NS   ## #
* *   NS
NS *

D14   D21   D28

C

Injected Tregs (x10³ cells/mL)

D14   D21   D28
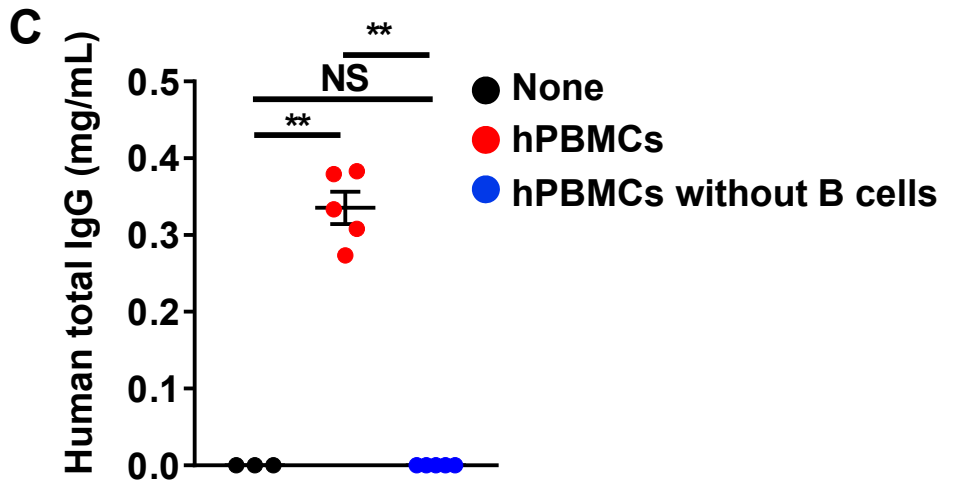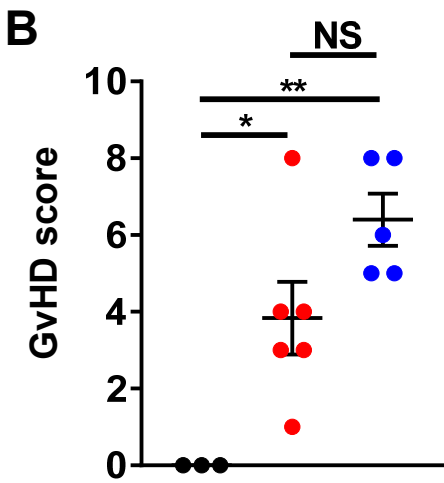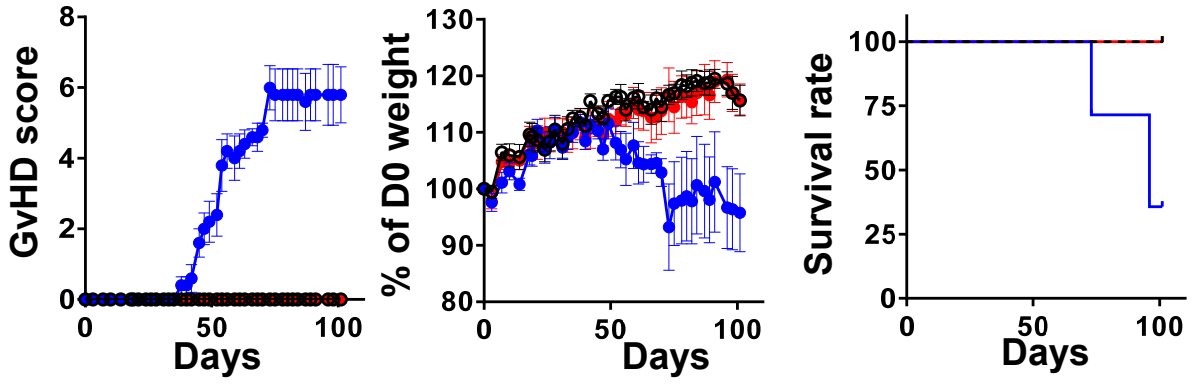
1 **Supplementary Figure 7.**

2 **Protective adoptive therapy of CD19-targeted CAR Tregs in vivo**

3 NSG mice were intravenously injected with 5×10^6 human PBMCs. Autologous

4 CD19-CAR Tregs and empty-Tregs (2×10^6) were adoptively transferred 6 h after

5 PBMC injection. (A) Total IgG antibody and IgM antibody levels in serum on day 14,

6 21, and 28 (n = 7–10). (B, C) The number of hCD45$^+$hCD20$^+$ B cells (B) and injected

7 Tregs (hCD45$^+$hCD4$^+$Venus$^+$) (C) in the peripheral blood on day 14, 21, and 28,

8 measured by flow cytometric analysis (n = 7–10). Data were collected from two

9 independent experiments. p values were determined using (C) a two-tailed Student's

10 t-test or (A) a one-way analysis of variance (ANOVA) (*$p < 0.05$, **$p < 0.01$; NS, not

11 significant compared red or blue symbols with each black symbol, $^#p < 0.05$ and $^{##}p <$

12 0.01 compared black symbols with each open symbol) Data are presented as the mean ±

13 SEM.

14

15

1   **Supplementary Figure 8.**

2   **Irrelevance of B cells on xenoGvHD**

3   (A) NSG mice were IV injected with 2×10^6 human PBMCs or CD19-CAR Tregs.

4   GvHD score, body weight, and survival from day 0 to day 101 are shown (n = 4 and 5).

5   (B and C) NSG mice were intravenously injected with 5×10^6 B cells–sufficient human

6   PBMCs or B cells–deficient human PBMCs. (B) GvHD score at day 28 is measured (n

7   = 3, 6 and 5). (C) The amount of total IgG antibody in serum on day 14 (n = 3, 5, 5) (B

8   and C) p values were determined using a one-way analysis of variance (ANOVA). Data

9   are presented as the mean ± SEM.

10

11

A
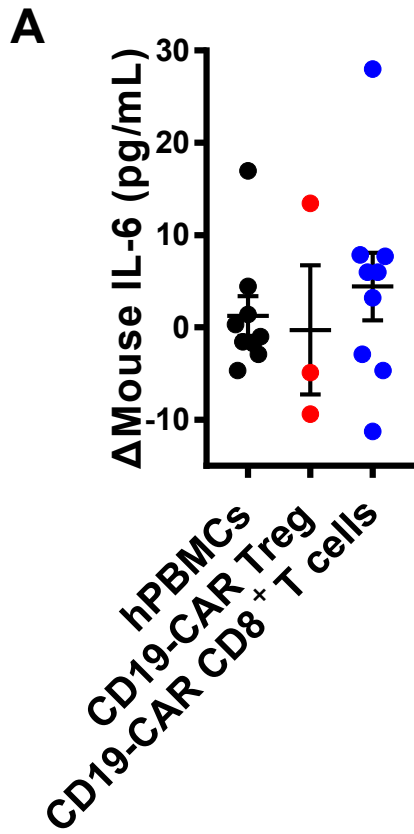
B (Gated on live hCD45⁺hCD4⁺ cells)
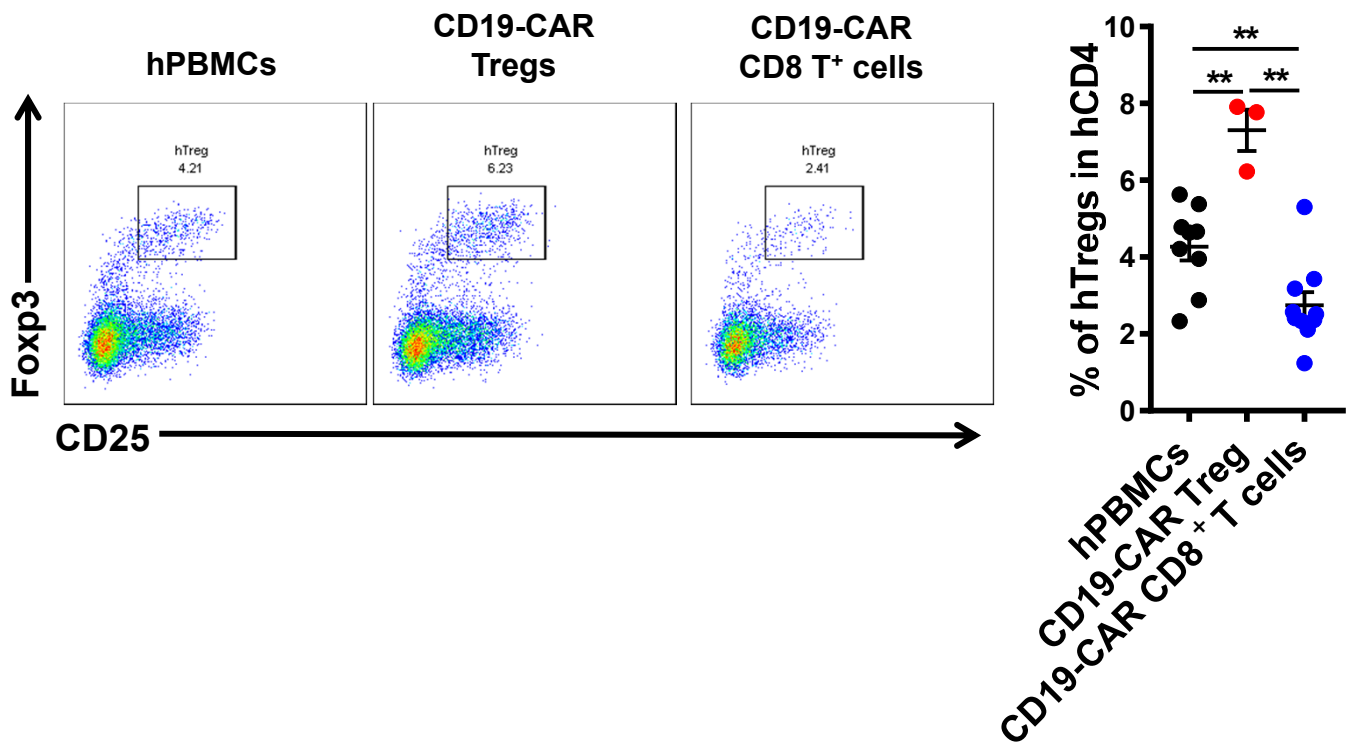
1 **Supplementary Figure 9.**

2 **Mouse IL-6 levels and Tregs affected by CD19-targeted CAR Tregs and**

3 **CD19-CAR-CD8$^+$ T cells**

4 NSG mice were intravenously injected with 5×10^6 human PBMCs. Autologous

5 CD19-CAR Tregs and CD19-CAR CD8$^+$ T cells (2×10^6) were adoptively transferred 6

6 h after PBMC injection. (A) Mouse IL-6 levels in serum at day 6 were measured by

7 ELISA. ΔIL-6 levels were calculated by subtracting the amount of IL-6 in mice not

8 injected (n = 9, 3 and 9). (B) Frequency of Tregs (CD25$^+$Foxp3^{hi}) in spleen

9 FVD$^-$hCD45$^+$CD4$^+$ T cells on day 6 were measured by flow cytometric analysis (n = 9,

10 3 and 9). (B) p values were determined using a one-way analysis of variance (ANOVA)

11 (**$p < 0.01$). Data are presented as the mean ± SEM.

12