SUPPLEMENTARY APPENDIX

Membrane protein CAR promotes hematopoietic regeneration upon stress

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Supplementary Methods

Mice

C57BL/6 CD45.2 and CD45.1 mice were purchased from the National Cancer Institute and the University of Texas Southwestern Medical Center animal breeding core facility. $CAR^{loxP/loxP}$ mice (target the 2^{nd} exon, stock #017359)[1] were purchase from Jackson Laboratory and were backcrossed 10 times to C57BL/6 CD45.2 mice. C57BL/6J inbred UBC-Cre-ERT2 mice (stock #000664) were also purchase from Jackson Laboratory. The two strains of mice were cross breed to obtain UBC-Cre-ERT2/$CAR^{loxP/loxP}$ or $CAR^{wt/wt}$ mice. Mice were maintained at the University of Texas Southwestern Medical Center animal facility. All animal experiments were performed with the approval of University of Texas Southwestern Committee on Animal Care. REDExtract-N-Amp™ Tissue PCR Kit was used for mice genotyping according to the manufacturer's instructions (Sigma). The following primers for genotyping: 5'-TCGATGCAACGAGTGATGAG-3'and 5'-TCCATGAGTGAACGAACCTG-3' for Cre, 5'-GGTGTGATGTTAGTGAGGAACG-3' and 5'-CTGCTCCAGATTCCCACAAT-3' for loxP sites in *CAR*, and 5'-GAGACTGGATTATGAGTTCCAGGCTTTAG-3' and 5'-CCTGCTCCAGATTCCCACAATTCC-3' for the *CAR* null allele. *CAR* conditional knockout was induced by treatment with tamoxifen (50 mg/kg I.P.) daily for 1 week and once (50 mg/kg) 1 week later.

Flow cytometry

Bone marrow cells were stained with a biotinylated lineage cocktail (anti-CD3, anti-

CD45R, anti-Gr-1, anti-Ter119) followed by staining with streptavidin-PE/Cy5.5, anti-Sca-1-FITC, anti-Kit-APC-Cy7, anti-CD135-PE, anti-CD34-eFlour450, anti-CD16/32-PE-Cy7, and anti-CD127-APC. LT-HSCs, ST-HSCs, and MPPs were identified as $Lin^-Sca\text{-}1^+cKit^+CD135^-CD34^-$, $Lin^-Sca\text{-}1^+cKit^+CD135^-CD34^+$, and $Lin^-Sca\text{-}1^+cKit^+CD135^+CD34^+$ cells, respectively, and CMP, GMP, MEP, and CLP cells were identified as $Lin^-Sca1^-cKit^+CD16/32^-CD34^+$, $Lin^-Sca1^-cKit^+CD16/32^+CD34^+$, $Lin^-Sca1^-cKit^+CD16/32^-CD34^-$, and $Lin^-Sca1^{low}cKit^{low}CD135^+CD127^+$ cells, respectively, essentially as we described [2]. To analyze the repopulation of mouse HSCs, peripheral blood cells were collected by retro-orbital bleeding, followed by lysis of red blood cells and staining with anti-CD45.2-FITC, anti-CD45.1-PE, anti-CD3-APC, anti-B220-APC, and anti-Mac-1-APC monoclonal antibodies (BD Biosciences). The percentage of repopulation was determined based on the staining results with anti-CD45.2-FITC and anti-CD45.1-PE. Cell-cycle analysis of HSCs with Hoechst and anti-Ki67 staining was performed as follows: Bone marrow cells were stained with a biotinylated lineage cocktail and Lin^- cells were isolated with Streptavidin Particles Plus – DM (BD Biosciences, cat. #557812). Lin^- cells were stained with streptavidin-PE/Cy5.5, anti-Sca-1-FITC, anti-Kit-APC-Cy7, anti-CD135-PE, and anti-CD34-eFlour450, then fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, cat. # 00-5523). After permeabilization, cells were stained with Hoechst and anti-Ki67 at room temperature. Before analysis with FACS, cells were washed and kept in cold PBS.

Hematology

Blood samples were obtained via facial vein and anticoagulated with EDTA tripotassium salt. WBC count, absolute differential WBC count, RBC count, hemoglobin concentration, and platelet count were determined using the Hemavet 950FS Hematology Analyzer within 1 hour of blood collection.

Quantitative RT-PCR

Total RNA was isolated from FACS-collected bone marrow HSCs, progenitors, or differentiated cells. First-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples were analyzed in triplicate 25 µl reactions (500 nM primers, 12.5 µl Master mix). The following primers were ordered from Sigma: 5'-TGGTTTGAGCATCACTACACC-3' and 5'-GCGTCGCCAGACTTGAC-3' to detect the second exon of *CAR*, 5'-GGAGAAGAGGCGAAGGG-3' and 5'-GCTCGGGTCTGTGCTGA-3' to detect *hes1*, 5'-GCCTTTGAGAAGCAGGG-3' and 5'-CTCCGATAGTCCATAGCCA-3' to detect *hey1*, and 5'-CTTTGGGCGTTGGAAACC-3' and 5'-CGCAGATGAAATAGGGCTGTA-3' to detect *myc*. The default PCR protocol was performed on an Applied Biosystems Prism 7000 Sequence Detection System. The mRNA level in each population was normalized to the level of *GAPDH* RNA transcripts present in the same sample.

Colony assays

Bone marrow cells were diluted to the indicated concentration in Iscove modified Dulbecco's medim with 2% FBS and were seeded into methylcellulose medium M3434 (StemCell Technologies) for GM and E colony formation assays or M3630 (StemCell

Technologies) for pre-B colony formation assays, according to the manufacturer's protocols and as we described previously [3, 4].

Homing analyses

Bone marrow cells were labeled with 5- and 6-carboxyfluorescein succinimidyl ester (CFSE) and 1-2×10^7cells were transplanted into lethally irradiated CD45.1 mice. After 12 hours, the total number of $CFSE^+$ cells in the bone marrow, spleen, and liver were determined by flow cytometry (3). When $CFSE^+$ LSK or LSKFC cells were analyzed, the bone marrow cells were stained with a biotinylated lineage cocktail followed by streptavidin-PE/Cy5.5, anti–Sca-1-FITC, anti–cKit-APC-Cy7, anti-CD135-PE, and anti-CD34-eFlour450 before analysis.

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Supplementary figure legends

Supplementary Figure 1. CAR expression (determined by qPCR) in HSPCs before or after 5-FU treatment.

Supplementary Figure 2. The commercial anti-mCAR can specifically bind BM cells expressing CAR on the membrane. The CAR^- and CAR^+ cells were sorted for qPCR of CAR expression (The mRNA levels were calculated based on CAR^+ group, and every group was repeated three times).

Supplementary Figure 3. Repopulation results of LSKFC CAR^- or CAR^+. Donor LSKFC cells were isolated from mice one and half days after treated with 5-FU. Peripheral Blood cells were collected from receipt mice 3 weeks after BM transplantation (n=5).

Supplementary Figure 4. Lineage cell numbers in peripheral blood after 5-FU treatment. WT （n=6）and *CAR* cKO (n=6) mice were treated with 250 mg/kg 5-FU at day 0. **, p<0.01.

Supplementary Figure 5. **CAR has no effect on hematopoietic cells, progenitors, or HSCs during homeostasis. A,** The percentages of indicated cells in bone marrow (BM), liver (LV), peripheral blood (PB), and spleen (SPL) in WT and *CAR* cKO mice. **B,** The number of indicated cells per 1 x 10^6 bone marrow cells.

Supplementary Figure 6. **CAR does not affect homing of HSCs. A,** The percentages of $CFSE^+$ cells in total cells of bone marrow (BM), spleen (SPL), and liver (LV). **B,** The

percentage of $CFSE^+$ LSK and LSKFC cells in BM. (n=4-6)

Supplementary Figure 7. Competitive repopulation assays for the donor BM cells from Scl-CreERT/CAR mice (with or without tamoxifen treatment). The bone marrow transplantation were performed with a 1:1 ratio of donor (CD45.2) and CD45.1 WT competitor bone marrow. Shown are the percentages of donor peripheral leukocytes (CD45.2) in total peripheral blood, the $Mac1^+$ population, and the $B220^+$ population. Data are means ± s.e.m. of 7 to 9 mice. *<0.05, ***<0.001.

Supplementary Figure 8. Competitive repopulation assays with CARcKO recipient mice. The bone marrow transplantation were performed with a 1:1 ratio of WT or CARcKO donor (CD45.2) and CD45.1 WT competitor bone marrow (500000 BM cells) into global CAR cKO recipient mice. Shown are the percentages of donor peripheral leukocytes (CD45.2) in total peripheral blood, the $Mac1^+$ population, and the $B220^+$ population. Data are means ± s.e.m. of 8 to 10 mice. **<0.01, ****<0.0001

Supplementary Figure 9. **CAR^+ HSCs was in cell cycle.** In the whole BM cells from mice with or without 5-FU treatment, the population of LSKFC was co-stained with anti-mCAR and Ki67.

Supplementary Figure 10. Numb expression in HSCs of WT and CARcKO mice after 5-FU treatment. A, Quantification of Numb negative staining in LSKFCs (n=3-6). **B,** Numb

mRNA levels in LSKFC cells (The mRNA levels were calculated based on 0d WT group, and every group was repeated three times).

Supplementary Figure 11. CAR influences T cells development. Repopulation assay with LTHSCs at day 170 in Figure 4A.

Supplementary Figure 12. **5-FU treatment increases notch signaling**. LSKFC cells from mice before or 1.5d after 5-FU treatment were isolated, and the expression levels of Notch target genes *hes1*, *hey1*, and *myc* were evaluated with qPCR.

Supplementary Figure 13. **Overexpression of CAR in HSCs in vitro enhances Notch1 signaling.** BM lin^- cells was transfected with full length CAR (CAR-FL) or CAR deleted intracellular domain (CAR del ICD), and the LSKFC CAR^+ cells were sorted for RNA extraction **(A)** and qPCR of Notch1 target genes **(B)**.

Supplementary Figure 14. Notch1 activator valproic acid could rescue CAR cKO mice dying from 5-FU treatment. Survival curves of WT (n = 10) and scl-creERT CAR cKO (n = 10 or 8) mice treated with 300 mg/kg 5-FU (at day0) with or without valproic acid (300mg/kg, twice a week)**.**

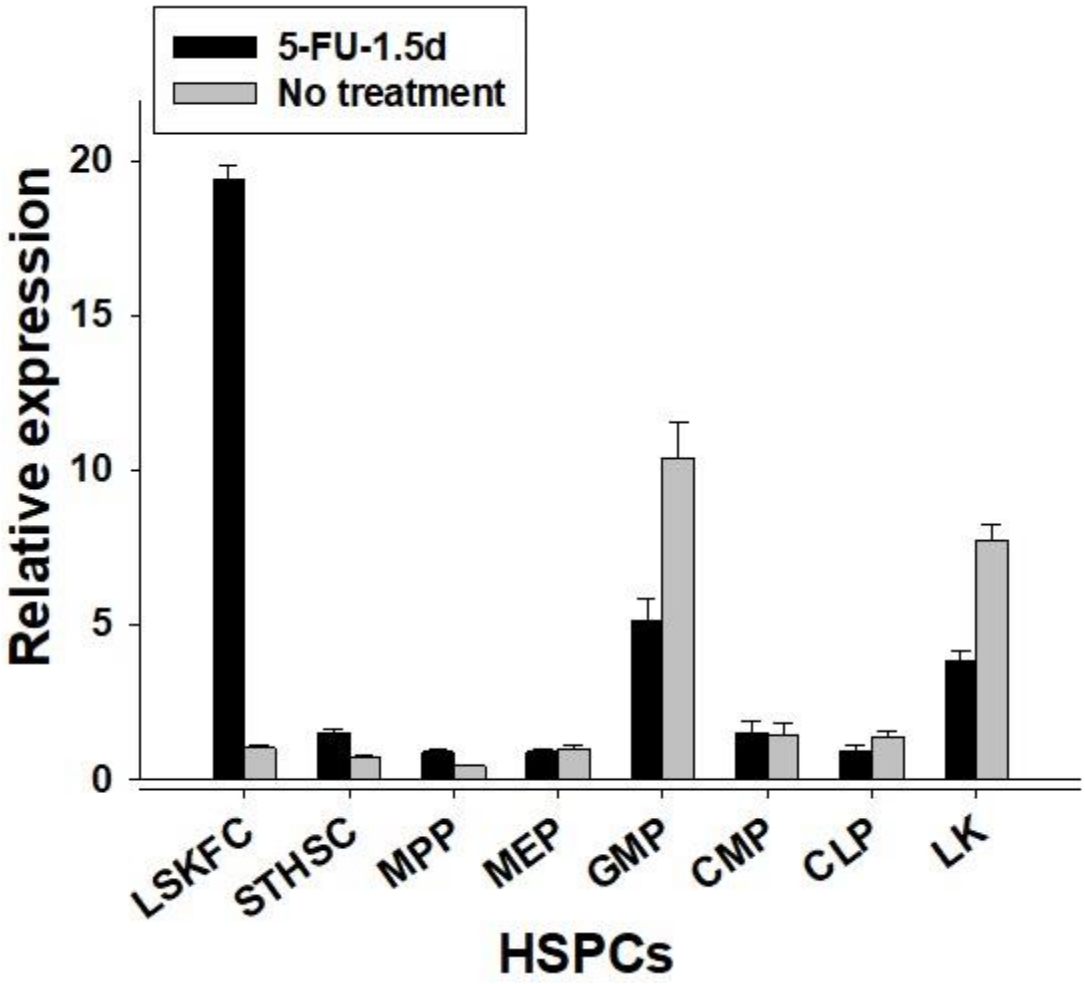
Supplementary Figure 15. Notch1 activator valproic acid could restore repopulation ability of HSCs without CAR. Competitive repopulation assays with bome marrow

transplantation were performed with a 1:1 ratio of donor (CD45.2) and CD45.1 WT competitor bone marrow. Shown are the percentages of donor peripheral leukocytes (CD45.2) in total peripheral blood, the $Mac1^+$ population, and the $B220^+$ population. Data are means ± s.e.m. of 5 mice. Mice were treated with PBS or valproic acid (300mg/kg, twice a week). *, $p<0.05$.

Supplementary Figure 16. Numb protein levels in LSK population with overexpression of CAR, LNX2 or DN-LNX2. Quantification of Numb negative staining LSK cells with FACS assay after overexpression of CAR, LNX2 or DN-LNX2 in Lin^- bone marrow cells in vitro. *, $p<0.05$.

Supplementary Figure 17. Proposed coupling between CAR and Notch in HSCs during regeneration.

Supplementary Figure 1



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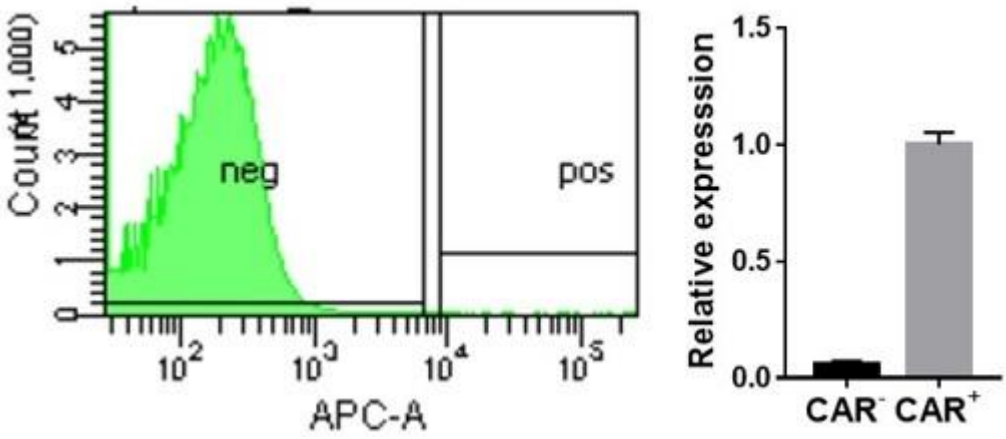

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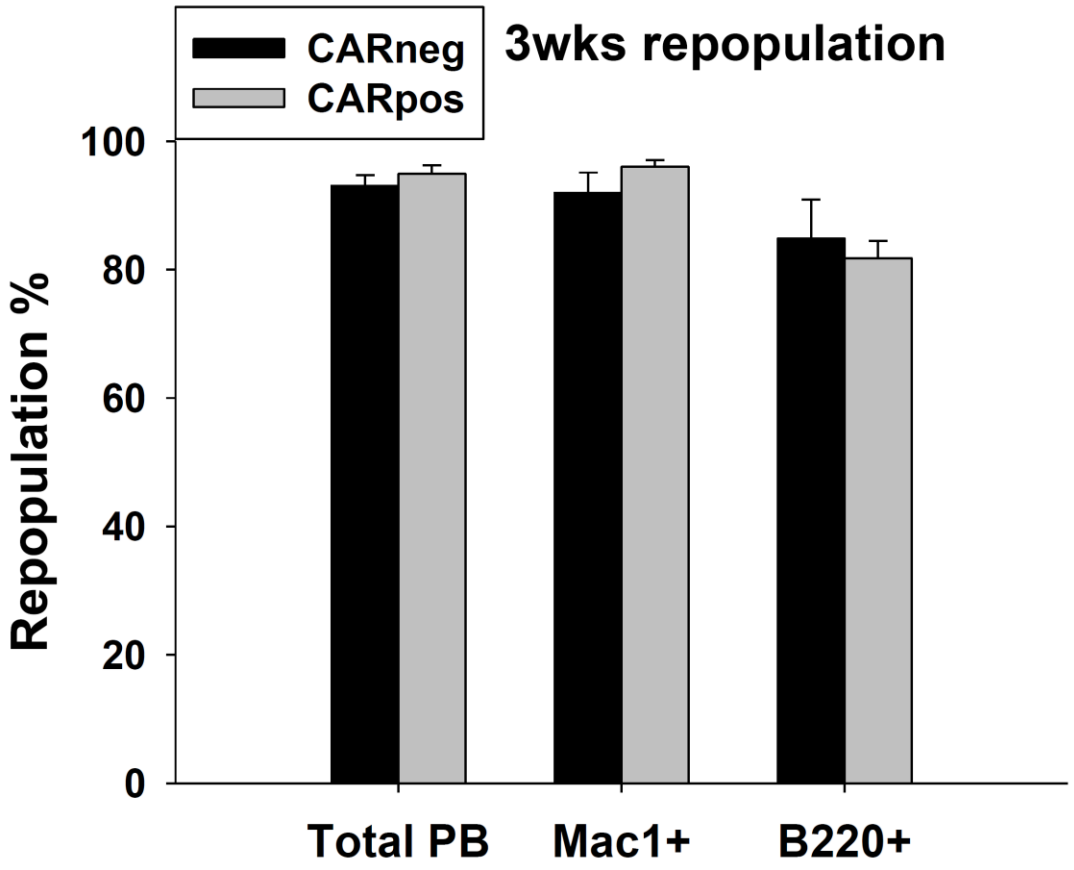

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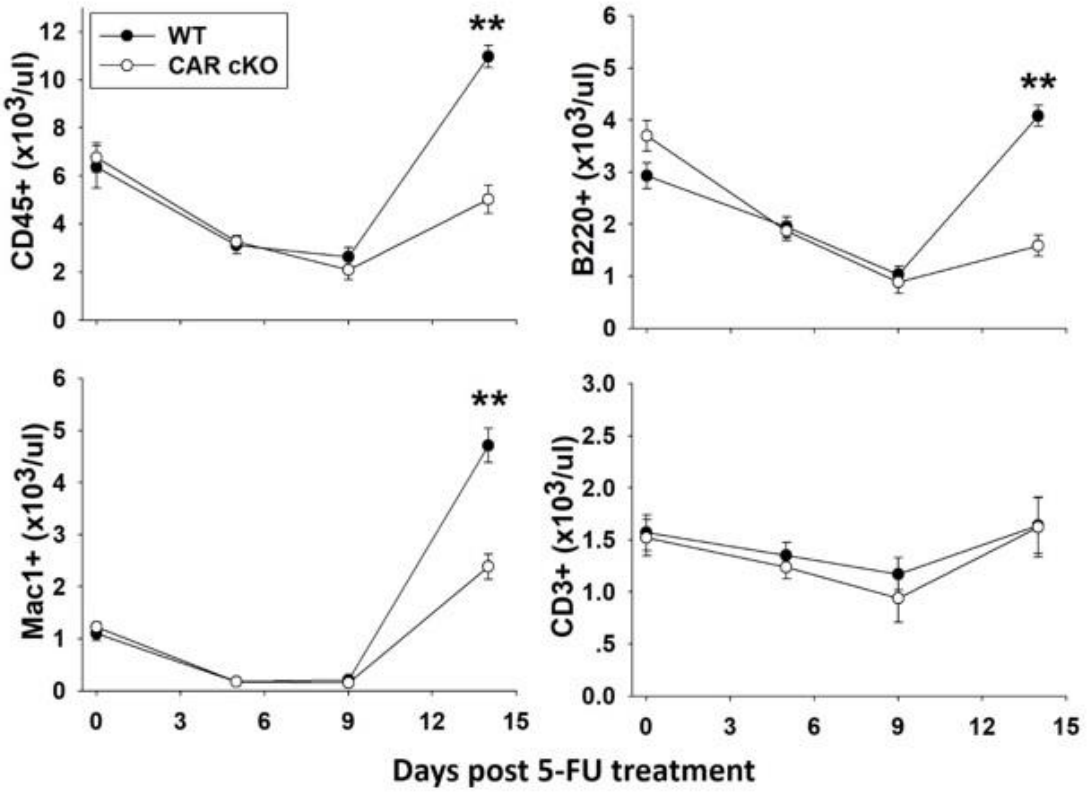

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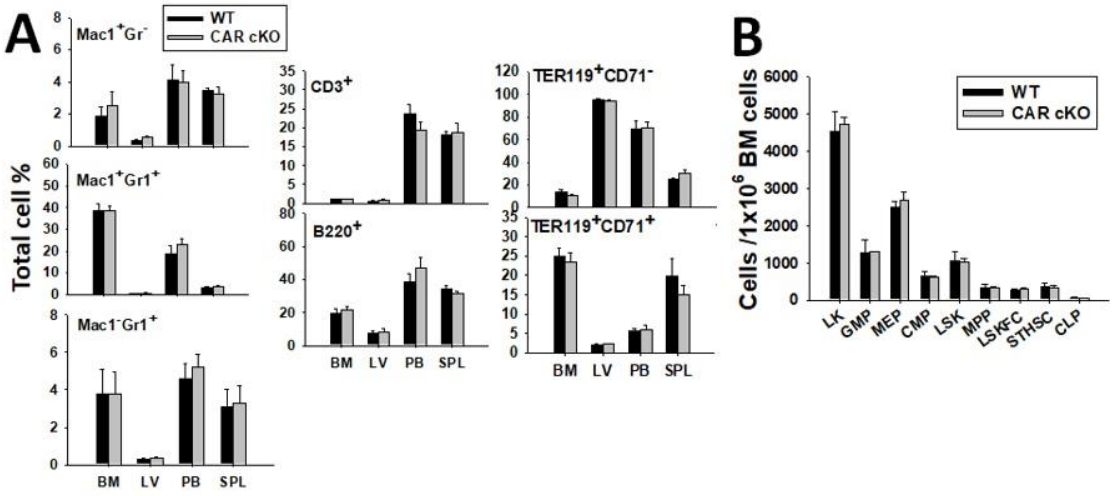

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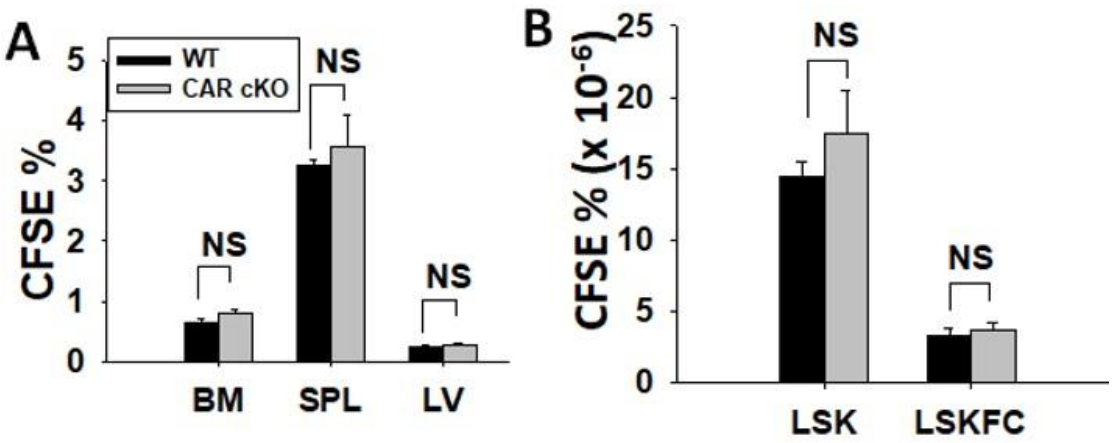

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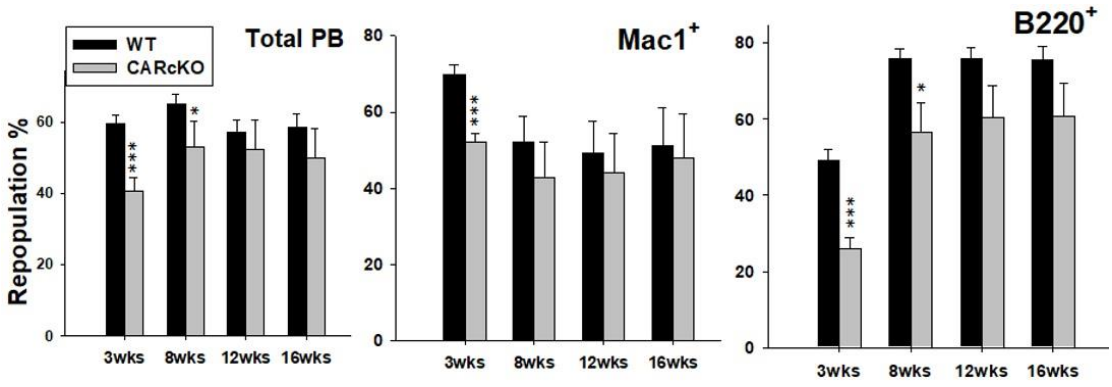

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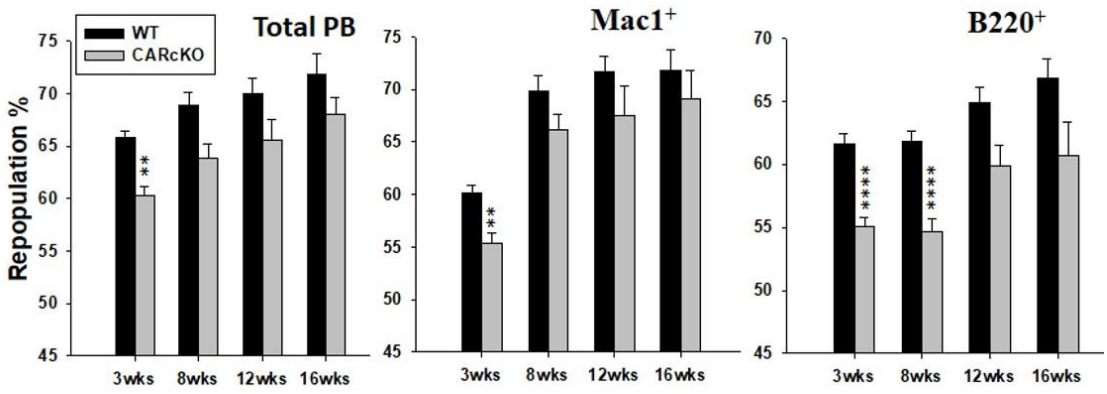

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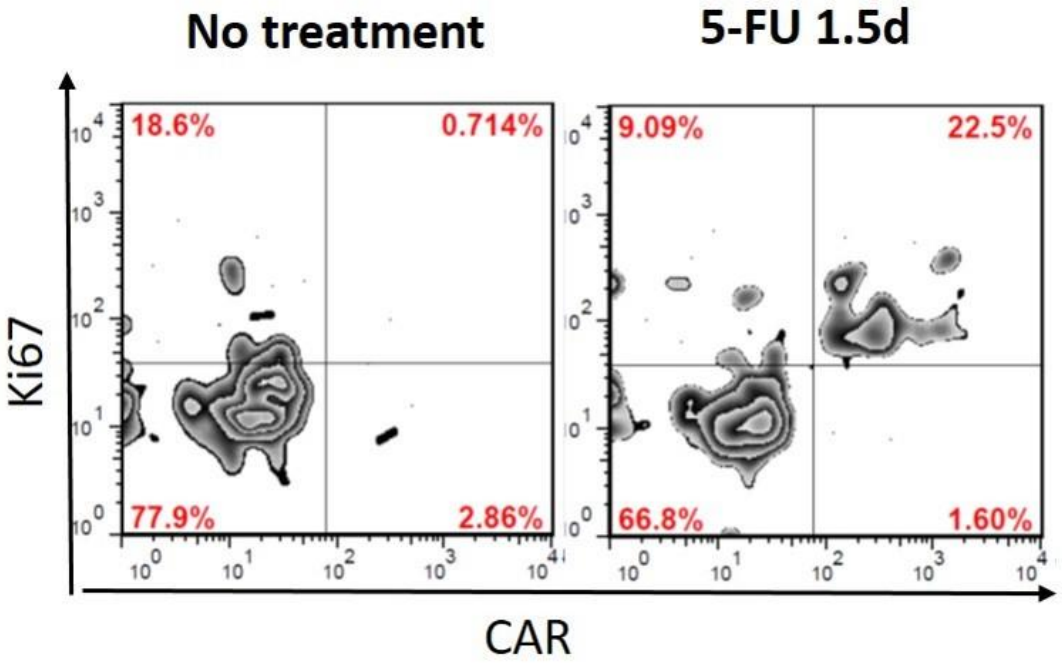

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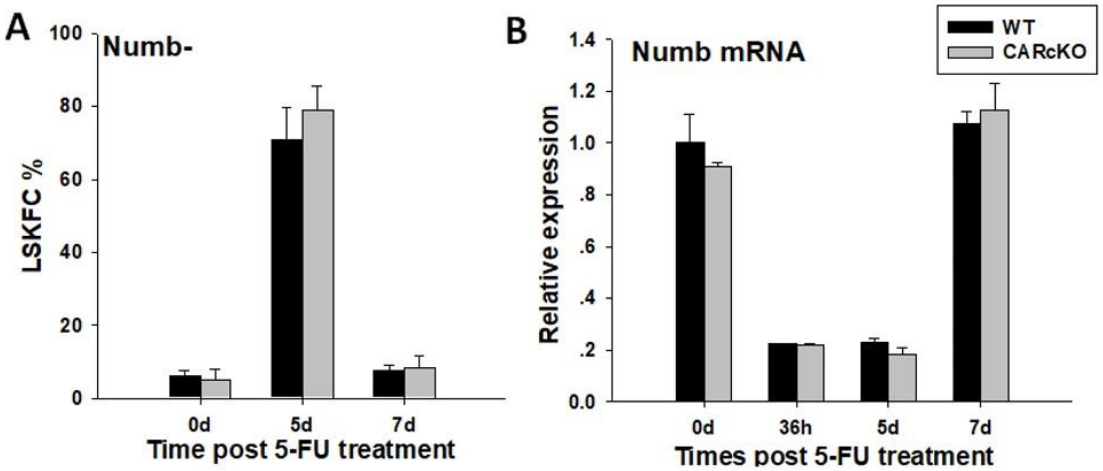

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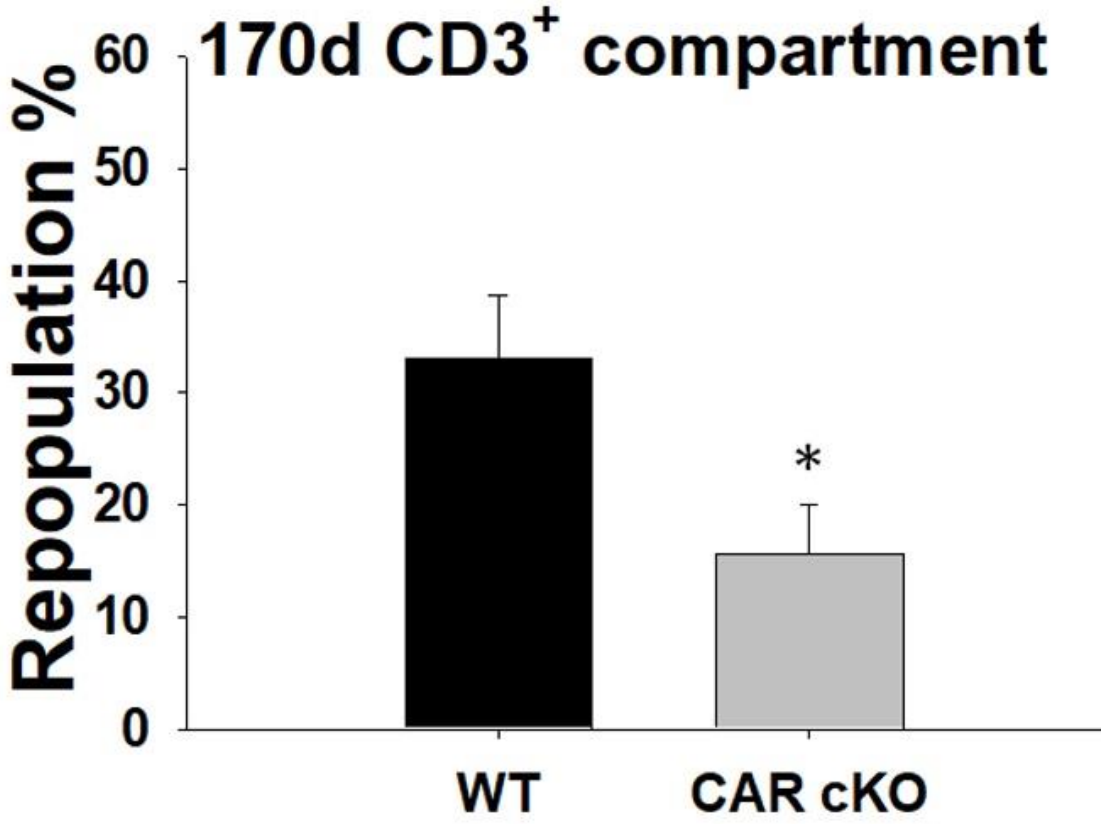

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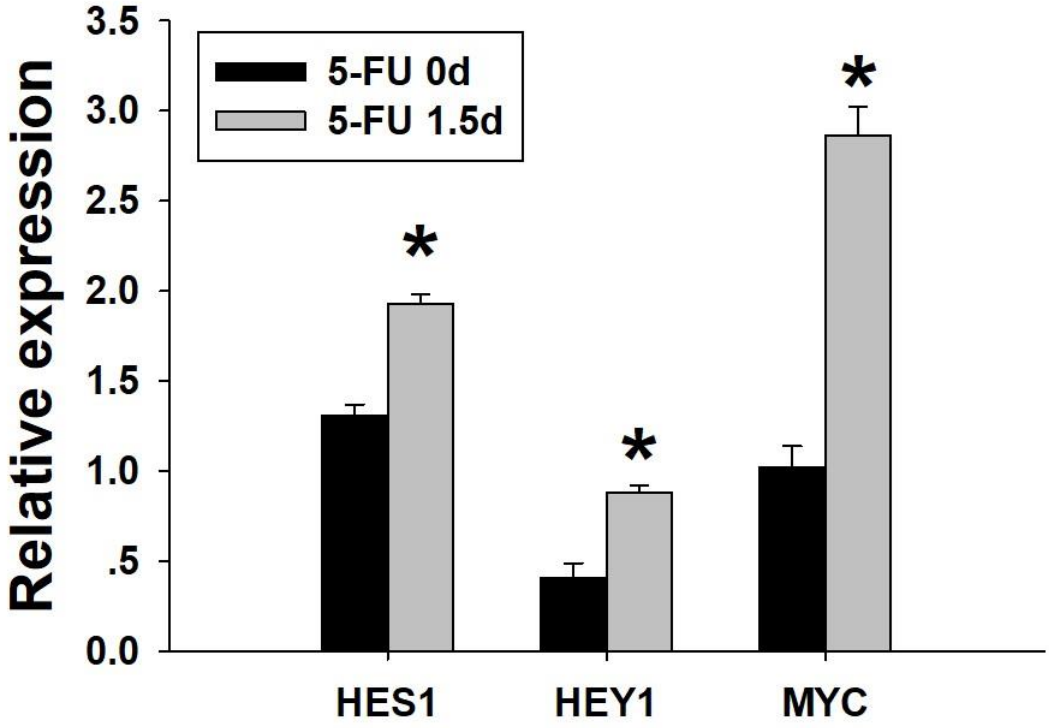

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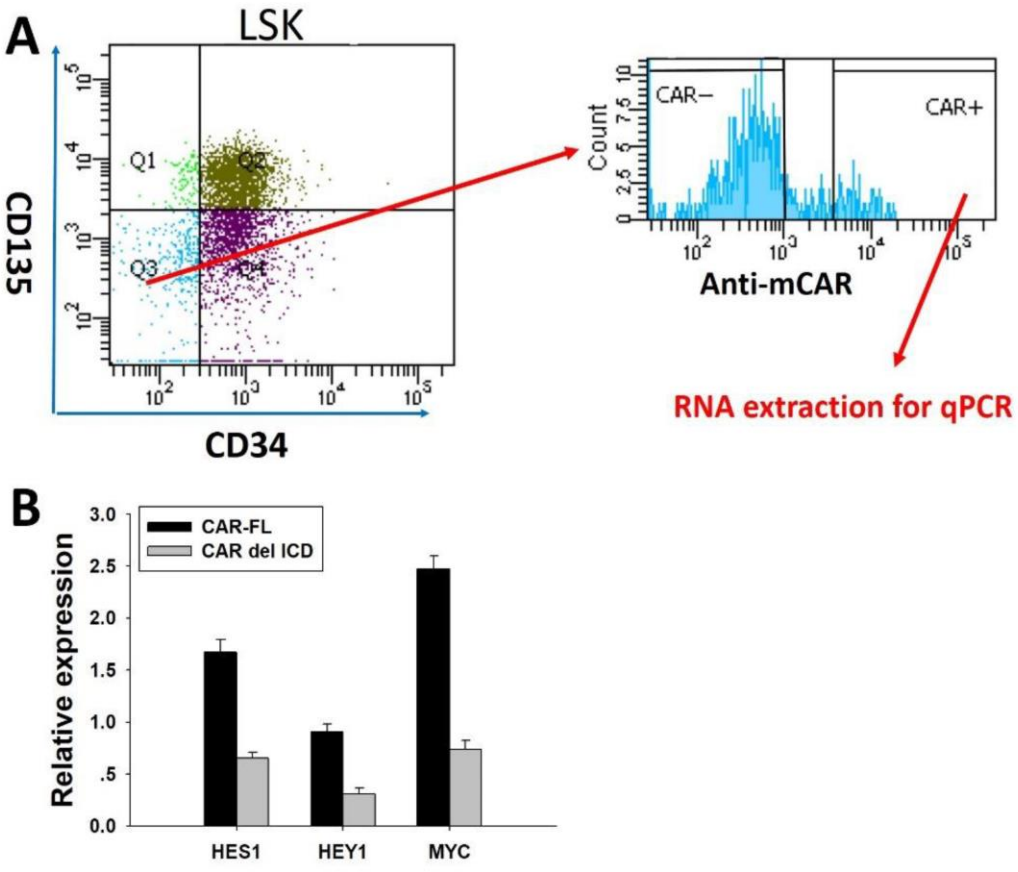

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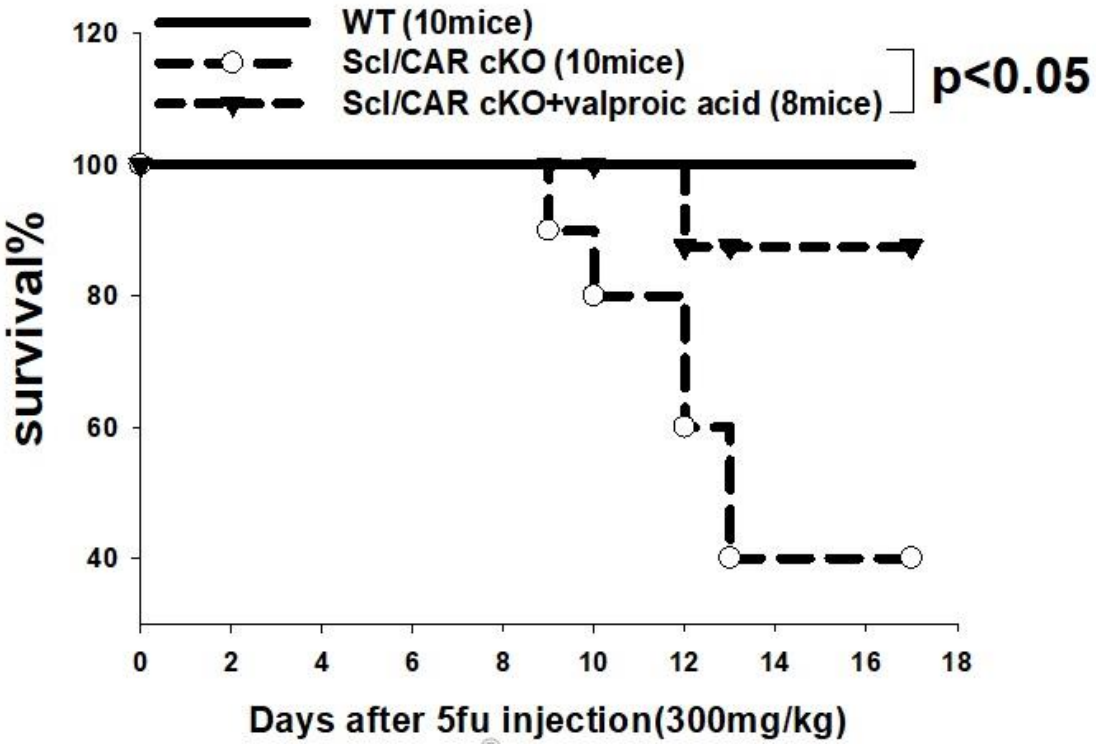

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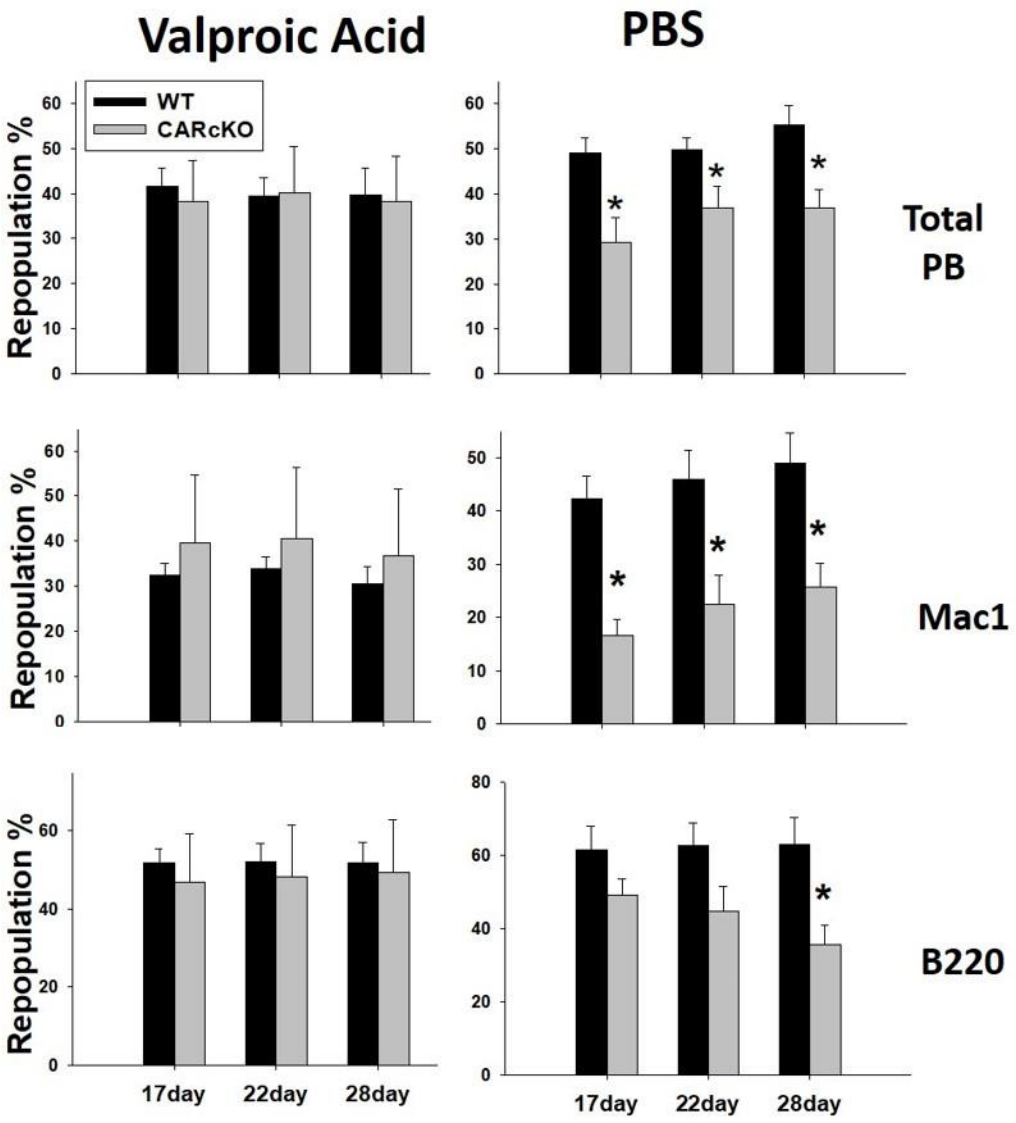

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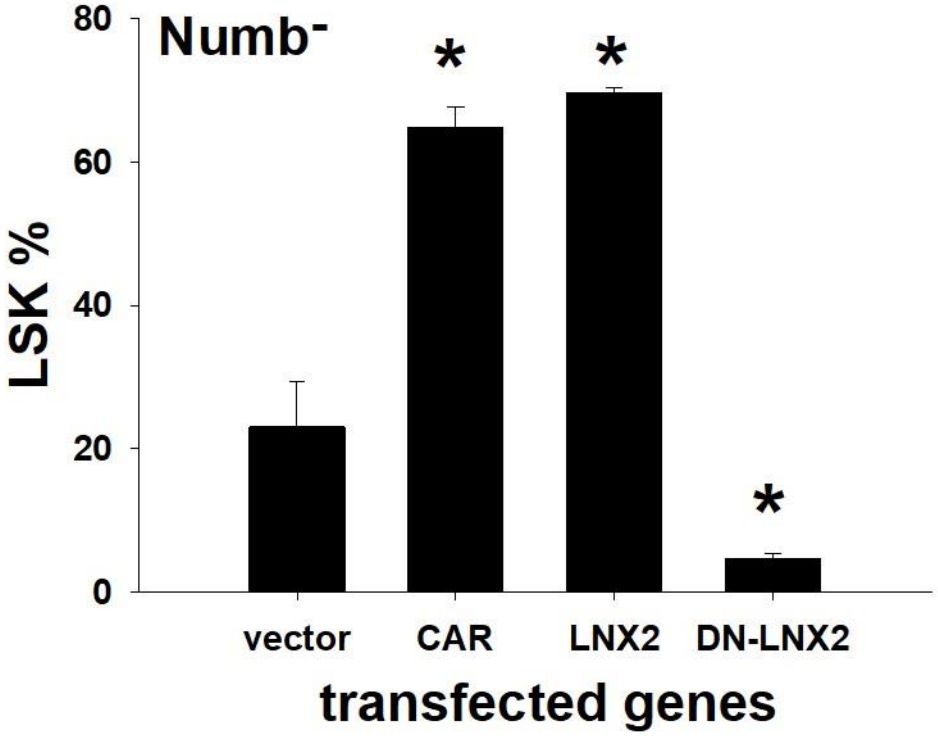

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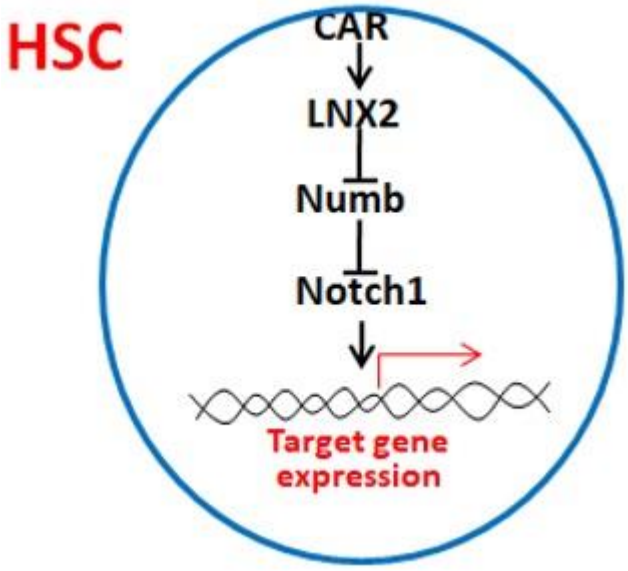

Supplemental Table 1. Limiting dilution analysis for estimating the frequency of hematopoietic stem cells in BM cells from Scl-CreERT/CAR mice (with or without tamoxifen treatment).

Group	HSC frequency*
WT	1/29,859 (1/22,404-1/39,795)
CARcKO	1/32,274 (1/24,171-1/43,091)

*L-Calc software was used to calculate the HSC frequency. *P*=0.85