SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Infection models

LM-OVA was kindly provided by H. Shen, (University of Pennsylvania, Philadelphia, PA). For primary Listeria infection, C57BL/6JOlaHsd were infected i.v. with $5x10^3$ cfu recombinant LM-OVA, followed subsequently by i.v. injection of $1x10^5$ OT-I T cells (with or without PD-1 KO). Spleens of the first two mouse groups were harvested for further analysis on day 8 and day 34 post-infection, while remaining mice were re-infected i.v. with $2x10^5$ cfu recombinant LM-OVA on day 35 after primary infection and spleens were analyzed 5 days later.

The $MCMV^{-ie2\text{-}SIINFEKL}$ (MCMV-OVA) was designed by fusing the SIINFEKL epitope at the C terminus of the ie2 sequence using en passant mutagenesis. In order to generate virus stocks, 3 weeks old male BALB/c mice were infected intraperitoneally (i.p.) with $2x10^5$ plaque-forming units (pfu) of MCMV-OVA. After 21 days, salivary glands were homogenized in 5% skim milk/DMEM using a dounce tissue grinder (loose pestle) and cells were pelleted (800 g, 4 °C, 5 min). In a next step, supernatants were transferred into a fresh dounce tissue grinder, homogenized again (tight pestle) and centrifuged. Finally, supernatants were aliquoted and stored at -80 °C. The virus concentration was determined by plaque-assay. For passaging MCMV, lower virus doses (500-2.500 pfu) were used. For infection experiments, 3 times passaged virus stocks were used *(1)*. For i.v. infection with MCMV, 10^5 OT-I T cells (with or without PD-1 KO) were injected i.v. one day before infection with a dose of 5×10^2 pfu of $MCMV^{IE2}$-OVA.

Cell culture

Mouse splenocytes were cultured in RMPI (Life Technologies) supplemented with 10% FCS, 0.025% L-glutamine, 0.1% HEPES, 0.001% gentamycin, 0.002% streptomycin and 15 ng/ml recombinant human (rh) IL-15 (Peprotech). The Platinum-E packaging cell line (PlatE) was cultured in DMEM (Life Technologies) supplemented with 10% FCS, 0.025% L-glutamine, 0.1% HEPES, 0.001% gentamycin, 0.002% streptomycin. All cells were grown in a humidified incubator at 37 °C and 5% CO_2.

CRISPR/Cas9-mediated PD-1 knock-out

crRNA sequence targeting the *Pdcd1* locus was 5′-ACAGCCCAAGTGAATGACCA-3′. 80 µM tracrRNA (IDT DNA) and 80 µM crRNA (IDT DNA) were incubated at 95 °C for 5 min and cooled to room temperature (RT). 20 µM high fidelity Cas9 (IDT DNA, Cat#1081061) was added slowly to the gRNA solution to yield RNPs with a final concentration of 12 µM Cas9, 20 µM gRNA and 20 µM electroporation enhancer (IDT DNA, Cat#1075916). RNPs were incubated for 15 min at RT and partially stored at -80°C.

Murine splenocytes were obtained from CD45.1 congenic OT-I $Rag^{-/-}$ mice (infection models) or CD45.1 congenic C57BL/6 mice (CAR-T cell model). Spleens were harvested and, after generation of single-cell suspensions and erythrocyte lysis, remaining cells were stimulated for 24 h at a concentration of $1x10^7$ cells/ml with purified anti–mouse CD3 (clone 145-2C11, 1:1000, BD Cat#553058) and anti–mouse CD28 (clone 37.51, 1:3000, BD Cat#553295) antibodies.

On the subsequent day, splenocytes were electroporated (pulse code DN-100) with Cas9 RNPs loaded with gRNA targeting the *Pdcd1* locus in nucleofector solution (20 µl per $3x10^6$ activated splenocytes; Lonza) with a 4D Nucleofector X unit (Lonza). After electroporation, cells were supplied with complete RPMI and 25 ng/mL rhIL-15 (Peprotech).

Retroviral transduction

The anti-CD19 CAR used in this study consists of a murine CD8α leader, an anti-murine CD19 single chain variable fragment, a spacer domain of three repetitive StrepTag-II sequences (W-S-H-P-Q-F-E-K) and a CD8α transmembrane domain. The intracellular domain comprises a CD28 and CD3ζ chain. Due to a P2A element, a truncated version of huEGFR was co-expressed with the CAR.

For the production of retrovirus, PlatE cells were transiently transfected by calcium phosphate precipitation with a retroviral expression vector (pMP72 encoding for the anti-CD19 CAR construct, gag/pol and amphotropic envelope). Virus-containing supernatant was harvested on day 3 and 4 after transfection, and purified from remaining cells by filtering (0.45 µm sterile filter).

Cells collected after CRISPR/Cas9-mediated PD-1 KO were directly retrovirally transduced via spinoculation. Therefore, tissue-culture untreated 24-well plates were coated overnight with RetroNectin (Takara Bio Europe SAS Cat#T100B) according to the manufacturer's protocol and purified anti–mouse CD3 and anti–mouse CD28 antibodies (dilution 1:1000 and 1:3000 respectively). Virus-containing supernatant was centrifuged at 3000 g at 32 °C for 2 h. Afterwards, stimulated and electroporated splenocytes were added to the plates, centrifuged at 800 g at 32 °C for 1.5 h and cultured in complete RPMI with 25 ng/mL rhIL-15 for 3 days prior i.v. transfer into sublethally irradiated C57BL/6 mice.

Adoptive T cell transfer

For the infection models, $1x10^5$ OT-I T cells (with or without PD-1 KO) harvested from the spleen of CD45.1$^{+/+}$ Rag$^{-/-}$ OT-I mice were transferred intravenously the same day (LM-OVA) or one day prior (MCMV-OVA) infection into immunocompetent C57BL/6 mice.

For the tumor model, CD45.1$^{+/+}$ immunocompetent C57BL/6 mice were inoculated intravenously with 1x10^6 CD45.2$^{+/+}$ ALL tumor cells, sublethally irradiated (5 Gy) at day 6, and either or not treated the following day with 3x10^6 CD8$^+$EGFRt$^+$ CAR-T cells obtained by the engineering of splenocytes harvested from CD45.1$^{-/+}$ Rag$^{-/-}$ OT-I mice. For long-term monitoring, 1.5x10^6 CD45.1$^{+/+}$ CAR-T cells were injected i.v. into sublethally irradiated C57BL/6 mice (2 Gy, 24 h before injection). Adoptively transferred CAR-T cells were analyzed for PD-1 and CAR expression by NGS (or Sanger sequencing when indicated) and flow cytometry before their injection, respectively. Combination of PD-1 KO together with CAR transduction resulted in four different mice groups (mock, PD-1 KO, CAR, PD-1 KO CAR). As injection products were not sorted, mice received T cells as bulk populations.

Cetuximab (1mg per mouse) (Bristol-Myers Squibb) was injected i.p. for *in vivo* depletion of CAR-T cells.

Flow cytometry and cell sorting

For blood screenings, blood from the tail vein was collected in heparin tubes before further preparation. Spleens and lymph nodes were harvested and mashed through a 40 µm cell strainer to generate single-cell suspensions. For bone marrow analysis, muscles of hind legs were removed and bone marrow was rinsed with complete RPMI and collected in falcons.

After red blood cell lysis (90% 0,17M NH_4Cl in Tris-HCl) of blood and tissues, remaining cells were initially stained with CD16/32 (Fc-block; 93 [unlabeled]; Biolegend Cat#101301, 1:400) for 20 min at 4 °C, followed by respective surface marker staining for 20 min at 4 °C in the dark. Depending on the respective experiment, a subset of the following fluorochrome-conjugated antibodies was used: CD19 (1D3; BD Bioscience Cat#562291), CD27 (LG7.F9, Life Technologies Cat#12-0271-83), CD3 (145-2C11; Biolegend Cat#100312), CD3 (17A2; Biolegend Cat#100221),

CD45.1 (A20, Life Technologies Cat#48-0453-82), CD45.1 (A20, Life Technologies Cat#17-0453-82), CD62L (MEL-14, Biolegend Cat#104427), CD69 (M1.2F3; Biolegend Cat#104511), CD8 (5H10; Life Technologies Cat#MCD0830), EGFR (AY13; Biolegend Cat#352904), PD-1 (J43; LifeTechnologies Cat#11-9985-81). Live/Dead discrimination was performed by addition of propidium iodide (LifeTechnologies Cat#P1304MP). Optional intranuclear staining with anti-Ki67 antibody (16A8; Biolegend Cat#652405) was performed using the eBioscienceTM Foxp3/Transcription Factor Staining Buffer Set, according to manufacturer's instructions.

Cell samples were acquired on a CytoFLEX S flow cytometer (Beckman Coulter). Flow sorting was performed on a FACSAriaIII (BD Bioscience), MoFlo I XDP (Beckman Coulter) or MoFlo Astrios (Beckman Coulter). Samples were sorted into 96-well plates filled with 50 µL 0,5% BSA in PBS, pelleted and processed for next-generation sequencing. All flow cytometry data were analyzed with FlowJo v10.

Antigen-specific *ex vivo* stimulation and intracellular cytokine staining

Spleens of mice infected with LM-OVA and injected with $1x10^5$ OT-I T cells (with and without PD-1 KO) were harvested on day 8 p.i. $1x10^6$ freshly isolated splenocytes were co-incubated with 10^{-6} M SIINFEKL peptide and 1x Golgi plug (BD PharMingen, Cat#555029) for 4 h at 37 °C. 25 ng/ml Phorbol-12-myristat-13-acetat (Sigma, Cat#P1585) and 1 µg ml ionomycin (Sigma, reference #I9657) were used as a positive control. After co-culture, both staining for live/dead discrimination with ethidium-monoazide-bromide (LifeTechnologies, Cat#E1374), as well as surface marker staining was performed for 15 min at 4 °C. For subsequent intracellular cytokine staining, antibodies for TNF-α (MP6-XT22, BD Cat#557644) and INF-γ (XGM1.2, LifeTech Cat#17-7311-82) were used after cell permeabilization with the BD Cytofix/Cytoperm™ Fixation/Permeablization Kit according to the manufacturer's protocol.

Analysis of CRISPR/Cas9-mediated *Pdcd1* disruption

Genomic DNA was extracted from flow cytometry-sorted CD45.1$^+$ OT-I T cells or EGFRt$^+$ CAR-T cells with the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer´s instructions, and the region surrounding the gRNA cutting site into the PD-1 locus was amplified via PCR. In this step, adapters for Illumina sequence were additionally added to the overall cDNA amplicon. Next-generation sequencing libraries were prepared using the Nextera XT index kit (Illumina) and sequenced on a MiSeq (Illumina). Fastq reads were filtered using trimmomatic (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:25 MINLEN:240) before CRISPR efficacy was calculated using CRISPResso2. CRISPResso2 was applied on reads with a minimum average phred score of 30, a minimum single base pair score of 20. The quantification window center was set to -17.

Guide sequence: TGGTCATTCACTTGGGCTGT

***Primer sequences for PCR*:**

Forward:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACAGTTTCCTTTCCGCTACA-3'

Reverse:

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTCAGACCTACCATCGCTTC-3'

Amplicon sequence:

GACAGTTTCCTTTCCGCTACAGACAACTCTGCCTGAGCAGCGGGGAGGAGGAAGAG
GAGACTGCTACTGAAGGCGACACTGCCAGGGGCTCTGGGCATGTGGGTCCGGCAGG
TACCCTGGTCATTCACTTGGGCTGTGCTGCAGTTGAGCTGGCAATCAGGGTGGCTTCT
AGGTATGTGGCTCCTAGGGATGTGGGATCAATTGGGGTTGGTGGCTGGGGCAGGGCT
TGAAGAAACGTTCACCCTCGGTTCCTATTACTGGAGGGGAGGAACACCTGTGTCCCT
GGTTACACGGATTGGCTCCTGGTTACACAGATTGGCTTTAGGTGGGTTTTACAGGTG
AAATGCCCCCTGGGGCTCAAAATCCACCCCCTCCTTGCTTTTAGACAGACAGAACAT
TTATCTAACGTCAGTTGATCTTCTGTGTCCCTCTCTGTGAAGCGATGGTAGGTCTGAA
C

REFERENCES

1. M. Zeeshan Chaudhry, R. Casalegno-Garduno, K. M. Sitnik, B. Kasmapour, A. K. Pulm, I. Brizic, B. Eiz-Vesper, A. Moosmann, S. Jonjic, E. S. Mocarski, L. Cicin-Sain, Cytomegalovirus inhibition of extrinsic apoptosis determines fitness and resistance to cytotoxic CD8 T cells. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 12961–12968 (2020).

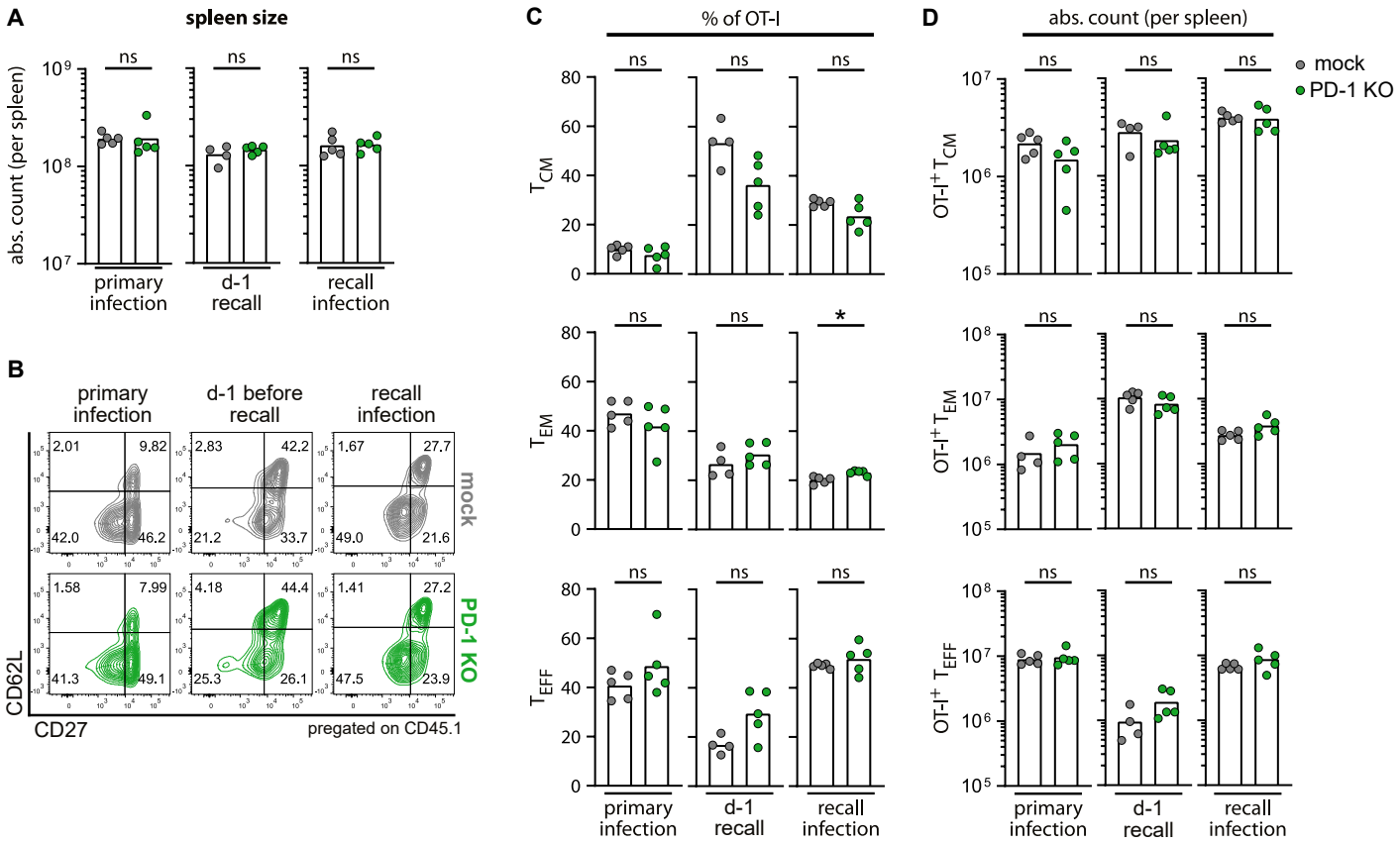

Fig. S1. Functional differentiation of PD-1 KO T cells after LM-OVA infection.
(A) Spleen size of individual mice treated with mock or PD-1 KO OT-I T cells after primary infection (day 8), one day before recall infection (d34) and after recall infection (day 40). Statistical testing by Mann-Whitney test, ns $p > 0.05$. **(B)** Representative flow cytometry plots of memory subset diversification after primary, one day before recall and after recall infection with LM-OVA. **(C and D)** Memory phenotype of individual mice treated with either mock or PD-1 KO OT-I T cells after primary, one day before recall and after recall infection with LM-OVA. T_{CM} ($CD62L^+$ $CD27^+$), T_{EM} ($CD62L^-$ $CD27^+$), T_{EFF} ($CD62L^-$ $CD27^-$). Percentages **(C)** and absolute counts per spleen **(D)** of individual T cell subsets were statistically tested by Mann-Whitney test, ns $p > 0.05$, *$p \leq 0.05$.

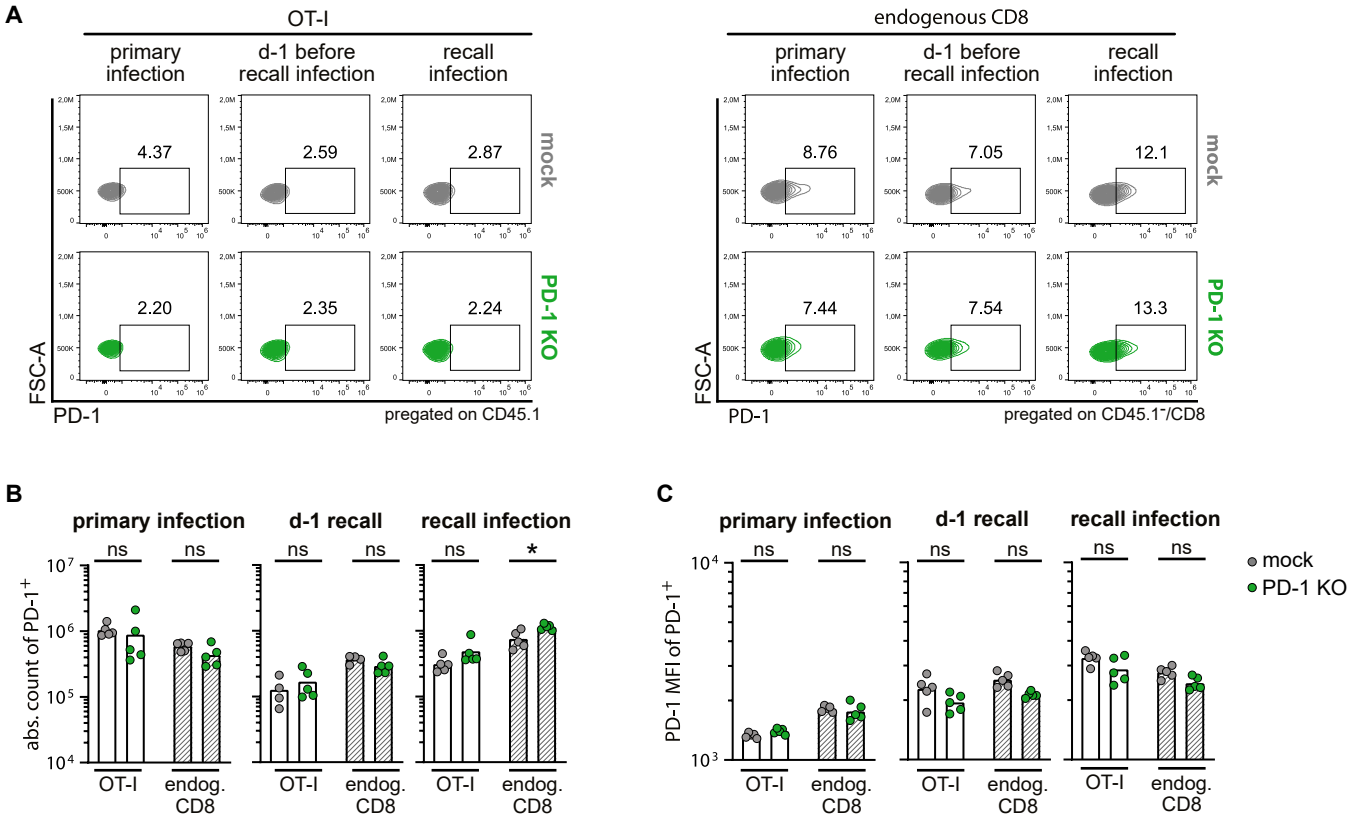

Fig. S2. PD-1 expression level after acute LM-OVA infection.
PD-1 surface expression was analyzed by flow cytometry in transferred CD45.1$^+$ OT-I T cells and endogenous CD8$^+$ T cells (CD45.1$^-$) recovered from spleens after primary, one day before and after recall LM-OVA infection. **(A)** Representative flow cytometry plots of PD-1 expression after primary (day 8), one day before recall (d34) and after recall (day 40) infection. **(B)** Absolute counts and **(C)** PD-1 MFI (mean fluorescence intensity) of PD-1 expressing cells. Statistical testing by two-way-ANOVA test, ns $p > 0.05$, *$p \leq 0.05$.

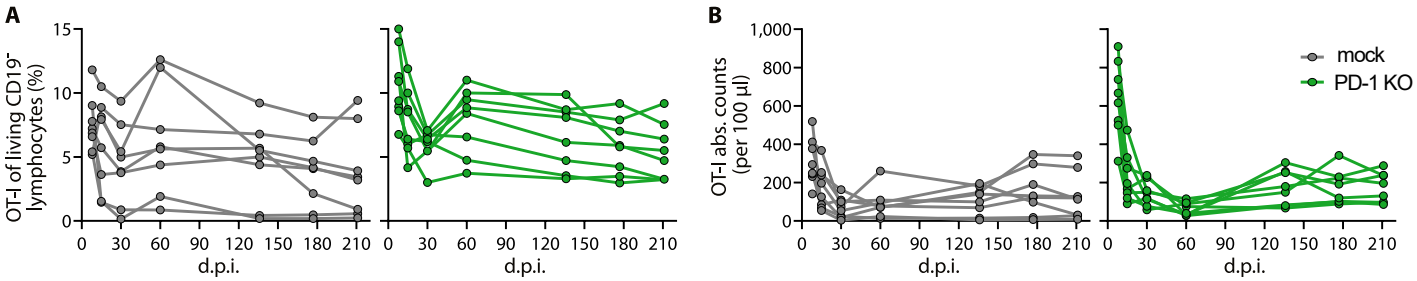

Fig. S3. Long-term persistence of antigen-specific T cells with PD-1 KO after chronic MCMV-OVA infection.

Longitudinal blood analysis of mice infected with $mCMV^{IE2\text{-}OVA}$ and treated with mock (grey) or PD-1 KO (green) OT-I T cells. **(A)** Frequencies of transferred $CD45.1^+$ OT-I T cells of $CD19^-$ living lymphocytes and **(B)** absolute counts of OT-I T cells in 100 µL blood are depicted for each individual mouse.

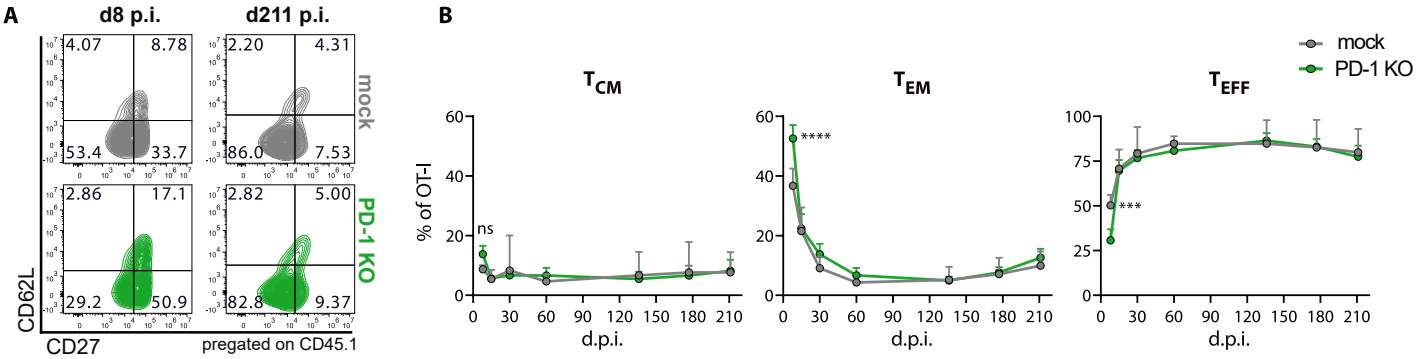

Fig. S4. Functional differentiation of PD-1 KO T cells after MCMV-OVA infection.
(A) Representative flow cytometry plots of phenotypic differentiation of CD45.1$^+$ OT-I T cells on day 8 and day 211 after infusion. **(B)** Longitudinal analysis of different phenotypic subsets of CD45.1$^+$ transferred OT-I T cells in the blood. Statistical testing by two-way ANOVA, ns $p > 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

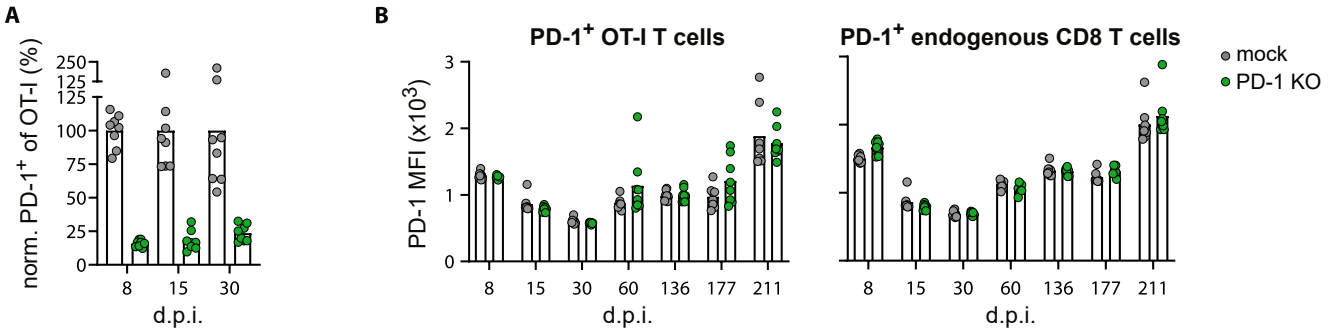

Fig. S5. Longitudinal analysis of PD-1 surface expression.
(A) Frequencies of PD-1$^+$ OT-I T cells were normalized to the mean frequency of PD-1$^+$ OT-I T cells of the mock treated group. **(B)** PD-1 MFIs (mean fluorescence intensity) of CD45.1$^+$PD-1$^+$ transferred OT-I T cells (left) and endogenous CD45.1$^-$ CD8$^+$ PD-1$^+$ (right) T cells for individual mice.

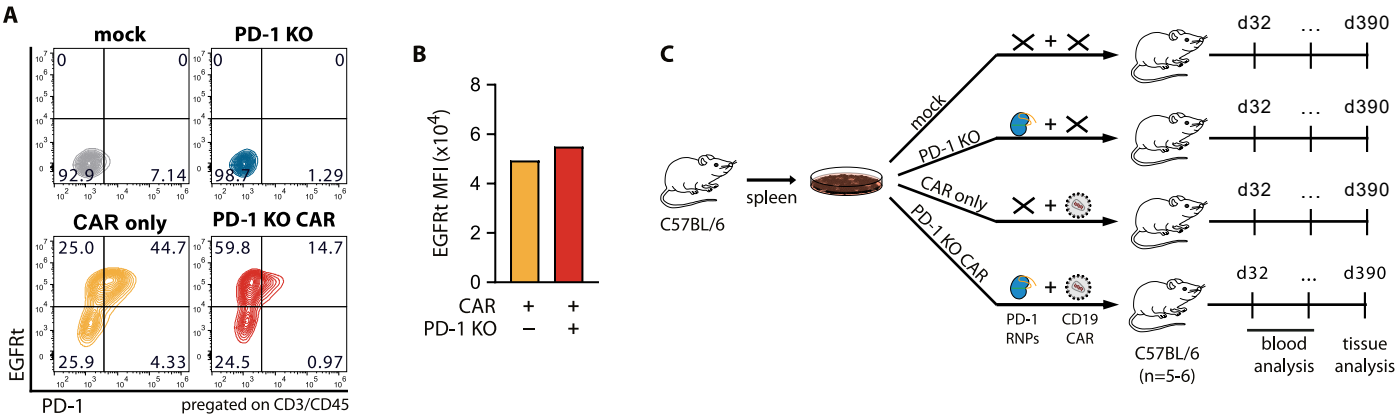

Fig. S6. Engineering of CAR-T cells.
(A) Representative flow cytometry plots of PD-1 and EGFRt (marker for CAR transduction) expression of the different generated T cell infusion products after engineering: control (mock, grey), CRISPR/Cas9-mediated PD-1 KO (PD-1 KO, blue), retroviral CAR transduction (CAR, yellow) or both (PD-1 KO CAR, red). **(B)** CAR expression level assessed by MFI (mean fluorescence intensity) of EGFRt. **(C)** Schematic overview of experimental layout. C57BL/6 mice received total body irradiation one the day before treatment with $1.5x10^6$ T cells of specific gene-editing (n = 5-6 mice for each group). Stimulated $CD45.1^+$ splenocytes were nucleofected with Cas9 RNPs targeting the *Pdcd1* locus, followed by retroviral transduction with an anti-CD19 CAR. Combination of both engineering methods resulted in four different T cell infusion products. Peripheral blood was tracked monthly for 390 days.

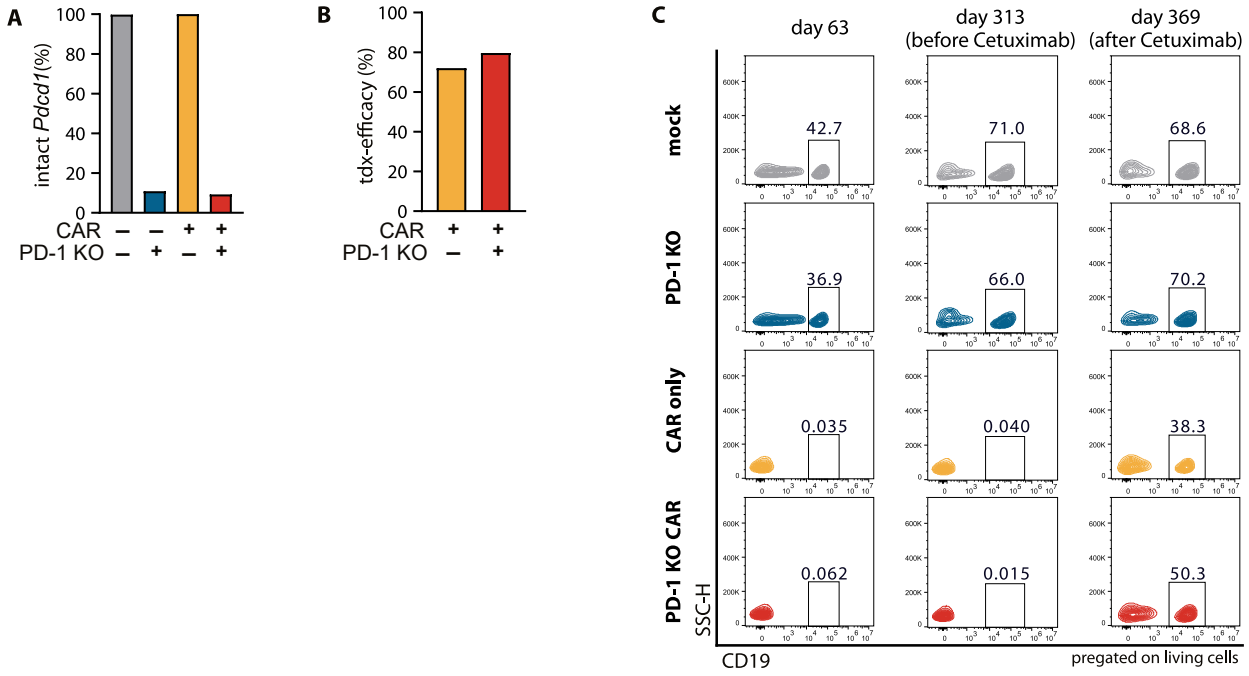

Fig. S7. B cell recovery after antibody-mediated depletion of CD19 CAR-T cells.
(**A)** NGS results of *Pdcd1* locus disruption of infusion T cell products. **(B)** Transduction efficacy of CAR-T cell products represented by EGFRt surface expression analyzed by flow cytometry. **(C)** Exemplary flow cytometry plots of B cell frequencies in the peripheral blood of different groups on day 63, day 313 and day 369.

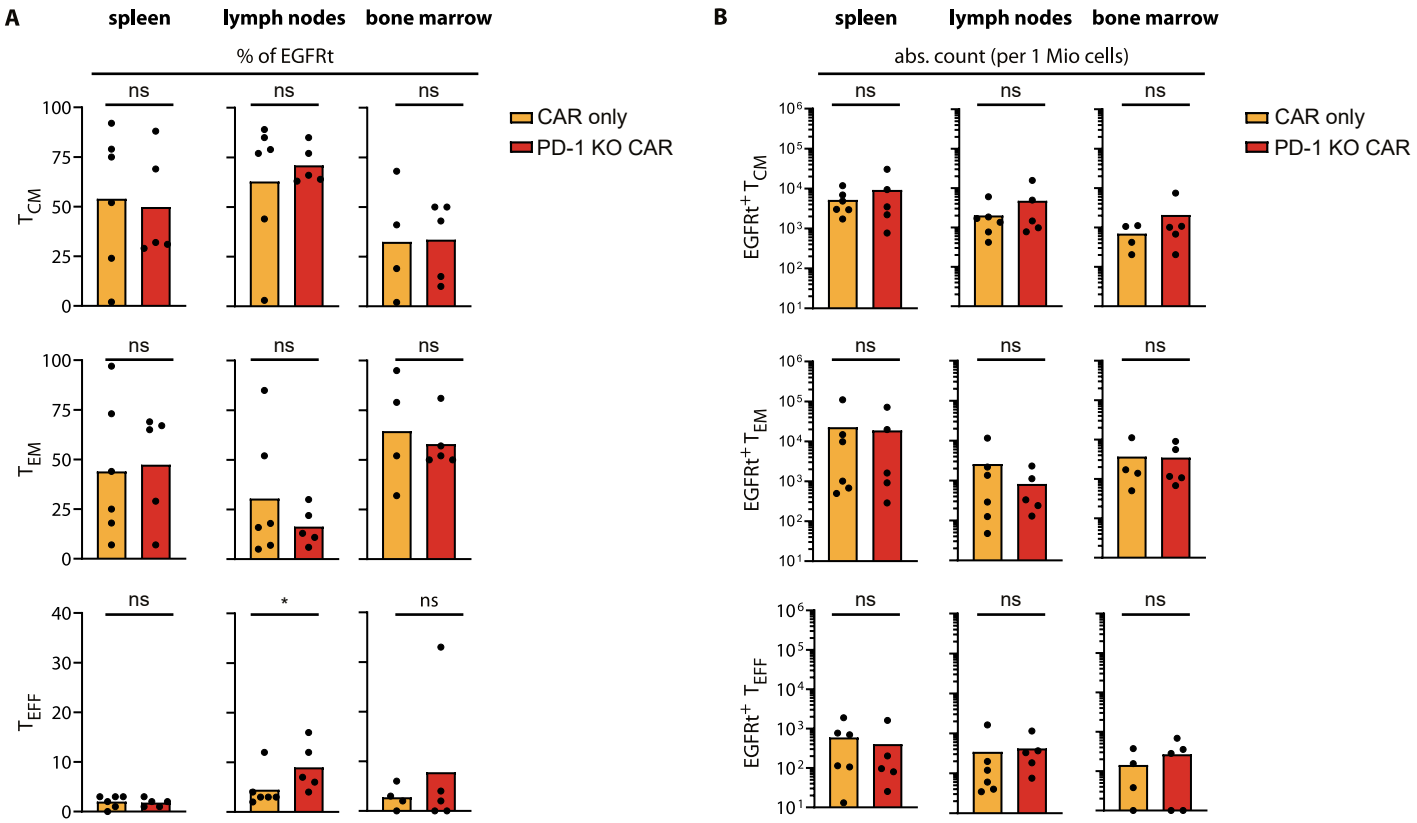

Fig. S8. Phenotype analysis of CAR-T cells in lymphoid organs 390 days post injection.
Phenotypic analysis of tissue-recovered CAR-T cells ($EGFRt^+$) on day 390 in individual mice. T_{CM} ($CD62L^+$ $CD27^+$), T_{EM} ($CD62L^-$ $CD27^+$), T_{EFF} ($CD62L^-$ $CD27^-$). **(A)** Frequencies and **(B)** absolute counts per $1x10^6$ living lymphocytes of T cell subsets of transferred CAR-T cells ($CD19^-CD45.1^+EGFRt^+$) in lymphoid organs. Statistical testing by Mann-Whitney test, ns $p > 0.05$, *$p \leq 0.05$.

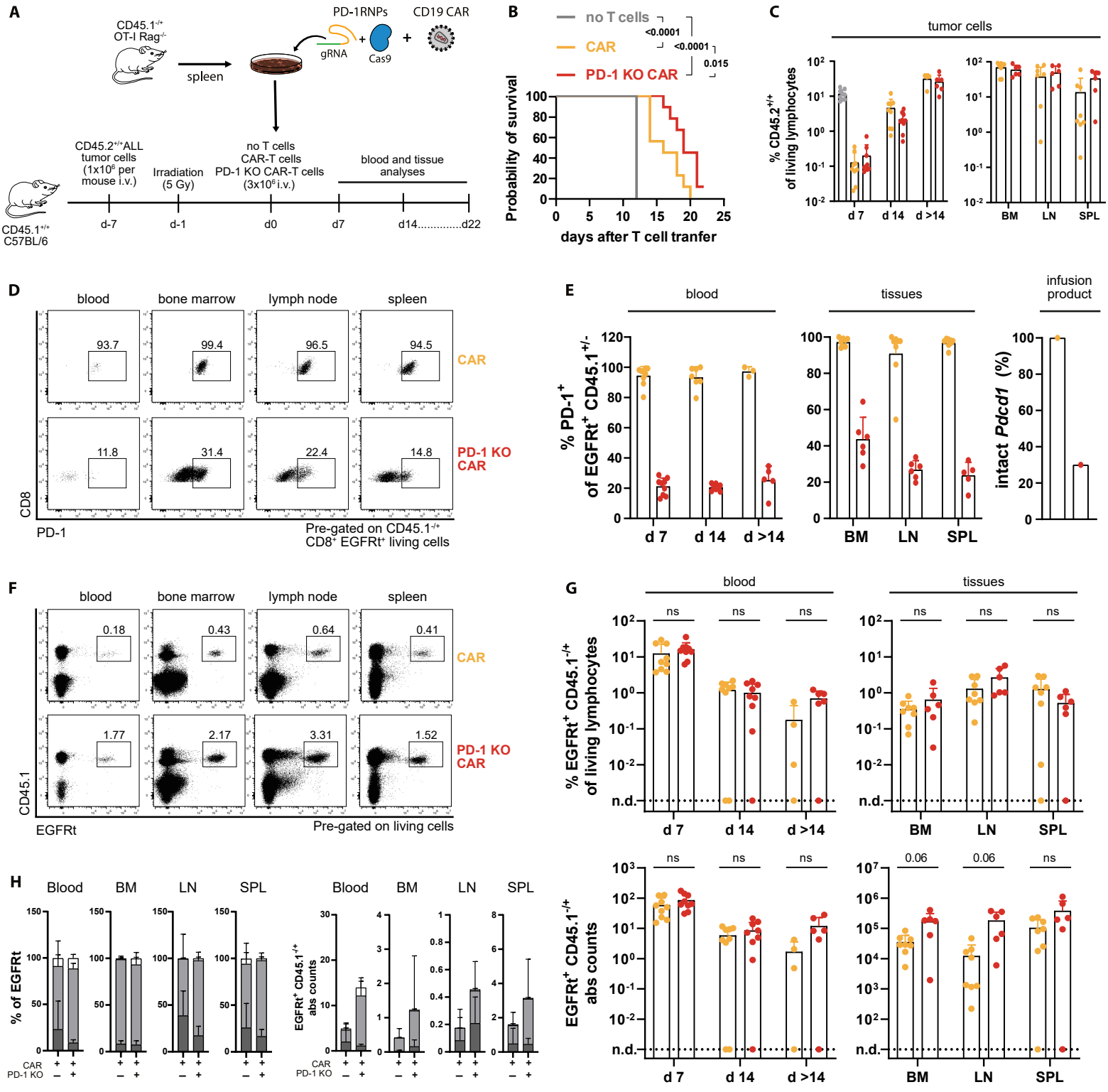

Fig. S9. Analysis of PD-1 KO CAR-T cells in a syngeneic tumor model.

(A) Schematic depiction of the experimental setting (n = 9). The amount of transferred cells refers to $CD8^+$ $EGFRt^+$ CAR-T cells. **(B)** Kaplan-Meier survival curves of ALL tumor-bearing mice either treated or non-treated with anti-CD19 CAR-T cells. Statistical significance was determined using log-rank Mantel-Cox test corrected for multiple comparisons. **(C)** Frequency of $CD45.2^{+/+}$ ALL tumor cells in the peripheral circulation at the indicated time points (left) and organs (right). **(D)** Representative flow cytometry data showing the frequency of PD-1-expressing CAR-T cells from peripheral blood and lymphoid organs of ALL tumor-bearing mice 18 days after T cell transfer. **(E)** Quantification of the frequency of PD-1-expressing cells in blood at the indicated time points and in tissues at the endpoint by flow cytometry (left and middle), and Sanger sequencing for the analysis of PD-1 genetic ablation in the infusion product (right). **(F)** Representative flow cytometry data showing the frequency of CAR-T cells from peripheral blood and lymphoid organs of ALL tumor-bearing mice 18 days after T cells transfer. **(G)** Quantification of frequencies (upper) and absolute numbers (below) of CAR-T cells in blood at the indicated time points and in organs at the endpoint. Statistical significance was determined using Multiple Wilcoxon test, ns $p > 0.05$. **(H)** Phenotypic analysis of circulating and tissue-recovered CAR-T cells ($CD45.1^{-/+}$ $EGFRt^+$) at the corresponding endpoint analysis. T_{CM} ($CD62L^+$ $CD27^+$), T_{EM} ($CD62L^-$ $CD27^+$), T_{EFF} ($CD62L^-$ $CD27^-$). Absolute numbers refer to µl of blood or total organ ($x10^5$). For (C), (D) and (G) blood analyses after day 14 refers to the endpoint analyses. Data are shown as mean+SD. BM = bone marrow; LN = lymph node; SPL = spleen.

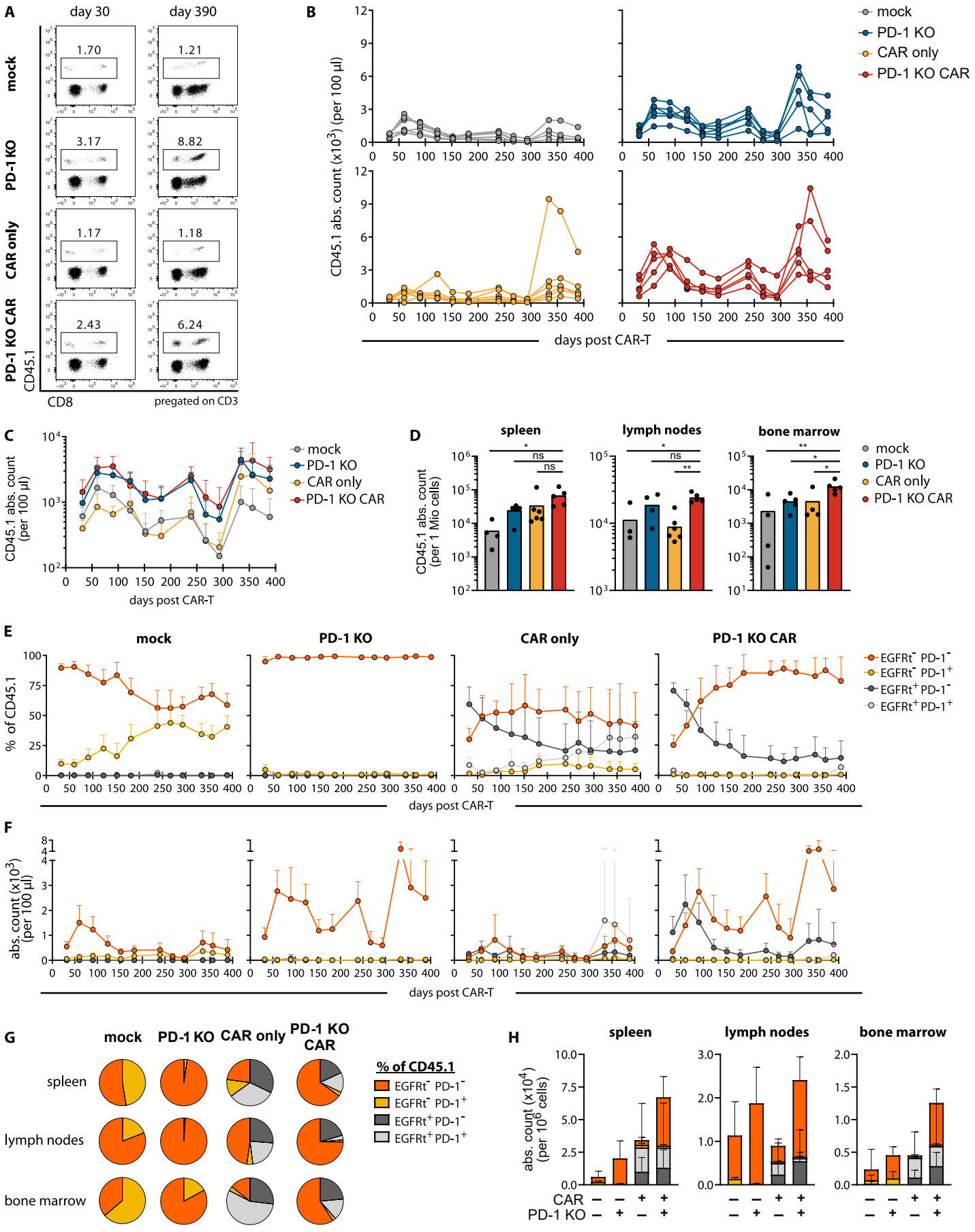

Fig. S10. Increased expansion of PD-1 KO T cells without expression of CAR.
(A) Exemplary flow cytometry plots of transferred CD45.1⁺ T cells in the peripheral blood of mice from the experiment described in Fig. S6C on day 30 and day 390 **(B)** Longitudinal tracking of absolute counts of CD45.1⁺ transferred cells per 100µl blood over 390 days. Data represents individual mice. **(C)** Absolute numbers of CD45.1⁺ transferred cells of all four groups in peripheral blood over time. Graph depicts mean+SD. **(D)** Tissue analysis of absolute counts of CD45.1⁺ transferred cells per 1x10⁶ living lymphocytes at day 390 after T cell injection. Statistical testing by one-way-ANOVA, ns $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$ using the mock group as a reference control. **(E-F)** Longitudinal blood analysis of PD-1 and EGFRt expressing transferred cells. Data represent frequencies (E) or absolute cell counts (F) as mean+SD. **(G-H)** Analysis of different subpopulations of transferred cells in lymphoid tissues on day 390 after CAR-T cell injection. Data represent frequencies of subpopulations of transferred cells as means (G) or absolute counts of different subpopulations per 1x10⁶ living lymphocytes (H) as mean+SD.