

## **Supplemental information**

### **Physiological lentiviral vectors**

#### **for the generation of improved CAR-T cells**

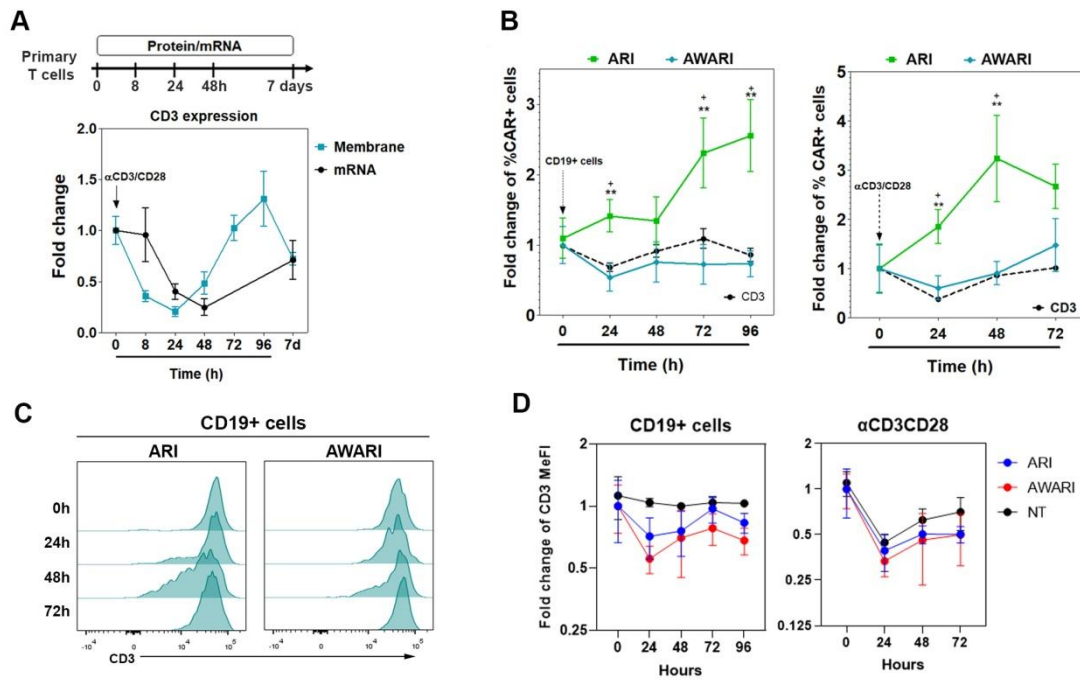
**María Tristán-Manzano, Noelia Maldonado-Pérez, Pedro Justicia-Lirio, Pilar Muñoz, Marina Cortijo-Gutiérrez, Kristina Pavlovic, Rosario Jiménez-Moreno, Sonia Nogueras, M. Dolores Carmona, Sabina Sánchez-Hernández, Araceli Aguilar-González, María Castella, Manel Juan, Concepción Marañón, Juan Antonio Marchal, Karim Benabdellah, Concha Herrera, and Francisco Martín**

## **Supplemental Figures and Materials**

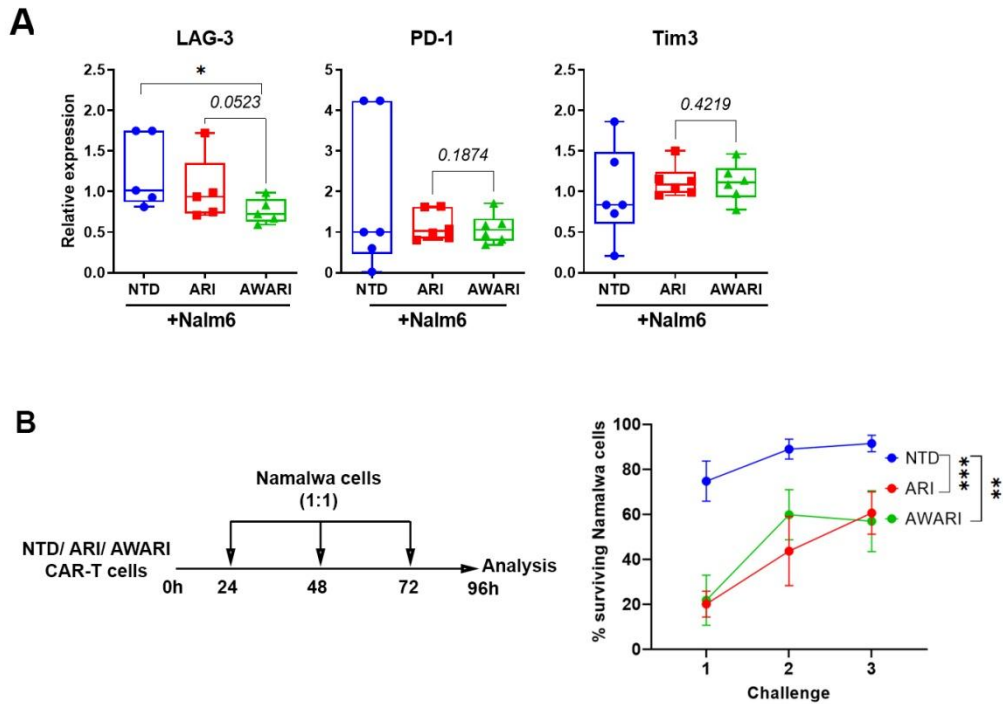
### **Title**

#### **Physiological (TCR-like) regulated lentiviral vectors for the generation of improved CAR-T cells**

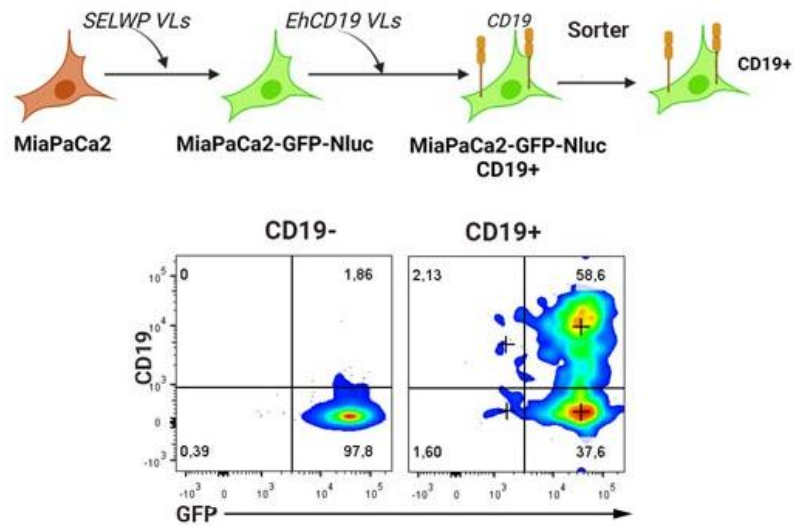
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**Supplementary Figure 1. A** CD3 kinetics after αCD3/CD28 stimulation at protein (membrane, n=10) and at mRNA level (n=5). **B** Fold change of the percentage of CAR+ cells driven by EF1-α (green) and AW-CAR19-BBzz (blue) CAR-T cells stimulated with CD19+ cells (**left**) and αCD3/CD28 (**right**) compared to CD3 expression pattern (black). Fold change indicates the percentage at different times related to those of 0h. ANOVA test, Bonferroni Post-test. \*, p<0.05. **C** Histograms of CD3 expression of ARI and AWARI CAR-T cells after the stimulation with CD19+ cells along the time. **D** Fold change of CD3 MeFI of Non-transduced T cells (NT, black) and ARI (blue) and AWARI (red) CAR-T cells after the stimulation with CD19+ (**left**) or αCD3/CD28 (**right**).

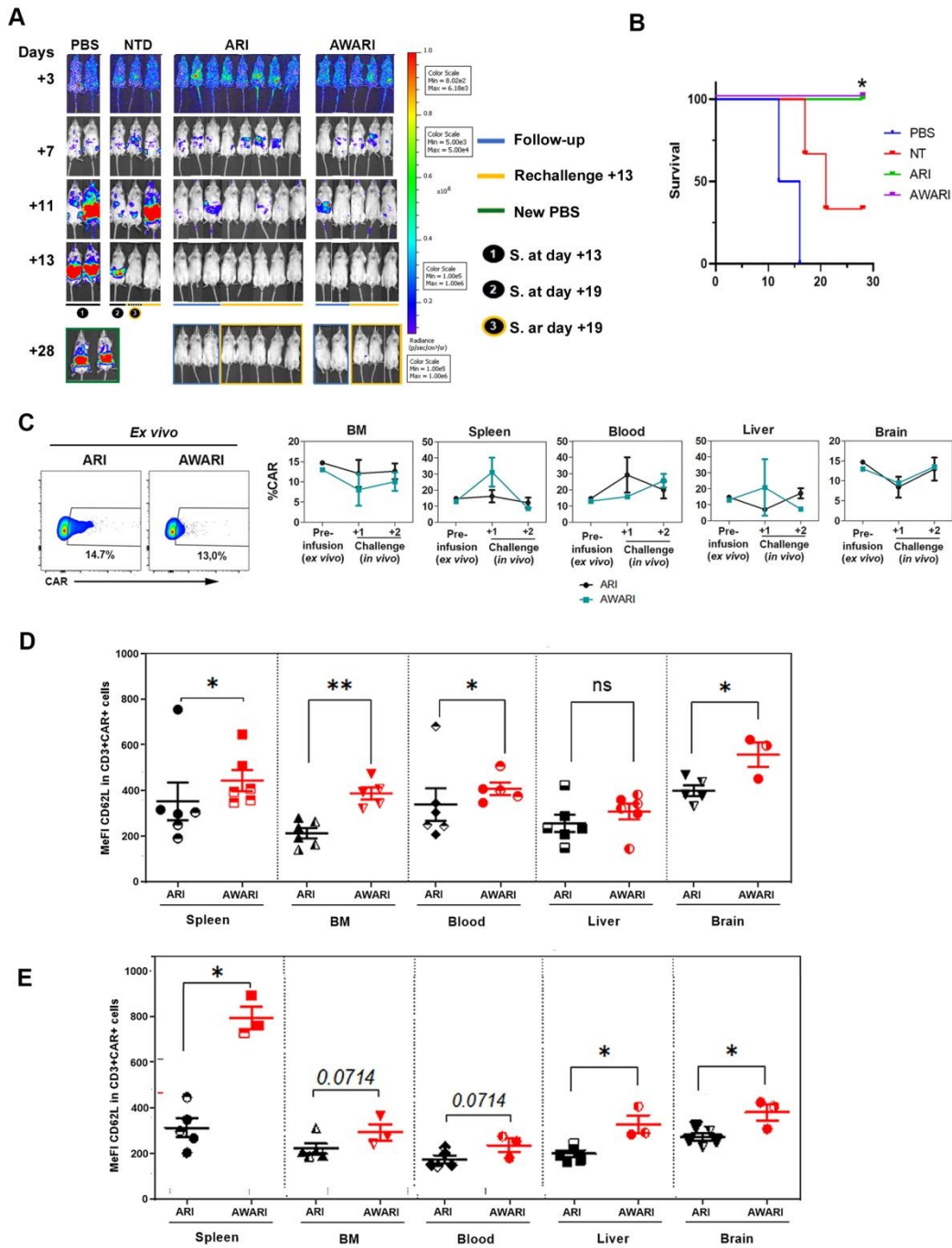


**Supplementary Figure 2.** **A)** Relative expression of LAG-3, PD-1 and Tim3 markers driven by ARI and AWARI CAR-T cells after being co-cultured with Nalm6 during 48h. Relative expression is referred to MeFI levels of every NT donor. One-tailed paired T-test ( $n \geq 5$ ). **B)** Left: work-flow of the anti-lymphoma efficacy of NT, ARI and AWARI CAR-T cells after with three challenges with Namalwa cells at an initial ratio 1:1 target:CAR-T cells. Right: percentage of surviving Namalwa CD19+ tumor cells in the culture after one, two or three challenges over NT or CAR-T cells. 2-way ANOVA, Tukey's multiple comparisons.

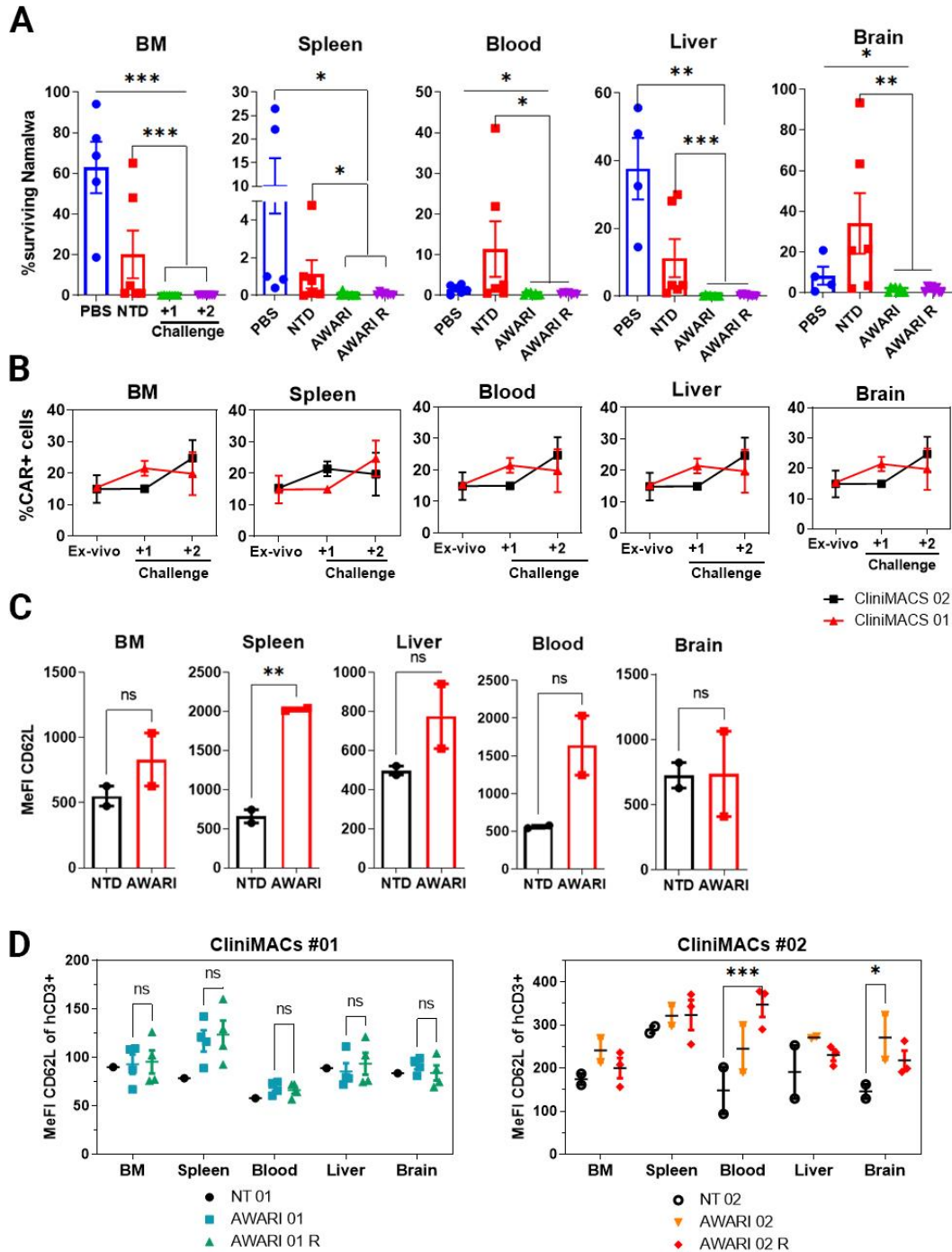


### Supplementary Figure 3

Top: Diagram of CD19+GFP+Nluc+MiaPaCa2 model's generation. MiaPaCa2 cells were first transduced with SELWP to generate GFP+Nluc+ cells. Then, the bulk population was transduced with EhCD19 LV and GFP+CD19+ cells were sorted. Bottom: Dot-plots of CD19- and CD19+ GFP+Nluc MiaPaCa2 cells used in the performed assays. Note that CD19 was downregulated or silenced in the sorted population by the time of the experiments.



**Supplementary Figure 4.** **A)** BLI images of PBS, NTD, ARI and AWARI treated mice for up to 28 days. Rechallenged mice are highlighted in yellow. **B)** Survival of the different groups of mice. Mantel-Cox test, \* indicates  $p=0,0476$ . **C) Left:** Dot-Plots of CAR expression of ARI and AWARI CAR-T cells *ex vivo*, prior to the infusion into de the mice. **Right:** Percentage of CAR+ cells analyzed in the hCD3+ population in the different organs from ARI and AWARI treated-mice after sacrificed one (+1) or two (+2) challenges with tumor cells compared to *ex-vivo* percentage before the infusion. No significance, 2-way ANOVA, Tukey's multiple comparisons. **D, E)** MeFi of CD62L in hCD3+CAR+ cells infiltrated in the different organs of mice with one (**D**) or two (**E**) Namalwa challenges. Half-full dots indicate mice from the  $5 \times 10^6$  dose experiment. Only when CAR+ population was  $>1\%$ , data was analyzed and included here. One-tailed nonparametric T test.



**Supplementary Figure 5.** A) Percentage of surviving Namalwa in both #01 and #02 CliniMACS experiments with one or two Namalwa inoculations analyzed by FACS at final points. One-tailed Mann-Whitney T- test. B) Percentage of CAR+ cells analyzed in the hCD3+ present in the different organs from mice inoculated with one or two challenges with tumor cells compared to *ex-vivo* percentage of AWARI CliniMACs #01 and #02 before the infusion. C) CD62L MeFI of hCD3+ from different analyzed organs of NT and AWARI CliniMACS #01 at day 13 post-inoculation. Two-tailed T-test. D) CD62L MeFI of hCD3+ from different analyzed organs of NT and AWARI mice with one or two (rechallenged, R) tumor inoculations of #01 (left) and #02 (right). 2-way ANOVA, Tukey's multiple comparisons.

## **Material and Methods**

### **RNA extraction and quantitative PCR**

Total RNA from T cells was extracted using Trizol (Ambion) and RNA samples were converted into cDNA using the high-capacity cDNA reverse transcription kit (ThermoFisher), complemented with RNase inhibitor (ThermoFisher). Q-PCR was performed in a Stratagene MX3005P system, using the Kappa SYBR FAST pPCR Master Mix (Kappa Biosystems) and primers against CD3: CD3-Fw 5'-AAGATGAAGTGGAAGGCG-3' and CD3-Rv 5'-CTCAGGAACAAGGCAGTG-3'. GAPDH as housekeeping gene: GAPDH-Fw 5'-ATGGGGAAGGTGAAGGTCG-3' and GAPDH-Rv 5'-GGGGTCATTGATGGCAACAATA-3'. Relative changes in gene expression were analysed using the  $2^{-\Delta\Delta C_t}$  method