CD28.OX40 co-stimulatory combination is associated with long *in vivo* persistence and high activity of CAR.CD30 T cells

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Supplemental Material and Methods Cell lines

Cell lines were obtained from Sigma-Aldrich [Karpas 299 cells (NHL)], DSMZ [HDML-2 (HL), L428 (HL) and CD30negative leukemia BV173] and ATCC (293T cells). All cell lines were authenticated by STR analysis (Eurofins, DE).

Description of the CAR.CD30 Constructs

Two "third" (III) generation clinical grade retroviral vectors have been designed, carrying the cassette anti-CD30 single-chain variable fragment (scFv), derived from a murine IgG antibody AC10, linked via an optimized human CD8 hinge-transmembrane domain, to the codon optimized signaling domains of the two costimulatory domains CD28 and 4-1BB or CD28 and OX40: (thereafter, 28.OX40.ζ) linked to CD3-ζ (for sequence details see Supplemental Table 1). For the second (II) generation constructs, we included as costimulatory domain either CD28 or 4-1BB. As a trackable marker we added a gene coding for a small molecule derived from the phosphoglycoprotein CD34 (ΔCD34) linked to human CD8-transmembrane domain (CD8aTM). We also included in the construct a new suicide gene, known as inducible Caspase 9 (iC9)(1), in frame with scFv to guarantee prompt elimination of the genetically modified T cells in case of life-threatening toxicity, uncontrollable with pharmacological therapy.

Transduction

Tumor cell lines were transduced using an eGFP-Firefly-Luciferase (eGFP-FFLuc) retroviral supernatant in 24-well plates pre-coated with recombinant human RetroNectin (Takara-Bio. Inc; Japan). Two days after transduction, tumor cell lines were expanded in specific medium. After 1 week, GFP positive cell lines they were selected by Cell Sorting (BD FACSAria III).

The retroviral supernatant carrying eGFP-FFLuc and/or CAR.CD30 were used to label primary T cells derived from peripheral blood mononuclear cells of healthy donors following standardized procedure (2, 3). Briefly, T lymphocytes were activated by OKT3/CD28 in the presence of recombinant human interleukin-2 (IL2, 100 U/ml; R&D), or human interleukin-7 (IL7, 10 ng/ml; R&D) and 15 (IL15, 5 ng/ml; R&D). Activated T cells were transduced on day 3 in 24-well plates pre-coated with recombinant human RetroNectin (Takara-Bio. Inc; Japan) using a specific retroviral supernatant (Retrovirus Copies equal to 10^9/0.5x10^6 T cells), and the above described cytokines. On day 5 after transduction, T cells were expanded in medium containing 45% RPMI1640 and 45% Click's medium (Sigma-Aldrich, Co.; USA) supplemented with 10% FBS and 2 mM Glutamax, and replenished twice a week.

Activation of suicide gene iCasp9

AP1903 was kindly provided by Bellicum-Pharmaceuticals (USA) and added at the indicated concentrations. The *in vitro* elimination of CAR+ T-cells was evaluated at 72hrs by FACS analysis.

In the first *in vivo* model (Supplemental Figure 7A-B), the activity of iCasp9 was performed as follows: Karpas 299-bearing NGS mice received i.v. 10×10^6 of NT or CAR.CD30 T-cells genetically modified with eGFP-FFLuc. Six days later, when the presence of circulating T-cells was confirmed by bioluminescence, mice were treated with 100 mg/mouse (i.p.) of AP1903 (day6 and day7) and residual CAR-T-cells were evaluated by IVIS imaging system

In the second *in vivo* setting, NHL Karpas 299-eGFP-FFLuc cells was systemically infused in NSG mice, at day -6. Effector T cells were infused *i.v.,* at the time of tumor establishment (day 0) assessed by IVIS Imaging. Specifically mice were divided in three cohorts and received injection of effector T cells (1e7 cell/mouse): NT T cells (10 mouse), 28.4-1BBζ T cells (10 mouse) or 28.OX40ζ T cells (10 mouse). Mice underwent periodical blood collection for FACS analysis. One month after the infusion, when circulating CAR-T cells were detected, mice were treated i.p. with AP1903 (100µg/mouse) for two days consecutively and evaluated circulating CAR-T cell before and after treatment.

TCR Vβ repertoire

T-cell receptor (TCR)-Vβ repertoire on NT and CAR-T cells, was evaluated at day+15 and day+30, using a panel of 24 different TCR Vβ- specific mAbs (IO TEST Beta Mark TCR-Vβ repertoire kit, BC) used in association with CD3 specific mAb (BD Biosciences) and isotype control (BD Biosciences). Samples were analyzed with a BD LSRFortessa X-20. Data were analyzed using the FACSDiva software (BD Biosciences). For each sample, we analyzed a minimum of 20,000 events.

Detection of tumor-infiltrating lymphocytes by staining and immunohistochemistry (IHC)

For IHC analysis, tumor sections were de-paraffinized with xylene, sequentially rehydrated in ethanol, and incubated in 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. Immunostaining was performed using the Envision Flex kit (Dako, USA). Nonspecific binding was blocked by incubation with BSA 5% for 60 minutes. Sections were incubated with anti-CD3 antibody (1:75, DAKO) at 4 °C overnight, detecting the presence of TILs (tumor-infiltrating lymphocytes). Sections were incubated with a biotinylated secondary antibody HRP for 20 minutes. Color was developed by adding peroxidase substrate diaminobenzidine. Sections are counterstained with Mayer hematoxylin Gill 2 (Bio Optica, Italy) and, finally, mounting solution and coverslips were added.

Proliferation assay thymidine

T or CD30.CAR T cells were plated in triplicate at 10^5 cells/well with γ-irradiated (30 Gy) autologous NT and CAR T cells, allogeneic PBMC derived from healthy donors (HD) and (45 Gy) CD30+ Karpas299 and CD30- Daudi cell lines at an effector-target ratio of 1:1. After 96 hours, NT or CD30.CAR T cells were pulsed with 1 µCi (0.037 MBq) methyl-3[H]thymidine (Perkin Elmer), cultured for additional 16 hours, and then harvested onto filters and dried. Thymidine uptake was measured as counts per minute (cpm) in a β-scintillation counter (liquid scintillation analyser Microbeta2; Perkin Elmer). Results were expressed as cpm.

Carboxyfluorescein Succinimidyl Ester (CFSE) proliferation assay

Primary T-cells were labeled with CFSE, using the CellTrace™ Proliferation Kit (Invitrogen, USA), following the manufacturer protocol. CFSE dilution of CAR T-cells was measured by FACS after 5 days of culture upon specific stimulation.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism (GraphPad Software). Differences between groups generating p-values <0.05 were considered significantly. The mouse survival data were analyzed using the Kaplan-Meier survival curve and Log-rank (Mantel-Cox) test was used to measure statistically significant differences. No valuable samples were excluded from the analyses. Animals were excluded only in the event of their death after tumor implant, but before T-cell infusion. Neither randomization nor blinding were done during the *in vivo* study. However, mice were matched based on the tumor signal for control and treatment groups before infusion of control or gene modified T cells. To compare the growth of tumors over time, bioluminescence signal intensity was collected in a blind fashion. Bioluminescence signal intensity was log transformed and then compared using a two-sample t-test. The analysis of the pathologist, aimed at quantifying tumor volume, was performed in a blind fashion.

Supplemental Results

Activation of the suicide gene iCasp9 in *vivo* model

We used two different approaches to test iCasp9 activity on CAR T-cells. In the first one, CAR.CD30 T-cells were transduced with GFP/FF-LUC and sorted for the subpopulation showing a double positivity (CAR+ GFP+) before infusion in NSG xenograft mice engrafted with CD30+ Karpas 299 cells. As shown in Supplemental Figure 7B-C, in mice treated with AP1903, we observed a significant

reduction in bioluminescence signaling, confirming the activation of the safety switch. As shown in Supplemental Figure 7B-C, the bioluminescence of Circulating NT T cells did not significantly change before (1.16e6 p/sec/cm2/sr (day0) and after AP1903 treatment (1.08e6 p/sec/cm2/sr (day13). The bioluminescence of CAR.CD30.CD28.4-1BB T cells without AP1903 significantly increased from 7,01E+05 p/sec/cm2/sr (day0) to 2,95E+07 p/sec/cm2/sr (day+13). Similar results were observed for CAR.CD30.CD28.OX40 T-cells [9.85E+05 p/sec/cm2/sr (day0) to 4.10E+07 p/sec/cm2/sr (day+13)]. In contrast, when mice were treated with 100µg of AP1903, the bioluminescence of CAR.CD30 T-cells did not significantly increase (CAR.CD30.CD28.4-1BB: 5.70E+05 before and 6.14E+05 after AP1903 administration; CAR.CD30.CD28.OX40: 1.44E+06 before and 7.60E+05 after AP1903 administration). The bioluminescence of negative control (mice that did not receive FireFly Luciferase T-cell treatment) was below to 8,5E+05 p/sec/cm2/sr (considered as background of the experiments).

To confirm these observations, we conducted a new experiment, where Karpas 299-bearing mice were treated with NT T or CAR.CD30 T-cells. One month after infusion, mice were treated with AP1903, and circulating T cells were evaluated before and after AP1903 treatment, as shown by the experimental design (Supplemental figure 7D). The level of circulating CAR+ T cells measured for CAR.CD30.CD28.OX40 was equal to 30.5%±10.5% and 2.2%±2.7%, before and after AP1903 administration, respectively (p=0,00003) (Supplemental figure 7E). Similarly, CAR+ T cells measured for CAR.CD30.CD28.4-1BB was equal to 18.6%±6.6% to 1.5%±1.5%, before and after AP1903 administration, respectively (p=0,0002). To evaluate the background of our CAR staining in mouse samples, we have also considered samples from mice infused with human un-transduced T cells (NT T cells). In this case, we observed non-specific staining equal to 3.1%±2.9% and 0.9%±1.6%, before and after AP1903 (no statistically significant difference between these values and those detected after AP1903 administration in both CAR T cell cohorts).

Additional references

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2. Quintarelli C, Orlando D, Boffa I, Guercio M, Polito VA, Petretto A, et al. Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma. Oncoimmunology. 2018;7(6):e1433518.
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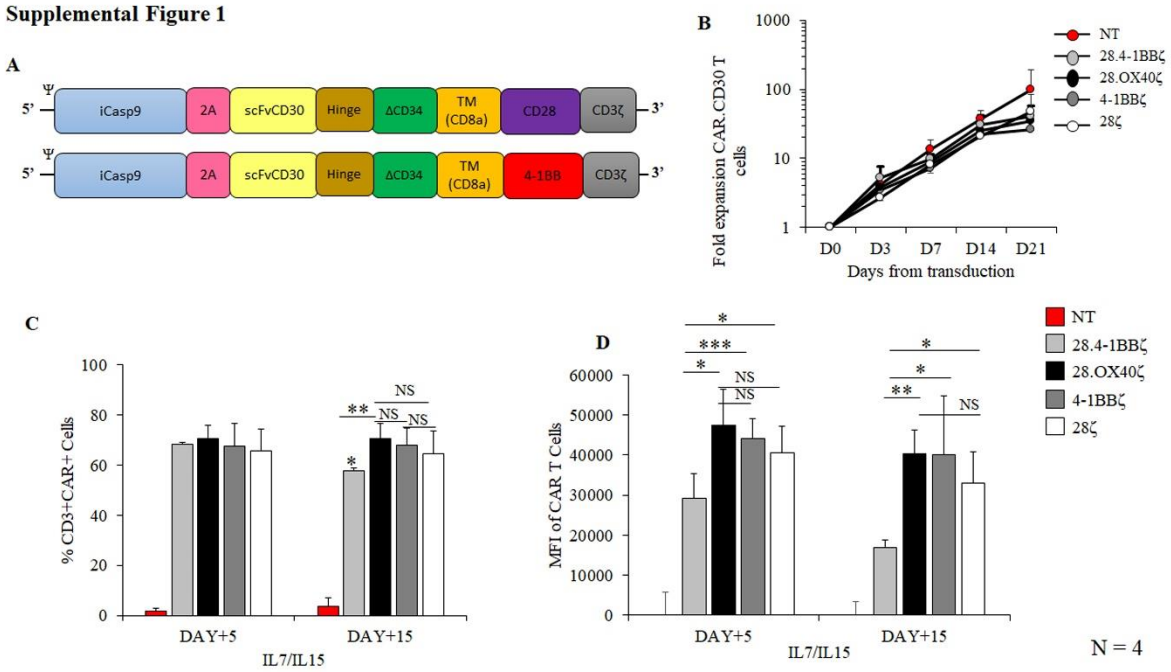
Supplemental Table 1

Region	Sequence
scFv-V_L	DIVLTQSPASLAVSLGQRATISCKASQSVDFDGDSYMNWYQQKPGQPPKVLIYAASNLESGIPA RFSGSGSGTDFTLNIHPVEEEDAATYYCQQSNEDPWTFGGGTKLEIK
scFv-V_H	QIQLQQSGPEVVKPGASVKISCKASGYTFTDYYITWVKQKPGQGLEWIGWIYPGSGNTKYNEK FKGKATLTVDTSSSTAFMQLSSLTSEDTAVYFCANYGNYWFAYWGQGTQVTVSA
CD34 epitope	CDIYIWAPLAGTCGVLLLSLVIT
CD8 hinge-transmembrane domain	PAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA
CD28	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS
4-1BB	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
OX40	RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI
CD3-ζ	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

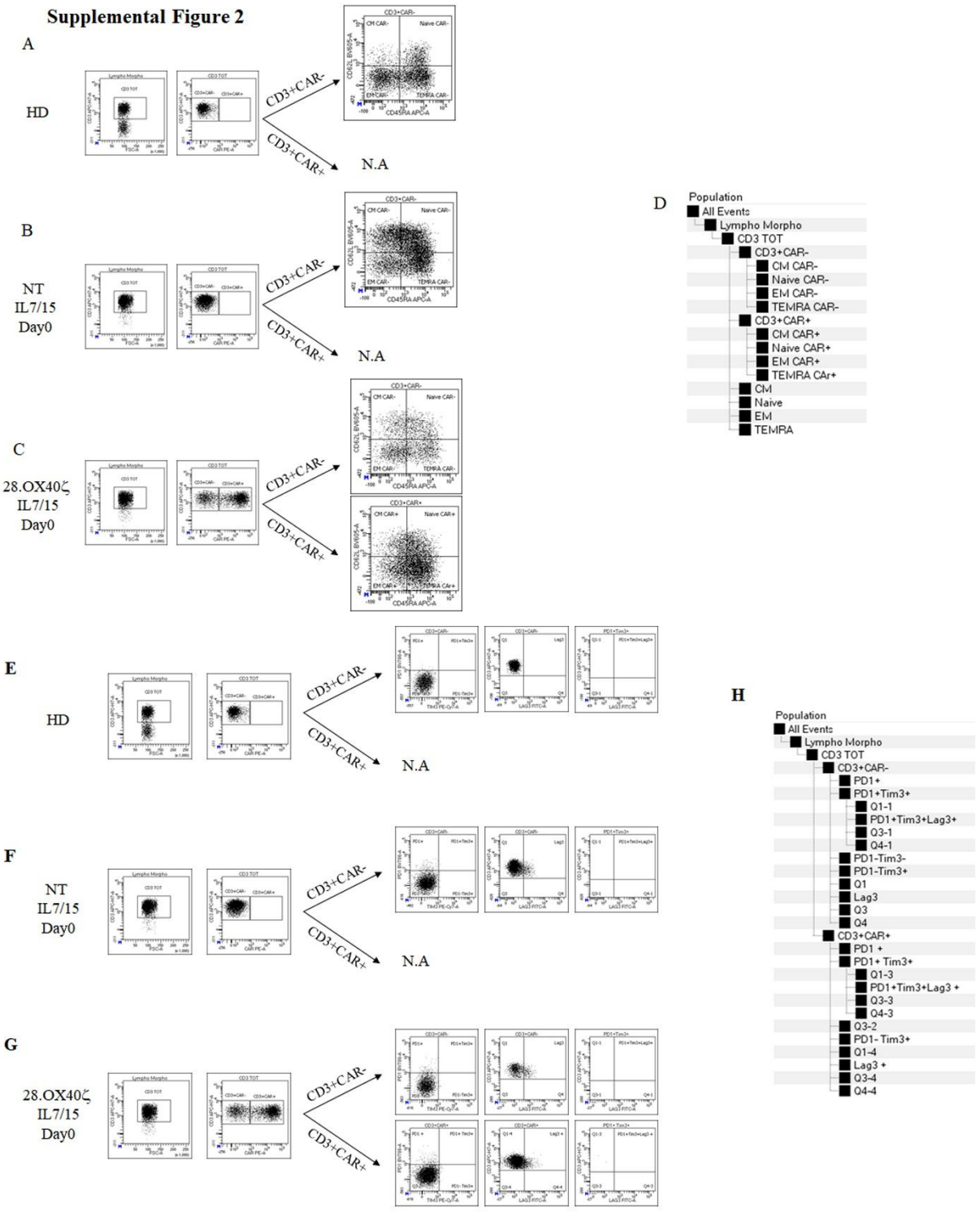
Supplemental Table 1

The illustration shows recombinant protein for ScFv, CD34 epitope, CD8 hinge transmembrane domain and intracellular components.



Supplemental Figure 1: CAR CD30 T-cells exhibit similar transduction level and *in vitro* proliferation upon cytokine stimulation.

(**A**) The panel shows the expression cassette of two second-generation CAR.CD30. The scFv of CD30 was cloned in frame with CD8aTM, CD28 or 4-1BB cytoplasmic moiety, as well as the signalling domain CD3-zeta chain (ζ). As a trackable marker, we added a peptide derived from human CD34 (ΔCD34). An inducible safety switch, namely inducible Caspase-9 (iCasp9), was also included in the vector constructs. (**B**) Fold expansion of NT (red circle), 28.4-1BBζ (light grey circle), 28.OX40ζ (black circle), 4-1BBζ (dark grey circle) and 28.ζ (white circle) grown in IL7/IL15. (**C**) Percentage of CAR+ T-cells and (**D**) Mean Fluorescence Intensity (MFI) of CAR during the time course of *in vitro* culture (day+5 and day+15) in NT (red bars) , 28.4.1BB.ζ (light grey bars) and 28.OX40.ζ (black bars), 4-1BBζ (dark grey bars) and 28.ζ (white bars) grown in IL7/IL15. Data represent results from 4 HDs. * p-value=<0.05, ** p-value=<0.01, *** p-value=<0.001.

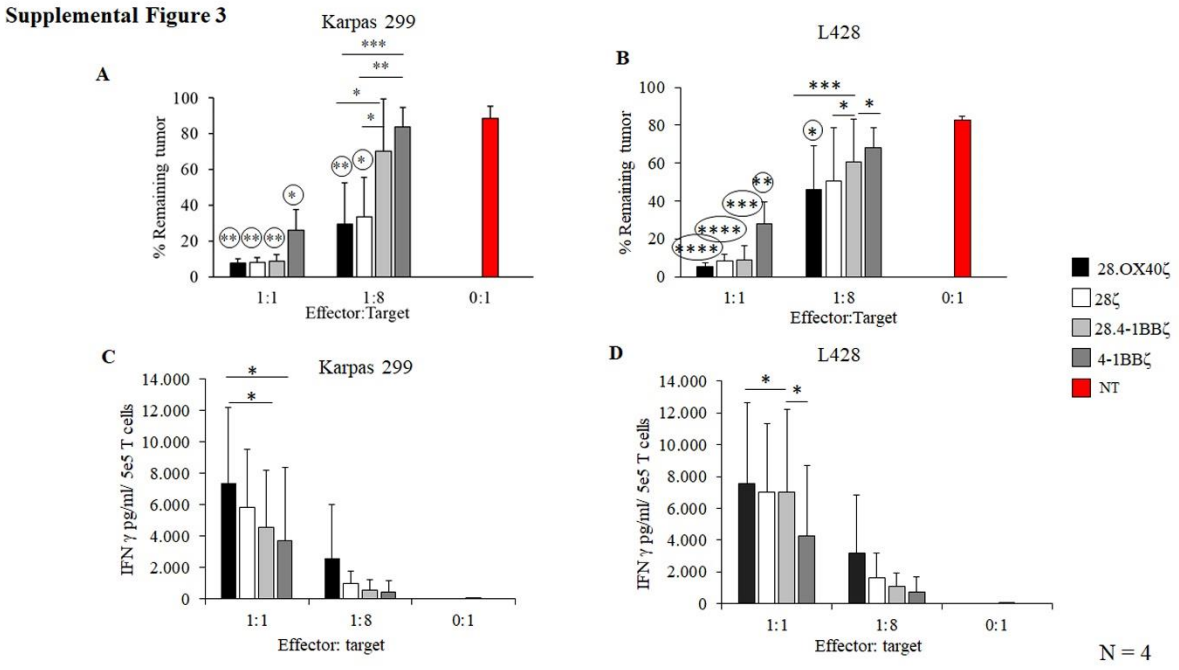


Supplemental Figure 2: Gating Strategy for CAR.CD30 T-cells

Memory profile on T cells was determined based on CD62L and CD45RA expression. We considered the following markers to define: *Naïve* T-cells (CD62L+CD45RA+), Central Memory (CM; CD62L+CD45RA-), Effector Memory (EM; CD62L-CD45RA-), and Terminal Effector cells (TEMRA; CD62L-CD45RA+). A) Gate setting for memory profile was performed on healthy donor samples. B) Gate strategy for the evaluation of memory profile in NT samples after 15 days from

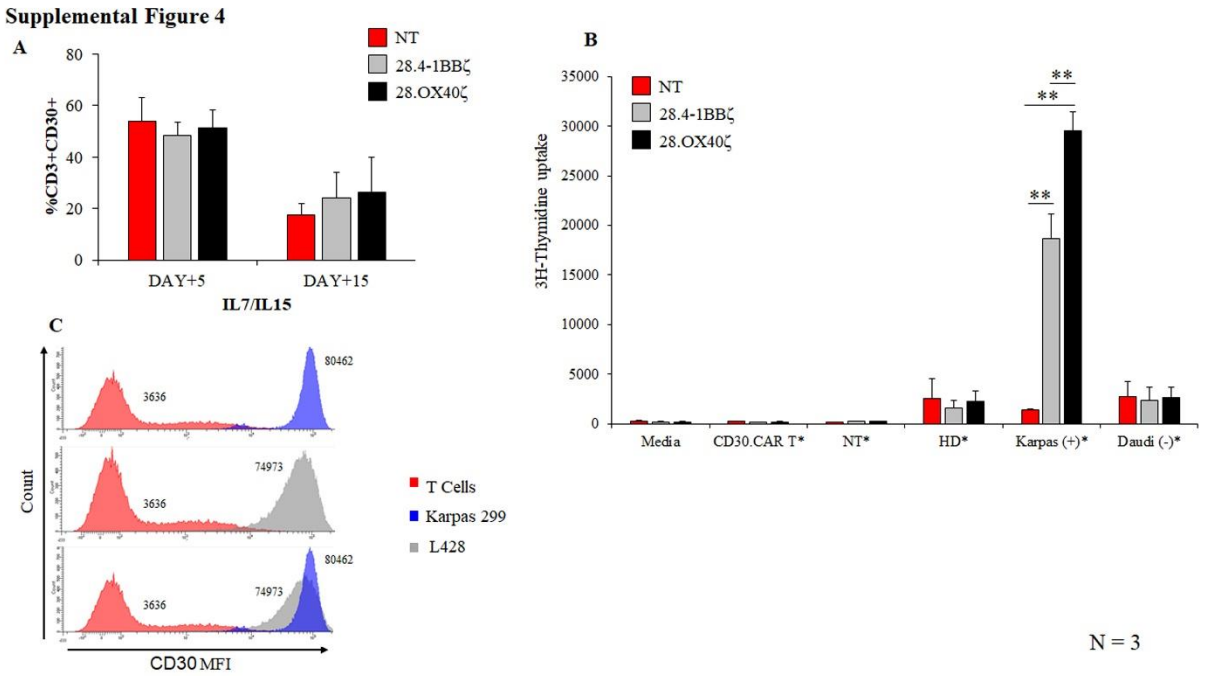
activation, reporting data of CAR negative cells in Figure 4A-B; C) Gate strategy for the evaluation of memory profile in CAR T-cell samples after 15 days from transduction, reporting data of CAR positive cells in Figure 4A-B. D) Gate strategy for the evaluation of memory profile in NT samples (Day 20 of stressed co-culture), reporting data of CAR negative cells in Figure 4B; E) Gate strategy for the evaluation of memory profile in CAR T-cell samples (Day 20 of stressed co-culture), reporting data of CAR positive cells in Figure 4B; F) Statistical hierarchy of the populations.

Exhaustion profiles were determined based on expression PD1, LAG3, TIM3 and triple co-expression on PD1+TIM+3 LAG3+. G) Gate setting for exhaustion profile was performed on healthy donor samples. H) Gate strategy for the evaluation of exhaustion profile in NT samples after 15 days from activation, reporting data of CAR negative cells in Figure 4A-B; I) Gate strategy for the evaluation of exhaustion profile in CAR T-cell samples after 15 days from transduction, reporting data of CAR positive cells in Figure 4A-B. J) Gate strategy for the evaluation of exhaustion profile in NT samples (Day 20 of stressed co-culture), reporting data of CAR negative cells in Figure 4B; K) Gate strategy for the evaluation of exhaustion profile in CAR T samples (Day 20 of stressed co-culture), reporting data of CAR positive cells in Figure 4B; L) Statistical hierarchy of the populations.



Supplemental Figure 3: Both 28.OX40ζ and 28ζ CAR T-cells exert an efficient lymphoma tumor control compared to 28.4-1BBζ and 4-1BBζ CAR T-cells at low effector/target ratios.

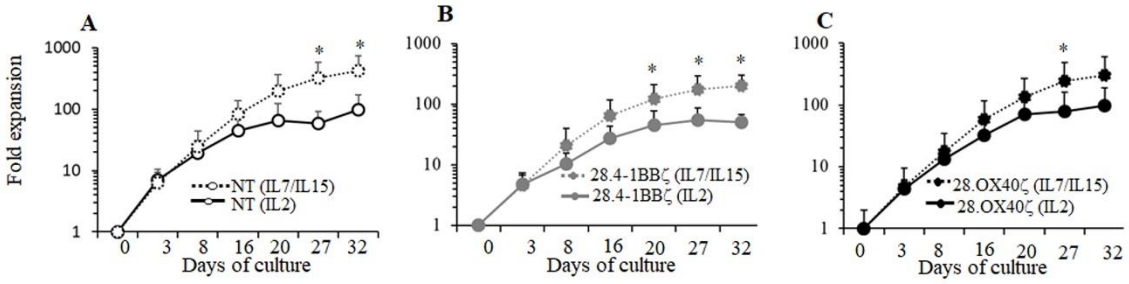
(**A**) Long-term 7-days co-culture assay was performed at effector target ratio of 1:1 and 1:8. In particular, 28.OX40ζ (black bars), 28.ζ (white bars), 28.4-1BB.ζ (light grey bars), 4-1BB.ζ (dark grey bars), and NT (red bars) T-cells were co-cultured with CD30+ Karpas 299 (**A**), L428 (**B**) cell lines. (**C-D**) IFN-γ production after 24h of co-culture was measured. Data from 4 HDs are expressed as average ± SD. * p-value≤0.05; ** p-value≤0.01; *** p-value≤0.001 and ****≤0.0001. Circled asterisks refer to the difference between the CAR T-cell and NT T-cell population.



Supplemental Figure 4: CAR.CD30 T-cells do not exert fratricide activity

(**A**) Percentage of CD30 positive T cells during the time course of *in vitro* culture (day+5 and day+15) in NT (red bars) , 28.4.1BB.ζ (light grey bars) and 28.OX40.ζ (black bars), grown in IL7/IL15. (**B**) Proliferation assay based on 3H-thymidine incorporation of T or III-CAR.CD30 T cells stimulated for five days with irradiated cells (*): CD30.CAR T, NT, PBMCs from Healthy Donor (HD) cells (30 Gy), and tumors: KARPAS 299 and DAUDI cells (45 Gy). CAR.CD30 T cells significantly proliferate only when exposed to CD30+ tumor cells, with minimal proliferation against CD30 negative, Daudi cell line and allogeneic PBMCs (HD), sign of basal alloreactivity. (**C**) The figure shows a figure exemplificative of MFI of CD30 expression in T cells (red) and tumors Karpas 299 (blue) and L428 (grey) presented as overlay. Data represent results from 3 HDs. * p-value=<0.05, ** p-value=<0.01, *** p-value=<0.001.

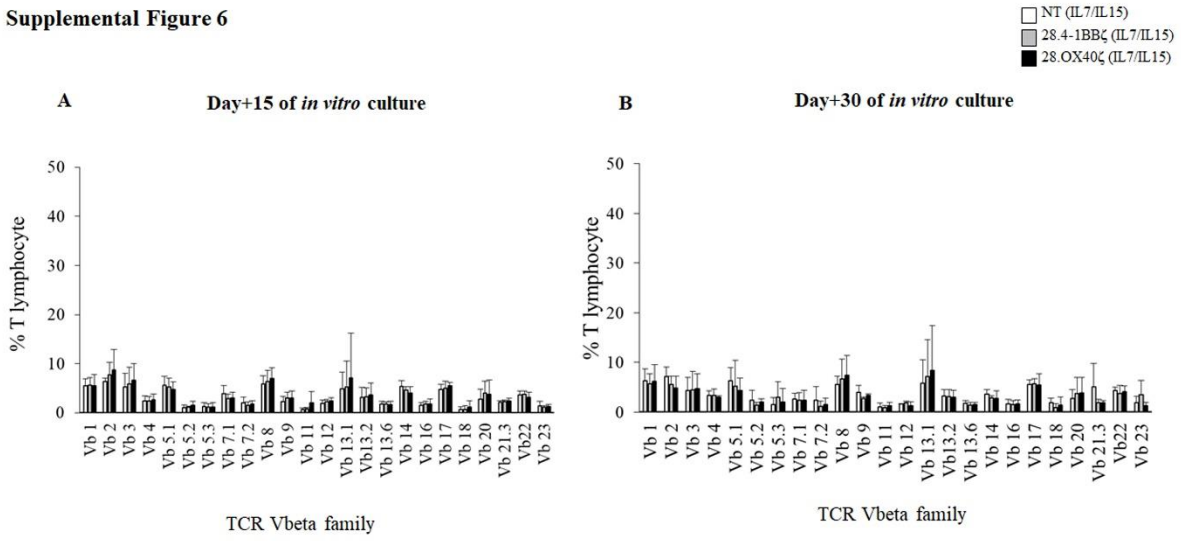
Supplemental Figure 5



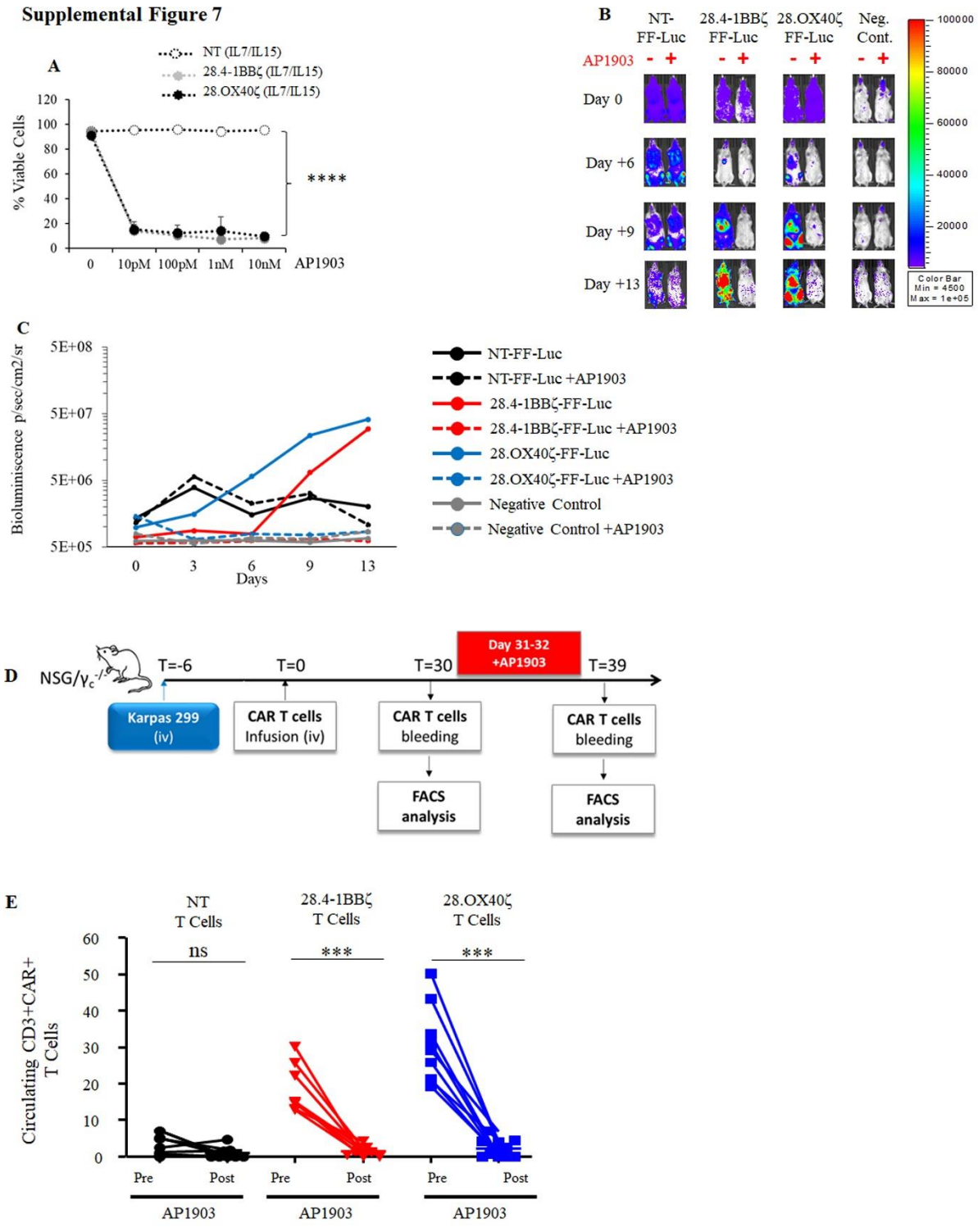
Supplemental Figure 5: Cytokine usage influence kinetic performance of NT and CAR.CD30 T-cells

(**A**) Fold expansion of NT T-cells grown in IL2 (continuous line) or IL7/IL15 (dotted lines). (**B**) Fold expansion of 28.4.1BB.ζ T-cells grown in IL2 (continuous line) or IL7/IL15 (dotted lines). (**C**) Fold expansion of 28.OX40.ζ T-cells grown in IL2 (continuous line) or IL7/IL15 (dotted lines). Data represent results from 7 HDs. * p-value=<0.05, ** p-value=<0.01, *** p-value=<0.001.

Supplemental Figure 6



Supplemental Figure 6: Safety profiles of CAR CD30 T-cells. (**A**-**B**) TCR Vβ repertoire analysis of NT and CAR CD30 T-cells grown in the presence of IL7/IL15 at Day +15 (**A**) and Day +30 (**B**) showing a regular polyclonal Vβ repertoire, without any preferential oligo or monoclonal selection

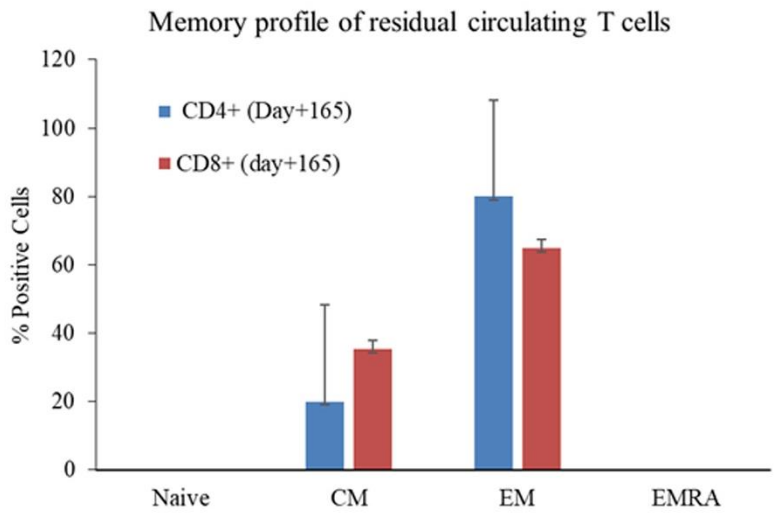


Supplemental Figure 7: Safety profiles of CAR CD30 T-cells.

(**A**) Evaluation of percentage of live (Annexin-V−/7AAD−) T-cells grown in the presence of IL7/15 and exposed to 0-10 nM AP1903 for 72 hours. The percentage of live NT (empty circle), 28.4.1BB.ζ (grey circle) and 28.OX40.ζ (black circle) after AP1903 exposure are shown. Data from four HDs are expressed as average ± SD. ****p-value=<0,0001. (**B**) Karpas 299-bearing NSG mice were infused at Day 0 with Control NT, 28.4.1BB.ζ and 28.OX40.ζ T-cells (two mice/cohort) modified with a

retroviral vector carrying the FireFly Luciferase. At Day +6 and +7, mice were treated *i.p.* with AP1903. The systemic administration of AP1903 allows a reduction of bioluminescence only in the mouse cohort receiving CAR.CD30 T-cells, but not in mice infused with the control NT T-cells (**B-C**). As negative control we treated also control mice (that did not received FireFly Luciferase T-cell treatment) to show the bioluminescence background (≤8,5E+05 p/sec/cm2/sr) **(D)** Illustration showing the *in vivo* xenograft immunodeficient mouse model, in which Karpas 299-eGFP-FFLuc cells were systemically infused in NSG mice (Day -6). Effector cells were infused *i.v.* at the time of tumor establishment (Day 0), as assessed by IVIS Imaging. Mice were divided in three cohort and received injection of T/CAR T-cells. Mice underwent periodical blood collections. One month after infusion, when circulating CAR T-cells were detected, mice were treated *i.p.* for two days consecutively with AP1903 (100mg/mouse) and circulating CAR T-cells were evaluated before and after treatment by FACS analysis. (**E**) The figure shows circulating human CD3+CAR+ T-cells in mice treated with NT (black lines; 10 mice), 28.4-1BBζ (red lines; 10 mice), 28-OX40ζ (blue line; 10 mice) before and 1 week after last AP1903 treatment. *** p-value=<0.001.

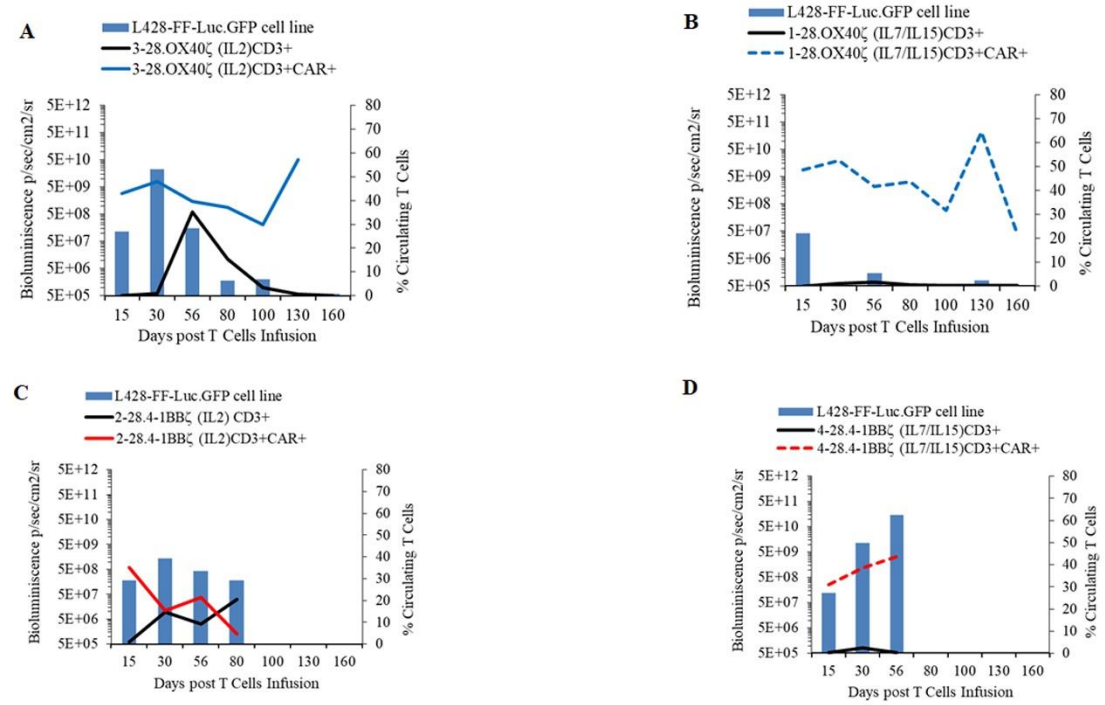
Supplemental Figure 8



Supplementary Figure 8: Memory profile of residual circulating T-cells in xenograft mice treated with 28.OX40.ζ (IL7/IL15) T-cells.

Analysis of circulating human CD4+ and CD8+ CAR+ T-cells in their memory subset for mice treated with 28.OX40.ζ T-cells at Day +165.

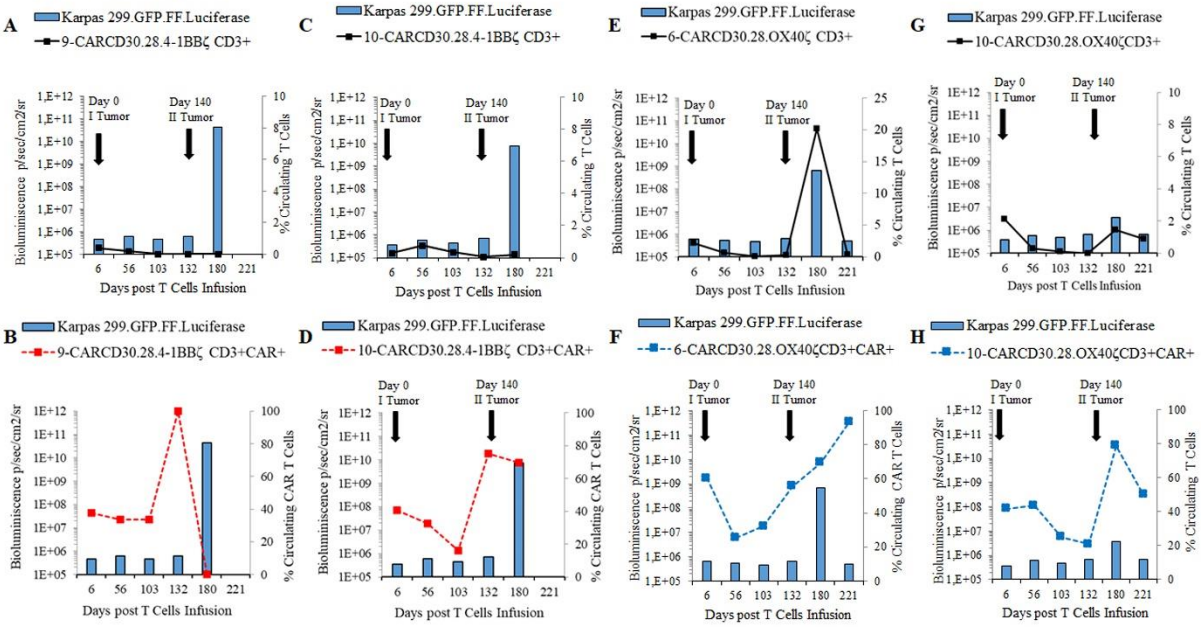
Supplemental Figure 9



Supplementary Figure 9: Correlation of kinetics of tumor control and CAR T-cell expansion in immune-deficient HL xenograft mouse model.

(A-D) Plots showing bioluminescence data (blue bars), circulating human CD3+ (black lines) and circulating CAR+ T-cells (28.OX40.ζ IL2, continuous blue line; 28.OX40.ζ IL7/IL15, dotted blue line; 28.4-1BB.ζ IL2, continuous red line and 28.4-1BB.ζ IL7/IL15, dotted red line). Figure shows one exemplificative mouse for each cohort.

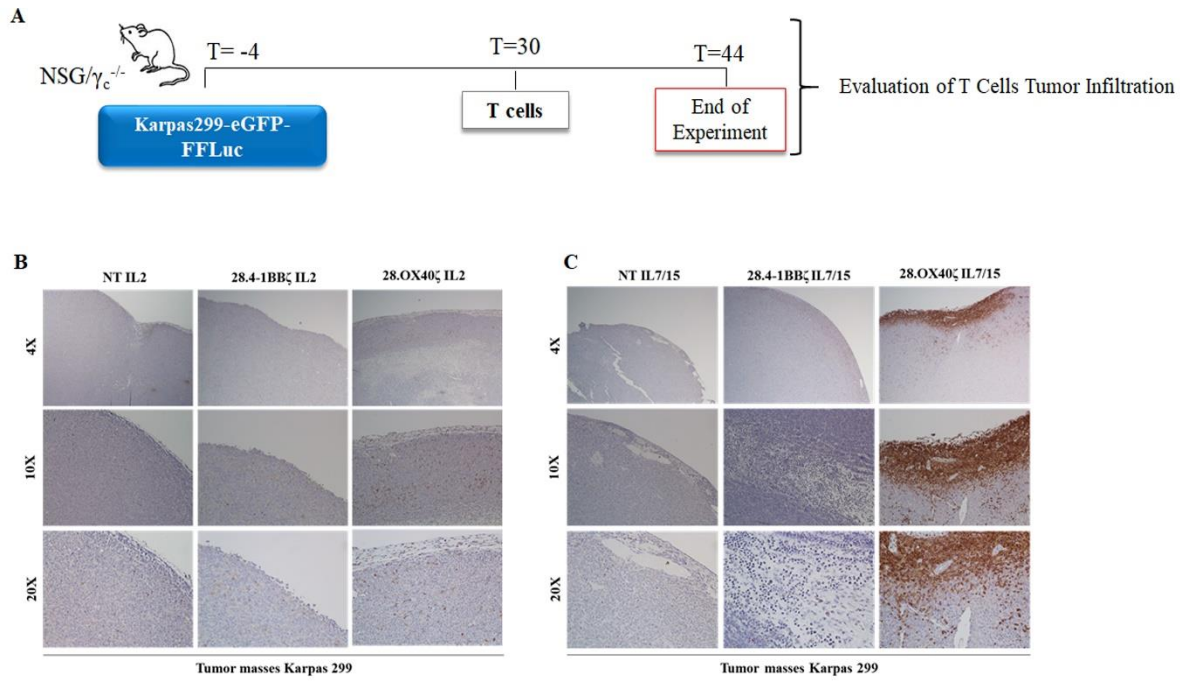
Supplemental Figure 10



Supplementary Figure 10: Correlation of the kinetics of tumor control and CAR T-cell expansion in immune-deficient NHL xenograft mouse in a tumor re-challenge model.

(A-H) Plots showing bioluminescence data (blue bars), circulating human CD3+ (black lines) and circulating CAR+ T-cells (dotted lines) of mice treated with 28.4-1BB.ζ (red lines) or 28.OX40.ζ (blue lines). Panels show two representative mice for each cohort.

Supplemental Figure 11



Supplementary Figure 11: CAR.CD30 T-cells infiltrating tumor masses in *in vivo* NHL mice model.

(**A**) Illustration showing the schematic model of the *in vivo* experiment to evaluate the penetration and proliferative capacity of CAR.CD30 T-cells. Mice received *i.v.* infusion of Karpas 299-eGFP-FFLuc cells and at Day +30, were treated with effector cells and sacrificed at Day +44. Tumor masses were excised and T-cell infiltrates were analyzed by IHC. (**B-C**) Slides of one representative mouse for each cohort are shown at 4x, 10x and 20x magnification.