

Supplementary information for the article entitled
Enhancing the iNKT cell immunotherapy platform by combining optimised
CAR endodomains with novel iNKT engagers

Kanagaraju Ponnusamy^{1,2*}✉, Klesti Karaxhuku^{1,2*}, Yuchao Jiang¹, Lyra Randzavola^{2,3}, Hongwei Ren², Ilia Leontari^{1,2}, Bryan Lye², Mehmood Zaidi^{1,2}, Edward J Bartlett⁴, Edward W Tate⁴, Vasileios Pardalis², Dimitrios Leonardos^{1,5}, Reza Nadafi⁶, Irene Sarkar⁶, Rogier M Reijmers⁶, Marco Bua⁷, Maria Atta⁷, Alexia Katsarou^{1,7}, Irene AG Roberts^{8,9}, Aristeidis Chaidos^{1,2,7}, Anastasios Karadimitris^{1,2,7}✉

¹Hugh & Josseline Langmuir Centre for Myeloma Research Centre for Haematology, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

²Centre for Haematology, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

³Centre for Inflammatory Disease, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

⁴Department of Chemistry, Natural Sciences, Imperial College London, London, United Kingdom.

⁵University of Ioannina Medical School, Ioannina, Greece.

⁶Lumicks, Amsterdam, Netherlands.

⁷Department of Haematology, Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom.

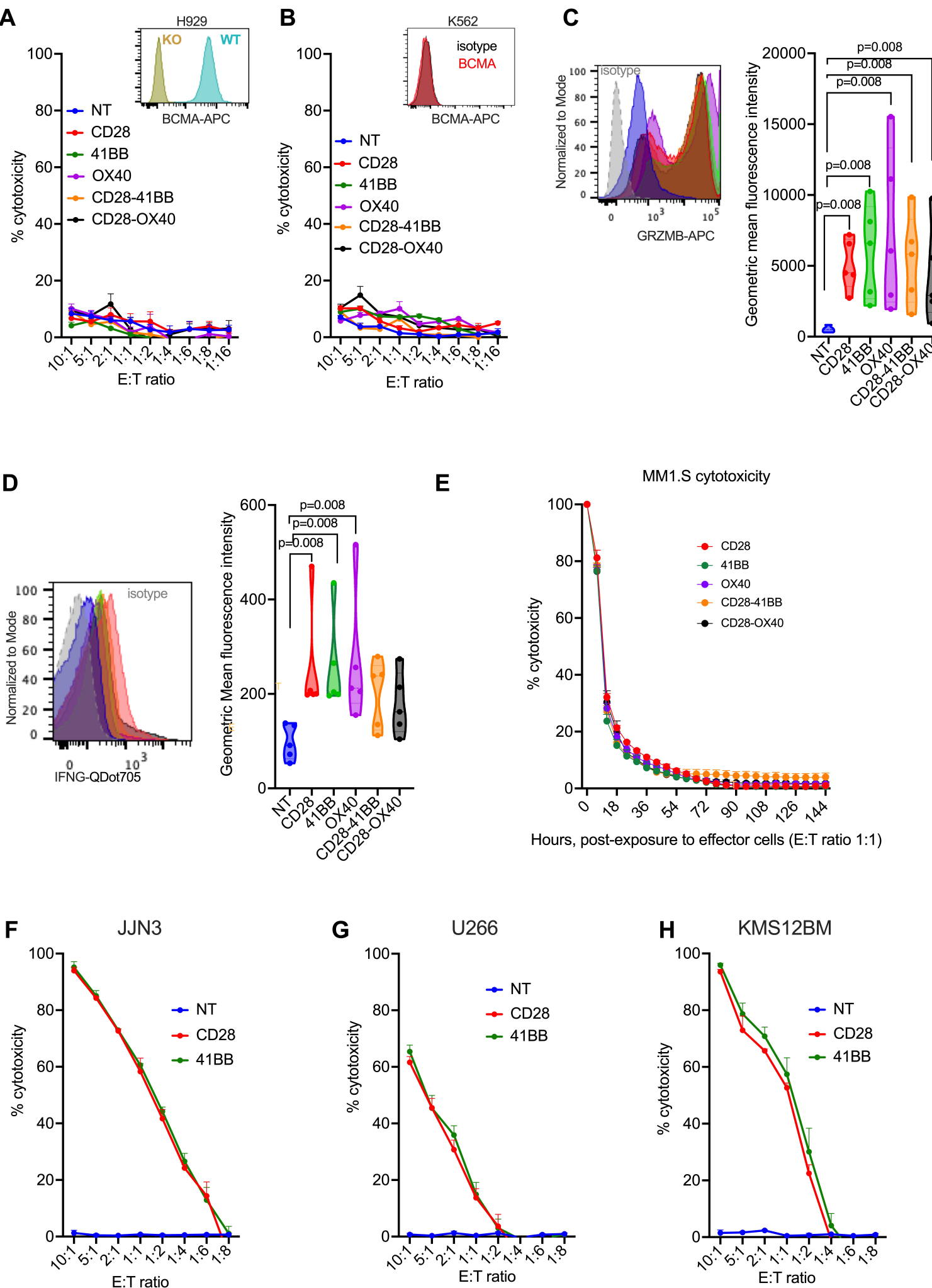
⁸Department of Paediatrics, University of Oxford, Oxford, United Kingdom & ⁹Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom.

Correspondence

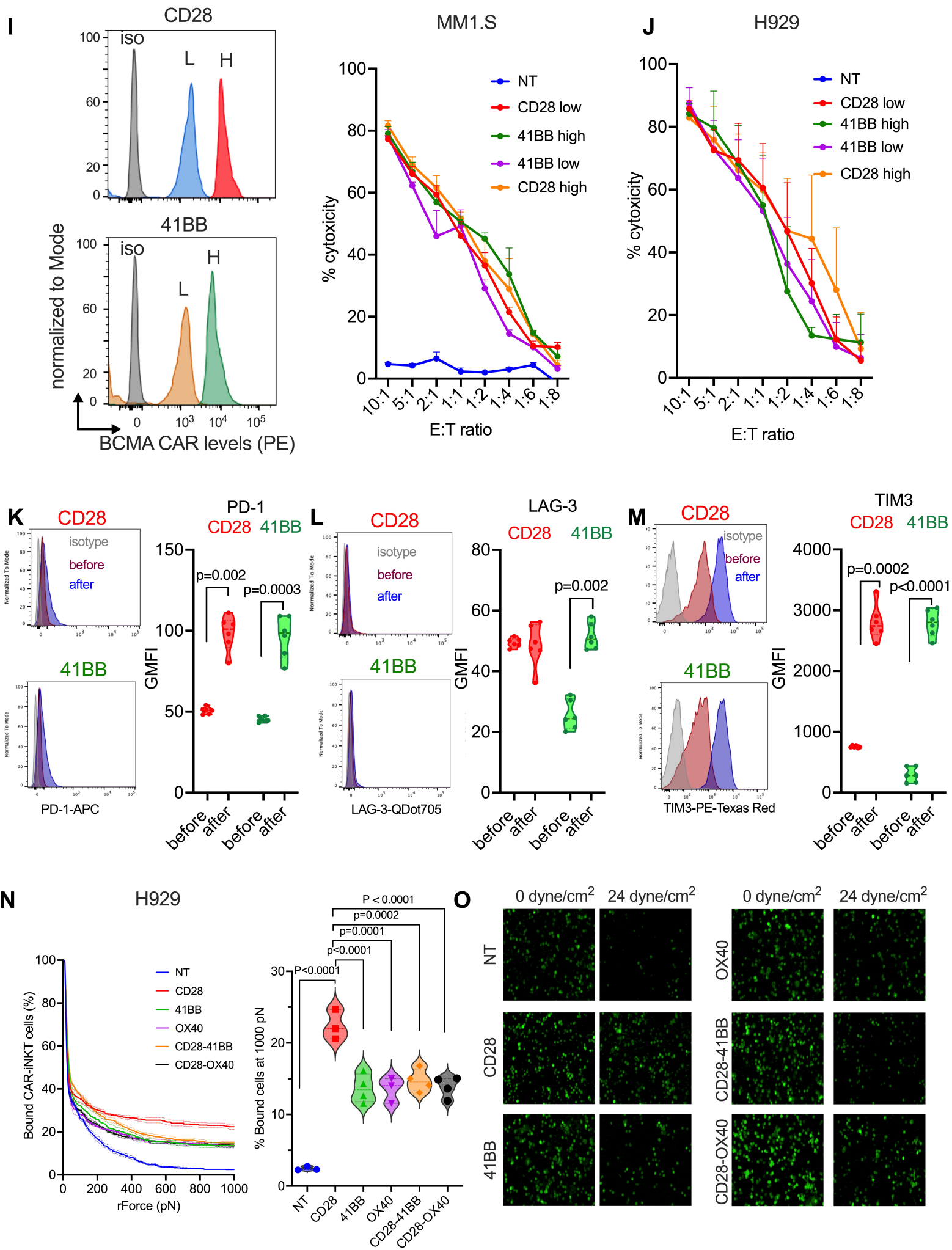
Anastasios Karadimitris a.karadimitris@imperial.ac.uk

Kanagaraju Ponnusamy k.ponnusamy@imperial.ac.uk

Suppl Fig. 1



Suppl Fig. 1



Supplementary Figure 1 (related to Figure 1)

A. BCMA expression in BCMA WT and BCMA KO H929 cells shown in histogram and their cytotoxicity by CAR iNKT cells at different E:T ratio. (n=4 donors).

B. BCMA expression in K562 erythromyeloid cells shown in histogram and CAR iNKT cell-mediated cytotoxicity against the same cells. (n=3 donors).

C. Left: Intracellular expression of granzyme B levels in CAR-iNKT measured by flow cytometry after exposing cells to the MM1.S myeloma cells for 16hours. Right: cumulative data from four different donors.

D. Left: Intracellular expression of interferon gamma levels in CAR-iNKT measured by flow cytometry after exposing the cells to the MM1.S myeloma cells for 16hours. Right: cumulative data from four different donors.

E. Cytotoxicity of MM1.S cells, that express tdTomato fluorescence marker, by BCMA CAR iNKT cells with different co-stimulatory molecules assessed by real-time Incucyte® imaging. The cytotoxicity levels determined by tdTomato fluorescence signals and normalized to hour 0 (n=duplicates of 3 donors).

F-H. Comparison of cytotoxic activity of CD28z and 41BBz CAR iNKT cells against JJN3 (F), U266 (G) and KMS12BM (H) myeloma cells is shown. (n=3 donors)

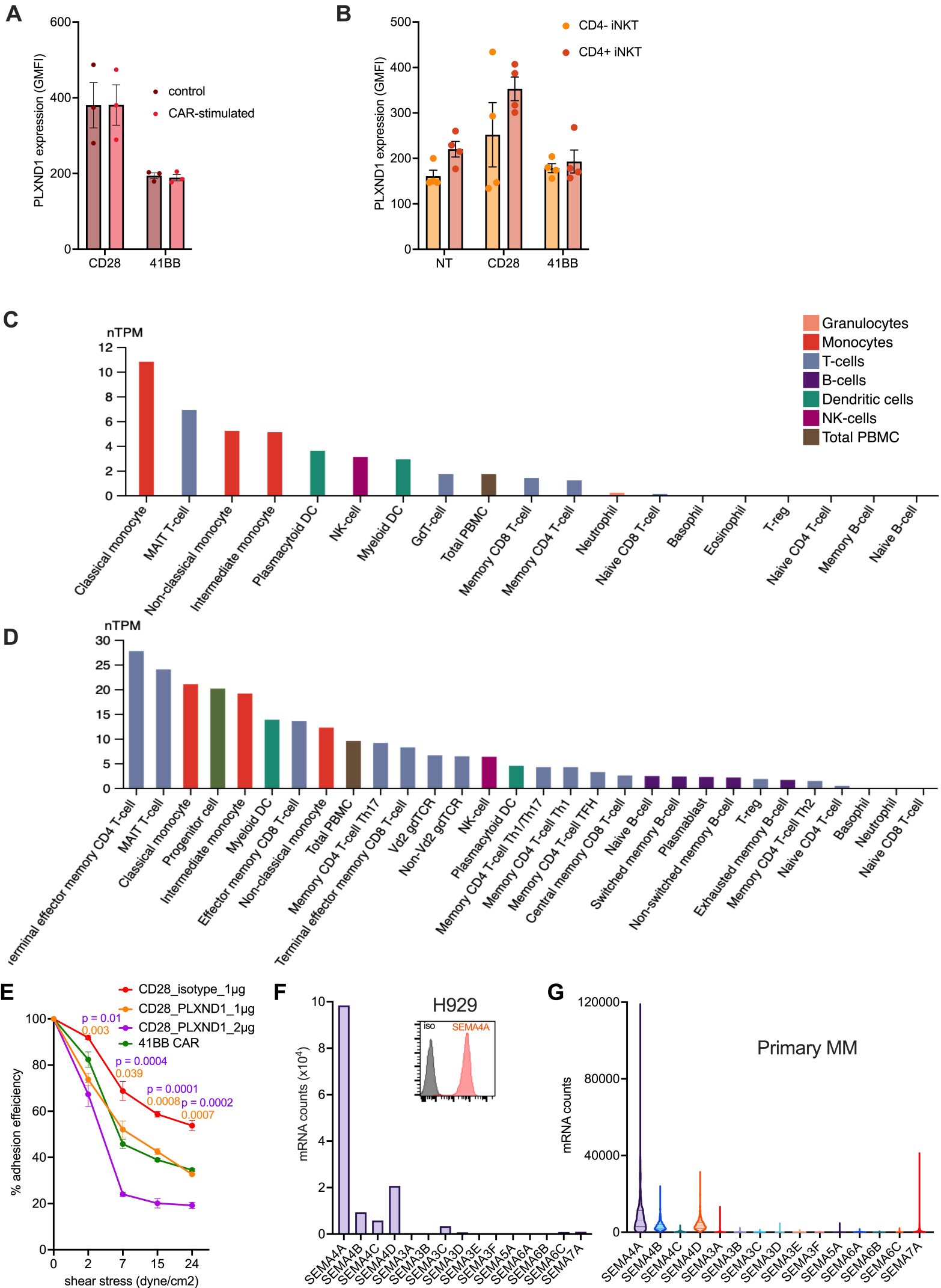
I-J. After CD28z and 41BBz BCMA CAR iNKT cells were FACS-sorted for low (L) and high (H) levels of CAR expression (I); their cytotoxic activity against MM1.S cells (I) and H929 cells (J) was tested. iso-isotype, L-low BCMA CAR iNKT, H-high BCMA CAR iNKT, NT-non-transduced iNKT cells. (n=3 donors).

K-M. PD-1 expression levels (K), LAG-3 (L) and TIM-3 (M) expression levels before exposing to target cells MM1.S and after exposing for 4 challenges between CD28z and 41BBz CAR iNKT cells. (n=duplicates of 3 donors). The p value calculated by the mean of each duplicates.

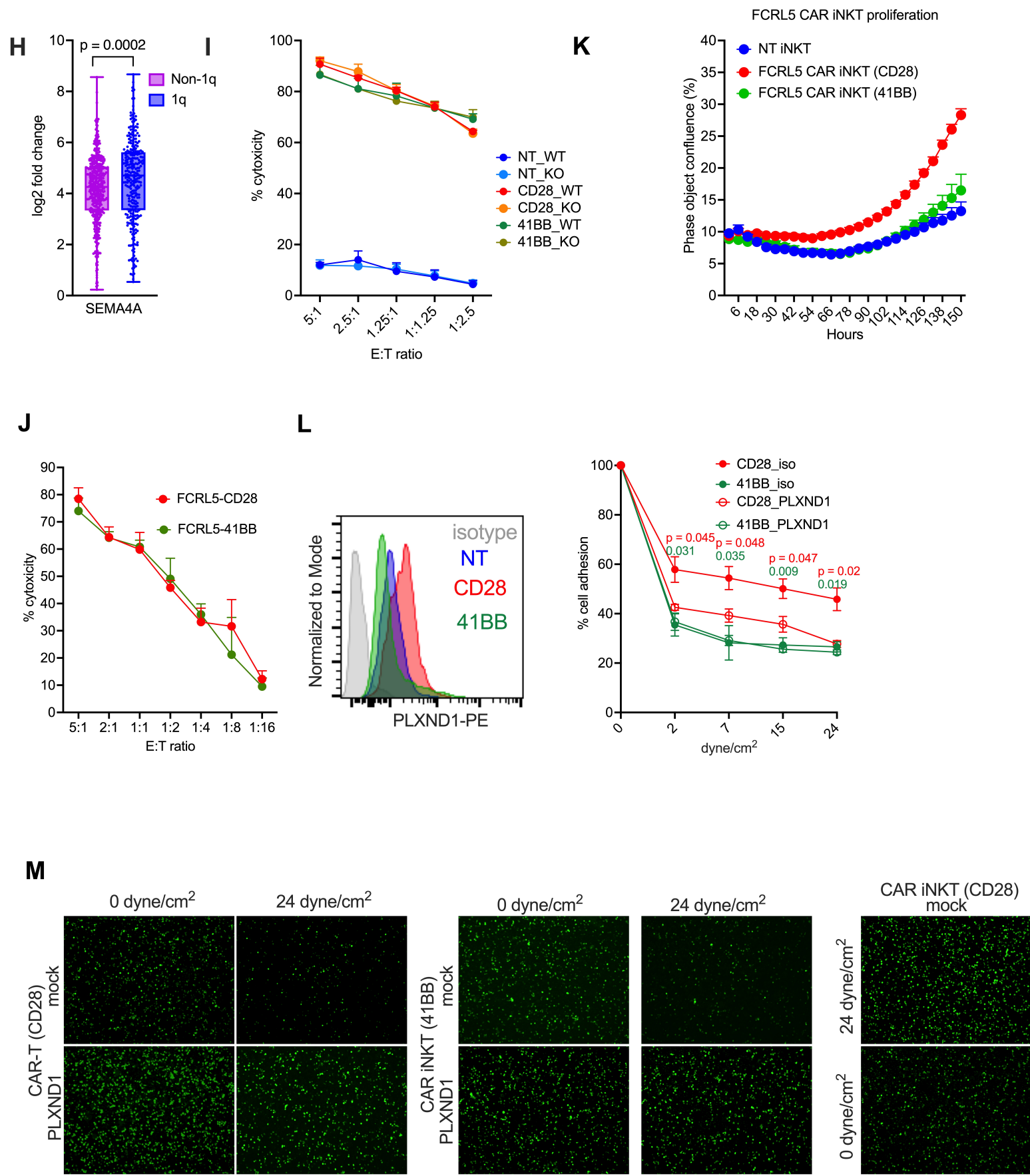
N. Left: CD28 CAR iNKT cell binding activity on H929 myeloma cells under increasing levels of acoustic force as compared to all other CAR- and NT iNKT; right: CAR-iNKT binding at rForce 1000 pN (data representative of n=2 donors).

O. Representative images of cell binding before shear stress (0 dyne/cm²) and after 24 dyne/cm² which are related to Figure 1K. Two-tailed statistical analysis Mann-Whitney U test (C & D), unpaired t-test (K-M) and ordinary one-way Anova (N) performed. All error bars show Mean±SEM, and box and whiskers show minimum to maximum values with all data points. p <0.0001 is referred as p < 0.0001. non significance measured by p > 0.05.

Suppl Fig. 2



Suppl Fig. 2



Supplementary Figure 2 (related Figure 2)

A. PLXND1 expression in CD28z CAR iNKT cells as assessed by flow cytometry after stimulation with MM1.S myeloma cells for 24hr (n=3).

B. PLXND1 expression in CD4+ and CD4- fractions of iNKT cells as assessed by flow cytometry shown as GMFI (n=4).

C-D. Expression of PLXND1 in subpopulations of immune cells. Data derived from Human Protein Atlas database^{46,47}.

E. Dose-dependent reduction of avidity of BCMA CAR iNKT with CD28z co-stimulatory molecules by PLXND1 blocking antibody. BCMA CAR with 41BBz shown as control for 2 donors.

F. SEMA4A mRNA count values and its family members in H929 cells (CCLE Dataset). The histogram shows SEMA4A expression on the surface of H929 cells.

G. Expression of SEMA4A mRNA counts and its family members in 770 primary myeloma cells from the MMRF CoMMpass study.

H. SEMA4A expression is higher in Chr 1q+ primary MM cells as compared to non-1q MM cells (MMRF CoMMpass Dataset).

I. No difference in cytotoxicity by BCMA CAR iNKT cells observed against SEMA4+ MM1.S (WT) and SEMA4- MM1.S (KO) cells (n=3 donors).

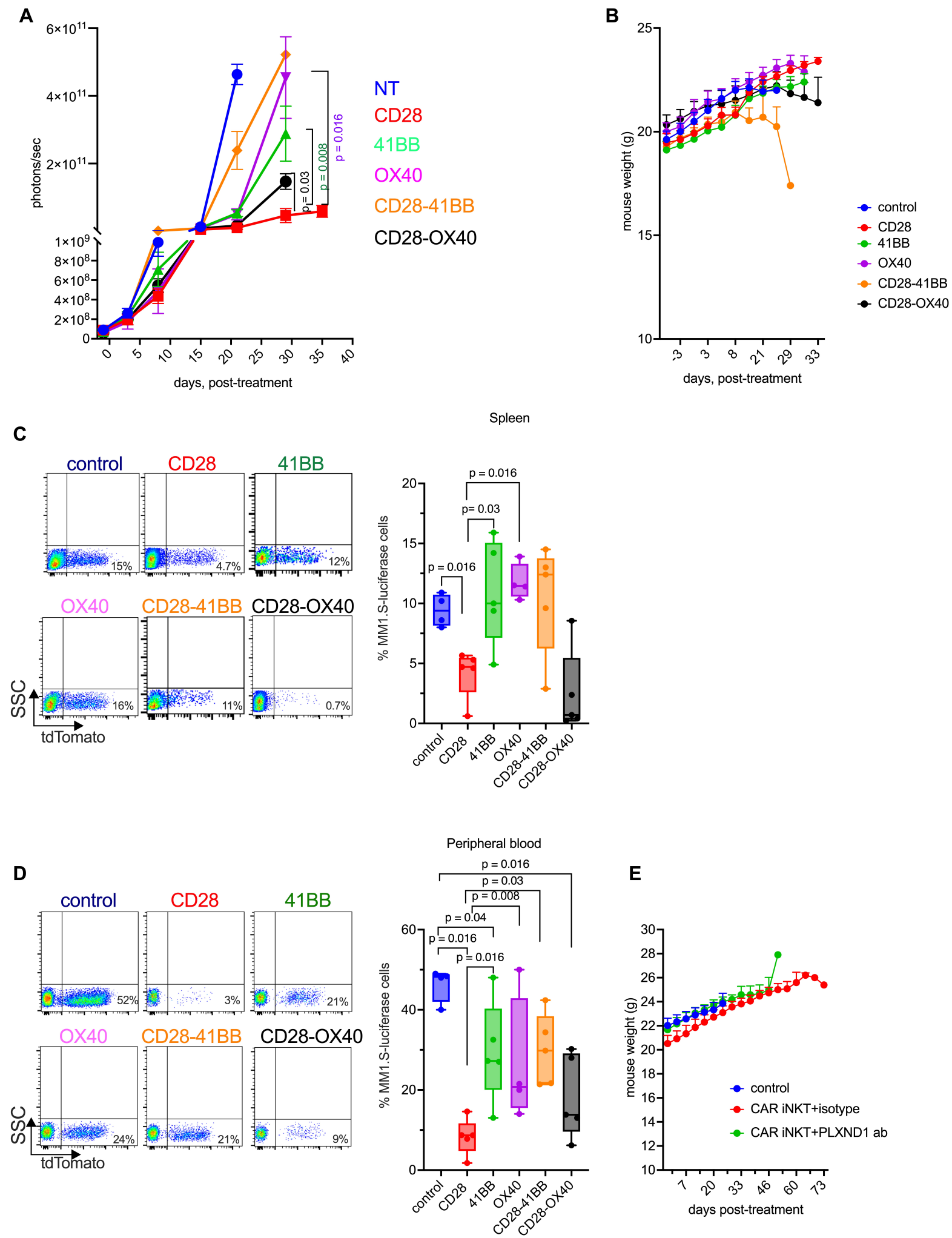
J. No difference in cytotoxic activity between FCRL5-CD28z and FCRL5-41BBz CAR iNKT cells against MM1.S-FCRL5+ target cells.

K. FCRL5-CD28z CAR iNKT cells shows higher proliferative potential than its 41BBz counterpart and NT iNKT cells in short-term proliferation assay in the presence of IL-15 as assessed by real-time Incucyte® imaging (n=3 donors). Unpaired t test shows $p < 0.001$ for CD28 compared to NT and 41BB from 60hr.

L. Left: Flow cytometry analysis of Plexin D1 expression in FCRL5 CAR iNKT cells with CD28z or 41BBz costimulatory molecules as compared to NT iNKT cells. Right: cell avidity of FCRL5-CD28z and FCRL5-41BBz CAR iNKT cells on MM1.S-FCRL5+ cells under shear stress with isotype control or PLXND1-blocking antibody at 1µg/ml concentration (n=3).

M. Representative images of indicated effectors bound to MM1.S myeloma cells at 0 and 24 dyne/cm² which are related Figure 2I. Two-tailed statistical analysis Mann-Whitney U test (B, H) and unpaired t test (E & L) performed. All error bars show Mean±SEM, and box and whiskers show minimum to maximum values with all data points. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Suppl Fig. 3



Supplementary Figure 3 (related Figure 3)

A. Cumulative photon strength of total body in animals treated with BCMA CAR iNKT with different co-stimulatory domains as also shown in Figure 3C (n=4-5 mice per group).

D. Body weights of the mice treated with CAR iNKT cells shown in Figure 3A-H (n=4-5 mice per group).

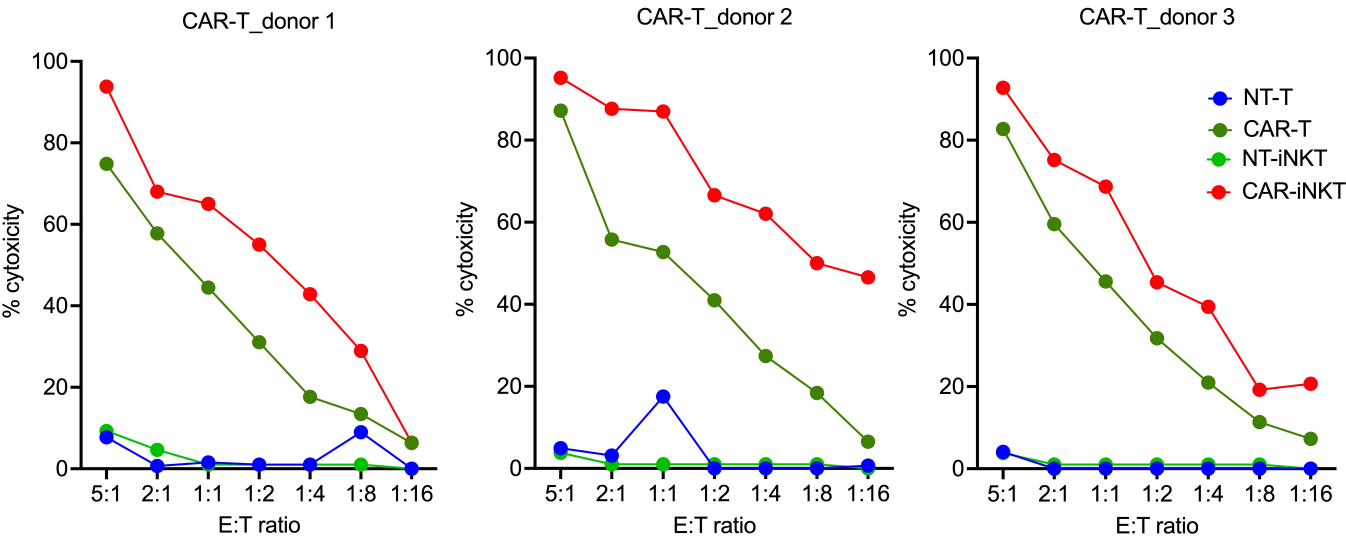
C-D. Frequency of MM1.S-luci tumor cells in the spleen (C), peripheral blood at end-point (D) (n=4-5 mice per group) as measured by tdTomato fluorescence marker using flow cytometry.

E. Body weights of the mice treated with CAR iNKT cells shown in Figure 3I-K (n=4-6 mice per group).

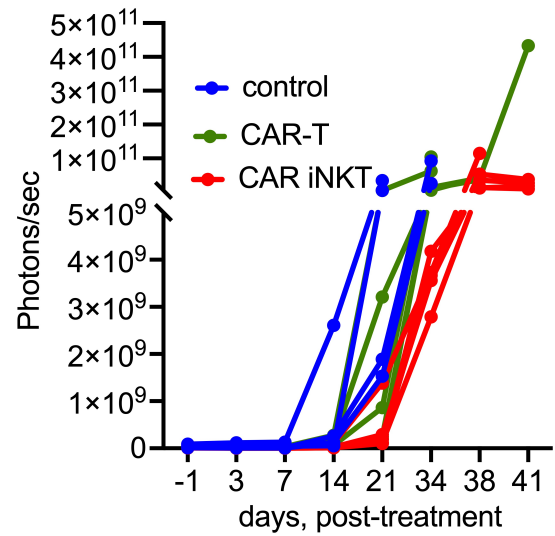
Two-tailed statistical analysis Mann-Whitney U test (A, C & D) performed. All error bars show Mean $\pm$ SEM, and box and whiskers show minimum to maximum values with all data points. p < 0.0001 is referred as p < 0.0001. non significance measured by p > 0.05.

Suppl Fig. 4

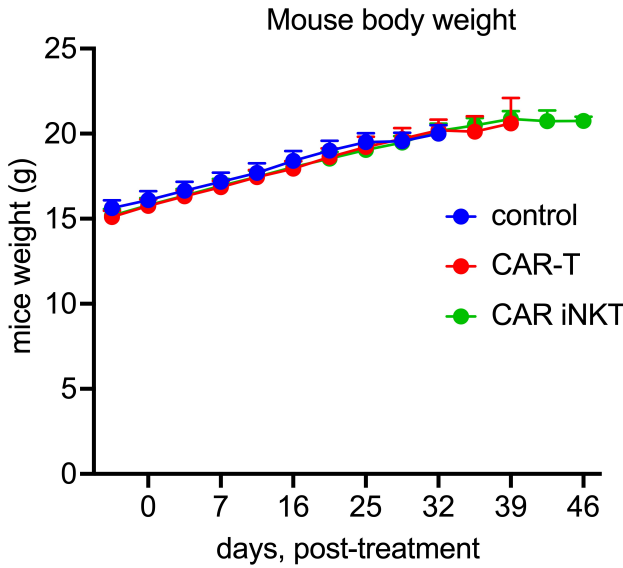
A



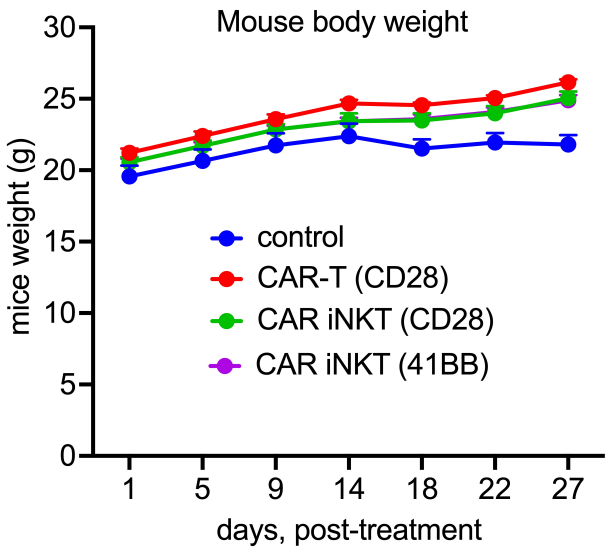
B



C



D



Supplementary Figure 4 (related for Figure 4)

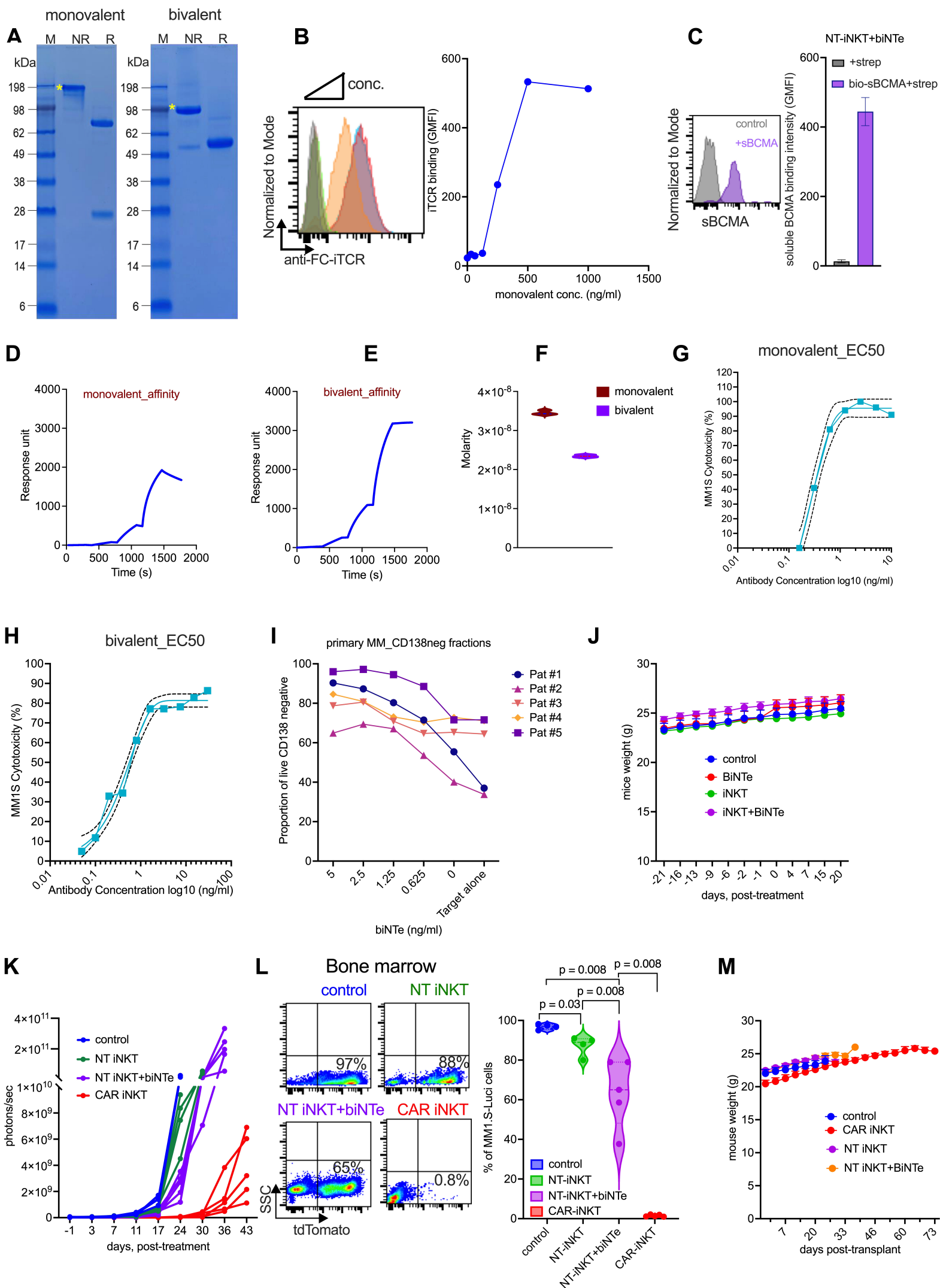
A. Cytotoxic potential of same donor BCMA-CD28z CAR iNKT and BCMA-CD28z CAR-T from 3 different donors against MM1.S cells.

B. Cumulative total body BLI signals shown for figure 4D.

C&D. Longitudinal measurement of weight of mice described in Figure 4A-G (C) and 4H-J (D).

All figure's error bars show Mean $\pm$ SEM. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Suppl Fig. 5



Supplementary Figure 5 (related to Figure 5)

A. SDS-PAGE gel analysis of purity of monovalent (left) and bivalent biNTE (right) under reducing (R) and non-reducing (NR). M indicates marker. * Indicates the band size ~198.9 kDa for monovalent and ~78.9 kDa for bivalent biNTE under non-reducing conditions.

B. Increasing levels of binding efficiency at increasing level of antibody concentration (0-1 μ g) for monovalent antibody to iNKT cells. Concentration range (0-1 μ g/ml).

C. Flow-cytometric analysis of pure iNKT cells after staining with bivalent biNTE and fluorescently labelled streptavidin (control: grey) or with bivalent biNTE, biotinylated soluble BCMA and fluorescently labelled streptavidin (purple). Representative histogram (left) and its cumulative data (right) shown as GMFI. (n=3 donors).

D-F. Affinity strength of monovalent (D), bivalent (E) antibodies on soluble BCMA against indicated time and their k_d values (F) as determined in SPR assay, (n=3).

G&H. EC50 values shown for monovalent (G) and bivalent engagers (H) against MM1.S by co-culture of NT iNKT at 1:1 E:T ratio plus indicated engagers. EC50 values were calculated by using non-linear fit, log(agonist) vs. response function in prism 10 software.

I. CD138 negative fractions of primary myeloma cells after incubating with NT iNKT cells and biNTE as part of the CD138+ fractions analysis shown in Figure 5G.

J. Body weight measurement for mice shown in Figure 5H.

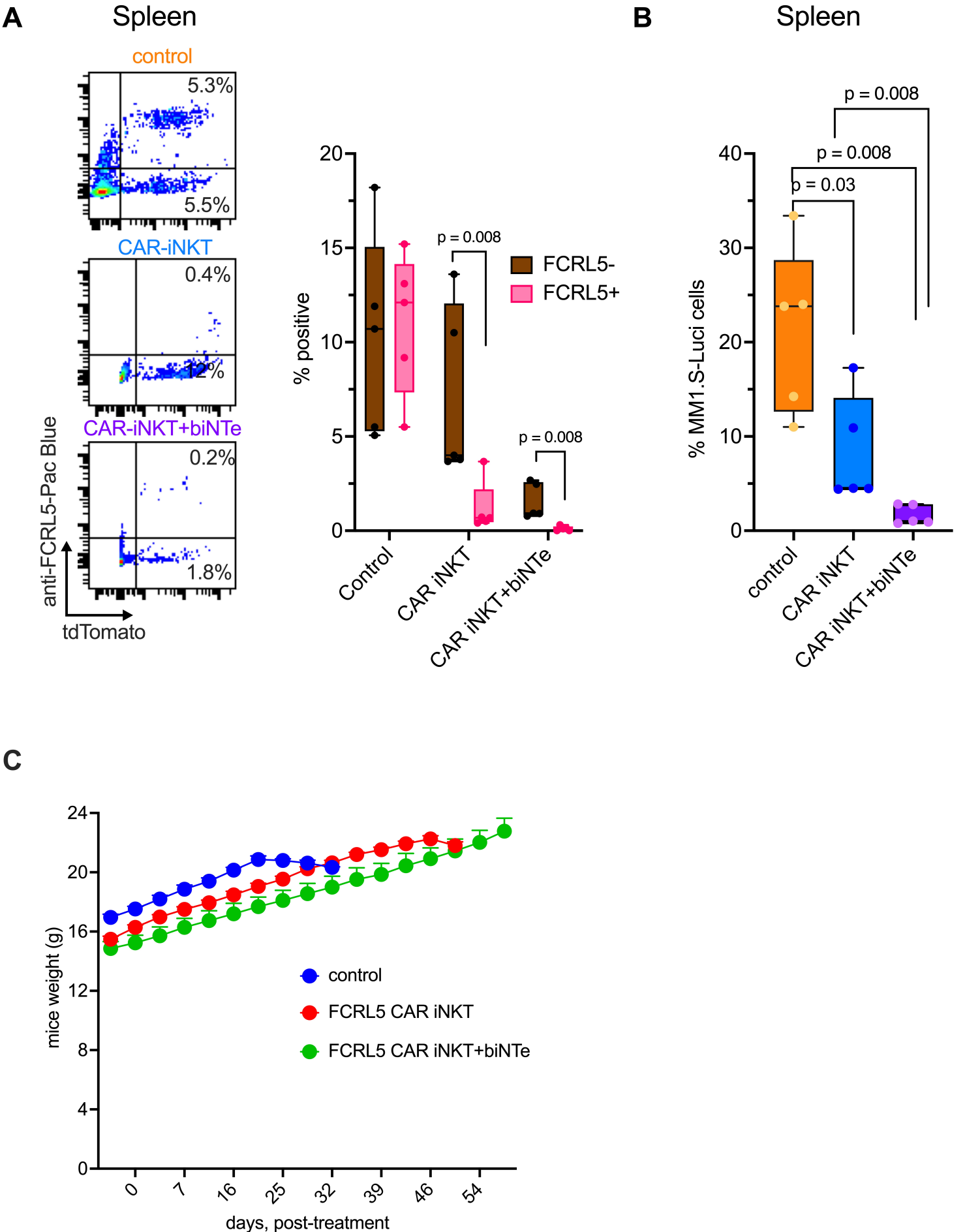
K. Cumulative BLI data for mice treated with NT iNKT and/or biNTE, and CAR iNKT and control group as shown in Figure 5J-L. (n=4-5 mice per group).

L. Flow cytometric analysis for frequency of MM1.S cells in the bone marrow from each group at end point. Left: representative flow cytometry plots; right: cumulative data. (n=4-5 mice per group).

M. Body weight measurement for mice shown in Figure 5J-L.

Two-tailed statistical analysis Mann-Whitney U test (L) performed. All error bars show Mean $\pm$ SEM. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Suppl Fig. 6



Supplementary Figure 6 (related to Figure 6)

A. Flow cytometry analysis of frequency of MM1.S_FCRL5⁻ and MM1.S-FCRL5⁺ cells in the spleen of treated and control mice at the end point shown as dot plot (left) and cumulative data for each group (right). (n=4-5 mice per group).

B. Flow cytometry analysis of total MM1.S myeloma burden as determined by tdTomato fluorescence marker in the spleen of treated and control mice at the end point. (n=4-5 mice per group).

C. Longitudinal weight measurement of animals from Figure 6B-H.

Two-tailed statistical analysis Mann-Whitney U test (A & B) performed. All error bars show Mean±SEM, and box and whiskers show minimum to maximum values with all data points.

All figure's error bars show Mean± SEM, and all the box and whiskers show minimum to maximum values with all the data points. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.