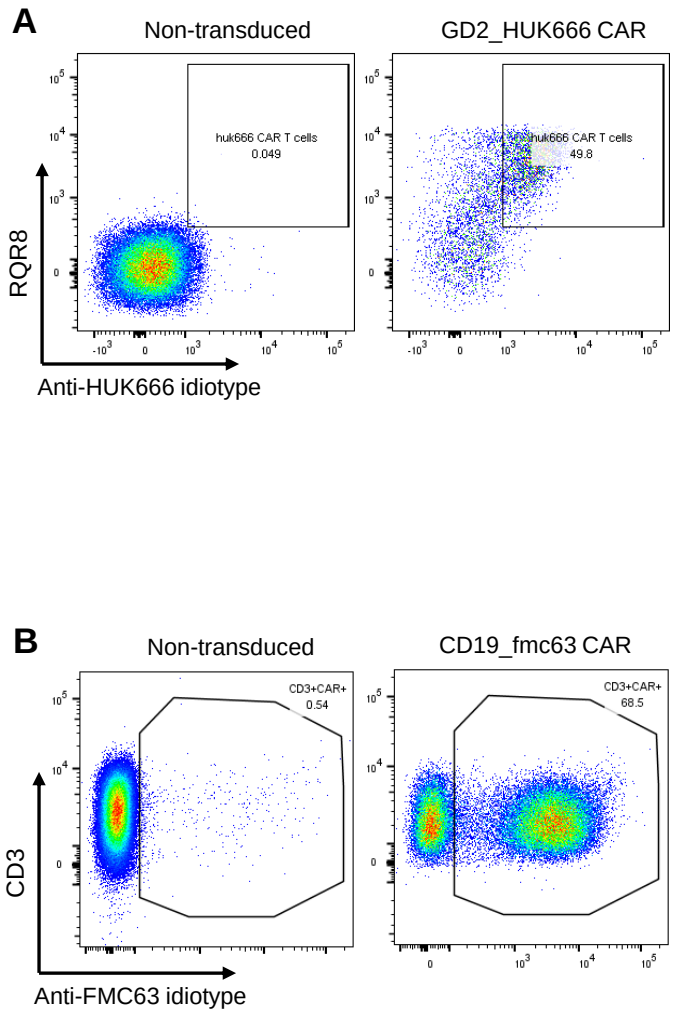
Supplementary Figures for Righi et al.

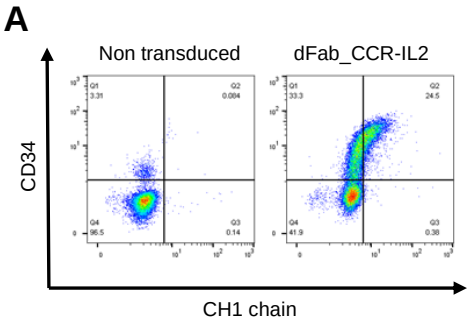
SUPPLEMENTARY FIGURE S1



Supplementary Figure S1. Anti-HUK666 scFv and anti-FMC63 scFv idiotype characterisation.

A) Non transduced and GD2_HUK666-scFv CAR T-cells were stained with anti CD34 and anti-HUK666-scFv idiotype-AF488 (20ug/mL). The anti-idiotype has been labelled with AF488 using the ThermoFisher Kit (Cat# A20181) according to manufacturer's protocol. **B)** Non transduced and CD19_fmc63-scFv CAR T-cells were stained with CD3 and anti-FMC63-scFv idiotype MuFc (20ug/mL). The anti-FMC63 scFv idiotype was stained with Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Thermo, A-21235, RRID: AB_2535804).

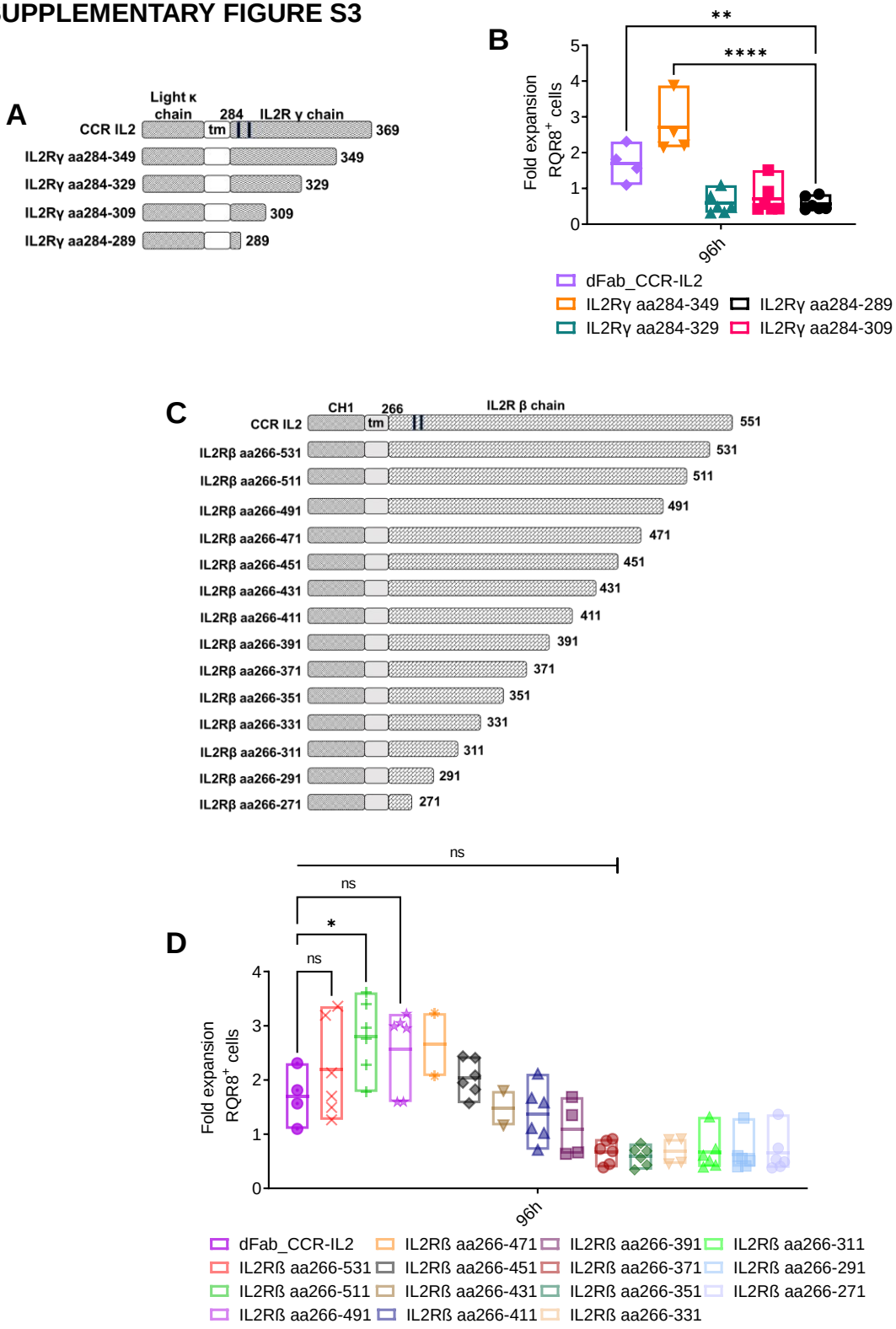
SUPPLEMENTARY FIGURE S2



Supplementary Figure S2. dFab_CCR-IL2 receptor hetero-pairs expression.

(A) Flow-cytometric analysis of the constant CH1 chain expression in dFab_CCR-IL2 transduced T cell, after fixation/permeabilization.

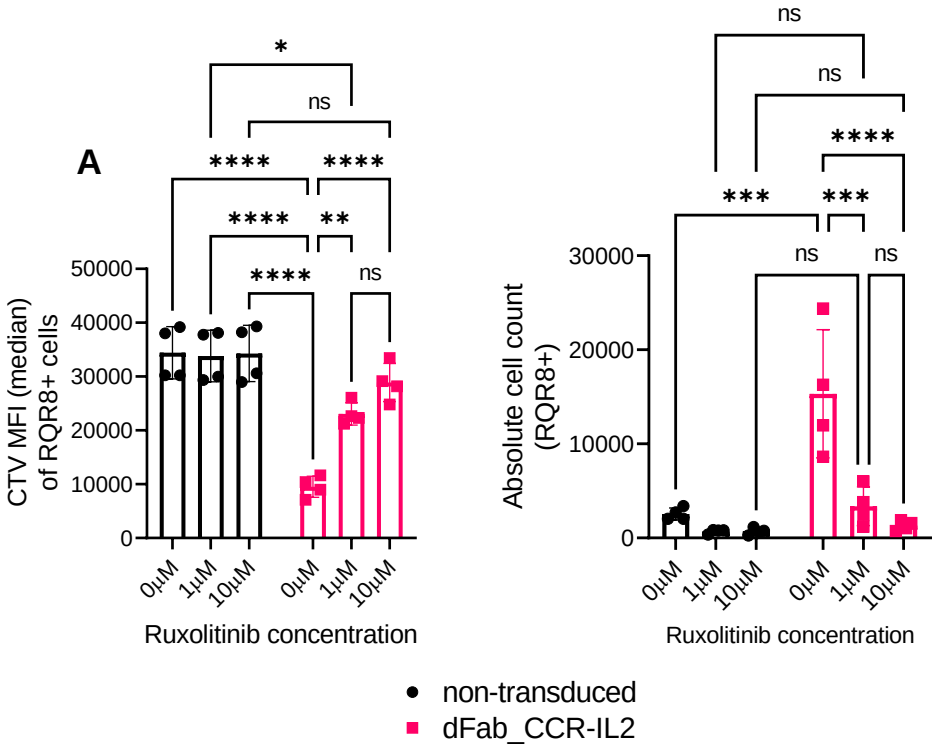
SUPPLEMENTARY FIGURE S3



Supplementary Figure S3. dFab_CCR-IL2 receptor endodomain signalling motifs influence dFab_CCR-IL2 signal potency.

(A) Schematic of the polypeptide sequence of the dFab_CCR CγC truncations. Each truncation was expressed with the full length IL2Rβ/CH1 chain. **(B)** Quantitated *in vitro* proliferation of

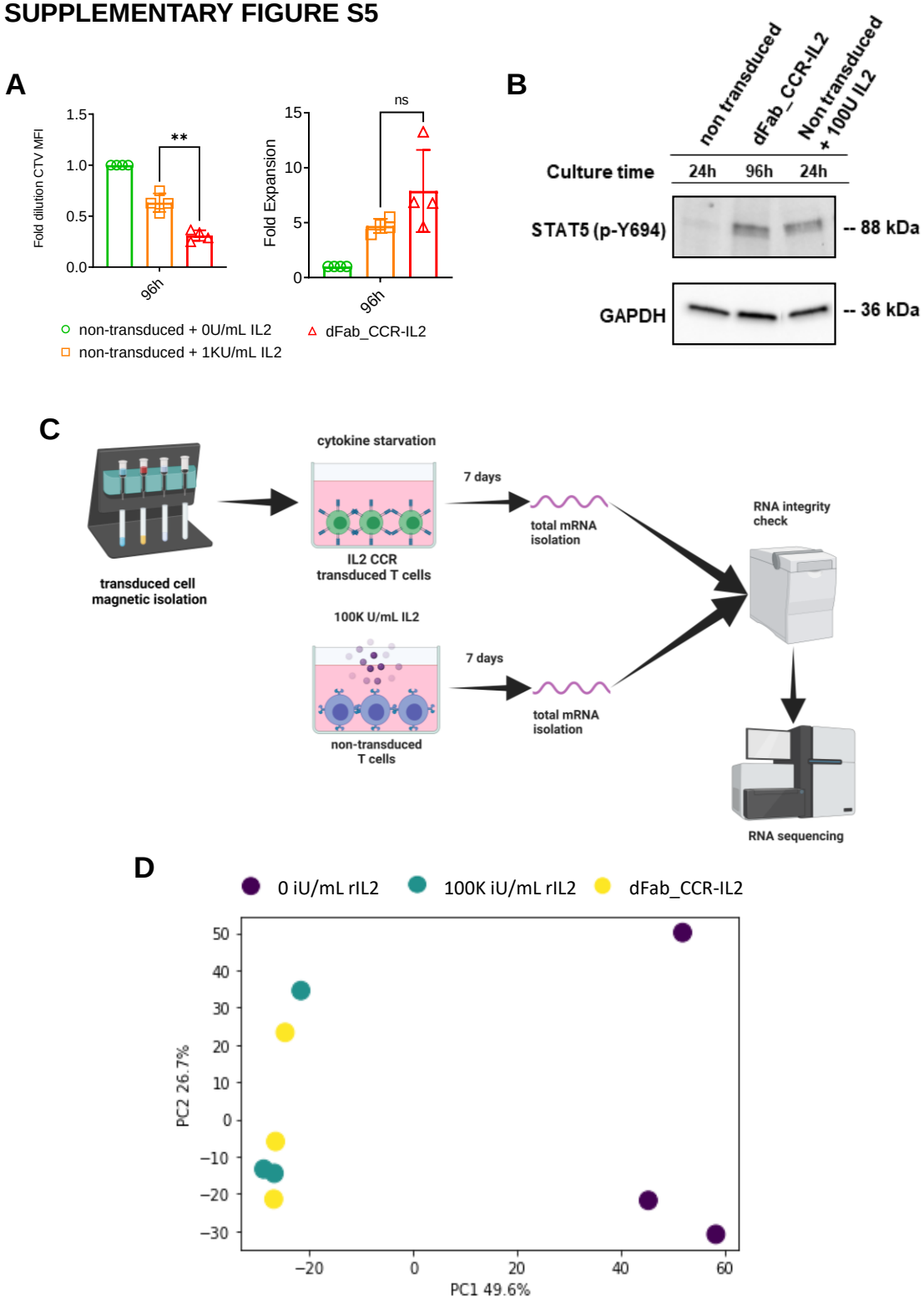
each dFab_CCR CγC truncations, expressed as $RQR8^+$ / $CD3^+$ T cells fold expansion (n=4, one-way ANOVA). **(C)** Schematic of the polypeptide sequence of the dFab_CCR IL2Rβ truncations. Each truncation was expressed with the full length CγC/CL chain. **(D)** Quantitated *in vitro* proliferation of the series of dFab_CCR IL2Rβ truncations, expressed as $RQR8^+$ / $CD3^+$ T cells fold expansion (n=4, one-way ANOVA). All data are presented as mean ± SEM.



Supplementary Figure S4. JAK kinase inhibitor Ruxolitinib tunes dFab_CCR-IL2 receptor signal.

A) Sensitivity of dFab_CCR-IL2 engineer T cell to Ruxolitinib. T cell were pre-treated with increasing concentration of Ruxolitinib for 4h. T cells were cultured for 96 hours in cytokine starvation condition. Proliferation expressed as CTV MFI dilution (left), and absolute count (right) of $RQR8^+$ / $CD3^+$ T cells. (n=4, two-way ANOVA). All data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S5



Supplementary Figure S5. dFab_CCR-IL2 and native IL2 receptor have comparable signal and transcriptome quality.

(A) Comparison of dFab_CCR-IL2 and natural IL2 receptor signal. dFab_CCR-IL2 T cells were cytokine starved for 96 hours. Non transduced T cells were cultured for 96 hours with or

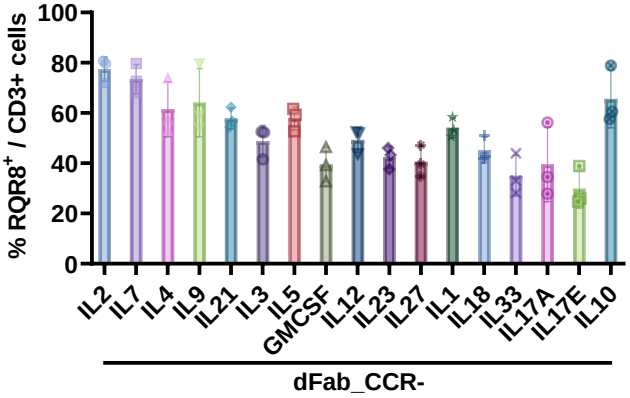
without 100000 iU/mL of IL2 for 96 hours. Fold CTV dilution and fold expansion were calculated normalizing the value with the non-transduced T cells in absence of IL2 (n=4, one-way ANOVA). (**B)** Western blot analysis of *in vitro* STAT5-Y694 phosphorylation. Non transduced T cells were culture in presence or absence of 100 IU/mL of IL2 for 24 hours. dFab_CCR-IL2 T cells were cultured in cytokine starvation for 96 hours. GAPDH was used as loading control in the same membrane. Data are representative of three independent experiments. **(C)** Schematic of the total mRNA sequencing experiments. dFab_CCR-IL2 transduced T cell were magnetically isolated using CD34 microbeads and subjected to 7 days of cytokine starvation. T cell were incubated for 7 days with 100000 IU/mL of IL2 or without IL2. IL2 was replenished after 4 days. Total mRNA was isolated and NGS sequenced. **(D)** PCA plot representing the differences between 7 days with 100000 IU/mL of IL2, without IL2 or dFab_CCR-IL2 expression.

SUPPLEMENTARY FIGURE S6

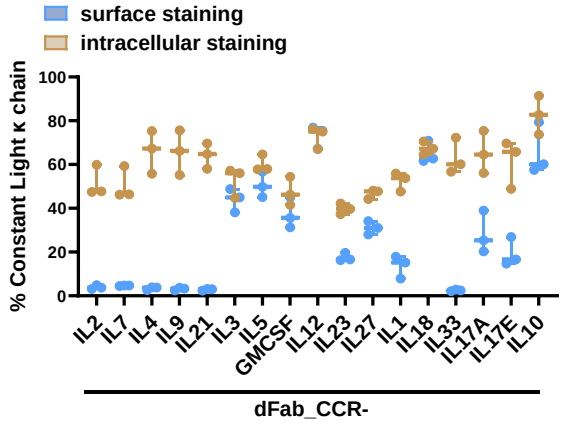
A

Human dFab_CCR vectors		
	Constant Light	
Cytokine receptor	κ-chain	CH1 chain
Common γ-chain family		
dFab_CCR-IL2	Common γ-chain	IL2 receptor β-chain
dFab_CCR-IL7	Common γ-chain	IL7 receptor α-chain
dFab_CCR-IL4	Common γ-chain	IL4 receptor α-chain
dFab_CCR-IL9	Common γ-chain	IL9 receptor α-chain
dFab_CCR-IL21	Common γ-chain	IL21 receptor α-chain
IL12 family		
dFab_CCR-IL12	IL12 receptor β1-chain	IL12 receptor β2-chain
dFab_CCR-IL23	IL23 receptor α-chain	IL12 receptor β1-chain
dFab_CCR-IL27	IL27 receptor β-chain	GP130
Common β-chain family		
dFab_CCR-IL3	IL3 receptor α-chain	Common β-chain
dFab_CCR-IL5	IL5 receptor α-chain	Common β-chain
dFab_CCR-GMCSF	GMCSF receptor α-chain	Common β-chain
IL10 family		
dFab_CCR-IL10	IL10 receptor β1-chain	IL12 receptor β2-chain
IL1 family		
dFab_CCR-IL11	IL1 receptor 1	IL1 receptor AP
dFab_CCR-IL18	IL18 receptor 1	IL18 receptor AP
dFab_CCR-IL33	IL1 receptor-like 1	IL1 receptor AP
IL17 family		
dFab_CCR-IL17A	IL17 receptor A	IL17 receptor B
dFab_CCR-IL17E	IL17 receptor A	IL17 receptor C

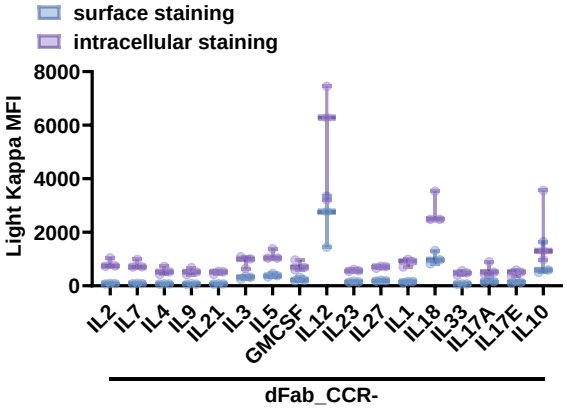
B



C



D

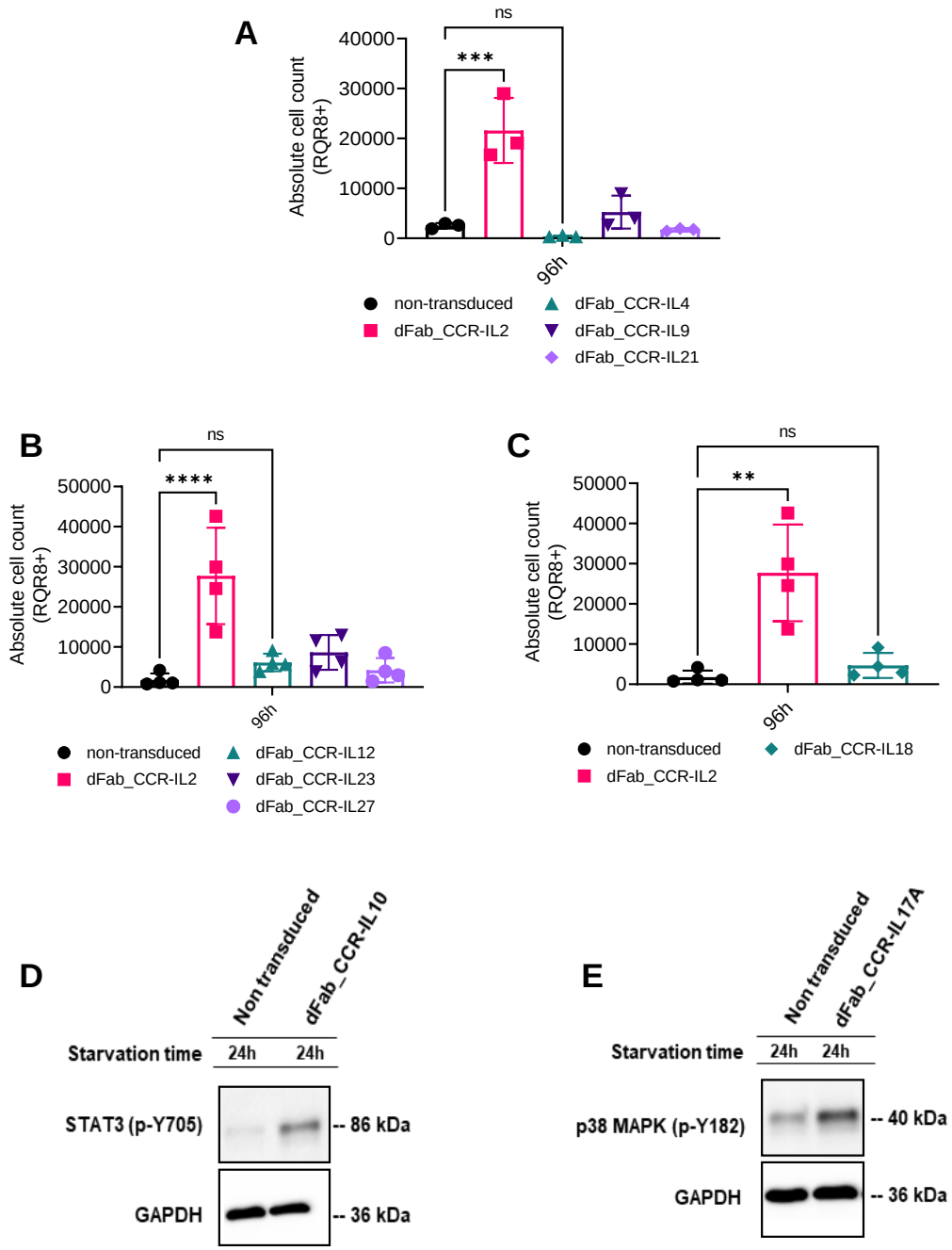


Supplementary Figure S6. dFab_CCR expression profile in primary T cells.

(A) List of dFab_CCRs cytokine receptor pairs utilized in Figure 2. **(B)** Flow cytometry analysis of dFab_CCRs T cells transduction efficiency as measured by CD34 staining of RQR8 marker gene. These cells were used for the experiments in figure 2. **(C)** Histograms representing the percentage of expression of light kappa chain based on surface staining (blue)

and intracellular staining (yellow), (n = 4) **(D)** Histograms representing the dFab_CCRs density expressed of MFI of light kappa chain based on surface staining (blue) and intracellular staining (purple), (n = 4), All data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S7

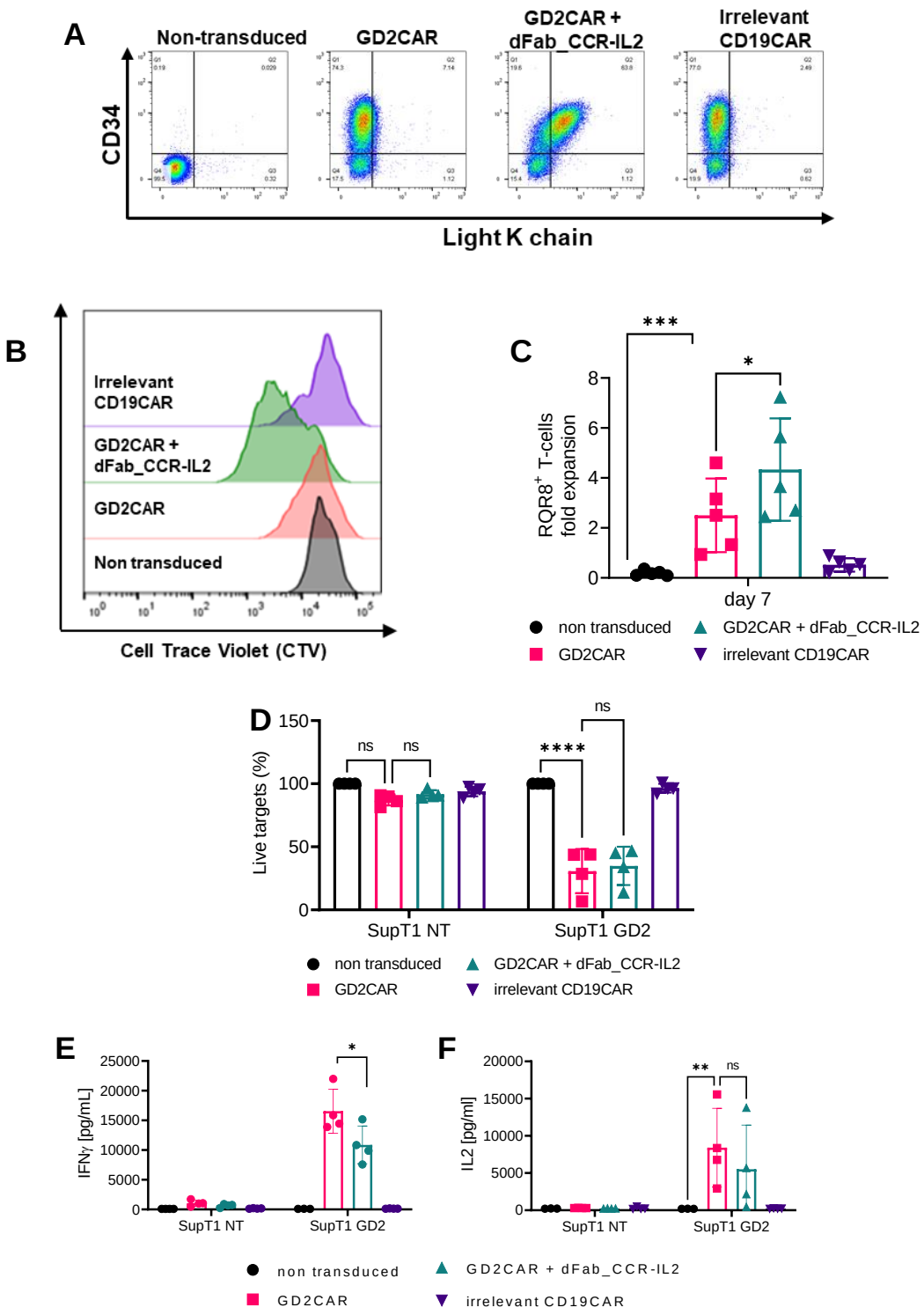


Supplementary Figure S7. dFab_CCRs function primary T cells.

(A, B, C) Quantification of *in vitro* proliferation after 96h of cytokine starvation of T cells engineered with IL4, IL9, IL21 (A), IL2, IL12, IL23, IL27 (B), IL18 (C) dFab_CCRs, expressed as absolute cell count of $RQR8^+$ / $CD3^+$ T cells (n=4, one-way ANOVA). **(D)** Immunoblot analysis of *in vitro* STAT3 phosphorylation, monitoring site-specific phosphorylation at Y705. Non-transduced T cells and dFab_CCR-IL10 T cells were cultured in cytokine starvation for 24 hours. GAPDH was used as loading control in the same membrane. Data are representative of three independent experiments. **(E)** Immunoblot analysis

of in vitro P38/MAPK phosphorylation, monitoring site-specific phosphorylation at Y182. Non-transduced and dFab_CCR-IL17A transduced T cells were cultured in cytokine starvation for 24 hours. GAPDH was used as loading control in the same membrane. Data are representative of three independent experiments. All data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S8

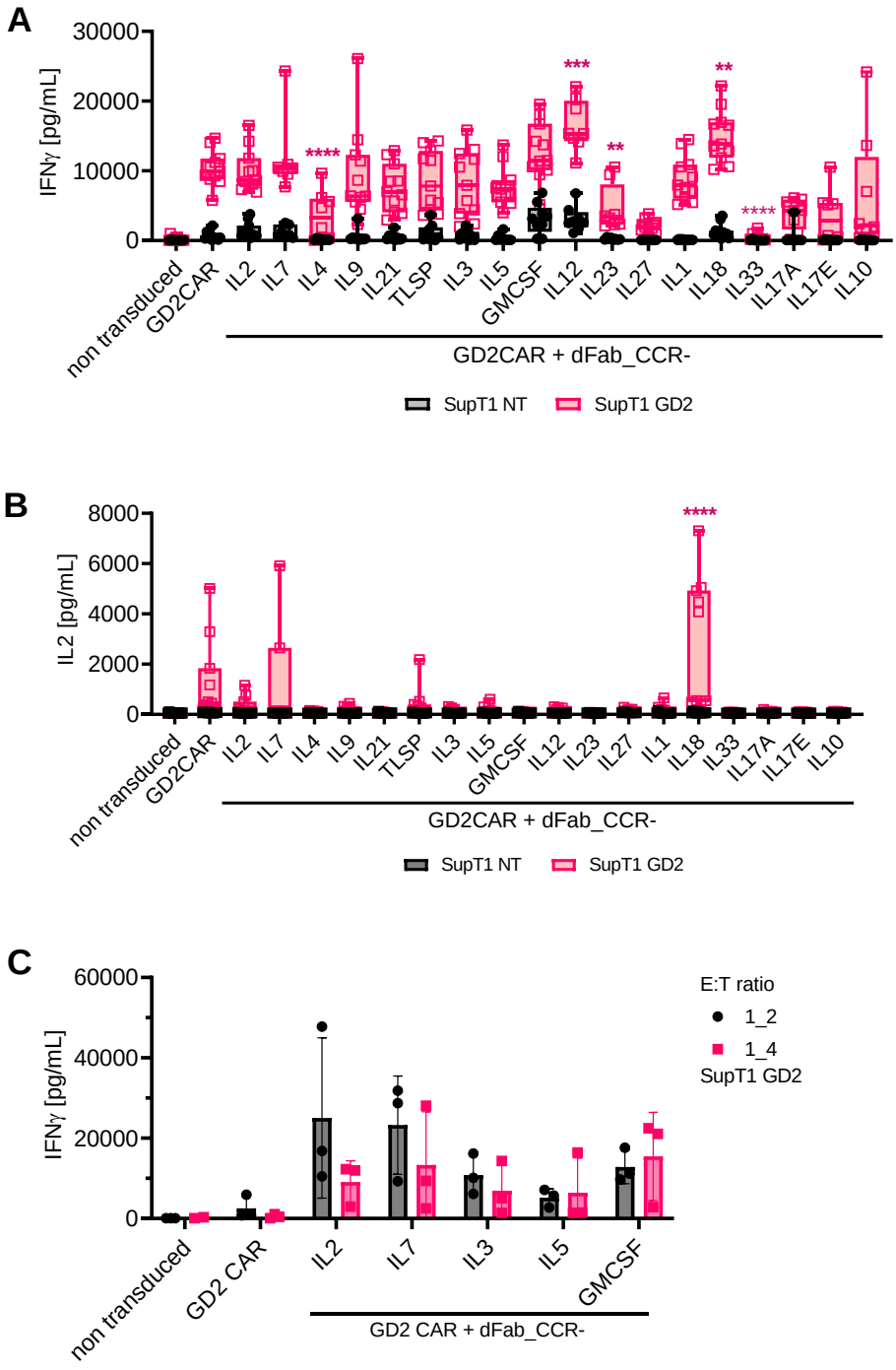


Supplementary Figure S8. dFab_CCR-IL2 maintains functionality when co-expressed with a second generation GD2 CAR in a bi-cistronic vector.

(A) Representative dot plot of the flow cytometry evaluation of T cells transduced with GD2 CAR, GD2 CAR co-expressing dFab_CCR-IL2 or irrelevant CD19 CAR. The graph represents CD34 and light κ chain expression. **(B)**. Representative histogram of Cell Trace Violet (CTV)

dilution of non-transduced, CAR only or CAR co-expressing dFab_CCR-IL2 after 96h of cytokine starvation. **(C)** Proliferation of either GD2 CAR alone or GD2CAR co-expressing dFab_CCR-IL2 T cells cultured for 7 days in cytokine starvation. Specific proliferation expressed as fold expansion (right) of $RQR8^+$ / $CD3^+$ T cells (n=4, one way ANOVA), **(D)** Killing of SupT1-NT or SupT1-$GD2^+$ after 48 hours co-culture with GD2 CAR alone or co-expressing dFab_CCR-IL2 T cells at a 1:1 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (n=4, two-way ANOVA). **(E, F)** Quantification of IFNγ (E) or IL2 (F) release from D. Data shows mean percentage (± SD) (n=4, two-way ANOVA). All data are presented as mean ± SEM.

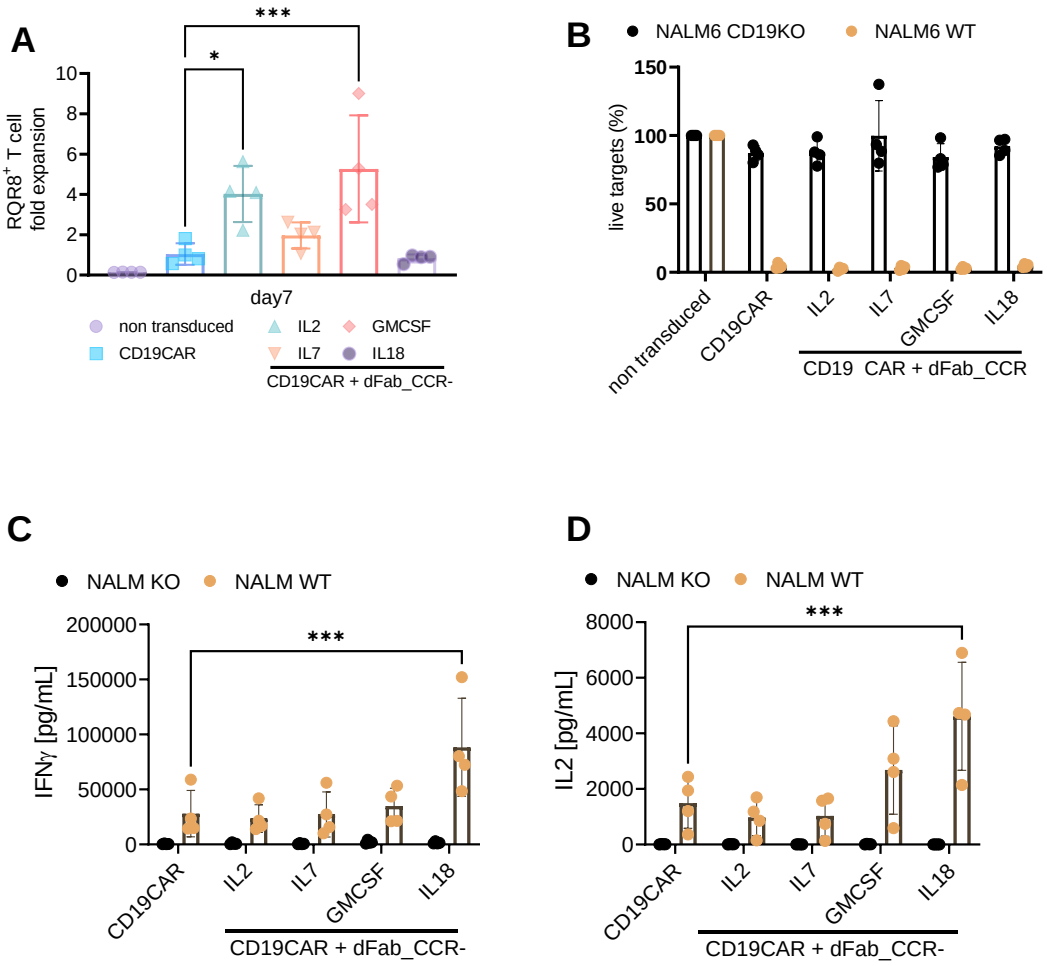
SUPPLEMENTARY FIGURE S9



Supplementary Figure S9. dFab_CCRs co-expression protect GD2 CAR T cells cytokine secretion.

(A, B) IFNγ (A) and IL2 (B) secretion evaluated from co-culture experiment in Figure 3C. (n=4, two-way ANOVA). All data are presented as mean ± SEM. **(C)** IFNγ (A) and IL2 (B) secretion evaluated from co-culture experiment in Figure 3C Figure 3E (n=4, two-way ANOVA).

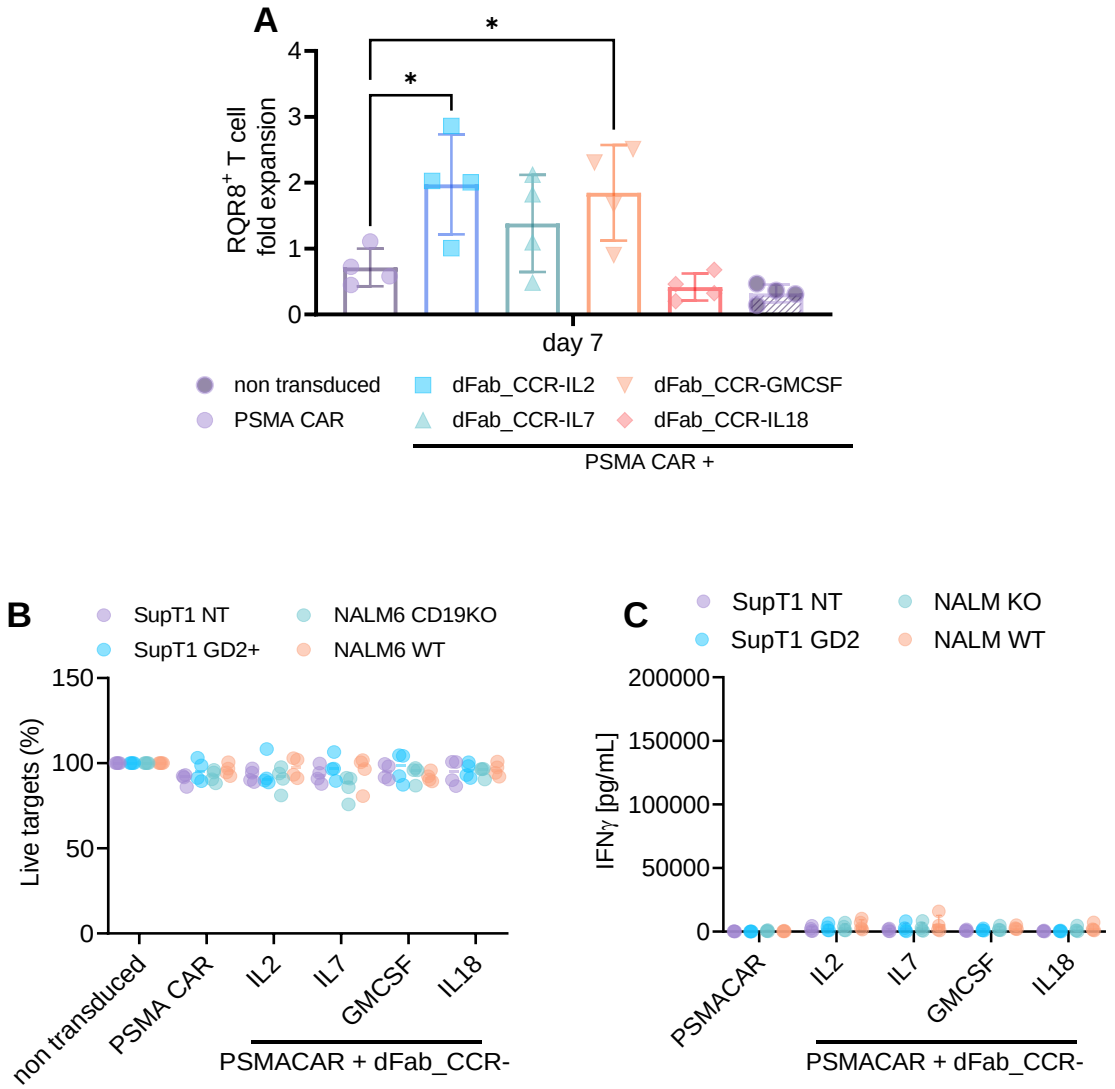
SUPPLEMENTARY FIGURE S10



Supplementary Figure S10. Characterisation of selected dFab_CCRs co-expressed with a second generation 41bbζ-CD19 CAR.

(A) Proliferation of either CD19 CAR alone or CD19CAR co-expressing dFab_CCR-IL2, IL7, GMCSF or IL18 cultured for 7 days in cytokine starvation. Specific proliferation expressed as fold expansion (right) of RQR8$^+$/CD3$^+$ T cells (n=4, one way ANOVA). **(B)** Killing of NALM6 CD19KO (black) or NALM6 WT (orange) after 48 hours co-culture with GD2 CAR alone or co-expressing dFab_CCR-IL2 T cells at 1:4 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (n=4). **(C, D)** Quantification of IFNγ (C) or IL2 (D) release from (B). (n=4, two-way ANOVA). All data are presented as mean ± SEM.

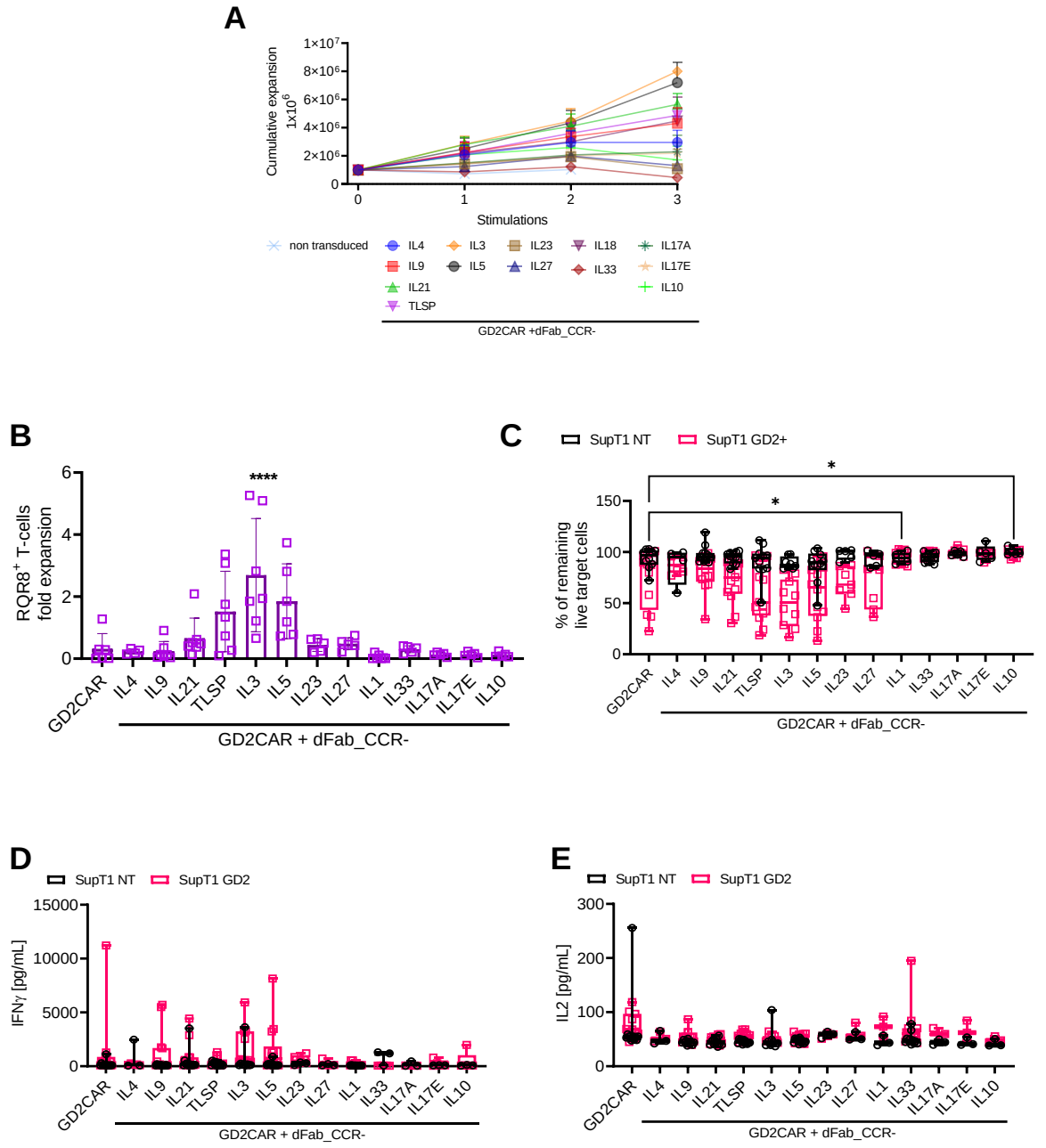
SUPPLEMENTARY FIGURE S11



Supplementary Figure S11. dFab_CCRs co-expression do not alter CAR specificity.

(A) Proliferation of either PSMA CAR alone or PSMACAR co-expressing dFab_CCR-IL2, IL7, GMCSF or IL18 cultured for 7 days in cytokine starvation. Specific proliferation expressed as fold expansion of RQR8$^+$ / CD3$^+$ T cells (n=4, one way ANOVA), **(B)** Killing of SupT1 NT (purple), SupT1 GD2 (cyan), NALM6 CD19KO (green) or NALM6 WT (orange) after 48 hours co-culture with GD2 CAR alone or co-expressing dFab_CCR-IL2 T cells at 1:4 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (n=4). **(C)** Quantification of IFNγ release from B. (n=4). All data are presented as mean ± SEM.

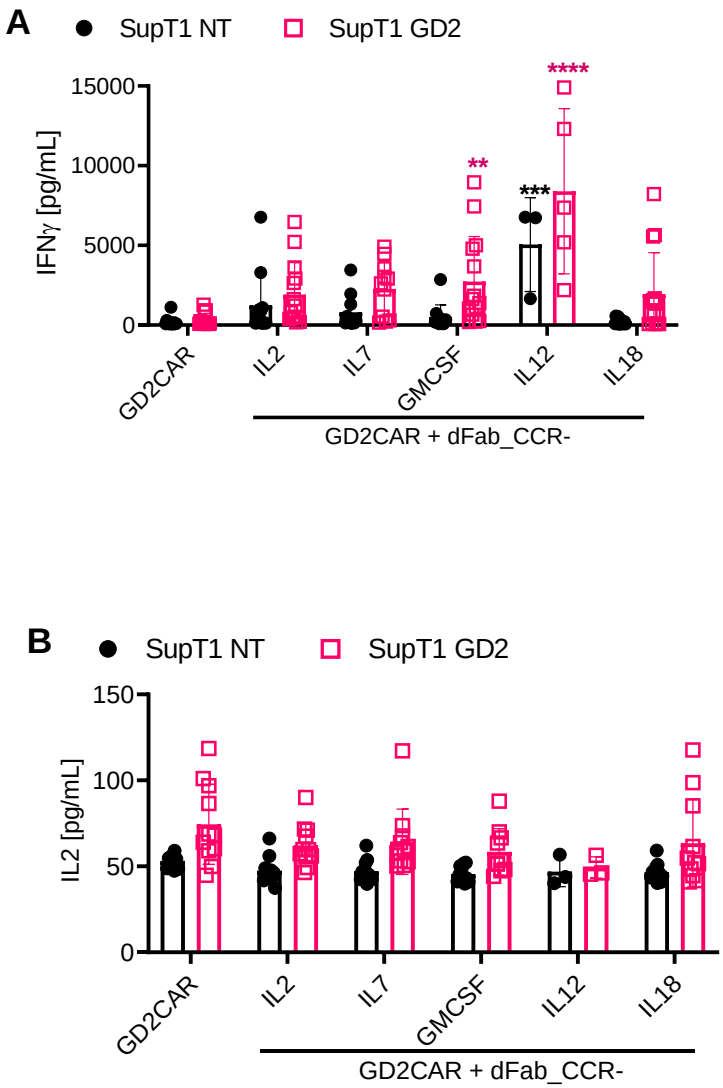
SUPPLEMENTARY FIGURE S12



Supplementary Figure S12. Differential effect on GD2 CAR functionality of the remaining dFab_CCRs library receptor upon antigen chronic stimulation.

(A) Expansion of GD2 CAR T cells co-expressing dFab_CCRs. Data are expressed as 10^6 cells cumulative expansion (n=6). **(B)** Quantitated *in vitro* proliferation of dFab_CCRs co-expressing GD2 CAR T cells, recovered after three rounds of stimulation (A) and cultured for 7 days in cytokine starvation condition. Proliferation expressed as fold expansion of $RQR8^+$ / $CD3^+$ GD2 CAR T cells (n=6, one-way ANOVA). **(C)** Killing of SupT1 NT (black) and $GD2^+$ (red) after 48 hours co-culture with GD2 CAR-T cells recovered after three rounds of co-culture (A) at 1:4 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (n=6, two-way ANOVA). **(D, E)** IFNγ (D), IL2 (E) secretion from (C) (n=6,). All data are presented as mean ± SEM.

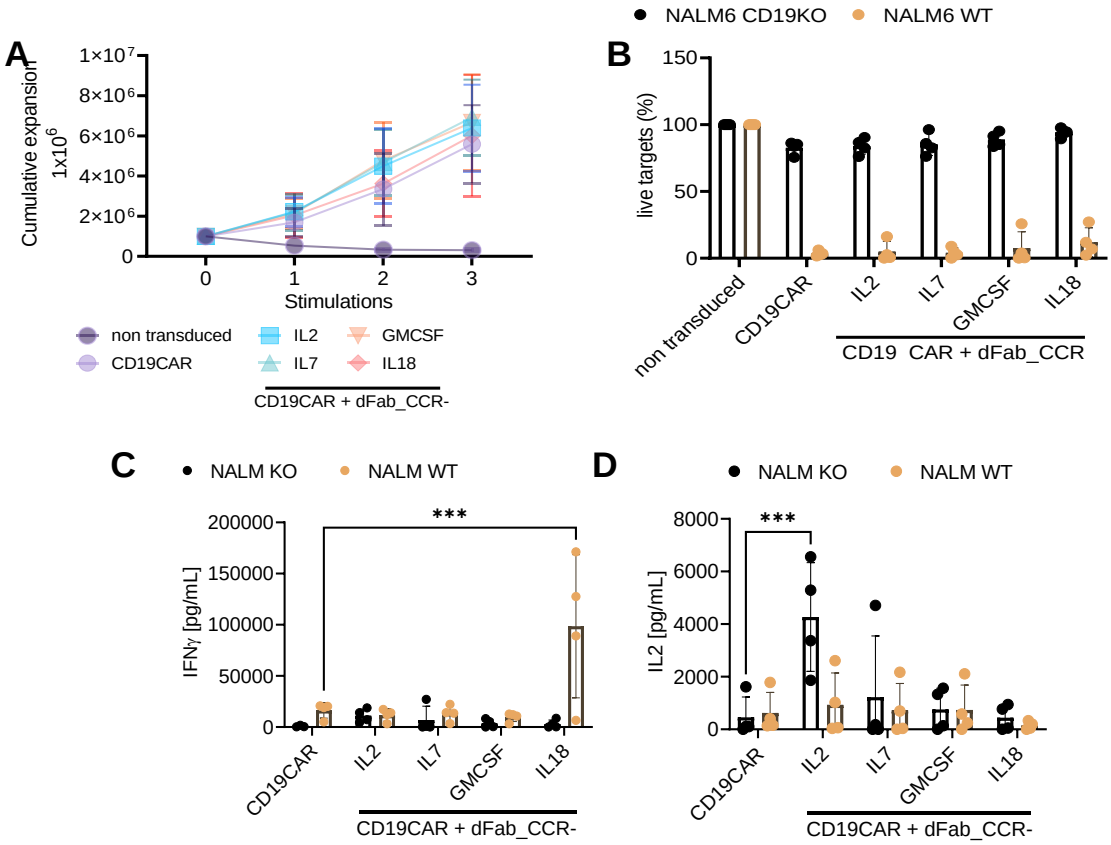
SUPPLEMENTARY FIGURE S13



Supplementary Figure S13. Selected dFab_CCRs library receptors protect GD2 CAR T cells cytokine secretion upon antigen chronic stimulation.

(A, B) Quantification of IFNγ (A) or IL2 (B) release from co-culture in Figure 4D (n=4, two-way ANOVA). All data are presented as mean ± SEM.

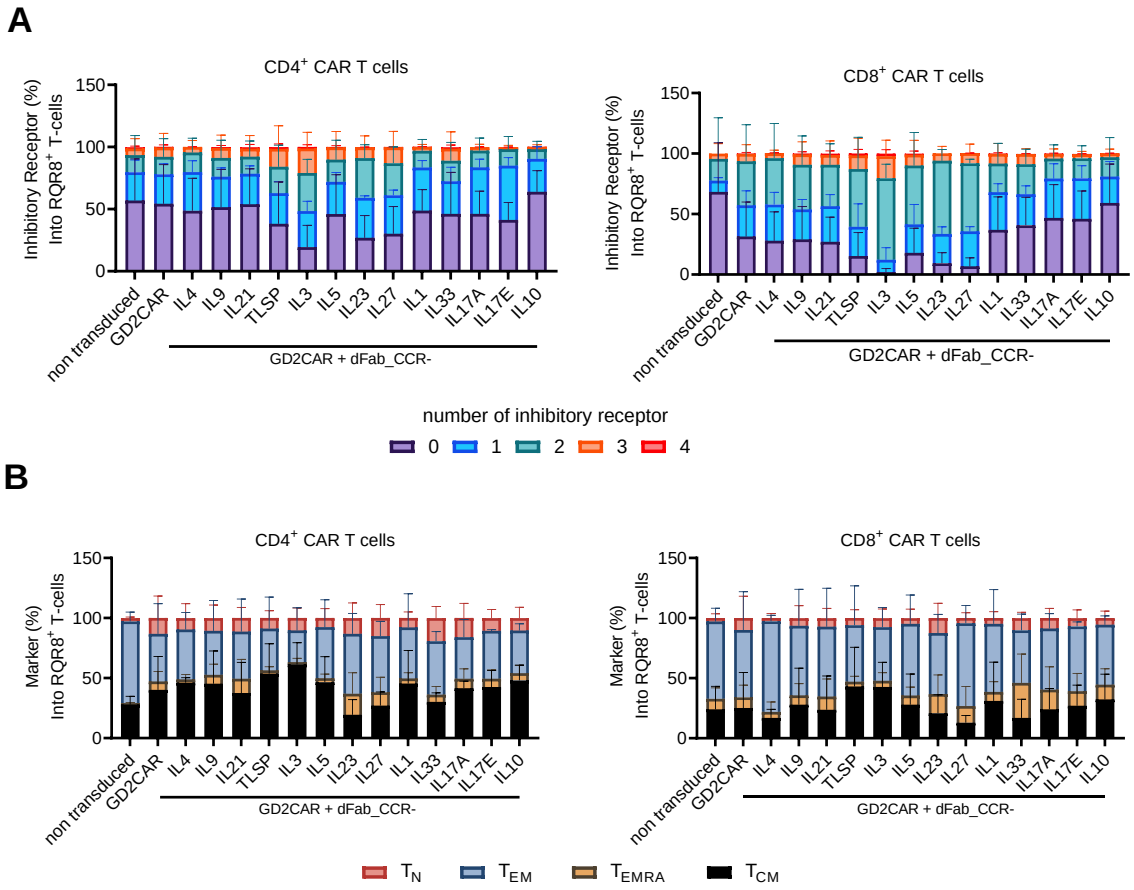
SUPPLEMENTARY FIGURE S14



Supplementary Figure S14. Chronic Antigen exposure effects on CD19 CAR T cells co-expressing selected dFab_CCRs.

A) Expansion of GD2 CAR T cells co-expressing dFab_CCRs. Data are expressed as 10^6 cells cumulative expansion (n=4). **(B)** Killing of NALM6-CD19KO (black) and NALM6 WT (orang) after 48 hours co-culture with cells recovered after three rounds of co-culture (A). at 1:4 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (n=4). **(C, D)** IFNγ (C), IL2 (D) secretion from (C) (n=4, two-way ANOVA).

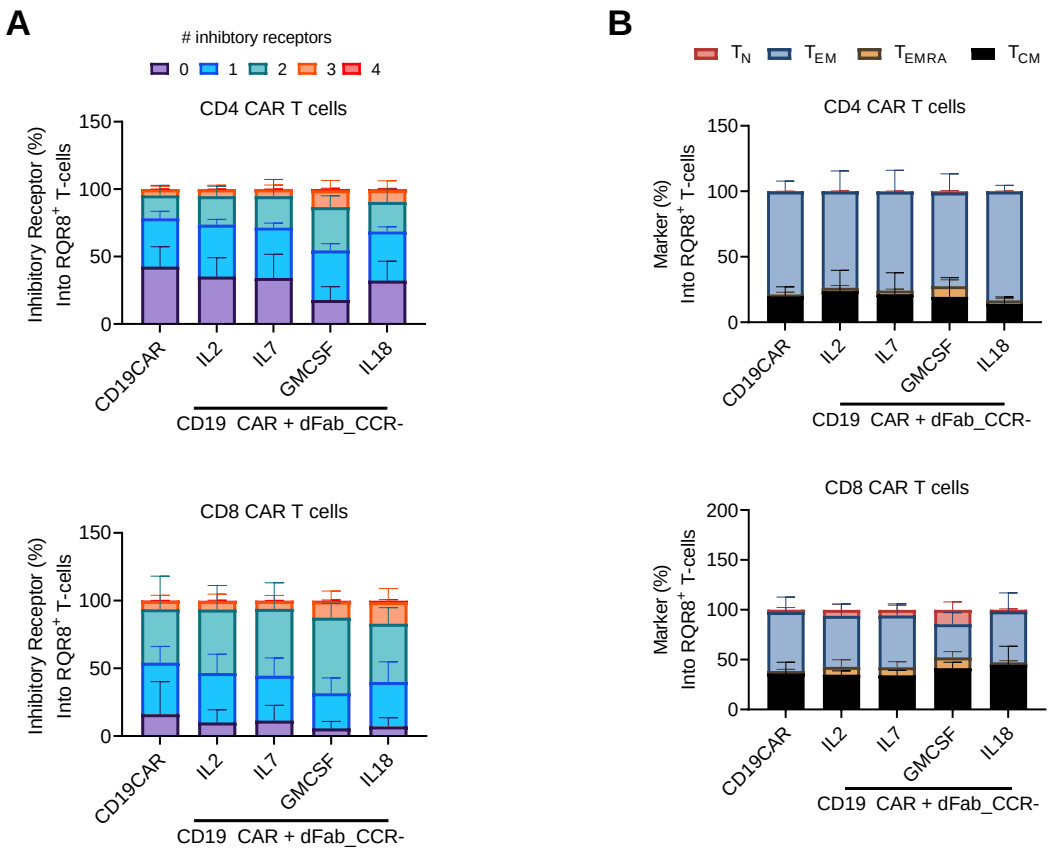
SUPPLEMENTARY FIGURE S15



Supplementary Figure S15. Differential effect of the remaining dFab_CCRs library receptors on exhaustion and memory phenotype after chronic antigen exposure.

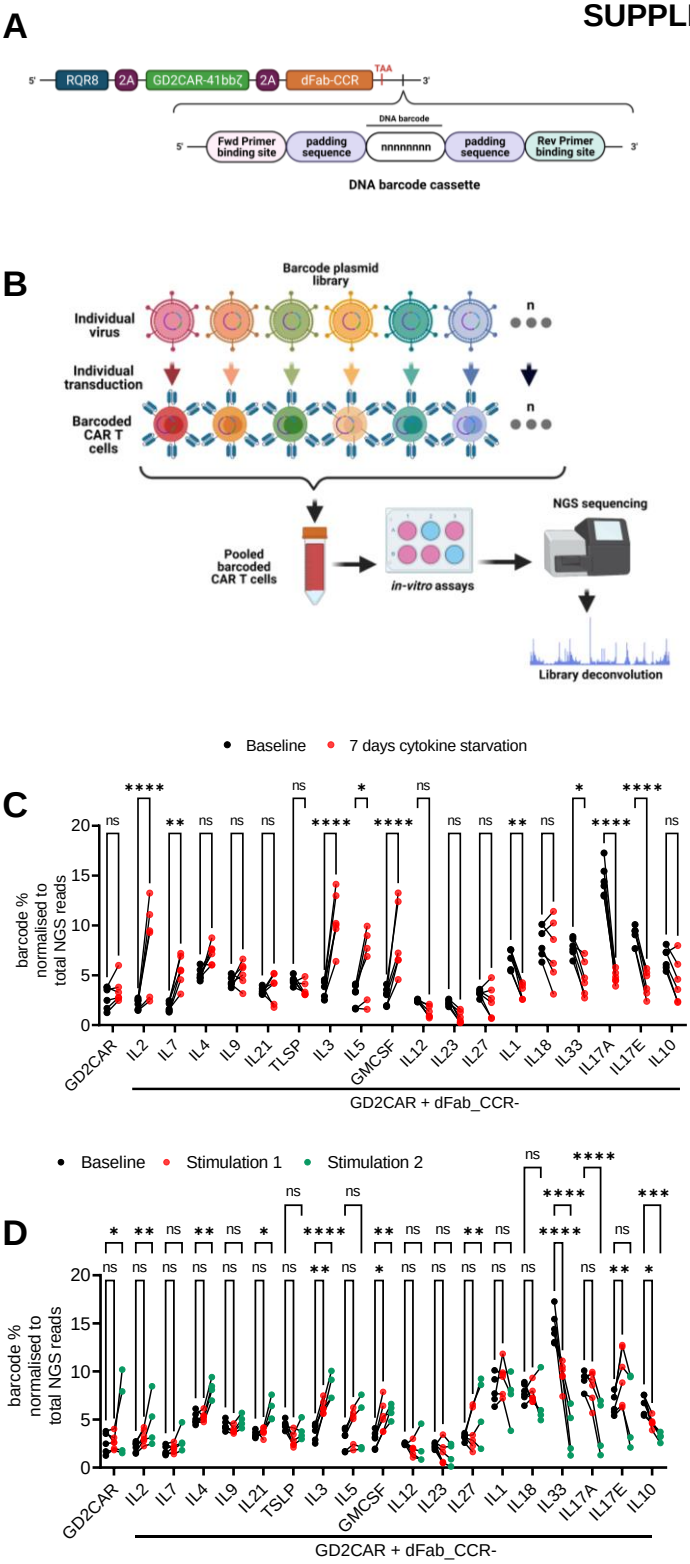
(A) Exhaustion phenotype of CAR T cells after three rounds of chronic antigen exposure, from Supp Fig S13A. Exhaustion evaluated by the expression of TIM3, LAG3, PD-1 or KLRG1 in either $CD4^+$ (left) or $CD8^+$ (right) T cells. Stacked bars show percentage of cells expressing 0, 1, 2, 3 or 4 markers per individual donors (n=11, one-way ANOVA). All data are presented as mean ± SEM. **(B)** Memory phenotype of CAR T cells after three rounds of chronic antigen exposure, from Supp Fig. S13A. Memory phenotype evaluated by the expression of CD45RA and/or CCR7 in either $CD4^+$ (left) or $CD8^+$ (right) T cells. Memory phenotype defined as: naïve T cells ($CD45RA^+$, $CCR7^+$), central memory (CM) T cells ($CD45RA^-$, $CCR7^+$), effector memory (EM) T cells ($CD45RA^-$, $CCR7^-$) and terminally differentiated (TEMRA) T cells ($CD45RA^+$, $CCR7^-$). Stacked bars show percentage of cells in each population markers per individual donor (n=11, one-way ANOVA). All data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S16



Supplementary Figure S16. Differential effect of selected dFab_CCRs library receptors on exhaustion and memory phenotype after CD19 CAR T cell chronic antigen exposure.

(A) Exhaustion phenotype of CAR T cells after three rounds of chronic antigen exposure, from A. Exhaustion evaluated by the expression of TIM3, LAG3, PD-1 or KLRG1 in either $CD4^+$ (top) or $CD8^+$ (bottom) T cells. Stacked bars show percentage of cells expressing 0, 1, 2, 3 or 4 markers per individual donors (n=4, one-way ANOVA). All data are presented as mean ± SEM. **(B)** Memory phenotype of CAR T cells after three rounds of chronic antigen exposure, from A. Memory phenotype evaluated by the expression of CD45RA and/or CCR7 in either $CD4^+$ (top) or $CD8^+$ (bottom) T cells. Memory phenotype defined as: naïve T cells ($CD45RA^+$, $CCR7^+$), central memory (CM) T cells ($CD45RA^-$, $CCR7^+$), effector memory (EM) T cells ($CD45RA^-$, $CCR7^-$) and terminally differentiated (TEMRA) T cells ($CD45RA^+$, $CCR7^-$). Stacked bars show percentage of cells in each population markers per individual donors (n=4, one-way ANOVA). All data are presented as mean ± SEM.

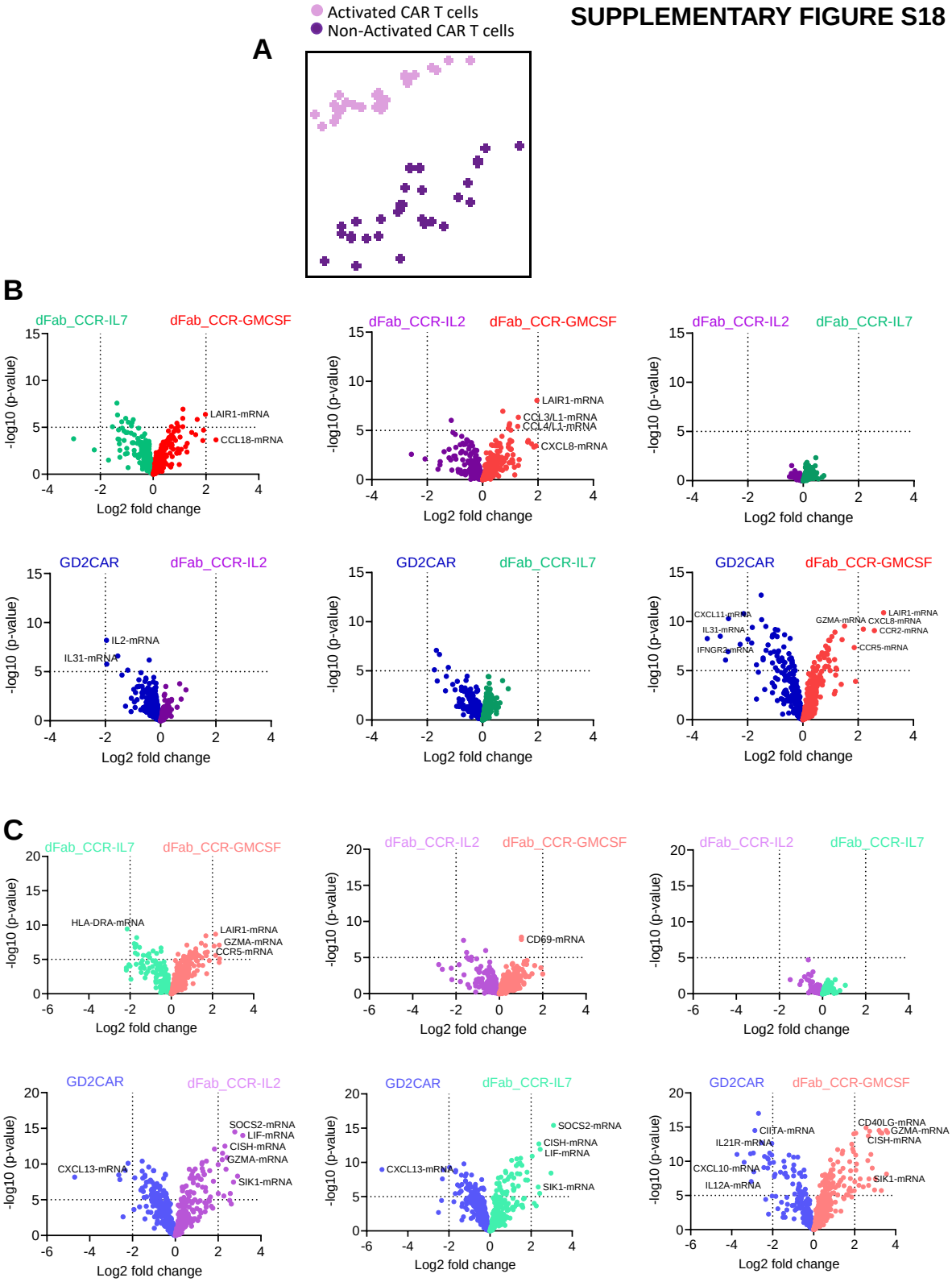


Supplementary Figure S17. dFab_CCR DNA barcoded dFab_CCR library deconvolute individual dFab_CCR functionality.

(A) Schematic of the Barcode DNA cassette. The individual DNA barcode is surrounded by two padding sequences and the primer binding sites**. (B)** Experimental set-up of *in vitro* and of the barcoded GD2CAR co-expressing dFab_CCRs pooled library. **(C)** Representation of

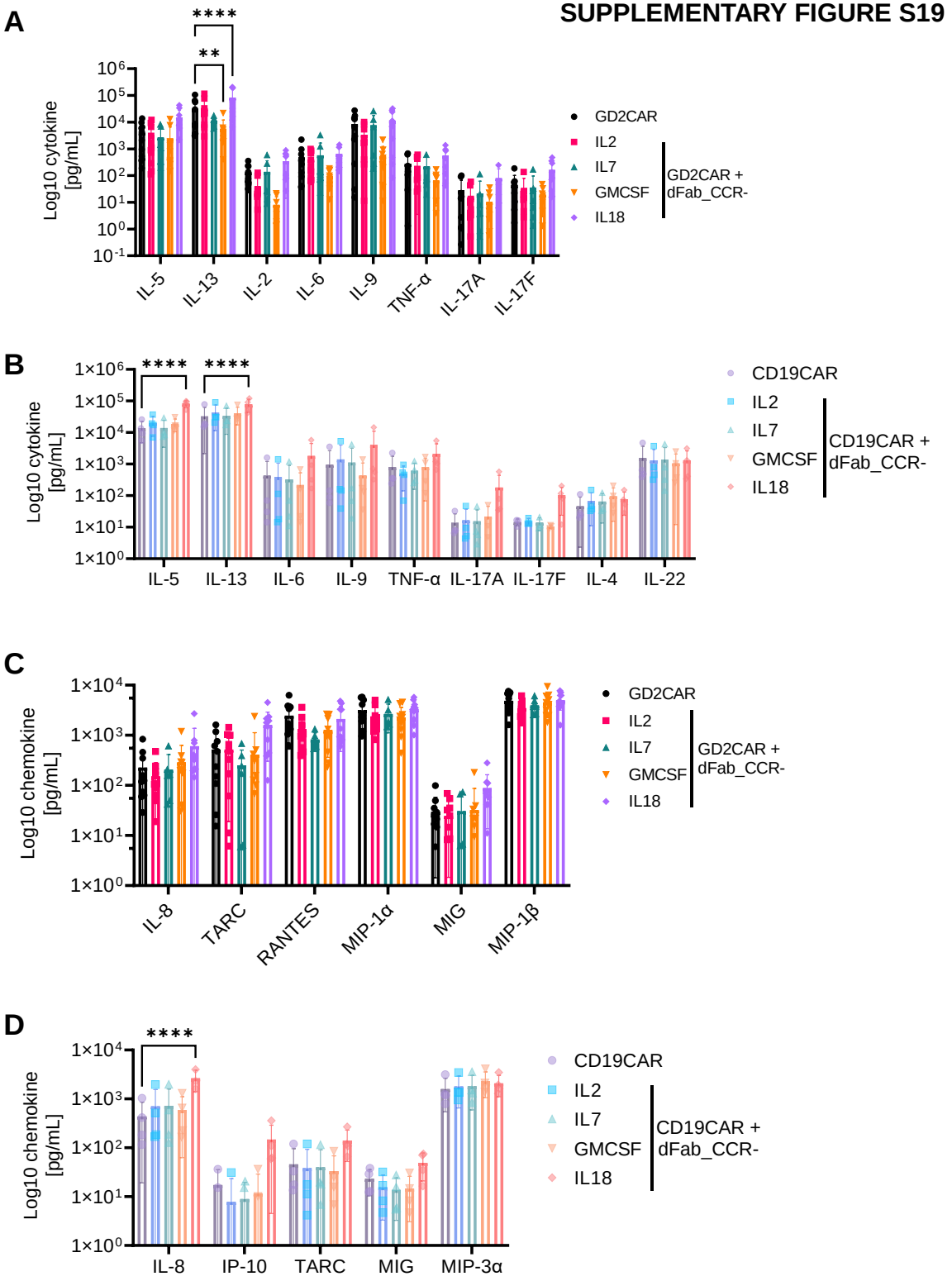
individual barcoded dFab_CCR CAR T cells within the mix population. The graph represents the barcode frequency at day 0 (baseline, black) and after 7 days of cytokine starvation (red) (n=6, two-way ANOVA). **(D)** Representation of individual barcoded CCRs within the mix population after two rounds of serial co-culture. The graph represents the barcode frequency at day 0 (baseline, black), after one stimulation (red) and after the second stimulation (green) (n=6, two-way ANOVA).

SUPPLEMENTARY FIGURE S18



Supplementary Figure S18. Transcriptomic profile of GD2 CAR T cells co-expressing a selection of dFab_CCRs.

(A) PCA analysis of transcriptome differences in activated CAR T cells (pink) or non-activated CAR T cells (purple) (n=6). **(B, C)** Differential gene expression volcano plot comparisons in activated CAR T cells (B) or non-activated CAR T cells (C) (n=6).

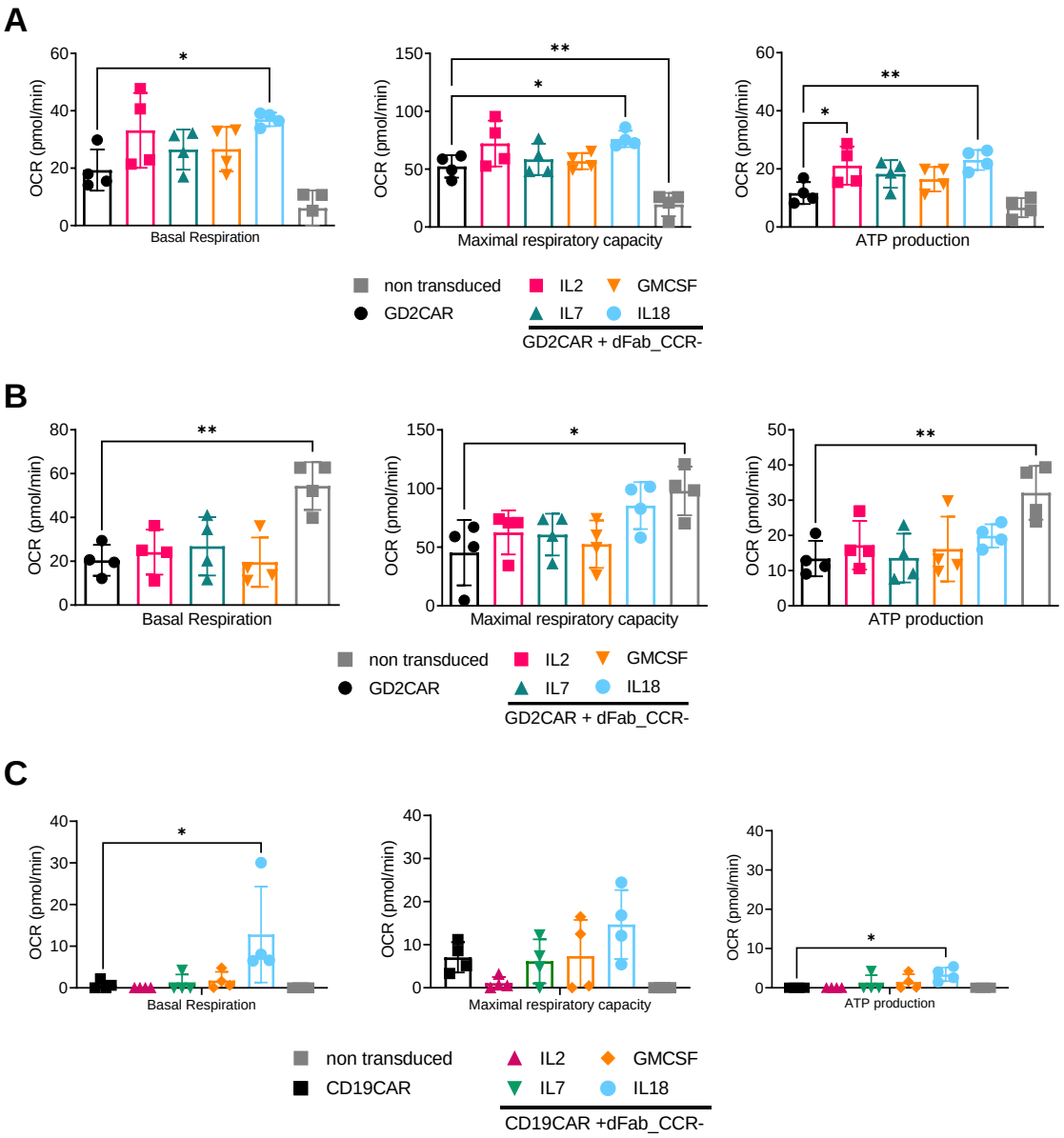


Supplementary Figure S19. Chemokines and cytokines secretion profile of GD2 and CD19 CAR T cells co-expressing a selection of dFab_CCRs.

(A, B) Quantitation of chemokines (A) or cytokines (B) of GD2CAR T cells co-expressing selected dFab_CCRs from co-culture with SupT1 GD2^{+} target cells in Figure 3C, analyzed with cytokine beads array (n=9). **(D, E)** Quantitation of chemokines (D) or cytokines (E) of

CD19CAR T cells co-expressing selected dFab_CCRs from co-culture with NALM6 WT target cells in Supplementary Figure S10B analyzed with cytokine beads array (n=4).

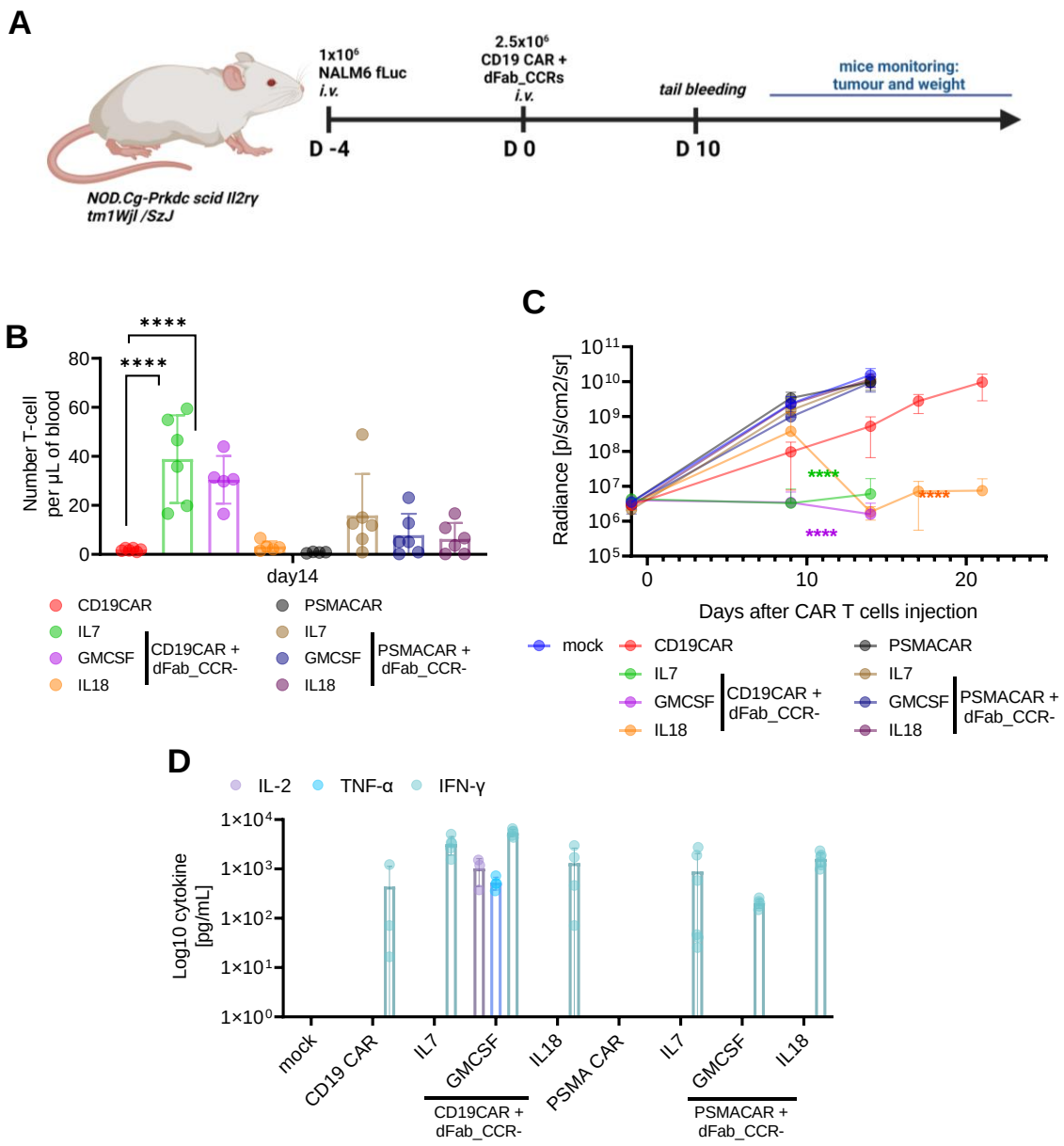
SUPPLEMENTARY FIGURE S20



Supplementary Figure S20. Metabolomic profile of GD2 and CD19 CAR T cells co-expressing a selection of dFab_CCRs.

(A, B) Evaluation of basal respiration, maximal respiratory capacity, and ATP production in resting GD2 CAR T cells (A) and activated GD2 CAR T cells (B) (n=4). **(C)** Evaluation of basal respiration, maximal respiratory capacity, and ATP production in resting CD19 CAR T cells (n=4). All data are presented as mean ± SEM.

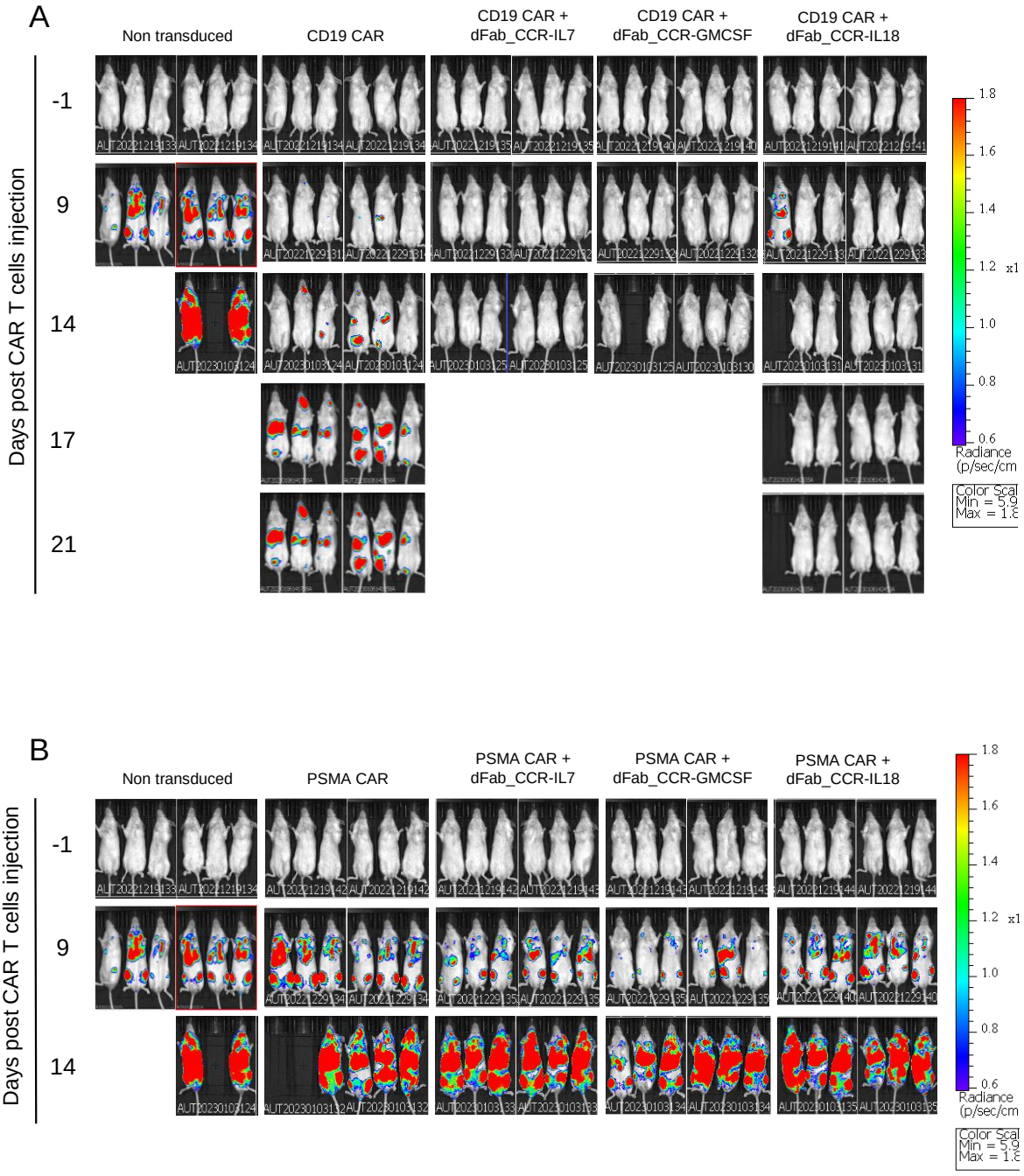
SUPPLEMENTARY FIGURE S21



Supplementary Figure S21. dFab_CCR co-expression augments CD19 CAR T cells efficacy in an establishes ALL NALM6 xenograph model.

(A) Experimental *in vivo* setup of the established NALM-6 ALL xenograph model. **(B)** CD19 CAR T cells peripheral engraftment at day 14, quantitated via flow cytometry by the expression human CD3 (n=6, one-way ANOVA). **(C)** Bioluminescence analysis of total flux (n=6, day 14 student t-test, day 21 student t-test). **(D)** Quantitation of serum cytokines at day 10, analyzed with cytokine beads array (n=6). All data are presented as mean ± SEM.

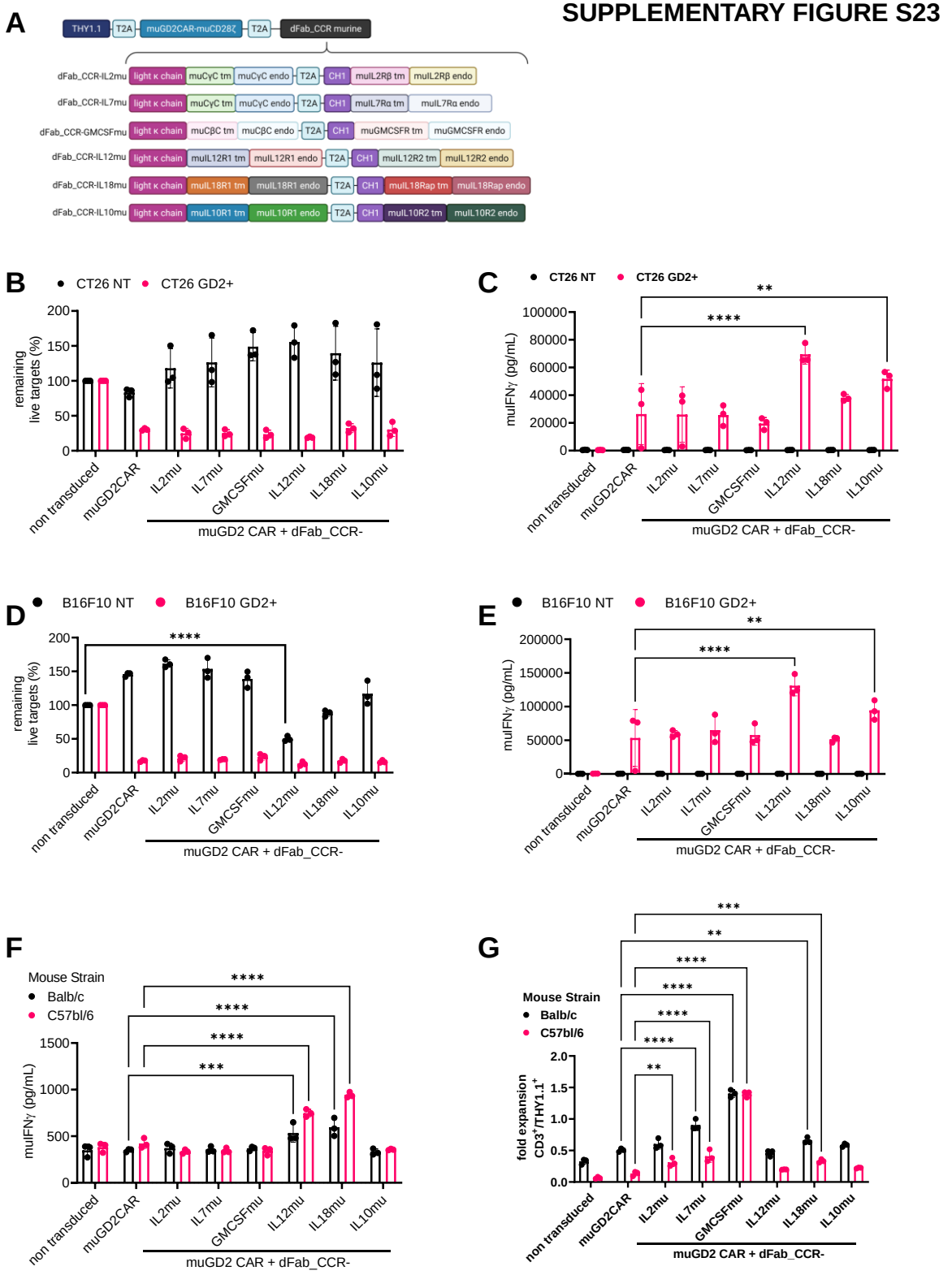
SUPPLEMENTARY FIGURE S22



Supplementary Figure S22. Bioluminescence imaging of the established ALL NALM6 xenograph model.

(A) Bioluminescence imaging of CD19-CAR and CD19-CAR co-expressing dFab_CCR-IL7, GMCSF and IL18 in NALM6 xenograph model. **(B)** Bioluminescence imaging of PSMA-CAR and PSMA-CAR co-expressing dFab_CCR-IL7, GMCSF and IL18 in NALM6 xenograph model.

SUPPLEMENTARY FIGURE S23

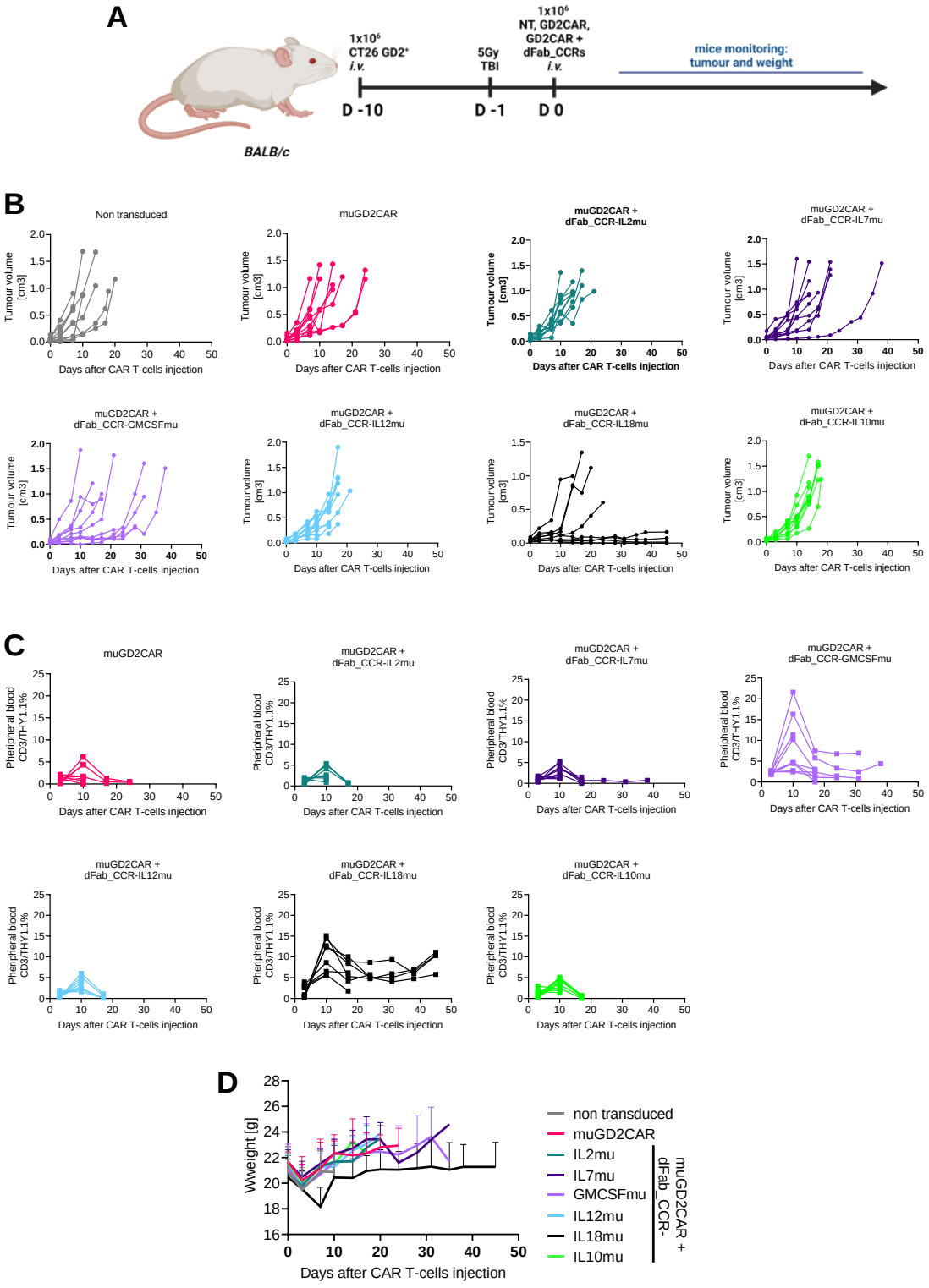


Supplementary Figure S23. ***In vitro*** **functionality of murinized dFab_CCR co-expressed with murine GD2CAR.**

(A) Schematic of the murine GD2 CAR plasmid DNA co-expressing selected murine optimized dFab_CCRs. **(B)** Killing of CT26-NT (black) and CT26-GD2^{+} (red) after 48 hours co-culture with murine GD2 CAR T cells at 1:4 effector:target ratio. Data shows mean

percentage (± SD) of live cells compared to non-transduced (NT) control (3 technical replicates, two-way ANOVA). **(C)** IFNγ secreted by co-culture from (A) (3 technical replicates, two-way ANOVA). **(D)** Killing of B16F10-NT (black) and B16F10-$GD2^+$ (red) after 48 hours co-culture with murine GD2 CAR T cells at 1:4 effector:target ratio. Data shows mean percentage (± SD) of live cells compared to non-transduced (NT) control (3 technical replicates, two-way ANOVA). **(E)** IFNγ secreted by co-culture from (C) (3 technical replicates, two-way ANOVA). **(F)** IFNγ secreted by murine GD2 CAR T cells culture for 48 hours in cytokine-free media (3 technical replicates, two-way ANOVA). **(G)** Quantitated *in vitro* proliferation of murine GD2 CAR T cells cultured for 96 hours in cytokine starvation condition. Proliferation expressed as fold expansion of $THY1.1^+$ / $CD3^+$ murine GD2 CAR T cells (3 technical replicates, two-way ANOVA). All data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S24

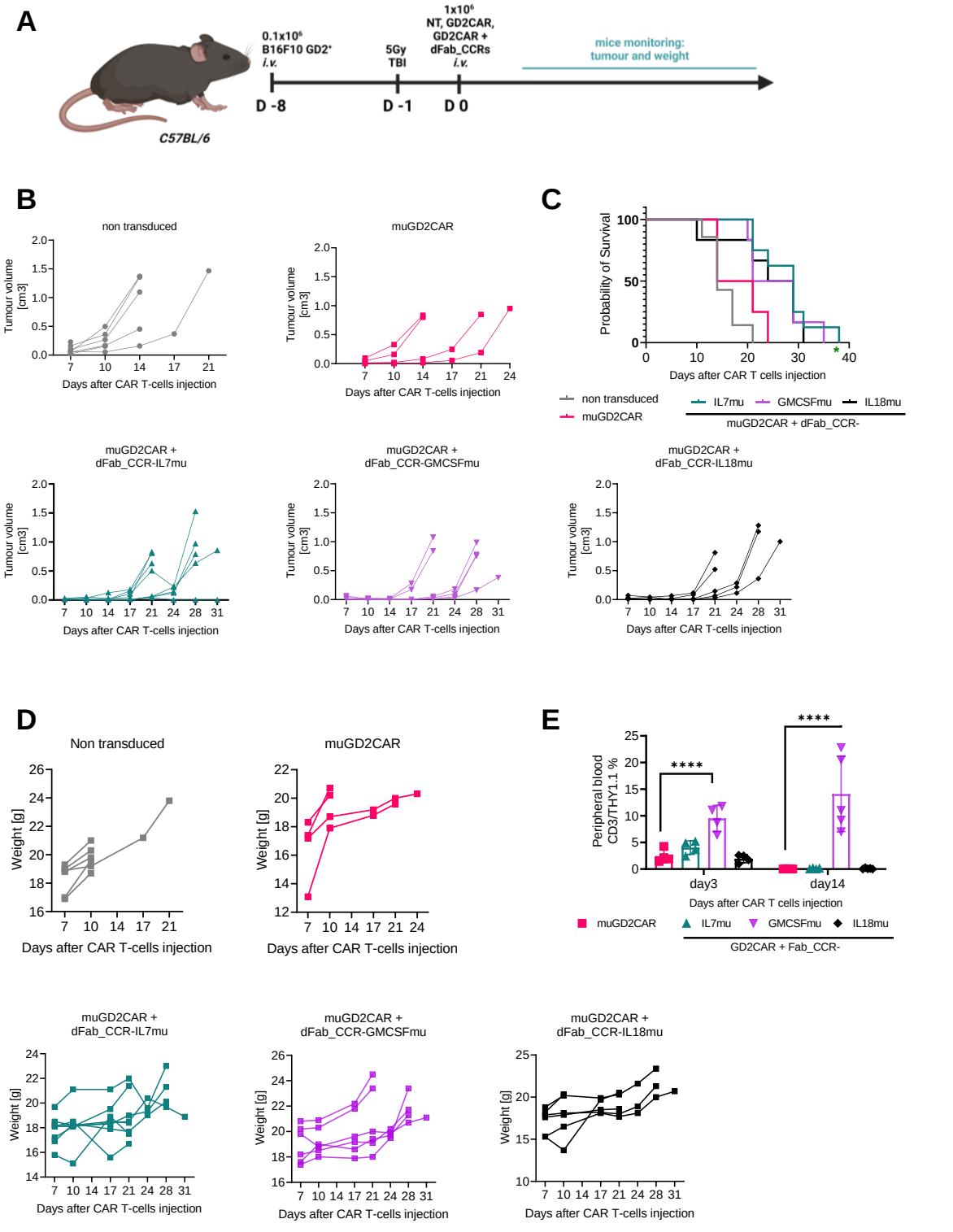


Supplementary Figure S24. *In vivo* functionality of murinized dFab_CCR co-expressed with murine GD2CAR in syngeneic CT26-GD2 colon carcinoma model.

(A) Experimental *in vivo* setup of the syngeneic CT26 GD2^{+} colon carcinoma model timeline, testing murine GD2 CAR T cells alone or co-expressing dFab_CCR-IL2mu, -IL7mu, -

GMCSFmu, -IL12mu, -IL18mu or -IL10mu. **(B**) Individual tumour growth traces (mm^3) (n=8) **(C)** Individual mice CAR T cell peripheral blood engraftment, evaluate as expression of transduction marker THY1.1 in $CD3^+/CD45^+/CD11b^-$ cells (n=8). All data are presented as mean ± SEM. **(D)** Mice weight measured bi-weekly and used as surrogate of CAR related toxicity (n-8).

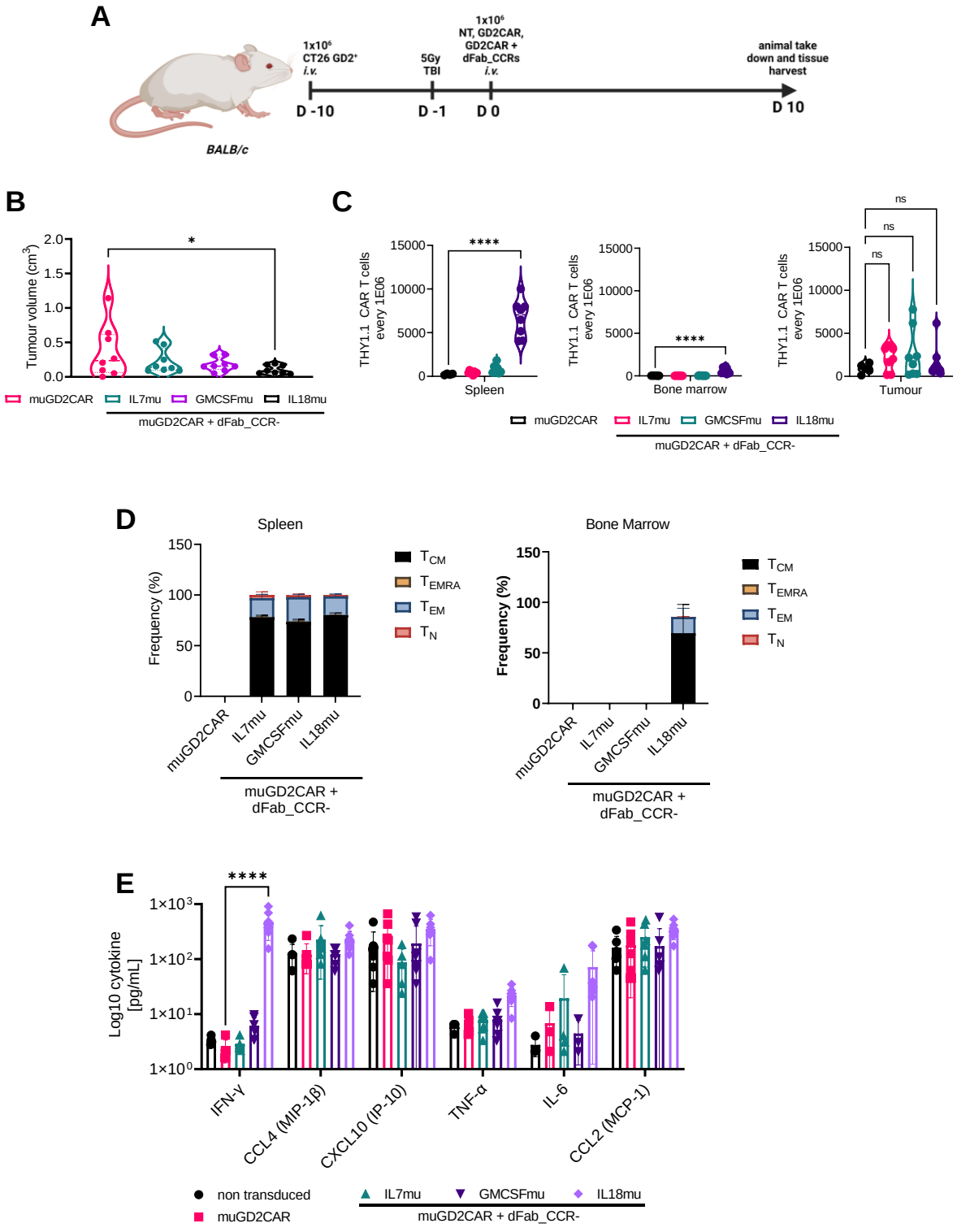
SUPPLEMENTARY FIGURE S25



Supplementary Figure S25. ***In vivo*** **functionality of murinized dFab_CCR co-expressed with murine GD2CAR in syngeneic B16-GD2 melanoma model.**

(A) Experimental *in-vivo* setup of the syngeneic B16F10 GD2^{+} melanoma model timeline, testing murine GD2 CAR T cells alone or co-expressing dFab_CCR-IL2mu, -IL7mu, -GMCSFmu, -IL12mu, -IL18mu or -IL10mu. **(B)** Individual tumour growth traces (n=8). **(C)**

Kaplan Mayer survival curve. **(D)** Individual mouse weight traces (n=8). **(E)** CAR T cell peripheral blood engraftment, evaluate as expression of transduction marker THY1.1 in $CD3^{+}/CD45^{+}/CD11b^{-}$ cells (n=8).



Supplementary Figure S26. dFab_CCR-IL18 co-expression improved CAR T cells engraftment in lymphoid tissues.

(A) Experimental timeline. **(B)** Tumour volume at day 10 (n=8, one-way ANOVA). **(C)** Murine GD2 CAR T cells engraftment in bone marrow, spleen and tumour expressed as THY1.1^{+} CAR

T cell number every 1E06 $CD3^+/CD45^+/CD11b^-$ T cells (n=8, one-way ANOVA). **(D)** Memory phenotype of murine THY1.1 CAR T cells in the bone marrow and spleen. Memory phenotype evaluated by the expression of CD62L and CD44. Naïve T cells ($C44^-$, $CD62L^+$), effector memory (EM) T cells ($CD44^+$, CD62L+), Central Memory (CM) T cells (CD44+, $CD62L^+$) and terminally differentiated (TEMRA) T cells ($CD44^-$, $CD62L^-$). Stacked bars show the percentage of cells in each population markers per individual donor (n=8) All data are presented as mean ± SEM. **(E)** Quantification of serum chemokines and cytokines (n=8, two-way ANOVA). All data are presented as mean ± SEM.