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Supplemental information

TGF-β1 potentiates Vγ9Vδ2 T cell adoptive immunotherapy of cancer

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Supplemental Information

TGF-β1 potentiates γδ T-cell adoptive immunotherapy of cancer

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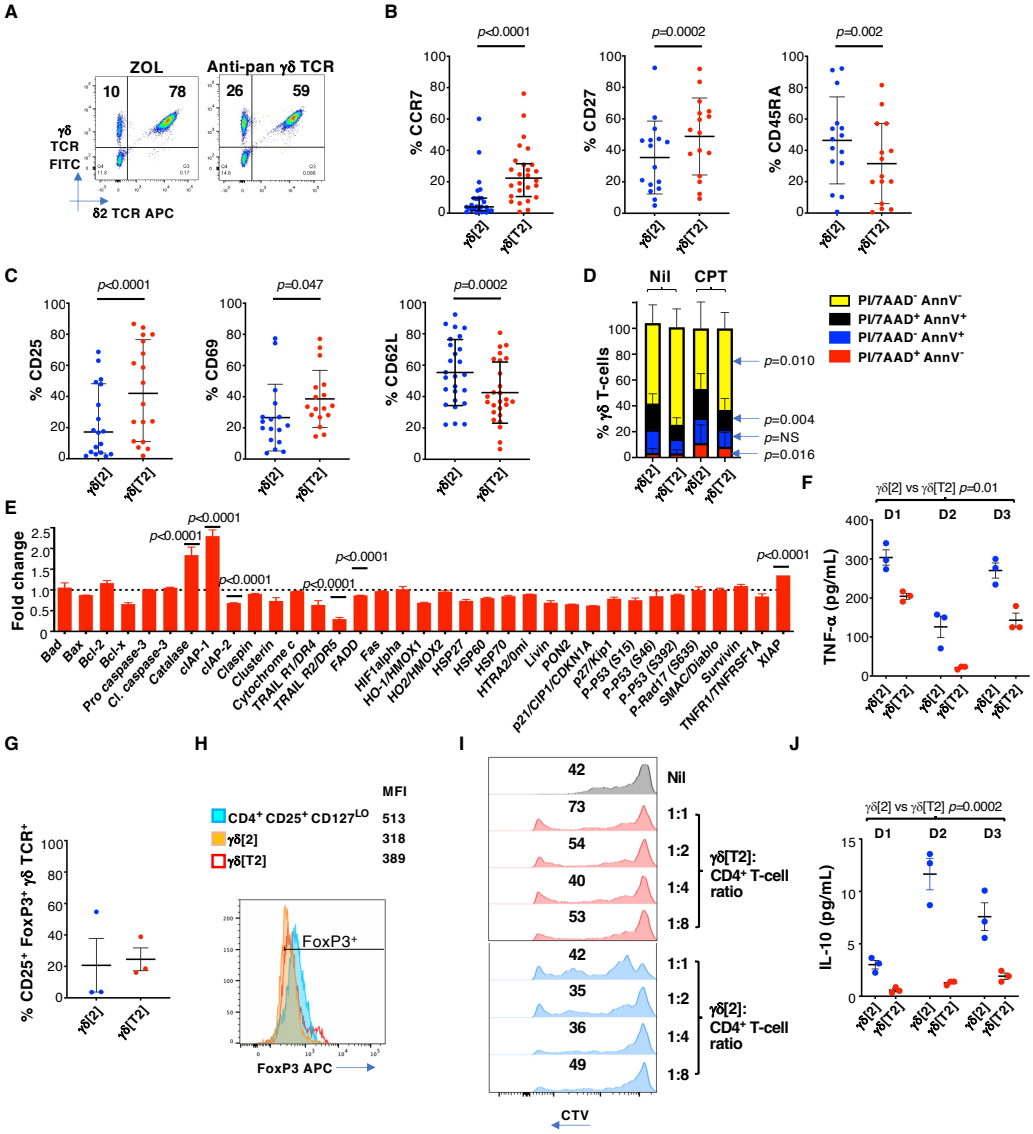

Figure S1. *In vitro* characterization of γδ[T2] cells

Healthy donor PBMCs were activated with ZOL (n=27) or immobilized anti-γδ TCR antibody (n=15) and then cultured for 14-17 days in SFM containing IL-2 alone (γδ[2] cells) or IL-2 + TGF-β (γδ[T2] cells). (**A**) Representative example of flow cytometric analysis of γδ TCR expression by expanded γδ[2] and γδ[T2] cells after co-staining with fluorochrome-conjugated non-cross reactive pan-γδ TCR and δ2 γδ TCR antibodies. (**B**) Following γδ T-cell expansion, the indicated markers of γδ T-cell differentiation were assessed in γδ[2] and γδ[T2] cells by flow cytometry. (**C**) Expanded cells were also assessed by flow cytometry for the indicated markers of γδ T-cell activation. In B-C, panels show mean +/- SD (CD27, CD45RA, CD69, CD62L) or median +/- interquartile ranges (CCR7, CD25), where data were or were not normally distributed respectively. Accordingly, statistical analysis was performed using a Student's *t*-test or Wilcoxon signed rank test respectively. (**D**) Expanded γδ[2] and γδ[T2] cells were cultured with camptothecin (CPT; 12μM) for 6h and then analyzed for viability by flow cytometry, after incubation with Annexin-V-FITC and 7AAD or PI (mean +/- SD, n=7). Statistical analysis compares camptothecin-treated γδ[2] and γδ[T2] cells using a paired Student's *t*-test or Wilcoxon signed rank test (PI+ AV-). (**E**) Expression of a panel of apoptosis-related proteins was quantified in γδ[2] and γδ[T2] cells using a Proteome Profiler Human apoptosis array kit. Data show pixel density (mean +/- SD, n=2 independent donors). Similar results were obtained in a second independent analysis. (**F**) TNF-α concentration was assessed in supernatant of γδ[2] and γδ[T2] cells from 3 separate donors (D1-D3) following expansion for 14 days. Statistical analysis was by unpaired Student *t*-test. (**G**) FoxP3 and CD25 co-expression in γδ[2] and γδ[T2] cells after 14d expansion (mean +/- SEM, n=3). (**H**) Representative example of FoxP3 expression in γδ[T2] cells (expanded for 14d) and $CD4^+$ $CD25^+$ $CD127^{LO}$ T-regs, enriched by negative selection. Gates were set with an APC-conjugated isotype control antibody. MFI – mean fluorescence intensity. (**I**) Representative suppression assay in γδ[2] and γδ[T2] cells were added to cell trace violet (CTV)-labeled $CD4^+$ cells (depleted of $CD25^+$ $CD127^{LO}$ T-regs) that had been activated with CD3+CD28 beads. Flow cytometry analysis was performed after 96h. (**J**) IL-10 concentration was assessed in supernatant of γδ[2] and γδ[T2] cells from 3 separate donors (D1-D3) following expansion for 14 days. Statistical analysis was by unpaired Student *t*-test. *Related to Figure 1.*

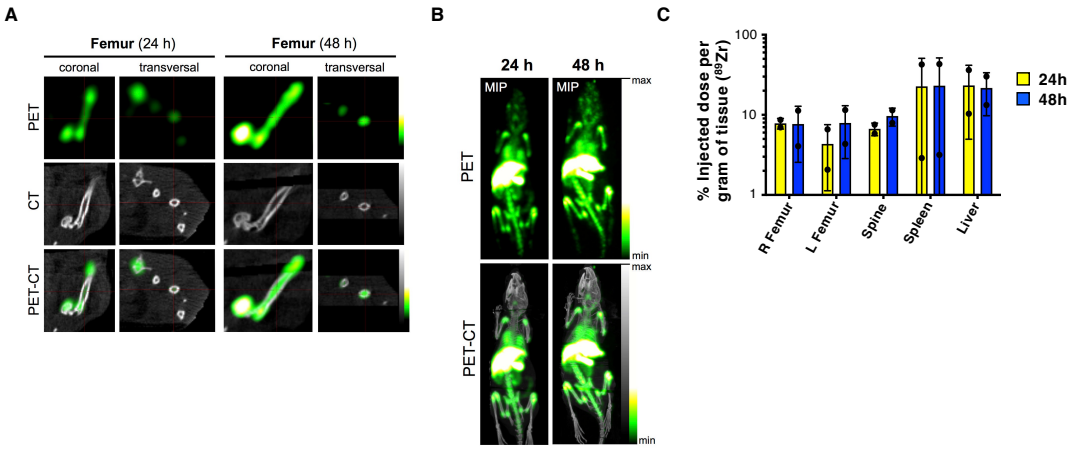

Figure S2. Analysis of trafficking of γδ[T2] cells

[^{89}Zr]Zr(oxinate)$_4$-labeled γδ[T2] T-cells were administered i.v. to NSG mice. Representative images of femur (centered on the medullary cavity; **A**) and whole-body following PET-CT imaging at the specified times (**B**). Proportion of total radioactivity in the indicated organs was determined by gamma counting (**C**). Data were normalized to total activity in each mouse (mean +/- SD n=2; Neg – negative control). *Related to Figure 1.*

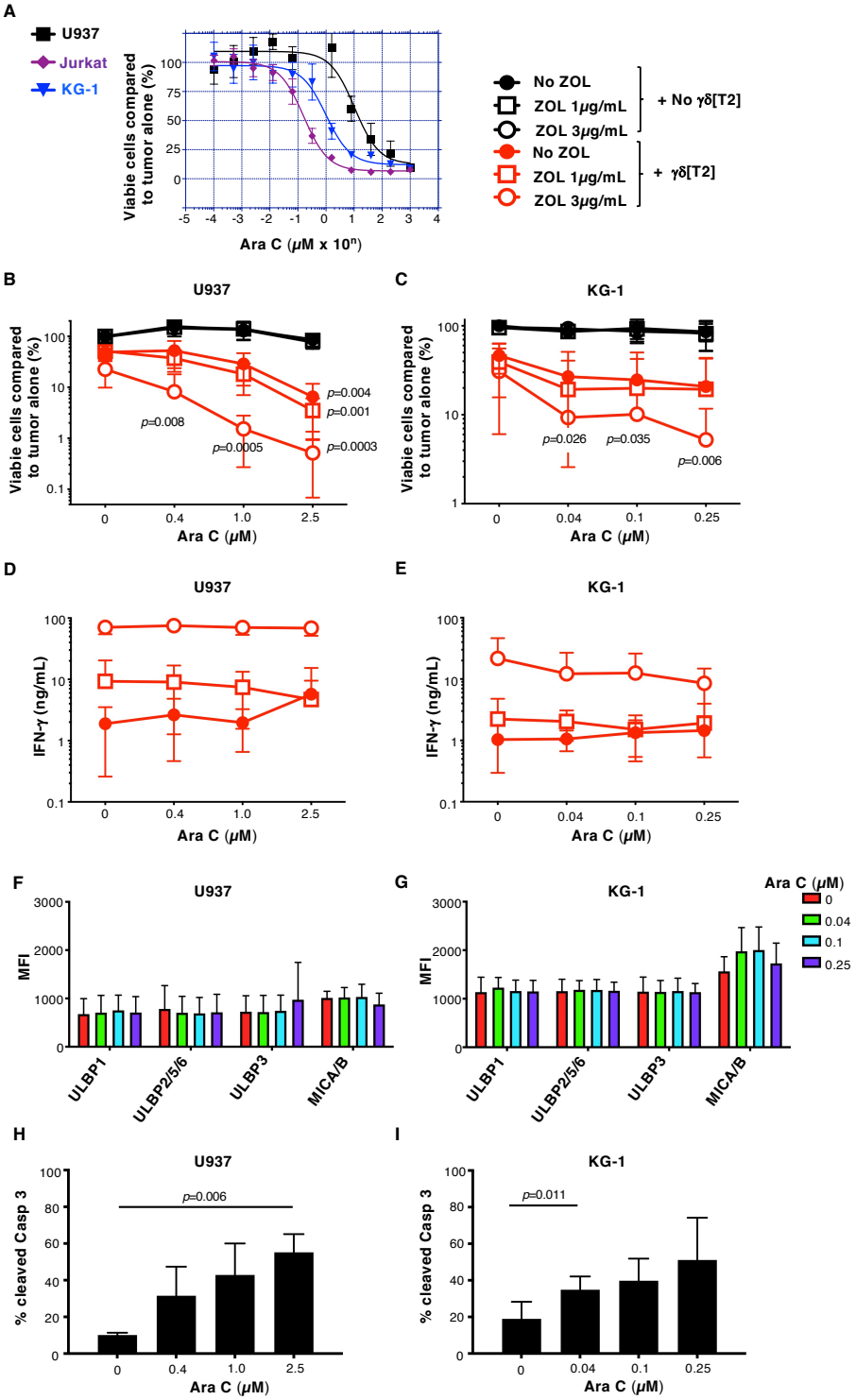

Figure S3. Sensitizing action of Ara C to cytotoxic activity of γδ[T2] cells

(**A**) Firefly luciferase-expressing leukemic cell lines were cultured in Ara C at the specified concentration for 48h. Viability was determined by luciferase assay (mean +/- SD, n=3-5 independent replicates). U937 (**B**) or KG-1 (**C**) cells were sensitized for 24h with the indicated concentrations of Ara C +/- ZOL followed by addition of γδ[T2] cells at a 1:1 E:T ratio. Leukemic cell viability was assessed after a further 24 hours by luciferase assay. Statistical analysis in B-C was performed by two-way ANOVA. *p* values show significance compared to cytotoxic effect of γδ[T2] cells alone, without additional ZOL or Ara-C. IFN-γ content of supernatants derived from U937 (**D**) and KG-1 co-cultures (**E**) was analyzed by ELISA (mean +/- SD, n=3-5 independent replicates). U937 (**F, H**) and KG1 leukemic cells (**G, I**) were treated with the indicated concentration of Ara C for 48 hours and then analyzed by flow cytometry for cleaved Casp(ase) 3 (**F, G**; mean +/- SD, n=3-4 independent replicates) or the indicated NKG2D ligands (**H, I**; mean +/- SD, n=4 independent replicates) . Statistical analysis in H-I was performed using one-way ANOVA. *Related to Figure 3.*

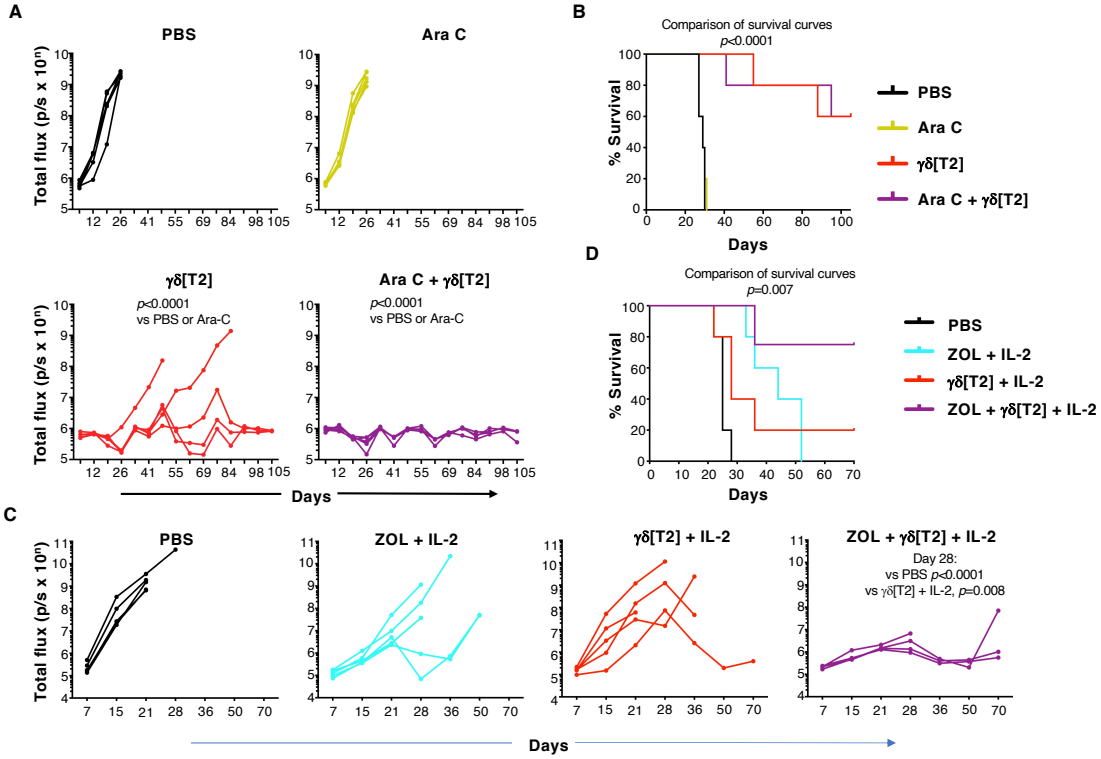

Figure S4. Immunotherapy of leukemia with γδ[T2] cells

(**A**) NSG mice were inoculated i.v. with 1 x 10^5 ffLuc$^+$ Jurkat cells on day 1. Indicated groups of mice received 110mg/kg of Ara C i.v. on day 5 and/or γδ[T2] T-cells (5 x 10^6 cells) i.v. on day 6. Serial BLI emission from individual mice is shown. (**B**) Kaplan Meier survival curve of mice shown in panel A. Two mice in the ZOL + Ara C + γδ[T2] group died of infection and were disease free. (**C**) SCID Beige mice were inoculated i.v. with 1 x 10^6 ffLuc$^+$ U937 cells (day 1). Groups of mice received: (i) ZOL (20μg) i.v. on day 2 and/or (ii) γδ[T2] T-cells (15 x 10^6 cells) i.v. on day 3 and (iii) IL-2 (5000i.u.) twice daily i.v. on days 3-5, making comparison with PBS alone. Serial BLI of individual mice is shown. (**D**) A Kaplan Meier survival curve of mice shown in panel C. All BLI data were analyzed using two-way ANOVA, and survival data were analyzed using the Log-rank (Mantel-Cox) test. *Related to Figure 3.*

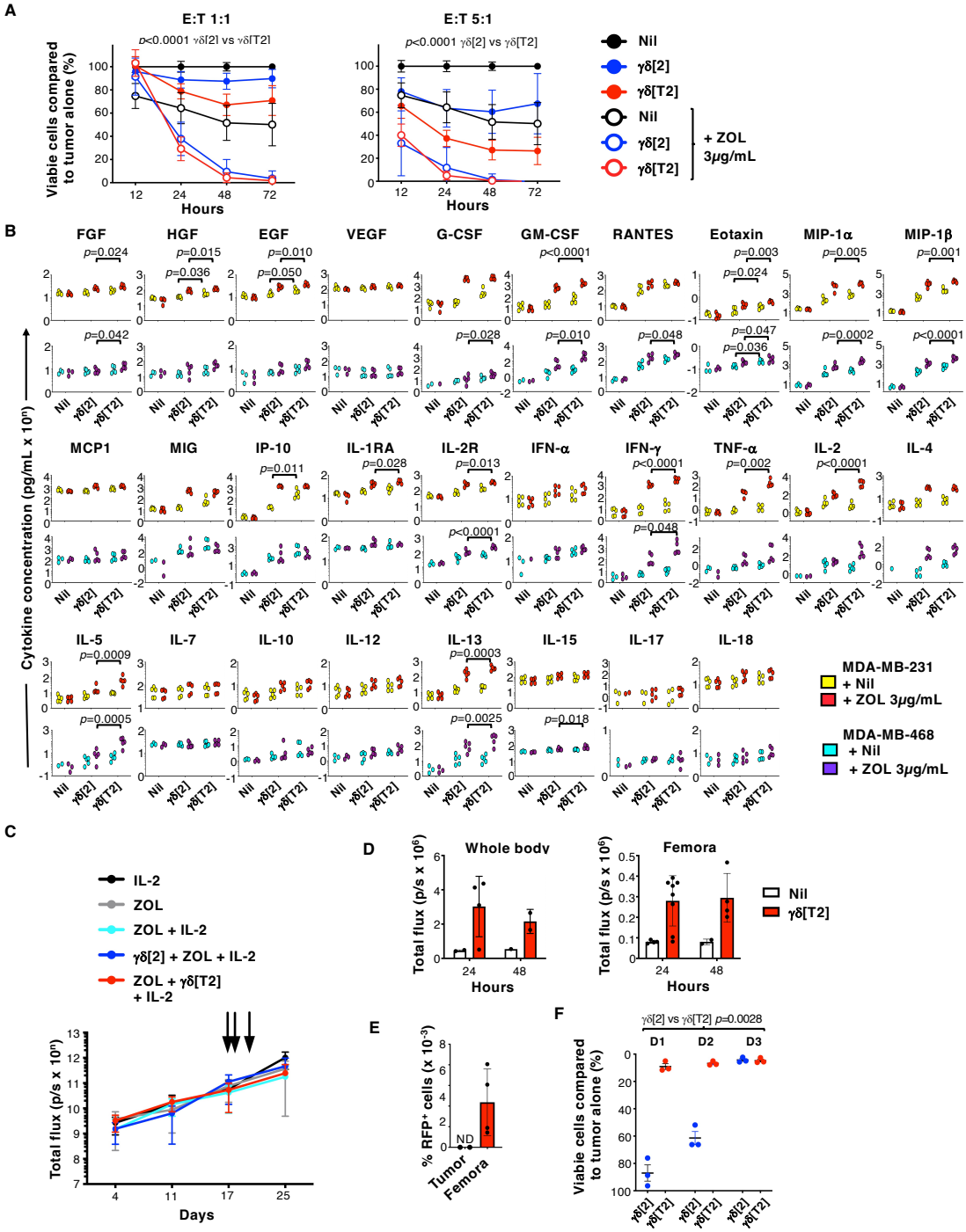

Figure S5. ***In vitro*** **analysis of γδ[2] and γδ[T2] γδ T-cells in solid tumor models**
(**A**) MDA-MB-231 cells ($2x10^4$ cells) were plated were plated on a 96 well electronic microplate. After 24h, cells were pulsed with ZOL (3μg/mL) or media alone. After a further 24h, γδ[2] or γδ[T2] cells were added at the indicated E:T ratio. Dynamic monitoring of adherent tumor viability/ proliferation was performed using an xCELLigence MP impedance analyzer (mean +/- SD n=5). Statistical analysis by two-way ANOVA. (**B**) Supernatants were collected after 24h co-culture of γδ T-cells with MDA-MB-231 and MDA-MB-468 TNBC cell lines at a 5:1 E:T ratio and analyzed for cytokine content using a Luminex array (mean +/- SD n=5). Data are presented on a Log_{10} scale and were analyzed using two-way ANOVA. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (**C**) SCID Beige mice with an orthotopic MDA-MD-231 tumor received ZOL (d17), γδ[2] or γδ[T2] cells (2×10^7 cells; d18) and IL-2 (1000 U d20) (all arrowed). Tumor status was imaged by BLI. (**D**) γδ[T2] T-cells were engineered to co-express ffLuc and RFP and 10 million cells injected i.v. into 4 SCID Beige mice with an orthotopic MDA-MB-231 tumor. BLI was performed after 24h and 48h to determine persistence of γδ T-cells in the whole body and in a femoral region of interest. (**E**) After 48h, the % RFP^+ γδ[T2] cells present in tumor (n=2) and bone marrow (n=4 femora) were determined. (**F**) SH-SY5Y neuroblastoma cells were pulsed with ZOL (1μg/mL). After 24h, γδ[2] or γδ[T2] cells (E:T ratio 5:1) from 3 separate donors (D1-D3) were added. Residual tumor cell viability was assessed after a further 24h. Statistical analysis by unpaired Student's *t*-test. All data are presented as mean +/- SD. NS – not significant. ND – not detected. *Related to Figure 4.*

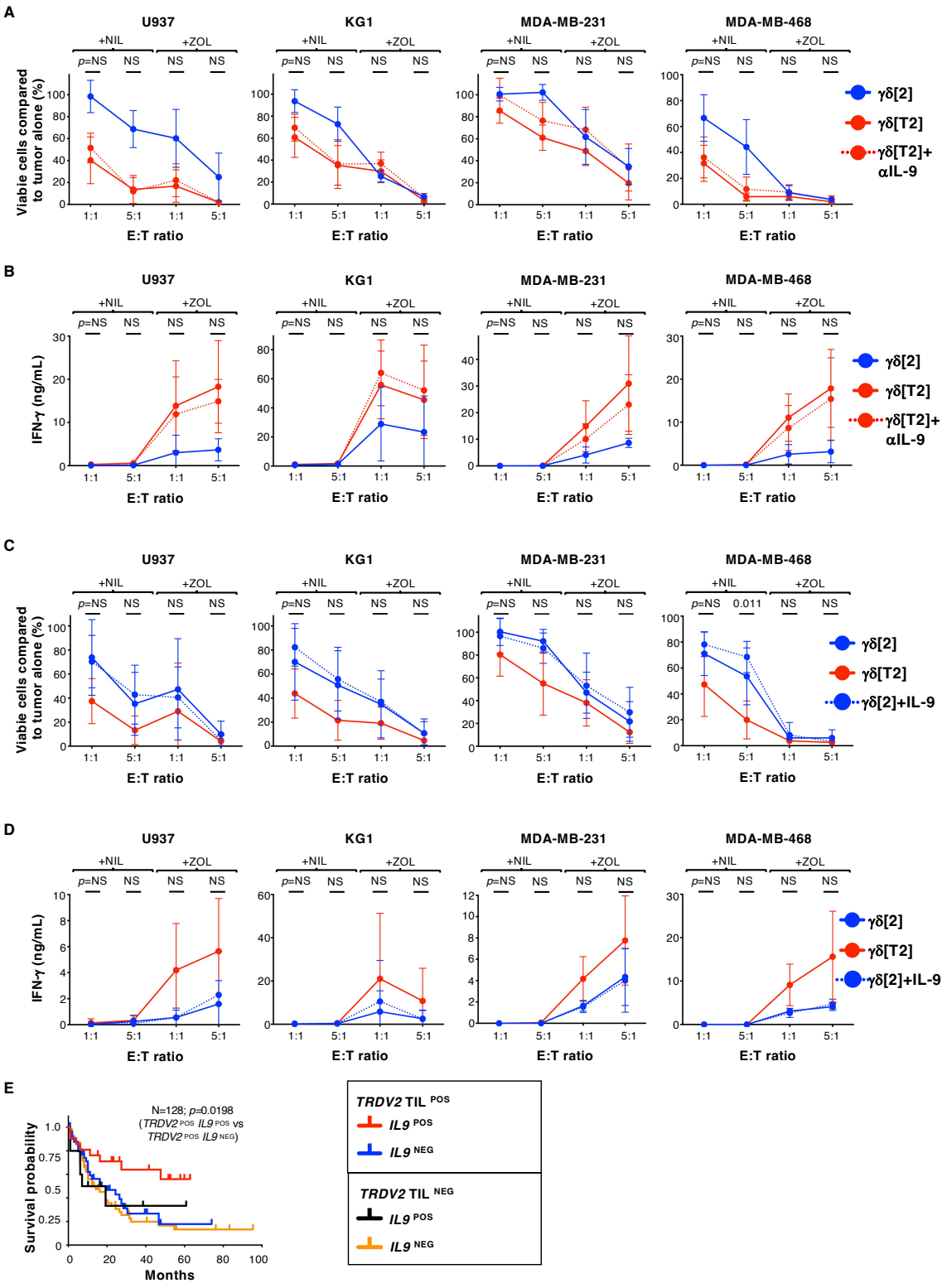

Figure S6. Role of IL-9 in function of γδ[T2] cells

Luciferase-expressing tumor cells were incubated +/- ZOL (3μg/mL) for 24h prior to co-culture for 24h at E:T ratios 1:1 and 5:1 with γδ[2] cells (**A**-**D**), γδ[T2] cells (**A**-**D**), γδ[T2] cells that had been expanded in the presence of αIL-9 blocking antibody (3μg/mL; concentration greater than ND50 for 5ng/mL IL-9; **A**-**B**) or γδ[2] cells that had been expanded in IL-9 (30ng/mL; **C**-**D**). Both IL-9 and αIL-9 blocking antibody were replenished in cultures 3 times per week. Percentage viability was determined by luciferase assay after 24h, making comparison with untreated tumor cells (**A**, **C**). Supernatants collected from these co-cultures after 24h were analyzed for IFN-γ production by ELISA (**B**, **D**). All data show mean +/- SD (n = 9-21 replicates in A, C; 5-14 in B, D) and were analyzed using two-way ANOVA. *p* values shown above each E:T ratio compare γδ[T2] versus γδ[T2]+αIL-9 (A,B) or γδ[2] versus γδ[2]+IL-9 (C,D). (**E**) The impact of *IL9* on survival in AML was determined using Kmplot and Log-rank test analysis for tumors in which *TRDV2* TILs were present or absent (numbers per cohort indicated). Top (POS) and bottom (NEG) thirds are shown for *IL9* in each case. *Related to Figure 6.*

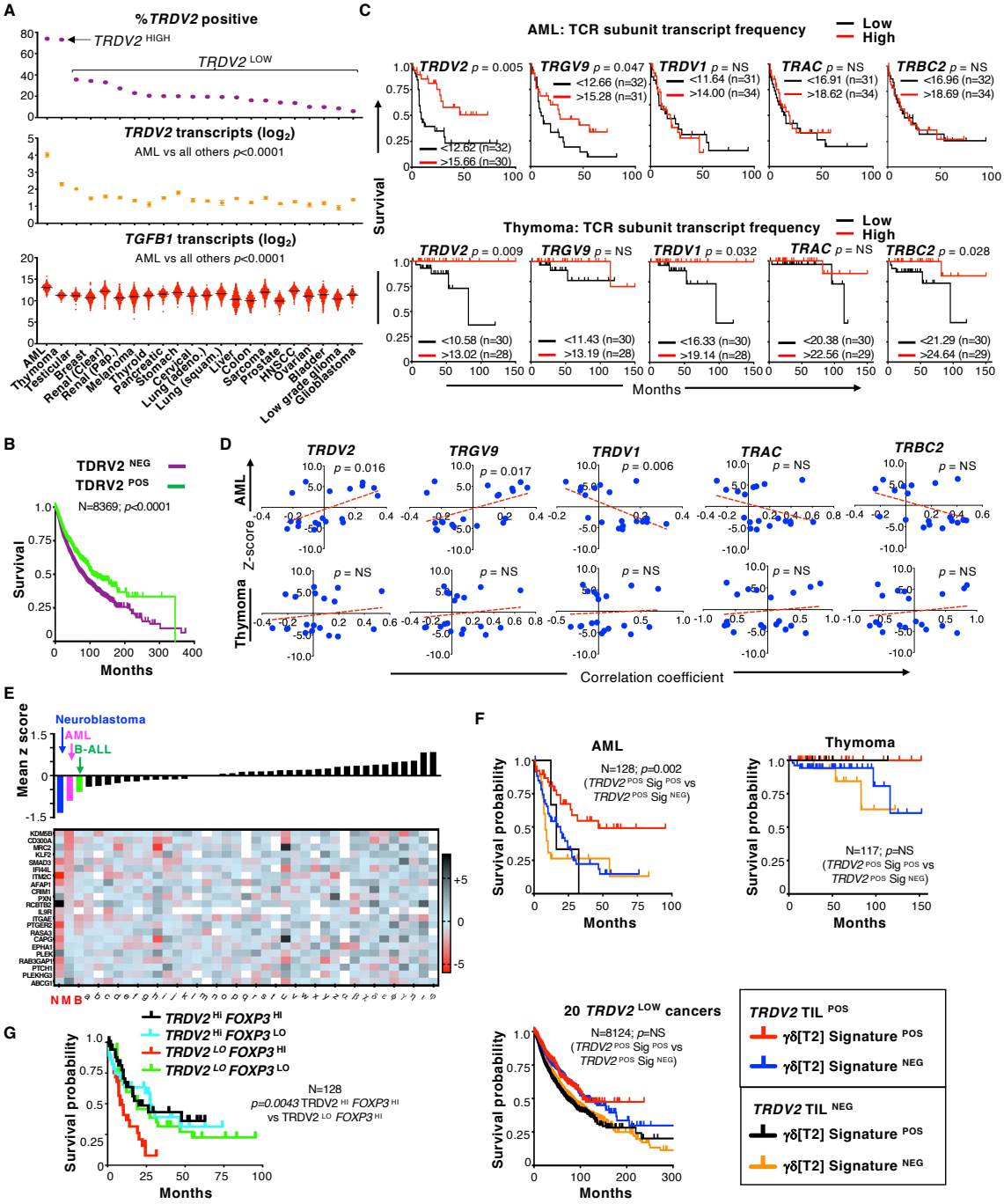

Figure S7. A γδ[T2] gene signature is present in AML and is associated with improved prognosis.
(**A**) Percentage of the indicated 22 cancers (N=8369) in which *TRDV2* transcripts were detected (TCGA; top panel). Transcript quantification is shown in the middle panel. Units are $\log_2$ fragments per kb transcript per million mapped reads upper quartile +1 (mean +/- SEM). Transcripts of *TGFB1* were similarly quantified in these cancers (mean +/- SEM; lower panel). Statistical analysis - one way ANOVA. Adeno – adenocarcinoma; AML, acute myeloid leukemia; clear – clear cell carcinoma; HNSCC, head and neck squamous cell carcinoma; Pap - papillary cell carcinoma; Squam – squamous cell. (**B**) Kaplan Meier curves and Log-rank test analysis showing the association between *TDRV2* expression and survival across 22 cancers listed in A. (**C**) Kaplan Meier curves and Log-rank test analysis showing the association between indicated TCR subunit transcript frequencies and survival in AML and thymoma (TCGA GDC). Patient numbers per cohort are indicated in each plot. (**D**) Correlation coefficient of indicated TCR transcripts and genes that are differentially expressed in γδ[2] and γδ[T2] cells (x axis; AML and thymoma TCGA GDC datasets) was plotted against z-scores of these differentially expressed genes. Statistical analysis was performed using simple linear regression. (**E**) Prediction of clinical outcomes from genomic profiles (PRECOG) analysis querying the association of a transcriptional 24 gene γδ[T2] cell signature in the following cancers: N neuroblastoma; M AML; B B-cell acute lymphoblastic leukaemia; a liver cancer; b mesothelioma; c bladder cancer; d multiple myeloma; e Burkitt lymphoma; f follicular lymphoma; g osteosarcoma; h DLBCL; i adenocarcinoma of lung; j colon cancer; k gastric cancer; l hypopharyngeal cancer; m pancreatic cancer; n small cell carcinoma of lung; o Ewing sarcoma; p oral squamous cell cancers; q medulloblastoma; r squamous cell carcinoma of lung; s meningioma; t kidney cancer; u glioma; v large cell carcinoma of lung; w melanoma; x esophageal cancer; y germ cell tumors; z prostate cancer; α mantle cell lymphoma; β ovarian cancer; χ head and neck cancers; δ breast cancer; ε glioblastoma; ϕ metastatic melanoma; γ astrocytoma; η adrenocortical cancer; ι chronic lymphocytic leukemia; φ liver cancer primary. Mean z scores are shown above the heatmap indicating expression of each differentially expressed gene. (**F**) The impact of γδ[T2] cell signature on survival in AML, thymoma and the remaining 20 *TRDV2* ^{LO} cancer types was determined using Kmplot and Log-rank test analysis for tumors in which *TRDV2* TILs were present or absent (numbers per cohort indicated). Top (POS) and bottom (NEG) thirds are shown for γδ[T2] cell signature in each case. (**G**) The impact of FoxP3 status on survival in AML, ranked for high or low content of *TRDV2* transcripts. Analysis was performed using Kmplot and Log-rank testing. *Related to Figure 6.*