Supplementary Figures: Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-G_{D2} CAR Expression and Inducible Cytokines

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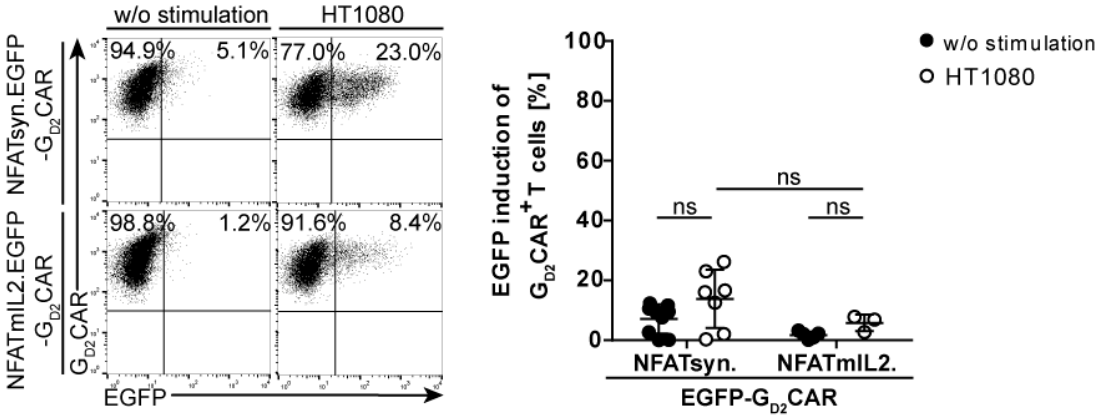

Figure S1. Co-culture of transduced T cells with GD2$^-$ target cells led to a moderate EGFP upregulation. Comparison of inducible promoters, namely NFATsyn and NFATmIL2. Primary human T cells were transduced with "all-in-one" EGFP constructs (NFATsyn.EGFP-G_{D2}CAR or NFATmIL2.EGFP-G_{D2}CAR), sorted and co-cultured with GD2$^-$ target cells. G_{D2}CAR-specific activation with HT1080 cells (GD2-negative) results in a moderate NFAT promoter-regulated EGFP expression similarly to the control without any target cells. Representative flow cytometric analysis of NFAT-driven EGFP expression in transduced T cells after 24 h co-culture with a 6:1 effector:target (E:T) ratio. Data shown are from the same experiment displayed in Figure 3. The graph summarizes multiple experiments of NFAT promoter-driven EGFP expression in "all-in-one" construct-positive ($G_{D2}CAR^+$) T cells post stimulation with HT1080 target cells. Shown are mean values ± SD; symbols indicate individual co-culture experiments with individual donors; n = 3–8. Indicated statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test; ns > 0.05).

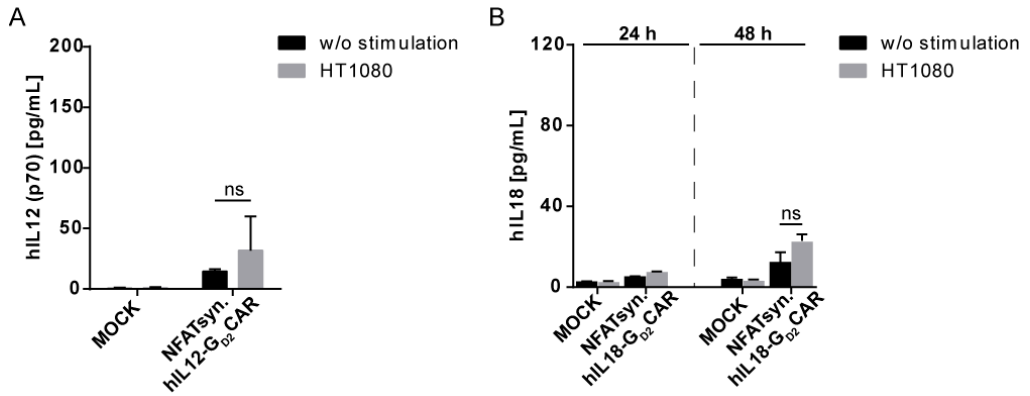

Figure S2. Human IL12 and human IL18 secretion after stimulation with GD2$^-$ target cells. (**A**) Human IL12 expression and (**B**) human IL18 expression in cell culture supernatants. Primary T cells were transduced with

"all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR constructs and co-cultured with GD2$^-$ target cells (HT1080) for 24 h (and 48 h) or without any cells. Inducible hIL12 or hIL18 secretion was determined via ELISA. ELISAs were performed in triplicates in two (hIL18) or three (hIL12) independent experiments; unsorted cells; 10:1 effector:target (E:T) ratio. Shown are mean values ± SD. Indicated significance was determined by unpaired two-tailed *t*-test; (ns > 0.05).

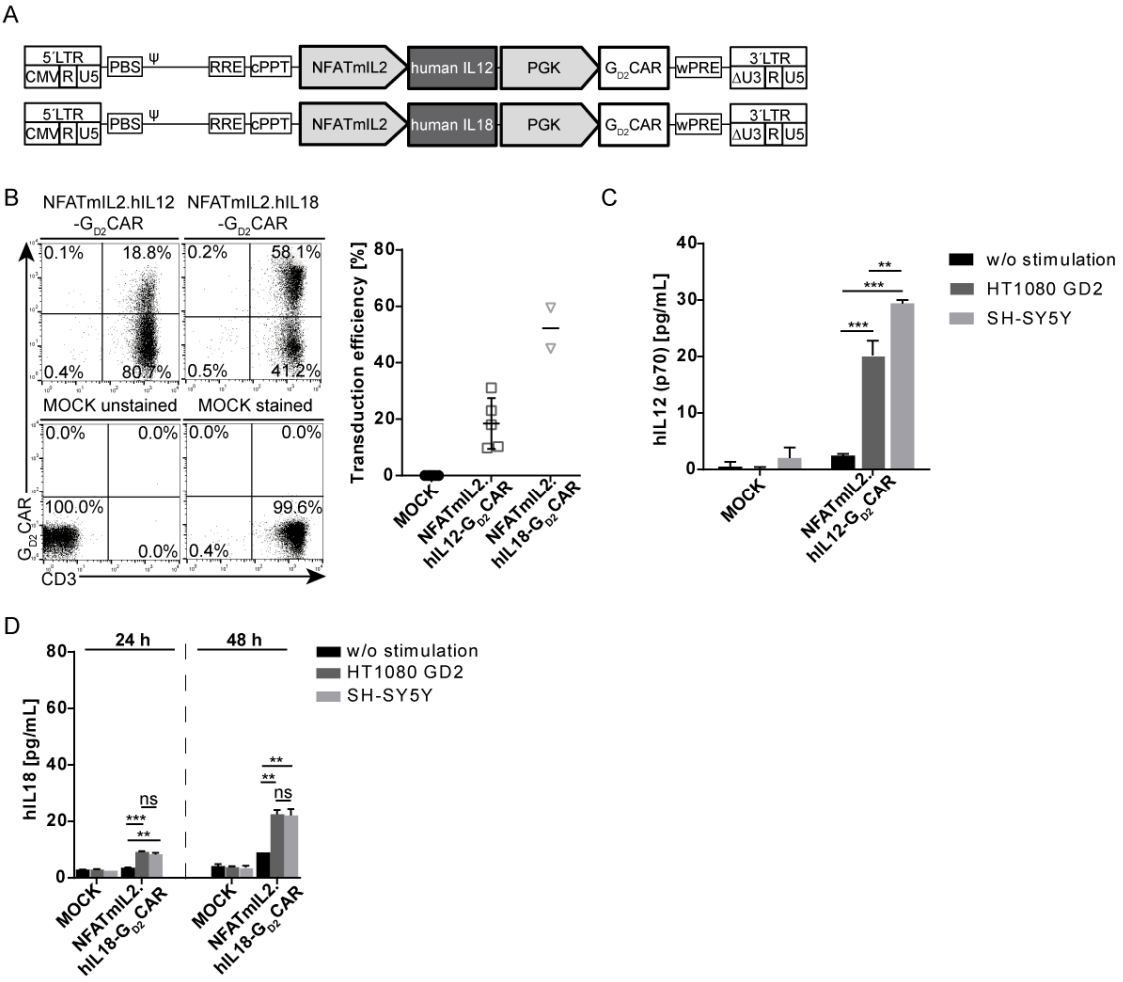

Figure S3. Inducible NFATmIL2-driven human IL12 and human IL18 secretion after CAR-specific stimulation. (**A**) Schematic design of the "all-in-one" NFATmIL2.hIL12-G_{D2}CAR and NFATmIL2.hIL18-G_{D2}CAR constructs. Indicated is the inducible promoter (NFATmIL2). (**B**) Representative flow cytometric analysis of the "all-in-one" NFATmIL2.hIL12-G_{D2}CAR and NFATmIL2.hIL18-G_{D2}CAR constructs in transduced primary human CD3$^+$ T cells (MOI 10). Transduction efficiency was determined six days post transduction via a G_{D2}CAR-specific antibody that recognizes the scFv region. Graph summarizes transduction efficiencies of the "all-in-one" NFATmIL2.hIL12-G_{D2}CAR and NFATmIL2.hIL18-G_{D2}CAR constructs in primary human T cells. Shown are mean values ± SD (symbols indicate individual donors; n = 2–5). (**C**) Human IL12 expression and (**D**) human IL18 expression in cell culture supernatants. Primary human T cells were transduced with "all-in-one" NFATmIL2.hIL12-G_{D2}CAR and NFATmIL2.hIL18-G_{D2}CAR constructs and co-cultured with GD2$^+$ target cells for 24 h (or 48 h) in a 10:1 effector:target (E:T) ratio. Inducible IL12 or IL18 secretion was determined via ELISA. ELISAs were performed in triplicates in two independent experiments. Shown are mean values ± SD. Indicated significance was determined by one-way ANOVA with Bonferroni´s multiple comparison test; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns > 0.05.

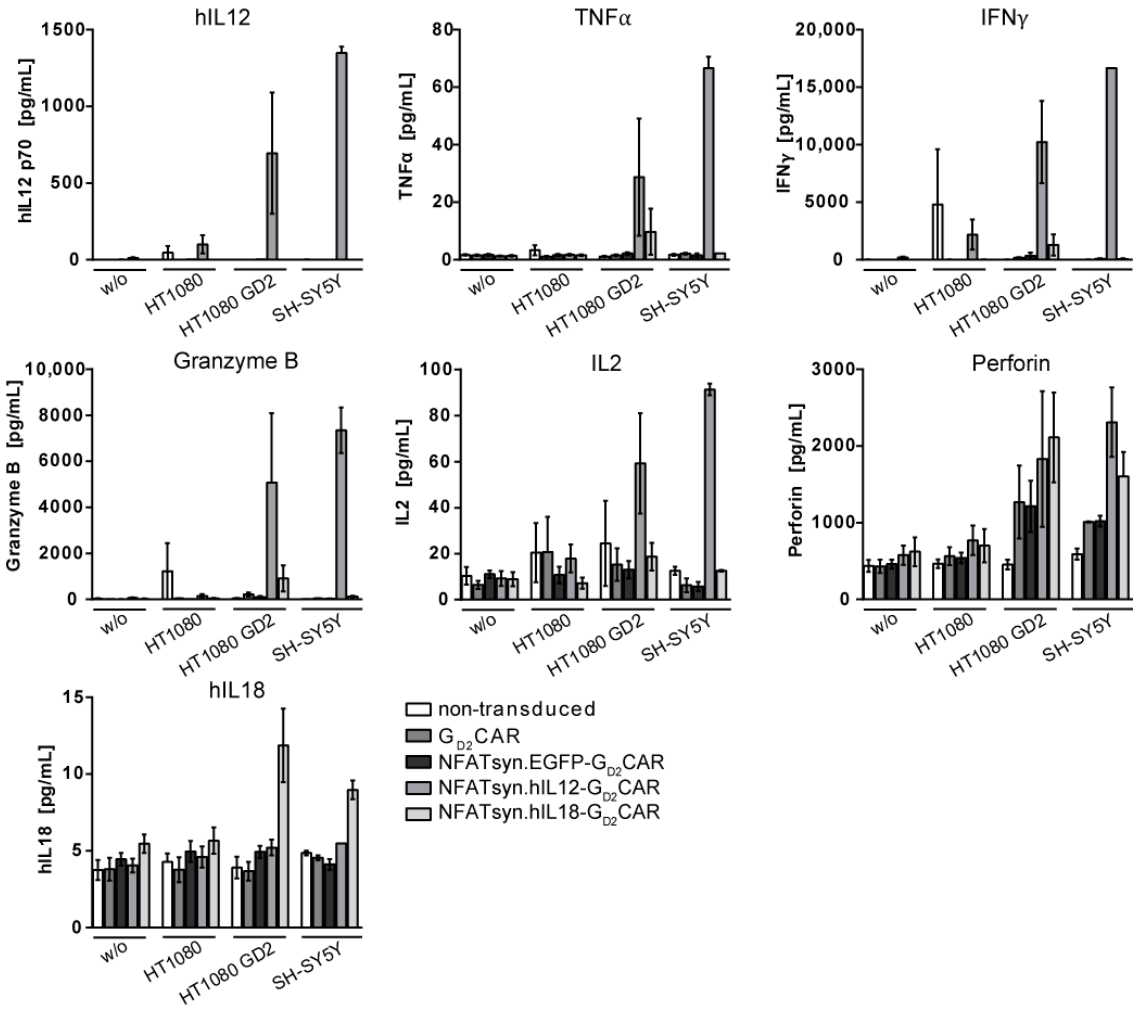

Figure S4. Cytokine analysis after G_{D2}CAR-specific activation in human T cells transduced with "all-in-one" vector constructs. Cytokine concentrations in co-culture supernatant were determined after 48 h co-culture experiments of transduced primary T cells stimulated with indicated target cells in a 6:1 effector:target (E:T) ratio using a customized LEDGENDplex™ Multi-Analyte Flow Assay (BioLegend), which allowed simultaneous detection of human IL12, TNFα, IFNγ, granzyme B, IL2, perforin and human IL18. Shown are mean values ± SD (symbols indicate individual donors; n = 2–5).