

1 **Knocking out CD70 rescues CD70-specific nanoCAR T cells** 2 **from antigen induced exhaustion**

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20 **Abstract**

21 CD70 is an attractive target for chimeric antigen receptor (CAR) T cell therapy as treatment for
22 both solid and liquid malignancies. However, functionality of CD70-specific CARs is only
23 modest. Here, we optimized a CD70-specific VHH based CAR (nanoCAR). We evaluated the
24 nanoCARs in clinically relevant models *in vitro*, using co-cultures of CD70-specific nanoCAR
25 T cells with malignant rhabdoid tumor organoids, and *in vivo* by using a diffuse large B cell
26 lymphoma (DLBCL) patient-derived xenograft (PDX) model. Whereas the nanoCAR T cells
27 were highly efficient in organoid co-cultures, they showed only modest efficacy in the PDX
28 model. Knocking out CD70 expression by the nanoCAR T cells resulted in dramatically
29 enhanced functionality in the PDX model, suggesting that endogenous CD70 interaction with
30 the nanoCAR induces exhaustion. Through single-cell transcriptomics, we obtained evidence
31 that CD70KO CD70-specific nanoCAR T cells are protected from antigen induced exhaustion.

32 Our data shows that CARs targeted to endogenous T cell antigens, negatively affect CAR T
33 cell functionality by inducing an exhausted state which can be overcome by knocking out the
34 specific target, in this case CD70.

35 Introduction

36 Adoptive transfer of chimeric antigen receptor (CAR) T cells has demonstrated great potential
37 for the treatment of various hematological malignancies¹⁻⁷. While response rates are high,
38 relapses still occur due to the emergence of antigen escape variants or due to the lack of long
39 term *in vivo* persistence of the CAR T cells^{1,4,5,8-10}. Furthermore, the efficacy in solid tumors
40 has yet to be demonstrated as the tumor suppressive microenvironment, homing inside the
41 tumor, tumor heterogeneity and the lack of optimal target antigens may compromise their
42 efficacy¹¹⁻¹³.

43 CD70 is an attractive and broadly applicable target antigen. It is a tumor necrosis factor (TNF)
44 superfamily member that acts as co-stimulatory ligand for CD27. CD70 has a broad expression
45 pattern on multiple solid and liquid malignancies such as clear cell renal cell carcinoma,
46 pancreatic cancer and some hematological malignancies such as acute myeloid leukemia (AML)
47 and diffuse large B cell lymphoma. Furthermore, expression has been reported on both primary
48 and metastatic tumor samples. Additionally, CD70 expression is associated with a poor
49 prognosis and a decreased survival¹⁴⁻¹⁶. CD70 has been extensively studied in the context of
50 AML, for which it has been shown by Riether *et al.* that the ligand-receptor pair CD70/CD27 is
51 expressed on AML blasts and AML stem/progenitor cells. This results in cell-autonomous
52 CD70/CD27 signaling which propagates the disease through activation of a stem cell gene
53 expression program, Wnt pathway activation and promotion of symmetric cell divisions and
54 proliferation¹⁷. Recently, cusatumab, an antibody targeting CD70, showed impressive
55 response rates in a phase I trial for AML patients unfit for traditional intensive-induction
56 chemotherapy^{18,19}. CD70 is not expressed in healthy tissues nor on hematopoietic stem cells
57 but is transiently upregulated on activated immune cells such as B cells, T cells and dendritic
58 cells¹⁵. In activated T cells, it provides survival signals and influences memory formation. CD70
59 specific CAR T cells were reported to inhibit anti-viral immune responses²⁰.

60 CARs against CD70 have been developed by several groups using different strategies. A first
61 strategy utilized a ligand-based targeting approach, the CD27 protein fused to the CD3 ζ
62 domain (fICD27- ζ), and showed efficacy towards multiple different B cell malignancies²¹. This
63 design was further optimized by fusing extracellular CD27 with intracellular 4_1BB (trCD27-
64 4_1BB: ζ) resulting in both a higher *in vivo* and *in vitro* functionality²². This construct is subject
65 of an ongoing phase I/II clinical trial (NCT02830724). A last optimization was performed by
66 Leick and colleagues: in a two way approach they increased the functional avidity and thus

67 functionality of the trCD27-4_1BB:ζ CAR²³. Next to the ligand-based targeting approach, a
68 second strategy uses single chain variable fragments (scFvs) as target antigen recognition
69 domains. A study by Sauer *et al.* showed that the original flCD27-ζ CAR outperformed a panel
70 of scFv based CARs²⁰. In a more recent study, multiple scFvs all targeting CD70 were
71 evaluated. Two distinct classes of scFvs were defined based on their CD70-specific binding
72 epitope. When used in CARs, these resulted in CAR T cells with different memory phenotypes,
73 activation statuses and cytotoxic activity²⁴.

74 While CD70 is an attractive target antigen due to its expression on multiple different tumor
75 types, CD70 expression on malignant cells both solid as liquid, is lower and less uniform
76 compared to the expression of CD19 in B cell malignancies. Since CAR T cell activity is
77 dependent on antigen load, it could be necessary to increase the expression level of CD70 on
78 the cell membrane^{1,8,25-27}. Indeed, it has been proven in a pre-clinical study that CD70-specific
79 CAR T cells have a higher efficacy when combined with the hypomethylating agent azacitidine
80 which acts as a nucleoside analog, inhibiting DNA methyltransferase and decreasing
81 methylation of the CD70 promotor, resulting in an increased surface expression of CD70²³.
82 The use of azacitidine has also been assessed in a clinical trial using the CD70 specific
83 monoclonal antibody cusatuzumab¹⁹. A similar approach has also shown its merit in a
84 preclinical model of a CD22-targeting CAR. Here, bryostatin 1, a macrocyclic lactone that acts
85 as modulator of kinase C, was used to increase the antigen density of CD22 on leukemic cell
86 lines, resulting in higher CD22 expression and a higher efficacy of CD22-specific CAR T cells²⁶.

87 While scFvs are commonly used as targeting domain in CAR T cells, also other antigen binding
88 moieties can be used. We have previously shown that nanobodiesTM, the variable regions of
89 heavy chain only antibodies (VHH), can be used as antigen recognition domains in CARs. We
90 termed these CARs as nanoCARs^{28,29}. Due to their monomeric structure they do not require a
91 conversion from immunoglobulin (Ig) towards single chain variable fragment (scFv).
92 Furthermore, they have a high solubility and lack aggregative properties which reduce the
93 likelihood of CAR clustering and tonic signaling³⁰. Lastly, due to their smaller size and high
94 sequence homology with the human VH3 gene family, they have a lower immunogenicity as
95 compared to murine scFvs^{31,32}. If needed, they can be easily humanized³³.

96 In this study, we optimized a CD70-specific nanoCAR. First, we generated multiple nanoCAR
97 constructs which differ in co-stimulatory moiety. The lead nanoCAR was selected based on
98 the best *in vivo* functionality and persistence. Next, we evaluated the lead nanoCAR *in vitro*
99 and *in vivo* against patient derived tumor cells in clinically relevant model systems. We
100 observed complete elimination of organoid cultures of malignant rhabdoid tumors at very low
101 effector to target (E:T) ratios. However, when tested in a DLBCL patient-derived xenograft
102 (PDX) model, only a modest efficacy could be observed. Using single-cell transcriptomics, we

103 could show that tumor-infiltrated CD70-specific nanoCAR T cells acquire an exhausted
104 phenotype during activation resulting in less potent nanoCAR T cells. *CD70* gene deletion in
105 the nanoCAR T cells dramatically altered the T cell transcriptome and improved *in vivo*
106 functionality. Our data shows that CARs targeted to T cell antigens could negatively alter CAR
107 T cell efficacy by inducing an exhausted state and that this could be overcome by knocking out
108 the specific target, in this case CD70.

109 **Results**

110 **JAK-STAT activation does not enhance CD70-specific nanoCAR functionality**

111 Out of a library of 33 llama VHH, the VHH with the highest affinity for CD70 was selected. It
112 was reported that addition of STAT3 and STAT5 docking sites to the cytoplasmic tail of the
113 CAR improves activity against solid tumors³⁴. Therefore, we generated a panel of four CD70-
114 specific nanoCAR constructs with different transmembrane and/or intracellular domains. Two
115 second generation nanoCARs were generated: i) nanoCAR with CD8 α as spacer and
116 transmembrane domain and 4_1BB as co-stimulatory domain (70-4-1BB: ζ) and ii) nanoCAR
117 with CD28 as spacer, transmembrane domain and co-stimulatory domain (70-28: ζ) (Figure
118 1A). In addition, we incorporated a truncated cytoplasmic domain of the interleukin -2 (IL-2)
119 receptor β between the cytoplasmic domains of 4_1BB (70-4-1BB: β : ζ^*) or CD28(70-28: β : ζ^*)
120 and CD3 ζ for STAT5 recruitment, and added a YXXQ motif at the C-terminus of CD3 ζ for
121 STAT3 recruitment, as described previously³⁴ (Figure 1A). NanoCAR T cells were generated
122 by retroviral transduction. After staining with a VHH specific antibody, we could validate
123 expression of all the nanoCAR constructs on the cell surface (Figure 1B). However, nanoCARs
124 containing the truncated IL-2 receptor β chain showed a lower mean fluorescent intensity (MFI)
125 (Figure 1C). Furthermore, 70-4_1BB: β : ζ^* showed a consistently lower transduction efficiency
126 as compared to the other three nanoCAR constructs (Figure 1D). We tested the functionality
127 of the different nanoCAR constructs *in vitro* against the CD70⁻ cell line Nalm6 and the CD70⁺
128 cell line SKOV3 (Figure S1A). IFN- γ and IL-2 were produced upon incubation with the CD70⁺
129 cell line, while there was no production when the nanoCAR T cells were incubated with Nalm6
130 (Figure 1E). All nanoCAR T cells showed cytokine production and the highest percentage of
131 IL-2⁺ cells could be observed in nanoCARs containing the CD28 co-stimulatory domain.
132 Interestingly, 70-4_1BB: β : ζ^* nanoCAR T cells showed an overall lower cytokine production
133 profile for both the CD4⁺ and the CD8⁺ T cells, suggesting a potential lower functionality. To
134 evaluate the *in vivo* antitumoral effect of the CD70-specific nanoCAR T cells, a SKOV3 murine
135 xenograft model was used, in which NSG mice were subcutaneously injected with the CD70⁺
136 ovarian cancer cell line SKOV3 (Figure 1F). Different concentrations of CD70-specific
137 nanoCAR T cells were injected and tumor growth was measured by caliper. At the highest

138 nanoCAR T cell doses, all four nanoCARs showed similar efficacy (Figure S1B-E). When the
139 dose was lowered, the 70-4_1BB:β:ζ* nanoCAR, combining both 4_1BB as JAK-STAT
140 signaling, was no longer capable of complete tumor eradication nor of tumor control (Figure
141 S1D-E). In addition, at the lowest dose, only second generation nanoCARs showed similar
142 high efficacy (Figure 1G-I). Antitumoral responses were long lasting for both second generation
143 nanoCARs but not for the 70-28:β:ζ* incorporating JAK-STAT signaling (Figure 1G-I). At day
144 63 post nanoCAR T cell injection, we assessed the presence of nanoCAR T cells in the spleen
145 of all treated mice (Figure 1H). We observed the highest frequency of nanoCAR⁺ T cells in
146 mice that were treated with a standard 4_1BB second generation nanoCAR, and the lowest
147 frequency in mice treated with the CD28 nanoCAR containing the domains for JAK-STAT
148 signaling. These data show that JAK-STAT signaling did not enhance nanoCAR T cell
149 functionality in this model and resulted in a less efficient nanoCAR T cell product. The 70-
150 4_1BB:ζ second generation nanoCAR outperformed all other nanoCAR T cell constructs in
151 terms of *in vivo* efficacy and persistence. As a result, we selected the 70-4-1BB:ζ CAR for
152 further optimization.

153 **Malignant rhabdoid tumor-derived organoids are efficiently killed by CD70-specific** 154 **nanoCAR T cells**

155 While cell lines are ideal for fundamental research, they are less relevant for translational
156 research due to loss of heterogeneity (e.g. target antigen expression) and divergence from the
157 original tumor present in the patient. To overcome this hurdle and show the potential of the
158 CD70-specific nanoCAR, we assessed the efficacy of CD70 nanoCAR T cells in CD70
159 expressing malignant organoid structures. These structures are derived from patient malignant
160 tissue and retain the tumor heterogeneity observed in patients. First, we analyzed CD70 mRNA
161 levels in Wilms tumor and malignant rhabdoid tumor (MRT) patient samples using data from
162 Calandrini *et al.*³⁵. While we observed no CD70 expression in primary Wilms tumor, mRNA
163 was expressed in all MRT samples to a variable degree (Figure 2A). Next, CD70 mRNA levels
164 were quantified in organoid lines derived from normal kidney, Wilms tumor and MRT³⁵. In line
165 with the tissues, normal kidney and Wilms tumor organoid lines showed no expression of CD70
166 while CD70 was expressed to variable degrees in MRT organoid lines, similar to the original
167 tumor (Figure 2B). CD70 membrane expression was further validated in four MRT organoid
168 lines using flow cytometry. This revealed that three out of the four lines display low to high
169 CD70 membrane expression (Figure 2C). One line (JD041T) did not show any CD70
170 expression and was taken as negative control in all subsequent experiments. NanoCAR T cells
171 were incubated with different organoid lines at multiple target to effector ratios. A CD20-specific
172 nanoCAR was used as negative control²⁹ (Figure 2D). Viable MRT cells were measured based
173 on luciferase activity over time. The CD70-specific nanoCAR T cells induced strong and

174 specific tumor lysis of all three antigen positive organoid lines even at target to effector ratios
175 of 100:1 (Figure 2E). The weakest cytotoxicity was seen after culture with the organoid line
176 60T2 when compared to the other organoid lines, although CD70 expression was high in these
177 organoids (Figure 2D-E). In addition, staining of the activation marker CD137 on nanoCAR T
178 cells showed strong expression after 20 hours of incubation with the organoid lines (Figure
179 2F). Expression of CD137 was in line with the killing efficiency towards the different organoid
180 lines, high expression correlated with high nanoCAR T cell efficacy. The CD70⁻ organoid line
181 JD041T was unaffected by the CD70-specific nanoCAR T cells. Some background nonspecific
182 killing activity was observed by both the CD70 as the CD20 CAR T cells, probably caused by
183 allogeneic reactivity (Figure 2D). Lastly, we assessed the motility of the CAR T cells in the
184 different organoid structures. We noticed interactions between the CD70-specific nanoCAR T
185 cells with organoid cells (Movie 1-3). These interactions resulted in killing of the CD70⁺
186 organoid cells and proliferation of the CD70-specific nanoCAR T cells (Figure 2G).

187 **CD70-specific nanoCAR T cells show efficacy in primary DLBCL PDX model**

188 Next, we explored the *in vivo* functionality of CD70-specific nanoCARs using clinical relevant
189 material. DLBCL were reported to express CD70¹⁵. We selected a DLBCL PDX that expressed
190 low levels of CD70 (Figure 3A). Fresh PDX cells were injected on day -28 and treated with 1 x
191 10e6 nanoCAR T cells on day 0. Treatment was evaluated by daily body weight measurements
192 (Figure 3B). Mice treated with CD70-specific nanoCAR T cells performed significantly better
193 compared to PBS control, although the antitumor effect was modest (Figure 3C). Although
194 mice were succumbing to disease, we still observed both nanoCAR T cells and DLBCL cells
195 40 days post nanoCAR T cell injection in the liver, an organ which is consistently infiltrated in
196 this DLBCL PDX model (Figure 3D), suggesting loss of nanoCAR T cell efficacy. Since we
197 selected a DLBCL PDX with low CD70 expression, we speculated whether antigen escape
198 was causing treatment failure. Antigen escape mutations are a common cause of CAR T cell
199 failure¹⁰. CD70 expression on blood DLBCL cells decreased over time in nanoCAR treated
200 mice. This reduction was observed starting at day 7 post nanoCAR T cell treatment (Figure
201 3E). At day 21 post nanoCAR T cell injection, virtually no CD70 expression could be detected
202 in the mice treated with nanoCAR T cells, suggesting antigen escape (Figure 3E). However,
203 sequencing of the CD70 cDNA showed no mutations (not shown). Next, the CD70⁻ DLBCL
204 cells of CAR T treated mice were isolated and injected in untreated NSG mice. After 28 days,
205 mice were sacrificed and CD70 expression was determined by flow cytometry. We observed
206 CD70 membrane re-expression comparable to the expression by DLBCL cells harvested from
207 untreated mice (Figure 3F). This suggests that the DLBCL cells in CAR T treated mice
208 expressed CD70 but membrane expression was downmodulated by interaction with the CAR
209 receptor as previously described³⁶.

210 **CD70 knock out in CD70-specific nanoCAR T cells reverses dysfunctionality**

211 Since antigen escape mutations were not causing CD70-specific nanoCAR T cell treatment
212 failure, we speculated whether CD70 expression by CAR T cells could influence their
213 functionality. It is known that CD70 is expressed on activated T cells, however, CD70
214 expression on activated CAR T cells has not been reported^{20,37}. CD70-specific nanoCAR T
215 cells were consistently negative for CD70 membrane expression, possibly caused by CD70-
216 CAR interaction (not shown). We therefore assayed nanoCAR T cells with a different specificity
217 (CD20) and stimulated these with CD20⁺ Raji cells (Figure 4A). Unstimulated CD20-specific
218 nanoCAR T cells already expressed CD70 at high levels. By 24 hours after stimulation, almost
219 all of the cells expressed CD70. Expression further increased with the highest levels of CD70⁺
220 cells observed at 48 hours post-incubation. Furthermore, there was a robust raise of CD70
221 MFI up to 48 hours after stimulation (Figure 4A). Since CD70 was expressed and strongly
222 upregulated upon activation of CD20-specific nanoCAR T cells, we argued that endogenous
223 CD70 expression on CD70-specific nanoCAR T cells may be causing nanoCAR T cell
224 dysfunctionality due to interaction with the nanoCAR either at the intracellular level and/or on
225 the membrane of the T cells. We first validated CD70 knock out (CD70KO) generation in CD20
226 nanoCAR T cells. No CD70 expression could be observed in unstimulated and stimulated
227 CD20 nanoCAR T cells in which CD70 was knocked out, showing the efficiency of CD70
228 targeted gRNA and CRISPR/Cas9 technology (Figure 4A). Next, CD70KO CD70-specific
229 nanoCAR T cells were generated. The functionality of CD70WT and CD70KO was assessed
230 in a long-term *in vitro* assay. CD70 wild type (WT) and CD70KO CD70-specific nanoCAR T
231 cells were stimulated with Raji cells every two days and cell numbers were determined.
232 CD70KO nanoCAR T cells showed a greater potential to control Raji cell outgrowth than
233 CD70WT nanoCAR T cells (Figure 4B). Furthermore, CD70KO CAR T cells showed a higher
234 survival rate following antigen exposure compared to CD70WT CAR-T cells (Figure 4B). Of
235 note, a similar enhancing effect of CD70KO was not observed for CD20 specific nanoCAR T
236 cells (Figure S2A). To determine the *in vivo* effect of CD70KO in CD70-specific nanoCAR T
237 cells, we injected NSG mice intravenously with CD70⁺ Raji cells followed by a low dose
238 CD70WT or CD70KO nanoCAR T cells (Figure 4C). Mice injected with CD70WT nanoCAR T
239 cells were unable to control Raji outgrowth and succumbed to disease similar to PBS injected
240 animals. In contrast, CD70KO nanoCAR T cells induced improved tumor control and prolonged
241 survival compared to the control groups indicating a robust increase in nanoCAR T cell efficacy
242 (Figure 4D-G). In the animals treated with CD70KO nanoCAR T cells that did show tumor
243 outgrowth, tumor cells were confined to the brain which suggests that the blood brain barrier
244 may hamper nanoCAR T cell efficacy against brain metastases (Figure 4G).

245 Next, we compared the CD70KO and CD70WT CD70-specific nanoCAR T cells in the patient
246 relevant DLBCL PDX model following the same workflow as described earlier (Figure 3B). The
247 weight of all mice dropped starting from day 5 post CAR T cell injection (Figure 5A-B). In mice
248 treated with PBS, all mice had to be sacrificed around day 20 due to severe weight loss. Mice
249 treated with CD70WT nanoCAR T cells experienced prolonged weight loss. Conversely, mice
250 treated with CD70KO nanoCAR T cells showed an initial weight drop, probably caused by
251 extensive nanoCAR T cell activation and the induction of cytokine release syndrome, but
252 gained weight steadily afterwards and fully recovered. Weekly blood monitoring by flow
253 cytometry revealed initial tumor control in the CD70WT nanoCAR T cell treated mice (Figure
254 5C-D). However, at day 21 post nanoCAR T cell injection, this control was lost. In contrast to
255 the CD70WT nanoCAR T cell treated mice, we could not detect any tumor cells around day 21
256 in mice treated with CD70KO nanoCAR T cells. Tumor control coincided with expansion of
257 nanoCAR T cells in the blood. This massive expansion of CD70KO nanoCAR T cells was not
258 detected in mice treated with CD70WT nanoCAR T cells (Figure 5C,E).

259 **CD70 KO nanoCAR T cells are protected from CAR induced exhaustion**

260 To decipher the mechanism underlying the functional superiority of CD70KO CD70 specific
261 nanoCAR T cells compared to their CD70WT counterparts, we analyzed the nanoCAR T cell
262 tumor infiltrate seven days after injection using single-cell transcriptomics. Mice were injected
263 with Raji cells and seven days later injected with either CD70WT nanoCAR T cells or C70KO
264 nanoCAR T cells. Another seven days later, mice were sacrificed, and bone marrow was
265 isolated (Figure 6A). At that time, Raji cells were present in both conditions (Figure S3A). The
266 resulting cell suspension was labelled with hashtags and sorted for nanoCAR T cells. Sorted
267 cells were pooled in roughly equal numbers and subsequently processed in a single run (Figure
268 6A). Uniform manifold approximation and projection (UMAP) analysis revealed twelve clusters
269 among the sorted nanoCAR T cells (Figure 6B, S3B). Cluster 11 (Table 1) was excluded from
270 further analysis since it was solely composed of mouse cells, caused by impurities from the
271 sorting process. Substantial heterogeneity was observed in the contribution of CD70WT and
272 CD70KO cells to each cluster. Cluster 0, 1, 4 and 9 were mainly composed of CD70KO
273 nanoCAR T cells while these were largely excluded from cluster 2, 3, 8 and 12 (Figure 6C-E).
274 For each cluster, the top 10 differential genes were plotted (Figure 6F). We focused on clusters
275 0 to 3 containing the majority of the cells (Figure 6E). Clusters 0 and 2 consisted of CD4 T cells
276 while clusters 1 and 3 were CD8 T cells (Figure 6E, S3C, table 1). We therefore labeled these
277 clusters CD4KO, CD8KO, CD4WT and CD8WT. Cluster CD4KO and CD8KO were compared
278 with CD4WT and CD8WT respectively. Over 600 differentially expressed genes were found
279 for each combination (Figure 6G, table 2-3). Interestingly, over 70% of the differentially
280 expressed genes were shared between the CD4 and CD8 populations (Figure S3D). Cluster

281 CD4KO and CD8KO showed high differential expression of *SELL*, *GZMK*, *ITGB1*, *GZMA*,
282 *CXCR3* while genes associated with terminal exhaustion such as *CCL1*, *CCL3*, *CCL4*,
283 *TNFSRF18*, *IL2RA*, *RGS16*, *SOCS1*, *TNFRSF9*, *LAG3*, *HAVCR2* are strongly downregulated
284 in CD70KO (Figure 6G, table 2-3). We speculated that this unique gene expression pattern
285 could be based on a more exhausted state of the CD70WT nanoCAR T cells. An unbiased
286 dysfunction gene signature of 30 genes that correlated with antigen induced CAR T cell
287 exhaustion³⁸ was tested against clusters CD4KO, CD8KO, CD4WT and CD8WT (Figure 6I).
288 As expected, clusters CD4WT and CD8WT highly expressed the dysfunction signature while
289 clusters CD4KO and CD8KO did express this signature to a lesser degree. Furthermore,
290 clusters CD4KO and CD8KO expressed more memory-associated genes such as *TCF7*, *IL7R*,
291 *SELL* and *KLF2* (Figure 6I). Of note, this enrichment was also strongly present in bulk CD70WT
292 and CD70KO nanoCAR T cells (Figure S3E). Exhausted CAR T cells are known to express
293 high amounts of activator protein 1 (AP-1)-related basic leucine zipper (bZIP)-interferon-
294 regulatory factor (IRF) families such as the activation factors JUNB, JUND, FOS, and
295 exhaustion-promoting factors basic leucine zipper ATF-like transcription factor (BATF), BATF3
296 and IRF4³⁹. We observed high expression of these factors in clusters CD4WT and CD8WT
297 while clusters CD4KO and CD8KO, containing mainly CD70KO nanoCAR T cells, showed
298 downregulation of these factors (Figure 6I). Lastly, analysis of the cell cycle revealed an
299 increased frequency of cells within the G2/M and S phases of the cell cycle in the CD70KO
300 condition (Figure 6J). Overall, our single cell analysis showed that CD70KO CD70-specific
301 nanoCAR T cells are less prone to exhaustion. Therefore, we believe that the overall increase
302 of CD70-specific nanoCAR T cell functionality of CD70KO T cells is not so much the result of
303 reduced fratricide but rather due to the prevention of exhaustion.

304 Discussion

305 In this study, we developed a nanoCAR T cells targeting CD70. First, we showed that
306 JAK/STAT signaling has no beneficial effect on CD70-specific nanoCAR T cells. Surprisingly,
307 the “conventional” 4_1BB-based second generation nanoCAR out performed JAK/STAT
308 signaling nanoCARs. Next, we assessed the functionality in clinically relevant models
309 expressing low to high levels of CD70. The CD70 nanoCAR T cells induced complete
310 elimination of pediatric MRT organoids *in vitro* and improved the survival of DLBCL PDX
311 injected mice *in vivo*. Importantly, we show that CD70 is upregulated on activated nanoCAR T
312 cells and inhibits the function of nanoCAR T cells specific for CD70 by inducing antigen-
313 induced exhaustion. Knocking out endogenous CD70 in CD70-specific nanoCAR T cells
314 resulted in an enhanced tumor eradication, a less-exhausted nanoCAR T cell phenotype with
315 retention of memory phenotype cell populations during *in vivo* stimulation.

316 CD70 is an attractive target for the treatment of various malignancies. Although its expression
317 is variable within a tumor, we could show here complete elimination both for MRT and DLBCL
318 patient-derived samples. These samples included cells with low to unmeasurable expression.
319 The fact that CAR-T cells were able to eliminate also these cells suggests that even the CD70
320 negative malignant cells may express the antigen at some point during the rejection phase. In
321 a clinical setting, pharmaceuticals such as azacitidine, which have been proven to increase
322 CD70 antigen density may synergize with CD-70 specific CAR T cell^{19,23}. Combining these
323 approaches of boosting antigen density and CAR functionality using the CD70KO, may be an
324 optimized strategy to target AML and solid tumors such as MRT.

325 Previous reports of CD70KO CD70-specific CAR T cells did not show improved CAR T cell
326 functionality²⁴. We believe that the timing of the KO is crucial for the beneficial effect to occur.
327 In contrast to the earlier study, we knocked out CD70 at the earliest stage of T cell activation
328 and before retroviral transduction, resulting in T cells with low to absent CD70 expression
329 before the CAR is expressed. Possibly, continuous CAR stimulation already during the CAR T
330 production process may predispose the T cells for exhaustion. In line with our results, others
331 have targeted membrane proteins expressed by T cells such as CD7 and CD5 either directly
332 by gene-editing or by protein expression blockers (PEBLs) and observed an increase in CAR
333 T cell functionality. This increased functionality was attributed to a decrease in fratricide. In
334 these studies, no assessment of dysfunctionality due to exhaustion was explored⁴⁰⁻⁴².
335 Although we did not address the occurrence of fratricide directly, our data suggest that
336 fratricide of nanoCAR⁺ cells is not a major factor contributing to the increased functionality,
337 since the nanoCAR T cells do not express CD70 on the membrane. The absence of CD70
338 may be due to retention of CD70-CAR complexes in the endoplasmic reticulum. Alternatively,
339 CD70 may traffic to the membrane and subsequently be removed by interaction with the CAR
340 T cells. This process was named trogocytosis⁴³.

341 We showed that tumor infiltrating CD70WT CD70-specific CAR shown clear signs of
342 exhaustion. CAR T cell functionality is limited by the induction of exhaustion, a progressive
343 state of dysfunctionality that develops when T cells are continuously exposed to antigen and
344 inflammatory stimuli. We hypothesized that the modest functionality of our CD70-specific CAR
345 is the result of increased CD70 mRNA translation upon T cell activation and subsequent
346 binding to and continuous triggering of the CAR, which ultimately results in exhaustion. This
347 exhaustion was evidenced by an increased expression of exhaustion-related transcription
348 factors from the bZIP/IRF and AP1 family³⁹. CD70WT nanoCAR T cells overexpressed in high
349 amounts *BATF*, *BATF3*, *IRF4*, *NR4A*, *JUNB* and *JUND*. In addition, we could observe a
350 downregulation of memory associated genes such as *TCF7*, *IL7R*, *SELL*, *CD27*, *KLF2*. Lastly,

351 a gene set that correlates with exhaustion in CAR T cells was highly enriched in the WT CD70-
352 specific CAR T cells³⁸.

353 Due to the upregulation of CD70 on activated DCs, B and T cells, toxicity can be expected in
354 a clinical setting. It has been shown that CD70-specific CAR T cells could hinder a successful
355 antiviral response²⁰. If this is the case, patients treated with CD70-specific CAR T cells could
356 be at risk for infectious diseases caused by CD70-specific immune defects. It is reassuring that
357 multiple groups have shown the safety profile of targeting CD70. For instance, in AML, the first
358 clinical trial with a monoclonal antibody targeted to CD70 in combination with the
359 hypomethylating compound azacitidine showed favorable responses, even complete
360 responses in patients unfit for intensive therapy. Most interestingly, no adverse effects
361 correlated with T cell toxicity were observed¹⁹. However, CAR T cells are living drugs which
362 make them harder to control compared to a monoclonal antibody and therefore a suicide gene
363 system should be included.

364 Our results show that CAR T cells targeting CD70 results rapidly in exhausted CAR T cells.
365 Using CRISPR/Cas9 gene-editing technology, we could overcome this exhaustion and
366 significantly increase CAR T cell functionality. These results could influence the optimization
367 of CARs targeting T cell-specific antigens such as CD5, CD4, CD7 and CD30.

368 **Material and methods**

369 **Culture of cell lines**

370 All the cell lines were cultured as per American Type Collection (ATCC) recommendation. Raji
371 wild type (WT), Raji knock out (KO) and Nalm6 were cultures in Rosewell Park Memorial
372 Institute (RPMI)-1640 medium (Gibco), supplemented with 10% fetal calf serum (FSC)
373 (Biowest), 2 mM L-glutamine (Gibco), 100 IU/mL penicillin (Gibco) and 100 IU/mL streptomycin
374 (Gibco). SKOV3 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco,
375 Invitrogen) supplemented with 10% FSC (Biowest), 2mM L-glutamine (Gibco), 100 IU/mL
376 penicillin (Gibco) and 100 IU/mL streptomycin (Gibco).

377 **Generation of nanoCAR plasmids**

378 The different constructs, as shown in Figure 1A, were generated by Gibson Assembly as
379 described earlier²⁹. The CAR backbone constructs were ordered as gBlock (IDT) and cloned
380 into the LZRS-IRES-eGFP retroviral plasmid by Gibson Assembly (NEBuilder HiFi DNA
381 Assembly Master Mix) (NEB). The VHH specific sequence was amplified using PCR (Phusion
382 High Fidelity PCR kit, NEB) and correct overhangs were incorporated. PCR products were
383 visualized on gel and purified using MinElute PCR Purification kit (Qiagen) as per fabricator
384 instructions. The LZRS-CAR-IRES-eGFP plasmids were overnight digested with BamHI (NEB)

385 and purified using Zymoclean Large Fragment DNA Recovery Kit (Zymo) according to
386 manufacture instructions. Subsequently, digested and purified plasmid was dephosphorylated
387 (rAPid Alkaline Phosphatase) (Roche) and used in a Gibson Assembly reaction together with
388 the purified PCR products. The Gibson Assembly reaction mix was transformed in bacteria
389 (NEB Stable Competent Escherichia coli (High Efficiency), NEB) and plated on agar. After
390 overnight incubation, colonies were selected and grown in liquid lysogenic broth (BD Difco)
391 overnight. Plasmids were isolated and sequenced. Colonies containing the correct plasmid
392 were further cultured and midpreps (Qiagen) were performed.

393 **Generation of retroviral particles**

394 Viral particles were produced as described earlier²⁹. In short, retroviral plasmids encoding for
395 the different CAR structures were transfected using calcium phosphate in the amphotropic
396 packing cell line Phoenix A. Every two days, medium was refreshed and every four days cells
397 were selected using puromycin. At day fourteen, retroviral supernatant was collected and
398 frozen at -80°C until use.

399 **RNP formation**

400 HiFi Cas9 protein was acquired from IDT. CD70 ribonucleoprotein (RNP) was prepared by
401 mixing Cas9 protein and CD70 gRNA (Synthego) at 2.5:1 molar ratio and incubated at 21°C
402 for 10 minutes in a thermocycler and immediately used for nucleofection.

403 **Generation of CAR expressing T cells**

404 Buffy coats from healthy donors were obtained from the Belgian Red Cross and used following
405 the guidelines of the Medical Ethical Committee of Ghent University Hospital, after informed
406 consent had been obtained, in accordance with the Declaration of Helsinki. Peripheral blood
407 mononuclear cells (PBMC) were isolated by Lymphrep (Axis-Shield) gradient centrifugation.
408 The percentage of CD3⁺ cells was determined by flow cytometry and T cells were stimulated
409 with Immunocult Human CD3/CD28/CD2 T cell activator (StemCell Technologies) per
410 fabricator instructions in IMDM (Gibco) supplemented with 10 % FCS (Biowest), 2 mM l-
411 glutamine (Gibco), 100 IU/mL penicillin (Gibco) and 100 IU/mL streptomycin (Gibco) (complete
412 IMDM, cIMDM), in the presence of 10 ng/mL IL-15 and IL-7 (Milteyni Biotec). After 48 hours,
413 cells were harvested and resuspended in retroviral supernatant and centrifuged for 90 minutes
414 at 1000g at 32°C on retronectin (TaKaRa) coated plates.

415 For the CD70KO CAR T cells, stimulated PBMC were nucleofected using the Lonza Amaxa
416 4D-nucleofector X unit (program EH-110). Cells were resuspended at a concentration of
417 1x10⁶ cells/20µl P3 buffer with 20 µM RNP. Following nucleofection, cells were harvested,
418 placed in fresh cIMDM + 10 ng/ml IL-7 + 10 ng/ml IL15 and allowed to rest for 24 hours at

419 37°C, 7% CO₂. After rest, cells were harvested and transduced as described earlier. For the
420 WT CAR T cells, cells were nucleofected but no RNP was added during nucleofection.

421 **Flowcytometric determination of cytokine production**

422 Detection of cytokine producing cells was performed as previously described. In short, CAR T
423 cells were incubated with target cells for 16 hours in the presence of BD Golgiplug (BD
424 Biosciences). Cells were harvested, labelled with antibodies specific for CD3, CD4 and CD8α
425 and a viability dye was added (Fixable Viability Dye-eFluor 506; Thermo Fisher Scientific).
426 Subsequently cells were fixed and permeabilized (BD Cytofix&Cytoperm, BD Biosciences)
427 following the supplier's protocol. Cells were analyzed on a LSR II device (BD Biosciences) and
428 data was analyzed using FACS DIVA software (BD Biosciences) and FlowJo v10.8.1 software
429 (BD Biosciences).

430 ***In vitro* stress test**

431 CAR T cells were added to 96-well plates containing Raji cells at an E:T ratio of 1:1 (1 x 10⁴
432 cells each) in the absence of exogenous cytokines. At the start of the experiment and every
433 two days, wells were harvested and cells were stained for CD3. Before measurement,
434 propidium iodide (Invitrogen) was added. Cells were measured on a LSR II device (BD
435 Biosciences) and data was analyzed using FACS DIVA software (BD Biosciences) and FlowJo
436 v10.8.1 software (BD Biosciences). The remaining wells were stimulated with 10⁴ Raji cells.

437 ***In vivo* experiments**

438 All animal experiments were performed after approval and in accordance with the guidelines
439 of the Ethical Committee for Experimental Animals at the Faculty of Health and Medicine and
440 Health Sciences of Ghent University (ECD 20-18, Ghent, Belgium).

441 Six to ten week old male or female in-house bred NOD.Cg-Prkdc^{scid}IL-2rg^{tm1Wjl}/SzJ (NSG) mice
442 were used in all the *in vivo* experiments. Experimental conditions are described within each
443 figure legend. Untransduced T cells, CAR T cells or PBS were injected intravenously via tail
444 vein at the indicated time points. All injected cells were resuspended in PBS and a maximum
445 volume of 200 µl was administered for tail vein injection. For subcutaneous injection, cells were
446 resuspended in 50 µl. Tumor burden was assessed by caliper measurement (SKOV3 model),
447 bioluminescence (Raji model) or tail vein bleeding (DLBCL PDX). Bioluminescence was
448 assessed after intraperitoneal injection of 150 mg/kg bodyweight D-luciferin (Perkin Elmer)
449 using an IVIS Lumina III *in vivo* imaging system. Animals were euthanized as per experimental
450 protocol or when the human endpoints were reached.

451 **Generation of DLBCL PDX**

452 The DLBCL PDX model (LYM024) was derived at first relapse after R-CHOP in a patient with
453 a DLBCL, non-GC subtype by IHC, BCL2 and cMYC double-expressor, without BCL2 or cMYC
454 rearrangements with negative CISH EBV (EBER). It was obtained from the registered biobank
455 “PDX Hematologie UZGent” (Belgian Registration number BB190094). Tumor cells were
456 isolated from early passage (P2) tumor bearing NSG mice and intravenously injected in FBS
457 in new NSG mice.

458 **Patient-derived MRT organoid culture**

459 MRT organoids were established, characterized, and cultured as previously described^{35,44}. In
460 brief, MRT organoids were cultured in droplets of growth factor-reduced basement membrane
461 extract (BME) Type 2 (R&D Systems) in kidney organoid medium (KOM) (Advanced
462 DMEM/F12 (Gibco) containing 1X GlutaMAX (Thermo Fisher Scientific), 10 mM HEPES
463 (Thermo Fisher Scientific) and 1X Penicillin-Streptomycin (P/S) (Merck Millipore) (AdDF+++),
464 supplemented with 10% R-spondin–conditioned medium, 1.5% B27 supplement (Gibco), 50
465 ng/mL epidermal growth factor (EGF) (PeproTech), 50 ng/mL fibroblast growth factor (FGF-2)
466 (PeproTech), 1.25 mM N-acetylcysteine (Sigma), 10 μ M Rho-associated coiled–coil containing
467 protein kinase (ROCK) inhibitor Y-27632 (Abmole) and 5 μ M A83-01 (Tocris Bioscience)).
468 Culture medium was refreshed every 3 to 4 days and organoids were dissociated mechanically
469 once a week.

470 **Bulk RNA sequencing analysis**

471 Bulk RNA sequencing data from pediatric renal tumor tissue and organoids as well as normal
472 kidney organoids were used to determine RNA expression of CD70⁴⁵.

473 **Flow cytometry analysis**

474 Staining of surface markers was performed in DPBS (Gibco) with 1 % FCS (Biowest) using the
475 antibody to cell ratio recommended by the supplier.

476 For all samples, after antibody incubation cells were washed once with FACS buffer and
477 propidium iodide (Invitrogen) was added. Flow cytometric analysis was performed on a LSR II
478 device (BD Biosciences) and data was analysed using FACS DIVA software (BD Biosciences)
479 and FlowJo v10.8.1 software (BD Biosciences).

480 *Staining on MRT organoid cultures*

481 To determine CD70 expression on MRT organoids, organoids were mechanically dissociated
482 into a single cell suspension, washed twice, and resuspended in FACS buffer (PBS, 2% FCS,
483 2 mM EDTA). Cells were stained with anti-CD70 (Cusatuzumab, MedChemExpress) labeled

484 with Alexa Fluor™ 647 NHS Succinimidyl Ester (Invitrogen) in a 1:1000 dilution for 30 min on
485 ice.

486 CD137 expression on CAR T cells was determined after 20 hours of co-culture with MRT
487 organoids. Cells were mechanically dissociated, washed twice, and resuspended in FACS
488 buffer. Cells were stained with CD137-APC (BD Biosciences) in a 1:25 dilution for 25 min on
489 ice.

490 For all samples (during MRT organoid cultures), after antibody incubation cells were washed
491 three times with FACS buffer and DAPI was added in a 1:200 dilution as a viability dye. Surface
492 antigen expression was measured with the CytoFLEX LX (Beckman Coulter) and data was
493 analyzed with the CytExpert Software Version 2.4 (Beckman Coulter) and FlowJo v10.8.1
494 software (BD Biosciences).

495 **Co-culture conditions**

496 MRT organoids were mechanically dissociated four days before the co-culture assay and
497 seeded out in BME droplets in KOM. One day prior to the co-culture 70- and 20-4_1BBζ cells
498 were defrosted and cultured in a density of 1×10^6 cells/mL in RPMI containing 1X GlutaMAX
499 (Gibco), 1X P/S and 10% FCS supplemented with 10 ng/mL IL7 and IL15 overnight. On the
500 day of co-culture, MRT organoids were carefully collected, washed with cold medium to
501 remove BME, and a fraction was dissociated into a single cell suspension. Single tumor cells
502 were counted to determine the number of cells present in the MRT organoid suspension. MRT
503 organoids were then seeded out in suspension in co-culture medium containing 10% FBS and
504 a 1:1 ratio of KOM and RPMI with 1X GlutaMAX and 1X P/S. Next, CAR T cells were washed,
505 counted and according to the chosen E:T ratio an appropriate amount was added to the MRT
506 organoids in co-culture medium. The cells were incubated for the indicated time points and
507 medium was refreshed on day 2 for the 3-day co-culture and on day 3 and 6 for the 7-day co-
508 culture.

509 **MRT organoid luciferase killing assay**

510 To determine tumor cell specific killing, a lentiviral transduction with pLKO.1-UbC-luciferase-
511 blast was performed on all MRT organoid lines, as described previously⁴⁶⁻⁴⁸. Two days after
512 transduction, luciferase-transduced cells were selected by addition of 10 µg/mL blasticidin. For
513 the co-culture assay, luciferase expressing MRT organoids were seeded in a flat-bottom 96-
514 well plate with an equivalent of 7500 single tumor cells per well and CAR T cells were added
515 in the indicated ratio in total amount of 100 µL of co-culture medium. Luciferase activity was
516 determined after washing the cells with PBS, using the luciferase assay system with the
517 Passive Lysis 5X buffer (Promega) according to manufacturer's instructions.

518 **Live cell imaging**

519 Live cell imaging was performed as described previously with minor modifications⁴⁹. In brief,
520 to visualize 70- and 20-4_1BB ζ CAR T cells in the co-culture assay, cells were washed with
521 PBS and stained with eBioscience Cell Proliferation Dye eFluor 450 (Thermo Fisher Scientific)
522 in PBS in a 1:1000 dilution for 10 minutes at 37°C. RPMI with 10% FBS was added, and cells
523 were further incubated on ice for 5 minutes. Then cells were washed, counted and seeded out
524 in black, glass-bottom 96-well plates (Greiner) together with MRT organoids in the indicated
525 ratio. The co-culture medium was supplemented with 2 drops/mL of NucRed Dead 647
526 ReadyProbes Reagent (Thermo Fisher Scientific) to fluorescently label live (excited by 561 nm
527 laser) and dead cells (excited by 633 nm laser). Cells were incubated in the incubation
528 chamber of the Leica TCS SP8 confocal microscope (5% CO₂, 37°C) and imaged every 30
529 minutes with a HC PL APO CS2 10x/0.4 dry objective for 72 hours. The following settings were
530 used: resonant scanner at 8000 Hz, bidirectional scanning, line averaging of 8, optimal Z-stack
531 step size, Z-stacks of 60-110 μ m in total and a resolution of 512 x 512 resulting in a Voxel size
532 of 1.82 μ m x 1.82 μ m x 2.409 μ m.

533 **scRNA-seq**

534 *Single cell library preparation and sequencing*

535 Mice were injected with 5x10⁵ Raji cells. After seven days, CAR T cells were intravenously
536 injected. Mice were sacrificed seven days post CAR T cell injection. Bone marrow was isolated
537 and processed to obtain a single cell suspension. Single cell suspension was labelled with
538 antibodies for human CD45, CD19 and hashtag antibodies. The suspension was sorted on a
539 BD ARIA II device based on positivity for human CD45, human CD19 and eGFP. Sorted cells
540 were pooled and resuspended in PBS + 0.04% BSA to obtain on average 1200 cells/ μ l and
541 loaded on a Chromium GemCode Single Cell Instrument (10x Genomics) to generate single-
542 cell gel beads-in-emulsion (GEM) at the VIB Single Cell Core. The scRNA-Seq libraries were
543 prepared using the GemCode Single Cell 5' Gel Bead and Library kit, version NextGEM 2 (10x
544 Genomics) according to the manufacturer's instructions. Sequencing libraries were loaded on
545 an Illumina NovaSeq flow cell at VIB Nucleomics core with sequencing settings according to
546 the recommendations of 10x Genomics.

547 *Processing of scRNA-seq and hashtag data*

548 The Cell Ranger pipeline (10x Genomics, version 6.0.0) was used to perform sample
549 demultiplexing and generation of FASTQ files for read 1, read 2 and the i7 sample index for
550 the gene expression and hashtag libraries. Read 2 of the gene expression library was
551 subsequently mapped to a combined reference genome of hg38 and mm10. The resulting

552 count matrices were loaded into R and processed using the Seurat package (version 4.0.5)⁵⁰.
553 The hashtag data was normalized via the “NormalizeData” function using the CLR (Centered
554 Log Ratio) method. Subsequently the “HTODemux” function was used to demultiplex the
555 different cell types i.e., WT and KO. Therefore, the positive.quantile parameter was set to 0.99.
556 After demultiplexing, only the singlet cells were retained for further analysis.

557 The singlet cells were filtered further based on their gene expression data using the following
558 criteria: i) nFeature_RNA > 600; ii) nCount_RNA > 1143; iii) nCount_RNA < 30000 and iv)
559 percent.mt < 10. Prior to further processing of the gene expression data, three gene sets were
560 removed, namely Cell Cycling and Histone genes⁵¹ and CD70. For removal of the Cell Cycle
561 genes, we relied on Table S8 of Park *et al.*⁵². Afterwards the gene expression data was
562 normalized with the “NormalizeData” function using the LogNormalize method and a
563 scale.factor of 10000. Then the highly variable features were identified with the
564 “FindVariableFeatures” function with selection.method set to vst and nfeatures to 4000. After
565 which the data was scaled with the “ScaleData” function.

566 For visualization of the data the “RunPCA” and the “RunUMAP” functions were used. After
567 which clustering was performed using the “FindNeighbors” and “FindClusters” functions.
568 Differential expression analysis was performed via the “FindMarkers” function for all clusters,
569 for comparison of the two cell types i.e., KO and WT and for comparison of specific clusters.

570 **Statistical analysis**

571 In general, data is shown as mean \pm standard error of the mean, unless otherwise noted in the
572 figure legend. Significance was considered at $p < 0.05$. All statistical tests are described in the
573 figure legends and p values are denoted either exact or with asterisk as follows: **** = $p <$
574 0.0001 ; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Number of replicates are described within
575 the relevant figure legend. Data was analysed and visualized using GraphPad Prism v9.5.0
576 (Dotmatics).

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584 **Author contributions**

585 Conceptualization: SDM, BVDK; Methodology: SDM, BVDK, JB, JD; Software: LDC, NV;
586 Validation: BVDK, JD; Formal analysis: SDM, LDC; Investigation: SDM, JB, AVP, GG;
587 Resources: SDM, JB, WD, ED, JT, JD, BVDK; Data curation: SDM, LDC, JB, BVDK; Writing –
588 original draft: SDM, BVDK; Writing – review & editing: SDM, JB, LDC, AVP, WD, EP, LD, JI,
589 GG, LB, MP, NV, JVD, FO, TK, ED, JT, JD, BVDK; Visualization: SDM, JB, LDC; Supervision:
590 TK, JB, BVDK; Project administration: SDM, BVDK; Funding acquisition: JD, BVDK.

591 **Conflict of interest**

592 AVP, ED, JT are affiliated with Orionis Biosciences BV (as a scientific advisor and/or an
593 employee) and holds equity interests in the Company. JT received financial research support
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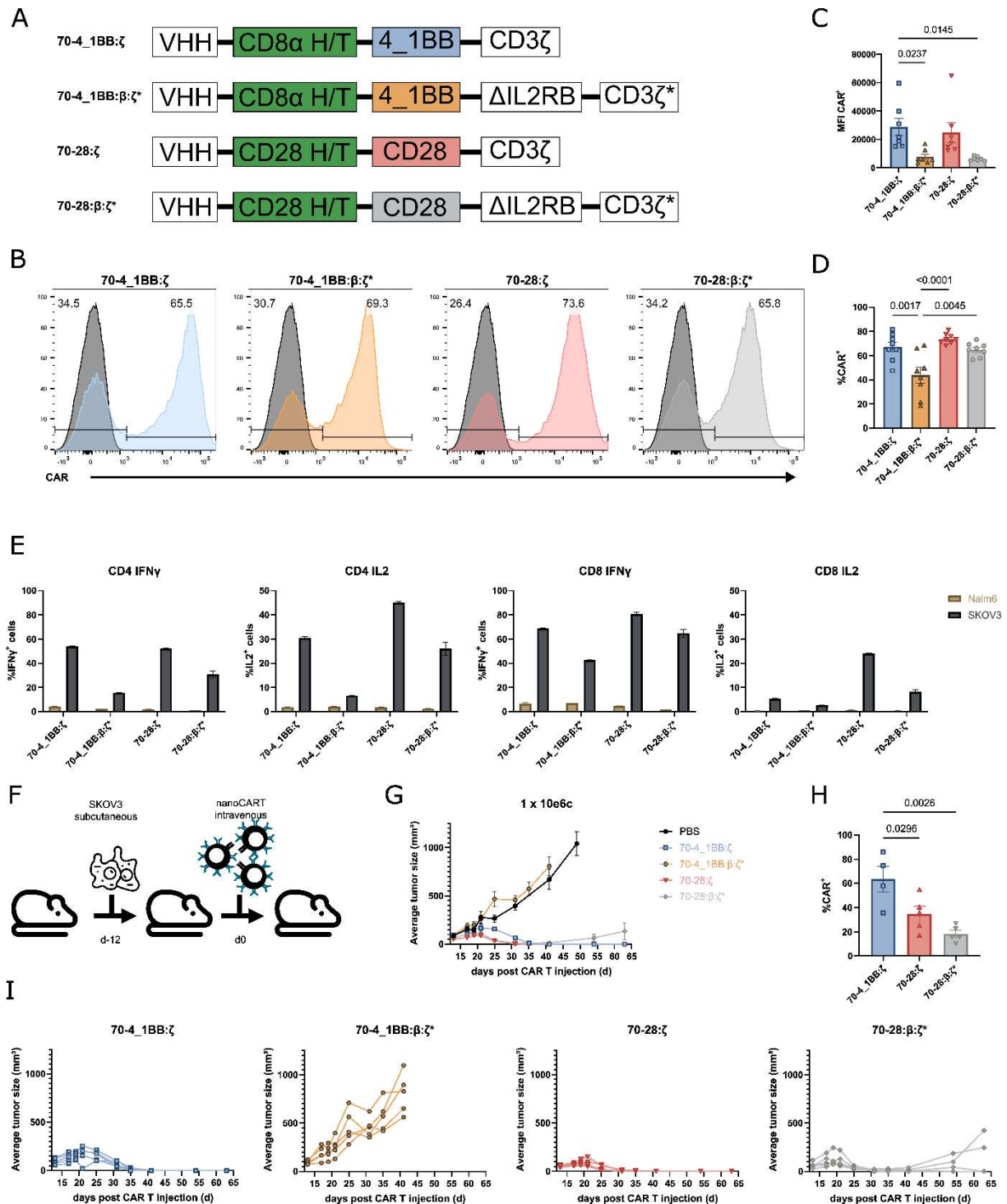


Figure 1: STAT signaling does not affect CD70-specific CAR T cell functionality. (A) Design of the different CAR T cell constructs used. **(B)** Representative histograms of CD70 CAR expression levels after transduction of activated T cells, nine days post transduction. **(C)** MFI of CAR positive population. **(D)** The frequency of CAR positive T cells of eight different donors. **(E)** Cytokine production by CD70-specific CAR T cells after stimulation with Nalm6 (CD70⁻) and SKOV3 (CD70⁺), determined by intracellular staining for IFN γ and IL2. **(F)** The experimental setup of the SKOV3 xenograft model. CD70-specific T cells (1×10^6) were injected intravenously twelve days after subcutaneous engraftment of SKOV3 cells (1×10^6) ($n=5$ for each treatment group). **(G)** SKOV3 tumor growth after injection of CAR T cells. Tumor growth is shown as average tumor size, error bars indicate SEM. **(H)** Tumor growth for each

individual mouse. **(I)** Percentage of CAR⁺ cells in the CD45⁺CD3⁺ in the spleen at day 63 post CAR T cell injection as assessed by flow cytometry. Mean values are shown, error bars indicate SEM.

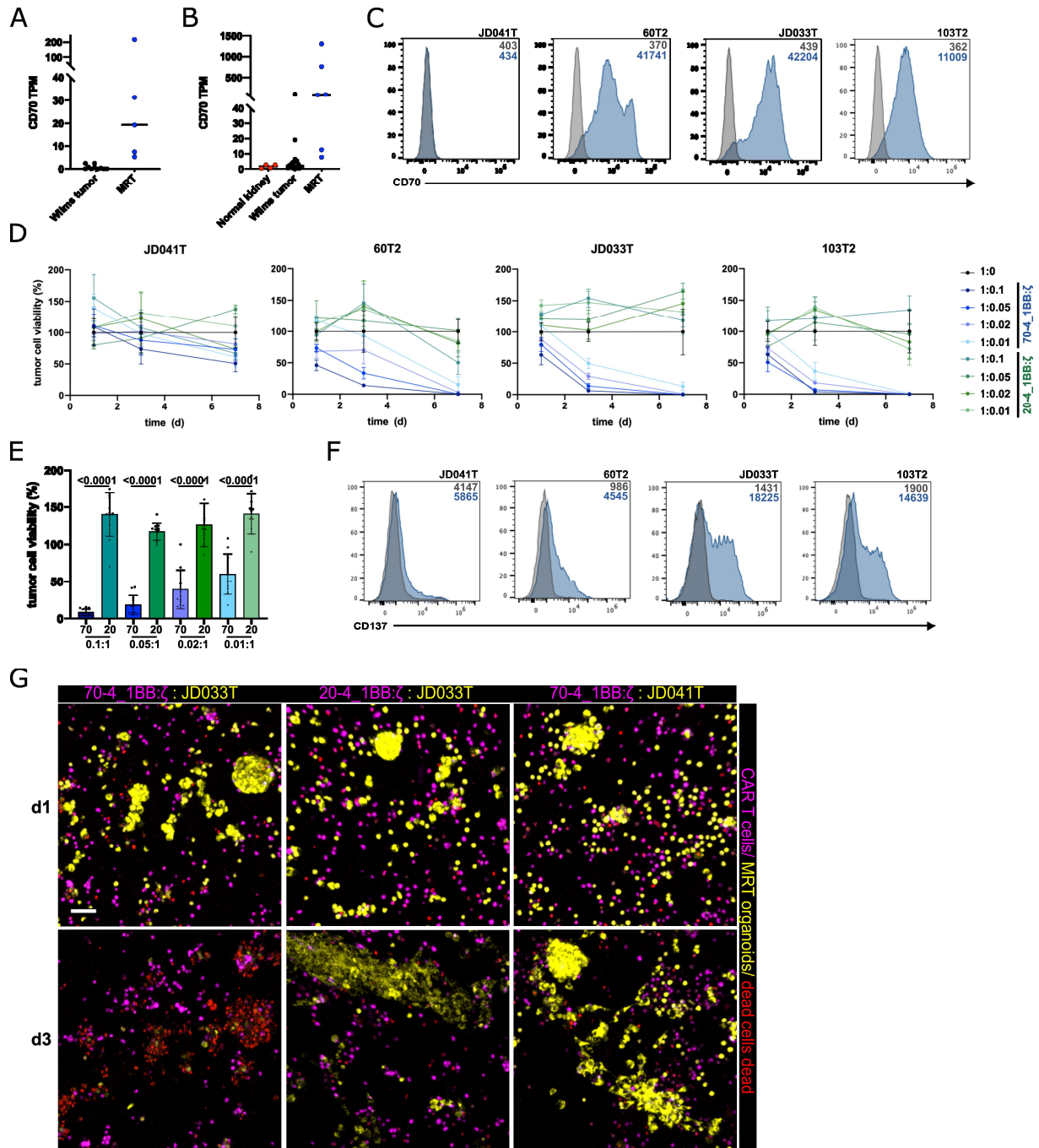


Figure 2: CD70 specific CAR T cells are reactive towards CD70 MRT organoids. (A) Normalized CD70 transcript per million (TPM) values in Wilms tumor and MRT patient samples. **(B)** Normalized CD70 transcript per million (TPM) values in normal kidney, Wilms tumor, and MRT organoid lines. **(C)** Flow cytometric analysis of CD70 expression in 4 MRT organoid lines (number indicate MFI, blue = CD70 expression, grey = unstained). Results are representative of at least n=3 experiments. **(D)** Remaining luciferase activity in 4 MRT organoid lines after co-culture with 70-4_1BB:ζ or 20-4_1BB:ζ for 1, 3 and 7 days in the depicted target to effector ratios. Values are normalized to MRT organoids in culture alone. Mean ± SD of n=4 technical replicates. **(E)** Remaining luciferase activity in 3 CD70 positive MRT organoid lines at day 3 after co-culture with 70-4_1BB:ζ or 20-4_1BB:ζ in the depicted effector to target ratios. Mean ± SD of n=3 biological replicates. Statistical significance was analyzed by unpaired t-test. **(F)** Flow cytometry analysis of CD137 expression on 70-4_1BB:ζ (blue) and 20-4_1BB:ζ (grey) after co-culture with the depicted MRT organoid lines for 20 hours in an effector to target cell ratio of 0.1:1 (number indicate MFI, blue = CD137 expression, grey = unstained). **(G)**

Immunofluorescence three-dimensional imaging of MRT organoid lines (yellow) JD033T (CD70 pos.) in co-culture with 70-4_1BB:ζ and 20-4_1BB:ζ (pink) as well as JD041T (CD70 neg.) with 70-4_1BB:ζ in a 1:1 E:T ratio on day 1 and 3. Dead cells are immunolabeled in red. Maximum intensity projections are shown. Scale bars: 50 μm.

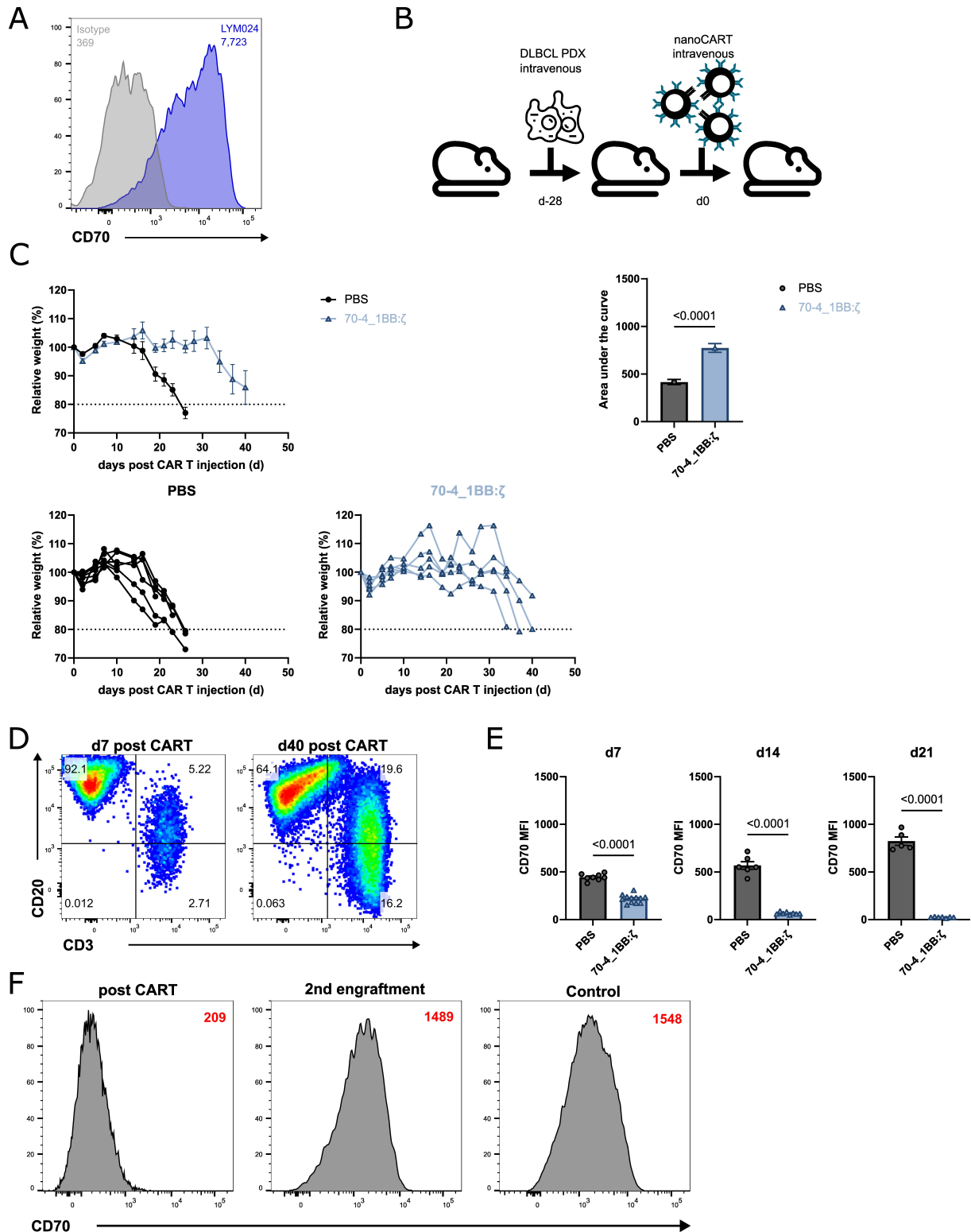


Figure 3: CD70 nanoCAR T cells are functional in a clinically relevant DLBCL PDX model. (A) CD70 expression relative to isotype control of the LYM024 DLBCL PDX. (B) The experimental setup of the DLBCL PDX model. CD70 CAR T cells (1×10^6) were injected intravenously 28 days after intravenous injection of DLBCL cells (1×10^6) ($n=5$ for each treatment group). (C) Relative weight after CAR T cell injection. Weight at the start of the experiment was 100%. Area under the curve was calculated and significance was tested. (D) Presence of T cells ($CD3^+CD20^{-}$) and DLBCL cells ($CD3^+CD20^+$) at day seven and day 42 post nanoCAR T cell injection in the liver. (E) CD70 expression levels assessed by flow

cytometry on circulating DLBCL cells at different time points post CAR T cell injection. Mean values are shown, error bars indicate SEM. **(F)** CD70 expression on DLBCL cells post CAR T cell treatment, after second engraftment and control. MFI is each time indicated.

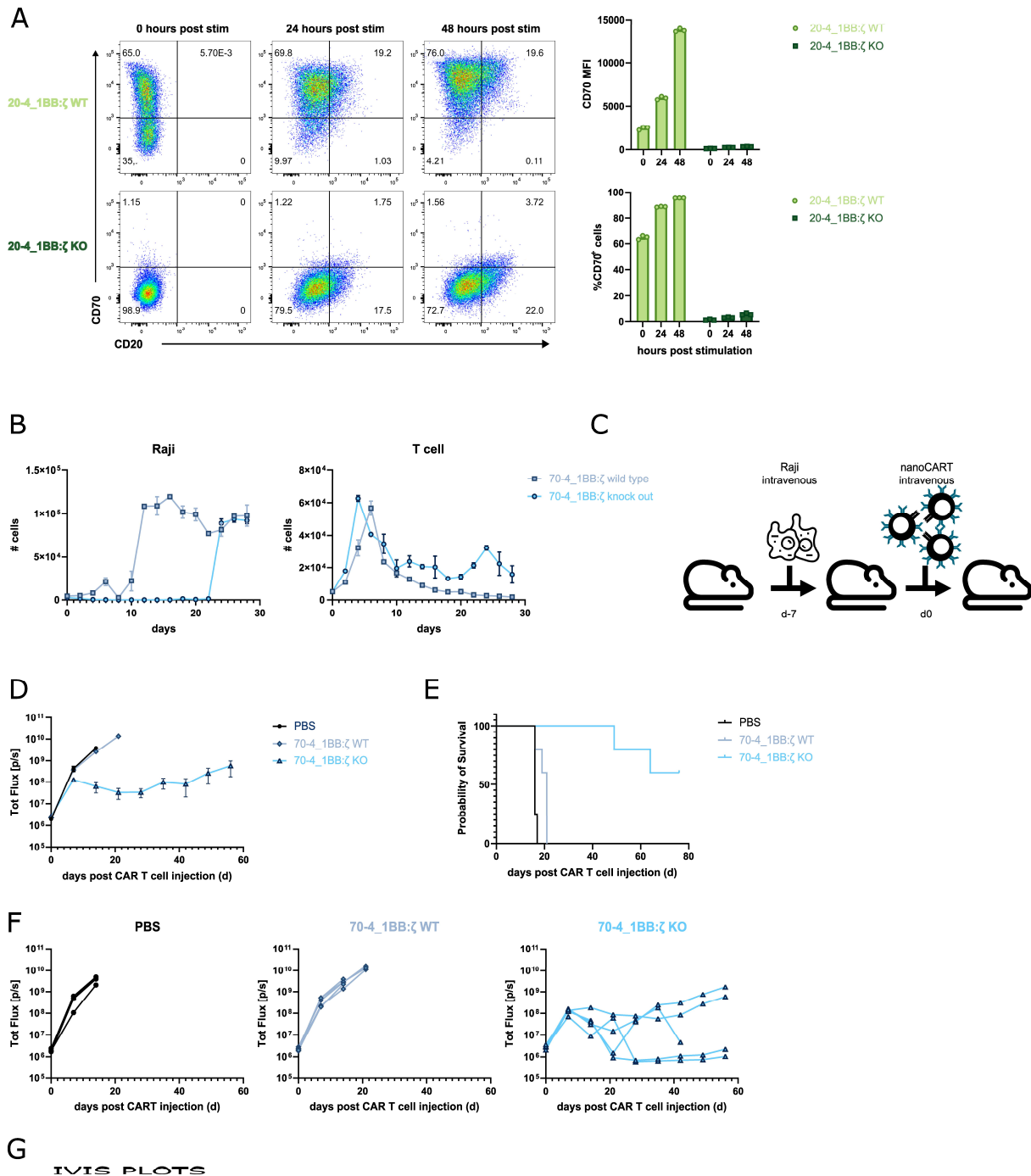


Figure 4: CD70 KO on CD70 specific nanoCAR T cells increases nanoCAR T cell functionality. (A) CD20 specific nanoCAR T cells, either WT or CD70 KO, were incubated with CD20⁺CD70⁻ Raji cells for zero, 24 or 48 hours. CD70 expression on the nanoCAR T cells was determined by flow cytometry. (B) CD70 specific nanoCAR T cells either WT or KO were incubated with WT Raji cells. Every two days the amount of cells were determined and the remaining wells were stimulated with fresh Raji cells. Median values are shown, error bars indicate SEM. (C) The experimental setup of the Raji xenograft model. CD70 nanoCAR T cells (0.75x10⁶) were injected intravenously 7 days after intravenous injection of Raji cells (0.5x10⁶). (D) Quantification of total flux (photons/s) in the experimental groups at the indicated time points. Points represent mean, error bars indicate SEM. (E) Kaplan-Meier survival curves for the different treatment groups. (F) Quantification of total flux (photons/s) for

each mouse in the experimental groups at the indicated time points. **(G)** Bioluminescence of Raji xenografts over time in the treatment groups.

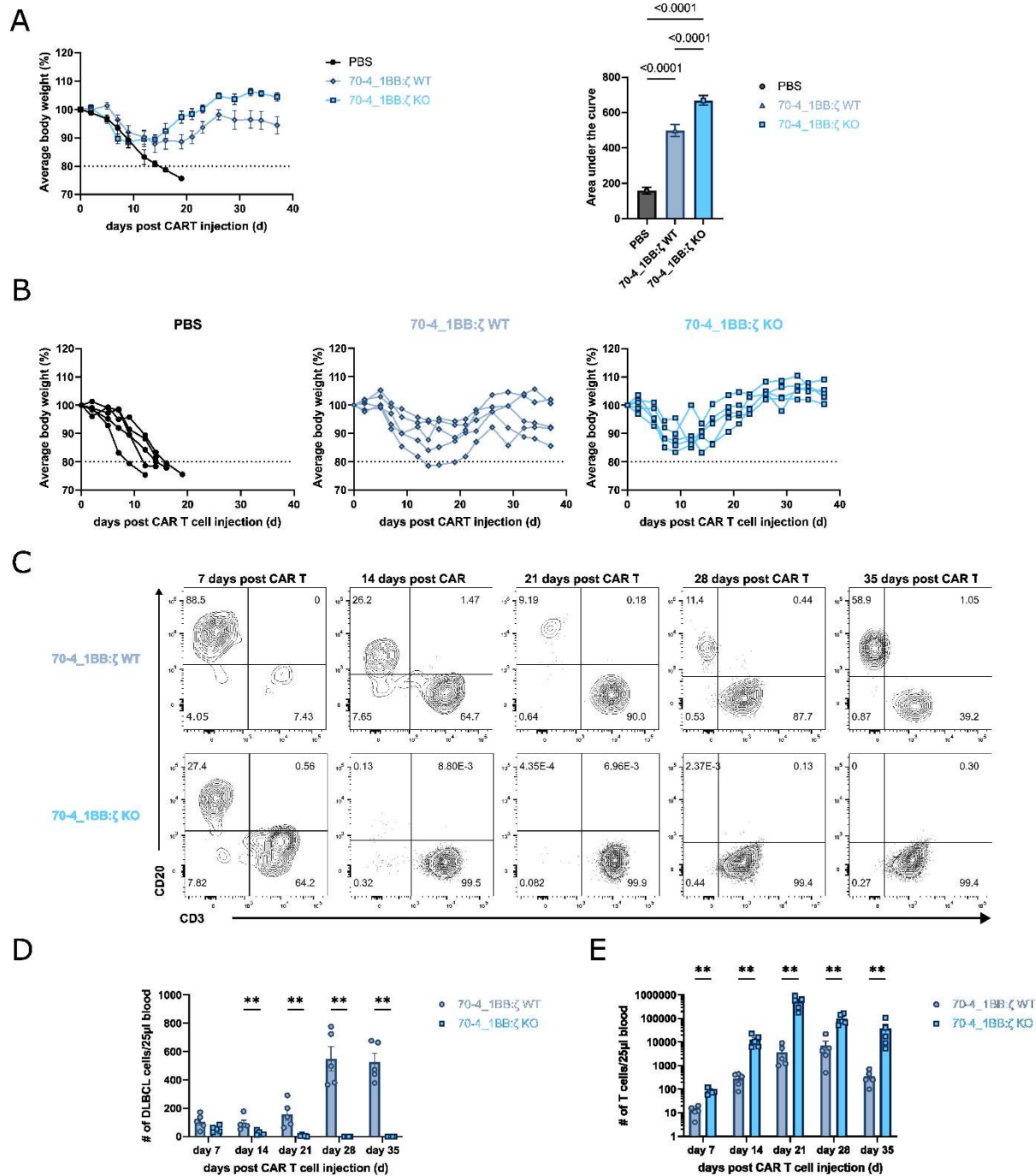


Figure 5: CD70 KO nanoCAR T cells eradicate DLBCL in a PDX model. (A) Relative weight after nanoCAR T cell injection. Weight at the start of the experiment was 100%. Mean number is shown, error bars indicate SEM. Area under the curve was calculated and significance was tested. **(B)** Relative body weight for individual mice for each treatment group. **(C)** Representative DLBCL and T cell expansion in blood at the indicated time points analyzed by flow cytometry. **(D)** Quantification of DLBCL and T cells in 25 μ l blood for each treatment group. Mean number of cells is shown, error bars indicate SEM.

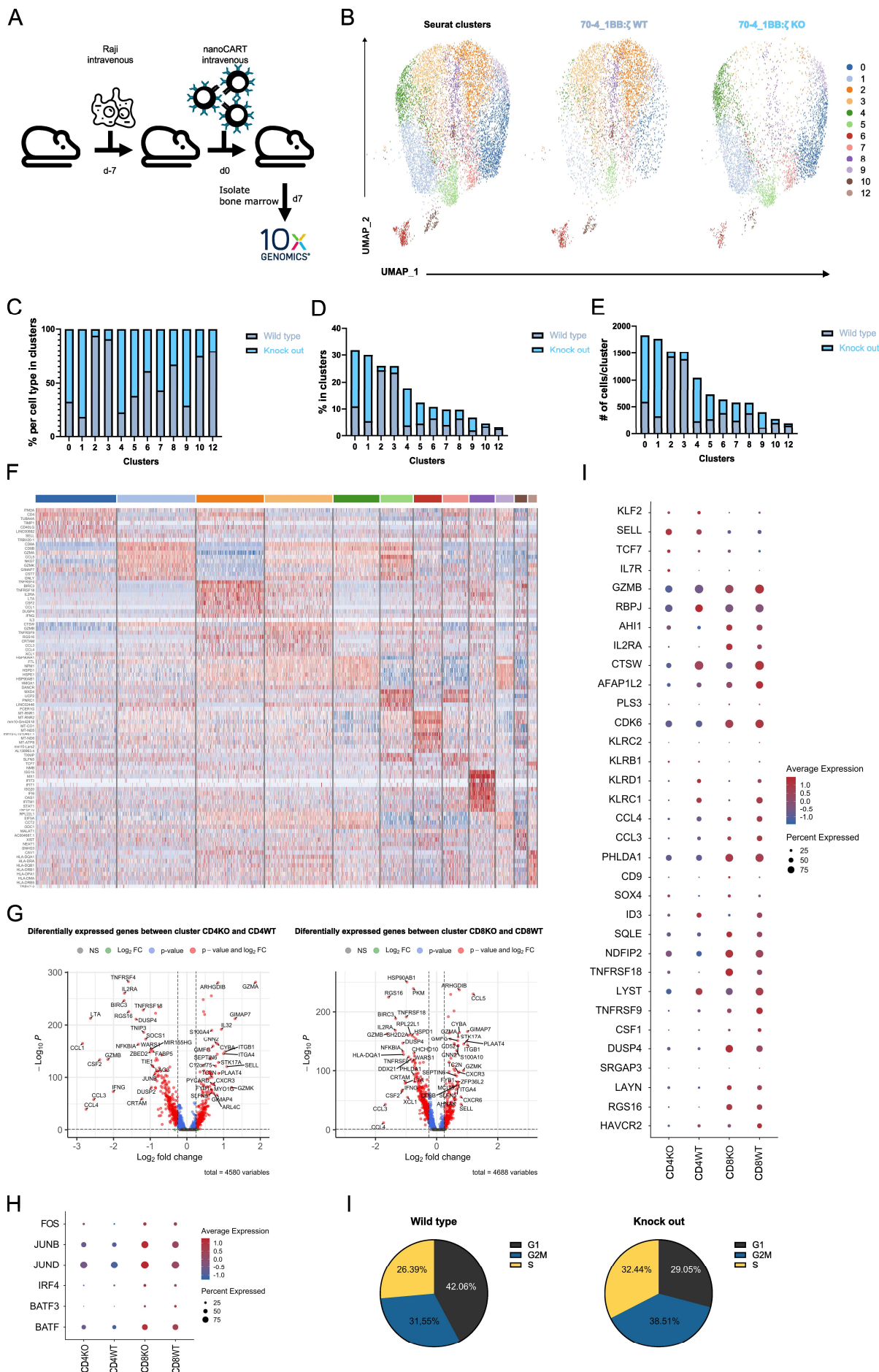


Figure 6: KO CAR T cells are protected from exhaustion. (A) The experimental setup of the Raji xenograft model. CD70 nanoCAR T cells (0.75×10^6) were injected intravenously 7 days after intravenous injection of Raji cells (0.5×10^6). At day seven post nanoCAR T cell injection, bone marrow was isolated and cells were sorted for CAR expression. Sorted cells were sequenced after 10x library construction. **(B)** UMAP representation of all the cells with exclusion of cluster 11. UMAP projection of CD70WT (middle) and CD70KO (right) are shown. **(C)** Bar graph representing the proportions of each CAR T cell condition in the different clusters. **(D)** Bar graph representing the contribution of each CAR T cell condition within the different clusters. **(E)** Absolute number of cells per cluster. **(F)** Heatmap of top 10 marker genes for each cluster defined in (B). **(G)** Volcano plot depicting differentially expressed genes between clusters CD4KO and C4WT (0 vs 1) and clusters CD8KO and CD8WT (1 vs 3). Genes upregulated in the KO clusters are on the right side. Red dots indicate significant genes with $p < 0.05$ and $\log_2FC > 0.2$. **(H)** Dot plot illustrating the expression level of naïve/memory genes and the dysfunction gene signature in cluster CD4KO, CD4WT, CD8KO and CD8WT. **(I)** Dot plot illustrating the expression level of *FOS*, *JUNB*, *JUND*, *IRF4*, *BATF* and *BATF3* in cluster CD4KO, CD4WT, CD8KO and CD8WT. **(J)** Relative frequency of sorted CD70WT and CD70KO nanoCAR T cells in each phase of the cell cycle as determined by single-cell RNA-seq.