

1 **IL-2-inducible T cell kinase deficiency sustains chimeric antigen receptor T cell therapy**
2 **against tumor cells**

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54 **Conflict of interests**

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59 **Abstract**

60 Despite the revolutionary achievements of chimeric antigen receptor (CAR) T cell therapy in
61 treating cancers, especially leukemia, several key challenges still limit its therapeutic efficacy. Of
62 particular relevance is the relapse of cancer in large part, as a result of exhaustion and short
63 persistence of CAR-T cells in vivo. IL-2-inducible T cell kinase (ITK) is a critical modulator of the
64 strength of T-cell receptor (TCR) signaling, while its role in CAR signaling is unknown. By
65 electroporation of clustered regularly interspaced short palindromic repeats (CRISPR) associated
66 protein 9 (Cas9) ribonucleoprotein (RNP) complex into CAR-T cells, we successfully deleted *ITK*
67 in CD19-CAR-T cells with high efficiency. Bulk and single-cell RNA sequencing (scRNA-seq)
68 analyses revealed down-regulation of exhaustion and up-regulation of memory gene signatures
69 in ITK-deficient CD19-CAR-T cells. Our results further demonstrated a significant reduction of T
70 cell exhaustion and enhancement of T cell memory, with significant improvement of CAR-T cell
71 expansion and persistence both in vitro and in vivo. Moreover, ITK-deficient CD19-CAR-T cells
72 showed better control of tumor relapse. Our work provides a promising strategy of targeting ITK
73 to develop sustainable CAR-T products for clinical use.

74

75 **Introduction**

76 Chimeric antigen receptor (CAR) T cell therapy, as an innovative cellular immunotherapy, has
77 been used in leukemia (1), solid tumors (2), and several other diseases such as autoimmunity (3,
78 4) and cardiac injury (5). Currently, a total of nine CAR-T products have been approved for clinical
79 therapy. Despite the remarkable clinical efficacy of CAR-T therapy in leukemia, especially in B
80 cell malignancy (1), there are still many challenges limiting its therapeutic efficacy. Relapse of
81 tumor remains the major obstacle to be addressed, especially in CD19-targeted CAR-T therapy
82 which even about 40%–60% of patients achieving a complete response (CR) eventually
83 experience relapse (1, 6, 7). For example, a recent study reported that CAR-T treatment for diffuse

84 large B-cell lymphoma (DLBCL) showed about 43% progression 8 months after CAR-T infusion
85 (8). The efficacy of CAR-T cell therapy remains largely to be improved in cancer treatment.
86 Severe T cell exhaustion in the tumor environment and the relatively short persistence of CAR-T
87 cells in vivo are among the key obstacles affecting the efficacy of CAR-T therapy (1, 6, 7, 9).
88 Recently, there have been many efforts towards decreasing exhaustion and promoting the
89 persistence of CAR-T cells. Combinations of CAR-T therapy with PD-1 blockade or knockout have
90 been investigated in several tumors and showed promising outcomes (10, 11). However, the
91 application of these strategies was restricted due to the limited PD-1 expression in certain tumors
92 and the systemic side effects of PD-1 blockade. In addition, targeted deletion or inhibition of genes,
93 such as *TGFBRII*, *NR4A*, *DNMT3A*, BET proteins, Ragnase-1 and Roquin-1, have been shown
94 to reduce T cell exhaustion, promote CAR-T cells persistence and/or enhance antitumor activity
95 (12-16). It has been reported that SRC family kinase (SFK) LCK, a critical kinase for T-cell
96 receptor (TCR) signaling, promotes strong signaling that tends to lead to exhaustion in CAR-T
97 cells (17). LCK-deficient CAR-T cells show enhanced therapeutic efficacy with reduced
98 exhaustion and enhanced memory in vivo (17). Additionally, previous studies have shown that
99 transient treatment with dasatinib, a multi-targeted tyrosine kinase inhibitor that targets ABL, SRC
100 and c-KIT, reduces the expression of exhaustion markers and increases the expression of
101 memory-associated markers through epigenetic remodeling (18-20). However, current knowledge
102 and approaches are still quite limited in addressing these obstacles.

103 The Tec family non-receptor tyrosine kinase IL-2-inducible T cell kinase (ITK), predominantly
104 expressed in T cells, is a crucial signaling mediator downstream of TCR and regulates the strength
105 of TCR signaling (21). ITK plays critical roles in T cell activation and differentiation (22). In *Itk*
106 knockout (hereafter as *Itk*^{-/-}) mice, T cells spontaneously develop a memory-like phenotype
107 independent of specific antigenic stimulation (23, 24), and can rapidly produce IFN- γ upon
108 stimulation (23, 25). In mouse models of antigen-specific CD8⁺ T cell development following
109 infections, naïve *Itk*^{-/-} CD8⁺ T cells also showed significantly enhanced memory development (26,

110 27). Similarly, naive *Itk*^{-/-} T cells expanded significantly better than WT cells under lymphogenic
111 conditions (28). In the clinic, ibrutinib, a Bruton's Tyrosine Kinase (BTK) inhibitor for B cell
112 leukemia treatment, has been used in combination with CD19-CAR-T cells for chronic lymphocytic
113 leukemia (CLL) therapy (29). An increased response rate of CAR-T cell therapy in CLL patients
114 with these combination therapy has been reported (30). Due to the C-terminal kinase domain has
115 high levels of structural similarity between ITK and other Tec family members, BTK inhibitors like
116 ibrutinib exhibit off-target effects on ITK. It has been reported that Ibrutinib could directly reduce
117 CD8⁺T cell exhaustion independent of BTK (31). Furthermore, chronic TCR activation drives T
118 cell exhaustion and tempering TCR signaling by ITK inhibition or deletion will reduce T cell
119 exhaustion in mice (32-34). However, the intrinsic role of ITK in CAR-T cells against tumors
120 remains unaddressed.

121 Here, by combining the clustered regularly interspaced short palindromic repeats (CRISPR)
122 associated protein 9 (Cas9) gene editing technology with multiple in vitro and in vivo models of
123 CD19-CAR-T cell therapy, we showed that ITK deficiency in CD19-CAR-T cells significantly
124 improved expansion, reduced cell exhaustion and enhanced memory of CAR-T cells. ITK-
125 deficient CD19-CAR-T cells showed better control of tumor relapse, leading to more sustainable
126 therapeutic effects. In addition, ITK deficiency in CAR-T cells derived from CLL patients
127 attenuated T cell exhaustion, a critical issue in CLL patients (35), thereby potentially addressing
128 a challenge in CAR-T cell therapy for CLL patients. Our results suggest that deletion of *ITK* during
129 CAR-T cell production may be a useful strategy for the development of sustainable and functional
130 CAR-T cell therapy, which will potentially benefit patients whose T cell functional quality are low,
131 as well as those who suffer from tumor relapses.

132

133 **Results**

134 **ITK deficiency attenuates immediate cytotoxicity of CD19-CAR-T cells**

135 CAR-T cells targeting human CD19 (hereafter referred to as CD19-CAR-T cells) were obtained

136 via lentiviral transduction of a third generation CAR (Figure 1A), and *ITK* deletion in CD19-CAR-
137 T cells was performed through electroporation of Cas9-single guide RNA (sgRNA)
138 ribonucleoprotein (RNP) complex (Figure 1B). Notably, we observed a modest impact on the
139 expression of several functional molecules in T cells following lentiviral transduction and
140 electroporation (Supplemental Figure 1, A-D). However, there was no significant impact on the
141 non-specific killing activity of T cells against MEC1 cells (a CD19-expressing CLL tumor cell line)
142 or on T cell expansion following these procedures (Supplemental Figure 1, E-G). The gene editing
143 efficiency of *ITK* was 86.6% for sgRNA1 and 96.9% for sgRNA2 (Figure 1C and Supplemental
144 Figure 1, H and I), with sgRNA1 displaying less off-target effects than sgRNA2 (Supplemental
145 Figure 1, J and K). Thus, sgRNA1 was used in the following experiments in this study unless
146 otherwise noted. Efficient deletion of *ITK* (*ITK*-KO) was further confirmed at the protein level by
147 western blotting (Figure 1D). Reduced activation of TCR downstream signaling molecules,
148 including phosphorylated PLC γ 1 (p-PLC γ 1), phosphorylated ERK1/2 (p-ERK1/2), and
149 phosphorylated p70S6 (p-p70 S6), were observed in *ITK*-KO CD19-CAR-T cells after co-cultured
150 with MEC1 cells (Supplemental Figure 1L). This indicates that *ITK* deficiency impairs TCR
151 downstream signaling in *ITK*-KO CD19-CAR-T cells. Continued monitoring of CAR-T cells co-
152 cultured with MEC1 cells suggested that both *ITK*-KO CD19-CAR-T cells and control CD19-CAR-
153 T cells were able to efficiently kill MEC1 cells (Supplemental Video 1-3). The cytotoxic activity of
154 *ITK*-KO CD19-CAR-T cells was further assessed in vitro against a panel of cell line models of B
155 cell malignancy (including two CLL cell lines expressing CD19 (MEC1 and HG3) and Raji cell line
156 derived from Burkitt lymphoma). The tumor killing ability of *ITK*-KO CD19-CAR-T cells was
157 compared to control CD19-CAR-T cells that were electroporated with a RNP complex containing
158 a non-targeting sgRNA (nt-KO). Deletion of *ITK* slightly reduced the cytotoxic function of CD19-
159 CAR-T cells compared to nt-KO CD19-CAR-T cells in all tested cell lines (Figure 1, E-G). However,
160 *ITK*-KO CD19-CAR-T cells showed no obvious changes in IFN- γ , TNF- α , and Granzyme B
161 expression by flow cytometric analyses, as compared to nt-KO CD19-CAR-T cells (Figure 1, H

162 and I, and Supplemental Figure 1, M and N). Furthermore, IL-17A, FOXP3 and Th2-associated
163 cytokines were expressed at low levels, with minimal differences observed between nt-KO and
164 ITK-KO CD19-CAR-T cells (Supplemental Figure 1O). These results suggest that ITK deficiency
165 in CD19-CAR-T cells attenuated the immediate cytotoxic effects against tumor cells but did not
166 profoundly affect the production of effector cytokines.

167

168 **ITK deficiency promotes long-term expansion of CD19-CAR-T cell in vitro**

169 To further determine the role of ITK in CD19-CAR-T cells, we examined the cell expansion,
170 proliferation and apoptosis in ITK-KO CD19-CAR-T cells and nt-KO CD19-CAR-T cells after co-
171 culture with tumor cells. ITK-KO CD19-CAR-T cells exhibited similar rates of cell expansion at the
172 early time points (e.g., before day 20 after co-culture) compared to nt-KO CD19-CAR-T cells
173 (Figure 2, A-C). However, we observed significant improvements in the expansion of ITK-KO
174 CD19-CAR-T cells at later time points compared to nt-KO CD19-CAR-T cells, (both CD4⁺ and
175 CD8⁺ CD19-CAR-T cells) (Figure 2, A-C, and Supplemental Figure 2). Annexin V staining
176 revealed that the apoptosis of nt-KO CAR-T cells significantly increased 45 days after in vitro
177 culture. In contrast, ITK-KO CD19-CAR-T cells displayed a significantly lower level of apoptosis
178 at this time point (Figure 2, D and E). Furthermore, Ki-67 staining demonstrated that the
179 proliferation of both nt-KO and ITK-KO CD19-CAR-T cells decreased over time, but this reduction
180 was smaller in ITK-KO CD19-CAR-T cells compared to nt-KO CAR-T cells at day 30 (Figure 2, F
181 and G). Overall, these data indicate that ITK deficiency enhances the long-term expansion with
182 significantly better survival and slower reduction of proliferation of CD19-CAR-T cells in vitro.

183

184 **Transcriptomic regulation by ITK in CD19-CAR-T cells**

185 To investigate how ITK regulates the transcriptome of CAR-T cells, we first conducted high-
186 throughput RNA sequencing (bulk RNA-seq) on ITK-KO and nt-KO CD19-CAR-T cells. A total of
187 1319 genes were significantly differentially expressed between nt-KO and ITK-KO CD19-CAR-T

188 cells (fold change ≥ 1.5 , $P \leq 0.05$) (Figure 3A and Supplemental Figure 3, A and B). Among these,
189 797 were increased, while 522 were decreased in the ITK-KO CD19-CAR-T cells (Figure 3A and
190 Supplemental Figure 3B). In line with the critical role of ITK in TCR signaling, we observed a
191 significant enrichment of differentially expressed genes involved in several immune signaling
192 pathways downstream of the TCR signaling, such as cytokine-cytokine receptor interaction and
193 cytokine-mediated signaling pathways (Figure 3B and Supplemental Figure 3C) by Kyoto
194 Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. In addition,
195 multiple genes involved in T cell activation, such as *TNFSF4*, *TNFSF14*, *XCL1*, *XCL2* and *TBX21*,
196 were significantly decreased in the ITK-KO CD19-CAR-T cells (Figure 3C). Furthermore,
197 transcription of several key JAK-STAT signaling genes, such as *STAT1*, *STAT2*, *STAT3* and
198 *STAT4*, but not *STAT6*, were significantly increased in ITK-KO CD19-CAR-T cells (Figure 3C).
199 Enhancement of *STAT1*, *STAT3* and *STAT5*, but not *STAT6*, had been shown to promote stem
200 cell memory and effector CAR-T cells (36). Our observations suggested that ITK deficiency
201 regulated TCR signaling in CD19-CAR-T cells and may promote effector/memory formation in
202 CAR-T cells. Indeed, several genes associated with naïve or progenitor-memory T cells, including
203 *SELL* (encoding CD62L), *IL7R* (encoding CD127), *TCF7* (encoding TCF1), *KLF2* and *LEF1*, were
204 significantly increased in the ITK-KO CD19-CAR-T cells (Figure 3, C and D). Gene set enrichment
205 analysis (GSEA) further indicated significant enrichment of genes associated with T cell
206 effector/memory phenotype in the ITK-KO CD19-CAR-T cells (Figure 3E). In contrast, genes
207 involved in T cell exhaustion(37), such as *PDCD1* (encoding PD-1) and *LAG3*, were significantly
208 decreased in ITK-KO CD19-CAR-T cells (Figure 3C). Together, our results suggested that ITK
209 may affect CAR-T cell activation, decrease T cell exhaustion, and promote memory formation.
210 To gain further insights into how ITK regulates the heterogeneity and transcriptomic profile of
211 CAR-T cells, we conducted single-cell RNA sequencing (scRNA-seq) on ITK-KO and nt-KO
212 CD19-CAR-T cells, following stimulation by MEC1 cells for 48 hours. By merging both nt-KO and
213 ITK-KO CD19-CAR-T cells, 8 T cell populations with different states were identified (Figure 3, F

214 and G and Supplemental Figure 3, D-F). Interestingly, while other populations were at similar level
215 between nt-KO and ITK-KO CD19-CAR-T cells, we observed that the memory-progenitor
216 population in ITK-KO CD19-CAR-T cells was almost two-fold higher compared to in nt-KO CD19-
217 CAR-T cells (33.74% in ITK-KO CD19-CAR-T cells vs 13.94% in nt-KO CD19-CAR-T cells)
218 (Figure 3, F and G and Supplemental Figure 3, D-F). In line with the observations from bulk RNA-
219 seq, lower expression of *LAG3* was observed in ITK-KO CD19-CAR-T cells (Figure 3H). In
220 contrast, multiple genes highly expressed in memory T cells, such as *TCF7* (encoding TCF1),
221 *KLF2* and *IL7R* (encoding CD127), showed increased expression in ITK-KO CD19-CAR-T cells,
222 especially within the memory-progenitor T cell population (Figure 3, I-K). This is consistent with
223 the observed increase of *TCF7*, *KLF2* and *IL7R* expression in ITK-KO CD19-CAR-T cells in the
224 bulk RNA-seq analysis (Figure 3, C and D and Supplemental Figure 3G). These findings
225 collectively indicate that ITK deficiency may reduce exhaustion and enhance T cell memory fate
226 in CD19-CAR-T cells.

227

228 **ITK deficiency reduces exhaustion and promotes memory phenotype in CD19-CAR-T cells** 229 **in vitro**

230 To further investigate the role of ITK in regulating T cell activation, exhaustion, and memory, we
231 determined the protein expression of multiple key molecules involved in these processes between
232 nt-KO and ITK-KO CD19-CAR-T cells under steady state or co-cultured with tumor cells. Despite
233 a slight decrease in CD69 expression in ITK-KO CD19-CAR-T cells under steady-state conditions
234 (Figure 4, A and B), we observed a significant up-regulation of CD69 expression in the ITK-KO
235 CD19-CAR-T cells upon stimulation with MEC1 cells, reaching levels comparable to those
236 observed in nt-KO CD19-CAR-T cells (Figure 4, A and B). These results indicated that ITK
237 deficiency did not affect the activation of CD19-CAR-T cells following stimulation by the targeted
238 tumor cells. Interestingly, we noted a significant down-regulation of the expression of multiple co-
239 inhibitory molecules associated with CAR-T cell exhaustion (37), including LAG-3, PD-1, and TIM-

240 3, in ITK-KO CD19-CAR-T cells compared to nt-KO CD19-CAR-T cells 48 hours after co-culture
241 with tumor cells (Supplemental Figure 4, A-D). This down-regulation was observable in ITK-KO
242 CD19-CAR-T cells both at the steady state and after co-culture with MEC1 cells or Raji cells
243 (Supplemental Figure 4, A-F). Moreover, down-regulation of co-inhibitory molecules, including
244 PD-1, TIGIT, TIM-3 and CTLA4, was observed in ITK-KO CD19-CAR-T cells 15 days after co-
245 culture with MEC1 cells (Figure 4, C and D, and Supplemental Figure 4, G and H). Remarkably,
246 the decreased expression of PD-1, LAG-3, and TIM-3 in ITK-KO CD19-CAR-T cells, compared
247 to nt-KO CD19-CAR-T cells, remained significant after multiple rounds of exposure to the targeted
248 tumor cells (Figure 4E). We also analyzed the cytotoxic potential of ITK-KO CD19-CAR-T cells
249 following repeated exposure to cancer cells. While we observed a limited but significant decrease
250 in their cytotoxic effect in the initial phases of antigen exposure (e.g., rounds 1 through 6, and
251 round 8), the tumor-killing ability of ITK-KO CD19-CAR-T cells reached comparable levels to nt-
252 KO CD19-CAR-T cells at later time-points (e.g., rounds 7 and 9) (Figure 4F and Supplemental
253 Figure 4I). These data suggest that ITK-KO CD19-CAR-T cells retain good anti-tumor activity
254 following repeated exposure to CD19⁺ cancer cells, probably due to less T cell exhaustion and
255 enhanced memory development. In fact, following exposure to tumor cells, there was a
256 significantly decreased fraction of terminally differentiated cells and an increased fraction of
257 central memory cells in ITK-KO CD19-CAR-T cells compared to nt-KO CD19-CAR-T cells (Figure
258 4, G and H, and Supplemental Figure 4J and K). Together, these results suggest that ITK
259 deficiency decreases T cell exhaustion and enhances memory fate of CD19-CAR-T cells in vitro.

260

261 **ITK deficiency enhances expansion and long-term persistence of CD19-CAR-T cells in vivo**

262 To test the long-term effects of *ITK* deletion on CD19-CAR-T cells in vivo, we first used a CLL
263 mouse model of *NOD-Prkdc^{scid}Il2ry^{null}* (NPG) mice bearing MEC1 tumor cells (injected
264 intraperitoneally) followed by infusion with ITK-KO or nt-KO CD19-CAR-T cells (Figure 5A). In this
265 tumor model, both ITK-KO and nt-KO CD19-CAR-T cells efficiently cleared tumor cells 14 days

266 after infusion (Supplemental Figure 5A). There was no significant difference in the body weight
267 of mice between ITK-KO and nt-KO CD19-CAR-T cell-injected groups (Supplemental Figure 5B).
268 Interestingly, upon tumor clearance, the abundance of ITK-KO CD19-CAR-T cells peaked at a
269 significantly higher level than nt-KO CD19-CAR-T cells (Figure 5, B and C), in agreement with the
270 enhanced expansion of ITK-KO CD19-CAR-T cells. In addition, while nt-KO CD19-CAR-T cells
271 rapidly contracted to levels that were nearly undetectable, ITK-KO CD19-CAR-T cells remained
272 in circulation at a significantly higher level even 77 days post-infusion (Figure 5, B and C).
273 Moreover, ITK-KO CD19-CAR-T cells showed significantly lower expression of multiple co-
274 inhibitory molecules that are associated with T cell exhaustion, including LAG-3, PD-1, TIM-3,
275 TIGIT and CTLA4 (Figure 5, D and E). As expected, significant decrease of apoptosis (indicated
276 by Annexin V) and significant increase of proliferation (indicated by Ki-67) were observed in ITK-
277 KO CD19-CAR-T cells compared to nt-KO CD19-CAR-T cells (Figure 5, F and G, and
278 Supplemental Figure 5, C and D). Notably, we observed a high level of apoptosis in CAR-T cells
279 (Supplemental Figure 5D), which may be because that these apoptotic CAR-T cells were in the
280 contraction phase at this time point and were not rapidly cleared in the immune-deficient recipients.
281 Remarkably, there were more effector memory and less terminally differentiated CAR-T cells in
282 mice injected with ITK-KO CD19-CAR-T cells compared to controls (Figure 5H, and Supplemental
283 Figure 5E). As enhanced memory cell phenotype has been correlated with improved long-term
284 CAR-T cell therapeutic effects (36, 38, 39), our results suggest that ITK deficiency enhances
285 expansion and long-term persistence of CD19-CAR-T cells due to ITK-mediated reduction of
286 exhaustion and improvement of memory in CAR-T cells in vivo.

287 The CLL cell line MEC1 could also be subcutaneously injected into NPG mice and form a solid
288 tumor (40). We further tested the impact of *ITK* deletion on CD19-CAR-T cells in this mouse model
289 of CLL with solid tumor (41) (Figure 5I). Consistent with the previous intraperitoneal MEC1
290 injection model, ITK-deficient CD19-CAR-T cells not only expanded to a higher peak level but

291 also persisted much longer in the peripheral blood after injection into the MEC1-bearing NPG
292 mice (Figure 5, J and K). Interestingly, we found that ITK-deficient CD19-CAR-T cells showed a
293 slight delay in the pattern of increase compared to nt-KO CD19-CAR-T cells (Figure 5, J and K).
294 This observation might be consistent with the results that mice from nt-KO CD19-CAR-T cell
295 injected groups showed a trend of faster tumor cell clearance, although with no significant
296 difference compared to mice from ITK-KO CD19-CAR-T cells injected groups (Supplemental
297 Figure 5F). The body weight of mice between ITK-KO and nt-KO CD19-CAR-T cell injected
298 groups also showed no significant difference in this tumor model (Supplemental Figure 5G).
299 Overall, above results show that ITK deficiency enables enhanced expansion and long-term
300 persistence of CD19-CAR-T cells in vivo in pre-clinical animal models of CLL.

301

302 **ITK-deficient CAR-T cells significantly improve control of tumor relapse in vivo**

303 As we observed significantly improved expansion and long-term persistence of ITK-deficient
304 CD19-CAR-T cells compared to nt-KO CD19-CAR-T cells in vivo, we speculated that mice
305 receiving ITK-KO CD19-CAR-T cells might be better protected against tumor relapse. The Raji
306 cell xenograft mouse model is known to relapse after the first wave of tumor clearance by CAR-T
307 cells (42, 43). Therefore, we used this model (Figure 6A) to assess the function of ITK in CD19-
308 CAR-T cell activity against tumor relapse. In this model, ITK-KO CD19-CAR-T cells showed
309 significantly enhanced expansion and long-term survival compared to nt-KO CD19-CAR-T cells
310 (Figure 6, B and C), as ITK-KO CD19-CAR-T cells displayed lower levels of apoptotic cells and
311 increased proliferation (Figure 6, D and E, and Supplemental Figure 6, A and B). In addition, ITK-
312 KO CD19-CAR-T cells showed significantly reduced levels of the T cell exhaustion molecules,
313 LAG-3 and TIGIT (Figure 6, F and G, and Supplemental Figure 6, C and D). Raji cell-bearing mice
314 that were treated without CAR-T cells showed progression of tumor growth and succumbed to
315 tumor growth before 40 days after Raji cell injection (Figure 6, H and I). In contrast, tumor-grafted
316 mice that received nt-KO CD19-CAR-T cells cleared tumors within 21 days but showed notable

317 cancer relapse approximately 56 days after CAR-T cell injection (Figure 6, H and I). This group
318 of mice also finally died of uncontrolled tumor relapse (Figure 6, H and I). Interestingly, even after
319 tumor relapse at day 56, animals that received ITK-KO CD19-CAR-T cells exhibited better control
320 of the relapsed tumor, leading to significantly improved survival (Figure 6, H and I, and
321 Supplemental Figure 6E). No significant difference in body weight was observed between groups
322 of mice that received either nt-KO or ITK-KO CD19-CAR-T cells (Supplemental Figure 6F). Above
323 results demonstrate that ITK deficiency in CD19-CAR-T cells promotes CAR-T cell expansion and
324 survival in vivo and enables better control of tumor relapse.

325 Higher frequency of effector memory and central memory CAR-T cell populations were observed
326 in mice that received ITK-KO CD19-CAR-T, compared to mice that received nt-KO CD19-CAR-T
327 cells (Figure 6J and Supplemental Figure 6G). To further validate the long-term effector-memory
328 function of ITK-KO CD19-CAR-T cells in vivo, we tested the memory-recall responses of ex-vivo
329 ITK-KO CD19-CAR-T cells, from Raji-grafted mice at day 50 after CAR-T cell injection (when the
330 first wave of Raji growth had been controlled). As shown, ex-vivo ITK-KO CD19-CAR-T cells
331 showed significant control of Raji cell growth and expansion when co-cultured with Raji cells
332 (Figure 6, K-M). Furthermore, we observed an increase of IFN- γ , TNF- α and Granzyme B
333 expression in ex-vivo ITK-KO CD19-CAR-T cells after co-culture with Raji cells (Figure 6N and
334 Supplemental Figure 6H). Together, our results suggest that ITK deficiency in CD19-CAR-T cells
335 promotes CD19-CAR-T cell memory fate associated with less T cell exhaustion and better in vivo
336 persistence, providing a potentially more sustainable CAR-T cell therapy.

337

338 **ITK deficiency attenuates exhaustion and promote memory phenotype in CD19-CAR-T** 339 **cells derived from CLL patients**

340 T cells from CLL patients show notable exhaustion (35), and prior treatment with ibrutinib, a BTK
341 inhibitor, was associated with an increased response rate of CAR-T cell therapy in CLL patients
342 (30). Since ibrutinib can also inhibit ITK (44), we speculate that the improved CAR-T therapeutic

343 effects in ibrutinib-treated CLL patients are in part due to the inhibition of ITK in CAR-T cells.
344 Therefore, we derived CD19-CAR-T cells from PBMCs of CLL patients (referred to as CLL-CAR-
345 T cells) and investigated the role of ITK in CLL-CAR-T cells. Notably, while the total cell number
346 of PBMCs from CLL patients was comparable to that from healthy donors (Supplemental Figure
347 7A), the proportion of T cells was significantly reduced in PBMCs from CLL patients
348 (Supplemental Figure 7, B-E). The viability of T cells from CLL patients was similar to that of T
349 cells from healthy donors (Supplemental Figure 7F). ITK-KO CLL-CAR-T cells showed slightly
350 increased TNF- α expression, following stimulation (Figure 7, A and B). In addition, a slight
351 decrease in T cell activation as indicated by CD69 was observed in ITK-KO CLL-CAR-T cells
352 when co-cultured with MEC1 cells (Figure 7, C and D). And as expected, ITK-KO CLL-CAR-T
353 cells showed a significant decrease in the expression of co-inhibitory molecules, including LAG-
354 3, PD-1 and TIM-3 (Figure 7, E and F). Furthermore, ITK-KO CLL-CAR-T cells exhibited
355 significantly lower apoptosis when co-cultured with MEC1 cells for 15 days compared to nt-KO
356 CLL-CAR-T cells (Figure 7, G and H). ITK-KO CLL-CAR-T cells also demonstrated enhanced
357 expansion in vitro compared to nt-KO CLL-CAR-T cells, particularly at later time points
358 (Supplemental Figure 7G), despite having attenuated cytotoxic function, especially at low E:T
359 ratio (Supplemental figure 7H).

360 To further determine whether inhibiting ITK kinase activity during the production of CAR-T cells
361 could mimic *ITK* deletion, we investigated the effects of PF-06465469 (a potent ITK inhibitor that
362 also inhibits BTK) and ibrutinib on CLL-CAR-T cells. Both PF-06465469 and ibrutinib significantly
363 decreased PD-1 and LAG-3 expression (Figure 7, I and J, and Supplemental Figure 7, I-N),
364 suggesting a potential decrease of exhaustion in CLL-CAR-T cells. Notably, CD69 expression
365 was also significantly decreased after PF-06465469 and ibrutinib treatment (Figure 7, I and J, and
366 Supplemental Figure 7, M and N), indicating reduced T cell activation. Consistent with the
367 expectation from previous results, there was a significantly increased in the fraction of central
368 memory cells in CLL-CAR-T cells 15 days after PF-06465469 treatment (Figure 7, K and L),

369 suggesting that ITK inhibition could also promote T cell memory in CD19-CAR-T cells generated
370 from CLL patient.

371 Of note, infusion of the inhibitor-treated CLL-CAR-T cells or control DMSO-treated CLL-CAR-T
372 cells into MEC1-bearing mice revealed only a trend but no significant increase of CLL-CAR-T
373 cells (Supplemental Figure 7, O-R). This data suggests that while inhibiting ITK during CAR-T cell
374 production can temporarily alleviate exhaustion and promote memory phenotype in CLL-CAR-T
375 cells in vitro, genetically targeting or continuously pharmacological inhibition of the T cell-intrinsic
376 ITK signaling is probably required for sustaining the long-term responses of CAR-T cells against
377 tumors.

378 To assess the long-term in vivo effects of *ITK* deletion on CLL-CAR-T cells, we employed a CLL
379 mouse model using NPG mice injected intravenously with MEC1 tumor cells, followed by infusion
380 with ITK-KO or nt-KO CLL-CAR-T cells (Figure 8A). The results showed that ITK-KO CLL-CAR-
381 T cells exhibited better expansion and long-term persistence compared to nt-KO CLL-CAR-T cells
382 (Figure 8, B and C). Additionally, ITK-KO CLL-CAR-T cells showed increased proliferation and
383 reduced apoptosis compared to nt-KO CLL-CAR-T cells (Supplemental Figure 8, A-D). There was
384 reduced exhaustion and enhanced memory phenotype in ITK-KO-CLL-CAR-T cells compared to
385 nt-KO CLL-CAR-T cells (Figure 8, D-G, and Supplemental Figure 8E). Furthermore, ITK-KO CLL-
386 CAR-T cells showed better control of tumor relapse (Figure 8, H and I, and Supplemental Figure
387 8F). Notably, mouse body weights were similar between the groups treated with ITK-KO and nt-
388 KO CLL-CAR-T cells (Supplemental Figure 8G). Above results suggest that CLL-CAR-T cells with
389 ITK deficiency exhibit enhanced efficacy in controlling tumor relapse.

390 Altogether, our results suggest that ITK deficiency could reduce exhaustion and promote memory
391 in CD19-CAR-T cells, improving the expansion and long-term persistence of CAR-T cells in vivo.
392 This contributes to better control of tumor relapse and potentially improves clinical outcomes.

393

394 **Discussion**

395 CAR-T therapy has emerged as a groundbreaking treatment for various hematologic
396 malignancies. However, the efficacy and long-term sustainability of CAR-T cells remain
397 challenging due to issues such as T cell exhaustion, relatively short persistence in vivo and
398 antigenic escape (45, 46). Furthermore, CAR-T cell therapy faces a substantial hurdle given its
399 autologous nature, which necessitates using a patient's own T cells for CAR-T cell production.
400 Obtaining a sufficient quantity and quality of T cells proves exceedingly difficult, primarily due to
401 the occurrence of T cell lymphopenia and exhaustion prevalent in cancer patients, including those
402 with chronic lymphocytic leukemia (CLL) (47). These limitations cause concerns about the safety,
403 efficacy, and accessibility of CAR-T cell therapy. Our data provide direct evidence for the cell
404 intrinsic role of ITK in regulation of expansion and memory development, as well as exhaustion in
405 CAR-T cells. Deletion of *ITK* during CAR-T cell production leads to enhanced CAR-T cell
406 expansion and memory development, along with reduced cell apoptosis and exhaustion upon
407 repeated exposures to tumor cells. This improved CAR-T cell sustainability is of particular interest
408 for controlling tumor relapse.

409 One of the interesting findings of this study is the attenuation of immediate cytotoxicity in ITK-
410 deficient CAR-T cells against tumor cells. While these cells exhibit slightly reduced cytotoxicity
411 against tumor cells, they do not show a significant decline in the production of critical effector
412 cytokines such as IFN- γ , TNF- α , and Granzyme B. This suggests that ITK deficiency primarily
413 affects the immediate cytotoxic effects of CAR-T cells, which may not be as critical for long-term
414 anti-tumor responses. Indeed, previous studies demonstrated that ITK is not required for T cell
415 activation, but it rather promotes the strength of TCR signaling and thus TCR-dependent cytotoxic
416 T cell function (48). This ITK-mediated strong TCR signaling can tune down T cell responses to
417 cytokines that can drive T cell expansion, survival and memory development, such IL-2 and IL-4
418 (49).

419 Our study reveals that ITK-deficient CAR-T cells exhibit enhanced expansion and long-term
420 survival in response to tumor antigen stimulation. This is crucial for maintaining therapeutic

421 efficacy over extended periods. While initial expansion rates are similar between ITK-deficient
422 and control CAR-T cells, the former outperform at later stages. This could be attributed to reduced
423 apoptosis and prolonged proliferation in ITK-deficient CAR-T cells. Regarding the in vitro
424 expansion of CAR-T cells, it appears that CD4⁺T cells exhibit greater improvement for expansion
425 compared to CD8⁺T cells following *ITK* knockout. This difference might be due to the varying
426 expression levels of IL-2 receptors on CD4⁺ T cells and CD8⁺ T cells (50). During the in vitro
427 expansion of CAR-T cells, relatively high level of IL-2 was added to the culture medium to produce
428 sufficient CAR-T cells for clinical use (51-54). Given the higher expression of IL-2 receptors in
429 CD8⁺T cells (50), the extend of IL-2 downstream signaling activation might be closer to saturation
430 in CD8⁺T cells than in CD4⁺T cells.

431 In addition, our current knowledge of the role of ITK in T cell exhaustion is very limited. Importantly,
432 this study shows that ITK deficiency leads to reduced T cell exhaustion, as evidenced by lower
433 expression of exhaustion markers such as LAG-3, PD-1, TIM-3, TIGIT, and CTLA4. Moreover,
434 the transcriptomic and single-cell RNA sequencing analyses indicate that ITK-KO CAR-T cells
435 are characterized by a more marked central memory phenotype, which could contribute to their
436 sustained anti-tumor activity. Recently it has been demonstrated that tumor-infiltrating TCF1⁺ T
437 cells have long-term memory, and are capable of self-renewal and persistent control of tumor
438 growth. It is possible that the up-regulation of TCF1, a dominant transcription factor negatively
439 regulating T cell terminal differentiation and exhaustion, observed in ITK-KO CAR-T cells might
440 prevent the progressive exhaustion and maintain a long-term memory.

441 We showed that while pharmacological ITK inhibition could mitigate T cell exhaustion and
442 promote memory phenotype in CAR-T cells derived from patients with CLL, it only showed a trend
443 but no significant increase of CLL-CD19-CAR-T cells in the tumor-bearing mouse model in vivo.
444 These observations may suggest that transient inhibition of ITK may not be enough to maintain
445 CAR-T cell status with low exhaustion and better memory. Genetic deletion or silencing of *ITK* at
446 least for a certain is probably required for efficiently and persistently reduce T cell exhaustion and

447 promote T cell memory in CAR-T cells. Of note, the control of relapse varied among individual
448 tumor-bearing mice, which might be attributed to biological variation in the recipient mice. This
449 variability suggests that clinical outcomes might differ among patients when using ITK-deficient
450 CAR-T cells for managing tumor relapse. Exploring host factors that might influence the
451 effectiveness of ITK-deficient CAR-T cells in controlling tumor relapse would be a valuable
452 direction for future research.

453 Previous studies have shown that transient treatment with dasatinib reduces expression of
454 exhaustion markers and increases expression of stem cell memory-associated markers, and
455 improves tumor clearance (18, 19). Investigate whether long-term dasatinib treatment or
456 permanent deletion of dasatinib targets could enhance CAR-T cell persistence and better control
457 tumor relapse in vivo would be an intriguing area for future research.

458 Since lentivirus transduction and electroporation, which might act as stimulatory factors affecting
459 T cell activation and function, are commonly used in CAR-T cell production and gene editing,
460 examining their overall impact on CAR-T cells in further studies would be beneficial. Additionally,
461 although we selected an ITK-targeting sgRNA with less overall off-target effects and with the top
462 predicted off-target sites outside the coding sequences of known genes, potential off-target effects
463 in introns or intergenic regions still should be considered, as these regions may affect gene
464 expression (55).

465 It has been reported that ITK deficiency may impact Th17 development and promote Treg
466 generation (56-60). Additionally, several studies have shown that *Itk*^{-/-} CD4 T cells exhibit defects
467 in producing Th2 cytokines in mice (61, 62). Our findings indicate that the expression of IL-17A,
468 IL-4, IL-13 and FOXP3 were low in the CAR-T cells, with minimal differences observed between
469 ITK-KO and nt-KO CAR-T cells. The low expression of these cytokines may be attributed to the
470 in vitro culture environment, where CAR-T cells are exposed to tumor cells, potentially favoring
471 Th1 cytokine expression. However, the effects of ITK on the expression of these molecules still
472 should be further considered in clinical applications involving human CAR-T cells, which may

473 encounter more complex environmental conditions. The expectation of inhibiting Th2, Th17 and
474 favoring Treg generation effects by ITK deficiency in CAR-T cells may potentially result in
475 attenuated cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity
476 syndrome (ICANS) in clinical settings. Moreover, the absence of ITK has been shown to attenuate
477 T cell migration to several peripheral organs, such as the intestine and brain (63-66), which may
478 further contribute to reduced CRS and ICANS during ITK-KO CD19-CAR-T cell therapy.
479 Investigating these aspects in future studies could provide valuable insights.

480 In conclusion, this study demonstrates that ITK deficiency enhances the expansion, with reduction
481 of exhaustion, and improvement of the long-term therapeutic effects of CAR-T cells. These
482 findings offer promising insights into the development of more effective and sustainable CAR-T
483 cell therapies for a broad spectrum of cancers. Further research and clinical trials are warranted
484 to validate the clinical applicability of targeting ITK in CAR-T cell therapy.

485

486 **Methods**

487 **Sex as a biological variable.**

488 Our study examined male and female animals, and similar findings are reported for both sexes.

489

490 **Mice**

491 NOD.Cg-Prkdc^{scid} Il2rg^{tm1Vst/Vst} (NPG) mice were from Beijing Vitalstar Biotechnology ranging
492 from 6 to 8 weeks of age. Experiments were performed with both male and female mice unless
493 otherwise indicated. For MEC1 cell-derived xenograft models, NPG mice were implanted with
494 1.0×10^7 MEC1 cells expressing firefly luciferase intraperitoneally in the left flank, or with 1.0×10^7
495 MEC1 cells subcutaneously, or with 5.0×10^6 MEC1 cells expressing firefly luciferase via the lateral
496 tail veins, using a 27-gauge needle. Five to ten days post-engraftment, mice bearing tumor were
497 intravenously administered 2.0×10^6 nt-KO or ITK-KO CAR-T cells via the lateral tail veins. For
498 Raji cell-derived xenograft models, NPG mice were implanted with 5×10^5 Raji cells expressing

499 firefly luciferase intraperitoneally in the left flank. Ten days later, mice bearing tumor were
500 intravenously injected with 5.0×10^6 nt-KO or ITK-KO CAR-T cells via the lateral tail veins.
501 Following CAR-T cell injection, blood samples were collected from the orbital venous plexus of
502 mice at indicated time points. Red blood cells were removed using ACK lysis buffer
503 (Cat#A1049201, Gibco). Then PBMC cells were proceeded to flow cytometry analysis. Mice
504 injected with MEC1 cells subcutaneously were monitored three times a week using a square
505 caliper to measure tumor growth (tumor volume = $\pi/6 \times \text{length} \times \text{width} \times \text{height}$) as previously
506 described (67). Mice injected with MEC1 or Raji cells intraperitoneally or intravenously were
507 monitored by in vivo imaging (IVIS Lumina III, PerkinElmer) at indicated timepoints. D-luciferin
508 potassium salt (Cat #122799, PerkinElmer) was dissolved in PBS to create a working solution at
509 15 mg/mL. All mice received an intraperitoneal injection of luciferin solution (150mg/kg) 10
510 minutes prior to in vivo imaging.

511

512 **Human T cell isolation and CAR-T production**

513 Blood samples were obtained from the Second Xiangya Hospital. Ficoll-Paque (Cat#25710,
514 Dongfang Huahui Co. Ltd., Beijing) was used for isolating peripheral blood mononuclear cells
515 (PBMCs) as previously reported (68). PBMCs were further used for T cell enrichment using an
516 EasySep Human T Cell Isolation Kit (Cat#17951, STEMCELL Technologies) following the
517 manufacturer's instructions. The enriched T cells were activated with Dynabeads Human T-
518 Activator CD3/CD28 (Cat#11132D, Gibco; T cells: beads = 1:2) for 24 hours and transduced with
519 CAR-encoding lentivirus (multiplicity of infection (MOI) =8). Twenty-four hours after CAR
520 transduction, the lentivirus-containing media was replaced with X-VIVO15 complete medium
521 supplied with IL-2 (Peprotech, Cat#200-02, 100 U/mL). Gene editing in CAR-T cells by
522 electroporation of RNP complex was performed 48 to 72 hours after CAR transduction. After gene
523 editing, CAR-T cells were cultured for additional 3 to 5 days before proceeding with in vitro

524 analysis or further expansion, or for additional 11 to 14 days before injection into mice in the in
525 vivo mouse experiments, unless otherwise noted.

526

527 **Gene editing in CD19-CAR-T cells**

528 CRISPR-mediated gene editing was used for deleting *ITK*. Single guide RNA (sgRNA) sequences
529 were designed using the CRISPick online tool
530 (<https://portals.broadinstitute.org/gppx/crispick/public>, Broad Institute) to achieve SpyCas9-
531 mediated CRISPR-KO. Two sgRNAs (named ITK-sg1 and ITK-sg2, see Supplemental Table 1)
532 were selected along with a non-targeting control sgRNA (named nt-sg1) (69), and synthesized
533 (GenScript Co., Ltd. Nanjing, China). Cas9 protein was purchased from Advanced Biomart (Cat#
534 CCN-066AB) and was delivered by nucleofection as a RNP complex with sgRNAs. Briefly, three
535 days after CAR transduction, anti-CD3/CD28 beads were removed and 5×10^6 CAR-T cells were
536 resuspended in 100 μ l electroporation buffer (P3 Primary Cell Solution 82 μ l mixed with
537 Supplement 18 μ l; Cat#V4XP-3024, Lonza). Cas9 protein (30 μ g) was gently mixed with specific
538 sgRNA (30 μ g) and placed at room temperature for 15 minutes, mixed with cells and transferred
539 to Nucleocuvett Vessels and electroporated using a 4D-Nucleofactor (Cat#AAF-1003X, Lonza)
540 with program EO115, followed by CAR-T cell culture and expansion in pre-warmed X-VIVO15
541 media. Three days later, genomic DNA was extracted and used for validation by Sanger
542 sequencing. Gene-editing efficiency and potential off-target effects were validated by PCR (see
543 Supplemental Table 1 and 2 respectively) and subsequently Sanger sequencing. The obtained
544 sequencing results were analyzed with the TIDE online tool (<http://shinyapps.datacurators.nl/tide/>)
545 to calculate gene editing efficiency, with a threshold set at a P-value of 0.001. SnapGene v6.01
546 was used for reading the Sanger sequencing results of DNA PCR products.

547

548 **In vitro killing assay**

549 MEC1 (CAT#CL-0761, Pricella), HG-3 (CAT#ACC765, DSMZ) and Raji cells (CAT#CL-0189,
550 Pricella) stably expressing firefly luciferase were obtained via lentiviral transduction with the
551 pLVX-Luc2-puro plasmid (Ningbo Testobio Co., Ltd (TSPLA10184)) following selection with 2
552 µg/ml puromycin (Cat# P8230, Solarbio, Beijing, China). CAR-T cells (effector, E) were co-
553 cultured with tumor cells (target, T) at indicated ratios in a 96-well cell culture plate for 48 hours,
554 and cancer cell lysis was detected using the Steady-Glo® Luciferase assay system (Cat#E2520,
555 Promega). The percentage of specific lysis was calculated as:

$$556 \quad \text{Specific lysis (\%)} = \left(1 - \left(\frac{\text{value of test well} - \text{value of CAR-T cell only well}}{\text{value of tumor cell only well}} \right) \right) \times 100\%$$

557 For serial tumor killing assays, CAR-T cells were co-cultured with MEC1 cells at an E: T ratio of
558 2:1 in a 96-well plate. Bioluminescence was measured every 48 hours and the E: T cell ratio was
559 re-adjusted to 2:1 for co-culture after each sampling. Nine rounds of analyses were performed.

560

561 **RNA sequencing and data analyses**

562 CAR-T cells were co-cultured with MEC1 cells at an Effector (E) to target (T) ratio of 2:1 in X-
563 VIVO 15 medium with 5% FBS at 37°C with 5% CO₂ for 48 hours. Following incubation, GFP⁺
564 CAR-T cells were FACS-sorted and the purified nt-KO or ITK-KO CAR-T cells were used for both
565 bulk and single cell RNA sequencing and analyses as previously described (70) and detailed in
566 the Supplemental Materials. Sequencing data is available in NCBI.

567

568 **Statistical analyses**

569 Two-tailed unpaired Student's *t*-test was performed for statistical analysis by GraphPad Prism
570 6.01 software unless otherwise noted. Log-rank (Mantel-Cox) test with Bonferroni's correction for
571 multiple comparisons was performed for the statistical analysis of the survival curves with
572 Graphpad Prism 6.01 software. Statistical analyses of solid tumor growth in mouse model with
573 subcutaneous injection of MEC1 cells were conducted by linear mixed-effect modeling (over the

574 whole-time course) with Bonferroni's correction for multiple comparisons as described previously
575 (71). Data for statistical analyses were presented as mean \pm SD unless otherwise noted. *, P <
576 0.05. **, P < 0.01. ***, P < 0.001. ****, P < 0.0001.

577

578 **Study approval**

579 All animal experiments were approved by the Institutional Animal Care and Use Committee of the
580 Second Xiangya Hospital and the Suzhou Institute of Systems Medicine, Chinese Academy of
581 Medical Sciences (CAMS-ISM) animal facility. This study involves human samples and was
582 approved by the Institutional Review Board at the Second Xiangya Hospital (Reference number:
583 052 (2018)). Participants in the study gave informed consent in accordance with the Declaration
584 of Helsinki before taking part.

585

586 **Data availability**

587 The bulk RNA-seq and scRNA-seq data in this study have been deposited in the NCBI database
588 under accession code GSE278601 and GSE278612, respectively. Values for data points in
589 figures are reported in the Supporting Data Values file. Other data and materials are available
590 upon reasonable request.

591

592 **Author contributions**

593 WH and ZF conceived the research idea. ZF, HP, and WH designed the study. HP, ZF, QS and
594 WH supervised this study. ZF, ZH, HX, QL, JL, YD, HZ, PW, HL, LH, YF, CW and KS acquired
595 the data. ZH, HX, QS, SKC, KY, AZ, KS, WH and ZF analyzed the data. ZF, WH, HP, QS, ZH,
596 AA, GV, AZ and KS prepared the manuscript. ZF was listed first among the co-first authors
597 because ZF was responsible for all experimental designs, performed the animal experiments,
598 analyzed and interpreted data, and drafted and revised the manuscript. ZH was listed second
599 among the co-first authors because ZH participated in all the research, collected, analyzed, and

600 interpreted data, and revised the manuscript.

601

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809

810 **Figure Legends**

811 **Figure 1 ITK deficiency attenuates immediate cytotoxicity of CAR-T cells**

812 Nt-KO indicates the control group of CAR-T cells electroporated with RNP complex containing
813 non-targeting sgRNA, while ITK-KO indicates the group that received *ITK*-targeting sgRNA. **(A)**
814 Schematic representation of the anti-human CD19-CAR molecule. CMV, cytomegalovirus
815 promoter. CD8 α SP, signal peptide of human CD8 α . anti-CD19-scFv, single chain fragment
816 variable of anti-human CD19 antibody (clone: FMC63). CD8 α TM, transmembrane domain of
817 human CD8 α . CD28, 4-1BB and CD3 ζ , signal transduction domains of human CD28, 4-1BB and
818 CD3 ζ , respectively. **(B)** Generation of ITK-deficient CAR-T cells. Briefly, T cells were enriched
819 from PBMCs and activated with anti-CD3/CD28 beads for 24 hours. Then, T cells were
820 transduced with CAR-encoding lentivirus. 48 hours post-transduction, CAR-T cells were
821 electroporated with RNP complex. **(C)** Gene editing efficiency of *ITK* locus by sgRNA1 targeting
822 *ITK* (ITK-sgRNA1). CAR-T cells were collected for analysis 3 days after electroporation of RNP
823 complex. **(D)** Validation of *ITK* deficiency at protein level by western blotting. CAR-T cells were
824 collected for western blotting 5 days after electroporation. **(E-G)** In vitro killing assay against the
825 indicated target tumor cells using control and ITK-KO CAR-T cells (n = 4). Luciferase-expressing
826 MEC1, HG3 and Raji cells were mixed at the indicated ratios with CAR-T cells and analyzed 48
827 hours after co-culture. **(H)** Representative flow cytometric plots of IFN- γ , TNF- α and Granzyme B
828 expression in CAR-T cells stimulated as indicated. E, effector (CAR-T cells). T, target (MEC1
829 cells). **(I)** Summary of percentages of CAR-T cells expressing different cytokines in **H** (n = 4).
830 Compiled data from one independent experiment for **E-G** and **I**. Technical replicates are shown
831 in **E-G** and **I**. Data represent results of at least two independent experiments in **C-I**.

832

833 **Figure 2 ITK deficiency promotes long-term expansion of CD19-CAR-T cell in vitro**

834 **(A-C)** Fold expansion of total **(A)**, CD4⁺ **(B)** and CD8⁺ **(C)** CD19-CAR-T cells at the indicated time

835 points, following 48 hours co-culture with MEC1 cells at the E:T ratio of 2:1 (n = 3). Fold expansion
836 values of the cell numbers were normalized to the average cell number of the CD19-CAR-T cells
837 at day 0. (D and E) Representative flow cytometric plots of Annexin V and 7-AAD (D) and
838 summary of percentages of Annexin V⁺ cells (E) in CAR-T cells at the indicated time points. (F
839 and G) Representative flow cytometric plots of Ki-67 (F) and summary of percentages of Ki-67⁺
840 cells (G) in CAR-T cells at the indicated time points. n = 3 for each group in E and G. Compiled
841 data from one independent experiment in A-C, E and G. Data represent results of at least two
842 independent experiments. Statistical differences were determined by two-tailed unpaired
843 Student's *t*-test.

844

845 **Figure 3 Transcriptomic regulation of CD19-CAR-T cells by ITK**

846 CAR-T cells were co-cultured with MEC1 cells for 48 hours and sort-purified for both bulk and
847 single-cell RNA sequencing (RNA-Seq). (A-E) Bulk RNA-seq analysis of the transcriptome in nt-
848 KO and ITK-KO CAR-T cells. (A) Differential gene expression (TPM ≥ 1, P ≤ 0.05, fold change ≥
849 1.5). Unadjusted P values are shown. (B) KEGG analysis of the differentially expressed genes.
850 (C) Heatmap of the indicated gene expression. (D) RNA-seq tracks of read coverage at the *TCF7*
851 (left), *KLF2* (middle) and *LAG3* (right) loci in CAR-T cells. (E) Gene set enrichment analysis
852 (GSEA) of the Effector-memory CD8 T cell gene set analysis using differentially expressed genes
853 shown in A. (F-K) Single-cell RNA-seq (scRNA-seq) analysis of the transcriptome in nt-KO and
854 ITK-KO CAR-T cells. (F) Clustering of functional T cell subsets of all CAR-T cells based on UMAP.
855 (G) Percentage of indicated T cell subsets within total T cells identified by scRNA-seq in F. (H-K)
856 Expression of indicated genes in nt-KO and ITK-KO CD19-CAR-T cells.

857

858 **Figure 4 ITK deficiency reduces exhaustion and promote memory phenotype in CD19-CAR-** 859 **T cells in vitro**

860 CAR-T cells were co-cultured with MEC1 following the serial killing assay protocol detailed in the

861 method section. **(A)** Representative flow cytometric plots of CD69 expression in the indicated
862 CAR-T cells in the presence or absence of MEC1 cells. CAR-T cells were analyzed after co-
863 culture with MEC1 cells (E:T = 2:1) for 48 hours. **(B)** Summary of percentages of CD69⁺ cells in
864 **A** (n = 3 for each CAR-T only group, and n = 4 for each CAR-T + MEC1 group) (two-way ANOVA
865 with Sidak correction for multiple comparisons; *, P < 0.05; ****, P < 0.0001). **(C)** Representative
866 flow cytometric plots of PD-1, TIGIT, TIM-3 and CTLA4 expression in CAR-T cells 15 days after
867 co-culture with MEC1 cells at the ratio of E:T = 2:1. **(D)** Summary of percentages of PD-1⁺, TIGIT⁺,
868 TIM-3⁺, and CTLA4⁺ cells as shown in **C** (n = 4). **(E)**. Summary of percentages of LAG-3⁺, PD-1⁺
869 and TIM-3⁺ cells in the indicated CAR-T cells following the indicated rounds of co-culture with
870 MEC1 cells (n = 4). CAR-T cells were co-cultured with MEC1 cells at 2:1 ratio for 48 hours for
871 each round for **E** and **F**. **(F)** Percentages of specific lysis determined by in vitro killing assay of
872 MEC1 cells by the indicated CAR-T cells at indicated rounds of co-culture (n = 4). **(G)**
873 Representative flow cytometric plots of CD45RA and CCR7 expression the in indicated CAR-T
874 cells 15 days after co-culture with MEC1 cells (E:T = 2:1). **(H)** Summary of percentages of CAR-
875 T cells expressing CD45RA and/or CCR7 in **G** (n = 3). Compiled data from one independent
876 experiment for **B**, **D-F** and **H**. Two-tailed unpaired Student's *t*-test in **D-F** and **H**. Data represent
877 results of at least two independent experiments.

878

879 **Figure 5 *ITK* deficiency enhances expansion and long-term persistence of CD19-CAR-T** 880 **cells in vivo**

881 **(A)** Experimental design of the CAR-T therapy against intraperitoneally (i.p.) injected MEC1 in
882 NPG mice. CAR-T cells were expanded for 11 days after electroporation before i.v. injection into
883 mice. PB, peripheral blood. **(B)** Representative flow cytometric plots of CAR-GFP and CD3 in the
884 PB samples collected from nt-KO or *ITK*-KO CD19-CAR-T cell recipients at the indicated time
885 points. **(C)** Summary of the percentages of CAR-T cells (CD3⁺GFP⁺) showed in **B** (n = 3 for day
886 77 *ITK*-KO group and n = 4 for the rest). **(D)** Representative flow cytometric plots of the indicated

887 molecules by CAR-T cells in the PB samples collected from nt-KO or ITK-KO-CAR recipients 28
888 days after CAR-T cell infusion. (E) Summary of the percentages of CAR-T cells that are LAG-3⁺,
889 PD-1⁺, TIM-3⁺, TIGIT⁺, or CTLA-4⁺, as shown in D (n = 4). (F and G) Summary of the percentages
890 of CAR-T cells that are Ki-67⁺ (F) and Annexin V⁺ (G) as shown in **Supplemental Figure 5, C**
891 and D (n = 5). (H) Statistical analysis of different populations of cells shown in **Supplemental**
892 **Figure 5E** (n = 4). (I) Experimental design of the CAR-T therapy against subcutaneously (s.c.)
893 injected MEC1 in NPG mice. CAR-T cells were expanded for 11 days after electroporation before
894 i.v. injection into mice. (J) Representative flow cytometric plots of CAR-GFP and CD3 in the PB
895 samples collected from nt-KO or ITK-KO CD19-CAR recipients at the indicated time points. (K)
896 Summary of the percentages of CAR-T cells (CD3⁺GFP⁺) showed in J (n = 4, mean ± SEM).
897 Compiled data from one independent experiment for C, E-H and K. Statistical differences were
898 determined by two-tailed unpaired Student's *t*-test. Data represent results of at least two
899 independent experiments.

900

901 **Figure 6 ITK-deficient CAR-T cells significantly improve control of tumor relapse in vivo**

902 (A) Experimental design of the CAR-T therapy against intraperitoneally (i.p.) injected Raji in NPG
903 mice. CAR-T cells were expanded for 11 days after electroporation before i.v. injection into mice.
904 (B) Representative flow cytometric plots of CAR-GFP and CD3 in the PB samples collected from
905 indicated recipients at the indicated time points. (C) Summary of the percentages of CAR-T cells
906 (CD3⁺GFP⁺) showed in B (n = 1 for day 95 nt-KO group, n = 3 for day 95 ITK-KO group, n = 4 for
907 the rest). (D) Representative flow cytometric plots of Annexin V and 7-AAD by CAR-T cells in the
908 PB samples collected from indicated recipients 28 days after CAR-T cell injection. (E) Summary
909 of the percentages of CAR-T cells that are Annexin V⁺ as shown in D (n = 4). (F) Representative
910 flow cytometric plots of LAG-3 and TIGIT expression by CAR-T cells in the PB samples collected
911 from indicated recipients 24 days after CAR-T cell infusion. (G) Summary of the percentages of
912 CAR-T cells that are LAG-3⁺ or TIGIT⁺, as shown in F (n = 4). (H) Representative bioluminescence

913 images of NPG mice xenografted with Raji cells as designed in **A**. Representative figures from
914 one independent experiment. Experiment was repeated twice. **(I)** Kaplan-Meier survival of Raji-
915 bearing NPG mice treated with PBS, nt-KO CAR-T or ITK-KO CAR-T cells (n = 9) (log-rank
916 Mantel-Cox test with Bonferroni's correction for multiple comparisons; **, P < 0.01; ****, P <
917 0.0001). Compiled data from two independent experiments. **(J)** Statistical analysis of CD45RO
918 and/or CCR7 expression by CAR-T cells shown in **Supplemental Figure 6G** (n = 4). **(K)**
919 Statistical analysis of ratio of Raji to CAR-T cells as shown in **Supplemental Figure 6H** (n = 4).
920 **(M)** Statistical analysis of fold change of CAR-T cell numbers in samples as shown in
921 **Supplemental Figure 6H** (n = 4). **(N)** Statistical analysis of percentage of IFN- γ , TNF- α and
922 Granzyme B expression in CAR-T cells shown in **Supplemental Figure 6I** (n = 3). Compiled data
923 from one independent experiment for **C, E, G, J** and **K-M**. Two-tailed unpaired Student's *t*-test
924 was performed in **C, E, G, J** and **K-M**. Data represent results of at least two independent
925 experiments.

926

927 **Figure 7 ITK deficiency attenuates exhaustion and promotes memory phenotype in CD19-**
928 **CAR-T cells derived from CLL patients**

929 **(A)** Flow cytometric analyses of IFN- γ , TNF- α , and Granzyme B expression in CLL-CAR-T cells.
930 CLL-CAR-T cells were co-cultured with MEC1 cells at a 2:1 ratio for 48 hours in **A-J**. **(B)** Statistical
931 analysis of the percentage of IFN- γ , TNF- α , and Granzyme B expression in CAR-T cells shown
932 in **A** (n = 5). **(C)** Flow cytometry analyses of CD69 expression in indicated CLL-CAR-T cells after
933 co-culture with or without MEC1 cells. **(D)** Statistical analysis of CD69 expression shown in **C** (n
934 = 3 for CAR-T only group, n = 4 for CAR-T + MEC1 group). **(E)** Flow cytometry analyses of LAG-
935 3, PD-1, and TIM-3 expression in indicated CLL-CAR-T cells. **(F)** Statistical analysis of the
936 percentage of LAG-3⁺, PD-1⁺, and TIM-3⁺ cells shown in **E** (n = 3 for CAR-T only group, n = 4 for
937 CAR-T + MEC1 group). **(G)** Flow cytometry analyses of Annexin V and 7-AAD expression in
938 indicated CLL-CAR-T cells at indicated time points. **(H)** Statistical analysis of Annexin V⁺ CAR-T

939 cells shown in **G** (n = 4). **(I)** Flow cytometry analyses of CD69, LAG-3, and PD-1 expression in
940 indicated CAR-T cells co-cultured with MEC1 cells with/without PF-06465469 (1 μ M) treatment.
941 **(J)** Statistical analysis of the percentage of CD69⁺, LAG-3⁺, and PD-1⁺ cells in CD19-CAR-T cells
942 shown in **I** (n = 4). **(K)** Representative flow cytometric plots of CD45RA and CCR7 expression in
943 indicated CAR-T cells 15 days after PF-06465469 treatment. **(L)** Summary of percentages of
944 CAR-T cells expressing CD45RA and/or CCR7 in **K** (n = 4). Compiled data from one independent
945 experiment for **B**, **D**, **F**, **H**, **J** and **L**. Statistical differences were determined by two-tailed unpaired
946 Student's *t*-test. Data represent results of at least two independent experiments.

947

948 **Figure 8 ITK-deficient CAR-T cells derived from CLL patients improve control of tumor**
949 **relapse in vivo**

950 **(A)** Experimental design of the CAR-T therapy against intravenously injected MEC1 cells
951 expressing luciferase in NPG mice via the lateral tail veins. CAR-T cells were expanded for 12
952 days following electroporation before i.v. injection into mice. **(B)** Representative flow cytometric
953 plots of CAR-GFP and CD3 in the PBMCs collected from CLL-CAR-T cell recipients at the
954 indicated time points. **(C)** Summary of the percentages of CAR-T cells (CD3⁺GFP⁺) as shown in
955 **B** (mean \pm SEM. n = 5 for the nt-KO groups on day 26, 33, and 40, as well as the ITK-KO groups
956 on day 40 and 52. n=4 for nt-KO day 52 group. n = 6 for the rest groups). **(D)** Representative flow
957 cytometric plots of TIM-3 expression on CAR-T cells in the PBMCs collected from nt-KO or ITK-
958 KO CLL-CAR-T recipients 26 days post-infusion. **(E)** Summary of the percentages of CAR-T cells
959 that are TIM-3⁺ as shown in **D** (n = 5). **(F)** Representative flow cytometric plots of CD62L and
960 CD45RA expression in the indicated CAR-T cells collected. PBMCs were collected on day 33
961 after CAR-T cell injection. **(G)** Statistical analysis of different cell populations as shown in **F** (n =
962 5). **(H)** Representative bioluminescence images of NPG mice xenografted with MEC1 cells as
963 designed in **A**. **(I)** Survival of MEC1-bearing NPG mice treated with PBS, nt-KO CLL-CAR-T or
964 ITK-KO CLL-CAR-T cells (n = 6) (log-rank Mantel-Cox test with Bonferroni's correction for multiple

965 comparisons; *, $P < 0.05$; **, $P < 0.01$). Compiled data from two independent experiments in **C**,
966 **E**, **G** and **I**. Statistical differences were determined by two-tailed unpaired Student's *t*-test in **C**, **E**
967 and **G**. Data represent results of two independent experiments.

Figure 1

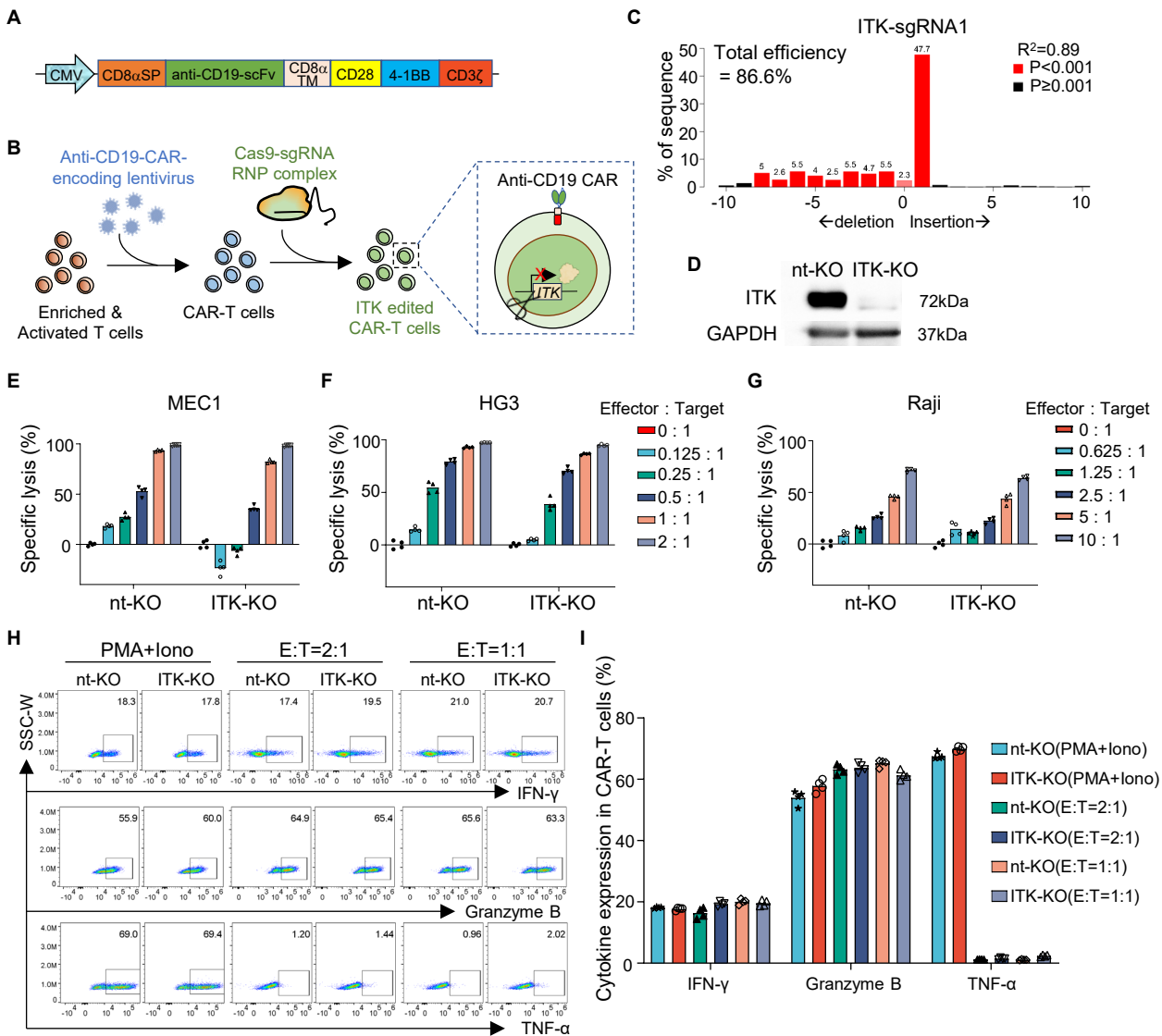


Figure 2

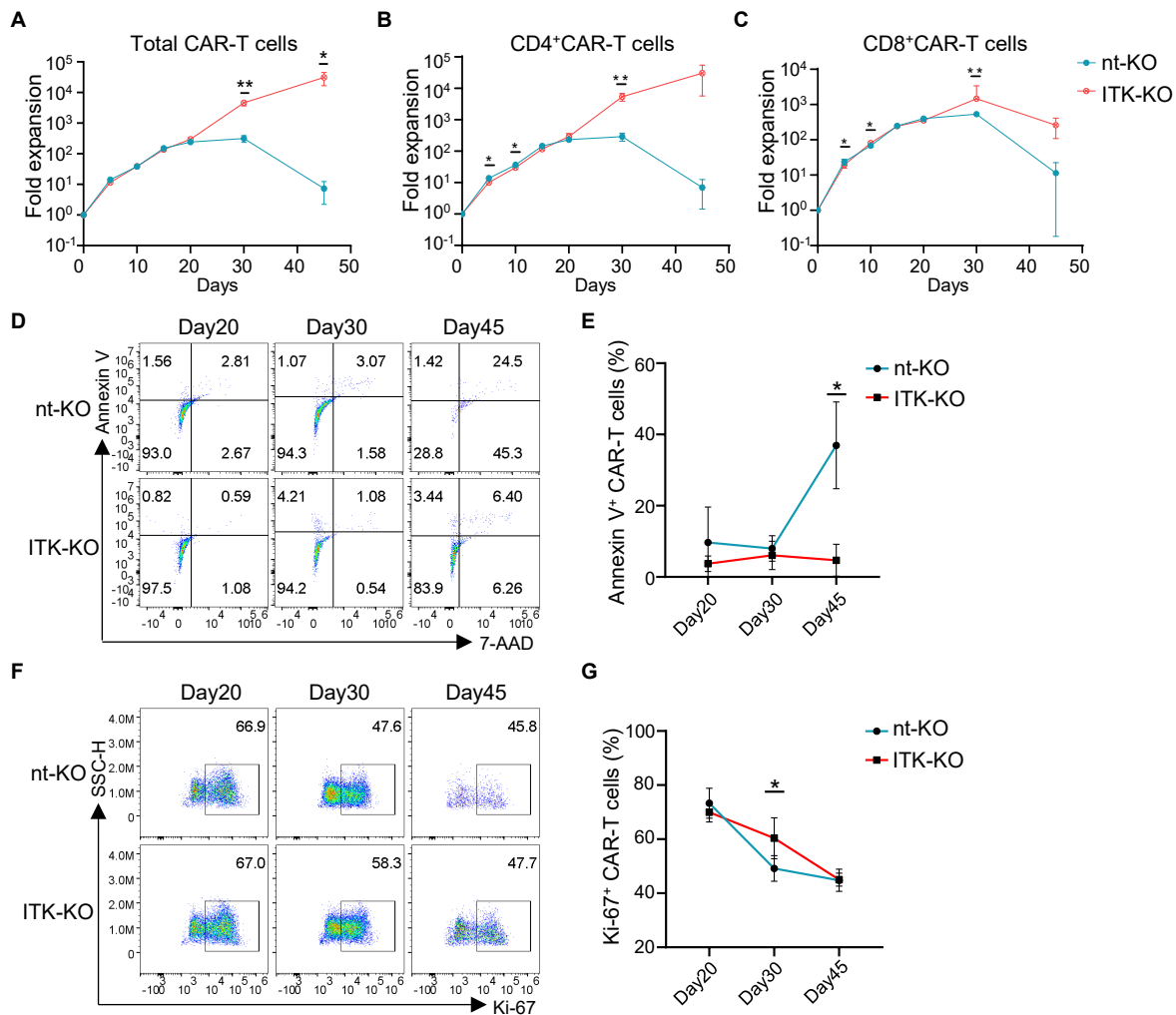


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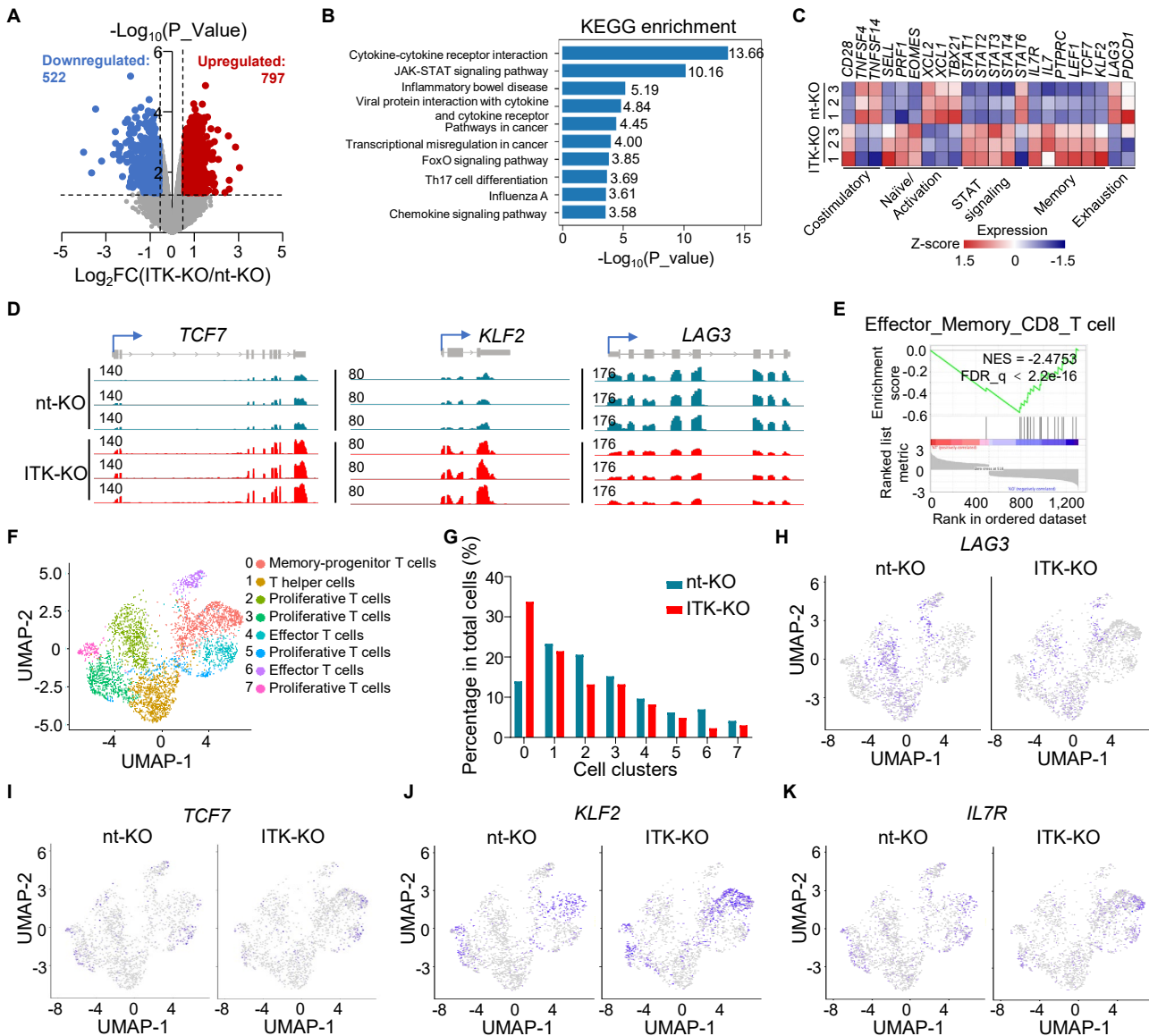


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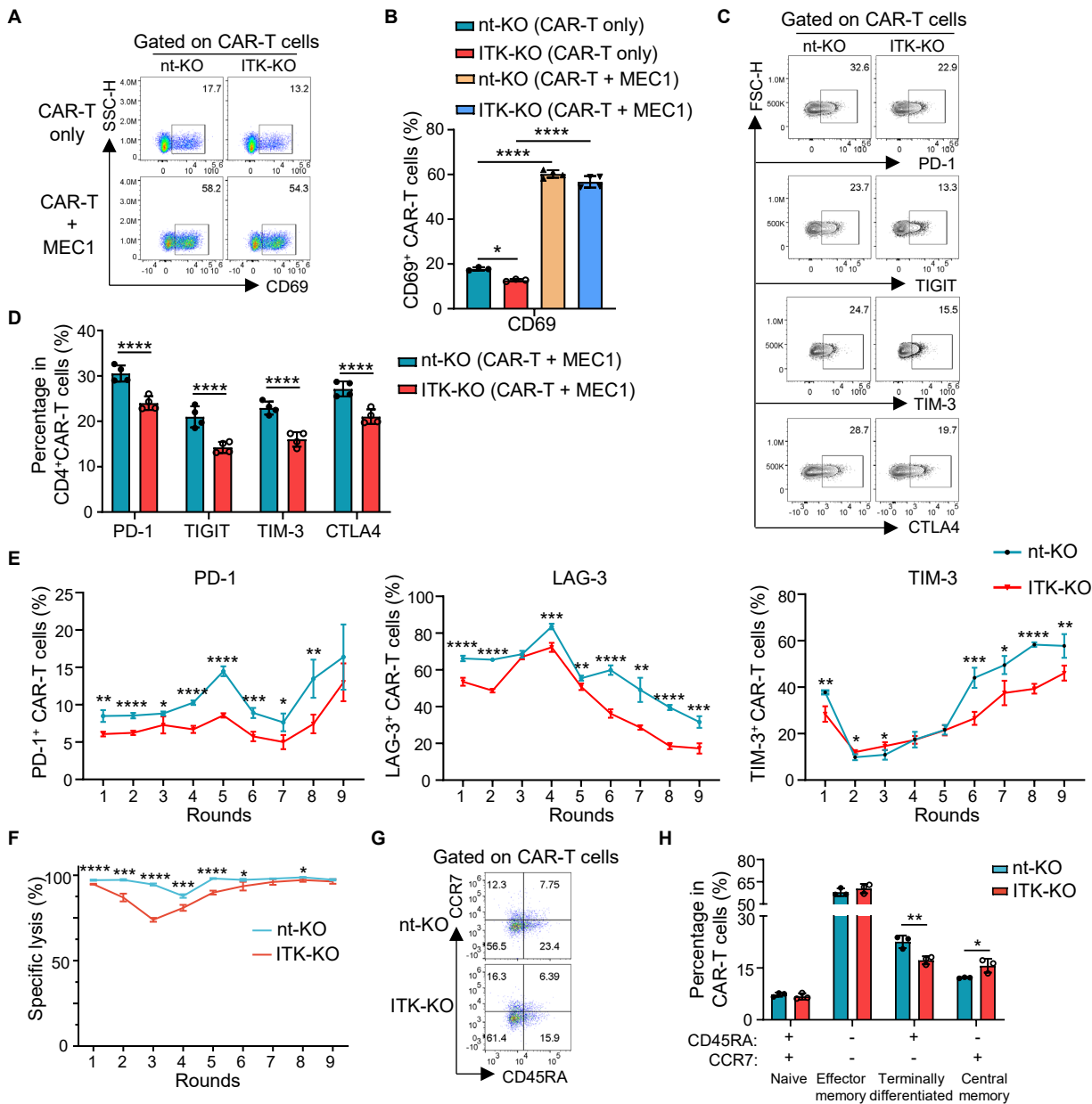


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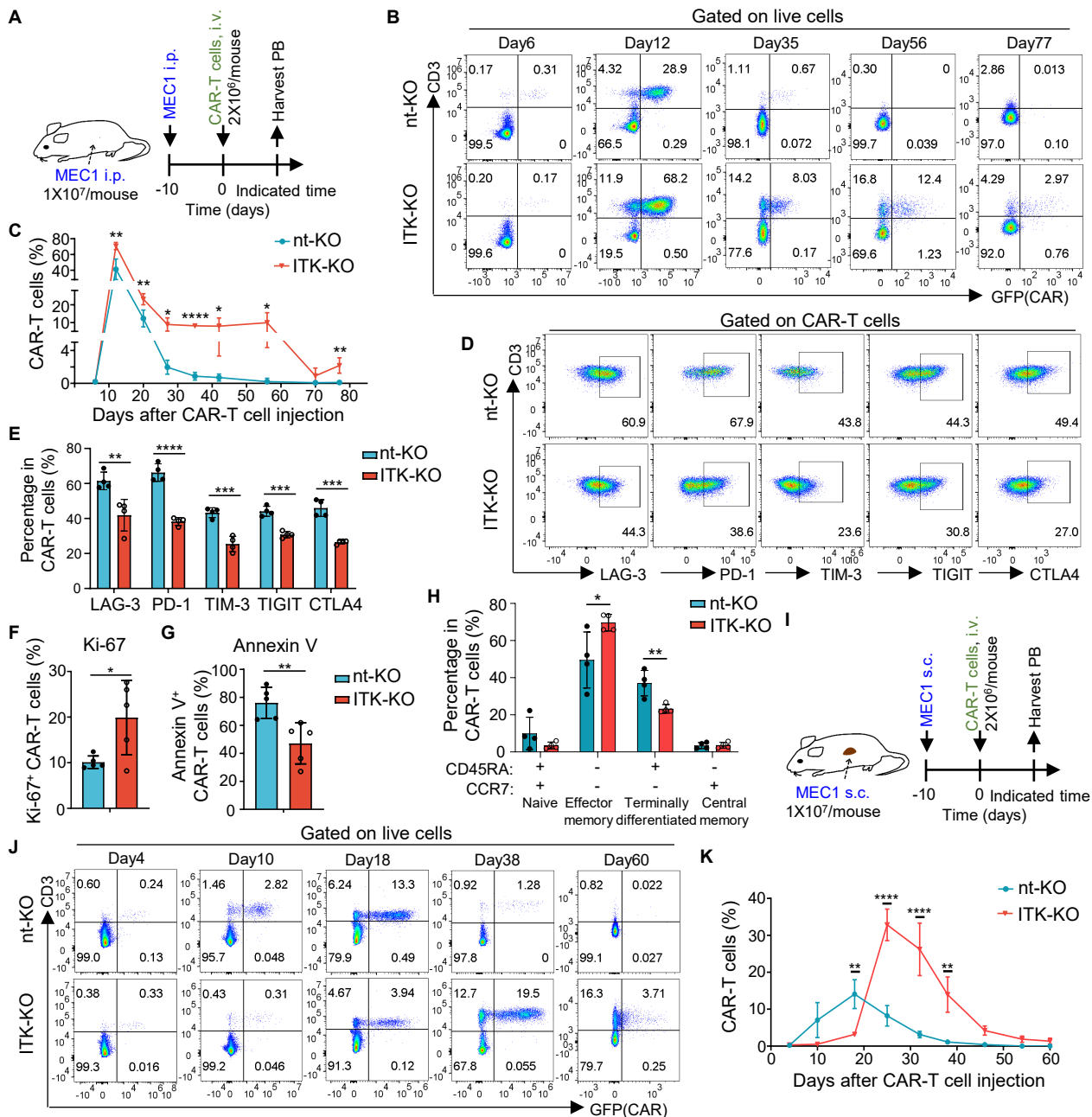


Figure 6

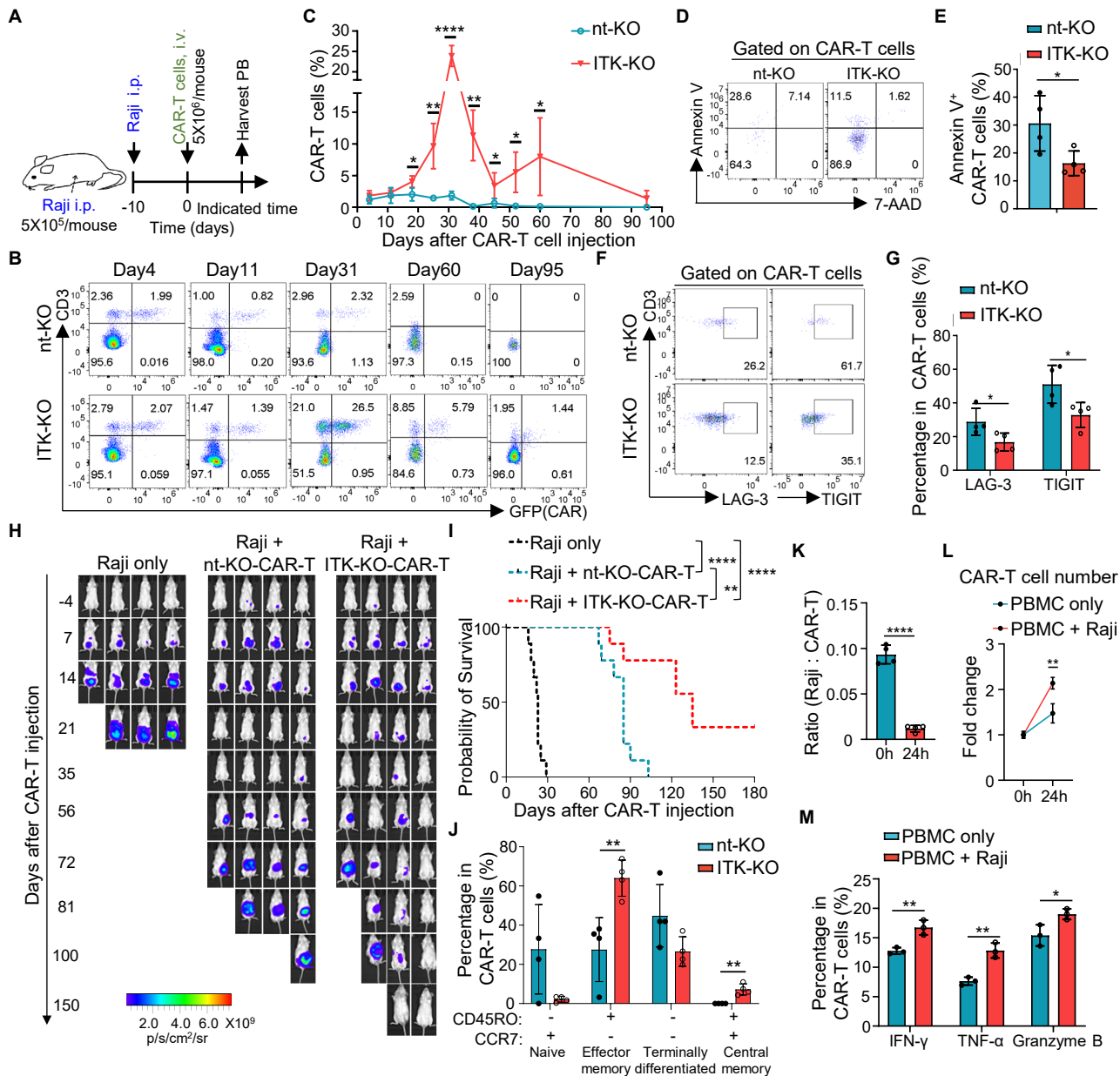


Figure 7

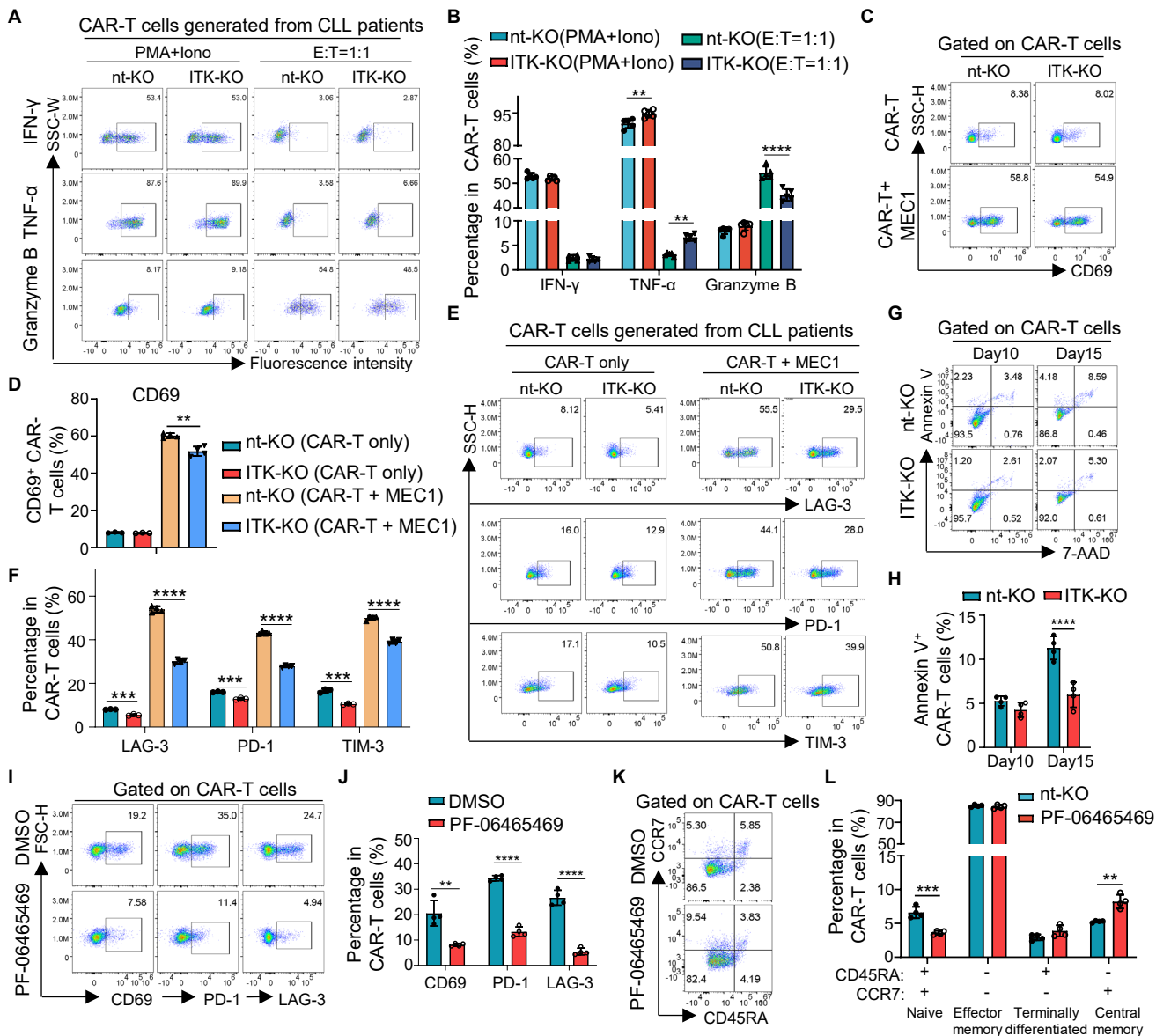


Figure 8

