CAR T Cells Secreting IL18 Augment Antitumor Immunity and Increase T Cell Proliferation and Costimulation

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Abstract

Interleukin 18 (IL18) is known to induce the expression of interferon-γ (IFNG), but its effects on T cell proliferation and costimulation are not completely understood. In this study, we demonstrate that ectopic expression of IL18 in CART cells caused significant T cell proliferation *in vitro* and *in vivo*, and enhanced antitumor effects in xenograft models. Moreover, IL18 mediated T cell expansion required neither tumor antigen nor CAR expression, and produced severe GVHD in NSG mice. Furthermore, recombinant IL18 costimulated IFNG secretion and proliferation of anti-CD3 beads treated T cells. Interestingly, IL18 costimulation could expand purified CD4 T cells, but not CD8 T cells. However, CD8 T cells proliferated greater than CD4 T cells in magnitude within bulk T cells, suggesting CD4 help effect was involved. Using CRISPR/Cas9 gene editing, we confirmed that IL18-driven expansion was both TCR and IL18 receptor (IL18R) dependent. Importantly, we demonstrated that TCR-deficient, IL18-expressing CD19 CART cells exhibited remarkable proliferation and persistent antitumor activity against CD19-expressing tumor cells in vivo. Finally, we describe APACHE T cells, a novel strategy for coupling IL18 expression in CART cells to antigen stimulation, thereby limiting potential toxicity associated with persistent IL18 production. In sum, our study supports human IL18 as a T cell costimulatory cytokine for fueling CART therapy.

Keywords

IL18, CAR, TCR, Costimulation, GVHD, CRISPR/Cas9, RNA electroporation, Knockout, Immunotherapy, NFAT

Abbreviation: T Cell Receptor –**TCR**; Chimeric Antigen Receptor –**CAR**; IL18 producing CART –**IL18-CART**; IL18 producing CD19 CART –**CD19-IL18 CART**; IL18 producing SS1 CART –**SS1-IL18 CART**; Tumor Infiltrating Lymphocytes –**TIL**; Graft-Versus-Host Disease –**GVHD**; Clustered regularly interspaced short palindromic repeats –**CRISPR**; CRISPR associated protein 9 –**Cas9**; Knockout–**KO**; IL18 Receptor –**IL18R**; Nuclear Factor of Activated T-cells–**NFAT**; artificial Antigen Presenting Cells –**aAPC**.

Introduction

Following the initial reports of clinical success using chimeric antigen receptor (CAR) T cell therapy to treat B cell leukemia and lymphoma (Kochenderfer et al., 2010; Porter et al., 2011), there have been intense efforts to improve the design and clinical translation of CART cells (Dotti et al., 2014; Jensen and Riddell, 2014; Kalos and June, 2013). It is well established that T cell receptor (TCR) engagement and costimulatory signaling provide the critical signals that regulate T cell activation, proliferation and cytolytic functions (Chen and Flies, 2013). In addition to TCR and costimulatory signals, exogenous cytokines also play a fundamental role in modulating T cell function (Curtsinger and Mescher, 2010), as reflected in the influence of cytokines produced by CD4+ T helper cells on the activity of CD8+ cytotoxic T cells(Bevan, 2004). For example, interleukin 2 (IL2) promotes clonal expansion of effector T cells in response to antigen stimulation, and is also required to maintain suppressive regulatory T (Treg) cell-mediated immune tolerance (Boyman and Sprent, 2012).

CART cells are among the most promising of multiple adoptive T cell transfer-based immunotherapies currently under development (Harris and Kranz, 2016; Hinrichs and Rosenberg, 2014; Lu et al., 2014; Robbins et al., 2013). The manufacture of sufficient tumor-specific T cells in a clinical setting has relied on the use of exogenous cytokines, including IL2, IL7 andIL15, to promote *ex vivo* expansion of genetically engineered T cells (Cha et al., 2010; Cieri et al., 2013). In addition, IL21 has been shown to promote expansion and engraftment of adoptively transferred T cells (Chapuis et al., 2013).

Although many immune cytokines have been evaluated for their ability to promote anti-tumor immunity when delivered exogenously (Dranoff, 2004; Lee and Margolin, 2011), reports of toxicity and/or lack of efficacy have limited their success. Several attempts to replace exogenous cytokine treatment with T cell-autonomous cytokine production have been reported. For example, tethering IL15 to CART cells was shown to enhance T cell persistence and antitumor activity (Hurton et al., 2016); however, there are concerns that this approach could lead to transformation of the T cells (Hsu et al., 2007; Sato et al., 2011). Similarly, IL12-expressing T cells have been investigated in multiple preclinical studies (Chinnasamy et al., 2012; Chmielewski et al., 2014; Chmielewski et al., 2011; Koneru et al., 2015; Pegram et al., 2012; Zhang et al., 2015), and IL12-expressing tumor infiltrating lymphocytes (TIL) were recently used to treat melanoma patients (Zhang et al., 2015). Although IL12-expressing TIL therapy resulted in effective anti-tumor response, toxicity from persistent, elevated serum IL12 concentrations was observed. More recently, interferon-β (IFNB) has shown efficacy in bridging innate and adaptive immune responses (Yang et al., 2014), and may be an attractive option for engineering cytokine producing CART in future (Zhao et al., 2015).

We hypothesized that including an IL18 gene expression cassette in the CAR lentiviral vector might overcome the requirement for exogenous cytokine stimulation, and promote improved antitumor CART cell effects. IL18 was initially characterized as an inducer of interferon-γ (IFNG) expression in T cells (Nakamura et al., 1993; Nakamura et al., 1989),and was shown to activate

lymphocytes and monocytes, without eliciting severe dose limiting toxicity, in a previous clinical study(Robertson et al., 2006). The combination of IL18 with IL12 treatment promotes Th1 and NK cell responses (Nakanishi et al., 2001), but also induces lethal toxicity in murine models (Carson et al., 2000; Osaki et al., 1998), reflecting the dynamic tension between achieving high potency and inducing toxicity in cytokine based immunotherapies. Despite initial indications that IL18 treatment alone may be only minimally effective in boosting anti-tumor immunity (Osaki et al., 1998), we previously reported that recombinant human IL18 could significantly enhance engraftment of human CD8 T cells in a xenograft model (Carroll et al., 2008).

In the current study, we demonstrate that T-cell autonomous IL18 expression, in combination with CAR or TCR signaling, promotes significant T cell proliferation *in vitro* and *in vivo*. Moreover, IL18 mediated T cell expansion in xenograft model required neither tumor antigen nor CAR expression, and produced severe GVHD in NSG mice. Interestingly, CD4+ T cells expanded preferentially in response to IL18 costimulation. Using CRISPR/Cas9 gene editing, we confirmed that IL18-dependent T cell expansion was both TCR and IL18 receptor (IL18R) dependent. Importantly, we demonstrated that TCR-deficient, IL18-expressing CD19 CART cells exhibited remarkable proliferation and persistent antitumor activity against CD19-expressing tumor cells and did not cause GVHD *in vivo*. These data support the redefinition of human IL18 as a T cell costimulatory cytokine and the utility of engineering CART cells to express IL18. We further describe an inducible expression system that couples IL18 expression levels to CAR stimulation, thereby reducing potential toxic effects of persistent IL18 expression in the absence of tumor antigen. Collectively, these studies identify a novel approach to enhancing CART cell expansion *in vivo*, which is a critical determinant of successful CART cancer immunotherapy.

Results

IL18 enhances proliferation of polyclonal antigen-specific and non-specific CAR T cells *in vivo*

To test the hypothesis that elevated IL18 signaling can enhance the anti-tumor effects of CART cell therapy, we introduced a gene expression cassette encoding human IL18 (or GFP as a negative control) into a previously described anti-mesothelin (SS1) CAR lentiviral construct (SS1-IL18) (Figure 1A). Similarly, the IL18 gene cassette was introduced into an anti-CD19 CAR construct (CD19-IL18) to serve as a control for antigen-specific effects (Figure 1A). SS1-IL18 CART cells efficiently expressed SS1 CAR and secreted active human IL18 into the culture medium, as assessed using an IL18 reporter cell line (Figure 1-figure supplement 1A,B,C).

Interestingly, elevated IL18 expression enhanced the lytic activity of SS1-CART cells when cultured *in vitro* with the mesothelin-expressing BxPC3 pancreatic tumor cell line (Figure 1-figure supplement 1D). As previous data indicates that recombinant human IL18 significantly enhances engraftment of human CD8 T cells in xenograft models (Carroll et al., 2008), we

127 investigated whether the IL18-expression could enhance CART cell-mediated antitumor effects
128 *in vivo*. AsPC1 cells expressing click beetle green (CBG) and GFP reporter proteins were injected
129 into NSG mice to generate xenograft tumors. Tumor-bearing mice were then injected with SS1,
130 SS1-IL18, CD19 or CD19-IL18 CART cells, and tumor burden was monitored by bioluminescence.
131 Surprisingly, both CD19-IL18 and SS1-IL18 CART cells suppressed tumor progression while
132 inducing significant loss of body weight (Figure 1B), and CD19-IL18 or SS1-IL18 T cells were
133 dramatically expanded 62-fold to 13993 cells/µl, or 74-fold to 38211 cells/µl, respectively
134 (Figure 1C). Moreover, the serum IL18 concentration was increased to 44291pg/ml in SS1-IL18
135 CART cells (Figure 1C). These results indicate that CART cell-autonomous IL18 expression can
136 drive expansion of both antigen-specific and non-specific CART cells in NSG mice.

137 To further analyze the specific effects of IL18 expression on CART cell proliferation, splenic T
138 cells were analyzed for CAR expression by flowcytometry. Interestingly, in mice receiving SS1
139 CART cells, 96.4% of the *in vivo* expanded CD8 T cells were CAR+, whereas only 50% of the CD8+
140 T cells in mice receiving SS1-IL18 CART cells were CAR+ (Figure 1D,E). This expansion is more
141 favorable to SS1 CAR+ T cells, as only approximately 24% of the expanded human T cells were
142 CAR+ in mice receiving CD19-IL18 CART cells (Figure 1D,E). The percentage of CAR+ cells within
143 CD4 or CD8 subsets are summarized in Figure 1E. These results suggested that IL18 promotes
144 proliferation of both CAR+ and CAR- T cells in autocrine and paracrine modes. Phenotyping of
145 TCRβ (TCRB) variants on expanded T cells reveals that all CART cell groups exhibit polyclonal
146 expansion (Figure 1-figure supplement 2), suggesting that IL18 expression does not select for
147 particular TCR specificities.

148 **IL18 mediated T cell proliferation induces lethal GVHD**

149 Our observation that CD19-IL18 CART cells elicited antitumor activity and loss of body weight, as
150 well as the expansion of untransduced T cells in mice receiving IL18-CART cells, suggested that
151 IL18 expression in these contexts might promote severe GVHD, consistent with our previous
152 report (Carroll et al., 2008). To test the severity of GVHD associated with IL18-CART cell therapy,
153 we injected tumor-free NSG mice with either SS1 or SS1-IL18 CART cells, and monitored
154 circulating human T cell levels, serum IL18 concentrations, body weight, and survival (Figure 1F).
155 As SS1-IL18 CART cells expanded, mice exhibited significant loss of body weight, and started to
156 die approximately 3 weeks after T cell injection (Figure 1F). Similarly, NSG mice bearing CD19-
157 expressing Nalm6 tumors treated with CD19-IL18 CART showed dramatic expansion of human T
158 cells in blood and spleen (Figure 1-figure supplement 3A,B). Remarkably, mice bearing AsPC1
159 cells succumbed quickly following injection with CD19-IL18 T cells, despite the absence of the
160 CD19 tumor antigen on AsPC1 cells (Figure 1-figure supplement 3C). IL18 expression had no
161 effect on the frequency of splenic regulatory T (Treg) cells (Figure 1-figure supplement 3D).
162 Histological analyses confirmed that treatment with either SS1-IL18 and CD19-IL18 CART cells
163 induced significant tumor destruction (Figure 1G). Immunohistochemical analysis for CD3
164 expression revealed the presence of T cells in the tumor treated with IL18-CART (Figure 1H), and
165 necropsy of mice receiving SS1-IL18 CART cells (Table S1), revealed remarkable GVHD in liver
166 and lung and atypical lymphocyte infiltration in pancreas (Figure 1I).

167 Collectively, our results suggest that expressing IL18 in the context of CART cell therapy can
168 enhance antitumor effects and xenogeneic GVHD, but may also produce unwanted toxicity due
169 to TCR-mediated autoimmune effects in an autologous setting, or GVHD in an allogeneic setting.
170 To optimize the use of IL18 in CART cell therapy, it was therefore important to dissect the
171 contribution of TCR signaling to IL18-mediated CART cell effects *in vitro* and *in vivo*.

172 **TCR activation and IL18 promotes CD4 helper dependent T cell expansion *in vitro* and *in vivo***

173 Based on our observation that IL18 expressing T cells proliferated in NSG mice independent of
174 CAR activation, and produced xenogeneic GVHD, we speculated that a functional synergy exists
175 between IL18 and TCR stimulation. To test this hypothesis *in vitro*, primary human T cells were
176 stimulated using anti-CD3 beads and recombinant IL18. Whereas combined exposure to anti-
177 CD3 beads and IL18 efficiently stimulated increases in T cell size and proliferation (Figure 2A,B),
178 neither treatment alone was effective, although anti-CD3 beads alone caused intermediate
179 increase in size without triggering T cell proliferation. These results were confirmed in T cells
180 isolated from two normal donors (Figure 2C).

181 To better characterize IL18 activation of T cells, we collected the culture supernatants at D5
182 from different conditions as indicated to analyze the profile of secreted cytokines. As expected,
183 low concentrations of IL18 combined with anti-CD3 beads, significantly enhanced IFNG
184 production compared to beads alone (Figure 2D). Although low concentrations of IL18 and anti-
185 CD3 beads treatment enhanced IL2 production, maximal IL2 levels were at least 20-fold lower
186 than those induced by standard anti-CD3/28 beads (Figure 2D). The production of other
187 cytokines was generally comparable between CD3/IL18 and CD3/D28 activation (Figure 2-figure
188 supplement 1). Multicolor flow phenotype revealed that IL18-expanded T cells had a primarily
189 central memory (CM) phenotype (CCR7+CD45RO+), similar to that obtained with anti-CD3/28
190 beads (Figure 2-figure supplement 2).

191 To address the specific roles of T cell subsets in these responses, T cells expanded by standard
192 anti-CD3/CD28 beads were sorted by magnetic-activated cell sorting (MACS) to generate CD4
193 and CD8 subpopulations. CD8 T cells failed to proliferate after restimulation with IL18 and anti-
194 CD3 beads despite a significant increase in size, and addition of IL2 had no additional effect
195 (Figure 2E,F). In sharp contrast, CD4 T cells or bulk T cells restimulated with IL18 and anti-CD3
196 beads proliferated efficiently, at a level comparable to that of combined CD4+CD8 T cells
197 following CD3/CD28 restimulation. Restimulated CD4 and CD8 T cells displayed transiently
198 elevated central memory (CM) markers, but ultimately expressed a primarily effector memory
199 (EM) phenotype (Figure 2-figure supplement 3A), similar to that observed for restimulated IL18-
200 CART cells (Figure 2-figure supplement 3B).

201 GFP-IL18 expressing CD8 T cells also failed to expand *in vivo* following injection into NSG mice
202 (Figure 2G). In contrast, GFP-IL18 expressing CD4 or bulk T cells exhibited greater than 400-fold
203 expansion between days 15 to 23 post T cell injection (Figure 2G), and were associated with
204 enlarged spleens (Figure 2H). The number of expanded CD4 T cells was comparable whether

205 pure CD4 T cells or total T cells were injected, suggesting that CD8 T cells do not have a
206 significant effect on CD4 T cell expansion (Figure 2I). Interestingly, CD8 T cells proliferated
207 greater than CD4 T cells in magnitude within bulk T cells, suggesting CD4 help effect was
208 involved (Figure 2J).

209 Serum cytokine levels were determined in mice receiving CD4, CD8, or bulk T cells expressing
210 GFP-IL18, or control GFP T cells. Consistent with our *in vitro* results, serum IL18 levels increased
211 in the GFP-IL18 T cell and GFP-IL18 CD4 T cell populations, but not in the isolated GFP-IL18 CD8 T
212 cell population (Figure 2K). IFNG levels also increased in proportion to T cell expansion (Figure
213 2L), and GM-CSF levels were significantly elevated in the expanding GFP-IL18 T cell and GFP-IL18
214 CD4 T cell populations, whereas other cytokines failed to show large changes (Figure 2M). In
215 summary, these results suggested that IL18 acts as a costimulatory cytokine that interacts
216 synergistically with TCR activation to drive efficient T cell proliferation, largely dependent on
217 effects through CD4 helper T cells.

218 **CAR or TCR signaling is required for IL18 mediated T cell proliferation *in vitro* or *in vivo***

219 To first evaluate the effect of IL18 coexpression on CART cell proliferation *in vitro*, we deleted
220 TCR mediated allogeneic effect in SS1-IL18 and CD19-IL18 CART cells using CRISPR/Cas9 gene
221 editing as previously described (Ren et al., 2016). TCR KO IL18-CART cells had only marginally
222 enhanced expansion following CD3/CD28 beads activation and RNA electroporation (Figure 3A).
223 When stimulated with irradiated K562 cells expressing cognate antigen and exogenous IL2, SS1-
224 IL18 CART cells displayed significantly increased proliferation relative to SS1 CART cells (Figure
225 3B), which were producing high level of IFNG and IL2, and polyclonal in TCRB variants
226 distribution (Figure 3-figure supplement 1A,B,C). However, CD19-IL18 CART cells expanded
227 slightly better than the CD19 CART cell control (Figure 3C). Interestingly, IL18 expression
228 promoted a modest increase in proliferation in both TCR-deficient SS1-IL18 and CD19-IL18 CART
229 cells relative to their TCR wild type counterparts (Figure 3B,C,D). Robust IL18 secretion was
230 observed in all cases (Figure 3E, Figure 3-figure supplement 2A,B), while IL18-CART displayed
231 enhanced cytokine production of IFNG and several others (Figure 3-figure supplement 2C). The
232 observation of extended proliferation in SS1-IL18 CART should be attributed to antigen
233 independent activation of SS1 CAR as previously found (Frigault et al., 2015). Moreover, the loss
234 of proliferation in TCR-deficient SS1-IL18 CART cells suggested that TCR structure might be
235 necessary to maintain the antigen independent activation of SS1 CAR. Nevertheless, this data
236 strongly suggested that IL18 could drive persistent T cell proliferation in presence of continuous
237 CAR activation *in vitro*.

238 To test whether continuous TCR activation by mouse MHC molecules was required for general
239 IL18-mediated T cell expansion *in vivo*, NSG mice were injected with TCR+ and TCR- GFP-IL18 T
240 cells. TCR+ GFP-IL18 expressing T cells expanded dramatically compared to TCR+ GFP control T
241 cells, whereas TCR- GFP-IL18 expressing T cells showed negligible IL18-induced expansion
242 relative to controls (Figure 3F,G). In addition, flowcytometry analysis of spleen cells indicated
243 that TCR+ GFP-IL18 expressing T cells displayed high levels of the IL18 receptor (IL18R) (Figure 3-

figure supplement 3A), and displayed a primarily effector memory phenotype (Figure 3-figure supplement 3B). Collectively these data indicate that CAR or TCR activation is required for IL18-mediated T cell proliferation *in vitro* or *in vivo,* respectively.

IL18 receptor is required for cell-autonomous IL18-medated T cell expansion *in vivo*

To further investigate the synergy between IL18 and TCR signaling, we generated IL18Rα (IL18RA) deficient T cells using CRISPR/Cas9 mediated gene editing (Figure 4-figure supplement 1A,B). We next employed a cell competition assay using four distinct populations of engineered T cells (Figure 4A). These included (1) WT CD19-IL18 T cells; (2) WT GFP T cells; (3) TCR KO T cells labeled with DsRed; and (4) IL18R KO T cells labeled with AmCyan. Successful deletion of the TCR and IL18R was confirmed by flowcytometry, and MACS depletion was performed to remove any residual T cells expressing either TCR or IL18R proteins (Figure 4B). Following *ex vivo* expansion, all four T cell populations were mixed and flowcytometry analysis confirmed the ratio of (WT CD19-IL18):(WT GFP):(TCR KO DSRED):(IL18R KO AMCYAN) cells to be 8.59% : 7.45% : 6.71% : 7.27% of total T cells (Figure 4C). The mixed population of T cells was injected into NSG mice and blood and spleen were collected for analysis when mice exhibited significant loss of bodyweight. Staining for IL18RA indicated that AmCyan labeled T cells partially regained low levels of IL18R expression (Figure 4D), and likely represent T cells that did not undergo successful gene editing and were not completely removed by MACS depletion.

Flowcytomety analysis revealed the final ratio of (WT CD19-IL18):(WT GFP):(TCR KO DSRED):(IL18R KO AMCYAN) to be 22.0% : 18.0% : 5.82% : 0.43% (Figure 4E). The number of CD45+ human T cells in individual mice ranged from 32800 to 79600 cells/µl blood (Figure 4F), confirming that all mice exhibited IL18-mediated T cell proliferation. Interestingly, the ratio of WT CD19-IL18 to WT GFP T cells following *in vivo* expansion was essentially unchanged (Figure 4G), consistent with the data in Figure 1D,E indicating that IL18 could drive equal expansion of CD19 CAR positive and negative T cells. In contrast, the proportion of IL18R KO AMCYAN T cells decreased significantly in blood and spleen, and it was even more dramatic for TCR KO DSRED T cells (Figure 4G). These data indicate that both TCR and IL18R signaling are required for IL18 mediated proliferation of human T cells as tested *in vivo* in the NSG model.

IL18 costimulation with CAR signaling significantly enhanced CART proliferation and antitumor activity in xenograft model

As our data indicate that IL18 expression promotes TCR-mediated T cell proliferation, we hypothesized that IL18 costimulation would also enhance CART cell proliferation and anti-tumor efficacy. To avoid GVHD, only TCR-deficient (TCR-) T cells were used in these experiments. NSG mice were engrafted with Nalm6 tumor cells expressing CBG and GFP reporter proteins, and subsequently treated with either TCR- CD19-IL18 or control TCR- CD19-GFP CART cells. Tumor burden was assessed by bioluminescent imaging. TCR- CD19-IL18 T cells effectively cleared detectable tumor cells by day 10 (Figure 5A), in contrast to TCR- CD19-GFP T cells (Figure 5A,B). In addition, TCR- SS1-IL18 CART cells had no effect on tumor progression (Figure 5B). Although

282 TCR- CD19-IL18 CART cells caused a transient decrease in body weight at D8, body weight
283 returned to baseline levels and remained stable thereafter (Figure 5C). Remarkably, mice
284 injected with TCR- CD19-IL18 CART cells remained viable for over 185 days post T cell injection,
285 except for one mouse that exhibited tumor relapse at day 101 (Figure 5A,D) due to
286 downregulation of CD19 expression (Figure 5-figure supplement 1A). Emergence of
287 contaminating TCR+ CD19-IL18 CART cells was not observed at any point during treatment
288 (Figure 5G).

289 From day 10 through day 21, mice receiving TCR- CD19-IL18 CART cells maintained
290 approximately 350 CD3-CD45+ T cells/µl blood (Figure 5E), whereas the number of CD19-GFP
291 CART cells varied between0.8 and 10.5 CD3-CD45+ T cells/µl. This approximately 100-fold
292 difference suggests that IL18 significantly enhanced of proliferation CD19 CART cells, resulting in
293 superior antitumor activity. Consistent with the expansion of CD19 CART cells, IL18
294 concentrations were high, averaging 77500pg/ml at D10 (Figure 5F). Subsequently, the number
295 of T cells and IL18 concentrations decreased to below 1 CD3-CD45+ T cell/µl and approximately
296 100pg/ml in tumor-free mice. Flowcytometric analyses revealed that the distribution of CD4 and
297 CD8 T cells rapidly evolved from equal balance at D10 (37% vs 55%, respectively) to CD4
298 domination at D21 (86% vs 11%) (Figure 5H). Interestingly, CD19-IL18 CART cells attained a
299 primarily EM phenotype in both CD4 and CD8 subsets (Figure 5I). In the single mouse that
300 developed a recurrent tumor associated with CD19 downregulation (Figure 5-figure supplement
301 1A), the number of T cells rose back to 373 CD3-CD45+ T cells/µl, and IL18 concentration back to
302 50209 pg/ml, while the percentage of CD3-CD45+ cells was still 99.6% (Figure 5-figure
303 supplement 1B). This observation suggested that TCR- CD19-IL18 CART cells were still capable of
304 expanding in response to tumor relapse, presumably in an antigen-dependent manner, while
305 retaining a primarily CD4 T_{EM} phenotype (Figure 5-figure supplement 1C).

306 These results collectively demonstrate that IL18 can significantly enhance CART cell proliferation
307 and induce superior anti-tumor activity. Equally important, the use of TCR-deficient CART cells
308 obviated concerns of developing TCR-mediated GVHD during therapy.

309 **Designing an NFAT-based inducible system for IL18 production: APACHE T cells**

310 Our data indicate that including the IL18 expression cassette within the CAR lentiviral design
311 strongly enhanced T cell proliferation in response to TCR or CAR stimulation, and may be
312 preferable to expanding CART cells that do not constitutively secrete cytokines. However, the
313 high concentration of systemic IL18 produced by CART cells could raise potential safety concerns
314 related to pathological activation or proliferation of bystander T cells reacting against foreign or
315 self-antigens, or induction of NK cell mediated inflammation. We therefore designed an
316 inducible system in which IL18 production is only activated after CAR engagement with tumor
317 antigen. Specifically, we generated a lentiviral vector in which the expression of CD19 CAR, GFP,
318 and IL18 is under the control of the NFAT promoter (Figure 6A). Once expressed, CD19 CAR will
319 engage with antigen to stimulate NFAT promoter activity. This, in turn, will maintain CD19 CAR
320 and IL18 expression through a positive feedback circuit. Once tumor antigen is cleared, CD19

321 CAR and IL18 expression levels should return to baseline, overcoming potential toxic effects of
322 long-term constitutive IL18 expression. Because the endogenous TCR can also activate NFAT
323 signaling pathways, we conducted these experiments in T cells rendered deficient in TCR
324 expression by CRISPR/Cas9. In order to initially activate the signaling cascade, the cells were
325 engineered to transiently express a CD19 CAR by mRNA electroporation into TCR-deficient T
326 cells already lentivirally transduced with the NFAT-driven cassette (Ren et al., 2016; Zhao et al.,
327 2010), as shown in Figure 6A. Deleting the TCR gene in these cells by CRISPR/Cas9 gene editing
328 avoids unwanted NFAT activation by endogenous TCR signaling (Figure 6A).

329 We tested the inducible NFAT-CD19-GFP-IL18 TCR-deficient T cells expanded as described in the
330 timeline shown in Figure 6A. TCR-/NFAT-CD19-GFP-IL18 T cells were restimulated under
331 different conditions, and analyzed for GFP expression and IL18 production. The results showed
332 that irradiated K562-CD19 aAPC and PMA/ionomycin treatment induced similar levels of GFP
333 expression and IL18 synthesis in TCR WT and TCR KO NFAT-CD19-GFP-IL18 T cells, whereas anti-
334 CD3/28 beads only activated GFP expression and IL18 synthesis in TCR WT NFAT-CD19-GFP-IL18
335 T cells (Figure 6B,C).This observation demonstrated that the inducible system, which we
336 deemed **A**ntigen **P**ropelled **A**ctivation of **C**ytokine **H**elper **E**nsemble (APACHE), operates as
337 intended. In NSG mice bearing Nalm6 xenograft tumors, APACHE-armored CD19 CART cells
338 suppressed tumor progression more effectively, and resulted in better survival, than TCR-/NFAT
339 CD19-GFP CART cells and TCR-/untransduced (UTD) T cells expressing mRNA encoded CD19 CAR
340 (Figure 6D,E). APACHE-armored CD19 CART cells did not induce significant loss of body weight
341 and only low levels of systemic IL18 were detected at day 10 and day 15 following T cell
342 injection (Figure 6E), suggesting that this inducible system could minimize the potential risk of
343 continuous systemic IL18 expression.

344

345 Discussion

346 Except for B cell tumors treated with CD19 CAR T cells, a major limitation of current CAR T cell
347 approaches has been insufficient expansion and persistence of the infused T cells, as
348 exemplified by trials in ovarian and brain cancer (Brown et al., 2015; Kershaw et al., 2006), and
349 reviewed in (Fesnak et al., 2016; Gilham et al., 2012; Jensen and Riddell, 2015). IL12, a cytokine
350 related to IL18, has shown promise in augmenting antitumor effects and T cell expansion
351 (Chmielewski et al., 2014; Pegram et al., 2012). There may be some advantages to the use of
352 IL18, including that it is a single chain cytokine in contrast to IL12, which is heterodimeric. In
353 addition, IL18 has been tested as a single agent in clinical trials and shown to have moderate
354 antitumor effects and an acceptable safety profile (Robertson et al., 2006). In contrast, IL12 has
355 been shown to have unacceptable toxicity in previous trials (Cohen, 1995).

356 IL18 can regulate both innate and adaptive immune responses through its effects on natural
357 killer (NK) cells, monocytes, dendritic cells, T cells, and B cells (Dinarello et al., 2013). The most
358 important biologic effect of IL18 is that it acts synergistically with other pro-inflammatory

359 cytokines to promote interferon-γ production by NK cells, T cells, and possibly other cell types.
360 Due to the pleiotropic functions of IL18, tissue-accumulated IL18 has been shown to impact both
361 the tumor stroma and the immune response, by sustaining T cell cytolytic activity, recruiting and
362 activating innate immune cells, and re-programming stroma associated immune suppressor cells
363 (Chmielewski et al., 2014). The rationale for our studies is that the accumulation of IL18 as a
364 transgenic pro-inflammatory cytokine is intended to recruit a second wave of immune cells to
365 the tumor microenvironment to mediate antitumor effects towards cancer cells that are
366 resistant to the direct effects of the CAR T cells.

367 Many studies have shown that efficacy of adoptive cell therapy with tumor targeted T cells is
368 enhanced by host conditioning using radiation or lymphodepleting chemotherapy. Intensive
369 chemotherapy or radiation given to enhance adoptive cell therapy has been reported to have
370 severe toxicity in some patients (Goff et al., 2016). Given the pronounced augmentation of CAR
371 T cell engraftment that we observed with T cells secreting IL18, it is possible that this may
372 decrease the need for prior host conditioning. In preclinical models with mouse CAR T cells
373 engineered to secrete IL-12, the need for lymphodepletion was obviated (Pegram et al., 2012).

374 One concern with CAR T cells armored with IL18 is the potential for systemic inflammation (Car
375 et al., 1999). To mitigate this possibility several strategies will be employed in clinical trials. First,
376 CAR-IL18 T cells will be given in low doses where the calculated maximal levels of IL18 could not
377 reach the levels shown to be safe in humans in previous trials (Robertson et al., 2006). Secondly,
378 if cytokine levels become a concern, they can be reduced by incorporation of various kill switch
379 strategies (Straathof et al., 2003). Finally, a more elegant approach was developed here where
380 the IL18 transgene was not constitutively expressed, but rather, was only induced and secreted
381 upon encountering with the surrogate tumor antigen. Presumably this would limit IL18 secretion
382 to the tumor microenvironment, and limit systemic exposure.

383 A limitation of our *in vivo* studies is that they were conducted in an immunodeficient NSG
384 mouse model. A strength of this model is that it enabled testing of the APACHE CAR T cells in a
385 robust tumor model. However, the tumor microenvironment is lacking in this model since the
386 tumor stroma is composed largely of mouse cells. In humans and in mice, the biologic activity of
387 IL18 is balanced by the presence of a high affinity, naturally occurring IL18 binding protein
388 (IL18BP). This binding occurs with a high affinity and has been reported to occur interspecies
389 between mouse and human IL18 (Aizawa et al., 1999). It is possible that the toxicity observed in
390 the mice manifested by systemic inflammation, weight loss and death in some cases, was
391 exaggerated or underreported based on the relative balance of mouse IL18BP and the
392 transgenic IL18 secreted by the APACHE T cells.

393 In summary, we demonstrate that CAR T cells can be engineered to secrete IL18 that supports *in*
394 *vivo* engraftment and persistence of the CAR T cells. Our observations demonstrate that IL18 has
395 a previously unappreciated form of costimulation on human CD4+ T cells, and therefore these
396 IL18 expressing CD4 T cells can be categorized as synthetic T helper cell producing IL18 (sTH18).
397 These findings may have therapeutic implications because the CAR IL18 T cells can support the

398 proliferation of CD8 T cells, and because increasing evidence points to the importance of
399 adoptively transferred CD4+ T cells in antitumor effects (Hunder et al., 2008; Hung et al., 1998).
400 Finally, CAR T cells with inducible IL18 may have a role in immune-surveillance and have a
401 therapeutic potential for inducing antigenic spread. Before translation of this technology into
402 clinical setting, further investigation is underway to address the IL18-CART toxicity and biology in
403 syngeneic models .

404 Materials and Methods

405 Mice and cell lines
406 NOD-SCID-G chain-/- (NSG) were purchased from Jackson Laboratories, and all animal
407 procedures were performed in animal facility at the University of Pennsylvania in accordance
408 with Federal and Institutional Animal Care and Use Committee requirements. Wild-type parental
409 AsPC1, K562 cells and Nalm-6 cells (purchased from American Type Culture Collection) were
410 engineered to express CBG-T2A-GFP as a reporter gene and trace of luciferase activity *in vitro*
411 and *in vivo*. K562 with CBG-T2A-GFP cells were further engineered to express human CD19
412 (K562-CD19) or human mesothelin (K562-CD19).
413
414 Molecular cloning
415 The parental vector pTRPE SS1BBz contains scFV recognizing human mesothelin, 4-1BB and CD3z
416 intracellular signaling domains. The sequence encoding mature human IL18 (Uniprot Q14116)
417 were synthesized from IDT and cloned into pTRPE SS1BBz along with a T2A (Kim et al., 2011)
418 linker (pTRPE SS1BBz-T2A-hIL18). GFP and Humanized CAR CD19BBz were cloned to replace
419 SS1BBz to generate pTRPE CD19BBz-T2A-hIL18 and pTRPE GFP-T2A-hIL18. GFP was cloned to
420 replace SS1BBz to generate pTRPE GFP. GFP was also cloned to replace hIL18 to generate pTRPE
421 SS1BBz-T2A-GFP and pTRPE CD19BBz-T2A-GFP. In order to generate the lentivector construct
422 pTRPE NFAT CD19-GFP-IL18, the parental vector pTRPE SS1BBz was depleted of the fragment
423 consisting EF1A promoter and SS1 BBz, and inserted with a reverse sequence of synthesized
424 fragment consisting of an NFAT promoter (Zhang et al., 2015) followed by GFP with a P2A (Kim
425 et al., 2011) linker. The resulting pTRPE NFAT GFP vector was modified by replacing GFP with
426 CD19BBz, which was further modified by inserting a fragment of GFP or GFP-T2A-IL18
427 downstream of P2A site to generate pTRPE NFAT CD19-GFP or CD19-GFP-IL18.
428
429 Primary T cell expansion
430 Primary donor T cells were transduced with lentivector encoding CAR or GFP with or without
431 hIL18 and expanded as previously reported (Milone et al., 2009). In brief, primary donor T cells
432 were activated with anti-CD3/CD28 Dynabeads (LifeTechnologies) at D0, and transduced with
433 lentivector at D1. Fresh medium was added at D3 and the activated T cells were de-beaded at
434 D5 and maintained at 7e5/ml until rested T cells were frozen in liquid nitrogen.
435
436 CRISPR/Cas9 gene knockout
437 Primary donor T cells were transduced with lentivector encoding CAR or GFP with or without
438 hIL18 and engineered with CRISPR/Cas9 system as previously reported (Cong et al., 2013; Jinek
439 et al., 2012; Mali et al., 2013; Ren et al., 2016). In brief, primary donor T cells were activated
440 with anti-CD3/CD28 Dynabeads (LifeTechnologies) at D0, and transduced with lentivector at D1.
441 The activated T cells were de-beaded at D3 and electroporated with sgRNA and Cas9 RNA at D3

and D4. Then the T cells were maintained at 7e5/ml until rested T cells were frozen in liquid nitrogen.

TCR-deficient T cells were achieved with sgRNA targeting GGAGAATGACGAGTGGACCC within TCRB region. For the IL18R knockout we designed 7 sgRNA targeting IL18RA (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013), and utilized RNA electroporation to induce CRISPR/Cas9 mediated gene editing following the procedure of generating TCR KO universal CART (Ren et al., 2015). The screening of IL18RA KO showed that a the A4 sgRNA produced the most efficient gene knockout (Figure 4-figure supplement 1A), and sequencing of the PCR product spanning the gene edited site confirmed mutations in the DNA sequence (GTACAAAAGCAGTGGATCAC) targeted by the A4 sgRNA (Figure 4-figure supplement 1B).

Contributions

CHJ and BH proposed the concept of IL18 expressing CART for cancer immunotherapy. BH designed all, and performed most, of the experiments. JR, BH, and YZ executed the knockout of TCR and IL18R by CRISPR/Cas9. BH and YL conducted live animal imaging, Trucount analyses and mouse necropsy. JS provided scientific resources, such as parental cloning vectors and research animals. BH, CHJ, BK and RMY wrote and edited the manuscript.

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B.H., C.H.J. and Y.Z. have financial interests due to a pending patent application. Conflicts of interest are managed in accordance with University of Pennsylvania policy and oversight. The other authors declare that they have no competing interests.

479 **References**

480 Aizawa, Y., Akita, K., Taniai, M., Torigoe, K., Mori, T., Nishida, Y., Ushio, S., Nukada, Y., Tanimoto,
481 T., and Ikegami, H. (1999). Cloning and expression of interleukin-18 binding protein. FEBS letters
482 *445*, 338-342.

483 Bevan, M.J. (2004). Helping the CD8(+) T-cell response. Nat Rev Immunol *4*, 595-602.

484 Boyman, O., and Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of
485 the immune system. Nat Rev Immunol *12*, 180-190.

486 Brown, C.E., Badie, B., Barish, M.E., Weng, L., Ostberg, J.R., Chang, W.-C., Naranjo, A., Starr, R.,
487 Wagner, J., Wright, C.*, et al.* (2015). Bioactivity and Safety of IL13Rα2-Redirected Chimeric
488 Antigen Receptor CD8+ T cells in Patients with Recurrent Glioblastoma. Clinical Cancer Research.

489 Car, B.D., Eng, V.M., Lipman, J.M., and Anderson, T.D. (1999). The toxicology of interleukin-12: a
490 review. Toxicol Pathol *27*, 58-63.

491 Carroll, R.G., Carpenito, C., Shan, X., Danet-Desnoyers, G., Liu, R., Jiang, S., Albelda, S.M.,
492 Golovina, T., Coukos, G., Riley, J.L.*, et al.* (2008). Distinct effects of IL-18 on the engraftment and
493 function of human effector CD8 T cells and regulatory T cells. PLoS One *3*, e3289.

494 Carson, W.E., Dierksheide, J.E., Jabbour, S., Anghelina, M., Bouchard, P., Ku, G., Yu, H., Baumann,
495 H., Shah, M.H., Cooper, M.A.*, et al.* (2000). Coadministration of interleukin-18 and interleukin-12
496 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon-
497 gamma production and STAT-mediated signal transduction. Blood *96*, 1465-1473.

498 Cha, E., Graham, L., Manjili, M.H., and Bear, H.D. (2010). IL-7 + IL-15 are superior to IL-2 for the
499 ex vivo expansion of 4T1 mammary carcinoma-specific T cells with greater efficacy against
500 tumors in vivo. Breast Cancer Res Treat *122*, 359-369.

501 Chapuis, A.G., Ragnarsson, G.B., Nguyen, H.N., Chaney, C.N., Pufnock, J.S., Schmitt, T.M.,
502 Duerkopp, N., Roberts, I.M., Pogosov, G.L., Ho, W.Y.*, et al.* (2013). Transferred WT1-reactive
503 CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. Sci Transl
504 Med *5*, 174ra127.

505 Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition.
506 Nature Reviews Immunology *13*, 227-242.

507 Chinnasamy, D., Yu, Z., Kerkar, S.P., Zhang, L., Morgan, R.A., Restifo, N.P., and Rosenberg, S.A.
508 (2012). Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates
509 multiple vascularized tumors in mice. Clin Cancer Res *18*, 1672-1683.

510 Chmielewski, M., Hombach, A.A., and Abken, H. (2014). Of CARs and TRUCKs: chimeric antigen
511 receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma.
512 Immunol Rev *257*, 83-90.

513 Chmielewski, M., Kopecky, C., Hombach, A.A., and Abken, H. (2011). IL-12 release by engineered
514 T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent

515 macrophage response on tumor cells that have shut down tumor antigen expression. Cancer Res
516 *71*, 5697-5706.

517 Cieri, N., Camisa, B., Cocchiarella, F., Forcato, M., Oliveira, G., Provasi, E., Bondanza, A.,
518 Bordignon, C., Peccatori, J., Ciceri, F.*, et al.* (2013). IL-7 and IL-15 instruct the generation of
519 human memory stem T cells from naive precursors. Blood *121*, 573-584.

520 Cohen, J. (1995). IL-12 deaths: explanation and a puzzle. Science *270*, 908.

521 Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini,
522 L.A.*, et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819-
523 823.

524 Curtsinger, J.M., and Mescher, M.F. (2010). Inflammatory cytokines as a third signal for T cell
525 activation. Current opinion in immunology *22*, 333-340.

526 Dinarello, C., Novick, D., Kim, S., and Kaplanski, G. (2013). Interleukin-18 and IL-18 binding
527 protein. Frontiers in immunology *4*, 289.

528 Dotti, G., Gottschalk, S., Savoldo, B., and Brenner, M.K. (2014). Design and development of
529 therapies using chimeric antigen receptor‐expressing T cells. Immunological reviews *257*, 107-
530 126.

531 Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer *4*, 11-
532 22.

533 Fesnak, A.D., June, C.H., and Levine, B.L. (2016). Engineered T cells: the promise and challenges
534 of cancer immunotherapy. Nature reviews Cancer *16*, 566-581.

535 Frigault, M.J., Lee, J., Basil, M.C., Carpenito, C., Motohashi, S., Scholler, J., Kawalekar, O.U.,
536 Guedan, S., McGettigan, S.E., Posey, A.D., Jr.*, et al.* (2015). Identification of chimeric antigen
537 receptors that mediate constitutive or inducible proliferation of T cells. Cancer Immunol Res *3*,
538 356-367.

539 Gilham, D.E., Debets, R., Pule, M., Hawkins, R.E., and Abken, H. (2012). CAR-T cells and solid
540 tumors: tuning T cells to challenge an inveterate foe. Trends Mol Med *18*, 377-384.

541 Goff, S.L., Dudley, M.E., Citrin, D.E., Somerville, R.P., Wunderlich, J.R., Danforth, D.N., Zlott, D.A.,
542 Yang, J.C., Sherry, R.M., Kammula, U.S.*, et al.* (2016). Randomized, Prospective Evaluation
543 Comparing Intensity of Lymphodepletion Before Adoptive Transfer of Tumor-Infiltrating
544 Lymphocytes for Patients With Metastatic Melanoma. J Clin Oncol *34*, 2389-2397.

545 Harris, D.T., and Kranz, D.M. (2016). Adoptive T Cell Therapies: A Comparison of T Cell Receptors
546 and Chimeric Antigen Receptors. Trends Pharmacol Sci *37*, 220-230.

547 Hinrichs, C.S., and Rosenberg, S.A. (2014). Exploiting the curative potential of adoptive T-cell
548 therapy for cancer. Immunol Rev *257*, 56-71.

549 Hsu, C., Jones, S.A., Cohen, C.J., Zheng, Z., Kerstann, K., Zhou, J., Robbins, P.F., Peng, P.D., Shen,
550 X., Gomes, T.J., *et al.* (2007). Cytokine-independent growth and clonal expansion of a primary
551 human CD8+ T-cell clone following retroviral transduction with the IL-15 gene. Blood *109*, 5168-
552 5177.

553 Hunder, N., Wallen, H., Cao, J., Hendricks, D., Reilly, J., Rodmyre, R., Jungbluth, A., Gnjatic, S.,
554 Thompson, J., and Yee, C. (2008). Treatment of metastatic melanoma with autologous CD4+ T
555 cells against NY-ESO-1. The New England journal of medicine *358*, 2698.

556 Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D., and Levitsky, H. (1998). The
557 central role of CD4(+) T cells in the antitumor immune response. J ExpMed *188*, 2357-2368.

558 Hurton, L.V., Singh, H., Najjar, A.M., Switzer, K.C., Mi, T., Maiti, S., Olivares, S., Rabinovich, B.,
559 Huls, H., Forget, M.A., *et al.* (2016). Tethered IL-15 augments antitumor activity and promotes a
560 stem-cell memory subset in tumor-specific T cells. Proc Natl Acad Sci U S A.

561 Jensen, M.C., and Riddell, S.R. (2014). Design and implementation of adoptive therapy with
562 chimeric antigen receptor-modified T cells. Immunol Rev *257*, 127-144.

563 Jensen, M.C., and Riddell, S.R. (2015). Designing chimeric antigen receptors to effectively and
564 safely target tumors. Curr Opin Immunol *33c*, 9-15.

565 Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A
566 programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*,
567 816-821.

568 Kalos, M., and June, C.H. (2013). Adoptive T cell transfer for cancer immunotherapy in the era of
569 synthetic biology. Immunity *39*, 49-60.

570 Kershaw, M.H., Westwood, J.A., Parker, L.L., Wang, G., Eshhar, Z., Mavroukakis, S.A., White, D.E.,
571 Wunderlich, J.R., Canevari, S., Rogers-Freezer, L., *et al.* (2006). A phase I study on adoptive
572 immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res *12*, 6106-6115.

573 Kim, J.H., Lee, S.R., Li, L.H., Park, H.J., Park, J.H., Lee, K.Y., Kim, M.K., Shin, B.A., and Choi, S.Y.
574 (2011). High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human
575 cell lines, zebrafish and mice. PLoS One *6*, e18556.

576 Kochenderfer, J., Wilson, W., Janik, J., Dudley, M., Stetler-Stevenson, M., Feldman, S., Maric, I.,
577 Raffeld, M., Nathan, D., Lanier, B., *et al.* (2010). Eradication of B-lineage cells and regression of
578 lymphoma in a patient treated with autologous T cells genetically-engineered to recognize CD19.
579 Blood *116*, 4099-4102.

580 Koneru, M., Purdon, T.J., Spriggs, D., Koneru, S., and Brentjens, R.J. (2015). IL-12 secreting
581 tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo.
582 Oncoimmunology *4*, e994446.

583 Lee, S., and Margolin, K. (2011). Cytokines in cancer immunotherapy. Cancers (Basel) *3*, 3856-
584 3893.

585 Lu, Y.C., Yao, X., Crystal, J.S., Li, Y.F., El-Gamil, M., Gross, C., Davis, L., Dudley, M.E., Yang, J.C.,
586 Samuels, Y.*, et al.* (2014). Efficient identification of mutated cancer antigens recognized by T
587 cells associated with durable tumor regressions. Clin Cancer Res *20*, 3401-3410.

588 Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M.
589 (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.

590 Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D., Samanta, M.,
591 Lakhal, M., Gloss, B., Danet-Desnoyers, G.*, et al.* (2009). Chimeric receptors containing CD137
592 signal transduction domains mediate enhanced survival of T cells and increased antileukemic
593 efficacy in vivo. Mol Ther *17*, 1453-1464.

594 Nakamura, K., Okamura, H., Nagata, K., Komatsu, T., and Tamura, T. (1993). Purification of a
595 factor which provides a costimulatory signal for gamma interferon production. Infect Immun *61*,
596 64-70.

597 Nakamura, K., Okamura, H., Wada, M., Nagata, K., and Tamura, T. (1989). Endotoxin-induced
598 serum factor that stimulates gamma interferon production. Infect Immun *57*, 590-595.

599 Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. (2001). Interleukin-18 regulates both
600 Th1 and Th2 responses. Annu Rev Immunol *19*, 423-474.

601 Osaki, T., Peron, J.M., Cai, Q., Okamura, H., Robbins, P.D., Kurimoto, M., Lotze, M.T., and Tahara,
602 H. (1998). IFN-gamma-inducing factor/IL-18 administration mediates IFN-gamma- and IL-12-
603 independent antitumor effects. J Immunol *160*, 1742-1749.

604 Pegram, H.J., Lee, J.C., Hayman, E.G., Imperato, G.H., Tedder, T.F., Sadelain, M., and Brentjens,
605 R.J. (2012). Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without
606 need for prior conditioning. Blood *119*, 4133-4141.

607 Porter, D.L., Levine, B.L., Kalos, M., Bagg, A., and June, C.H. (2011). Chimeric antigen receptor-
608 modified T cells in chronic lymphoid leukemia. N Engl J Med *365*, 725-733.

609 Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H., and Zhao, Y. (2015). Multiplex Cripsr/Cas9 Genome
610 Editing to Generate Potent Universal CART and PD1-Deficient Cells Against Leukemia. Blood
611 *126:4280*.

612 Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H., and Zhao, Y. (2016). Multiplex genome editing to
613 generate universal CAR T cells resistant to PD1 inhibition. Clin Cancer Res.

614 Robbins, P.F., Lu, Y.C., El-Gamil, M., Li, Y.F., Gross, C., Gartner, J., Lin, J.C., Teer, J.K., Cliften, P.,
615 Tycksen, E.*, et al.* (2013). Mining exomic sequencing data to identify mutated antigens
616 recognized by adoptively transferred tumor-reactive T cells. Nat Med *19*, 747-752.

617 Robertson, M.J., Mier, J.W., Logan, T., Atkins, M., Koon, H., Koch, K.M., Kathman, S., Pandite,
618 L.N., Oei, C., Kirby, L.C.*, et al.* (2006). Clinical and biological effects of recombinant human
619 interleukin-18 administered by intravenous infusion to patients with advanced cancer. Clin
620 Cancer Res *12*, 4265-4273.

621 Sato, N., Sabzevari, H., Fu, S., Ju, W., Petrus, M.N., Bamford, R.N., Waldmann, T.A., and Tagaya, Y.
622 (2011). Development of an IL-15-autocrine CD8 T-cell leukemia in IL-15-transgenic mice requires
623 the cis expression of IL-15Ralpha. Blood *117*, 4032-4040.

624 Straathof, K.C., Spencer, D.M., Sutton, R.E., and Rooney, C.M. (2003). Suicide genes as safety
625 switches in T lymphocytes. Cytotherapy *5*, 227-230.

626 Yang, X., Zhang, X., Fu, M.L., Weichselbaum, R.R., Gajewski, T.F., Guo, Y., and Fu, Y.X. (2014).
627 Targeting the tumor microenvironment with interferon-beta bridges innate and adaptive
628 immune responses. Cancer Cell *25*, 37-48.

629 Zhang, L., Morgan, R.A., Beane, J.D., Zheng, Z., Dudley, M.E., Kassim, S.H., Nahvi, A.V., Ngo, L.T.,
630 Sherry, R.M., Phan, G.Q.*, et al.* (2015). Tumor-infiltrating lymphocytes genetically engineered
631 with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma.
632 Clin Cancer Res *21*, 2278-2288.

633 Zhao, Y., Moon, E., Carpenito, C., Paulos, C.M., Liu, X., Brennan, A., Chew, A., Carroll, R.G.,
634 Scholler, J., Levine, B.L.*, et al.* (2010). Multiple injections of electroporated autologous T cells
635 expressing a chimeric antigen receptor mediate regression of human disseminated tumor.
636 Cancer Res *70*, 9062-9072.

637 Zhao, Z., Condomines, M., van der Stegen, S.J., Perna, F., Kloss, C.C., Gunset, G., Plotkin, J., and
638 Sadelain, M. (2015). Structural Design of Engineered Costimulation Determines Tumor Rejection
639 Kinetics and Persistence of CAR T Cells. Cancer Cell *28*, 415-428.
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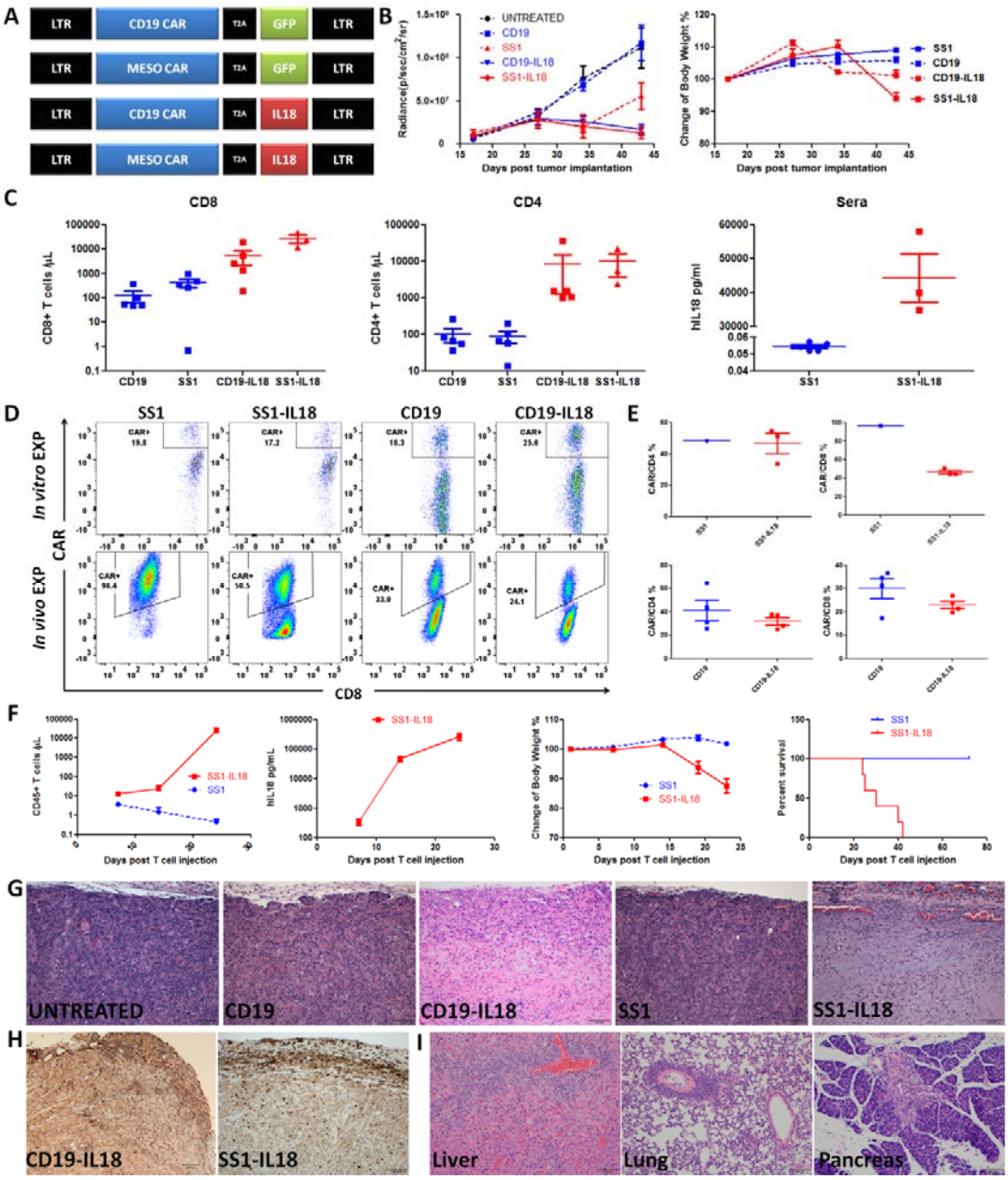

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656 **Figure 1. IL18 dependent T cell proliferation *in vivo*, and associated GVHD in NSG mice. (A)** The
657 construction designs of CART are listed as CD19-GFP, SS1-GFP, CD19-IL18, and SS1-IL18. **(B,C)**
658 NSG mice (n=5) were inoculated with 2.5e6 AsPC1 expressing CBG-T2A-GFP. 3 weeks later, the
659 mice received 2e6 of different CART as indicated. **(B)** Tumor growth and body weight. **(C)** CD8+
660 and CD4+ T cells in peripheral blood. IL18 levels were assessed by ELISA. **(D)** Representative
661 FACS plots showing percentages of CAR+/CD8+ in CART cells during expansion *in vitro* and in the
662 spleens of mice. Each data point represents cells from a single spleen, except that 5 spleens
663 from mice receiving SS1 CART were pooled. **(E)** The percentage of CAR+/CD4+ or CAR+/CD8+ T
664 cells in spleens from (D). **(F)** Tumor free NSG mice (n=5) were inoculated with 5e6 SS1 or SS1-
665 IL18 CART cells, and monitored for circulating T cells, serum IL18 concentrations, body weight
666 and survival. **(G)** H&E staining of tumors treated with different CART cells as indicated. **(H)** IHC
667 staining for human CD3+ T cells in tumor sections from mice treated with CD19-IL18 or SS1-IL18
668 CART cells. **(I)** Representative pictures of liver, lung and pancreas from mice receiving SS1-IL18
669 CART cells demonstrated T cell infiltration and GVHD (see also Table S1). All data with error bars
670 are presented as mean±SEM.

671 The following figure supplements are available for figure 1:

672 **Figure supplement 1.** SS1-IL18 CART cells displayed enhanced cytotoxicity.

673 **Figure supplement 2.** IL18 expanded T cells are polyclonal through phenotyping of TCRB variants.

674 **Figure supplement 3.** CD19-IL18 CART cells mediate dramatic T cell expansion and lethal GVHD
675 in xenograft model.

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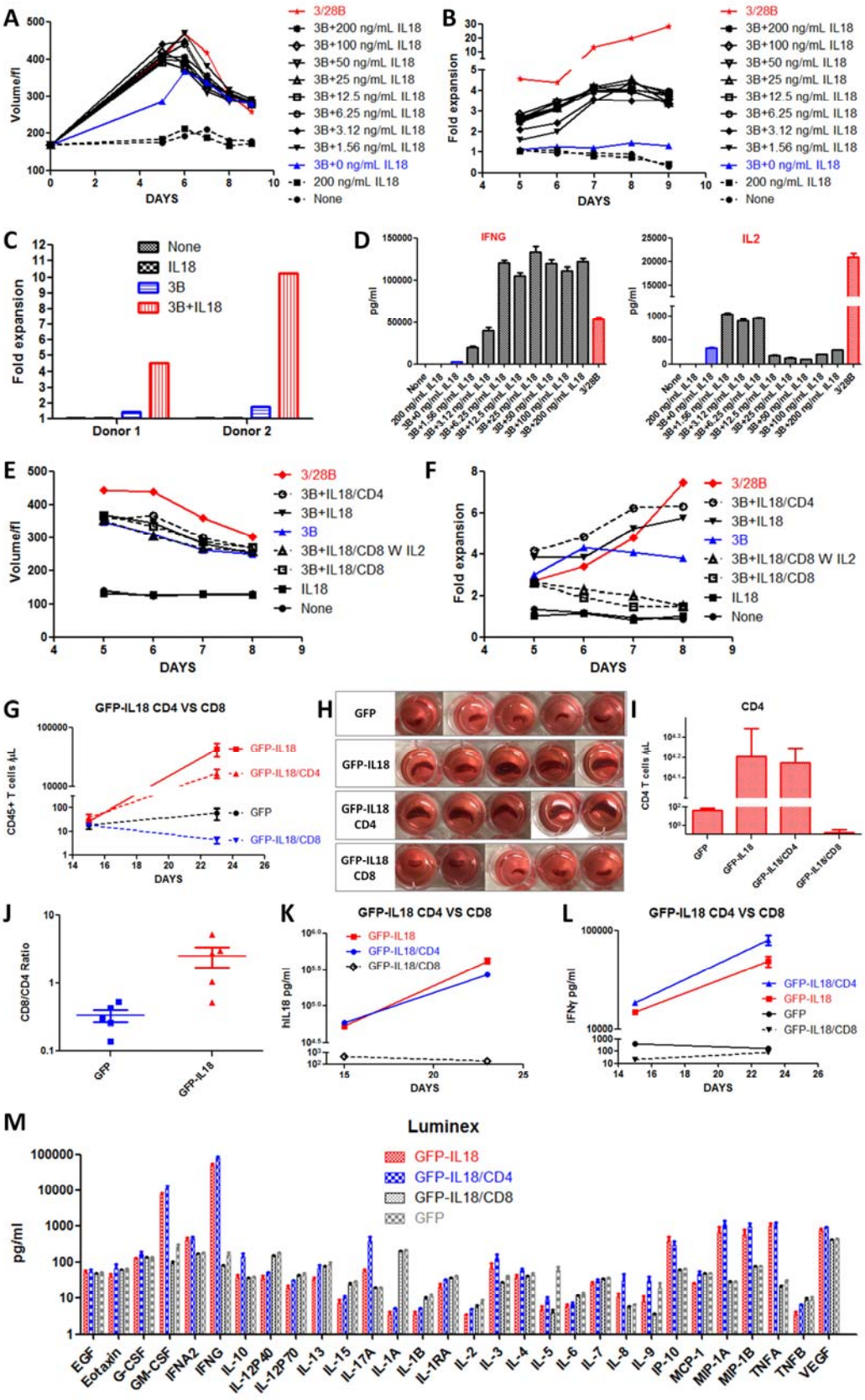
A
Volume/fl
DAYS
3/28B
3B+200 ng/mL IL18
3B+100 ng/mL IL18
3B+50 ng/mL IL18
3B+25 ng/mL IL18
3B+12.5 ng/mL IL18
3B+6.25 ng/mL IL18
3B+3.12 ng/mL IL18
3B+1.56 ng/mL IL18
3B+0 ng/mL IL18
200 ng/mL IL18
None
B
Fold expansion
DAYS
C
Fold expansion
None
IL18
3B
3B+IL18
Donor 1
Donor 2
D
IFNG
IL2
pg/ml
E
Volume/fl
DAYS
3/28B
3B+IL18/CD4
3B+IL18
3B
3B+IL18/CD8 W IL2
3B+IL18/CD8
IL18
None
F
Fold expansion
DAYS
G
GFP-IL18 CD4 VS CD8
CD45+ T cells µL
DAYS
GFP-IL18
GFP-IL18/CD4
GFP
GFP-IL18/CD8
H
GFP
GFP-IL18
GFP-IL18 CD4
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CD4
CD4 T cells µL
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GFP-IL18
GFP-IL18/CD4
GFP-IL18/CD8
J
CD8/CD4 Ratio
GFP
GFP-IL18
K
GFP-IL18 CD4 VS CD8
hIL18 pg/ml
DAYS
GFP-IL18
GFP-IL18/CD4
GFP-IL18/CD8
L
GFP-IL18 CD4 VS CD8
IFNγ pg/ml
DAYS
GFP-IL18/CD4
GFP-IL18
GFP
GFP-IL18/CD8
M
Luminex
pg/ml
GFP-IL18
GFP-IL18/CD4
GFP-IL18/CD8
GFP
EGF
Eotaxin
G-CSF
GM-CSF
IFNA2
IFNG
IL-10
IL-12P40
IL-12P70
IL-13
IL-15
IL-17A
IL-1A
IL-1B
IL-1RA
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IP-10
MCP-1
MIP-1A
MIP-1B
TNFA
TNFB
VEGF

687

688 **Figure 2. Combined TCR and IL18 signaling promotes CD4 helper dependent T cell expansion.**
689 **(A-D)** Primary T cells from normal donors were activated with anti-CD3 beads and varying
690 concentrations (1.56 doubled up to 200 ng/ml) of recombinant IL18 (added at D0 and D3).
691 Activated T cells were de-beaded at D5 and counted every day afterwards. **(A)** changes in T cell
692 size and **(B)** proliferation kinetics. **(C)** IL18 dependent expansion of T cells from two different
693 donors. **(D)** Luminex analysis of secreted IFNG and IL2. Additional cytokine data are shown in
694 Figure 2-figure supplement 1. **(E,F)** Normal donor T cells expanded with anti-CD3/CD28 beads
695 were selected by MACS columns to purify CD8 and CD4 T cells. Purified CD8, CD4 T cells, or bulk
696 CD8+CD4 T cells were restimulated with anti-CD3 beads and 100ng/ml IL18 (added at D0 and
697 D3). Cell size **(E)** and proliferation kinetics **(F)** were monitored after stimulation with anti-CD3
698 beads and recombinant IL18. **(G-M)** T cells were transduced with lentivector encoding GFP or
699 GFP-IL18 at D1 during standard T cells expansion and GFP-IL18/CD8 and GFP-IL18/CD4 T cells
700 were selected using MACS columns. NSG mice (n=5) were injected intravenously with $2X10^6$
701 GFP-IL18 T cells, GFP-IL18/CD8, or GFP-IL18/CD4 T cells. GFP T cells were included as a negative
702 control. The data shown is representative of two independent experiments. **(G)** Circulating
703 CD45+ T cells at d15 and D23 after T cells injection. **(H)** Spleens from various groups, indicating
704 splenomegaly in mice receiving GFP-IL18/CD4 or bulk T cells. **(I)** Circulating CD4+CD45+ T cells at
705 D23 following T cell injection. **(J)** The ratio of CD8 to CD4 T cells. Circulating Serum levels of IL18
706 **(K)** and IFNG **(L)** at D15 and D23. **(M)** Serum at D23 was analyzed by luminex (duplicate) to
707 assess cytokine profile.

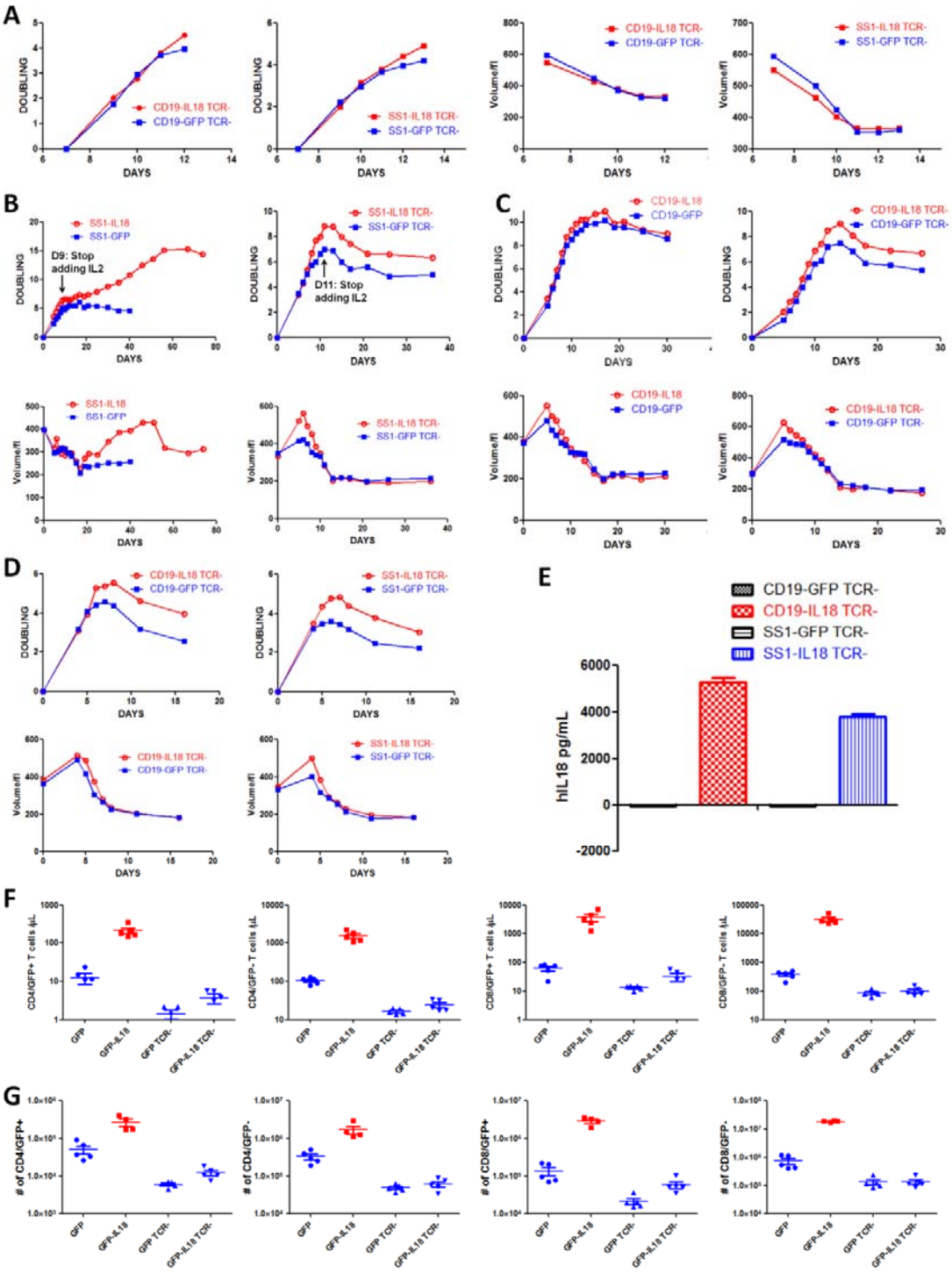
708 The following figure supplements are available for figure 2:

709 **Figure supplement 1.** Cytokine profile of IL18 and anti-CD3 beads activated T cells.

710 **Figure supplement 2.** Memory phenotype of T cells treated with recombinant IL18 and anti-CD3
711 beads.

712 **Figure supplement 3.** Memory phenotype of anti-CD3/IL18 restimulated T cells and aAPC
713 restimulated IL18-CART cells.

714


A
DOUBLING
DAYS
CD19-IL18 TCR-
CD19-GFP TCR-
SS1-IL18 TCR-
SS1-GFP TCR-
Volume/fl
B
SS1-IL18
SS1-GFP
D9: Stop adding IL2
D11: Stop adding IL2
C
CD19-IL18
CD19-GFP
D
E
CD19-GFP TCR-
CD19-IL18 TCR-
SS1-GFP TCR-
SS1-IL18 TCR-
hIL18 pg/mL
F
CD4/GFP+ T cells µL
CD4/GFP- T cells µL
CD8/GFP+ T cells µL
CD8/GFP- T cells µL
GFP
GFP-IL18
GFP TCR-
GFP-IL18 TCR-
G
# of CD4/GFP+
# of CD4/GFP-
# of CD8/GFP+
# of CD8/GFP-

719 **Figure 3**. **IL18 mediated T cell proliferation requires CAR or TCR signaling. (A)** Expansion
720 kinetics and size of TCR-deficient (TCR-) CD19-GFP or CD19-IL18 CART cells following stimulation
721 with anti-CD3/CD28 beads and RNA electroporation. **(B)** Expansion kinetics and size of TCR+ and
722 TCR-deficient SS1-GFP or SS1-IL18 CART cells following restimulation with irradiated mesothelin-
723 expressing K562 cells in the presence of exogenous IL2 (until day 9 or day 11, as indicated). **(C)**
724 Expansion kinetics and size of TCR+ and TCR-deficient CD19-GFP or CD19-IL18 CART cells
725 following restimulation with irradiated CD19-expressing K562 cells in the presence of exogenous
726 IL2. **(D)** TCR-deficient CART cells expanded from (B) and (C) were further restimulated with aAPC
727 in the absence of exogenous IL2. **(E)** IL18 secretion (duplicate) of indicated CART cells following
728 restimulation with cognate antigen. **(F,G)** NSG mice (n=5) were injected with $2X10^6$ TCR+ or TCR-
729 deficient T cells expressing either GFP or GFP-IL18. After 18 days, the number of GFP+ and GFP-
730 CD4+ and CD8+ cells in blood **(F)** or spleens **(G)** was assessed. All data with error bars are
731 presented as mean±SEM.

732 The following figure supplements are available for figure 3:

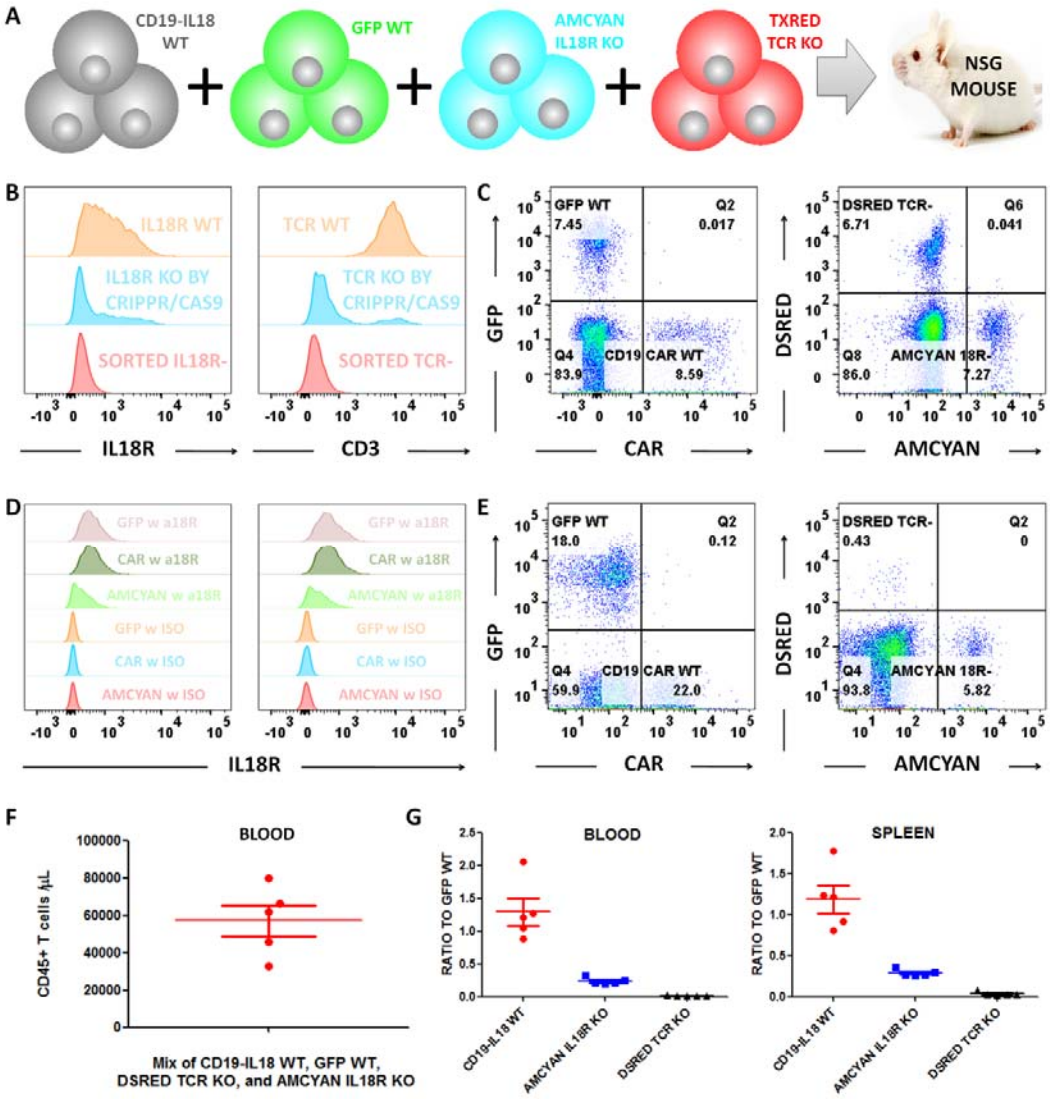
733 **Figure supplement 1.** Characterization of SS1-IL18 CART cells during extended proliferation.

734 **Figure supplement 2.** Cytokine production by IL18-CART cells stimulated with aAPC.

735 **Figure supplement 3.** IL18RA expression and memory phenotype of IL18 expanded T cells in
736 xenograft model.

737

738


A
CD19-IL18 WT
GFP WT
AMCYAN IL18R KO
TXRED TCR KO
NSG MOUSE
B
IL18R WT
IL18R KO BY CRIPPR/CAS9
SORTED IL18R-
TCR WT
TCR KO BY CRIPPR/CAS9
SORTED TCR-
IL18R
CD3
C
GFP WT 7.45
Q2 0.017
Q4 83.9
CD19 CAR WT 8.59
GFP
CAR
DSRED TCR- 6.71
Q6 0.041
Q8 86.0
AMCYAN 18R- 7.27
DSRED
AMCYAN
D
GFP w a18R
CAR w a18R
AMCYAN w a18R
GFP w ISO
CAR w ISO
AMCYAN w ISO
IL18R
E
GFP WT 18.0
Q2 0.12
Q4 59.9
CD19 CAR WT 22.0
GFP
CAR
DSRED TCR- 0.43
Q2 0
Q4 93.8
AMCYAN 18R- 5.82
DSRED
AMCYAN
F
BLOOD
CD45+ T cells /µL
Mix of CD19-IL18 WT, GFP WT, DSRED TCR KO, and AMCYAN IL18R KO
G
BLOOD
SPLEEN
RATIO TO GFP WT
CD19-IL18 WT
AMCYAN IL18R KO
DSRED TCR KO

749 **Figure4. IL18 receptor is required for cell-autonomous IL18-mediated T cell expansion *in vivo*.**
750 **(A)** Cell competition assay design. CD19-IL18 WT, GFP WT, Amcyan IL18R KO, and DsRed TCR KO
751 T cells were mixed together and injected into NSG mouse (n=5) intravenously, to evaluate the
752 expansion of each cell population in response to IL18 secretion. **(B)** Normal human primary T
753 cells were activated and transduced with lentiviral expression vectors by standard protocols,
754 then electroporated with sgRNA targeting TCRB or IL18RA, and Cas9 RNA as described in
755 Methods. Three days after electroporation, T cells were stained with anti-CD3 or anti-IL18RA to
756 confirm gene knockout. T cells with TCR or IL18R knockout were stained with anti-CD3, or anti-
757 IL18RA microbeads and negatively sorted by MACS. **(C)** $1X10^6$CD19-IL18 CART cells, $1X10^6$GFP T
758 cells, $1X10^6$IL18R deficient Amcyan T cells, and $1X10^6$TCR-deficient DsRed T cells were mixed
759 together and injected intravenously into NSG mice. **(D)** Expression levels of IL18RA on GFP WT,
760 CD19 CAR WT, and Amcyan IL18R- cells in blood (left) and spleen (right). **(E)** Frequencies of
761 CD19-IL18 WT, GFP WT, IL18R-deficient Amcyan, and TCR-deficient DsRed T cells in blood. **(F)**
762 Total circulating CD45+ cells, indicating robust expansion. **(G)** The ratios of CD19 CART WT,
763 IL18R-deficient Amcyan, and TCR-deficient DsRed to GFP T cells in blood and spleen. All data
764 with error bars are presented as mean±SEM.

765 The following figure supplements are available for figure 4:

766 **Figure supplement 1.** CRISPR/Cas9 targeted knockout of IL18RA expression.

767

768

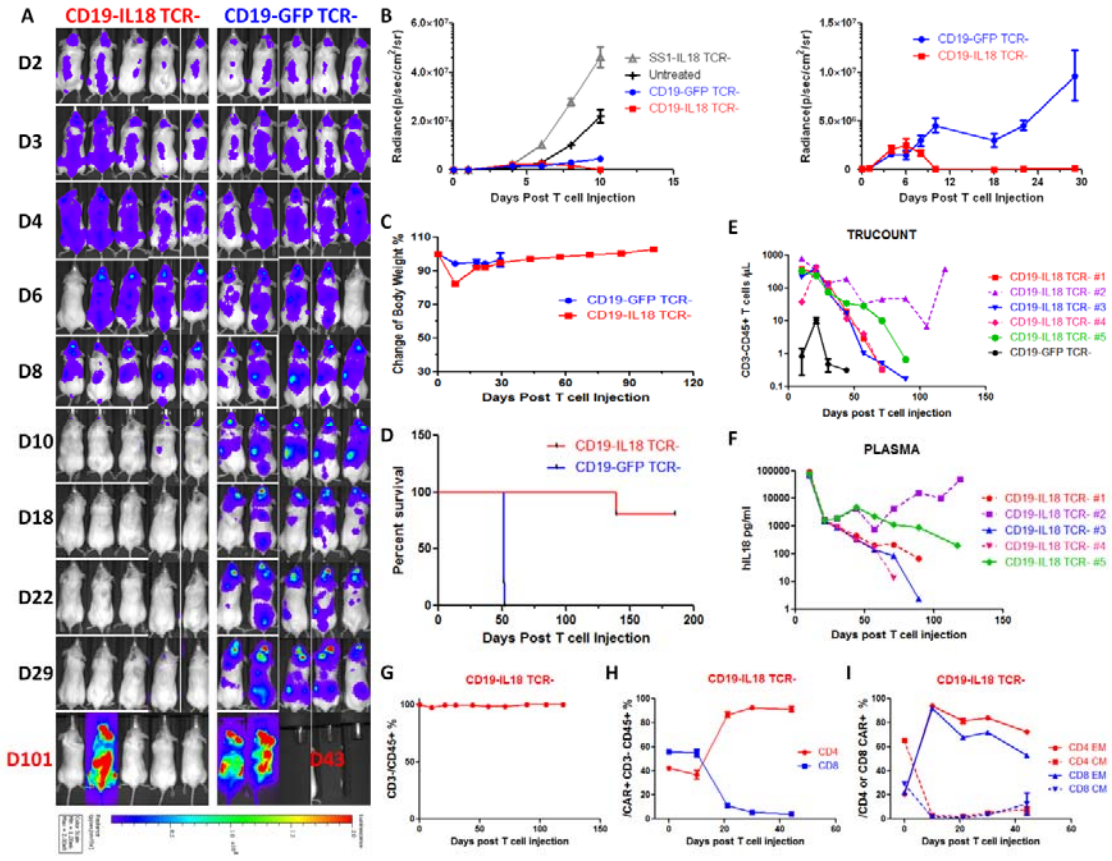


Figure5. IL18 costimulation significantly enhanced proliferation and antitumor tumor activity of CD19 CART cells. NSG mice were injected intravenously with 1X10^6 Nalm6 cells expressing CBG and GFP reporter proteins. At D7 post tumor injection, mice (n=5) were imaged and randomized into groups to receive 1X10^{6}TCR-deficient CD19-GFP T cells or CD19-IL18 CART cells. Mice receiving no CART cells or TCR-deficient SS1-IL18 CART cells were included as controls. The data shown is representative of two independent experiments. **(A)** Live animal imaging of CBG expressing tumors in mice receiving CD19-GFP or CD19-IL18 TCR-deficient CART cells. **(B)** Left panel: tumor growth curves for mice injected with CD19-GFP CART cells, SS1-IL18 CART cells, or CD19-IL18 CART cells (all TCR-deficient). Untreated control received no T cells. Right panel: tumor growth in mice receiving TCR-deficient CD19-GFP CART cells or TCR-deficient CD19-IL18 CART cells. Mice were monitored for body weight **(C)** and survival **(D)**. The number of CD3-CD45+ T cells **(E)** and IL18 concentration **(F)** in blood were monitored over time. **(G)** The percentage of CD3- cells among total CD45+ cells, distribution of CD4+ and CD8+ T cells within CD19 CART cells **(H)**, and memory phenotype in CD4+ and CD8+ CART cells **(I)** in mice receiving TCR-deficient CD19-IL18 CART cells. All data with error bars are presented as mean±SEM.

The following figure supplements are available for figure 5:

Figure supplement 1. Characterization of relapsing Nalm6 cells and re-expanded CD19-IL18 TCR-CART cells.

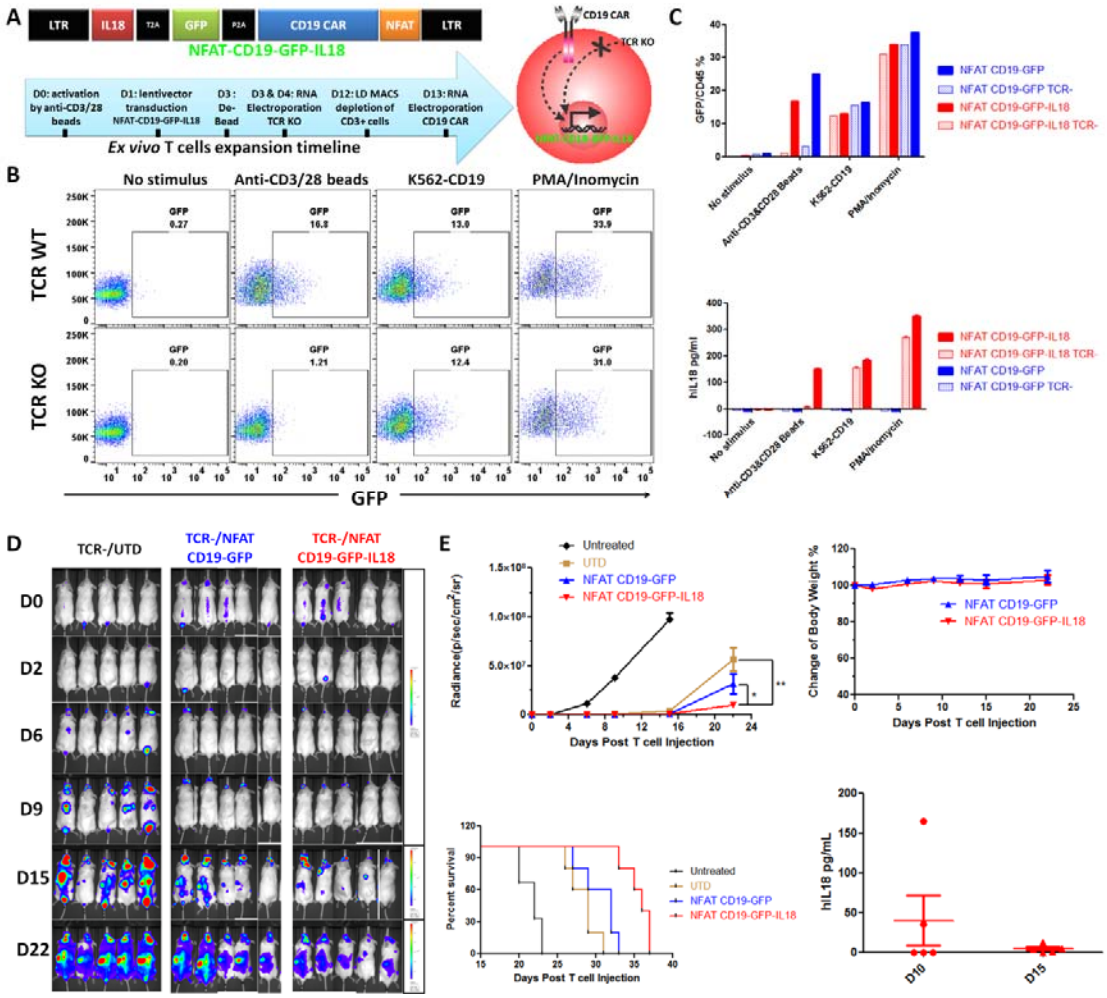


Figure6. An inducible system for IL18 expression in CART cells. (A) Upper panel: schematic of lentiviral construct in which the NFAT promoter drives expression of CD19-GFP-IL18. Lower panel: the *ex vivo* timeline to generate TCR-deficient T cells transduced with NFAT-CD19-GFP-IL18 and electroporated with mRNA CD19 CAR (designated as TCR-/NFAT-CD19-GFP-IL18). **(B)** TCR-/NFAT-CD19-GFP-IL18 CART cells were stimulated with anti-CD3/28 beads, K562-CD19, or PMA/ionomycin, after which CD45GFP+ cells were estimated by flowcytometry. **(C)** Summary of GFP+/CD45+ percentages (upper panel) and IL18 concentrations (lower panel) under different conditions as indicated. **(D)** NSG mice (n=5) were inoculated with $1X10^6$ Nalm6-CBG/GFP cells, and treated 1 week later with TCR-deficient untransduced, NFAT-CD19-GFP, or NFAT-CD19-GFP-IL18 CART (all bearing mRNA encoded CD19 CAR). Tumor progression was monitored by live animal imaging. **(E)** Tumor growth kinetics (Student's t-test: **p=0.0057, *p=0.0359), survival rate after T cells infusion, change of body weight, and IL18 concentrations at D10 and D15 following T cell injection. All data with error bars are presented as mean±SEM.