

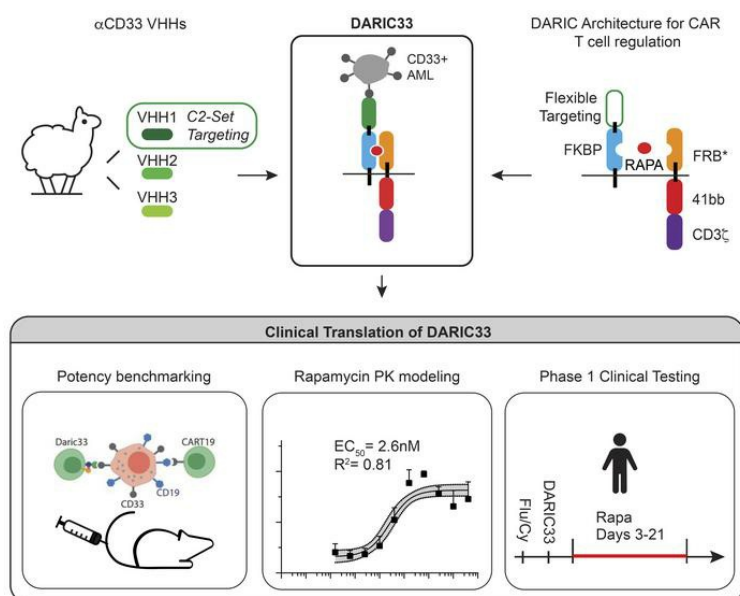
Drug-regulated CD33-targeted CAR T cells control AML using clinically optimized rapamycin dosing

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1 **Title: Drug-regulated CD33-targeted CAR T cells control AML using**
2 **clinically optimized rapamycin dosing**

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29 **Abstract:** Chimeric antigen receptor (CAR) designs that incorporate pharmacologic control are
30 desirable, however designs suitable for clinical translation are needed. We designed a fully
31 human, rapamycin-regulated, drug product for targeting CD33+ tumors called dimerization agent
32 regulated immunoreceptor complex (DARIC33). T cell products demonstrated target specific
33 and rapamycin-dependent cytokine release, transcriptional responses, cytotoxicity, and in vivo
34 antileukemic activity in the presence of as little as 1nM rapamycin. Rapamycin withdrawal
35 paused DARIC33-stimulated T cell effector functions, which were restored following re-
36 exposure to rapamycin, demonstrating reversible effector function control. While rapamycin-
37 regulated DARIC33 T cells were highly sensitive to target antigen, CD34+ stem cell colony
38 forming capacity was not impacted. We benchmarked DARIC33 potency relative to CD19 CAR
39 T cells to estimate a T cell dose for clinical testing. In addition, we integrated in vitro and
40 preclinical in vivo drug concentration thresholds for OFF-ON state transitions, as well as murine
41 and human rapamycin pharmacokinetics, to estimate a clinically applicable rapamycin dosing
42 schedule. A phase 1 DARIC33 trial has been initiated (PLAT-08, NCT05105152), with initial
43 evidence of rapamycin-regulated T cell activation and anti-tumor impact. Our findings provide

44 evidence that the DARIC platform exhibits sensitive regulation and potency needed for clinical

45 application to other important immunotherapy targets.

46

47 **Main Text:**

48 **INTRODUCTION**

49 Chimeric antigen receptor (CAR) T cell products are potent living drugs that dramatically
50 expand in the days following adoptive transfer into patients. For existing CD19 and BCMA CAR
51 T cell products, T cell engraftment, expansion, function, and persistence characteristics are
52 product autonomous, such that, at their peak, CAR T cells may number 100- to 1000-fold greater
53 than the number of cells initially infused (1). Toxicities, such as cytokine release syndrome
54 (CRS), immune effector cell associated neurotoxicity syndrome (ICANS), and rare late effects,
55 such as marrow hypoplasia, have also been reported (2). Technologies that allow physicians to
56 control the activity of engineered T cell therapies following patient infusion may address some of
57 the safety concerns associated with this promising class of drugs.

58 In many applications, such as acute myeloid leukemia (AML) and solid tumors, on-
59 target/off-tumor product reactivity may negatively impact therapeutic index. In the case of B-cell
60 malignancies, broad targeting and elimination of both normal and malignant CD19+ cells is
61 generally well tolerated and clinically manageable. However, in the case of AML, no known
62 target allows selective ablation of malignant myeloid cells without the simultaneous loss of
63 essential nonmalignant cell types (3). Despite this caveat, hematopoietic stem/progenitor cell
64 (HSPC) antigens with increased expression on AML blasts, such as CD33, CD123 and CLL1
65 (CLEC12A) have emerged as potential targets. Thus, targeting AML antigens with constitutively
66 active CAR T cell products may eliminate HPSCs, resulting in prolonged or permanent marrow
67 hypoplasia. A technological platform that allows for recursive cycles of tumor kill interspersed

68 with periods of myeloid recovery, akin to cycles of cytotoxic chemotherapy, is a conceptually
69 attractive approach to target this class of AML antigens.

70 Drug-induced dimerization of split CAR designs may be a general approach to allow
71 physicians to modulate CAR activity in a time scale matched to clinical need. Previously
72 reported dimerizing agent-regulated immunoreceptor complexes (DARICs) are composed of
73 separate antigen targeting and T cell signaling components, with embedded extracellular
74 rapamycin-dependent heterodimerizing domains (4). Targeting and signaling components
75 dimerize in the presence of rapamycin, resulting in antigen-responsive T cell activation (4, 5).
76 Prior studies demonstrated that CD19-targeted DARIC T cells (DARIC19) display OFF to ON
77 (e.g. quiescent to antigen responsive) functional transitions in the presence of sub-
78 immunosuppressive concentrations of rapamycin (e.g. ≤ 1 nM), well below the range
79 recommended for immunosuppression in patients following solid organ transplant (6). Further,
80 DARIC19 T cells in the presence of nanomolar concentrations of rapamycin exhibited potency
81 equivalent to conventional CD19 CAR T cells in preclinical in vitro and in murine disease
82 models.

83 Here, we describe the assembly and validation of a CD33 targeted DARIC chimeric
84 immunoreceptor. From a single domain V_HH antibody library, we identified candidate CD33-
85 binders that redirect T cell effector functions to target cells expressing CD33, an established
86 AML antigen (7). Among several high affinity single domain antibodies, we identified one
87 candidate capable of recognizing an epitope within the membrane proximal C2-set domain of
88 CD33, which is a conserved domain across CD33 splice isoforms (8). DARIC receptors
89 incorporating the C2 epitope-specific V_HH (DARIC-V_HH1) displayed rapamycin-dependent
90 recognition and activation against multiple gene modified and AML cell lines, as well as an in

91 vivo antitumor effect against established CD33+ human tumor xenografts in NSG mouse
92 models. Following manufacture of clinical scale DARIC33 T cell lots using GMP manufacturing
93 methodologies, we found donor-matched DARIC33 T cells and control CD19 CAR T cells both
94 exhibited similar expression of phenotypic markers associated with engraftment fitness while
95 neither expressed markers associated with tonic signaling or exhaustion. Finally, correlation of
96 dose-exposure and activity relationships establish rapamycin concentration thresholds required
97 for DARIC33 T cell activity in vivo. Based on these findings, we have initiated a phase I
98 DARIC33 trial in pediatric patients with relapsed/refractory AML and demonstrated initial signs
99 of rapamycin-mediated T cell activation and tumor response. Together, these observations will
100 serve as a model for the development of additional drug-regulated T cell therapies.

101 **RESULTS**

102 The DARIC architecture utilizes a split CAR design in which the antigen targeting and T cell
103 signaling domains are separated into distinct transmembrane receptors that contain extracellular
104 cognate rapamycin-dependent heterodimerization domains (**Fig. 1A**). This bipartite design
105 leverages a highly energetically favorable ternary complex between rapamycin and the
106 rapamycin binding domains derived from *FK506-binding protein 12kd (FKBP12)* and
107 mammalian target of rapamycin (mTOR) *FKBP12-rapamycin-binding (FRB)* domain. The FRB
108 domain was modified to incorporate the T2098L mutation, which destabilizes domain folding in
109 the absence of rapamycin and increases the rate of protein turnover(9), promoting a stringent off
110 state. We previously reported potent and reversible rapamycin-dependent anti-tumor responses
111 by CD19-DARIC T cells (4) and sought to adapt this design for an AML therapeutic approach by
112 engineering the DARIC to target CD33.

113 Construction of rapamycin-dependent CD33-targeted DARICs

114 To identify CD33-targeting single domain antibodies, a library screen of heavy chain only
115 (V_{HH}) binders isolated following alpaca immunization and yeast surface display was conducted.
116 Three lead V_{HH} candidates that bound recombinant human CD33 protein were identified. To
117 confirm hits, binders were tested against CHO cells transiently expressing CD33 using
118 increasing amounts of purified recombinant anti-CD33 V_{HH} -Fc fusion proteins and secondary
119 antibodies. Evaluation of binding isotherms revealed apparent affinities ranging from 0.9nM to
120 249nM (**Fig. S1A**). The binding characteristics of clone V_{HH1} were further characterized by
121 surface plasmon resonance (SPR) (**Fig. S1B**).

122 To determine whether CD33 specific V_{HH} domains are capable of redirecting DARIC T cell
123 effector functions, codon optimized V_{HH} domains were embedded as the targeting moiety of the
124 DARIC architecture, resulting in DARIC- V_{HH1} -3 (4). Peripheral blood mononuclear cells
125 (PBMCs) were activated with CD3/CD28 antibodies, transduced with lentiviral vectors and
126 expanded for *in vitro* analysis (**Fig. 1B**). T cell products contained an average of 1.5 to 2
127 integrated lentiviral genomes per cell, independent of construct (**Fig. S1C**). Untransduced (UTD)
128 T cells, both in the presence or absence of rapamycin, exhibited low to undetectable levels of
129 interferon gamma ($IFN\gamma$) release following coculture with CD33+ tumor cells in media with and
130 without rapamycin (Fig. 1C), reflecting minimal T cell activation. In addition, while none of the
131 DARIC- V_{HH} T cell products responded to CD33+ stimulator cells in the absence of rapamycin
132 each produced 120 – 240 μ g/mL $IFN\gamma$ when 1nM rapamycin was added to the coculture, an
133 increase of 50-74-fold above UTD cells (**Fig. 1C**). This data demonstrates that DARIC- V_{HH} T
134 cells are stringently dependent on rapamycin for effector cytokine release.

135 **Rapamycin stabilizes the surface expression of DARIC components.**

136 The FRB domain has been shown to act as a rapamycin sensitive degron (10). Therefore, we
137 assessed the effect of rapamycin exposure on surface expression of both targeting and signaling
138 polypeptide receptors on DARIC-V_HH T cells. Following incubation of DARIC-V_HH T cells in
139 standard media or media containing 1nM rapamycin, we evaluated surface expression of
140 DARIC-V_HH components using flow cytometry by staining cells with biotinylated CD33
141 antigen, anti-FRB, or anti-V_HH antibodies. Across all DARIC-V_HH constructs, rapamycin
142 exposure increased the proportion of T cells binding soluble CD33 antigen by 26%-38%
143 (ANOVA $p = 0.0232$), and the mean fluorescence intensity (MFI) by 2-4 fold (ANOVA $p <$
144 0.0001) (**Fig. 1D**). We also observed increased surface expression of both FRB and V_HH
145 domains after rapamycin exposure (ANOVA $p < 0.001$, **Fig. 1E, F**). While the percentage of
146 DARIC⁺ cells diverged among the various detection methods, the MFI ratio with +/- rapamycin
147 were similar with all analytic approaches. In addition and similar to DARIC19 (4), these results
148 demonstrate that rapamycin increases surface expression of DARIC33 components to facilitate T
149 cell responses.

150 **DARIC-V_HH T cells are sensitive to low levels of rapamycin and CD33 antigen.**

151 To determine rapamycin concentration thresholds required for DARIC-V_HH T cell activation,
152 we assayed cytokine release from 24 hour co-cultures of DARIC-V_HH T cells and CD33⁺ AML
153 target cells, including a rapamycin concentration range up to 4 nM (**Fig. 2A**). Release of IFN γ
154 and IL-2 followed a sigmoidal response to increasing rapamycin concentrations, reaching a
155 maximum in the presence of 0.25 nM rapamycin and remaining unchanged at higher rapamycin
156 concentrations. DARIC-V_HH2 induced more cytokine release than the other two V_HH clones.

157 The rapamycin EC₅₀ for DARIC-V_HH T cell activation, determined by cytokine release, ranged
158 from 15.8 pM to 74.2 pM for IFN γ and IL-2 (**Fig. 2A**), and 17 pM to 52 pM for TNF α (**Fig.**
159 **S2A**). Based on these data, the rapamycin concentration required for DARIC-V_HH T cell
160 activation in the presence of CD33⁺ target cells is near or below the IC₅₀ of mTORC1 (62 pM)
161 or mTORC2 (534 pM)(11).

162 We next assessed CD33 antigen sensitivity by co-culturing DARIC-V_HH T cells with target
163 cells expressing a range of CD33 antigen densities in the presence or absence of rapamycin.
164 HEK293T cells electroporated with escalating amounts of CD33 mRNA exhibited dose-
165 dependent levels of cell surface CD33 protein as determined by flow cytometry (**Fig. S2B**). In
166 the presence of rapamycin and target cells with increasing CD33 antigen density, IFN γ release by
167 DARIC-V_HH T cells increased between 19 and 140-fold and IL-2 release increased between 408
168 and 618-fold (**Fig. 2B**). Among samples treated with CD33 mRNA, CD33 expression increased
169 38-fold, while IFN γ release by DARIC-V_HH T cells increased only 2- to 3-fold, suggesting
170 saturation of DARIC-V_HH signaling outputs at low densities of CD33. We further evaluated
171 rapamycin-dependent induction of IFN γ release following stimulation of DARIC-V_HH T cells by
172 target cells expressing lower densities of CD33 (**Fig. S2D,E**). One construct, DARIC-V_HH2,
173 released substantial amounts of IFN γ in the presence of rapamycin and unmanipulated HEK293T
174 cells, suggesting antigen-independent signaling of this construct (**Fig 2B** and **Fig S2E**). Addition
175 of soluble CD33 protein to coculture experiments did not inhibit rapamycin-dependent
176 DARIC33 stimulation of T cell responses (**Fig. S2F**). Together these results demonstrate
177 rapamycin-activated DARIC-V_HH T cells exhibit sensitivity to AML target cells with low CD33
178 densities.

179 **DARIC-V_HH T cells exhibit rapamycin-dependent antileukemic activity in vivo.**

180 To evaluate anti-tumor activity in vivo, we used xenograft tumor models in which
181 immunodeficient NSG mice are intravenously inoculated with luciferase-tagged AML cell lines
182 in the context of a range of rapamycin doses and administration schedules. MOLM14, a cell line
183 derived from a secondary AML, exhibits robust CD33 expression (**Table S1**) and, following
184 modification for bioluminescence imaging (BLI), grows rapidly when inoculated into NSG mice.
185 Following intravenous inoculation of MOLM14 AML cells, we treated mice with DARIC-V_HH
186 or UTD control T cells followed by rapamycin delivered at a dose of 0.1mg/kg by intraperitoneal
187 (IP) injection three times weekly for the duration of the study (**Fig. 2C**). Mice treated with UTD
188 T cells demonstrated logarithmic increases in tumor burden and developed tumor associated
189 symptoms within 3 weeks (**Fig. 2D**). Mice treated with DARIC-V_HH T cells without rapamycin
190 exhibited equally rapid tumor progression. In contrast, mice treated with DARIC-V_HH T cells
191 and rapamycin displayed delayed tumor growth and significantly extended survival.

192 As a second xenograft tumor model to evaluate DARIC-V_HH anti-tumor activity, we used the
193 CD33-expressing acute promyelocytic leukemia-like cell line HL60, modified for BLI j(**Fig.**
194 **2E**). We similarly observed DARIC-V_HH T cell anti-tumor activity that was fully rapamycin
195 dependent (**Fig. 2F**). None of the mice in either HL60 or MOLM14 models lost weight following
196 adoptive transfer of DARIC-V_HH T cells, either alone or followed by rapamycin administration
197 (**Fig. 2F** and data not shown). Across both AML tumor models, the rank order of anti-tumor
198 activity exhibited by the DARIC-V_HH constructs was preserved. Together, these studies
199 demonstrate that the DARIC-V_HH chimeric immunoreceptor architecture elicits *in vivo* anti-
200 tumor activity in the presence of rapamycin.

201 **DARIC-V_HH1 T cell activation is specific to the membrane proximal domain of CD33**

202 Because alternative splicing impacts CD33 expression (8, 12, 13), we included cDNAs
203 encoding both full length CD33M and the major alternative shorter transcript CD33m, which
204 lacks the membrane distal Ig-like IgV2 sialic acid-binding domain encoded by exon 2 (14–16) in
205 our screening library. Expression of CD33M resulted in a strong fluorescent signal following
206 staining with all three V_{HH}-Fc fusion proteins, while expression of CD33m resulted in a strong
207 fluorescent signal only following staining with V_HH1-Fc (**Fig. S1A and S3A**). To verify the
208 CD33 membrane proximal epitope specificity we stained CHO cells transiently expressing
209 CD33m with increasing concentrations of purified V_{HH}-Fc fusions and secondary antibodies.
210 V_HH1-Fc fusion bound CD33m expressing CHO cells with an apparent $K_d = 162$ nM, whereas
211 no binding of V_HH2Fc or V_HH3Fc to CD33m was detected (**Fig. S3A**).

212 To evaluate potential off-target activity, we screened CD33-targeting V_{HH} domains for
213 binding to a library of transgenes encoding 5,528 secreted and transmembrane proteins.
214 HEK293T cells expressing library transgenes were spotted on slides, fixed, and stained with anti-
215 CD33 V_{HH} domain-Fc protein fusions followed by fluorescently labeled anti-Fc secondary
216 antibodies (**Fig. 3A**). This screen did not identify strong binding of the CD33-specific V_{HH}
217 clones to non-CD33 cell surface molecules in the library. We did observe weak fluorescent
218 signal of V_HH1 towards samples expressing Siglec-6 (NM_198845), a sialic acid binding protein
219 recently identified as a potential antigen target for AML (17) that shares substantial homology
220 with CD33. We also observed weak V_HH3 reactivity towards MMP13 (**Fig. 3B**).

221 To determine whether DARIC-V_HH1 T cells respond to CD33m or Siglec-6, we cultured
222 DARIC T cells with HEK293T cell lines electroporated with CD33M-, CD33m-, or Siglec6-
223 encoding mRNA (**Fig. 3C-D**). Consistent with the findings above, DARIC-V_HH1 T cells

224 exhibited rapamycin-dependent IFN γ release following coculture with HEK293T cells
225 expressing CD33M or CD33m, whereas T cells expressing DARIC-V_HH3 T cells responded
226 only to HEK293T cells expressing CD33M (**Fig. 3C**). Similarly, we cultured DARIC-V_HH1 or
227 DARIC-V_HH3 T cells with HEK293T cells, electroporated with titrated amounts of Siglec-6
228 mRNA, in the presence or absence of rapamycin (**Fig. 3D**). When rapamycin was present,
229 HEK293T cells electroporated with the highest amounts of Siglec6 mRNA stimulated release of
230 40-60 ng/mL IFN γ from DARIC-V_HH1 T cells, which corresponds to approximately 10% of the
231 amount released following coculture with CD33⁺ AML cells. No IFN γ release was observed
232 when DARIC-V_HH1 T cells were cultured with HEK293T cells expressing lower levels of
233 Siglec-6. Transgenic expression of CD33 in lung cancer cells that do not endogenously express
234 CD33 (**Fig S3B**) resulted in rapamycin-dependent DARIC-V_HH1 T cells proliferation *in vitro*,
235 whereas targeted deletion of CD33 from CD33⁺ AML cell lines eliminated IFN γ responses *in*
236 *vitro* (**Fig. S3C-D**) and *in vivo* (**Fig. S3G**) In addition, DARIC-V_HH1 T cells exhibited anti-
237 tumor activity in a CD33^{low} Nalm6 xenograft tumor models (**Fig. S3E-F**). Together, these data
238 show that stimulation of T cell effector functions by DARIC-V_HH1 is specific to the membrane
239 proximal domain of CD33 present in both CD33M and CD33m isoforms. The strict target
240 specificity, promising affinity characteristics, rapamycin dependence, and recognition of both
241 CD33M and CD33m led us to select DARIC-V_HH-Clone 1 as a lead clinical candidate for
242 subsequent development. Below, we refer to DARIC-V_HH1 simply as DARIC33, and T cell
243 products manufactured by Seattle Children's Therapeutics at clinical scale using good medical
244 practice (GMP) compatible reagents and techniques as SC-DARIC33.

245 **CD33m is a prevalent isoform of CD33 expressed by AML**

246 Four separate single nucleotide polymorphisms (SNPs) have been reported to influence
247 splicing of CD33 by SRSF2(14, 16), including rs12459419 C>T in the splice enhancer region
248 that regulates exon 2 skipping and rs2455069 A>G resulting in protein modification (14–16).
249 Though controversial (18), the rs12459419 T/T genotype has been associated with predominance
250 of the shortened transcript (lacking exon 2) encoding CD33m, and poor responses to CD33-
251 targeted therapeutics that recognize the IgV2 domain missing from CD33m (14, 19). We
252 reviewed transcriptional profiles of 577 AML cases, evaluating the proportion of CD33
253 transcripts lacking exon 2. We identified strong correlations between SNPs and CD33m
254 transcript expression among AML cases (**Fig. S4A-E**). We also identified CD33m transcripts
255 among profiles of healthy tissues, though with less abundance than among AML cases (**Fig.**
256 **S4F**). While attempts at targeting the CD33m isoform for AML immunotherapy are being
257 developed (20, 21), these strategies have also faced challenges. As a potential control, some
258 studies have shown that antibody HIM3-4 is specifically reactive to the CD33m isoform, we
259 observed minimal reactivity of this clone towards CD33m over-expressing cells (**Fig. S4G**).
260 Coculture of DARIC33 T cells with AML cells of various rs12459419 SNP genotypes (21, 22)
261 including OCI-AML3 (T/T), U-937 (C/C), HL-60 (C/C), MV4-11 (C/T), and MOLM14 (C/C)
262 stimulated similar rapamycin-dependent release of IFN γ and IL-2(**Fig. 3E**), despite different
263 CD33M expression density (assessed using IgV2-targeted p67.6 antibody, see **Supplemental**
264 **Table S1**). Combined, these findings demonstrate the challenges associated with targeting the
265 CD33m epitope with established antibody clones and support the use of DARIC33 to target
266 CD33⁺ cells across a range of expression and isoform usage.

267 **DARIC33 T cells do not impact hematopoietic colony forming capacity.**

268 CD33 is expressed by granulocyte precursors (23) as well as hematopoietic stem/progenitor
269 cells (HSPC) with potential for multilineage engraftment in immunodeficient mice (24).
270 Elimination of HSPC may result in intolerable myeloablation (25, 26). However, recent studies
271 found the number of cells with the potential to form multilineage colonies in stem cell plating
272 assays is not decreased by exposure to CD33 CAR T cells (27). To assess hematopoietic safety
273 of DARIC33, we plated purified CD34+ HSPCs in colony forming assays following overnight
274 incubation alone or together with a 10-fold excess of DARIC33 cells or comparator T cell
275 products and rapamycin. As expected, colony forming units (CFU) of the granulocyte/monocyte
276 lineage (CFU-GM) and of multilineage precursors (CFU-GEMM) were markedly reduced
277 following incubation with CD123 CAR T cells (28), but not following incubation with UTD
278 control cells (**Fig. 3F**). Compared to UTD T cells, the number of CFU-GM and erythroid burst
279 forming units (BFU-E) were slightly reduced following coculture with DARIC33 effector T cells
280 in the presence of rapamycin, but not when rapamycin was omitted from the overnight culture.
281 These data suggest that while activated DARIC33 T cells have some impact on hematopoietic
282 colony formation, this is a rapamycin-dependent process that can be controlled by withdrawing
283 the drug.

284 **Rapamycin exposure drives an antigen-dependent CAR T cell activation signature in**
285 **DARIC33 T cells.**

286 Transcriptional programs are tightly associated with T cell differentiation and functional
287 status (29, 30). We interrogated transcriptional changes of sorted CD4+ and CD8+ DARIC33
288 cells following antigen exposure in the presence or absence of rapamycin (see **Fig. 4A** for

289 schema). We then modeled transcriptional changes to identify a “DARIC active” profile distinct
290 from either “antigen without rapamycin” or “rapamycin-only” transcriptional profile, restricting
291 our analysis to a subset of genes informative of changes in T cell states (see Supplemental
292 Methods). Of the 2,792 queried genes, 228 genes showed transcriptional regulation specific to
293 the *DARIC active* condition in either the CD4 or the CD8 population, or both (genes with $FDR <$
294 0.05 and fold change > 2.8 over the combined individual effects of rapamycin and antigen, **Fig.**
295 **4B**, see also **Fig. S5**). Following rapamycin exposure and antigen stimulation, DARIC33 cells
296 transcriptional profiles showed significant enrichment of CAR T cell activation genes (Fisher
297 exact test; CD4 $p = 0.024$ and CD8 $p = 0.005$), including *GZMB*, *IL2RA* and *TNFRSF9*
298 (encoding 4-1BB) (**Fig. 4C**), which was also reflected by changes in protein abundance as
299 measured by flow cytometry (**Fig. 4D**). Taken together, these results reveal a transcriptional
300 activation signature of DARIC33 CD4⁺ and CD8⁺ T cells in the presence of both target antigen
301 and rapamycin. Notably, this signature is consistent with conventional CAR T cells activated by
302 antigen in the absence of rapamycin (31–33).

303 **DARIC33 and CAR33 T cells have similar functionality and activation signature.**

304 We have previously shown equivalent functional activity when the same CD19-targeting
305 scFv was placed in either a CAR vs a DARIC backbone (4). To investigate whether DARIC33 T
306 cells had similar activity as a CAR, we generated CD33-targeting CAR T cells using the
307 identical CD33-specific V_HH1 binder. Both CAR33 and DARIC33 T cells had similar
308 expression and virus integration profile (**Fig S6A**). When co-cultured with CD33⁺ HL60 tumor
309 cells, CAR33 T cells had robust IFN γ production in the presence or absence of rapamycin, while
310 DARIC33 only secreted cytokines in the presence of rapamycin (**Fig S6B**). In addition, both
311 CAR33 and rapamycin-exposed DARIC33 T cells had similar rates of cytotoxicity in vitro (**Fig**

312 **S6C**). Next, we analyzed the phenotype of both CAR33 and DARIC33 T cells with or without
313 activation. We observed some evidence of tonic signaling in the CAR33 T cells, characterized by
314 increased CD69 and CD25 expression compared to unstimulated DARIC33 T cells (**Fig. S6D-**
315 **E**). Following co-culture with CD33+ tumor cells, both CAR33 and DARIC33 T cells exhibited
316 a similar activation profile, however the CAR33 cells had higher expression of PD1, LAG3,
317 CD69 and CD25, suggesting greater activation following T cell activation (**Fig. S6D-E**).
318 Together, these data suggest similar rates of tumor reactivity for both CAR33 and DARIC33
319 platforms, with the DARIC33 cells demonstrating lower rates of tonic signaling compared to the
320 CAR33 platform.

321

322 **GMP manufacturing at scale generates SC-DARIC33 cell products with similar features as**
323 **CD19 CAR T cell products.**

324 To evaluate the performance of DARIC33 generated at clinical scale (SC-DARIC33), we
325 generated donor-matched (n.= 2) SC-DARIC33 and CD19 CAR T cells using designs and
326 manufacturing methods previously deployed in clinical trials at Seattle Children’s Hospital. In
327 these trials, CD19 CAR T cell administration resulted in complete remission rates of >90% in
328 children and young adults with R/R B-cell malignancies (34, 35). Compared to control CD19
329 CAR T cell products, SC-DARIC33 showed similar expansion kinetics, CD4/CD8 ratios,
330 proportions of CAR/DARIC+ cells and CAR/DARIC cell yields (**Fig. 5A**). The frequency of
331 cells expressing both CD62L+ and CD45RO+, a phenotype associated with preserved
332 engraftment fitness and antitumor potential(36), was >90% within each of the UTD, CD19 CAR,
333 and SC-DARIC33 T cell products (**Fig. 5B**).

334 Previous preclinical studies performed by our group found control of Raji xenograft tumor
335 burden progression correlated with clinical activity of CD19 CAR T cell designs (37). To
336 compare anti-tumor activity across CD19- and CD33-targeted T cell therapies, we generated Raji
337 cells with matched levels of CD19 and CD33 expression via lentiviral transduction
338 (Raji.CD33.ff/luc). Progression of intravenously injected Raji.CD33.ff/luc xenograft tumor
339 burden was monitored following administration of CD19 CAR, SC-DARIC33 (tested at two
340 doses), and CD19-specific DARIC T cell products manufactured using a GMP process (see **Fig.**
341 **5C** for schema). As expected, no Raji tumor burden progression was observed following infusion
342 of 1×10^7 CD19 CAR T cells per animal, whereas mice receiving no treatment, or rapamycin
343 alone (0.1mg/kg qMWF delivered by intraperitoneal injection) showed rapid tumor growth and
344 developed tumor-associated symptoms requiring euthanasia within 12 days (**Fig. 5D**). Infusion
345 of either 3×10^7 or 1×10^7 SC-DARIC33 T cells followed by rapamycin dosing suppressed
346 tumor growth and prolonged survival of mice compared to control animals not receiving
347 rapamycin (**Fig. 5E**, $p = 0.028$ and $p = 0.004$). Administration of CD19-specific DARIC cells
348 also resulted in rapamycin-dependent tumor suppression and prolonged survival in the Raji
349 xenograft tumor model. Together, these data suggest SC-DARIC33 may require higher T cell
350 doses to achieve similar potency as CD19 CAR T cells.

351 **DARIC33 and SC-DARIC33 T cells display recursive rapamycin-dependent ON-OFF-ON**
352 **functional state transitions.**

353 The capacity to temporarily pause DARIC T cell effector function in patients following SC-
354 DARIC33 administration represents a potential control feature for mitigating potential toxicities
355 and permitting hematopoietic recovery. Moreover, therapeutic T cells that are intermittently
356 rested may be less prone to functional exhaustion and capable of repopulating memory cell

357 compartments (29). We therefore developed systems to probe pharmacologic control of
358 DARIC33 T cells. Kinetic assessments of DARIC33-induced cancer cell cytotoxicity showed
359 rapid and complete rapamycin-dependent cancer cell killing only when the target antigen CD33
360 is expressed in cancer cells (**Fig. S7A,B**). Killing rates increased immediately following
361 rapamycin addition, and was maximal after 37 hours (**Fig. S7C,D**). To define kinetic effects of
362 rapamycin removal, DARIC33 T cells cultured with rapamycin for 24 hours were washed and
363 rested for increasing periods of time in rapamycin-free media prior to challenge with CD33+
364 MV4-11 AML target cells. At early time points, pre-activated SC-DARIC33 T cells showed high
365 levels of IFN γ release. Increasing durations of rest resulted in a progressive decline of IFN γ
366 release that returned to baseline after 96 hours, following first-order kinetics characterized by a
367 half-life of 17 hours (**Fig. 6A**).

368 To evaluate the reversibility of SC-DARIC33 T cell activation in vivo, we treated mice
369 bearing AML xenografts derived from MV4-11 cells modified for BLI with SC-DARIC33 T
370 cells and rapamycin delivered following continuous (Days 1-150), interrupted (Days 1-14 and 28
371 – 150), or abbreviated (Days 1-14) schedules (see **Fig. 6B** for schema). Mice receiving UTD
372 control cells (with or without rapamycin) exhibited tumor growth and tumor associated
373 symptoms by day 50, whereas mice treated with SC-DARIC33 T cells and rapamycin exhibited
374 delayed tumor burden progression (**Fig. 6C**), and prolonged symptom-free survival (**Fig. 6F**).
375 Four of 5 mice receiving the abbreviated rapamycin schedule exhibited tumor relapses
376 approximately 21 days after rapamycin was discontinued. In contrast, when rapamycin was
377 reinitiated on Day 28, 4 of 5 mice controlled the tumor through the end of the observation period.
378 Estimates of tumor growth kinetics, modeled using linear mixed effects, revealed similar tumor
379 growth rates in negative control groups and animals treated with SC-DARIC33 and abbreviated

380 rapamycin (**Fig. 6D, E**). However, among animal receiving either continuous or intermittent
381 rapamycin, SC-DARIC33 T cells suppressed tumor growth rates and extended survival (**Fig.**
382 **6F**). Together, these data are consistent with a model wherein discontinuation of rapamycin
383 pauses SC-DARIC33 anti-tumor activity, which may be restored by resuming rapamycin
384 administration.

385 **Preclinical models define blood rapamycin concentrations associated with SC-DARIC33**
386 **activation *in vitro* and *in vivo*.**

387 To support rapamycin dose selection for first-in-human testing of SC-DARIC33, we sought
388 to define rapamycin concentrations required for DARIC33 activation both *in vitro* and in a
389 mouse xenograft tumor model. We used Förster Resonance Energy Transfer (FRET) to
390 characterize the dimerization kinetics of the DARIC33 system *in vitro*. A PE-labeled anti-V_HH
391 antibody was used as a donor fluorophore while an AlexaFluor 647-labeled anti-FRB antibody
392 was used as an acceptor (**Fig. S8B**). Labeling of DARIC33 cells with both antibodies, but not
393 with either antibody alone, resulted in rapamycin-dependent fluorescence emission signal in the
394 PE-Cy5 channel (**Fig S8A**), indicating heterodimer-dependent FRET. We quantified rapamycin-
395 mediated dimerization parameters by culturing DARIC33 cells in a gradient of rapamycin
396 concentrations in media and determined the rapamycin EC₅₀ for DARIC33 dimerization was
397 135pM in T cell media (**Fig S8C**). To determine the time between rapamycin dosing and peak
398 DARIC33 activation, we cultured DARIC33 T cells in rapamycin and analyzed the FRET signal
399 at selected times after administration. The FRET signal, reflecting the combined effects of
400 surface expression and dimerization, peaked at 8 hours post rapamycin addition, suggesting that
401 DARIC33 activation is time dependent and reaches maximal levels soon after rapamycin
402 addition (**Fig S8D**).

403 In patients, rapamycin is highly sequestered by erythrocytes due to highly abundant
404 cytoplasmic FKBP-related proteins (38) and is bound to plasma proteins (39, 40) which both act
405 to reduce the amount of unbound rapamycin available to bind to DARIC33. We therefore sought
406 to understand the rapamycin concentrations required for DARIC33 heterodimerization and
407 DARIC33 T cell activation in the presence of anticoagulated whole blood using both a FRET-
408 based dimerization assay and an AML-stimulated cytokine release assay, respectively. Similar to
409 T cell media (**Fig. S8C**), DARIC33 T cells cultured in whole blood exhibited a rapamycin-
410 dependent increase in FRET signal ($EC_{50}=11.4\text{nM}$, **Fig. S8E**). Next, we performed overnight co-
411 cultures of CD33⁺ MV4-11 cells and DARIC33 T cells in either T cell culture media, human, or
412 mouse whole blood samples. The DARIC33 samples exhibited rapamycin-dependent increases
413 in IFN γ release that were similar in either species (IFN γ release, human blood, $EC_{50} = 2.6\text{ nM}$
414 while mouse blood $EC_{50} = 2.8\text{ nM}$, **Fig. 7A**) and among human T cell donors (EC_{50} range of 1.5
415 nM – 6.3 nM across three T cell donors and two blood donors, examined in duplicate, $n = 12$
416 total). The T cell activation assays and FRET dimerization assays showed higher concentrations
417 of rapamycin (~20-fold and ~100-fold, respectively) are required for half-maximal DARIC33
418 activity in whole blood as compared to media. These data define a target range of whole blood
419 rapamycin concentrations capable of activating DARIC33 T cells in the presence of CD33-
420 expressing tumor cells.

421 We next measured rapamycin exposure following single and repeat intraperitoneal (IP)
422 administrations in tumor-bearing mice using a quantitative whole blood assay. Blood
423 concentrations of rapamycin were generally dose proportional, peaking within 2 hours of
424 administration and decaying with an elimination half-time between 16 and 24 hours (**Fig. 7B**).

425 Peak rapamycin concentrations ranged from 10 ng/mL at doses of 0.02mg/kg to near 100 ng/mL
426 at a dose of 0.1 mg/kg (**Supplemental Table S2**).

427 To determine the impact of various rapamycin dose levels and dosing schedules on the anti-
428 tumor activity of SC-DARIC33 T cells, we treated MV4-11 tumor bearing mice with SC-
429 DARIC33 T cells followed by different rapamycin dosing and administration schedules (see **Fig.**
430 **7C** and **Fig. S9A** for detailed schema). Among mice receiving dosing regimens predicted to be
431 inactive (e.g. rapamycin alone, UTD T cells with or without rapamycin, or SC-DARIC33 cells
432 alone) tumor growth was similar compared to mice receiving no treatment ($\log[\text{Flux}]/\text{day} = -.26$
433 $- 0.27$, **Fig. 7D-E**). In contrast, treatments predicted to be active (e.g., SC-DARIC33 product
434 followed by rapamycin) exhibited lower rates of tumor growth, with the lowest rate observed
435 among mice receiving 0.01mg/kg rapamycin IP daily ($\log[\text{Flux}]/\text{day} = 0.058$). Tumor growth
436 rates correlated with survival: while control mice developed tumor-associated symptoms near
437 day 45, none of the mice receiving active treatment (DARIC33 + rapamycin) exhibited signs of
438 tumor progression at this time point. All rapamycin doses tested prolonged survival ($p < 0.001$
439 log rank test): at the end of the 90-day observation period, treatment with SC-DARIC33 and
440 0.01mg/kg rapamycin daily continued to control tumor outgrowth in 5 of 10 mice (**Fig. S9B**).

441 Interestingly, while *in vitro* mouse and human whole blood assays showed similar
442 rapamycin-dependent DARIC33 activation (**Fig. 7A**), we identified species differences in
443 rapamycin red blood cell (RBC) partitioning and plasma protein binding (PPB) (**Table S3-4**). In
444 humans, 94.5% of rapamycin is bound to RBCs while only 3.1% is found in plasma (39). In
445 human plasma, rapamycin is highly protein bound (92%). In contrast, in mice we observed that
446 rapamycin has 5.5% RBC partitioning and is greater than 99% PPB (**Table S5**). Despite these
447 species-specific differences in rapamycin distribution in blood compartments, we observed

448 similar rapamycin EC₅₀'s for DARIC33 T cell activation in the presence of human or mouse
449 whole blood (**Fig. 7A**), indicating that unbound rapamycin available to interact with DARIC33
450 was similar, and suggests this may also occur *in vivo*. Taken together, these data support
451 DARIC33 activity across a wide range of rapamycin dosing in vivo and inform a target
452 rapamycin trough (C_{24h}) blood concentration range of 1.5-3 ng/mL for DARIC33 T cell
453 activation in humans.

454 **First in human clinical experience demonstrates feasibility of rapamycin activation of SC-** 455 **DARIC33**

456 We designed PLAT08 (NCT05105152), as a first-in-human phase 1 trial evaluating the
457 safety of escalating doses of SC-DARIC33 in pediatric and young adult patients with relapsed
458 and refractory AML (**Fig. 8A**). In this trial, subjects receive lymphodepleting chemotherapy
459 followed by SC-DARIC33 T cell products and rapamycin. To identify rapamycin doses and
460 schedules that maximize the likelihood of achieving rapamycin blood concentrations troughs
461 within the target range of 1.5-4 ng/mL, we simulated rapamycin dose/exposure relationships
462 from adult and pediatric patients by using population pharmacokinetic models(38, 41) and
463 sampled anthropomorphic measurements for children (**Fig. S10A-B**). Among evaluated dosing
464 schedules (**Fig. S10C-H**), rapamycin daily dosing of 0.50 mg/ m² (for patients ≤1.5 m²) or 0.75
465 mg (for patients >1.5 m²) 0.5mg/m² is predicted to achieve target rapamycin trough
466 concentrations of above 1.5 ng/mL, and peak concentrations below 8ng/mL in 90% of the
467 pediatric population (**Fig. 8B**). The mean population rapamycin peak and trough (C_{24h}) levels are
468 predicted to be between 2ng/mL and 4ng/mL, well below the range of rapamycin typically used
469 for immunosuppression in solid organ transplant recipients (12-24ng/mL, (42)). We therefore
470 selected the dose schedule of daily oral rapamycin at 0.5mg/m² for initial evaluation in pediatric
471 patients.

472 To evaluate whether rapamycin exposure is associated with evidence of SC-DARIC33 tumor
473 reactivity in patients, we evaluated infusion products and blood samples obtained from the first
474 three patients enrolled on PLAT08 and treated at dose level 1 (DL1 = 10^6 DARIC⁺ cells per kg).
475 Infusion products contained between 49% and 57.9% DARIC⁺ T cells (defined as those
476 expressing surface V_HH). We developed a high dimensional flow cytometry panel capable of
477 simultaneously evaluating markers of myeloid and T cell identity (CD33, CD3, CD4, and CD8),
478 lentiviral transduction (V_HH and FRB), and T cell activation state (CD101, PD-1, and 4-1BB) in
479 patient samples. We found similar proportions of DARIC⁺ and DARIC⁻ populations of CD8 T
480 cells expressed activation markers following incubation of patient infusion products in media
481 alone or media supplemented with rapamycin, demonstrating that exposure to rapamycin alone
482 was insufficient to stimulate T cell activation (**Fig. 8D**). Using continuous rapamycin monitoring
483 and dose adjustment, rapamycin concentrations in blood samples among the three subjects
484 (S001, S002 and S004) were within the target range in 0/2 timepoints, 8/15 timepoints, and 14/18
485 timepoints, respectively (**Fig. 8E**). Finally, we monitored temporal trends in serum levels of a
486 broad panel of cytokines enriched for analytes associated CAR T cell activation(34, 35) and
487 successful CAR T cell therapy of lymphocytic leukemia (43). Serum samples from patient S004,
488 which had the highest proportion of rapamycin concentration in blood falling within the target
489 range, exhibited dramatic increases in IFN γ , TNF α and IL-6, peaking around day 10 post CAR
490 infusion, followed by steady declines (**Fig. 8F**). These initial observations indicate that
491 successfully achieving the target rapamycin concentration is associated with elevated levels of
492 cytokines affiliated with CAR T cell activation.

493 Following observations that rapamycin stabilized surface FRB on DARIC33 cells (**Fig. 1E**),
494 we hypothesized that FRB expression would correlate with rapamycin exposure. As expected,

495 evaluation of healthy donor T cell products using our clinical flow cytometry panel showed
496 overnight exposure to rapamycin resulted in increased V_HH and FRB expression (**Fig. 9A**).
497 Patient S002 exhibited choromas, some of which developed increased hemorrhagic necrosis
498 following SC-DARIC33 and rapamycin administration (**Fig. 9B**). Flow cytometric evaluation of
499 choroma and peripheral blood tissue demonstrated preferential accumulation of VHH⁺FRB⁺
500 cells within the choroma tissue (**Fig. 9C**). These cells had an activated phenotype, as the
501 proportion of PD1⁺ and TIM3⁺ cells was higher among the VHH⁺FRB⁺ CD8 T cells compared to
502 VHH⁻ (DARIC33⁻) CD8 T cells (**Fig. 9D**). We next analyzed T cell expansion and functionality
503 in patient S004. Among peripheral blood samples from patient S004, the proportion of
504 circulating blast-like CD33^{hi} side scatter (SSC^{low}) cells decreased by 99.8%, from 88% to 0.23%
505 from day 7 to day 15 (**Fig. 9E**). This was accompanied by concurrent expansion of DARIC33⁺ T
506 cells within the peripheral blood, peaking at 6% of total lymphocytes and 20.5% of circulating T
507 cells on day 9 post CAR infusion, before contracting (**Fig. 9F**). Evaluation of surface phenotypes
508 of V_HH⁺FRB⁺ ('RAPA exposed') CD8 T cells showed progressively increasing expression of
509 activation markers, including PD-1, TIM3 and 4-1BB, within RAPA exposed CD8⁺ T cells from
510 days 9 through 21 (**Fig. 9G**). In contrast, DARIC33-negative CD8 T cells presented with a
511 transient increase in surface TIM3 and PD-1 at day 15 that was not sustained. These data show
512 that, in the presence of tumor antigen and rapamycin, SC-DARIC33 expands, engrafts, and
513 acquires activated states. SC-DARIC33 activation and expansion was temporally coincident with
514 increases cytokine markers of T cell activation and transient depletion of CD33^{hi} cells in
515 peripheral blood. Together, these findings provide initial in human evidence that the DARIC33
516 platform achieves rapamycin responsive antigen dependent T cell activation.

517

518 DISCUSSION

519 In this report we describe preclinical characterization and first clinical data from a distinct
520 CAR T cell platform that aims to solve difficult challenges in treating AML through a drug
521 regulated DARIC architecture. Targeting AML with CAR T cells presents specific challenges, as
522 overlapping expression of target antigens on myeloid cells and hematopoietic stem cells limits
523 the therapeutic window for constitutively active CARs. Here, we describe the development of a
524 regulated anti-AML CAR T cell therapy that targets a membrane proximal domain CD33
525 epitope. Rapamycin-dependent heterodimerization of DARIC components results in a stringent
526 OFF state in the absence of rapamycin and acquisition of an effector ON-state T cell in the
527 presence of low nanomolar rapamycin concentrations. As a benchmark, we compared DARIC33
528 T cells to a CD19 CAR production design that has achieved clinical efficacy (34, 35). The two
529 architectures demonstrate similar potency *in vitro* and comparable potency in challenging *in vivo*
530 models. The DARIC33 system, composed exclusively of human or humanized domains, and
531 using clinically tolerable dosing of a FDA approved drug (rapamycin), represents a substantial
532 advance over other regulated CAR formats. We have initiated clinical testing of the DARIC33
533 system and observed endpoints consistent with T cell activation, expansion, and early signs of
534 anti-tumor activity.

535 Precise control of CAR T cell activity may help mitigate toxicities associated with CAR T
536 engraftment syndromes such as cytokine storm and/or the aplasia that occurs from targeting a
537 cell lineage specific antigen such as CD19 and CD33. Following infusion, CAR T cells can
538 proliferate dramatically after synchronous activation by abundant tumor cells or their antigen
539 expressing non-malignant counterparts and release of effector cytokines resulting in potentially
540 fatal cytokine release syndrome and neurotoxicity (2, 44). These adverse effects limit CAR

541 dosing but could potentially be mitigated by pausing CAR activity such that DARIC products
542 infused in the OFF-state may be subject to pulses of rapamycin induction to drive engraftment
543 and incrementally reduce tumor burden. While the risks of B-cell aplasia following CD19 CAR
544 T cell therapy may be mitigated by immunoglobulin infusions, indefinite elimination of cells
545 expressing AML associated antigens such as CD33, CD123, CLL1/CLEC12A, or CD38 is likely
546 to result in clinically intolerable myelosuppression. Thus, when treating AML, strategies to
547 mitigate hematopoietic toxicity are likely to be a requirement.

548 Individualizing OFF-state ON-state sequencing may tailor therapeutic windows to patient-
549 specific circumstances (45) and represents a useful feature of the DARIC platform. Intermittent
550 cycling of DARIC33 activity through metronomic rapamycin dosing may enable episodic
551 hematopoietic recovery between cycles of active leukemic targeting. While the half-life of
552 rapamycin precludes rapid cessation of DARIC T cell function, pharmacologic inhibitors of
553 proximal antigen receptor signaling, such as dasatinib (46, 47), in combination with rapamycin
554 withdrawal may represent an alternative strategy for managing acute toxicities arising from
555 unrestrained T cell activation. In addition, periods of alternating signaling and quiescence may
556 enhance the efficacy of anti-tumor T cells by preventing T cell exhaustion(48–50) and allowing
557 effector T cells to transition to memory states(29, 30, 51–54) after periods of prolonged antigen
558 exposure. We are currently evaluating transcriptional and epigenetic changes in SC-DARIC33
559 cells following rapamycin interval dosing to analyze the impact of paused T cell activity on T
560 cell memory state transitions. Temporal pauses of CAR activity may therefore be a general
561 method to promote or sustain the fitness of engineered therapeutic T cells.

562 Controllable CAR designs may open new paradigms of CAR T cell therapy that directly
563 address both prevailing failure mechanisms and risks to patients. For example, controllable CAR

564 T cells may allow administration of higher cell doses followed by individualized titration of the
565 activating drugs, widening therapeutic windows (45). In addition, whereas constitutive potency
566 enhanced CARs risk runaway reactivity that may be difficult to bring back under control (55, 56),
567 regulated CAR designs may promote the safety of genetic potency enhancement strategies that
568 attempt to further CAR T cell survival, expansion or reactivity. Finally, if intermittent T cell
569 activation leads to a formation of a long-lived DARIC T cell niche, patients could be re-dosed
570 with rapamycin to control any tumor recurrence following the initial remission. Overall, clinical
571 validation of a controllable CAR T cell designs will impact multiple research questions and
572 clinical outcomes within the cellular therapy field. The on-going first-in-human trial of SC-
573 DARIC33 for children and young adults with relapsed or refractory CD33+ AML will provide
574 clinical and correlative data supporting the pharmacologic control of CAR T products, as well as
575 AML- and myeloid cell- targeting attributes of this next-generation cellular therapeutic.

576

577 **MATERIALS AND METHODS**

578 **Sex as a biological variant**

579 Murine xenograft studies used female mice to minimize size variation. Results are expected
580 to be relevant to all humans.

581 **Cell Lines**

582 Cell lines were obtained from the following sources: MOLM-14 (ACC 777), and MV4-11
583 (ACC 102) were purchased from DSMZ; A549 (CCL-185) and THP1 (TIB-202) were purchased
584 from ATCC; HL-60 was provided by the Bhatia lab (Fred Hutchinson Cancer Research Center,
585 Seattle, WA, USA). HL60, MV4-11 and MOLM14 were engineered to express GFP and firefly
586 luciferase via lentiviral transduction. GFP+ cells were FACS sorted to generate uniformly
587 positive cell population. Raji cells engineered to express GFP and firefly luciferase were further
588 modified to express CD33M (NM_001772) by lentiviral transduction, followed by FACS sorting
589 and limiting dilution cloning to select expression of equivalent CD19 and CD33 antigen
590 concentrations. K562 was transduced with lentivirus to express membrane bound OKT3 as a
591 positive control for in vitro T cell activity assays. HL-60, MOLM14, MV4-11, THP1 cells were
592 cultured in RPMI-1640 supplemented with 10% FBS and 1% L-glutamine, referred to as
593 complete RPMI.

594 **CD33-Targeted DARIC-V_HH Lentiviral Vector Design and Production**

595 DARIC lentiviral vectors were generated as previously described(4). Briefly, transgenes
596 encoding the CD33-specific V_HH binders were synthesized incorporating sequence modifications
597 that optimized codon usage and enhanced human immune tolerance and cloned into the

598 previously described CD19-DARIC transfer plasmid (4) using Gibson cloning (NEB). Cloned
599 products were verified using Sanger sequencing.

600 A four-plasmid self-inactivating lentiviral production system was used. Briefly, the DARIC
601 transfer vectors mixed with envelope and packaging vectors were transfected into 293T cells
602 using TransIt transfection reagent (Mirus Bio). Vector-containing supernatant was collected,
603 passed through a 0.2 micron filter and either used immediately or stored at -80 until use. In some
604 cases, vector supernatant was concentrated by centrifugation at 10,000g x 4 hours prior to
605 cryopreservation. Analysis of Virus Copy Number (VCN) was performed as described
606 previously (4).

607 **DARIC T Cell Manufacture**

608 Thawed PBMCs were resuspended in TCGM supplemented with 250 IU/ml recombinant
609 human IL-2 (Stemcell, catalog 78220.3) prior to activation with 50ng/ml anti-CD3 (clone OKT3)
610 and anti-CD28 (clone 15E8) antibodies (Miltenyi Biotec). Lentivirus supernatants were added to
611 PBMC cultures 24 hours later (multiplicity of infection [MOI] = 10). 72 hours after activation,
612 transduced PBMC were collected, washed, and resuspended in complete TCGM with human IL-
613 2 at 0.5×10^6 cells/ml and transferred to gas permeable culture vessels (G-REX, WilsonWolf).
614 PBMC cultures were expanded in vitro at cell density of $0.5-2 \times 10^6$ cells/ml maintained by the
615 addition of fresh media every 2-3 days for a total of 10-11 days until cryopreservation. Clinical T
616 cell product manufacture was completed as essentially as described (57), except T cell cultures
617 were initiated with a 1:1 ratio of CD4 and CD8 T cells.

618 **Cytokine Release Assay**

619 For cytokine production analysis, 0.1×10^6 T cells were cocultured with 5×10^4 target cells
620 (effector:target ratio = 2:1) for 24 hours with or without rapamycin (1 nM, unless otherwise
621 specified) in TCGM. Culture supernatants were evaluated using the V-PLEX Proinflammatory
622 Panel 1 Human kit (Meso Scale Diagnostic) and analyzed by the MESO QuickPlex SQ 120
623 Instrument (Meso Scale Diagnostic) according to the manufacturer's instructions.

624 **Murine Xenograft Models**

625 Female adult (8-12 week-old) NOD/Scid IL-2R^{null}(NSG) mice were bred in house or
626 purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen free
627 conditions with as 12-hour light/dark cycle and monitored daily by veterinary staff or research
628 scientists. All experiments were carried out following Institutional Animal Care and Use
629 Committee (IACUC) approved protocols. Mice exhibiting hunched posture, decreased mobility
630 that impaired feeding, single or multiple tumors totaling >1cm in diameter, >20% weight loss or
631 loss of skin integrity were humanely euthanized. Development of tumor associated symptoms
632 requiring euthanasia was considered an event for the purposes of Kaplan Meier analyses.

633 Cultures of tumor cells modified for bioluminescence imaging (BLI) were washed and
634 resuspended in phosphate buffered saline (PBS). Cell densities were adjusted to contain the
635 following cell doses within 200uL as follows: HL60 (5×10^6), MV4-11 (1×10^6), MOLM14 ($1 \times$
636 10^5), or Raji (0.5×10^6). Tumor cell suspensions were administered via lateral tail vein injection.

637 BLI was performed by intraperitoneal or subcutaneous injection of 4.29mg per mouse of D-
638 luciferin (Xenogen) at various timepoints prior to and after tumor inoculation. Prior to treatment,
639 mice were distributed so that treatment groups had similar median bioluminescence. Mice

640 exhibiting tumor signal only within the tail were excluded from studies. Imaging of isoflurane
641 anesthetized mice occurred 15min after D-luciferin injection using the IVIS Spectrum Imaging
642 System (PerkinElmer). Luciferase activity was analyzed using Living Image Software version
643 4.5.2 (PerkinElmer).

644 Prior to administration to recipient mice, cryopreserved T cell products were thawed into
645 human AB serum, washed with PBS, counted, and resuspended in PBS such that a single dose of
646 T cells was administered in a total volume of 200uL. Cells suspensions were maintained on ice
647 until injection via the lateral tail vein of recipient mice. Mice received a single injection of cells.
648 To determine cell dosing, the total number of DARIC33 T cells and CD19 DARIC T cells
649 administered to mice were calculated on the basis of FRB+ cells, i.e. $total\ cells = desired\ cell$
650 $dose / (proportion\ FRB+)$. Similarly, the total number of CD19 CAR T cell products were
651 determined on the basis of the proportion of cells expressing the EGFRt marker(58). The total
652 cell dose of untransduced (UTD) control T cell products was matched to highest total number of
653 T cells administered within a given experiment.

654 Mice assigned to rapamycin treatment received rapamycin by intraperitoneal injection either
655 daily or every Monday, Wednesday, and Friday as specified within schemas and/or figure
656 legends. Solutions of rapamycin for injection were prepared by dilution of a 10mM DMSO stock
657 into PBS immediately prior to administration such that final concentration of DMSO was < 0.2%
658 (v/v). For weight-based dosing, mouse weights were determined weekly and used to adjust
659 rapamycin dosing. The total volume of rapamycin solution administered ranged from 50uL to
660 200uL.

661 **Statistical Analysis**

662 Statistical significance was determined by a P value of < 0.05 using GraphPad Prism 9
663 software or the lme4 package of the R statistical computing package. Tumor symptom free
664 survival of mice within studies were compared using Kaplan Meier method and the log rank test.
665 Global comparisons for studies with more than 2 groups were conducted, and if significant,
666 pairwise comparisons were examined using a false discovery rate of 0.05. Logistic dose response
667 curves were evaluated in GraphPad Prism 9.

668 **Evaluation of blood, serum and chloroma samples from patients**

669 PLAT08 is an ongoing phase 1 study of CD4⁺ and CD8⁺ T cells lentivirally transduced to
670 express the DARIC33 transgene, delivered via intravenous infusion following lymphodepleting
671 chemotherapy in pediatric and young adult patients (<30 years old) with relapsed or refractory
672 acute myeloid leukemia (NCT050105152). The study is conducted in accordance with FDA and
673 international conference on harmonization guidelines for good clinical practice, the declaration
674 of Helsinki and applicable institutional review board guidelines (study protocol approved by
675 Seattle Children's Institutional Review Board). All patients or their guardians provided written
676 informed consent for trial participation. Written informed consent was received for the use of
677 photographs and the record of informed consent has been retained at Seattle Children's.
678 Following enrollment, CD4⁺ and CD8⁺ T cells isolated from cells collected by leukopheresis
679 were combined in a 1:1 ratio to manufacture SC-DARIC33 as described (57). Freshly obtained
680 blood, marrow, or chloroma samples from patients following SC-DARIC33 infusion were
681 evaluated by immunophenotyping following RBC lysis using standard staining and flow
682 cytometry techniques (see Supplementary Methods for additional details).

683 **Data and materials availability**

684 Requests for materials will be fulfilled following requests to corresponding authors and
685 completion of appropriate material transfer agreements. High throughput sequencing data
686 (RNAseq) has been deposited in the Genome Expression Omnibus at NCBI, accession
687 #GSE255002. Code used to evaluate CD33 splicing from sequence read archives will be made
688 available following request to A.A. Supporting Data Values associated with each figure is
689 provided in a supplemental spreadsheet.

690 See *supplementary materials* for additional study details and descriptions.

691

692 **List of Supplementary Materials**

693 Supporting Data Values

694 Supplemental Materials and Methods

695 Fig S1 to S10

696 Table S1 to S5

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834

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845 **Author contributions:**

846 Conceptualization: JSA, AEP, KO, GT, MF, JJ, MP, JAG, AA, MCJ

847 Methodology: JSA, AP, KO, GT, MF, MH, DEZ, SRR, NT, SH, MP, JAG, AA

848 Investigation: KO, JZ, WHL, UM, ARK, DX, PPLS, SKH, CE, SS, RL, PL, MF,

849 RAC, SS, KJ, AS, WC, JT, AH, BE, SB, JW, SRR, NT

850 Visualization: JSA, AA, SRR, NT, DEZ

851 Funding acquisition: JSA, MCJ, JJ, PDG

852 Project administration: JSA, JJ, JAG, MCJ

853 Supervision: JSA, JJ, JAG, AA, MCJ

854 Writing – original draft: JSA, AA

855 Writing – review & editing: JSA, JJ, MP, PDG, JAG, AA, MCJ

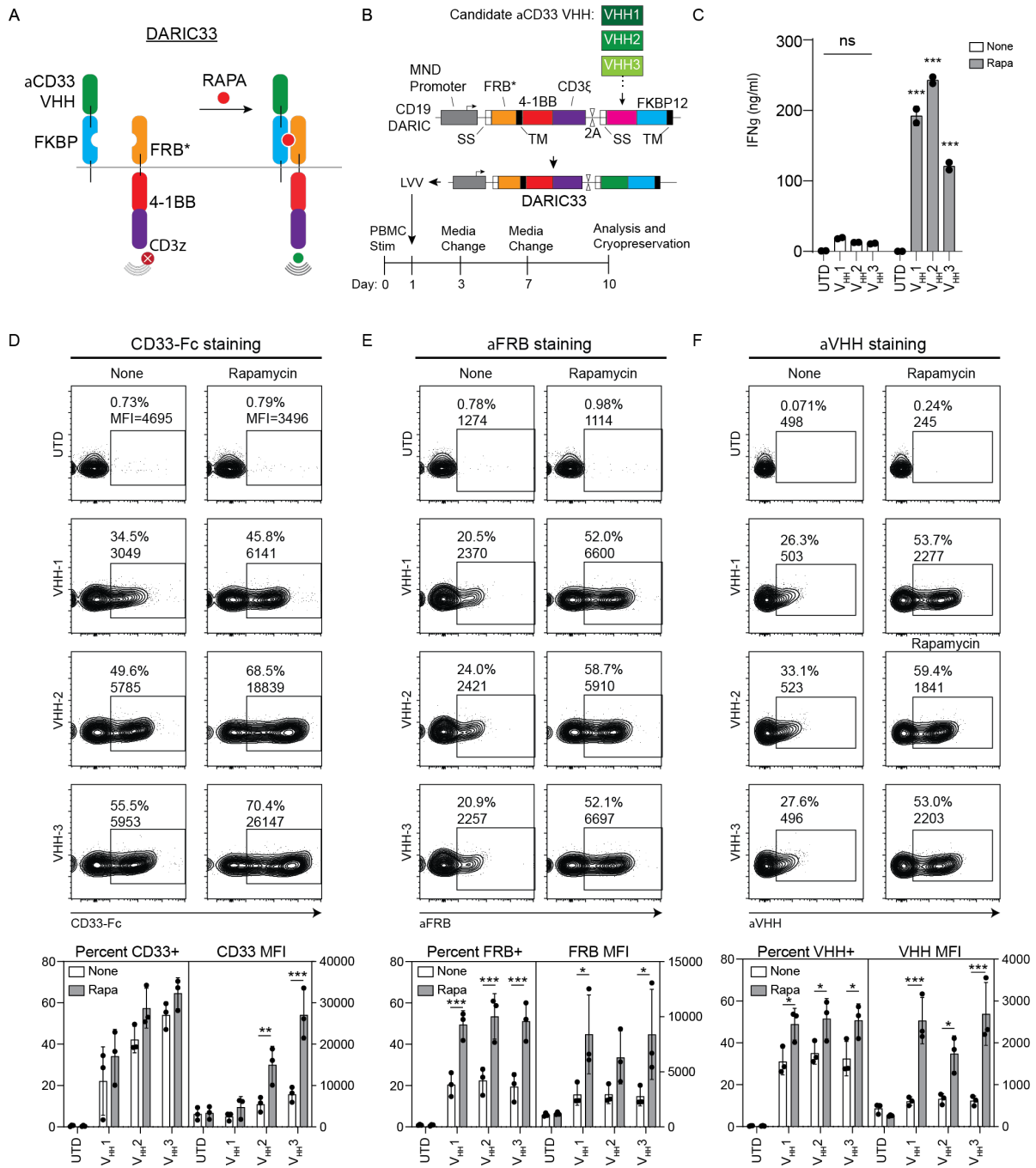
856 **Competing interests:** WHL, AA, MP and JJ are holders of patents related to DARIC. MCJ is a

857 holder of patents related to CD19 CAR T cells. AEP, JZ, WHL, DX, PPLS, SKH, UM,

858 ARK, DEZ, PL, PDG, JJ, MP and AA are current or former employees of 2seventy

859 bio and own equity in 2seventy bio.

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863 **Figure 1. Rapamycin licenses antigen dependent DARIC33 T cell responses and stabilizes**

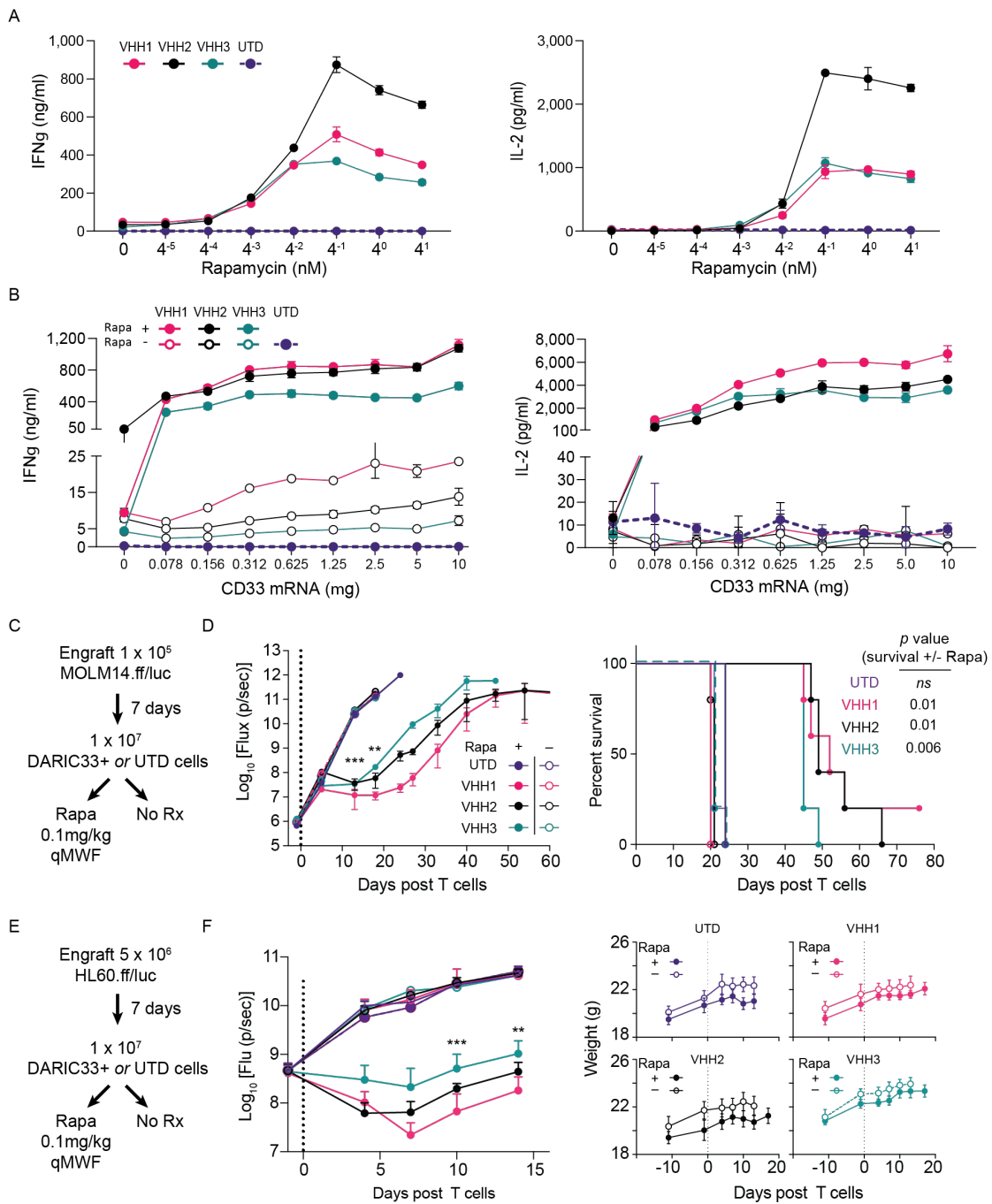
864 **surface expression of DARIC33 components. (A) Schematic depicting rapamycin (rapa)**

865 dependent activation of DARIC33. In the absence of rapa, the two DARIC components are split
866 and do not respond to antigen. Following rapa addition, heterodimerization of DARIC
867 components enables antigen dependent T cell responses. **(B)** Schematic depicting generation of
868 DARIC33 candidates and T cell production. DNA sequences encoding modified VHH sequences
869 are incorporated into DARIC33 lentiviral expression vectors. **(C)** IFN γ release by DARIC33 cell
870 products following coculture with CD33+ MV4-11 AML cells. One of $n = 3$ donors shown.
871 **** $p < 0.0001$, ANOVA with Tukey's multiple comparison correction. **(D-F)** Rapamycin
872 stabilizes surface expression of DARIC33 components. DARIC33 cell products were cultured in
873 media or media containing 1nM rapa overnight prior to staining and evaluation by flow
874 cytometry. Representative flow cytometry plots from one of three donors (*above*) with
875 quantitation of %pos and median fluorescence intensity from all three donors (*below*) *** $p <$
876 0.001, ** $p < 0.01$, * $p < 0.05$, 2-way ANOVA with Sidak's multiple comparison correction, $n =$
877 3 donors. **(D)** Rapa increases antigen binding capacity of DARIC33 cells. **(E)** Rapa increases
878 surface expression of the antigen signaling arm of DARIC. **(F)** Rapa increases surface expression
879 of the antigen recognition arm of DARIC.

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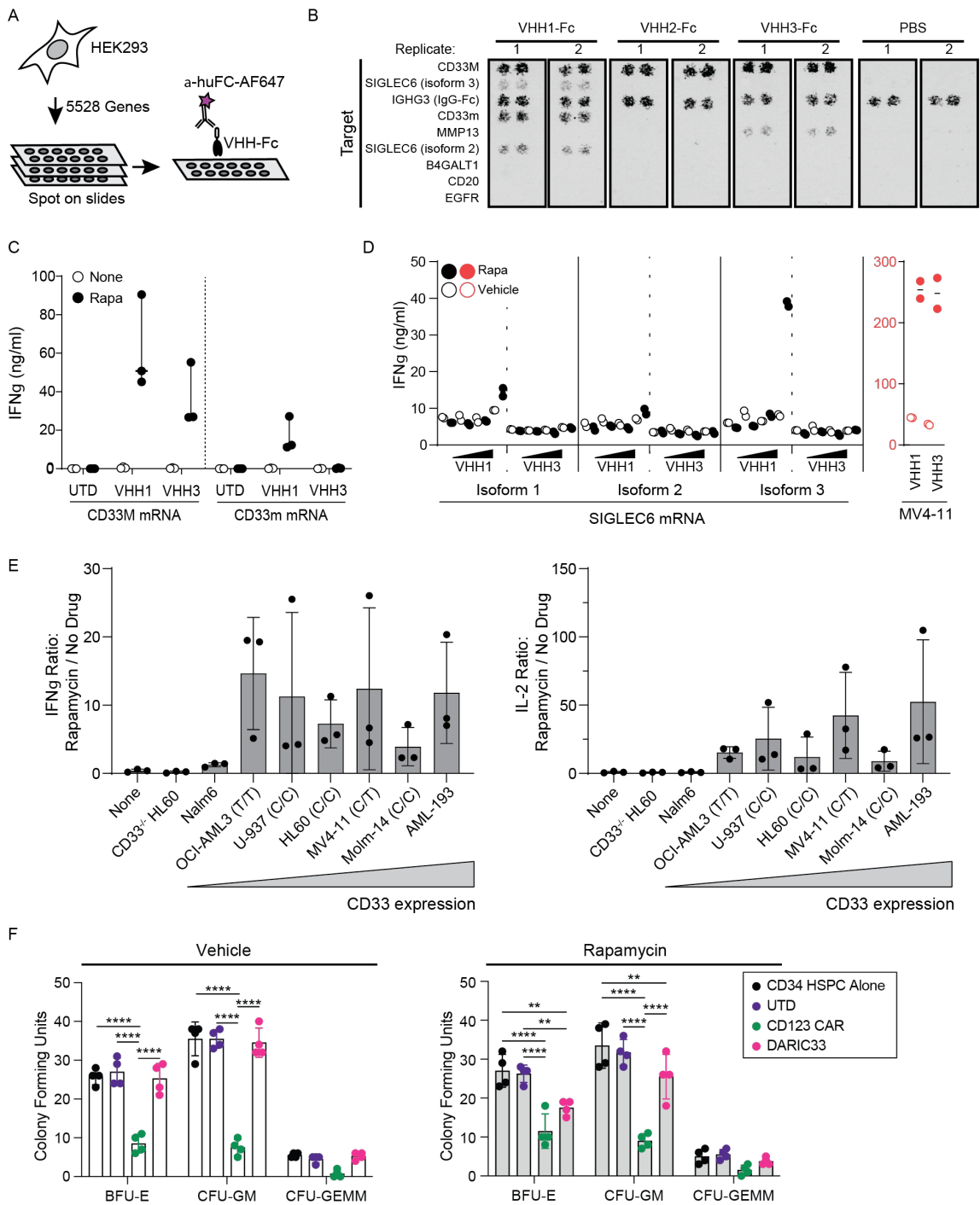
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884 **Figure 2. DARIC33 stimulated T cell responses require low levels of target antigen and low**
 885 **concentrations of rapamycin. (A) Cytokine release by DARIC33 cells following coculture with**
 886 **MV4-11 AML target cells in the presence of increasing concentrations of rapa. IFN γ is shown**

887 *left*, IL2 is shown *right*. **(B)** Cytokine release by UTD (control) or DARIC33 cells following
888 coculture with or without rapa and HEK293 T cells electroporated with increases amounts of
889 CD33 mRNA. **(C-D)** 10^7 DARIC33+ cells, or an equivalent number of UTD control cells were
890 infused intravenously (IV) in NSG mice 7 days after engraftment of 1×10^5 MOLM14.ff/luc
891 leukemia cells per animal. Following T cell infusion, mice were treated 3 times per week with
892 0.1mg/kg rapamycin or were observed. **(D)** Quantification of tumor growth by BLI *left*; mean +
893 s.e.m., $n = 5$ mice per group (*left*) and symptom-free survival (*right*) with comparisons by
894 Mantel-Cox (Log-rank) test. **(E-F)** 10^7 DARIC33+ cells, or an equivalent number of UTD
895 control cells were infused intravenously (IV) in NSG mice 7 days after engraftment of 5×10^6
896 HL-60.ff/luc leukemia cells per animal. Following T cell infusion, mice were treated 3 times per
897 week with 0.1mg/kg rapamycin or were observed. **(F)** Quantification of tumor growth by BLI,
898 *left*, mean + s.e.m., $n = 5$ mice per group and mouse weight (*right*). Time points where all
899 DARIC33 formats meet the p -value threshold when compared to UTD cells + rapa (panels *D* and
900 *F*) are indicated as *** $p < 0.001$, ** $p < 0.01$, , using repeated measures ANOVA with
901 Dunnett's multiple comparison correction.
902



903

904 **Figure 3. DARIC33 is specific for CD33 antigen and does not inhibit HSPC colony**

905 **formation. (A-B) Evaluation of CD33-specific V_HH-Fc fusion proteins used in DARIC33**

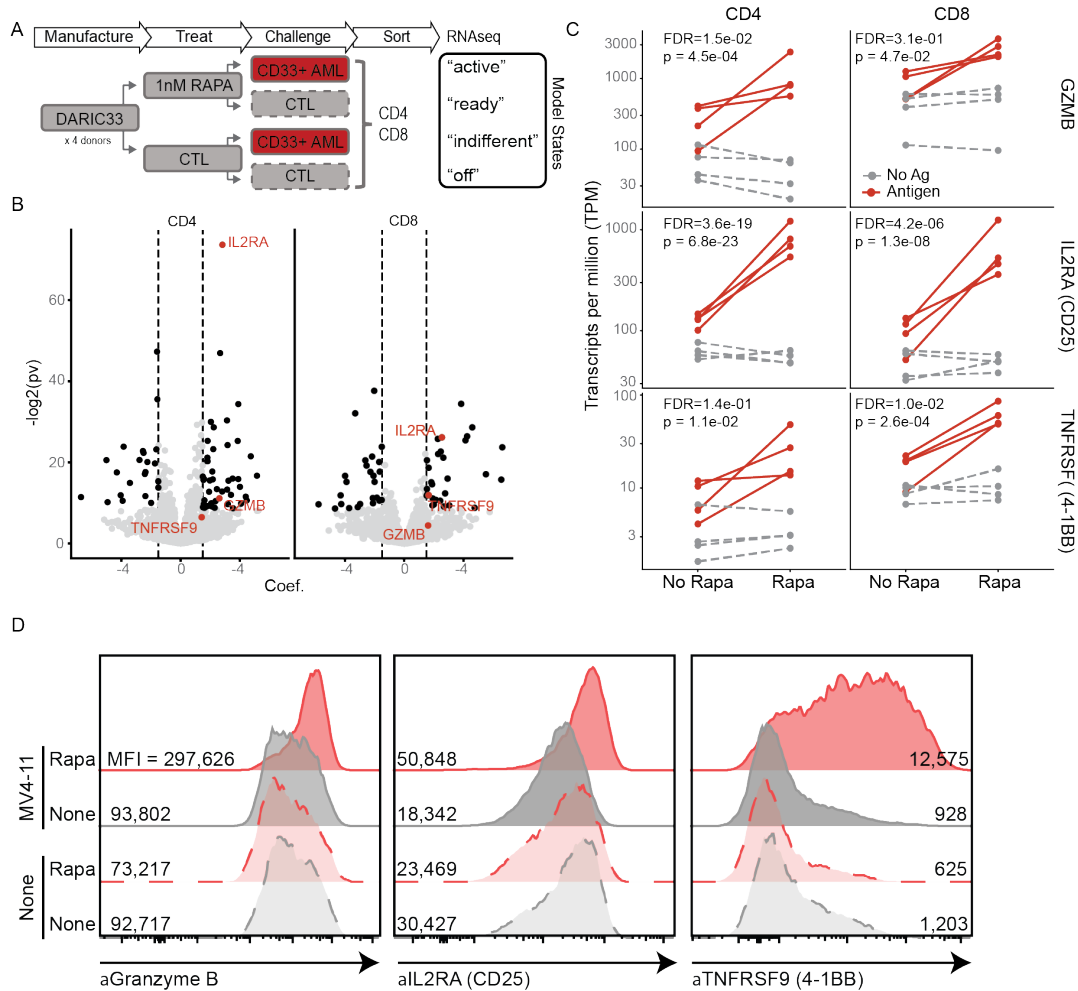
906 **designs. (A) Schematic depicting detection strategy of V_HH-Fc fusions binding to HEK293 cells**

907 expressing one of 5,528 surface-bound or secreted proteins. Following reverse transfection
908 HEK293 cells are spotted onto slides then stained with V_HH-Fc proteins (or PBS a control) and
909 alexa647-labeled anti-human-Fc secondary antibodies. **(B)** Secondary screen of selected hit and
910 control transgenic HEK293 samples ($n = 2$ replicates shown). **(C)** Stimulation of T cell IFN γ
911 release by DARIC33 designs in the presence of rapa following exposure to HEK293 cells
912 electroporated with mRNA encoding CD33M (*left*) and CD33m (*right*). **(D)** Stimulation of T cell
913 IFN γ release by DARIC33 designs in the presence of rapa following exposure to HEK293 cells
914 electroporated with mRNA encoding Siglec6 (*left*). Release of IFN γ following coculture of
915 DARIC33 with MV4-11 AML cells is shown for comparison (*right*). **(E)** Correlation of CD33
916 density (expressed as the logarithm of the antigen binding capacity) with release of IFN γ (*left*)
917 and IL-2 (*right*). **(F)** Colony forming units following culture of CD34⁺ cells alone or with T cells
918 in the presence or absence of rapa. Colonies were enumerated after 14 days of growth. $n = 2$ T
919 cell donors. **** $p < 0.0001$, ** $p < 0.01$, ANOVA with Tukey's multiple comparison
920 correction.

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924

925 **Figure 4. DARIC33 stimulates T cell transcriptional responses in the presence of antigen**

926 **and rapamycin and without hallmarks of tonic signaling. (A-D) DARIC33 cells derived from**

927 $n = 4$ healthy donors were incubated with 1nM rapamycin or media alone prior to culture alone

928 or with CD33+ MV4-11 AML target cells. Following coculture, CD4+ and CD8+ cells were

929 sorted and evaluated by RNA-Seq. (A) Schema for the experiment. DARIC33 cells resting in the

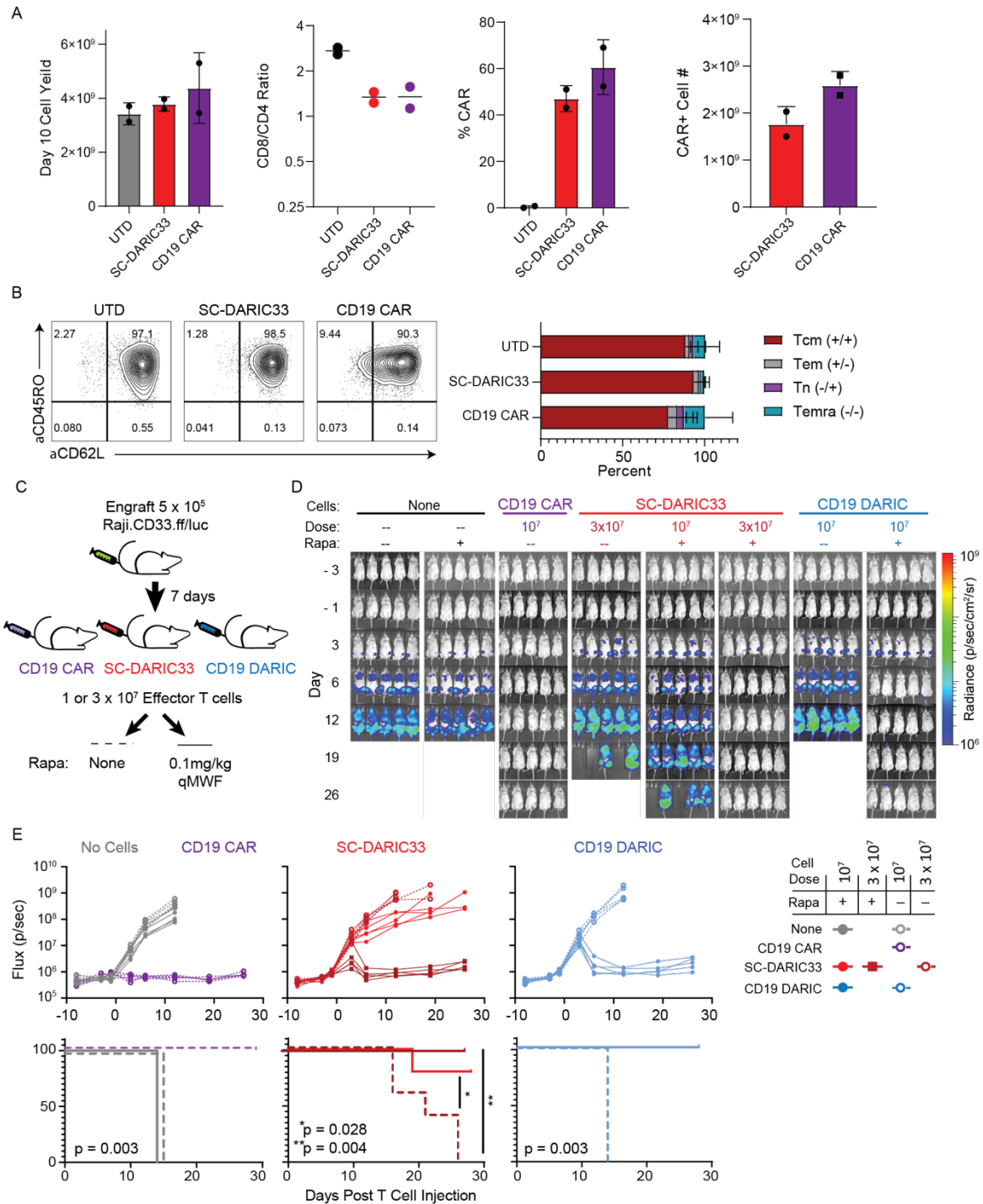
930 absence of rapamycin or antigen are considered 'off', whereas DARIC33 cells incubated in rapa

931 without antigen and with antigen exposure are labeled 'ready' and 'active' respectively. (B)

932 Transcriptional responses among selected genes associated with early T cell activation. (C)

933 Volcano plot of the magnitude of statistical significance (y -axis) vs magnitude of rapamycin and

934 antigen (e.g. 'DARIC active') effect (x -axis, labeled 'Coef.' in the figure). GZMB, IL2RA, and
935 TNFRSF9 are shown in red, additional genes exhibiting significant 'DARIC active' regulation
936 are shown in black, with more detail provided in a heatmap shown in Fig S5. **(D)** Flow
937 cytometric confirmation that transcriptional changes are reflected in protein abundance. Median
938 fluorescence intensity (MFI) for each sample is shown.



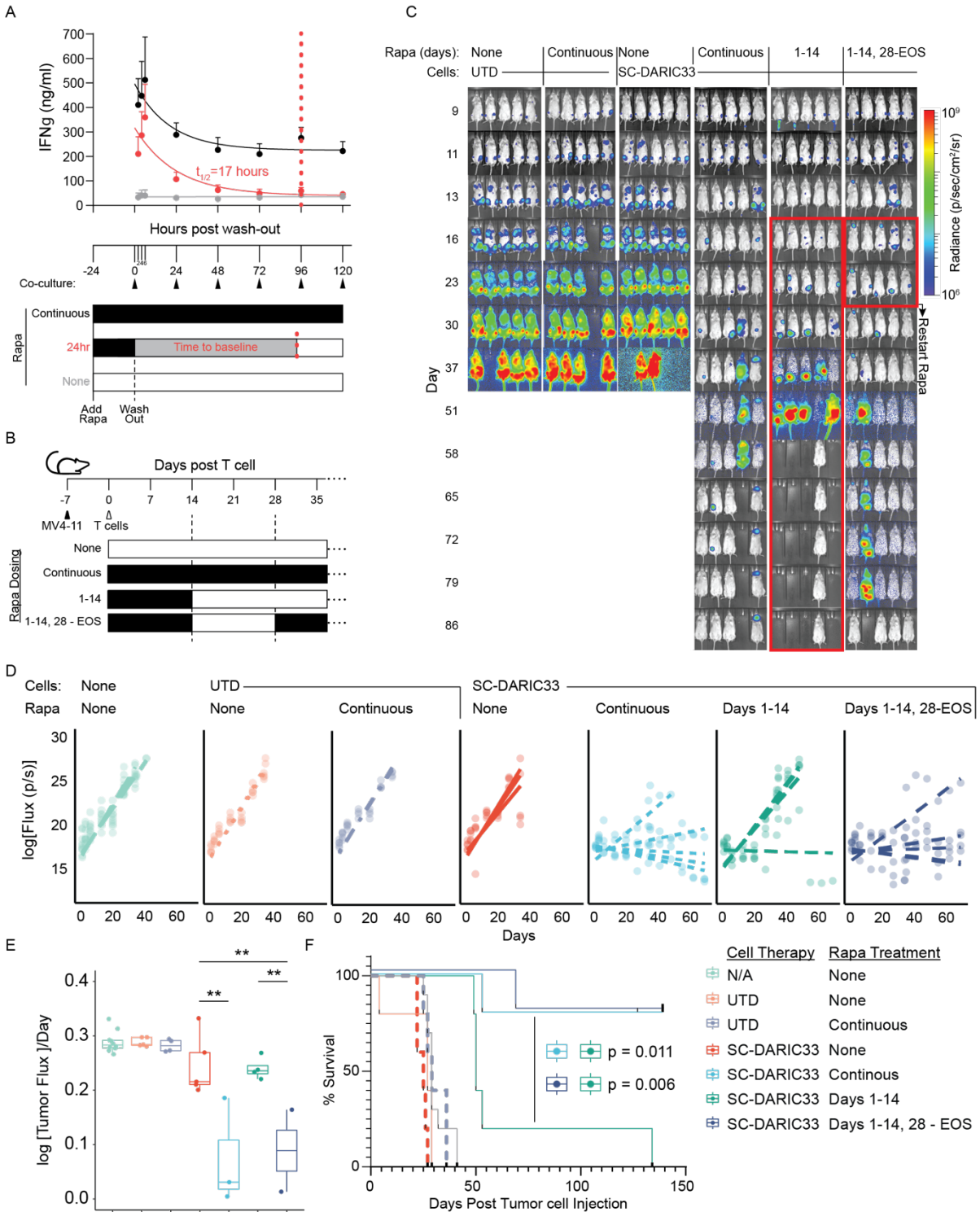
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940 **Figure 5. Clinically appropriate manufacture of donor matched DARIC33 and CD19 CAR**

941 **allows comparisons of manufacture feasibility and cell potency. (A) Yields of UTD, CD19**

942 **CAR and DARIC33 cell products following manufacture using reagents and techniques**

943 appropriate for clinical application from $n = 2$ donors. **(B)** Surface expression of CD45RO and
944 CD62L of clinical cell product facsimiles. Representative flow plot is shown *left*, with
945 quantitation from $n = 2$ donors shown *right* (stacked bars indicate mean \pm sd). **(C-E)** $1-3 \times 10^7$
946 DARIC33+ cells, CD19 CAR T cells, CD19 DARIC+ cells or an equivalent number of UTD
947 control cells were infused intravenously (IV) in NSG mice 7 days after engraftment of 5×10^5
948 Raji.CD33.ff/luc leukemia cells. Following T cell infusion, mice were treated with 0.1mg/kg
949 rapa 3 times weekly for the indicated durations or were observed. **(C)** Schematic depicting
950 experimental design. To compare cell potency with benchmark immunotherapy products, two
951 doses of DARIC33+ cells were used. **(D)** Tumor progression monitored by bioluminescence, $n =$
952 5-8 mice per group. **(E)** Quantitation of tumor growth (*above*), with points representing
953 measurements of individual mice. Kaplan Meier survival estimates (*below*), log-rank test p
954 values.
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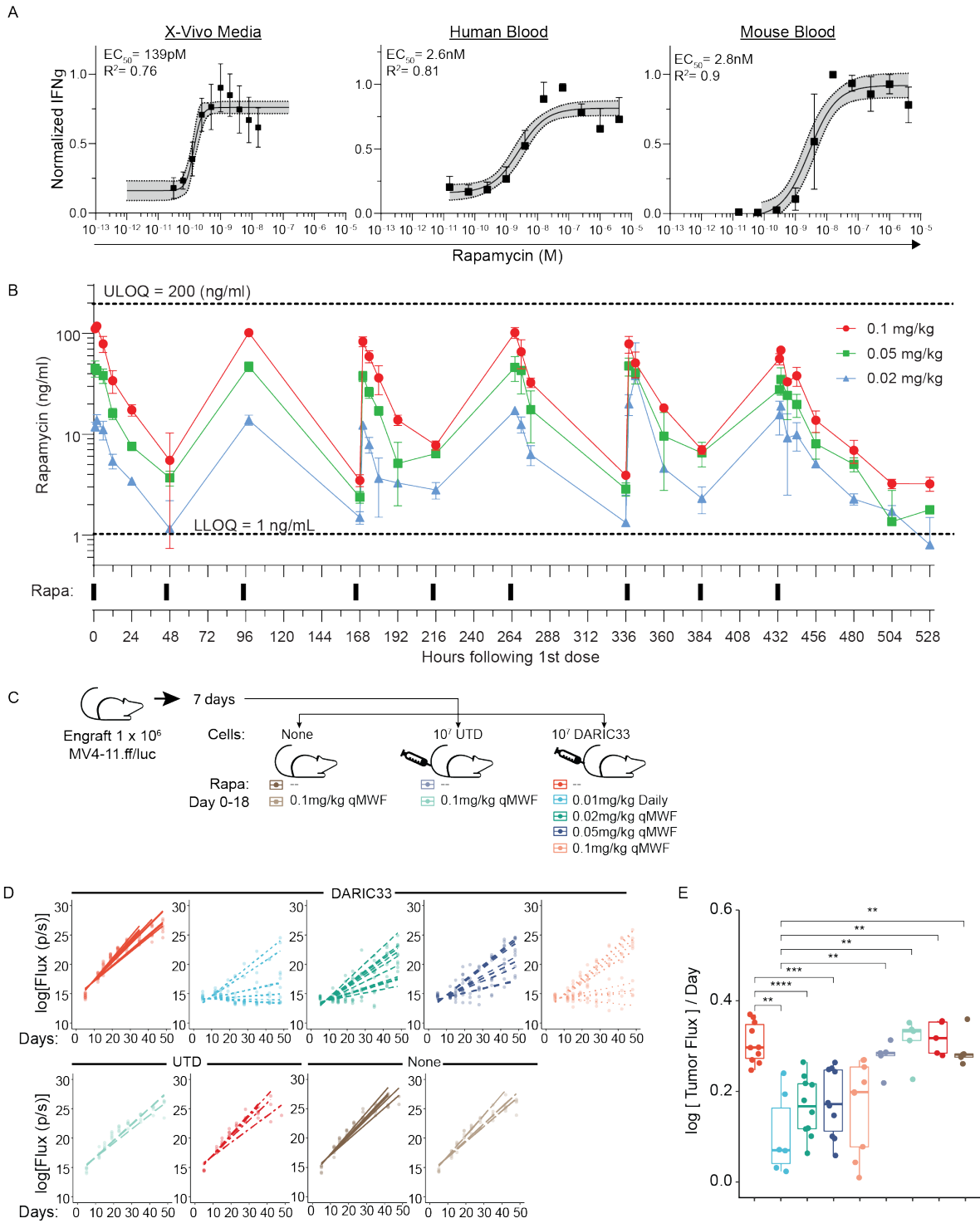


956

957 **Figure 6. Activation of SC-DARIC33 is reversible. (A)** DARIC33 cell cytokine responses to

958 antigen at various times following wash out from rapa containing media. DARIC33 cells

959 replaced into rapa containing media or DARIC33 cells previously cultured in media not
960 containing rapa were used as comparators. The $t_{1/2}$ is determined by fitting a single-phase
961 exponential decay. **(B-F)** 10^7 SC-DARIC33+ cells, or an equivalent number of UTD control cells
962 were infused intravenously (IV) in NSG mice 7 days after engraftment of 1×10^6 MV4-11.ff/luc
963 leukemia cells. Following T cell infusion, mice were treated with 0.1mg/kg rapa 3 times weekly
964 for the indicated durations or were observed. **(C)** Tumor progression monitored by
965 bioluminescence, $n = 5$ mice per group. Images taken during a 'pause' in rapa dosing are
966 outlined in red. **(C)** Quantitation of tumor growth. Points are measurements of individual mice,
967 best-fit tumor growth trajectories (see supplemental methods). **(D)** Tumor growth rates. Points
968 are growth rates fit for individual mice, box and whiskers show mean and standard deviation,
969 asterisks indicate ** $p < 0.01$, t -tests, with Benjamini-Hochberg correction for multiple
970 comparisons. **(E)** Survival after infusion of DARIC33 cells or UTD cells following by treatment
971 with various rapa schedules. Mantel-Cox log-rank p values are shown uncorrected.
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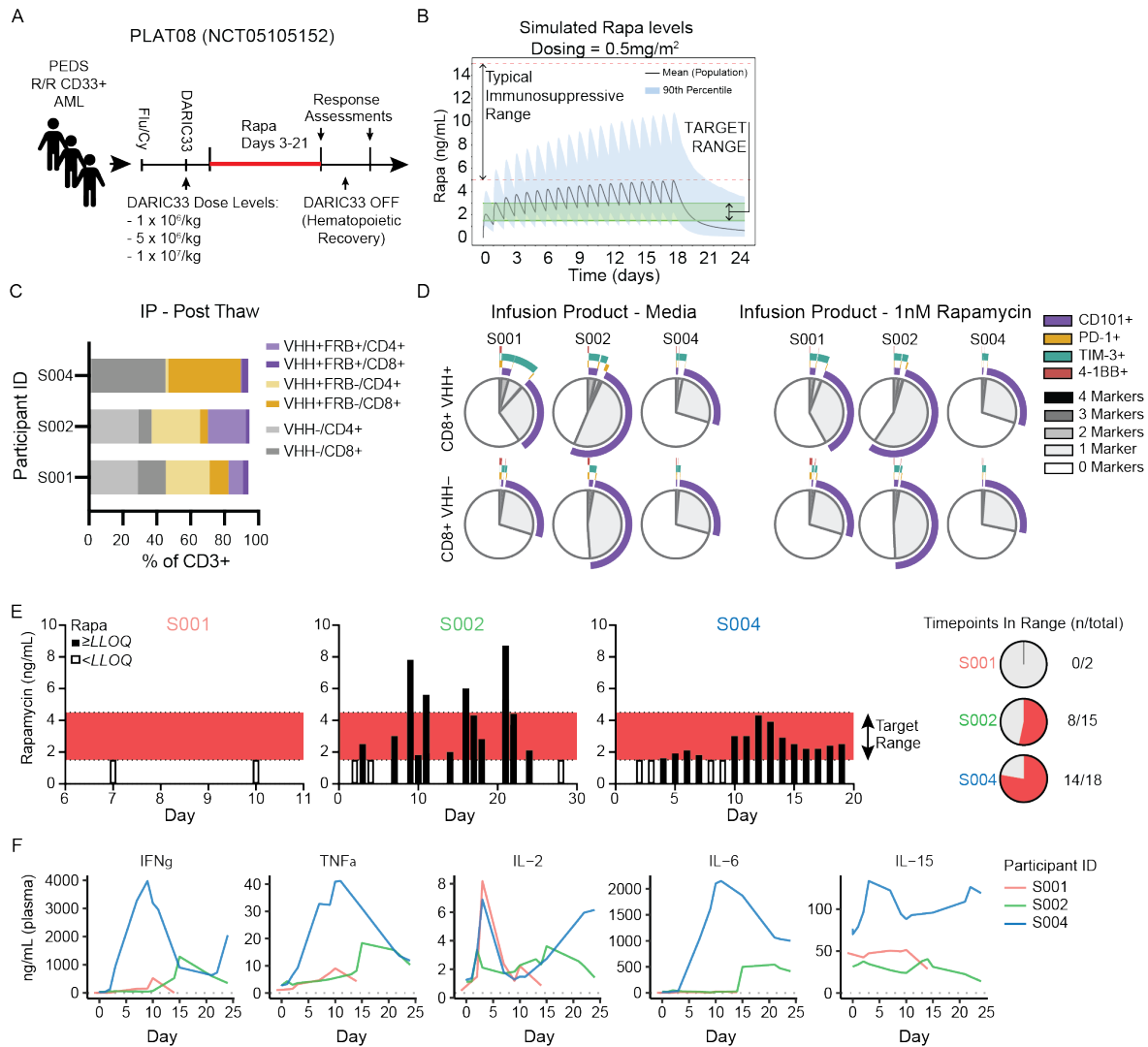
Figure 7. In vitro modeling of SC-DARIC33 rapamycin response allows targeted

975

rapamycin dosing in vivo. (A) Cytokine release following stimulation of DARIC33 cells with

976 MV4-11 AML cells in media or whole blood in the presence of increasing rapamycin
977 concentrations. IFN γ responses are normalized per donor and apparent EC50s determined using a
978 four-parameter logistic dose response curves are reported. **(B)** Determination of rapamycin
979 pharmacokinetics in mice. Concentrations of rapa in whole blood obtained during administration
980 of various rapa doses 3 times weekly are shown above, along with the timing of IP rapa
981 injections, *bars, below*. Upper limit of quantitation (ULOQ = 200ng/mL) and lower limit of
982 quantitation (LLOQ = 1ng/mL) are indicated. **(C-D)** AML tumor progression in mice following
983 treatment with DARIC33 and various dose schedules of rapa days 0-18 post T cell infusion. **(C)**
984 Schematic illustrating experimental design. **(D)** Quantitation of tumor growth kinetics. Points
985 represent bioluminescence measures of individual mice ($n = 5-10$ per group) and lines indicate
986 tumor growth trajectories modeled using linear mixed effects. **(E)** Modeled tumor growth rates
987 (slopes of lines in *D*). Points are growth rates modeled for individual mice, box and whiskers
988 show mean and standard deviation (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, *t*-tests with
989 Benjamini-Hochberg correction for multiple comparisons.).

990



991

992 **Figure 8. Clinical SC-DARIC33 exhibits activity in patients following accurate targeting of**

993 **rapamycin levels. (A)** PLAT08 clinical treatment schema. After SC-DARIC33 manufacturing,

994 subjects receive lymphodepletion fludarabine and cyclophosphamide (Flu/Cy) and SC-DARIC33

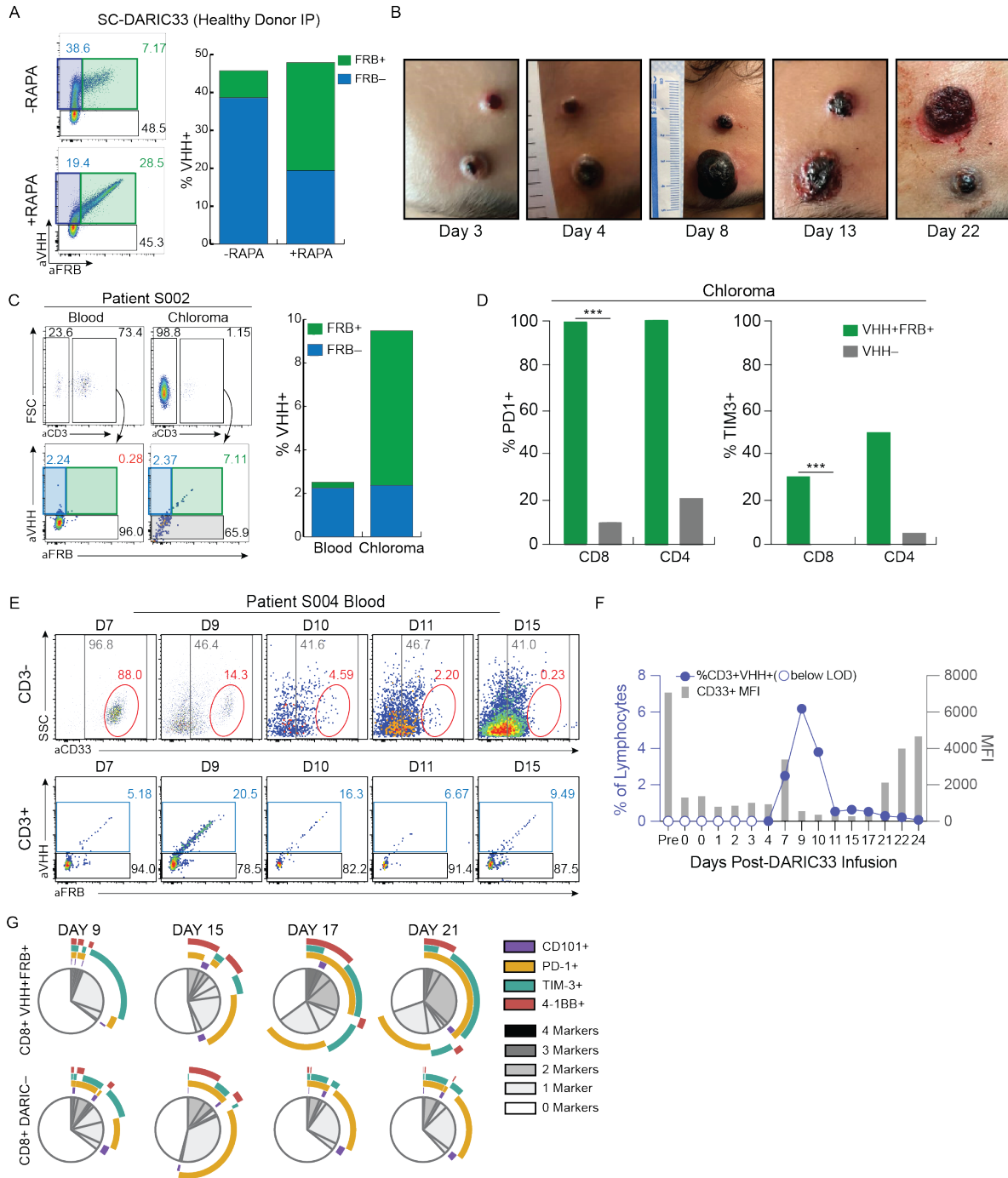
995 at one of three assigned dose levels on day 0. Rapamycin is administered on days 3-21. Bone

996 marrow biopsies are conducted for response assessments on days 21 and 28. **(B)** Simulated

997 serum rapamycin concentrations using population pharmacokinetic modeling. Daily

998 administration of 0.5mg/m² rapamycin achieves trough concentrations above the target range for

999 SC-DARIC33 activation and peak concentrations below immunosuppressive doses of rapamycin
1000 for most pediatric subjects. (C) Characteristics of thawed clinical SC-DARIC33 cell products
1001 administered to trial participants. The proportion of cells expressing surface DARIC components
1002 as assessed by flow cytometry are shown. (D) Expression of activation markers by clinical
1003 infusion cell products following overnight culture in media alone or media supplemented with
1004 1nM rapamycin. (E) Frequent re-evaluation enables successful targeting of serum rapamycin
1005 levels in patients. The proportion of timepoints (both peak and trough levels) within the target
1006 range (1.5 – 4ng/mL) are shown on the right. (F) Elevation of serum cytokines associated with T
1007 cell activation is observed following administration of SC-DARIC33. Traces show cytokine
1008 levels for samples obtained from each patient. Values reported are the mean of $n = 2$ replicates.
1009



1010

1011 **Figure 9. Clinical activity of rapamycin-activated SC-DARIC33 in patients.** (A) Expression

1012 of FRB by SC-DARIC33 is correlated with rapamycin exposure. SC-DARIC33 manufactured

1013 from a healthy donor was cultured overnight in media alone or media supplemented with 1 nM

1014 rapamycin. The proportion of VHH+ and FRB+ cells are shown in the bar graph. Note the
1015 rightward shift of VHH+ cells following rapamycin exposure. **(B)** Progressive inflammatory
1016 changes and hemorrhagic conversion of a chloroma following administration of SC-DARIC33 to
1017 subject S002. Samples from chloroma tissues are shown in panels C and D. Photographs with
1018 permission. **(C)** Rapamycin-activated FRB+ DARIC33 T cells are expanded within chloroma
1019 tissue. Paired blood and chloroma tissue from patient S002 were evaluated by flow cytometry. T
1020 cells expressing CD3 were analyzed for VHH and FRB expression. The proportion of VHH+ and
1021 FRB+ cells among CD3+ cells are shown in the bar graph. **(D)** Rapamycin-activated DARIC33
1022 cells within chloroma tissue obtained from patient S002 express increased markers of activation
1023 including PD1 and TIM3. The proportion of either VHH+FRB+ cells (green bars) or VHH- cells
1024 (grey bars) expressing PD1 or TIM3 are shown (***) $p < 0.001$, chi square with Bonferroni
1025 correction for multiple tests). **(E)** Peripheral blood from patient S004 shows concurrent
1026 expansion of DARIC33 cells and reduction of CD33^{hi} cells. **(F)** Quantification of antigen
1027 abundance, as measured by MFI, and expansion of SC-DARIC33 cells within blood samples.
1028 Peak SC-DARIC33 expansion is followed by decreased CD33 antigen expression. **(G)**
1029 Expression of activation/exhaustion markers by rapamycin-activated SC-DARIC33 cells, as
1030 assessed by flow cytometry. Boolean gating results are shown are pie graphs with overlapping
1031 arcs indicating multi-antigen expression. At later time points (days 17 & 21), expression of
1032 activation markers is increased among VHH+FRB+ cells.
1033