# Anti-CCR9 Chimeric Antigen Receptor T cells for T Cell Acute Lymphoblastic Leukemia

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**Abstract:**
T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of immature T lymphocytes, associated with higher rates of induction failure in comparison to B-ALL. The potent immunotherapeutic approaches applied in B-ALL, which have revolutionized the treatment paradigm, have proven more challenging in T-ALL, largely due to a lack of target antigens expressed on malignant but not healthy T cells. Unlike B cell depletion, T cell aplasia is highly toxic. Here, we demonstrate that the chemokine receptor CCR9 is expressed in >70% of cases of T-ALL, including >85% or relapsed/ refractory disease, and only on a small fraction (<5%) of normal T cells. Using cell line models and patient-derived xenografts, we show chimeric antigen receptor (CAR)-T cells targeting CCR9 are resistant to fratricide and have potent anti-leukemic activity both *in vitro* and *in vivo*, even at low target antigen density. We propose anti-CCR9 CAR-T cells could be a highly effective treatment strategy for T-ALL, avoiding T cell aplasia and the need for genome engineering that complicate other approaches.

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## KEY POINTS:

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1. CCR9 is expressed on the majority of cases of T-ALL, but not on normal T cells or other essential tissues
2. Anti-CCR9 CAR-T were highly potent against T-ALL *in vitro* and *in vivo*

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## ABSTRACT:

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T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of immature T lymphocytes, associated with higher rates of induction failure in comparison to B-ALL. The potent immunotherapeutic approaches applied in B-ALL, which have revolutionized the treatment paradigm, have proven more challenging in T-ALL, largely due to a lack of target antigens expressed on malignant but not healthy T cells. Unlike B cell depletion, T cell aplasia is highly toxic. Here, we demonstrate that the chemokine receptor CCR9 is expressed in >70% of cases of T-ALL, including >85% or relapsed/ refractory disease, and only on a small fraction (<5%) of normal T cells. Using cell line models and patient-derived xenografts, we show chimeric antigen receptor (CAR)-T cells targeting CCR9 are resistant to fratricide and have potent anti-leukemic activity both *in vitro* and *in vivo*, even at low target antigen density. We propose anti-CCR9 CAR-T cells could be a highly effective treatment strategy for T-ALL, avoiding T cell aplasia and the need for genome engineering that complicate other approaches.

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## INTRODUCTION

T-acute lymphoblastic leukemia (T-ALL) is an aggressive cancer arising from the malignant transformation of immature T cell precursors. It accounts for approximately 15% and 25% of cases of ALL in children and adults[1] respectively, and typically presents with leucocytosis or cytopenia(s), with frequent extra-medullary manifestations including central nervous system (CNS) infiltration and a mediastinal mass. Treatment is with multi-agent cytotoxic chemotherapy[2]. Historically, outcomes have been worse than for patients with B-ALL, but with contemporary, minimal residual disease (MRD)-directed approaches[3], survival in children with B- and T-ALL is now similar, with cure rates of >90%[4,5]. In adults, long-term survival is much lower, approaching 50% in patients who can tolerate intensive chemotherapy[1,6,7]. However, just under half of patients relapse after or fail to respond to standard therapy. These patients have poor prognosis with a median OS of ~8 months[8]. New treatment options which impart meaningful survival benefits are lacking, with <50% of children and <10% of adults attaining sustained remissions[9,10].

In relapsed/refractory (r/r) T-ALL, the standard approach to attaining remission is with intensive re-induction chemotherapy followed by allogeneic transplantation, with regimens typically associated with significant toxicity and high failure rates. Unlike B-ALL, where highly potent immunotherapies such as the bispecific T cell engager blinatumomab[11–13], the antibody-drug conjugate inotuzumab ozogamicin[14], and chimeric antigen receptor (CAR)-T cells[15] have revolutionized the treatment paradigm, no specific immunotherapies are available for T-ALL. Perhaps the most promising advance in r/r B-ALL is CAR-T cells, which lead to high rates of deep and sustained remissions, even in advanced and refractory disease[15,16]. Application of CAR-T therapy to patients with T-ALL is highly desirable.

Due to a lack of tumor-specific antigens, in B-ALL, CAR-T cells target pan-B cell antigens such as CD19 or CD22, leading to loss of normal B cells. However, targeting a pan-T cell antigen requires additional considerations. First, unlike B cell aplasia, which is well-tolerated, depletion of normal T cells may induce life-threatening immunodeficiency[17]. Secondly, CAR-T cells may target each other during manufacture and after administration[18]. This so-called 'fratricide' precludes CAR-T targeting of pan-T cell antigens without the use of complex genome-editing or protein-retention techniques to prevent CAR-T cell expression of the cognate antigen.

To avoid these problems, identification of an antigen selectively expressed on T-ALL blasts, but not normal T cells or other essential cell types is critical. Here, we propose the

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88     chemokine receptor CCR9 (C-C Motif Chemokine Receptor 9, or CD199) as such a target.

89     CCR9 is a seven-pass transmembrane G-coupled receptor (GPCR) for the natural ligand

90     CCL25[19] (Figure 1d), and in mice is expressed in gut intraepithelial $\gamma\delta$ T-cells, but less than

91     5% of normal circulating T cells and B cells[20]. We demonstrate CCR9 is expressed on a high

92     proportion of cases of r/r T-ALL, but <5% of normal T cells. Further, we generate anti-CCR9

93     CAR-T cells and show robust anti-tumor efficacy in multiple *in vitro* and *in vivo* models of T-

94     ALL, without evidence of fratricide or lysis of normal T cells.

## METHODS

### Cell lines and maintenance

HEK-293T cell line was cultured in IMDM (Lonza, Switzerland) and other cell lines were cultured in complete RPMI (Lonza, Switzerland), each supplemented with 10% FBS and 2 mM GlutaMAX. All cell lines were routinely tested for mycoplasma using EZ-PCR Mycoplasma Detection Kit (Biological Industries) and the identity of T-ALL cell lines were verified by short tandem repeat analysis using the PowerPlex 1.2 system (Promega) in June 2017. All cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

### Generation of CCR9-KO cells by CRISPR/ Cas-9 Nicking Strategy

CCR9 negative versions of P12-Ichikawa and PF382 T-ALL cell lines were generated using CRISPR/Cas9 genome engineering. Guide RNAs (gRNA) were designed targeting exon 4 of the CCR9 gene (https://chopchop.cbu.uib.no/), choosing the guides with the top quality and least off-target score; gRNA 1: TGGAAGACTACGTTAACTTC; gRNA 2: GTACTGGCTCGTGTTCATCG. The Alt-R CRISPR RNP system (IDT) was used as per manufacturer's instructions. Cells were electroporated using AMAXA Nucleofector (Lonza). Once confirmed by flow cytometry, knockout cells were validated at DNA level. DNA was extracted using Qiagen DNeasy kit followed by PCR amplification using specific primers (forward: 5'- CCCTTGCAGAGCCCTATTCC; reverse: 5'- ACCTTCAGGGTCAAGACAGC).

### Flow cytometry and primary T-ALL samples

Primary T-ALL tumor samples were obtained from the UK Cellbank collection or from local biobanks at Great Ormond Street Hospital (GOSH) or University College London Hospital (UCLH). The primary T-ALL samples used in primary *in vitro* killing experiments were all bone marrow samples collected at the time of initial diagnosis and were respectively derived from an adult male with high count T-ALL (genetics unknown), an adult female with high count T-ALL (genetics unknown) and a 14-year old male with STIL-deleted T-ALL. Quantification of CCR9 antigen density was undertaken using BD Quantibrite beads (BD Biosciences, NJ) according to manufacturer's instructions. Flow cytometry was performed on an BD Fortessa LSR II instrument. A list of antibodies is included in Supplementary Material.

128 ***Retroviral transduction of T cells***

129 CAR constructs were expressed in the SFG vector backbone. Viral supernatant was

130 generated and PBMC transductions performed as previously described[21,22].

131

132 ***FACS-based co-culture and cytotoxicity assays***

133 Target cells were pre-labelled with carboxyfluorescein succinimidyl ester (CFSE) and

134 effector cells with CellTrace Violet (CTV; both Invitrogen, Carlsbad, CA). Co-cultures were

135 performed with 50000 target cells/ well in a 96-well plate (25000 for primary samples).

136 After 48hrs (72hrs for primary tumor samples), the plate was spun and 100μl of supernatant

137 was removed from the 1:8 E:T ratio wells for cytokine assays. Cytokines were measured by

138 ELISA (Biolegend) according to manufacturer' instructions.

139 After staining with appropriate antibodies and fixable viability dye, cells were resuspended in

140 100ul of 0.4% paraformaldehyde (PFA)/ PBS. Data acquisition was made on the BD

141 Cytoflex instrument. Assays were performed in triplicate. To minimise the impact of

142 alloreactivity, cytotoxicity for CAR-T cells was normalized to that for NT cells at the same E:T

143 ratio.

144

145 ***T cell proliferation assay***

146 Effector NT or CAR-T cells were labelled with CFSE by incubating the cells for 5 minutes at

147 a concentration of 1uM in complete media, before 2 x washes with complete media. A

148 sample of cells was fixed in 0.4% PFA and kept at 4C, for later FACS. Cells were then

149 plated at 2.5 x $10^5$/ml, 100ul in wells of a 96-well plate (25000 cells/well), in triplicate.

150 Irradiated (40Gy) target cells (MOLT4 or SupT1) were then added to the wells at a 1:2 ratio.

151 After 7 days, effector cells were counted, and a sample taken for FACS. T cell proliferation

152 was assessed by CFSE dilution compared to baseline sample and by fold-expansion.

153

154 ***Mouse models of T-ALL***

155 This work was performed under a UK Home Office–approved project license and was

156 approved by the UCL Biological Services Ethical Review Committee. Female NSG mice

157 aged 6-12 weeks were obtained from Charles River and assigned randomly to control and

158 experimental groups.

159    The MOLT4-Fluc cells used in the assay were generated by lentiviral transduction of the
160    parental cell line with a plasmid expressing luciferase. PDX samples were developed at the
161    Institute of Child Health by Professor Owen Williams, and were derived from an 11-year old
162    boy with hyperdiploid T-ALL (PDX 1078), a 5-year old boy with TLX3-rearranged T-ALL
163    (PDX 1139), a 14-year old boy with STIL1-deleted T-ALL (PDX 782), a 1-year old girl with
164    ATM-deleted T-ALL (PDX 682), a 10-year old boy with biallelic CDKN2A-deleted T-ALL
165    (PDX 602), and an 8-year old boy with CDKN2a/ STIL1-deleted T-ALL (PDX 352).

166    Mice were intravenously injected with cell suspensions via the tail vein and tail vein bleeds of
167    50ul were undertaken as indicated in the text. Blood, spleen and bone marrow was analyzed
168    by FACS. Human T cells were identified as CD45brightCD3bright and T-ALL cells as
169    CD45dimCD3dim/negative.

170    For experiments with a survival endpoint, mice were weighed at least twice weekly. Animals
171    with >10% weight loss or those displaying evidence of graft-versus-host disease or disease
172    progression, including hunched posture, poor coat condition, reduced mobility, pilorection or
173    hindlimb paralysis, were killed. Bioluminescence imaging of mice was performed using the
174    IVIS system (PerkinElmer, Buckinghamshire, UK). General anesthesia was induced and
175    maintained using inhaled isoflurane. Following induction, intraperitoneal injection of luciferin
176    (200ul via 27-gauge needle) was undertaken. After 2 min, mice were placed in the imaging
177    chamber. Simultaneous optical and bioluminescence imaging was performed.

178

179    ***Statistical analyses***

180    Unless otherwise noted, data are summarized as mean +/- standard deviation. Student's t-
181    test was used to determine statistically significant differences between samples for normally
182    distributed variables, and the Mann–Whitney U-test was used for nonparametrically
183    distributed variables.  Paired analyses were used when appropriate. When three or more
184    groups were compared, one-way ANOVA with Dunnett's test for multiple comparisons with
185    $\alpha$ = 0.05 were used. For longitudinal outcomes, comparisons were made using 2-way
186    ANOVA or a mixed effects model, with multiple comparisons between groups made by
187    Sidak's test, $\alpha$ = 0.05. Survival curves were generated using the Kaplan–Meier method.
188    Graph generation and statistical analyses were performed using GraphPad Prism v9
189    (GraphPad, La Jolla, CA).

190    **RESULTS**

191

192    ***CCR9 is highly expressed in T-ALL blasts with limited expression on normal immune***

193    ***cells***

194    We sought to identify potential immunotherapy targets for T-ALL. We first reasoned that any

195    tractable T-ALL target must be expressed in T-ALL cells, but not normal tissues. Thus, we

196    analysed the collated gene expression profiles of 35 normal tissues (n=172 samples) as

197    compared to MOLT-4 cells, a TAL1-positive T-ALL cell line included in the Protein Atlas

198    Cancer compendium. Using subtractive transcriptomics, we identified 12 transcripts uniquely

199    expressed in MOLT-4 cells but in no other normal tissue (Figure 1a)[23]. Of these, CCR9 was

200    the most attractive, being predicted to reside on the cell surface and thus amenable to

201    immunotherapy.

202    Further, analysis of the largest published pediatric T-ALL dataset shows CCR9 is expressed

203    in 80% of T-ALL cases at the RNA-level at diagnosis, with notable expression in most

204    HOXA-positive patients, half of whom have MLL gene rearrangements (Figure 1b,c). CCR9

205    was expressed in 12 of 19 ETP T-ALL patients in this cohort, thus highlighting the potential

206    of CCR9-directed therapy in the highest-risk patients. There was no significant difference in

207    the mutation profile of CCR9 positive patients as compared to CCR9 negative patients, apart

208    from a lower incidence of chromosome 6q deletions (p=0.002) and higher incidence of

209    NOTCH1 mutations (p<0.0001; Supplementary Figure 1)[24].

210    We examined CCR9 status of primary cases of T-ALL using flow cytometry. 74/102 cases

211    (73%) expressed CCR9 (defined as expression on > 20% of blasts), with expression

212    enriched in cases of relapsed/ refractory disease – 38/59 (65%) diagnostic v 11/13 (85%)

213    relapsed v 26/30 (86%) primary refractory (Figure 1d, Supplementary Figure 2). The 20%

214    threshold was selected to defined positivity, as blast expression of CCR9 was typically dim

215    but homogenous by flow cytometry (Supplementary Figure 2). The median number of copies

216    of CCR9 per cell was 1732 (1320 diagnostic v 1889 relapsed v 2175 refractory, Figure 1e).

217    Expression was similar in pediatric (72% CCR9+) and adult cases (75% CCR9+). True

218    biphenotypic expression with CCR9-positive and CCR9-negative blast populations was

219    noted in only 3/102 cases (Supplementary Figure 2, highlighted in red). In diagnostic

220    samples where full immunophenotyping was available, 6/32 cases were identified as ETP-

221    ALL phenotype. Four of six (67%) ETP cases were CCR9+. In 3 cases where matched

222    diagnostic and relapse samples were available, CCR9 expression was preserved or

223    increased upon relapse.

224 Next, to examine the potential for hematological toxicity when targeting CCR9, we examined
225 CCR9 expression on peripheral blood cells isolated from healthy donors. We found low
226 levels of expression, limited to 11% of B cells and less than 5% of CD3+ cells. CCR9 was
227 not expressed on monocytes, granulocytes, NK cells or peripheral blood gamma-delta T
228 cells (Figure 1f). The median copy number on both positive B and T cells was lower than
229 that seen in primary T-ALL, at <500 copies/ cell. Expression of CCR9 on T cells was not
230 clearly linked to CD4/CD8 identity, markers of differentiation (CD45RA/CCR7/CD95) (Figure
231 1f), or activation (HLA-DR) and did not change on stimulation of T cells with CD3/ CD28
232 antibodies (data not shown).

233 We also evaluated *CCR9* expression in thymic subsets and CD34+ marrow precursor cells
234 from healthy donors, using qPCR. As previously described, no *CCR9* expression was seen
235 in CD34 cells, with minimal expression in single-positive peripheral blood CD4 and CD8
236 cells, confirming FACS data. *CCR9* was expressed at low levels (< 5% of GAPDH signal) in
237 early thymic T cell precursors (DN1 – SP CD3-) with somewhat higher expression in DP
238 CD3+ (22% GAPDH signal) and SP CD3+ CD4 (8% GAPDH signal) and CD8 (8% GAPDH
239 signal) thymic cells (Supplementary Figure 3).

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242 ***Anti-CCR9 chimeric antigen receptor (CAR)-T cells are effective against T-ALL cell***
243 ***lines in vitro***

244 We developed a novel binder against CCR9 by gene-gun vaccination of rats with a plasmid
245 encoding human CCR9, followed by hybridoma generation from lymphoid tissue of
246 seroconverted animals. A single CCR9-specific hybridoma clone (P4T1) was identified, from
247 which we generated a single-chain variable fragment.  We cloned anti-CCR9 scFv as a
248 second-generation CAR, incorporating CD8 stalk/ transmembrane domain and 4-1BB-
249 CD3zeta endodomain[25] (Figure 2a). This was encoded in a gamma-retroviral viral vector
250 with RQR8 marker/ sort-suicide gene[26] and used to transduce primary human T cells.  CAR
251 was detected directly on the surface of transduced cells using an anti-Fab antibody (Figure
252 2b). T cells transduced with anti-CCR9 CAR expanded similarly to those transduced with a
253 control CAR targeting CD19, with no evidence of fratricide (Figure 2c). No CCR9+ cells were
254 detected in the transduced cell product, suggesting 'purging' of CCR9+ T cell
255 (Supplementary Figure 4a). Further, there were no differences in expression of markers of
256 differentiation (CD45RA, CCR7), exhaustion (TIM-3, LAG-3, PD-1) or activation (CD71,
257 HLA-DR, forward scatter) between CAR19 and CCR9 CAR-T cells following manufacture
258 (Supplementary Figure 4b,c).

259 We co-cultured anti-CCR9 CAR-T cells or control CAR-T targeting CD19 for 48hrs with a
260 panel of T-ALL cell lines, which express CCR9 at varying surface densities (Figure 2d). In
261 order to confirm specific anti-CCR9 functions, we included CCR9-negative variants of P12-
262 Ichikawa and PF382 cell lines (designated P12-KO and PF382-KO), generated using
263 CRISPR-Cas9. We showed specific cytotoxicity of anti-CCR9 CAR-T against CCR9+ cell
264 lines (Suppl Fig 5d, Fig 2e), including at low target density of approximately 400 molecules/
265 cell (SupT1-CD19, Fig 2d). A small degree of non-specific kill (~10%) above CD19 CAR was
266 seen against PF382-KO cell line but not against P12-KO cell line, perhaps due to slightly
267 higher basal activation. We also examined cytokine secretion and showed that anti-CCR9
268 CAR-T specifically secreted the pro-inflammatory cytokines interferon-gamma (IFN-$\gamma$) and
269 interleukin-2 (IL-2) only in co-culture with CCR9+ cell lines (Figure 2f). Further, in 7-day
270 CFSE dilution assays, anti-CCR9 CAR-T but not anti-CD19 CAR-T proliferated in response
271 to CCR9+ target cells (Figure 2g,h,i), with > 10-fold expansion seen over this period (Figure
272 2i).

273

274 ***Anti-CCR9 CAR-T were effective against primary T-ALL blasts in vitro***
275 We isolated blasts from peripheral blood of 3 patients with newly diagnosed T-ALL and
276 confirmed CCR9 expression (89% v 55% v 56% blasts CCR9+ respectively) and antigen
277 density by flow cytometry (2478 v 952 v 545 molecules per cell respectively). We incubated
278 blasts at a 1:1 ratio for 72 hrs with NT, CAR19 or CARCCR9 cells, generated from healthy
279 donor T cells. In comparison with NT or CAR19 cells, CARCCR9 showed potent cytotoxicity
280 and secretion of interferon-gamma (Figure 3a-d).

281

282 ***Anti-CCR9 CAR-T were effective in murine cell-line and patient-derived xenograft***
283 ***models of high-burden T-ALL***
284 To test the anti-tumor potency of anti-CCR9 CAR-T *in vivo*, we intravenously (IV) injected
285 NSG mice with 3 x 10$^6$ MOLT-4 cells, engineered to express firefly-luciferase (MOLT4-Fluc)
286 (Figure 4a). Engraftment and exponentially increasing disease signal in marrow was
287 confirmed by bioluminescence imaging (BLI) at D4 and 9 following injection (Figure 4b).
288 Mice were treated on D9 (CAR D+0) with 8 x 10$^5$ IV NT, CAR19 or CARCCR9 cells, and
289 disease was tracked by BLI and clinical assessment. While untreated mice and those
290 receiving NT or CAR19 cells experienced disease progression, rapid weight loss and death
291 by CAR D+16, mice receiving CARCCR9 had disease regression, continued weight gain and
292 prolonged survival beyond CAR D+80 (Figure 4c,d). Further, in order to confirm durable T

293    cell memory, mice were re-injected with 1 x 10$^6$ MOLT4-Fluc on CAR D+40. In 3/4 (75%) of

294    mice, no increasing signal was detected in marrow, suggesting continued anti-CCR9

295    immunosurveillance (Figure 4c,d). The remaining mouse died of progressive CCR9+

296    disease in the absence of detectable human T cells.

297    We also tested CARCCR9 in  patient-derived xenograft (PDX) models of T-ALL (Figure 5a-

298    h), with antigen densities of 1078 (Figure 5b) and 1139 (Figure 5i) molecules/ cell

299    respectively. NSG mice were injected with 1 x 10$^6$ primary blasts, then 8 x 10$^5$ NT, CAR19 or

300    CARCCR9 cells were administered IV on D+20 (CAR D+0) (Figure 5a,h). In PDX model

301    1078 (Figure 5a,b), disease was slowly progressive, with NT or CAR19 recipients displayed

302    increasing blast percentage in peripheral blood (Figure 5c). Leukemic death occurred in

303    most animals by CAR D+120 (Figure 5d), in association with massive splenomegaly (Figure

304    5e) and heavy infiltration of spleen (Figure 5f)/ marrow (Figure 5g) with T-ALL. Late tumor

305    regression associated with development of xeno-GvHD was seen in one recipient of CAR19.

306    By contrast, in recipients of CARCCR9, no tumor was detected in peripheral blood until the

307    end of the study (Figure 5c), although low level infiltration of T-ALL blasts was seen at

308    necropsy in both spleen and marrow in 1/4 animals (Figure 5f,g). Blasts were CCR9+ and no

309    T cells were detected.  PDX model 1139 was more aggressive (Figure 5h,i), with recipients

310    of NT or CAR19 displayed increasing ALL burden in peripheral blood over time (Figure 5j),

311    with eventual leukemic death and massive splenomegaly in all animals (Figure 5k,l). By

312    contrast, all CARCCR9 recipients had undetectable leukemia and disease-free survival until

313    CAR D+60, when mice were culled due to development of graft versus host disease (GvHD)

314    in some animals (median survival NT 42 days, CAR19 42 days, CARCCR9 NR, p = 0.0032,

315    Figure 5k). At the time of cull, all CCR9 CAR recipients had normal-sized spleens, with no

316    detectable leukemia either in spleen (Figure 5l, m) or marrow (Figure 5o). Instead, human T

317    cell infiltration was seen (Figure 5n,o).

318    Finally, given the high potency of CARCCR9 thus far demonstrated, we sought to investigate

319    performance *in vivo* against low density targets. Thus, PDX with antigen densities of 782,

320    682, 602 and 352 CCR9/ cell respectively were engrafted in NSG mice as before, with IV

321    adminstration of 8 x 10$^5$ CAR19 or CARCCR9 cells on D+20 (Figure 6a). Even at these low

322    densities, tumour clearance and long term survival until CAR D+60 was seen in all recipients

323    of CARCCR9, other than those engrafted with the lowest density PDX (352/cell) (Figure 6b-

324    e). All surviving mice were culled at D60 due to development of GvHD in the majority of

325    animals. Notably, even in PDX 352, which was the most aggressive model tested, initial

326    disease control was seen in 4/5 recipients of CARCCR9, followed by rapid relapse in all

327    mice. This was associated with a survival benefit for CARCCR9 recipients (Figure 6e).

328

**DISCUSSION**

In this study, we have demonstrated that CCR9 is a viable immunotherapy target for T-ALL, expressed in the majority of patients with T-ALL, but on <5% of peripheral blood T cells. Anti-CCR9 CAR-T cells were not prone to fratricide, specifically lyzed T-ALL cell lines and primary tumors *in vitro* and were highly potent in multiple *in vivo* models of T-ALL. Importantly, most patients with ETP-ALL, a group with a particularly high risk of induction failure, and >85% of patients with r/r T-ALL expressed CCR9, suggesting CCR9 CAR-T cell therapy could be a valuable approach in those patients most at need of novel treatment approaches.

Currently, treatment options in r/r T-ALL are limited, and there is no standard of care. Most patients receive combination salvage chemotherapy, typically including a purine analogue such as nelarabine. However, salvage regimens are associated with considerable morbidity and mortality, and remission rates of <40%[27]. For the minority of patients who enter remission, allogeneic hematopoietic stem cell transplant (allo-HSCT) is often considered as a curative option. However, allo-HSCT is only suitable for younger, medically fit patients and is associated with a mortality of up to 20%[28].

Few investigational approaches to T-ALL are available. The anti-CD38 mAb daratumumab is being tested in a clinical study in children with r/r T-ALL [NCT03384654], but clinical data to date is limited. A single case report of off-label use in MRD-positive T-ALL showed 2 patients attained MRD-negative remission, sustained to 10 months with continuous treatment[29], but no long-term data is currently available. Although CD38 is expressed by >70% of T-ALL cases[30], it is not an ideal immunotherapy target since it is also expressed by activated T cells, haematopoietic stem cells (HSCs) and monocytes[31].

Given the success of CAR-T in B-ALL, there is great interest in development of CAR-T for T-ALL. Multiple CAR-T targets have been proposed, most of which are pan-T cell antigens (CD3[32], CD4[33], CD5[34], CD7[18,35]) or are expressed on activated T cells (CD38[36]). We previously described targeting of TRBC1 or TRBC2 alleles at the TCR beta constant region, an approach which may spare a substantial proportion of normal T cells[21]. However, this strategy is better suited to mature T cell malignancies, since surface expression of the T cell receptor is limited to ~15-20% of T-ALL[1]. CD1a is a potential target absent from normal T cells[37], but expression defines cortical T-ALL, which constitutes a good prognostic group. Hence, it is infrequently expressed in r/r disease (~10% of r/r T-ALL cases)[30].

13

362     The most clinically advanced CAR-T targets for T-ALL are CD7 and CD5. CD7 is expressed

363     on >90% of r/r T-ALL, but also on both natural killer cells and normal (and CAR) T cells. To

364     prevent CAR-T fratricide, strategies to knock-out CD7 by genome editing or protein retention

365     are therefore needed, introducing complexity to manufacture[18,35]. An initial study has been

366     published. This used allogeneic donor-derived CAR-T cells, limiting use to patients pre/post

367     allo-HSCT[38]. Of 20 reported patients, 18 (90%) attained CR at 30 days. 7/19 responders

368     (37%) then received allo-HSCT. Nine of twelve patients not transplanted remained in

369     remission with median F/U of 6.3 months.  Depletion of normal T and NK cells was

370     observed, and some patients experienced opportunistic infections/ viral reactivations.

371     Unexpectedly, most patients had recovery of CD7-negative peripheral T and NK cells,

372     although in markedly reduced numbers.

373     Another clinically-tested target is CD5, expressed on >70% of r/r T-ALL but also on all

374     normal T cells. Preclinical data demonstrated that anti-CD5 CAR-T cells showed relative

375     sparing of normal T cells, so knock-out of CD5 might not be needed[34]. However, early

376     clinical data showed no responses in patients with T-ALL[39], perhaps due to reported

377     exhaustion from chronic self-stimulation[34].

378     Here, we have identified CCR9 as another potential CAR target in T-ALL, which is not

379     expressed on most normal T cells: thus, T cell fratricide and T cell aplasia are unlikely to be

380     risks of this strategy. However, other potential on-target off-tumor risks must be considered.

381     In mature tissues CCR9 is expressed mainly on gut-resident immune cells, including gut-

382     homing CD4+ and CD8+ T cells[40], gamma-delta T cells[41], plasmacytoid dendritic cells

383     (pDCs)[42], IgA plasmablasts and plasma cells[43], and intraepithelial lymphocytes (IELs)[44]. It is

384     important to note that CCR9 is not, however, expressed on gut epithelium. Its only known

385     ligand is CCL25, which is constitutively expressed in thymic and intestinal epithelial cells,

386     and is overexpressed in the intestine during gut inflammation and autoimmunity[20].

387     CCL25/CCR9 interactions play a role in infiltration of effector T cells to the small intestinal

388     mucosa, and are also involved in thymic T cell migration and maturation[45], with maximal

389     CCR9 expression found on double-positive thymocytes[46].

390     Human and murine CCR9 share similar function, expression patterns and 86% sequence

391     homology, and so evidence from murine models may be instructive. *Ccr9 -/-* mice display

392     normal T cell development and, although diminished numbers of gamma-delta IELs are

393     present in the small intestine, this observation is not associated with any adverse

394     phenotype[19,41], probably due to some functional redundancy with other receptors including

395     CD103 and Ccr7[45]. Some clinical data also suggests CCR9 may be safely targeted in

396     humans. The CCL25/ CCR9 axis has been implicated in the pathology of inflammatory

397  bowel disease[4748], leading to clinical trials of a small-molecule inhibitor of CCR9 in Crohn's

398  disease. Although these trials did not demonstrate efficacy, anti-CCR9 therapy was not

399  associated with significant toxicity, in the gut or elesewhere[48,49]. Indeed, gut-resident T cells

400  are also CD7+, and no early gut toxicity has been seen in initial studies of anti-CD7 CAR-T,

401  despite presumed targeting of these cells[38]. Further, although CCR9-directed

402  immunotherapies may lead to thymic ablation, a recent meta-analysis of children who have

403  undergone total thymectomy during cardiothoracic procedures has shown no evidence of

404  clinical immunocompromise, increased risk of cancer or autoimmunity[50].

405  Whether CCR9 has a role in the pathophysiology of T-ALL or simply reflects expression by

406  the underlying normal thymocyte counterparts of T-ALL blasts is unknown. CCR9 is a

407  downstream target of NOTCH1[51], which is physiologically expressed in normal thymocytes

408  but affected by oncogenic activating mutations in ~60% of cases of T-ALL[52], potentially

409  explaining the higher rate of *NOTCH1* mutations identified in the CCR9+ cohort (Suppl Fig

410  1). In our dataset, CCR9 expression was enriched in patients with r/r disease, suggesting

411  CCR9 may identify patients with worse prognosis. Previous reports described that CCR9

412  expression in T-ALL may confer a proliferative phenotype[51] and resistance to apoptosis[53].

413  Further, some T-ALL cell lines or primary blasts can signal through CCR9, demonstrating

414  CCL25-mediated chemotaxis. However, no clear evidence demonstrates the CCL25/ CCR9

415  axis is required for disease initiation or progression[51,54]. Indeed, in our study CRISPR/Cas9-

416  mediated knockout of CCR9 from P12-Ichikawa and PF382 cell lines did not reduce blast

417  survival or growth, indicating CCR9 signalling is not likely a requirement for survival *in vitro*.

418  Of note, no CCR9 loss was seen in the cell line or PDX models used in this study, despite

419  prolonged selection pressure, suggesting that neither *de-novo* antigen downregulation nor

420  selection of a pre-existing CCR9-negative clone occurred. This is important as in B-ALL

421  patients treated with anti-CD19 CAR-T[55], CD19 downregulation is an important cause of

422  relapse. Ultimately, clinical testing will be required to determine if CCR9 antigen loss is seen

423  in T-ALL patients treated with CARCCR9.

424  In our analysis, we found relatively low expression of CCR9 on primary T-ALL compared to

425  that reported for other CAR targets in ALL. CCR9 median surface density was 1732

426  molecules/ cell on T-ALL blasts, compared to ~10000 for CD19 and ~14000 for CD22 on B-

427  ALL[56]. Antigen-low escape was recently seen in a trial of anti-CD19/22 CAR-T in B-ALL and

428  diffuse large B cell lymphoma (DLBCL): in this study, 'low' density was defined as <3000

429  molecules/cell[57]. Despite low target density, we found anti-CCR9 CAR-T cells displayed

430  potent cytokine secretion and cytotoxicity against both cell line and primary T-ALL targets.

431  Indeed, in co-culture with the SupT1-CD19 cell line, which natively expresses CCR9 at only

432  ~450 molecules/ cell, and transgenically expresses CD19 at 80 000 molecules/cell,

15

433 production of interferon-gamma and IL-2 was similar between anti-CCR9 and anti-CD19

434 CAR-T cells. In addition, no evidence of antigen-low escape was seen in multiple PDX

435 models with very low CCR9 density, including one model extending >100 days. Indeed, only

436 in a highly aggressive PDX model with extremely low antigen density (352/ cell) was initial

437 clearance followed by tumor escape seen. Precedent for targeting of a low-density molecule

438 comes from the clinical success of CAR-T targeting B cell maturation antigen (BCMA) in

439 myeloma[58,59], which is expressed at a median density of only 1061 molecules/ cell[60].

440 In conclusion, we have demonstrated that CCR9 is a viable potential CAR-T target for r/r T-

441 ALL. Clinical exploration of anti-CCR9 CAR-T for T-ALL is warranted, and a phase 1 clinical

442 trial of anti-CCR9 CAR-T is planned. CAR-T cells against CCR9 and other targets may

443 potentially bring the potent therapeutic potential of cellular immunotherapy to this neglected

444 disease area.

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16

**CONTRIBUTIONS**

PM designed, led and performed the *in vitro* and *in vivo* experiments, analysed the data, prepared the figures and wrote the manuscript. PAW led on hybridoma screening, antibody and CAR subcloning, and designed and performed *in vitro* and *in vivo* experiments. NCM, AB and DOC sourced T-ALL samples and performed and analysed flow cytometry data on primary samples. SD and MH assisted with i*n vitro/ in vivo* data collection. TK performed ELISA, molecular cloning, and co-culture experiments. TL and TRD generated and maintained CCR9-negative and Fluc+ cell lines used in the experiments, and assisted in hybridoma screening. S Rahman and RP assessed *CCR9* expression on thymic subsets by qPCR. DM, SR and TC supplied sorted thymic subset cells. GG supplied primary T-ALL samples. OW provided T-ALL PDX samples. LL provided support for the experiments and animal work was carried out on a Home Office license held by LL. MP supported the experiments and wrote the manuscript. MRM conceived the study, obtained funding and wrote the manuscript.

**CONFLICTS OF INTEREST**

PMM and LL own stock and received research funding from Autolus Ltd. MAP is employed by and owns stock in Autolus Ltd. No other authors declare conflicts of interest.


**REFERENCES**

1. Marks DI, Paietta EM, Moorman AV, et al. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). *Blood.* 2009;114(25):5136–5145.

2. Litzow MR, Ferrando AA. How I treat T-cell acute lymphoblastic leukemia in adults. *Blood.* 2015;126(7):833–841.

3. O'Connor D, Enshaei A, Bartram J, et al. Genotype-Specific Minimal Residual Disease Interpretation Improves Stratification in Pediatric Acute Lymphoblastic Leukemia. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 2018;36(1):34–43.

4. Vora A, Goulden N, Wade R, et al. Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. *Lancet Oncol.* 2013;14(3):199–209.

5. Schrappe M, Valsecchi MG, Bartram CR, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. *Blood.* 2011;118(8):2077–2084.

6. Goldstone AH, Richards SM, Lazarus HM, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood.* 2008;111(4):1827–1833.

7. Sive JI, Buck G, Fielding A, et al. Outcomes in older adults with acute lymphoblastic leukaemia (ALL): results from the international MRC UKALL XII/ECOG2993 trial. *Br. J. Haematol.* 2012;157(4):463–471.

509   8.  Fielding AK, Richards SM, Chopra R, et al. Outcome of 609 adults after relapse of acute
510       lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. *Blood*.
511       2007;109(3):944–950.

512   9.  Ko RH, Ji L, Barnette P, et al. Outcome of patients treated for relapsed or refractory
513       acute lymphoblastic leukemia: a Therapeutic Advances in Childhood Leukemia
514       Consortium study. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 2010;28(4):648–654.

515   10. Samra B, Alotaibi AS, Short NJ, et al. Outcome of adults with relapsed/refractory T-cell
516       acute lymphoblastic leukemia or lymphoblastic lymphoma. *Am. J. Hematol.*
517       2020;95(9):E245–E247.

518   11. Kantarjian H, Stein A, Gökbuget N, et al. Blinatumomab versus Chemotherapy for
519       Advanced Acute Lymphoblastic Leukemia. *http://dx.doi.org/10.1056/NEJMoa1609783*.
520       2017;

521   12. Brown PA, Ji L, Xu X, et al. Effect of Postreinduction Therapy Consolidation With
522       Blinatumomab vs Chemotherapy on Disease-Free Survival in Children, Adolescents,
523       and Young Adults With First Relapse of B-Cell Acute Lymphoblastic Leukemia: A
524       Randomized Clinical Trial. *JAMA*. 2021;325(9):833–842.

525   13. Locatelli F, Zugmaier G, Rizzari C, et al. Effect of Blinatumomab vs Chemotherapy on
526       Event-Free Survival Among Children With High-risk First-Relapse B-Cell Acute
527       Lymphoblastic Leukemia: A Randomized Clinical Trial. *JAMA*. 2021;325(9):843.

528   14. Kantarjian HM, DeAngelo DJ, Stelljes M, et al. Inotuzumab Ozogamicin versus Standard
529       Therapy for Acute Lymphoblastic Leukemia. *N. Engl. J. Med.* 2016;375(8):740–753.

530   15. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in Children and Young
531       Adults with B-Cell Lymphoblastic Leukemia. *N. Engl. J. Med.* 2018;378(5):439–448.

532   16. Ghorashian S, Kramer AM, Onuoha S, et al. Enhanced CAR T cell expansion and
533       prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19
534       CAR. *Nat. Med.* 2019;25(9):1408–1414.

535   17. Notarangelo LD, Kim M-S, Walter JE, Lee YN. Human RAG mutations: biochemistry and
536       clinical implications. *Nat. Rev. Immunol.* 2016;16(4):234–246.

537   18. Gomes-Silva D, Srinivasan M, Sharma S, et al. CD7-edited T cells expressing a CD7-
538       specific CAR for the therapy of T-cell malignancies. *Blood*. 2017;130(3):285–296.

19

539 19. Uehara S, Grinberg A, Farber JM, Love PE. A Role for CCR9 in T Lymphocyte
540     Development and Migration. *J. Immunol.* 2002;168(6):2811–2819.

541 20. Wurbel M-A, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine
542     receptor show a mild impairment of early T- and B-cell development and a reduction in
543     T-cell receptor γδ+ gut intraepithelial lymphocytes. *Blood.* 2001;98(9):2626–2632.

544 21. Maciocia PM, Wawrzyniecka PA, Philip B, et al. Targeting the T cell receptor β-chain
545     constant region for immunotherapy of T cell malignancies. *Nat. Med.* 2017;89:3909–12.

546 22. Wawrzyniecka PA, Ibrahim L, Gritti G, Pule MA, Maciocia PM. Chimeric antigen receptor
547     T cells for gamma–delta T cell malignancies. *Leukemia.* 2021;1–3.

548 23. Uhlen M, Zhang C, Lee S, et al. A pathology atlas of the human cancer transcriptome.
549     *Science.* 2017;357(6352):.

550 24. Liu Y, Easton J, Shao Y, et al. The genomic landscape of pediatric and young adult T-
551     lineage acute lymphoblastic leukemia. *Nat. Genet.* 2017;49(8):1211–1218.

552 25. Imai C, Mihara K, Andreansky M, et al. Chimeric receptors with 4-1BB signaling capacity
553     provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia.*
554     2004;18(4):676–684.

555 26. Philip B, Kokalaki E, Mekkaoui L, et al. A highly compact epitope-based marker/suicide
556     gene for easier and safer T-cell therapy. *Blood.* 2014;124(8):1277–1287.

557 27. DeAngelo DJ, Yu D, Johnson JL, et al. Nelarabine induces complete remissions in
558     adults with relapsed or refractory T-lineage acute lymphoblastic leukemia or
559     lymphoblastic lymphoma: Cancer and Leukemia Group B study 19801. *Blood.*
560     2007;109(12):5136–5142.

561 28. Hamilton BK, Rybicki L, Abounader D, et al. Allogeneic Hematopoietic Cell
562     Transplantation for Adult T Cell Acute Lymphoblastic Leukemia. *Biol. Blood Marrow*
563     *Transplant.* 2017;23(7):1117–1121.

564 29. Ofran Y, Ringelstein-Harlev S, Slouzkey I, et al. Daratumumab for eradication of minimal
565     residual disease in high-risk advanced relapse of T-cell/CD19/CD22-negative acute
566     lymphoblastic leukemia. *Leukemia.* 2020;34(1):293–295.

567 30. Leong S, Inglott S, Papaleonidopoulou F, et al. CD1a is rarely expressed in pediatric or
568     adult relapsed/refractory T-ALL: implications for immunotherapy. *Blood Adv.*
569     2020;4(19):4665–4668.

570    31. Lund FE, Cockayne DA, Randall TD, et al. CD38: a new paradigm in lymphocyte
571         activation and signal transduction. *Immunol. Rev.* 1998;161(1):79–93.

572    32. Rasaiyaah J, Georgiadis C, Preece R, Mock U, Qasim W. TCRαβ/CD3 disruption
573         enables CD3-specific antileukemic T cell immunotherapy. *JCI Insight.*
574         2018;3(13):56219–14.

575    33. Pinz K, Liu H, Golightly M, et al. Preclinical targeting of human T-cell malignancies using
576         CD4-specific chimeric antigen receptor (CAR)-engineered T cells. *Leukemia.*
577         2016;30(3):701–707.

578    34. Mamonkin M, Rouce RH, Tashiro H, Brenner MK. A T-cell-directed chimeric antigen
579         receptor for the selective treatment of T-cell malignancies. *Blood.* 2015;126(8):983–992.

580    35. Png YT, Vinanica N, Kamiya T, et al. Blockade of CD7 expression in T cells for effective
581         chimeric antigen receptor targeting of T-cell malignancies. *Blood Adv.* 2017;1(25):2348–
582         2360.

583    36. Bride KL, Vincent TL, Im S-Y, et al. Preclinical efficacy of daratumumab in T-cell acute
584         lymphoblastic leukemia. *Blood.* 2018;131(9):995–999.

585    37. Sánchez-Martínez D, Baroni ML, Gutierrez-Agüera F, et al. Fratricide-resistant CD1a-
586         specific CAR T cells for the treatment of cortical T-cell acute lymphoblastic leukemia.
587         *Blood.* 2019;133(21):2291–2304.

588    38. Pan J, Tan Y, Wang G, et al. Donor-Derived CD7 Chimeric Antigen Receptor T Cells for
589         T-Cell Acute Lymphoblastic Leukemia: First-in-Human, Phase I Trial. *J. Clin. Oncol.*
590         2021;

591    39. Hill LC, Rouce RH, Smith TS, et al. Safety and Anti-Tumor Activity of CD5 CAR T-Cells
592         in Patients with Relapsed/Refractory T-Cell Malignancies. *Blood.*
593         2019;134(Supplement_1):199–199.

594    40. Kunkel EJ, Campbell JJ, Haraldsen G, et al. Lymphocyte CC chemokine receptor 9 and
595         epithelial thymus-expressed chemokine (TECK) expression distinguish the small
596         intestinal immune compartment: Epithelial expression of tissue-specific chemokines as
597         an organizing principle in regional immunity. *J. Exp. Med.* 2000;192(5):761–768.

598    41. Uehara S, Song K, Farber JM, Love PE. Characterization of CCR9 Expression and
599         CCL25/Thymus-Expressed Chemokine Responsiveness During T Cell Development:

600     CD3highCD69+ Thymocytes and γδTCR+ Thymocytes Preferentially Respond to
601     CCL25. *J. Immunol.* 2002;168(1):134–142.

602  42. Wendland M, Czeloth N, Mach N, et al. CCR9 is a homing receptor for plasmacytoid
603     dendritic cells to the small intestine. *Proc. Natl. Acad. Sci. U. S. A.* 2007;104(15):6347–
604     6352.

605  43. Pabst O, Ohl L, Wendland M, et al. Chemokine Receptor CCR9 Contributes to the
606     Localization of Plasma Cells to the Small Intestine. *J. Exp. Med.* 2004;199(3):411–416.

607  44. Svensson M, Marsal J, Ericsson A, et al. CCL25 mediates the localization of recently
608     activated CD8αβ$^{+}$ lymphocytes to the small-intestinal mucosa. *J. Clin. Invest.*
609     2002;110(8):1113–1121.

610  45. Svensson M, Agace WW. Role of CCL25/CCR9 in immune homeostasis and disease.
611     *Expert Rev. Clin. Immunol.* 2006;2(5):759–773.

612  46. Carramolino L, Zaballos A, Kremer L, et al. Expression of CCR9 beta-chemokine
613     receptor is modulated in thymocyte differentiation and is selectively maintained in
614     CD8(+) T cells from secondary lymphoid organs. *Blood.* 2001;97(4):850–857.

615  47. Trivedi PJ, Bruns T, Ward S, et al. Intestinal CCL25 expression is increased in colitis
616     and correlates with inflammatory activity. *J. Autoimmun.* 2016;68:98–104.

617  48. Wendt E, Keshav S. CCR9 antagonism: potential in the treatment of Inflammatory Bowel
618     Disease. *Clin. Exp. Gastroenterol.* 2015;8:119–130.

619  49. Feagan BG, Sandborn WJ, D'Haens G, et al. Randomised clinical trial: vercirnon, an oral
620     CCR9 antagonist, vs. placebo as induction therapy in active Crohn's disease. *Aliment.*
621     *Pharmacol. Ther.* 2015;42(10):1170–1181.

622  50. Roosen J, Oosterlinck W, Meyns B. Routine thymectomy in congenital cardiac surgery
623     changes adaptive immunity without clinical relevance. *Interact. Cardiovasc. Thorac.*
624     *Surg.* 2015;20(1):101–106.

625  51. Mirandola L, Chiriva-Internati M, Montagna D, et al. Notch1 regulates chemotaxis and
626     proliferation by controlling the CC-chemokine receptors 5 and 9 in T cell acute
627     lymphoblastic leukaemia. *J. Pathol.* 2012;226(5):713–722.

628  52. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic
629     leukemia. *J. Clin. Invest.* 2012;122(10):3398–3406.

630    53. Qiuping Z, Jei X, Youxin J, et al. CC chemokine ligand 25 enhances resistance to
631        apoptosis in CD4+ T cells from patients with T-cell lineage acute and chronic
632        lymphocytic leukemia by means of livin activation. *Cancer Res.* 2004;64(20):7579–7587.

633    54. Chamorro S, Vela M, Franco-Villanueva A, et al. Antitumor effects of a monoclonal
634        antibody to human CCR9 in leukemia cell xenografts. *mAbs.* 2014;6(4):1000–1012.

635    55. Sotillo E, Barrett DM, Black KL, et al. Convergence of Acquired Mutations and
636        Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy. *Cancer
637        Discov.* 2015;5(12):1282–1295.

638    56. Majzner RG, Rietberg SP, Sotillo E, et al. Tuning the Antigen Density Requirement for
639        CAR T-cell Activity. *Cancer Discov.* 2020;10(5):702–723.

640    57. Spiegel JY, Patel S, Muffly L, et al. CAR T cells with dual targeting of CD19 and CD22 in
641        adult patients with recurrent or refractory B cell malignancies: a phase 1 trial. *Nat. Med.*
642        2021;

643    58. Berdeja JG, Madduri D, Usmani SZ, et al. Ciltacabtagene autoleucel, a B-cell maturation
644        antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or
645        refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. *The
646        Lancet.* 2021;398(10297):314–324.

647    59. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or
648        Refractory Multiple Myeloma. *N. Engl. J. Med.* 2019;380(18):1726–1737.

649    60. Lee L, Draper B, Chaplin N, et al. An APRIL based chimeric antigen receptor for dual
650        targeting of BCMA and TACI in Multiple Myeloma. *Blood.* 2017;131(7):blood-2017-05-
651        781351-28.

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653

654    **FIGURE LEGENDS**

655

656    **Figure 1: CCR9 is expressed on T-ALL blasts with limited expression on normal
657    peripheral blood cells** (a) Heatmap showing genes that are solely expressed in MOLT-4 T-
658    ALL cells (red) as compared to 35 normal tissue (blue; n=172 samples) using subtractive
659    transcriptomics from data from the Protein Atlas[23] (b,c) CCR9 gene expression as

660 determined by RNA-seq from pediatric T-ALL patients from St Jude's Hospital, Memphis[24].

661 Pie chart (b) shows distribution of patients considered CCR9 positive (FPKM >2.0), while

662 scatter chart (c) shows CCR9 expression according to genetic subgroup (UNK=unknown).

663 (d) Expression of CCR9 on 102 cases of primary T-ALL, proportion of positive blasts (e)

664 CCR9 antigen density of positive samples, antibodies bound per cell.

665

666 **Figure 2: Anti-CCR9 CAR has potent cytotoxicity against T-ALL cell lines *in vitro*** (a)

667 Structure and vector design of anti-CCR9 and control anti-CD19 CAR used in the study,

668 utilising 'Campana' architecture with RQR8 marker/ sort-suicide gene (b) Expression of anti-

669 CCR9 CAR on the surface of transduced T cells, detected by anti-murine Fab. (c) Fold

670 expansion of non-transduced (NT), CAR19 or CARCCR9 cells 5 days following transduction

671 (d) Antigen density of CD19 and CCR9 on cell lines used in study (e) Cytotoxicity of CAR19

672 v CARCCR9 against primary T-ALL cell lines, data normalised to NT condition, 48hr co-

673 culture, data shown at 1:8 E:T ratio (f) Secretion of interferon gamma (left) and IL-2 (right) in

674 48hr co-culture, 1:8 E:T ratio as in (e). (g) Example flow plot of CFSE dilution on T cells after

675 7 day incubation with irradiated MOLT-4 cells at 1:2 ratio (h) Quantification of T cell CFSE

676 dilution, 3 donors (i) Fold expansion of T cells after 7-day co-culture with irradiated SupT1

677 cells at 1:2 ratio, 3 donors * p,0.05, **p <0.01, ***p<0.001, ****p<0.0001

678

679 **Figure 3: Anti-CCR9 CAR has potent cytotoxicity against primary T-ALL blasts *in***

680 ***vitro*.** NT, CAR19 or CARCCR9 cells from healthy donor T cells were incubated for 72hrs, at

681 1:1 ratio with T-ALL blasts obtained from 3 separate patients (a,c,d) Flow cytometry of

682 sample CCR9 density (left), quantification of remaining blasts (middle) and interferon

683 gamma secretion (right) in patient 1-3 respectively (b) Example flow cytometry gating from

684 patient 1 at end of co-culture

685

686 **Figure 4: Anti-CCR9 CAR has potent anti-tumor activity in a MOLT4 xenograft model**

687 **of T-ALL** (a) Schematic of murine MOLT4 model (b) Bioluminescence signal in mice in

688 study (c) Mass of mice in study, expressed as percentage of starting mass. (d) Survival of

689 mice in study. n= 4/group. Experiment performed twice, data shown from representative

690 experiment.

691

692 **Figure 5: Anti-CCR9 CAR has potent anti-leukemic activity in patient-derived**
693 **xenograft models of T-ALL** (a) Flow diagram of PDX model 1, n = 4/group (b) Flow
694 cytometry of CCR9 expression in blasts of PDX model 1 (c) Serial bleeds of mice, % tumor
695 of total CD45+ cells (d) Survival of mice in PDX model 1 (e) Spleen mass at necropsy in
696 PDX model 1 (f) Tumor  in spleen, % of total CD45+ cells (g) Tumor in marrow, % of total
697 CD45+ cells (h) Flow diagram of PDX model 2, n = 5 (NT), 3 (CAR19) and 5 (CARCCR9) (i)
698 Flow cytometry of CCR9 expression in blasts of PDX model 2 (j) Serial bleeds of mice in
699 PDX model 2, % tumor of total CD45+ cells (k) Survival of mice in PDX model 2 (l) Spleen
700 mass at necropsy in PDX model 2 (m) Tumor in spleen in PDX model 2, % of total CD45+
701 cells (n) T cells in spleen in PDX model 2, % of total CD45+ cells (o) T cells and tumor in
702 marrow of CARCCR9 recipients in PDX model 2, % of total CD45+ cells

703

704 **Figure 6: Anti-CCR9 CAR has potent anti-leukemic activity in patient-derived**
705 **xenograft models of T-ALL with low CCR9 antigen density** (a) Flow diagram of low
706 density PDX models (b-e) PDX models 3-7. CCR9 antigen density in PDX blasts before
707 injection, molecules per cell (left), leukemic burden in peripheral blood in PDX models
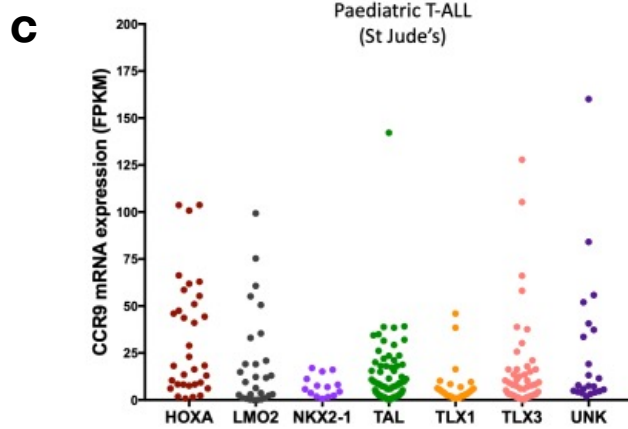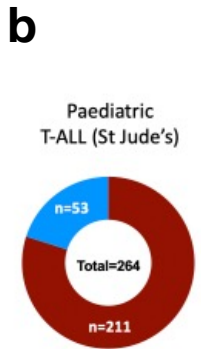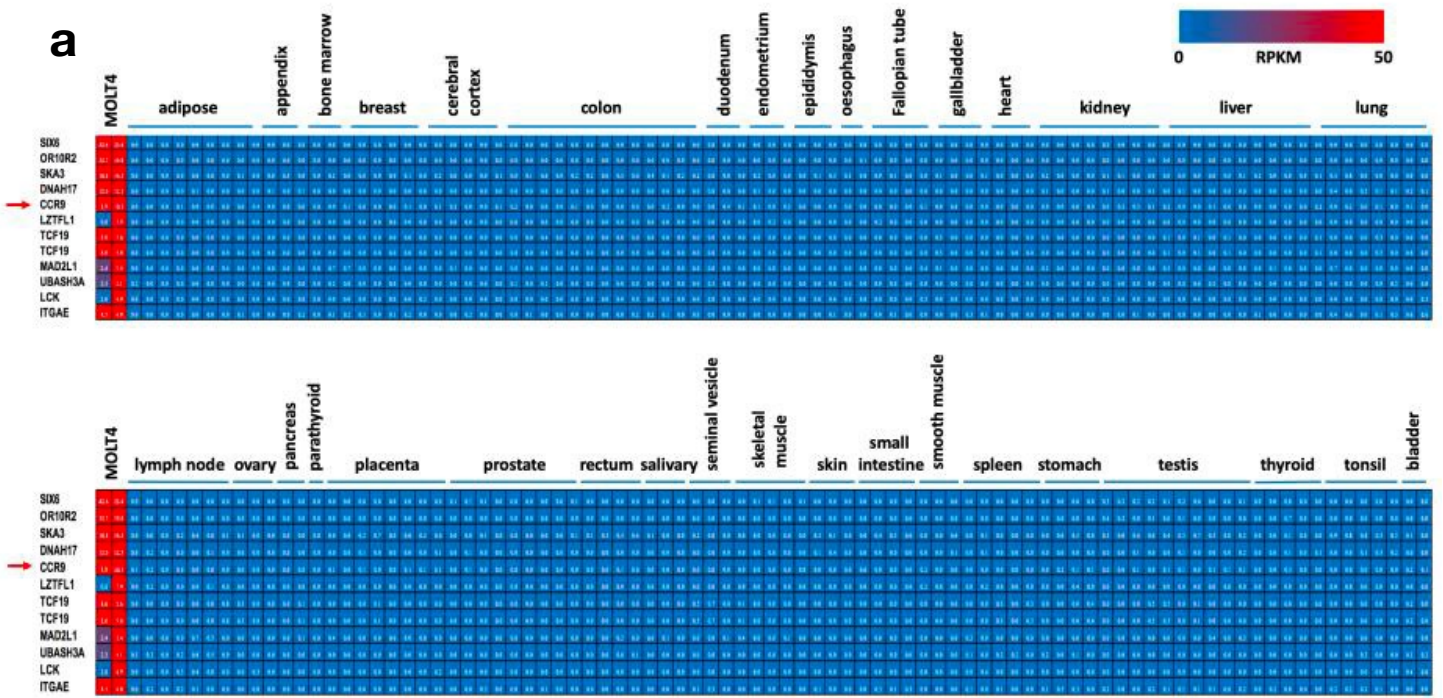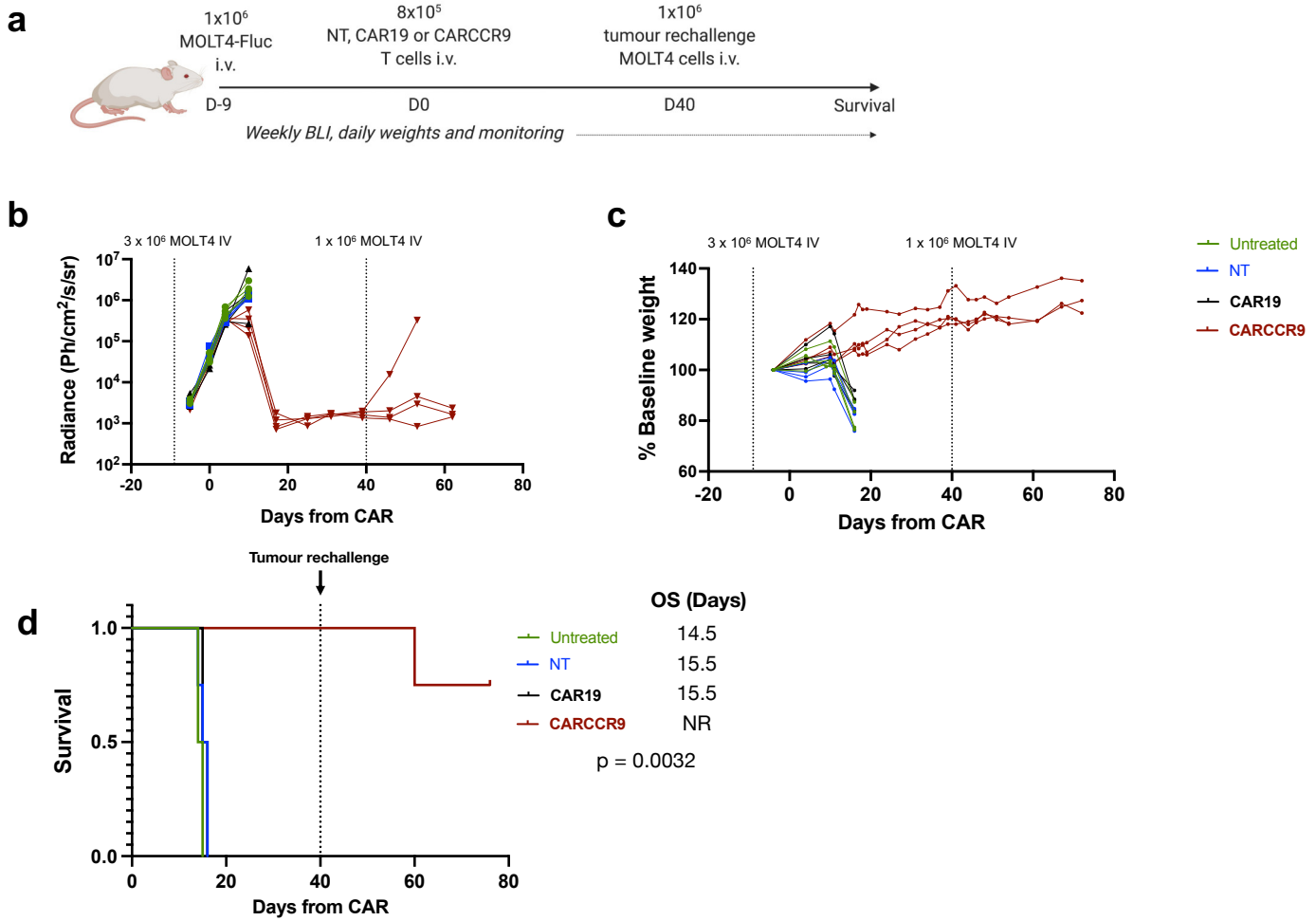708 (centre) and survival curves of animals in PDX models (right). n = 5/ group in all models.

709

710

711

712
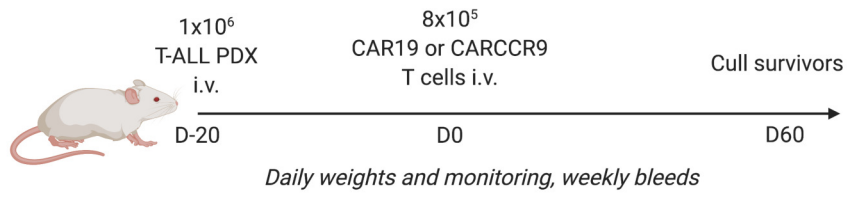
# FIGURE 1

# FIGURE 2

# FIGURE 3

**a**



2478/cell





**b**

| Untreated | NT | CAR19 | CARCCR9 |



Blasts 89.9 | Blasts 70.0 | Blasts 22.5 | Blasts 0.54

**c**

952/cell







**d**

545/cell

# FIGURE 4

**a**



1x10^6
MOLT4-Fluc
i.v.

8x10^5
NT, CAR19 or CARCCR9
T cells i.v.

1x10^6
tumour rechallenge
MOLT4 cells i.v.

D-9      D0      D40      Survival

*Weekly BLI, daily weights and monitoring*

**b**



3 x 10^6 MOLT4 IV    1 x 10^6 MOLT4 IV

Radiance (Ph/cm²/s/sr)

Days from CAR

**c**



3 x 10^6 MOLT4 IV    1 x 10^6 MOLT4 IV

% Baseline weight

Days from CAR

- Untreated
- NT
- **CAR19**
- **CARCCR9**

**d**



Tumour rechallenge

Survival

Days from CAR

**OS (Days)**

| | |
|---|---|
| Untreated | 14.5 |
| NT | 15.5 |
| **CAR19** | 15.5 |
| **CARCCR9** | NR |

p = 0.0032

# FIGURE 5

# FIGURE 6