

TITLE: Preclinical development of anti-CD21 chimeric antigen receptor T cells to treat T cell acute lymphoblastic leukemia

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ONE SENTENCE SUMMARY:

Fratricide-resistant anti-CD21 CAR-T cells are effective in pre-clinical models of T cell acute lymphoblastic leukemia (T-ALL).

ABSTRACT:

Patients with relapsed/refractory (r/r) T-cell acute lymphoblastic leukemia (T-ALL) have a dismal prognosis, highlighting the urgent need for effective therapies. CAR-T approaches targeting pan-T antigens may be limited by T cell aplasia and fratricide, necessitating ‘rescue’ allogeneic hematopoietic stem cell transplantation. In this study, we identify CD21, a pan-B-cell marker, as a promising target for T-ALL immunotherapy. CD21 is expressed in 50% of T-ALL cases at diagnosis but in fewer than 10% of mature T cells. We observed that CAR-T cells targeting membrane-distal CD21 epitopes were ineffective, likely due to the bulky, glycosylated nature of the antigen. However, when we engineered CAR-T cells to target membrane-proximal CD21 epitopes using a Fab-CAR design, we demonstrated robust activity against T-ALL cell lines, primary tumors, and patient-derived xenografts in both in vitro and in vivo models. The enhanced efficacy of this Fab-CAR design was driven by its high stability and reduced surface expression, addressing limitations of traditional CAR constructs. Additionally, pharmacological inhibition of the Phosphatidylinositol 3-kinase (PI3K) axis upregulated CD21 expression in T-ALL, further enhancing the potency of anti-CD21 CAR-T cells in vitro and in a patient-derived xenograft in vivo model. This study establishes CD21 as a viable CAR-T target and highlights advances in CAR design for bulky antigens, as well as the potential for pharmacological strategies to augment target expression. Anti-CD21 CAR-T cells represent a promising therapeutic option for improving outcomes for patients with T-ALL.

Main Text:

INTRODUCTION

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T cell precursors, accounting for ~15% of ALL diagnoses in children and 25% in adults (1, 2). Outcomes have been historically poor, but recent advances in treatment have brought them in line with B-ALL (3, 4). However, the prognosis for patients with relapsed or refractory (r/r) disease is dismal, with only 6.5% long-term survival in adults and < 25% in children (2, 5).

CAR-T cell therapy is effective for patients with r/r B-ALL, inducing sustained responses in ~40-50% of patients (6). Early studies suggest CAR-T cell therapies may also be useful for patients with T-ALL. However, CAR-T cell targeting of T-ALL is more complex: analogous approaches to CD19 CAR-T cell therapy, where T cell lineage antigens, such as CD5 and CD7 (7–9), are targeted, carry risks of fratricide and T cell aplasia. This may require additional engineering and possible rescue by hematopoietic stem cell transplant (HSCT).

Targeting antigens only expressed on T-ALL blasts, without expression on normal T cells, such as TRBC1, CD1a, and CCR9 (10–12), is more desirable. However, this latter approach is limited since only a proportion of T-ALL cases express target-selective antigens. Identification of additional targets is required to maximize the proportion of patients with T-ALL who can benefit from CAR-T cell therapies. Moreover, in B-ALL, antigen-negative escape has emerged as a major mechanism of resistance. Dual-targeting approaches are being explored in clinical trials and it is likely that analogous strategies will be required for treatment of T-ALL (13, 14).

CD21 (CR2, complement receptor 2) is a type-1 transmembrane glycoprotein containing 15/16 highly conserved short consensus repeats (SCRs). It is expressed on mature B lymphocytes and follicular dendritic cells (FDCs), where it binds complement within the B cell co-receptor

complex alongside CD19, CD81 and CD225 (*15, 16*). It has no known expression on non-immune tissues.

Here, we explore CD21 as a promising CAR-T target for treatment of patients with T-ALL and demonstrate that CD21 is expressed in 50% of patients with T-ALL but only in 10% of normal T cells. CD21 is a complex CAR-T target due to its large, flexible and highly glycosylated extracellular domain. Despite this, we show that anti-CD21 CAR-T cells targeting membrane-proximal epitopes and using a Fab (fragment of antigen binding) antigen binding domain have robust in vitro and in vivo anti-tumor activity in multiple T-ALL models. Further, we show that anti-CD21 CAR-T efficacy can be enhanced through phosphatidyl inositol-3 kinase (PI3K)-mediated pharmacological upregulation of CD21 antigen density, representing a promising new potential treatment strategy for patients with T-ALL.

RESULTS

CD21 is aberrantly expressed in T-ALL

We first aimed to investigate the expression of CD21 (Fig.1A) across published datasets of T-ALL. CD21 (*CR2*) RNA expression was detected in 80% (210/264) of T-ALL cases in a published pediatric cohort (Fig.1B), across all molecular subtypes (Fig. 1C) (17). In this pediatric dataset (17) and a second adult dataset (UKALL14) (18) *CR2* RNA expression correlated with expression of the *NOTCH1* target *DTX1*, with only limited correlation with *CCR9*, consistent with the known NOTCH control of *CR2* expression (19) (Fig. S1A-D). Next, we evaluated expression across immortalized T-ALL cell lines. Seventy percent (11/16) of T-ALL cell lines we tested by flow cytometry were CD21-positive (defined as >20% of cells positive), with a mean antigen density (antibodies bound per cell, ABC) of 2538 (Fig. S2A-B). 11/13 cell lines with *NOTCH1* mutations expressed CD21, whereas three *NOTCH1* wild-type cell lines were CD21-negative (Fig.S2A).

Next we evaluated primary patient samples that we collected from the UK CellBank, Great Ormond Street Hospital, or University College London Hospital. Fifty percent (29/58) of T-ALL bone marrow samples obtained at diagnosis (43/54 where age data was available were from individuals aged <18 years) expressed CD21. In most positive samples, CD21 was uniformly expressed, with only a few cases showing heterogeneity of expression. (Fig.1D, fig.S3A). The mean ABC of CD21 surface expression on diagnostic samples was 1265 (range 408-2875). CD21 was minimally expressed on normal T cells in these samples (>10-fold lower than lymphoblasts, mean of 80 antigens/cell, fig.1E).

Next we evaluated if CD21 expression varied according to maturation stage in these same patient samples. We found the highest expression was seen in cortical (80%), pre-T (72%), and mature T-ALL (67%), and lesser expression was present in early T precursor (ETP)-ALL

(25%) and pro-T-ALL (17%) (Fig.1F). We also evaluated CD21 expression in pediatric and adult cases of relapsed or refractory T-ALL collected from similar sources. Forty-five percent (5/11) of marrow samples obtained from patients with relapsed T-ALL were CD21+ by flow cytometry (mean ABC 820). Seventeen percent (5/30) of samples from patients with primary progressive T-ALL expressed CD21 (mean ABC 969) (Fig.1G, fig.S3A).

To evaluate the potential for CD21 CAR-T in a dual-targeting approach, we used flow cytometry to compare CD21 expression to previously described T-ALL targets CD7, CCR9 and CD1a in 50 of these already described diagnostic T-ALL samples, where extended phenotyping was available. Sixty-nine percent were CCR9+, 57% CD21+ and 20% CD1a+. In this cohort, 14% of cases were CD21+ but CCR9-negative, 26% were CCR9+ but CD21-negative and 8% were CD1a+ but CD21-negative. Considering co-expression with CD21, 43% of cases were CD21+CCR9+ whereas only 12% were CD21+ CD1a+. Only 16% of cases did not express either CCR9 or CD21. As expected, 48/50 (96%) cases were CD7+ (Fig.S4). Thus, dual-targeting anti-CCR9 and anti-CD21 could allow T cell-sparing CAR-T treatment for 84% of patients in this cohort.

CD21 expression is limited to B cells and a minor fraction of T cells

Next, we examined the expression of CD21 (*CR2*) in normal tissues. Analysis of the Human Protein Atlas showed that *CR2* RNA expression in peripheral blood is limited to B cells and a small fraction of T cells (Fig.S5A). No expression of *CR2* on non-lymphoid normal tissues was seen (Fig.S5B). To explore expression in thymic subsets, we obtained thymic tissues from children undergoing incidental thymic resection during cardiac surgery. *CR2* was expressed on developing thymocytes: highest on immature double-negative cells, before becoming lost in mature double- and single-positive subsets (20) (Fig.S5C). Next, we used flow cytometry to

assess protein expression of CD21 in blood from healthy individuals (n = 12). CD21 was highly expressed on B cells, but was not detected on granulocytes, natural killer (NK) cells, NK-T cells or monocytes. CD21 was detected on few $\alpha\beta$ -T cells with similar expression in CD4 (mean 10% CD21+) and CD8 cells (mean 11% CD21+, fig.1H). CD21+ T cells in these healthy donor samples expressed CD21 at low surface density (mean T cell CD21 ABC 316), 10-fold lower than B cells and 4-fold lower than T-lymphoblasts. CD21 expression was highest in $\gamma\delta$ and naive T cell subsets (mean 15 and 20% respectively), still at low density (mean naïve T cell ABC 316, mean $\gamma\delta$ -T cell ABC 335, mean B cell ABC 4250) (Fig. 6A-B). CD21 was not upregulated on activated T cells and showed no correlation with the activation marker HLA-DR (Fig.S6C-D). These experiments suggested that CD21 is a T-ALL-selective immunotherapy target without expression on most T cells or other essential tissues.

CARs targeting CD21 membrane-distal epitopes are ineffective

Next we attempted to make CAR-T cells targeting CD21. Six anti-CD21 single-chain variable fragments (scFvs) were selected from an immune phage library, generated by immunizing Wistar rats with the full-length CD21 ectodomain (19). All six scFvs exhibited nanomolar binding affinities (Fig.2A). To map their binding epitopes on CD21, we engineered variants of Raji wild-type (WT) cells, a B cell lymphoma line that natively expresses CD21. Using CRISPR-Cas9, we deleted CD21 expression to create Raji-CD21KO cells and then retrovirally transduced these with truncated CD21 constructs, each expressing one of three variants: the five membrane-proximal short consensus repeats (SCRs) (Raji TrCD21 SCRs 11–15), the five middle SCRs (Raji TrCD21 SCRs 6–10), or the five membrane-distal SCRs (Raji TrCD21 SCRs 1–5) (Figure 2B). All six scFvs bound full-length CD21 on Raji WT cells, with five out

of six recognizing epitopes within the membrane-distal SCRs 1–5, whereas none bound to the membrane-proximal regions (Fig.2B).

From these scFvs, second-generation CARs were constructed (Fig.2C) incorporating a CD8 stalk spacer, a CD8 transmembrane domain, and a 41BB-z endodomain. To test their efficacy, SUPT1 cells, a T-ALL cell line natively expressing CD21, were retrovirally transduced to express CD19, generating CD19⁺ SUPT1 cells (CD21 ABC: 4535; CD19 ABC: 84,794). This setup enabled benchmarking of CAR performance against a control anti-CD19 CAR. To generate negative control cells, CD21 expression was separately deleted in SupT1 cells using CRISPR-Cas9, creating SupT1-CD21KO cells (CD21 ABC: 0; CD19 ABC: 0). Initial CAR-T constructs showed no in vitro functionality, with no antigen-specific secretion of interferon-gamma (IFN- γ) (Fig.2D) or interleukin-2 (IL-2) (Fig.2E) in co-culture with CD19⁺ SUPT1 cells (orange). Testing two CAR constructs (C43 and C48) with either a short IgG1 hinge spacer or a long IgG1 CH2-CH3 spacer (mutated to prevent Fc binding) failed to improve function (Fig.S7).

To investigate the underlying cause of poor efficacy, we engineered SUPT1-CD21KO cells to express a truncated CD21 variant limited to the membrane-distal SCRs 1–5 (SupT1-21 SCRs 1–5; CD21 ABC: 3948; CD19 ABC: 0). This configuration was expected to create a shorter cell-to-cell distance and potentially improve immunological synapse formation. Indeed, the same CARs demonstrated antigen-specific cytokine secretion only in co-culture with SupT1-21 SCRs 1–5 cells (blue), which could not be attributed to higher CD21 antigen density compared to CD19⁺ SUPT1 cells (Fig.2E). These results suggest that the membrane-distal location or accessibility of the epitope was responsible for the poor in vitro functionality observed with these initial constructs.

CARs targeting CD21 membrane-proximal epitopes show improved in vitro function

To optimize CAR signaling a new immune library was generated from Wistar rats immunized with truncated CD21 (membrane-proximal domains, SCRs 11-15). Sixteen scFvs with nanomolar binding affinities were identified through bio-panning followed by manual screening (NM1/NM2) or PacBio next-generation sequencing of the panned library (Ph-derived) (21) (Fig.S8A). One scFv (H1) was derived from a hybridoma generated from the same rats. All but one scFv bound to the 5 most membrane-proximal domains on CD21 (Raji TrCD21 SCRs 11-15). (Fig.S8B) A further scFv was derived from the mouse anti-CD21 antibody clone Bu32, which bound to SCR 11-15 with a higher binding affinity than any phage display-derived scFv. (Fig.S8C). Binder diversity was relatively limited with each of the 16 scFvs belonging to 1 of 9 highly similar heavy chain CDR (complementarity-determining region) 3 families (Fig.S9).

All scFvs were cloned into the same CD8STK-CD8TMD-41BBz CAR architecture as previously (Fig.2C). Here, 4 CARs (NM2, Ph9, Ph10, Ph12) had CD21-specific cytotoxicity (Fig.S10A). NM2, Ph9 and Ph10 have similar VH CDR3 suggesting a similar binding epitope (Fig.S9). Multiple CARs triggered in vitro IFN- γ secretion against SupT1-WT (CD21+); however, high basal IFN- γ secretion in co-culture with SupT1-21KO (CD21-negative) cell line was also present (Fig.S10B). No CD21-specific IL-2 secretion was observed (Fig.S10C). We confirmed that several aCD21 CAR-T cells secreted high amounts of IFN- γ even when cultured in the absence of targets Fig.S10D). These results suggested to us that the high basal IFN- γ secretion seen was due to ligand-independent tonic signaling.

Anti-CD21 Fab-CARs exhibit CD21-specific cytotoxicity and cytokine secretion

To attempt to improve CAR function, selected scFvs recognising membrane-proximal epitopes were reformatted into 41BBz Fab CARs, using a bi-cistronic vector to express anti-CD21 VH fused to human IgG1-hinge and CD28 transmembrane domain/ 41BBz endodomain; and anti-CD21 VL fused to Ig-K constant (Fig.3A-B). We hypothesized that this backbone would provide greater CAR stability and efficacy. To further investigate tonic signaling and the effect of Fab architecture upon this, we first expressed both NM2 scFv-CAR and NM2 Fab-CAR in a CD21-knockout Jurkat NFAT (nuclear factor of activated T cells) reporter cell line. The scFv-CAR showed high ligand-independent signaling, which was significantly reduced with Fab architecture ($P = <0.0001$) (Fig.3C). We then selected 4 scFv-CARs (NM2, Ph9, Ph10, Ph12) which had anti-CD21 cytotoxicity but also basal cytokine secretion (Fig.S10B and D), cloned their Fab-CAR counterparts, then expressed these constructs in T cells. In a 7-day tonic signaling assay where CAR-T were plated without target cells, the antigen-independent expansion noted for all scFv-CARs was significantly reduced in 3 of 4 Fab-CARs (NM2 $P=0.0420$, Ph10 $P=0.0101$, Ph12 $P=0.0367$) (Fig.S11A). Ph9 Fab-CAR also had significantly reduced antigen-independent proliferation ($P=0.0138$) and expression of the activation/exhaustion marker LAG3 ($P=0.0011$) (Fig.S11C). Two Fab-CARs (NM2 $P=0.0003$, Ph9 $P=<0.0001$) also had significantly reduced basal IFN- γ secretion (Fig.S11D). No difference was seen in T cell differentiation between scFv- and Fab-CARs (Fig.S11E).

We next evaluated the efficacy of these 4 Fab-CARs against CD21-positive and CD21-negative T-ALL cell lines. All demonstrated CD21-specific cytotoxicity and cytokine secretion (Fig.3D-F), with NM2 Fab-CAR showing the greatest potency across multiple donors (Fig.3G-H). As expected, the cytotoxicity and cytokine secretion of the NM2 Fab-CAR were lower than those of the control anti-CD19 CAR, given the 20-fold higher density of CD19 on SUPT1-19 cells

(CD19 ABC: 84,794) compared to CD21 (CD21 ABC: 4,535). The potency of the NM2 Fab-CAR was further confirmed in experiments using SUPT1-WT cells, which are CD19-negative (Fig.S12A-B).

Fab CARs had lower surface expression and enhanced stability

We next investigated multiple engineering approaches to enhance the potency of anti-CD21 CAR-T cells and reduce tonic signaling. Initially, we hypothesized that phage display-derived scFvs might inherently promote tonic signaling. However, CARs generated from the high-affinity, proximal-binding antibody Bu32 (KD: 2.46×10^{-10}), derived from a mouse hybridoma, also showed high non-specific IFN- γ secretion and were non-functional in both scFv- and Fab-CAR formats (Fig.S12C-D). Next, we examined whether high tonic signaling was linked to overexpression from the SFG gammaretroviral vector. However, switching the NM2 scFv-CAR to a lentiviral vector (pCCL) with an EF1a promoter did not improve cytotoxicity, cytokine secretion, or tonic signaling (Fig.S12E-F). We then explored construct-specific reasons for the improved performance of the NM2 Fab-IgG1-CD28TMD-41BBz (NM2 Fab) CAR. Using whole murine IgG2a, where the binder is naturally in a Fab format, NM2, Ph9, Ph10 and Ph12 and their corresponding scFv-Fc binders had equivalent binding characteristics (Fig.4A). We also evaluated thermal stability of binders and showed that our lead candidate NM2 Fab binder was uniquely stable, remaining folded up to 95°C (Fig.4B). Additionally, NM2 Fab-CAR showed significantly lower cell-to-cell avidity than NM2 scFv-CAR ($P < 0.0001$) or Bu32 scFv-CAR ($P = 0.0028$) (Fig.4C).

The impact of CAR surface expression density was also investigated. NM2 Fab-CARs had lower surface expression than scFv-based CARs, regardless of whether lentiviral or

gammaretroviral expression systems were used (Fig.4D-E, fig.S12E). To explore the functional impact of reduced CAR expression further, we generated NM2 scFv-CAR constructs with an upstream internal ribosome entry sequence (IRES), resulting in significantly reduced CAR surface expression, $P<0.0001$ (Fig.4F). This was associated with significantly decreased basal cytokine secretion when CARs were plated alone, $P=0.0465$ (Fig.4G). However, although CD21-specific cytotoxicity was comparable between IRES-modified NM2 scFv-CAR and NM2 scFv-CAR (Fig.4H), IRES NM2 scFv-CAR had less specific cytokine secretion (Fig.4I). Thus, reduced surface expression could not fully explain the enhanced potency of NM2 Fab-CAR.

We also evaluated the role of the spacer and transmembrane domains (TMD) in potency and tonic signaling. The NM2 Fab-CAR employed a human IgG1 hinge spacer and CD28 transmembrane domain, whereas initial scFv-CARs used a CD8 spacer and CD8 TMD. NM2 CAR constructs incorporating either scFv or Fab architecture and varying spacer/TMD combinations were assessed. Both NM2 scFv-CARs were poorly effective: NM2 scFv-8-8 exhibited low cytotoxicity against SUPT1-19 targets, and NM2 scFv-H-28 showed non-specific killing of SUPT1-CD21KO cells (Fig.S13A). These scFv-CARs also demonstrated high basal IFN- γ secretion (Fig.S13B) and elevated surface expression compared to Fab-CARs (Fig.S13C). Among Fab-CARs, NM2 Fab-8-8 displayed specific cytotoxicity but reduced IFN- γ and undetectable IL-2 secretion. NM2 Fab-H-28 achieved the best performance, demonstrating specific cytotoxicity and robust secretion of both IFN- γ and IL-2 (Fig.S13D). Our findings suggest that the optimal performance of NM2 Fab-H-28 CAR-T cells is attributed to the targeting of a membrane-proximal epitope, enhanced binder stability, and reduced CAR surface density.

NM2 CD21 Fab CAR is effective against CD21 low-density T-ALL cell lines and PDX

NM2 Fab-IgG1-CD28TMD-41BBz CAR, hereafter referred to as simply NM2 Fab-CAR, was selected as our lead candidate for further evaluation. NM2 Fab-CAR-T cells proliferated (Fig.5A-B) with no difference in expansion, exhaustion, or differentiation compared to control anti-CD19 CAR-T cells (CAR19) (Fig.5C-D) in a 7-day proliferation assay with irradiated CD21⁺ SUPT1 cells. NM2 Fab-CAR-T cells were CD21-negative (Fig.S14A) and expanded post-transduction comparably to CAR19 (Fig.5E) indicating limited fratricide of CD21-positive T cells. NM2 Fab-CAR showed CD21-specific cytotoxicity and IFN- γ secretion against 3 CD21-low density T-ALL cell lines: Jurkat (ABC 618), MOLT-4 (ABC 1196), P12 (ABC 601) (Fig.5F). Further, when co-cultured with 2 CD21-low (474 and 889 CD21 ABC respectively) patient-derived xenograft (PDX) models of T-ALL (PDX1, PDX2), NM2 Fab-CAR-T specifically eliminated both T-ALL lymphoblast populations (Fig.5G). As expected, NM2 Fab-CAR-T cells were cytotoxic to B cells but not to normal T cells when co-cultured with autologous PBMCs (Fig.S14B). Moreover, when NM2 Fab-CAR-T cells were co-cultured with and without ‘spiked-in’ normal mature autologous B cells, which express higher amounts of CD21 than T-ALL blasts, there was no significant attenuation of anti-CD21 cytotoxicity (Fig.S14C).

NM2 CD21 Fab CARs exhibit anti-leukemic activity in vivo

NM2 Fab-CAR-T (CAR21) were next evaluated in a Jurkat xenograft model of T-ALL. Three million IV Jurkat cells were injected into NSG (NOD scid gamma) mice followed by 1×10^6 IV CAR21 or NT (non-transduced) T cells on day 6 (Fig.6A). Jurkat cells had low but homogenous expression of CD21 (CD21 ABC 600-750, fig.6B). CAR21-treated mice had significantly improved tumor control by BLI ($P < 0.0001$ by simple linear regression of transformed

radiance, fig.6C), and prolonged survival compared to mice which received NT T cells (median 41 versus 22 days, HR 0.05323, $P = 0.0034$ by log-rank test, fig.6D). Residual tumor at necroscopy in CD21-CAR recipients showed CD21 downregulation in spleen ($P=0.0013$ by unpaired t test) but not bone marrow (NS by unpaired t test, $P=0.1085$) compared to control mice (Fig.S15A). There were no persistent detectable T cells at necroscopy.

We next tested CAR21 in 2 clinically relevant PDX models of T-ALL. In the first PDX (PDX1), 0.8×10^6 CAR21 or CAR19 cells were injected IV on D+20 after 1×10^6 IV PDX cells in NSG mice (Fig.6E). CD21 was expressed on the cell surface at an ABC of 888 (fig.6F). CAR19 recipients experienced rapid disease progression with death by D40. All CAR21 recipients had no detectable leukemia in blood by day 48 (Fig.6G). CAR21 mice had a significant survival advantage and were all alive and well at experiment-end ($P=0.0023$ by log-rank, HR = 0.042, fig.6H). Most mice had no detectable CAR-T cells by day 41 (Fig.S15B).

The second PDX (PDX2) expressed CD21 at lower antigen density (CD21 ABC 404). In this model, 1×10^6 T-ALL lymphoblasts were followed by 4×10^6 CAR21 or CAR19 cells on day 20 in NSG mice. Mice were monitored with bi-weekly bleeding (Fig.6I-J). CAR19-treated mice rapidly progressed, and all had died by day 48. CAR21-treated mice had tumor control, with complete disease eradication in the blood by day 57 (Fig.6K) and improved survival (median survival 48 versus 115 days, HR 0.257, $P=0.0114$ by Log-Rank test, fig.6L, fig.S15E). There were late relapses in CAR21 mice in this model leading to death due to increased leukemic burden, with tumor cells detected in spleens of 3/4 mice at necroscopy. Tumor relapse was associated with loss of detectable CAR-T cells in this immunodeficient model. Tumor control at this stage was achieved in a single mouse with detectable T cells. (Fig.S15C). Tumor cells at relapse remained CD21-positive (Fig.S15D).

PI3K/mTOR inhibition increases CD21 expression and enhances CAR-T function.

In primary T-ALL samples tested, CD21 exhibited a range of antigen density, with some samples potentially falling below the threshold required for effective CAR-T targeting. To address this, we investigated pharmacological strategies to enhance CD21 expression and evaluated their impact on CAR-T cell performance. We focussed on the Phosphatidylinositol-3 Kinase (PI3K) pathway, as PI3K inhibition of T-ALL cell lines has been demonstrated to activate the NOTCH-MYC pathway and upregulate CD21 (22).

We initially investigated the effect of incubation with PI3K/mTOR/AKT pathway inhibitors (AZD5363 [Akt], GDC0941 [pan PI3K], BEZ235 [pan PI3K+mTOR] and rapamycin [mTOR]) on CD21 expression across six T-ALL cell lines. Rapamycin produced a significant increase in CD21 expression ($P = 0.0314$) with a trend to upregulation with AZD5363, GDC0941 and BEZ235. Conversely, the γ -secretase inhibitor L685 significantly downregulated CD21 expression ($P = 0.0248$) (Fig.S16A). CD21 modulation persisted up to 48hrs after drug removal (Fig.S16B). CD21 modulation did not occur in a CD21-negative cell line, on normal B or T cells (Fig.16C), or in activated T cells (Fig.16D).

We next investigated the clinically-approved dual PI3K $\alpha\delta$ inhibitor copanlisib in vitro, and saw significant at least 2-fold increases of CD21 expression across 4/5 T-ALL cell lines tested (MOLT-4 $P = 0.0197$, CUTLL-1 $P = <0.0001$, SUPT-1 $P = 0.0069$, RPMI-8402 $P = 0.0013$, fig.7A). The impact of copanlisib was specific to CD21, as, excepting CCR9 on SUPT1, there was no change in CCR9 or CD7 expression across 4 T-ALL cell lines (Fig.S16E). This effect could also be reproduced in a CD21-low PDX T-ALL sample (PDX1) in vitro (Fig.7B-C).

We next explored whether PI3K-mediated CD21 upregulation would improve in vitro CAR21 functionality. In MOLT-4 cells, an increase in CD21 after 48hr pre-incubation with copanlisib

was associated with significantly increased IFN- γ ($P=0.0093$) and IL-2 ($P=<0.0001$) production by CAR21 T cells (Fig.7D). This was replicated in co-cultures with 2 further T-ALL cell lines, CUTLL-1 and RPMI 8402 (Fig.S16F). Conversely, and as expected given its known inhibitory effect on T cells, when copanlisib was added directly to the co-culture rather than removed after pre-incubation, there was a significant attenuation of IFN- γ ($P=<0.0001$) and IL-2 ($P=0.0004$) production (Fig.S16G).

We finally investigated the effects of copanlisib on CD21 expression in vivo. CD21 upregulation after copanlisib treatment was seen in NSG mice engrafted with either MOLT-4 cells (Fig.7E) or a PDX T-ALL sample (PDX2, Fig.7F). To confirm the hypothesis that copanlisib pre-treatment may increase low CD21 expression above a threshold required for CAR-T efficacy, we identified a PDX T-ALL sample with very low CD21 density (PDX3, 207 ABC) and engrafted a further cohort of NSG mice. After 3 weeks, mice were treated with either copanlisib or PBS control. Upregulation of tumor CD21 expression with copanlisib was confirmed (mean CD21 ABC 207 PBS versus 338 copanlisib, fig.7H, $P = 0.0159$). After a 48hr washout period (~ 8 half-lives), mice were treated with a low dose (0.5M) of CAR21 T cells. Tumor control at experiment end was seen only in those mice pre-treated with copanlisib ($P = <0.0001$) (Fig.7I), associated with significant T cell expansion from day 10 onwards, $P=0.0032$ (Fig.7J,) only in these mice. These findings were confirmed in both bone marrow (Fig.S16H) and spleen (Fig.S16I) of mice at necroscopy.

DISCUSSION

Patients with r/r T-ALL face a poor prognosis (2, 5, 23). Salvage chemotherapy followed by allogeneic stem cell transplantation (allo-HSCT) is the only current curative approach but is often unsuccessful and limited to younger patients without co-morbidities (24, 25). In contrast, anti-CD19 CAR-T cell therapy for r/r B-ALL can induce durable remissions (13). This success has spurred interest in CAR-T cell approaches for r/r T-ALL, where there is a clear unmet need.

An analogous approach to anti-CD19 CAR-T cell therapy is targeting pan-T cell antigens, with CD7 being the most investigated (8, 9, 26–28). A recent phase I study reported 95% bone marrow MRD (minimal residual disease)-negative complete responses (26). However, targeting pan-T cell antigens may cause CAR-T cell fratricide and T cell aplasia. Early anti-CD7 CAR-T data showed expansion of naturally-occurring CD7-negative T cells, but overall T cell numbers remained low with opportunistic infections and viral reactivation described, as well as deaths due to monocytopenia (8, 29, 30). Phase-II data with longer follow-up revealed that most patients relapsed without consolidative transplant, and 30-50% experienced CD7 antigen loss (8, 29).

An alternative approach is targeting selective antigens expressed by T-ALL blasts but not by normal T cells. CD1a is one such selective target, although it has only been identified as expressed by cortical T-ALL which tends to have a good prognosis (11). Anti-TRBC1/2 CAR-T cells are more relevant to mature T cell malignancies as only 1/3 of patients with T-ALL express surface TCR (T cell receptor) (10). We recently proposed CCR9 as a CAR target for T-ALL; however, it is not expressed in all T-ALL cases, with 65% positivity at diagnosis (19). Given the lack of a ubiquitously expressed selective T-ALL target antigen and the likelihood of target antigen modulation, identifying additional T-ALL targets is necessary.

Here we explored CD21 as a leukemia-selective CAR target in T-ALL. CD21 is also found in mature B cell malignancies such as CLL (chronic lymphocytic leukemia), DLBCL (diffuse large B cell lymphoma) and follicular NHL (non-hodgkin's lymphoma) (31–33). Reports of CD21 expression in T cell malignancies are limited, although CD21-positivity of T-ALL cell lines and T-ALL patient samples has been previously reported (34, 35). Recent studies by the Tokyo Children's Cancer Study Group described CD21 expression in T-ALL, with highest expression in cortical T-ALL (36). Our study confirms uniform CD21 expression in approximately half of T-ALL patient samples but on <10% of normal T cells.

We compared the expression of CD21 to other T-ALL selective targets currently undergoing clinical exploration, CCR9 and CD1a. Expression of CD21 with either antigen was only partially overlapping. A co-targeting approach with CD21 CAR and either CCR9 or CD1a CAR would both increase the proportion of patients who could receive antigen-restricted CAR-T treatment, and potentially reduce the risk of antigen-negative escape.

CD21 is expressed on normal B cells and follicular dendritic cells (FDCs), so B cell aplasia is an expected limitation of CD21-directed CAR-T therapy, but it is generally well-tolerated (37). Depletion of FDCs could impair responses to infections. In mature $\alpha\beta$ T cells, CD21 was expressed at very low density on only <10%, with highest expression in naïve subsets, as described in the literature (38). Accordingly, anti-CD21 CAR-T cells were not affected by fratricide and T cell aplasia is an unlikely consequence of therapy. The functional consequences of CD21⁺ T cell depletion are unknown, but CR2 knockout mice (which lack both CD21 and CD35) exhibit impaired IgM and IgG responses without a T cell deficit. Previous antibody-based CD21 therapies for B cell malignancies resulted in minimal toxicity (39, 40).

Of note, CD21 was expressed on a small proportion (mean 15%) of $\gamma\delta$ -T cells, suggesting anti-CD21 CAR-T could lead to partial depletion of these cells. There is no data on the role of CD21 in $\gamma\delta$ -T cells, or if CD21+ $\gamma\delta$ -T represent a unique subtype. Further, pan- $\gamma\delta$ -T cell depletion presents largely unknown clinical consequences. Theoretically, there may be a compromise of early-stage immune responses against intracellular pathogens and tumors, though murine models suggest that the phenotype may be relatively mild. Detailed exploration of this issue will require clinical testing.

CD21 is expressed at relatively low density in T-ALL (approx. 1300 ABC) compared to CD19 in B-ALL (>10000 ABC) (41) but similar to other successful CAR targets like BCMA (~1500 ABC) (42). Although anti-CD21 CAR-T cells were effective at low CD21 density, there is likely a correlation between antigen density and CAR-T potency (41). We found that inhibiting the PI3K/mTOR pathway upregulated CD21 in T-ALL, enhancing CAR-T cell efficacy both in vitro and in vivo. Indeed, copanlisib pre-treatment enabled CAR-T control of a PDX sample with CD21 ABC of only 207, in which CAR-T cells alone were not effective. Previously we showed that PI3K inhibition led to increased activation of NOTCH target genes, increased *MYC* RNA expression and increased surface CD21 expression in T-ALL cells (22). This was likely mediated by glycogen synthase kinase-3 (GSK-3) (43–45). However, PI3K inhibitors can impair T cell function, (46) so careful clinical strategies will be needed to balance these effects. These could include sequential targeting / intermittent use of PI3K inhibition to upregulate CD21 density, whilst minimizing inhibitory effects on T cells.

A further challenge for CD21 as a CAR-T target is its long, flexible, highly glycosylated structure (47, 48). At 146kDa, it is larger than well-studied CAR-T targets like CD19 and even CD22 (49). Our initial scFv-based anti-CD21 CARs targeting membrane-distal epitopes were not functional, mirroring findings with CARs targeting membrane-distal CD22 epitopes (50).

We therefore tested CARs with scFvs recognising CD21 membrane-proximal epitopes. These resulted in some anti-CD21 activity but remained considerably less potent than anti-CD19 CAR-T.

We next combined the targeting of membrane-proximal epitopes with a Fab-CAR architecture, hypothesizing that the increased stability of the Fab format would enhance potency. CD21 Fab-CARs indeed demonstrated improved efficacy, prompting further investigation into the underlying mechanisms. Initially, we considered whether increased stability might lead to higher expression of Fab-CARs; however, Fab-CARs were found to be expressed at lower surface densities compared to scFv-CARs, likely due to the bicistronic expression system required. We then questioned whether this reduced expression could paradoxically enhance function. To test this, we generated an scFv-CAR with an upstream IRES element, which reduced surface expression and resulted in enhanced cytotoxicity, but also diminished cytokine secretion. This suggested that reduced expression alone does not account for the superior performance of anti-CD21 Fab-CARs.

We observed that multiple CD21 scFv-CARs exhibited degrees of tonic signaling that were reduced in Fab-CARs, which contributed to enhanced anti-tumor efficacy (49, 50). This reduction was likely due to the increased thermal stability of Fab binders (51) and their reduced tendency for clustering, which can occur in scFvs due to VH-VL domain instability and unfolding (52–54).

Additionally, we demonstrated that the use of a CD28 transmembrane domain (TMD) instead of a CD8 TMD enhanced CAR potency, as previously reported, although this did not mitigate tonic signaling (39). CD21 Fab-CARs exhibited equivalent binding affinity but lower cell-to-cell avidity compared to their scFv counterparts. Although higher avidity is often considered

beneficial for CAR-T efficacy, this is not consistently supported across all data sets (55). Our findings highlight the complexities of CAR design, emphasizing that although some principles for optimal CAR-T constructs are established, empirical testing remains essential, particularly when targeting challenging antigens.

A final anti-CD21 Fab-CAR was effective against multiple T-ALL cell lines and patient samples in vitro, and in clinically relevant in vivo models. In a Jurkat xenograft model, CAR21-treated mice had statistically improved survival over control. CD21 downregulation in the absence of CAR-T cells was seen in the spleen of mice at relapse, suggesting possible antigen-negative escape. Encouragingly this was not seen in more clinically relevant PDX models. Antigen-negative escape is a potential risk of all single antigen-targeting CAR-T and has emerged in recent clinical trials of CD7-targeting CAR-T. Ultimately, clinical testing is required to fully evaluate this risk in patients.

Our study has some limitations. The majority of the patient samples analyzed were from pediatric patients and it is possible that the expression of CD21 may differ in an adult population. In addition, due to the rarity of the disease, the number of relapsed and refractory patient samples that were available to us was relatively limited. Lastly, although our findings demonstrate the efficacy of anti-CD21 CAR-T cells in relevant pre-clinical models of T-ALL, well-designed clinical trials will be necessary to confirm their potential in patients.

Early CAR-T data in T-ALL shows promise, but current clinical-stage approaches which target pan-T cell antigens require allo-HSCT to consolidate responses and mitigate toxicity. Use of antigens selectively expressed by T-ALL blasts may avoid the limitations of these approaches. CD21 adds to such targets since it is expressed by approximately half of T-ALL cases, but only on a small population of normal T cells. CD21 is a large and bulky antigen. However, anti-

CD21 Fab-CAR-T cells showed potent anti-CD21 activity, and pharmacological upregulation of CD21 further enhanced efficacy. Anti-CD21 CAR-T cell targeting may open new avenues in the treatment of T-ALL, while avoiding fratricide and T cell aplasia.

MATERIALS AND METHODS

Study Design

This study was designed to validate expression of CD21 in T-ALL and normal tissues using cell lines, patient samples and healthy donors. The study additionally involved identification of CD21-directed antibody fragments followed by pre-clinical testing of anti-CD21 CAR-T cells using in vitro assays (cell lines, patient samples, patient-derived xenografts) and in vivo models of cell lines and patient-derived xenografts. Except where stated, all in vitro experiments were carried out on at least two separate occasions and had three technical replicates for each condition. P values were determined using methods described in the figure legends. For all in vivo experiments, a minimum of four mice were used per arm in each animal model. Mice were randomly assigned to experimental groups. Investigators were blinded to experimental circumstances during assessment and data analysis. All mice were cared for in an unbiased fashion by animal technicians. Given the discovery research nature of the study, no predefined power calculations for sample size were used.

Cell lines and maintenance

HEK-293T cell line was cultured in IMDM (Lonza) with 10% FBS (HyClone, GE) and 2mM GlutaMAX (Invitrogen). All others were cultured in RPMI (Lonza) with 10% FBS and 2mM GlutaMAX. All underwent routine mycoplasma testing and were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Samples and flow cytometry

UCL Research Ethics Committee granted ethical approval. Whole blood was obtained from consenting healthy donors or leucocyte cones from NHS Blood and Transplant. Peripheral

blood mononuclear cells (PBMCs) were isolated using Ficoll Paque (GE Healthcare) and SepMate tubes (STEMCELL technologies) following manufacturer's protocol.

T-ALL peripheral blood and bone marrow samples were obtained from UCL/UCLH Biobank, Great Ormond Street Biobank, Blood Cancer UK Leukaemia CellBank and the Biobank of Ospedale Papa Giovanni XXIII, Bergamo, Italy.

Flow cytometry was performed on a BD LSR Fortessa instrument (BD Biosciences), a CytoFLEX (Beckman Coulter) or NovoCyte (Agilent) 96-well flow cytometer. CD21 antigen density (antibodies bound per cell – ABC) was determined using Quantibrite PE beads (BD) as per manufacturer's instructions.

CD21-scFv generation

Genetic rat vaccination for CD21 was performed by Aldevron. Three rats were DNA vaccinated with a pVAC (DNA vaccine) plasmid engineered to express the full ectodomain and TMD of CD21 with a truncated endodomain (Uniprot P20023-1 AA sequence 1-1014, Supplementary Figure 17A). In a second vaccination, a further three rats were vaccinated with a pVAC plasmid expressing only SCRs 11-15 of the CD21 ectodomain with a truncated endodomain (Uniprot P20023-1 AA sequence 660-1014, Supplementary Figure 17B). Animals were culled once seroconversion was confirmed. Immune phage libraries were created using an in-house protocol. Anti-CD21 scFvs were identified through bio-panning then screening of these immune phage display libraries both manually and using PacBio next-generation sequencing (Genewiz), as previously described (21). Briefly, phage libraries were incubated with CD21 coated streptavidin beads for two/three rounds to enrich the libraries for anti-CD21 antibodies. Once bulk library enrichment for CD21 was confirmed, supernatant from individual bacterial colonies was assessed for binding to CD21 by flow cytometry. DNA was extracted

from colonies showing greatest binding to CD21 and sequenced using sanger sequencing. In addition, DNA from the second round of bio-panning was amplified using pHEN1 phagemid vector specific primers. Amplicon DNA was then sequenced using Pacific Biosciences sequencing technology by Genewiz.

Surface plasmon resonance

Kinetic analysis was performed on a Biacore T200 (GE Healthcare) using recombinant human CD21 protein covalently coupled to a CM5 sensor chip according to manufacturer's recommendations. Serial dilutions of scFvs with a murine IgG2a Fc were captured and injected over the flow cells at a rate of 30ul/minute and equilibrium binding analysis was performed. Kinetic rate constants were obtained by curve fitting according to a Langmuir 1:1 binding model. Data analysis was performed using Biacore Insight Evaluation software v3.0 (GE Healthcare).

Differential scanning fluorimetry

A Prometheus NT.48. (NanoTemper) was used. The emission of fluorescence radiation was measured at wavelengths 330nm and 350nm across a temperature range from 20°C to 95°C at a rate of increase of 1°C min⁻¹ and used to determine the protein melting temperatures.

Avidity measurements

MOLT4 cells were seeded on poly-L-lysine coated z-Movi microfluidic chips at a density of 160 x10⁶ cells/mL. Cells were incubated for 0.5 hour in serum free media followed by 2-hour incubation in complete media. Effector cells were stained with Cell Trace far-red (ThermoFisher Scientific, C34564) at 1:000 dilution. Labelled effector cells were introduced on to the chip at a density of 10 x10⁶ and incubated for 10 minutes prior to acoustic force

application using z-Movi cell avidity analyser. Detachment of effector cells was analysed using Ocean 1.4 software.

Generation of CAR-T cells

CAR constructs were expressed in the SFG vector unless otherwise stated and retroviral supernatant generated through transient transfection of HEK293T cells. Donor PBMCs were transduced using a standardized protocol as previously described. (19)

Generation of engineered cell lines

Raji and SUPT1 CD21 knockout (KO) cell lines created using CRISPR/Cas9 engineering were donated by Autolus Ltd. Retroviral vectors containing CD21 SCRs 1-5, 6-10 and 11-15 and a BFP marker were used to transduce SUPT1 and Raji CD21 KO cell lines. Cells were bulk-sorted for CD21⁺ BFP⁺ populations, then single-cell cloned.

In vitro cytotoxicity and cytokine release assays

Target cells were stained with CellTrace Violet (CTV) (Thermofisher), plated at 25-50,000 per well with three technical repeats. CAR-T cell to target cell ratios of 1:1, 1:2, 1:4, 1:8 and 1:16 were used. Controls were non-transduced (NT) cells, a non-binding aCD21 CAR (Ph2) and aCD19 FMC63 CAR. At 48hrs, enzyme-linked immunosorbent assay (Biolegend) for IFN- γ and IL-2 was performed on cell supernatant. 72hr co-culture of primary T-ALL cells were performed using allogeneic donor T cells. Residual lymphoblasts were identified using human CD45, murine CD45, CD3 and CTV staining.

In vitro proliferation assays

CAR-T cells labelled with CTV were co-cultured with irradiated (30Gy) target cells in a 1:2 E:T ratio for 7 days. CTV MFI on days 0 and 7 determined CTV dilution and fold-expansion

from baseline. CAR-T cell counts were established by staining with aCD34 antibody for the RQR8 marker gene.

Jurkat NFAT reporter cell line assays

Jurkat cells were engineered to express enhanced green fluorescent protein (GFP) under the control of the NFAT (nuclear factor of activated T cells) promoter. *CR2* gene was deleted from cells by CRISPR-Cas9 and single-cell cloned to produce a Jurkat_NFAT-GFP_CD21KO reporter cell line. Anti-*CR2* guide-RNA was designed using CRISPOR (CTCTAGGACGATTTCCCAAT). This line was transduced with CAR constructs and GFP surface expression in transduced cells was assayed on day 7.

Tonic signaling assays in PBMCs

Healthy donor PBMCs were transduced with CAR constructs and stained for CTV as previously described. 100,000 transduced cells/well were plated without targets or exogenous cytokines. Readouts of CTV MFI, cytokine secretion and exhaustion/differentiation were performed on days 0, 7, 9, 12 and 14.

In vivo models of T-ALL

Work was performed under a UK Home Office-approved project license (PP8379762) and was approved by the UCL Biological Services Ethical Review Committee.

PDX1 was derived from a 10-year old boy with biallelic *CDKN2A*-deleted T-ALL. PDX2 was derived from a 1-year old girl with *ATM*-deleted T-ALL. PDX3 was from a 14-year old boy with a *STIL* deletion. All were developed at the Institute of Child Health by Professor Owen Williams or in-house.

Female and male NSG mice aged > 8 weeks (Charles River) were used, with groups matched for sex. Mice were culled over behavioural concerns, >15% weight loss or at pre-specified time points. Cell suspensions were injected by tail vein. 50ul tail vein bleeds were taken at set timepoints. Tumor cells were identified as human CD45+ CD3-negative. Bioluminescence (BLI) imaging was performed using the IVIS system (PerkinElmer). General anesthesia was induced using inhaled isoflurane. Following induction, intraperitoneal injection (IP) of luciferin (200 µl) was undertaken.

Pharmacological manipulation of CD21 antigen density

Drug concentrations used: L685 (1uM), GDC0941 (1uM), BEZ235 (1uM), AZD5363 (5uM), rapamycin (20nM), copanlisib (100nM). Target cells at 5×10^5 /ml were incubated with drugs in 24-well or 6-well T cell coated plates for 24, 48 or 72 hours. T lymphoblasts were maintained in culture using the HS5 stromal cell line, donated by Professor Kwee Yong. Blasts were identified as CD7+ BFP-negative. Drugs were washed off three times using sterile PBS. *In vivo*, 14mg/kg / 19mg/kg copanlisib or DMSO/PBS control was injected intraperitoneally on two consecutive days and CD21 expression was assessed at 24hours post-treatment.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v9 for Windows (GraphPad Software). Student's t test or 2-way ANOVA were used for samples with normally-distributed variables as indicated in the text. Survival curves were generated using the Kaplan-Meier method with hazard ratios calculated by the log-rank method. $p < 0.05$ showed statistical significance. Data is summarized as mean +/- standard deviation unless otherwise specified.

LIST OF SUPPLEMENTARY MATERIALS

Materials and Methods

fig S1 – S17)

MDAR Reproducibility Checklist

Data File S1

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AK, MP and NM conceptualized and designed the study. Funding for the study was acquired by AK and MP. Research supervision was provided by MP, MM, AK and PM. The animal license for in vivo experiments was provided by LL. NM performed in vitro and in vivo experiments. MH performed in vitro and in vivo experiments. CA and AB performed in vitro experiments. FN undertook phage display work. PW undertook phage display work and performed avidity experiments. TK, BW, SR, MS, SN performed in vitro experiments. MF performed SPR experiments. TM assisted with immunohistochemistry. SO performed PacBio NGS analysis. DO performed flow cytometry on relapsed/refractory patient samples.

GG provided some primary patient samples. The manuscript was written by NM and edited by NM, MP and PM.

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P.M.M. and L.L. own stock and received research funding from Autolus Ltd. M.A.P., S.O and M.F. are current or previous employees of and own stock in Autolus Ltd. The remaining authors declare no competing financial interests. MP, NM and AK are listed co-inventors on a filed patent on CD21-directed CAR-T cell therapies (WO2019220108A1).

DATA AND MATERIALS AVAILABILITY:

All data associated with this study are in the manuscript or supplementary materials. All plasmids used in the study including CAR-T constructs and CD21-truncation expression vectors are available from the corresponding author upon request and completion of a Material Transfer Agreement.

FIGURES:

Figure 1: T-ALL derived cell lines and primary tumors express surface CD21 by flow cytometry with only limited expression seen on normal T cells

(A) Schematic diagram of structure of CD21 – extracellular domain consisting of 15/16 highly conserved short consensus repeats (SCRs) (B) *CR2* (CD21) RNA expression in 264 primary T-ALL cases. Data from St Jude’s dataset (Liu et al) (C) *CR2* RNA expression in same cohort according to molecular subtype. Horizontal bars represent mean value. (D) Example flow plots and histograms of three primary T-ALL samples (red = CD21, blue = isotype control) including ABC of each sample (E) Flow cytometric evaluation of CD21 antigen density (ABC) for

CD21-positive diagnostic T-ALL cases, $n = 27$ alongside CD21 density on corresponding normal T cells. Horizontal bar represents median value. **(F)** CD21 expression in diagnostic T-ALL samples by maturation status - ETP, Pro-, Pre-, cortical and mature T-ALL. $n=50$. Horizontal bars represent mean value. **(G)** ABC of CD21-positive relapsed and refractory T-ALL cases. Horizontal bars represents mean value. **(H)** CD21 expression on peripheral blood mononuclear cells (PBMCs). Six experimental repeats, $n=14$ donors. ABC = antibodies bound per cell. G-D T cells = gamma-delta T cells, TREGS = regulatory T cells, NK = natural killer, ETP - Early thymocyte precursor.

Figure 2: Chimeric Antigen Receptor (CAR)-T cells targeting CD21 distal epitopes are not functional in vitro

(A) Surface plasmon resonance (SPR) measurements for six anti-CD21 scFvs. k_{on} (k_a) = association rate constant, k_{off} (k_d) = dissociation rate constant, K_D (k_d/k_a) = equilibrium dissociation constant. **(B)** Epitope mapping of anti-CD21 scFvs against Raji CD21 mutant cell lines. Dark Green = Raji 21SCR11-15 (5 most membrane-proximal domains). Light Green = Raji 21SCR6-10 (5 middle domains). Orange = Raji 21SCR1-5 (5 most membrane-distal domains). Blue = Raji WT (naturally expresses full-length CD21 (SCRS 1-15)). Grey = Raji 21 KO (engineered to eliminate surface expression of CD21). **(C)** SFG retroviral vector with second-generation chimeric receptor architecture (CD8 stalk spacer (STK), CD8 transmembrane domain (TMD), 41BB co-stimulatory domain and CD3 ζ signaling domain). RQR8 = marker/suicide gene. **(D)** IFN- γ and **(E)** IL-2 secretion by ELISA from NT (non-transduced), anti-CD19 and anti-CD21 CAR-T cells against SUPT-1 19 (CD21 and CD19 positive), SUPT-1 21KO (CD19 and CD21 negative), and SUPT1 21 SCRs1-5 after 48hr co-

culture. $n = 3$, one experiment. Statistical comparisons by repeated measures 2-way ANOVA.

* $P = <0.05$, ** $P = <0.01$ *** $P = <0.001$ **** $P = <0.0001$, ns = not significant.

Figure 3: A Fab-CAR architecture reduces tonic signaling and enhances in vitro cytotoxicity

(A) Schematic of Fab (fragment of antigen binding) antigen-binding domain compared to scFv.

(B) Schematic of anti-CD21 Fab-CARs (SFG retroviral vector with second-generation CAR architecture (hinge spacer, CD28 TMD, 41BB co-stimulatory domain, CD3 ζ signaling domain)). LTR = long terminal repeat, 2A = 2A peptide, SP = signal peptide, VL = variable light domain, VH = variable heavy domain, CK = constant kappa domain, CH1 = constant heavy domain 1, SAR = scaffold attachment region (C) NM2 scFv- and Fab-CARs expressed in a Jurkat_NFAT-GFP-CD21KO reporter cell line. % GFP expression by flow cytometry on day 7. $n=3$. Dotted lines represent positive (14G2A) and negative (FMC63) controls. Analysed by unpaired t test **** $P = <0.0001$. (D) Cytotoxicity ($n=6$), (E) IFN- γ ($n=8$) and (F) IL-2 ($n=8$) secretion by NM2, Ph9, Ph10 and Ph12 Fab-CARs by ELISA after 48hr co-culture with SupT-19 (orange) and SupT1-21KO (grey) cells. E:T ratio = 1:8. Comparisons made using 2-way ANOVA. (G) IFN- γ and (H) IL-2 secretion from NM2 Fab-CARs and NM2 scFv-CARs after 48hr co-culture against SupT1-21KO (grey) and SupT-19 (orange). NM2 $n=9$ and NM2 Fab $n=6$. 3 experimental repeats. E:T ratio = 1:4. Pairwise comparisons by 2-way ANOVA. * $P = <0.05$, ** $P = <0.01$, **** $P = <0.0001$, ns = not significant.

Figure 4: CD21 Fab CARs show lower surface expression and enhanced stability compared to scFv-CARs

(A) Binding kinetics of Ph9, Ph10, Ph12 and NM2 scFvs and Fab (IgGs) by SPR. (B) Thermal stability of Ph9, Ph10, Ph12 and NM2 scFvs and Fab binders (IgGs) by DSF. $T_m1/T_m50 = 50\%$ protein unfolding corresponding to variable domain unfolding, T_m50 for NM2 IgG was not reached as protein had not completely unfolded by 95°C . Mean of two experimental repeats. NR = not reached (C) Immune synapse-binding avidity to MOLT4 WT (CD21+) assessed by acoustic force microfluidic microscopy. Experiment represents 1 donor CAR-T cells and un-transduced (UNT) control on each out of 4 separate chips (D) NM2 scFv versus Fab CAR21 surface expression donor T cells. $n=3$. Representative data from one experiment repeated with the same results. (E) Median IgG2a MFI between NM2 scFv-CAR (orange) and NM2 Fab-CAR (teal). $n=3$. Compared using unpaired t-test. (F) Median IgG2a MFI between NM2 scFv-CAR (grey) and NM2 IRES scFv-CAR (orange). Compared using unpaired t-test, $P < 0.0001$, $n=6$, (G) basal IFN- γ secretion (turquoise), (H) cytotoxicity, (I) IFN- γ secretion of NM2 scFv-CAR and NM2 IRES scFv-CAR against SUPT1-19 (orange) and against SUPT1-21KO (grey) after 48hr co-culture, 1:4 E:T ratio. Compared using 2-way ANOVA. * $P = < 0.05$, ** $P = < 0.01$, **** $P = < 0.0001$, ns = not significant.

Figure 5: In vitro functional evaluation of NM2 Fab anti-CD21 Chimeric Antigen Receptor (CAR)-T cells

(A) Representative histograms of CTV MFI on day 7 of proliferation assay after 1:2 co-culture with irradiated CD21+ SupT1 cells. $n=3$. (B) Day 0-7 fold-increase in CAR-T numbers in proliferation assay comparing NM2 Fab to CD19 CAR-T, $n=6$. (C) Exhaustion and (D) differentiation marker expression on day 7 (NM2 Fab and CD19 CAR-T). Data analysed by 2-

way ANOVA. $n=6$. **(E)** Fold-change in cell numbers pre- and post- CAR transduction between NM2 Fab and CD19 CAR-T. $n=11$, 4 experimental repeats. Comparisons made using a paired t-test. **(F)** 48hr cytotoxicity (left) and IFN- γ secretion (right) by ELISA of NM2 Fab-CAR (orange) and Ph2 negative control CAR (grey) against three CD21-low density cell lines, including IFN- γ secretion from CARs plated without targets, 1:4 E:T. Comparisons made using 2-way ANOVA. $N=3$ donors from one experimental repeat. **(G)** Residual cells from two CD21-low patient-derived xenograft (PDX) T-ALL samples after 72hr co-culture with allogeneic NM2 Fab-CAR T cells, CAR19 T cells and NT cells, 1:1 E:T ratio. $n=3$ donors. Comparisons made using paired t-test. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$ **** $P < 0.0001$, ns = not significant. CTV = CellTrace Violet, ABC = antibodies bound per cell

Figure 6: In vivo evaluation of NM2 Fab anti-CD21 CAR-T cells

(A) Jurkat murine efficacy model. NSG mice were injected intravenously with 3 million Jurkat cells followed by 1 million CAR-T 21 or non-transduced T cells on day 6. $n=5$ /group. **(B)** CD21 expression on Jurkat cell line. Gated on isotype control. **(C)** Bioluminescence of NT- and CAR21-treated mice over time. Data log-transformed then NT and CAR21 slopes compared using simple linear regression. ($P < 0.0001$). **(D)** Kaplan-Meier survival curve for NT- or CAR21-treated mice, compared by log-rank test. $P = 0.0034$. Hazard ratio (logrank) 0.05323, 95% CI of ratio = 0.007492 to 0.3782. **(E)** PDX1 murine efficacy model of T-ALL. 1 million T-ALL PDX cells were injected intravenously until engraftment followed by 0.8 million CAR-T 21 or CAR-T 19. $n=5$ /group. **(F)** CD21 expression on PDX1 cells. **(G)** Residual tumor % in blood of CAR19- and CAR21-treated mice **(H)** Kaplan-Meier survival curves for PDX1 mice treated with CAR19 or CAR21 cells. Curves significantly different by Log-Rank

$P=0.0023$, Mantel-Haenszel HR 0.04195, 95% CI of ratio 0.005457 to 0.3225. **(I)** PDX2 murine efficacy model. 1 million T-ALL PDX cells were injected intravenously until engraftment followed by 4 million CAR-T 21 or CAR-T 19 cells. $n=4/\text{group}$. **(J)** CD21 expression on PDX2 tumor cells. **(K)** Residual tumor cells in blood for CAR19- or CAR21-treated mice post-CAR-T. **(L)** Kaplan-Meier survival curves for PDX2 mice treated with CAR19 or CAR21 cells. Comparison by Log-rank test. $P = 0.0114$.

Figure 7: PI3K inhibition increases CD21 expression in T-ALL, enhancing in vitro CD21 CAR-T efficacy

(A) CD21 ABC change from baseline on T-ALL cell lines incubated with (orange) and without (grey) copanlisib for 48hrs. MOLT4-4 $n=4$, CUTLL-1 $n=3$, Supt-1 $n=5$, RPMI 8402 $n=3$, Jurkat $n=2$ experimental repeats. Comparisons made using 2-way ANOVA. ABC = antibodies bound per cell. **(B)** CD21 expression on T-ALL PDX2 lymphoblasts after 48hrs with a range of PI3K inhibitors and L685 in vitro. **(C)** Representative flow cytometry plots and histogram showing expression of CD21 on T-ALL PDX1 lymphoblasts before (left, grey histogram) and after (right, orange histogram) incubation with copanlisib. **(D)** IFN- γ and IL-2 secretion by NM2 Fab CAR21 cells against MOLT4 cells and MOLT4 cells with and without copanlisib. $n= 5$. Comparisons made using 2-way ANOVA. Grey = MOLT4 untreated cells. Orange = MOLT4 cells pre-treated with copanlisib. **(E)** CD21 MFI of bone marrow lymphoblasts in MOLT-4 murine model of T-ALL with or without treatment with two doses of 14mg/kg IP copanlisib, $n=3/\text{cohort}$. **(F)** CD21 ABC on bone marrow lymphoblasts in a T-ALL PDX (PDX1) engrafted in NSG mice with and without IP copanlisib, $n=4/\text{cohort}$ $P=0.17$ by unpaired t-test **(G)** Experimental timeline of a combined copanlisib and CAR21 model in NSG mice

engrafted with CD21 low T-ALL PDX3 **(H)** CD21 ABC of T-ALL lymphoblasts in blood with and without two doses of 19mg/kg IP copanlisib, $n=4/\text{cohort}$, $P=0.0159$ by unpaired t-test **(I)** % blasts in peripheral blood before and after CAR21 in control (grey) and copanlisib (orange) treated mice. **(J)** % T cells in peripheral blood before and after CAR21 in control (grey) and copanlisib (orange) treated mice. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$, ns = not significant.