Leukemia (2017) **31**, 1087–1095

**www.nature.com/leu**

**ORIGINAL ARTICLE**

# Vaccination to improve the persistence of CD19CAR gene-modified T cells in relapsed pediatric acute lymphoblastic leukemia

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Trials with second generation CD19 chimeric antigen receptors (CAR) T-cells report unprecedented responses but are associated with risk of cytokine release syndrome (CRS). Instead, we studied the use of donor Epstein–Barr virus-specific T-cells (EBV CTL) transduced with a first generation CD19CAR, relying on the endogenous T-cell receptor for proliferation. We conducted a multi-center phase I/II study of donor CD19CAR transduced EBV CTL in pediatric acute lymphoblastic leukaemia (ALL). Patients were eligible pre-emptively if they developed molecular relapse ($>5\times10^{-4}$) post first stem cell transplant (SCT), or prophylactically post second SCT. An initial cohort showed poor expansion/persistence. We therefore investigated EBV-directed vaccination to enhance expansion/persistence. Eleven patients were treated. No CRS, neurotoxicity or graft versus host disease (GVHD) was observed. At 1 month, 5 patients were in CR (4 continuing, 1 *de novo*), 1 PR, 3 had stable disease and 3 no response. At a median follow-up of 12 months, 10 of 11 have relapsed, 2 are alive with disease and 1 alive in CR 3 years. Although CD19CAR CTL expansion was poor, persistence was enhanced by vaccination. Median persistence was 0 (range: 0–28) days without vaccination compared to 56 (range: 0–221) days with vaccination ($P=0.06$). This study demonstrates the feasibility of multi-center studies of CAR T cell therapy and the potential for enhancing persistence with vaccination.

*Leukemia* (2017) **31,** 1087–1095; doi:10.1038/leu.2017.39

## INTRODUCTION

Clinical trials of second generation CD19 chimeric antigen receptors (CAR) T-cells have shown impressive outcomes in relapsed/refractory ALL[1–7] with 70–90% complete response rates and 1 year disease-free survival rates of 50–60% even in patients who have relapsed after allogeneic stem cell transplant (SCT). This approach however, has been associated with severe cytokine release syndrome (CRS) in ~30%, reflecting supraphysiological activation through CAR signaling, which may limit broader application. In addition, the persistence of CD19CAR T-cells has been limited in some studies.[4,6] Strategies to enhance the persistence of CD19CAR T cells will be critical in determining their role, in particular whether CAR T cells are used as a bridge to transplant or a stand-alone therapy.

Here, we report the results of the first multi-center, European CAR T-cell trial, CD19TPALL. Rather than using unmanipulated T cells as effectors, we have investigated utilizing donor Epstein–Barr virus-specific cytotoxic T-cells (EBV CTL) redirected with a CD19CAR. This strategy has a number of theoretical advantages. EBV CTL persist long term in SCT recipients[8] without causing graft versus host disease (GVHD). Moreover, signaling through the endogenous EBV TCR upon encounter with viral antigens should drive more physiological expansion of CAR CTL without the cytokine-mediated toxicities observed with second generation CARs.[1,4–6] Proof of concept was established In a phase I clinical trial in patients with neuroblastoma, where autologous EBV CTL engineered with a first generation GD2 CAR circulated at higher levels than non-specifically activated T-cells.[9] Finally, in case stimulation through the endogenous TCR was insufficient, we tested whether the novel strategy of vaccination with irradiated EBV transformed lymphoblastoid cell lines (LCL) could improve the expansion/persistence of CD19CAR CTL and thereby boost their anti-leukemic efficacy. Our study utilized an integrated immunotherapy strategy incorporating the use of CD19CAR transduced donor EBV CTL as effectors, lymphodepletion, administration in the context of minimal residual disease (MRD) and vaccination.

The primary study objectives were to evaluate the feasibility/safety of adoptive transfer of donor CD19CAR CTL, and to determine their biological effect on residual leukemia as assessed by bone marrow (BM) MRD. Secondary objectives were to determine *in vivo* persistence of CD19CAR CTL, whether LCL

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Received 17 August 2016; revised 21 November 2016; accepted 5 January 2017; accepted article preview online 27 January 2017; advance online publication, 10 March 2017;

vaccination improved their expansion/survival and 1 year relapse/disease-free survival after immunotherapy.

## MATERIALS AND METHODS

### Study design

This open-label phase I/II multi-center trial of CD19CAR CTL therapy in children with acute lymphoblastic leukaemia (ALL) aged ⩽18 years was conducted across six sites in the UK and Germany. It was approved by the UK/German regulatory authorities and national/local ethical review boards. Local IRB approval was obtained for patients treated off study. Written informed consent was given by parents. The study had two arms (Figure 1a). In the pre-emptive arm patients in first or subsequent CR who were at high (>50%) risk of relapse post SCT (inclusion criteria: Supplementary Table 1) were monitored in the BM for emergence of MRD monthly (months 1–6) and then 6-weekly (months 7–12) for 1 year post SCT. CD19CAR CTL were generated from stem cell donors before SCT and administered in case of MRD positivity $\geqslant 5x10^{-4}$. In the prophylactic arm, patients who had relapsed after first SCT were treated with CD19CAR CTL 60–70 days after second SCT following withdrawal of immunosuppression. Stem cell donors had to be EBV seropositive and 7–8/8 HLA-matched with the recipient. Patients in the pre-emptive arm received myeloablative conditioning (BFM protocol[10]), whereas those in the prophylactic arm received conditioning with fludarabine/treosulphan/thiotepa for their second transplant and serotherapy in those receiving grafts from unrelated donors. CD19CAR CTL infusion was contraindicated in patients with acute GVHD ⩾grade II or chronic GVHD requiring systemic steroids.

An interim analysis of safety and CTL persistence was planned after the first cohort of patients received CD19CAR CTLs alone. If CD19CAR CTL were not detectable in 50% of patients at 2 months post infusion, subsequent patients would be vaccinated with LCL.

### Generation of CD19CAR CTL

EBV-specific CTL were generated from 80 ml donor blood as described[11] by repetitive stimulation of donor T cells with LCL except that autologous and human AB serum were used. EBV CTL were then retrovirally

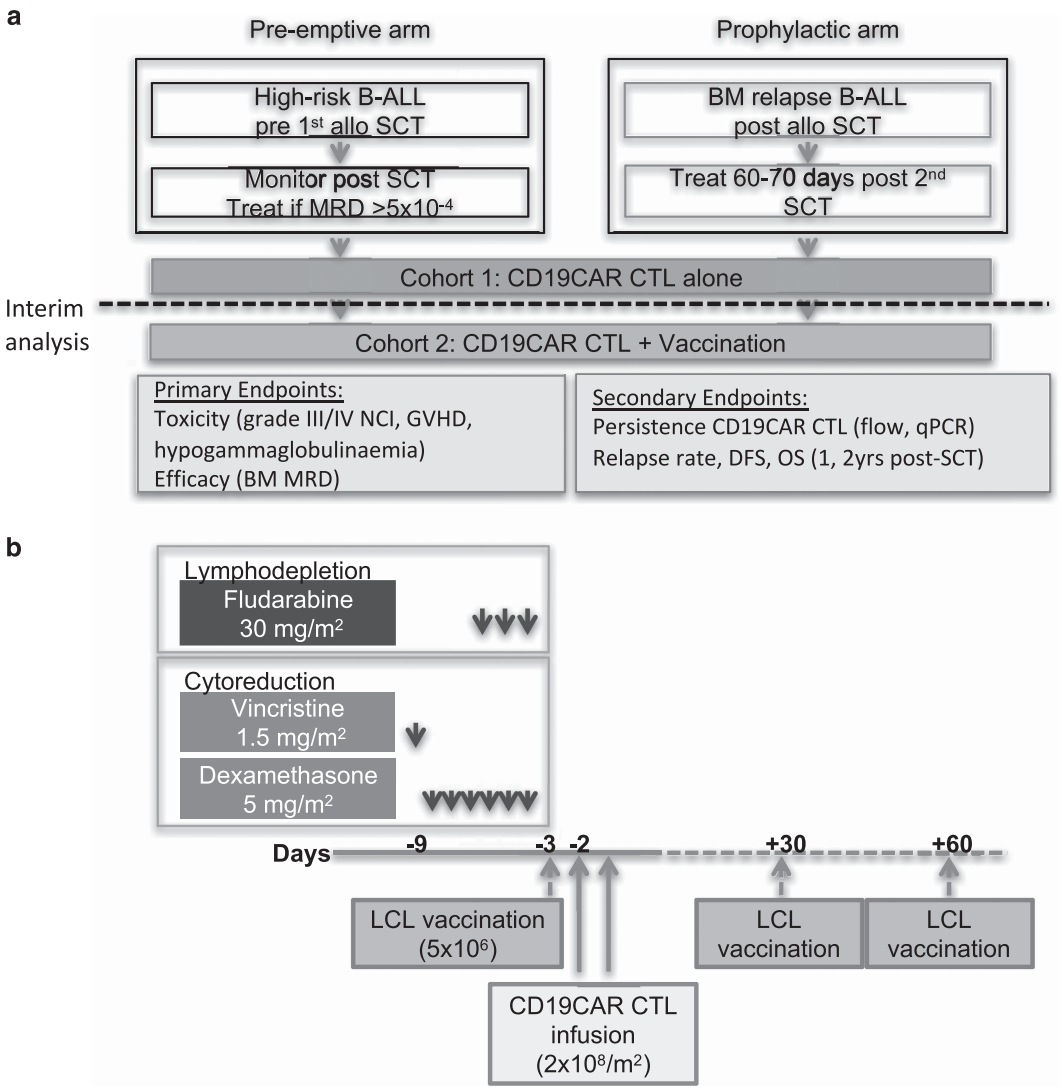


**Figure 1.** Study design and treatment. (**a**) Study design. The study had 2 arms: patients in the pre-emptive arm were in first or subsequent CR and at high (>50%) risk of relapse post SCT. These patients were monitored for emergence of MRD closely for 1 year post SCT. CD19CAR CTL were then administered only in case of MRD positivity $\geqslant 5\times10^{-4}$ in bone marrow. The prophylactic arm included patients with ALL relapsing after SCT who had achieved morphological remission and were candidates for a second SCT. They received CD19CAR CTL after withdrawal of immune suppression at day 60–70 post transplant. (**b**) Study treatment. All patients received lymphodepletion with fludarabine on days − 5 to − 3, and cytoreductive treatment with vincristine and dexamethasone was additionally given to those with MRD recurrence or frank relapse. The CTL infusion was given over 2 days at a total dose of $2\times10^8/m^2$. Vaccination for cohort 2 consisted of three doses of $5\times10^6$ irradiated (70 Gy) donor-derived LCL given on 2 days before CTL infusion and on days 30 and 60 post CTL infusion.

transduced with the CD19CAR transgene.[12] Our CD19CAR comprises the heavy and light chains of the α-CD19 antibody FMC63 in frame with a human IgG1 $CH_2$–$CH_3$ hinge linked to the transmembrane of CD28 and the endodomain of CD3ζ. A GALV-pseudotyped SFG retroviral vector was used to express the CD19ζ CAR gene in EBV CTL. Clinical grade vector supernatant was generated by EUFETS (Idar-Oberstein, Germany). EBV CTL generation, transduction, testing and cryopreservation were performed centrally at Unité de Therapie Cellulaire et Genetique (Nantes, France). Release criteria for CD19CAR CTL included sterility, transduction efficiency ($\geqslant$ 15% expression of CD19CAR by flow) and $\geqslant$ 25% lysis of the $CD19^+$ cell line SupB15 in $^{51}Cr$ release assays. Irradiated donor LCL used for vaccination were tested for the absence of viable proliferating cells after 3 weeks in culture.

### Trial procedures

All patients received lymphodepletion consisting of fludarabine 30 mg/$m^2$ on day − 5 to − 3 before CTL infusion (total 90 mg/$m^2$; Figure 1b). Patients with detectable residual disease also received cytoreduction with vincristine (1.5 mg/$m^2$ i.v., max 2 mg on Day − 9) and dexamethasone (10 mg/$m^2$ per day orally, day − 9 to − 3) before infusion of cryopreserved CD19CAR CTL. Patients were treated with a target dose of $2\times10^8$/$m^2$ thawed CD19CAR CTL split into 2 doses of $4\times10^7$/$m^2$ and $1.6\times10^8$/$m^2$ administered i.v. over consecutive days. Cell dose was based on total cells regardless of CAR expression. A split dose schedule was used to avoid early infusional toxicity and patients who developed grade 4–5 toxicity with the first dose were precluded from receiving the second dose. Vaccination consisted of three doses of $5\times10^6$ irradiated (70 Gy) donor LCL administered subcutaneously 2 days before and at 30 and 60 days post CD19CAR CTL infusion.

### Assessment of response

Disease status was assessed by morphology, cytogenetics and qPCR MRD analysis of clone-specific IgH or TCR gene rearrangements with a sensitivity of at least $10^{-4}$ at Euro-MRD reference laboratories in Frankfurt and London[13] on BM samples taken 1 month post CD19CAR CTL infusion. Complete response was defined as undetectable MRD, partial response as reduction in MRD level by $>1$ log, stable disease by unchanged MRD, and progressive disease by MRD increased by $>1$ log or frank relapse. Duration of response was assessed by monitoring the BM 1, 2, 4, 6 and 12 months post CD19CAR CTL infusion.

### Assessment of CD19CAR CTL persistence

To assess the persistence of CD19CAR CTL, we analyzed blood samples at 2, 7 and 14 days after infusion, then at monthly intervals. Peripheral blood mononuclear cells on day 0 before CTL infusion were analyzed as controls. CTL persistence was analyzed by flow cytometry and qPCR. Flow cytometry detected the Fcγ hinge domain of the CD19CAR using FITC-labeled goat antimouse IgG Fcγ-specific antibody (Jackson ImmunoResearch, Newmarket, UK) with a sensitivity of 1:10 000 determined by spiking sorted CD19CAR CTL into peripheral blood mononuclear cells (Supplementary Figure 1). As an alternative method, we used FLAG-tagged soluble CD19 (Origene, Rockville, MD, USA) followed by detection with a PE-labeled anti-FLAG antibody (Miltenyi, Bergisch-Gladbach, Germany).

### Flow cytometry, intracellular cytokine, ELISPOT, cytotoxicity, CD107a granule release and HAMA assays

These are outlined in Supplementary Methods.

### Toxicity

The incidence of severe toxicity (defined as combined incidence of Grade 4–5 toxicity that may be attributable to CD19CAR CTL within 12 weeks of infusion and Grade III/IV acute GVHD occurring by day 100 post transplant) was determined. A rate of 30% was considered too unsafe.

## RESULTS

### Patients and CD19CAR CTL manufacture

Between May 2012–November 2015, 29 patients were enrolled, 19 on the pre-emptive arm and 10 on the prophylactic arm. Of the pre-emptive patients, 15 were not treated either because they remained MRD-negative for the first year post SCT ($n=7$), donor refusal ($n=5$), transplant related mortality ($n=2$) or problems with CTL manufacture ($n=1$). Of the patients in the prophylactic arm, 4 died or relapsed before CD19CAR CTL treatment and 1 was treated off study as he was not fit for second transplant. One further patient was treated on the prophylactic arm off study before registry approval. One patient (P010) who had a transient response to CD19CAR CTL alone was retreated with CD19CAR CTL and LCL vaccination. Thus, a total of 12 treatments were administered to 11 patients, 4 in the pre-emptive arm and 7 in the prophylactic arm. The initial 6 patients received CD19CAR CTL alone and the subsequent cohort (6 patients including the patient who was retreated) CD19CAR CTL+LCL vaccination. Patient characteristics are shown in Table 1. The median age was 9 years (range: 2–12). Six patients were transplanted from matched sibling and 5 from matched unrelated donors. All patients were heavily pre-treated: at the time of CD19CAR CTL infusion, 7 patients had active disease (1 cytogenetic relapse, $3\times$2nd, $2\times$3rd and $2\times$4th molecular relapse) and 4 patients were in remission ($1\times$2nd CR, $3\times$3rd CR). The median follow-up of the treated patients is now 12 months (range: 1–37 months). Eight patients have died (0.6–26 months since CD19CAR CTL infusion), and the 3 alive with follow-up of 9,13 and 37 months.

Manufacturing of the target dose of CD19CAR CTL ($2\times10^8$/$m^2$ recipient body surface area) was successful in 18/23 patients in whom donor blood was available (78%). In the 11 treated patients, the median transduction efficiency by flow cytometry was 29% (range: 12–58.9%) and the median gene copy number/cell by qPCR was 0.37 (range: 0.14–1.6, Table 1). CD19CAR CTL effectively lysed $CD19^+$ SUPB15 leukemic cells (median 53.2% lysis at 20:1) but showed negligible alloreactivity against patient PHA blasts (median lysis 2%).

### Cohort 1: CD19CAR CTL therapy alone

The first cohort of 6 patients received donor CD19CAR CTL alone. Four patients were treated in the prophylactic arm and 2 in the pre-emptive arm after developing BM MRD $\geqslant5\times10^{-4}$. Four patients received the target dose of $2\times10^8$/$m^2$ and 2 received only the first dose of $4\times10^7$/$m^2$ because it was only possible to generate this dose (P010) or physician choice (P000). Toxicity and outcomes are summarized in Table 2. Infusion of CD19CAR T cells was well-tolerated with no significant infusional toxicity. In particular, none of the patients experienced CRS, neurotoxicity or GVHD attributable to CD19CAR CTL. Two patients developed transient grade 4 neutropenia following lymphodepletion/cytoreduction, 1 patient experienced grade 3 febrile neutropenia, 1 patient had a grade 1 bacterial infection and 1 patient transient grade 2 increase in liver enzymes (likely drug-induced, Table 2). Only 1 patient (P010) had EBV viremia (11 000 copies/ml) at the time of CD19CAR CTL infusion. Persistence studies by flow cytometry, based on detection of the Fcγ hinge domain of the CAR, failed to detect CD19CAR CTL in peripheral blood from day 2 post infusion in all 6 patients. Using the more sensitive qPCR methodology, CD19CAR CTL were detectable in 2 patients for 1 week and 1 month post infusion, respectively (Figures 2a and b). Two patients (P004, P010) lacked B cells in peripheral blood at 1 month. However, B-cell aplasia as a functional parameter for CTL persistence is of limited value in our setting as the patients had low or undetectable B-cell numbers already at the time of CTL therapy. At 1 month post CD19CAR CTL infusion, 2 patients treated on the prophylactic arm were in CCR, 1 patient treated pre-emptively had a PR, 2 patients (1 in the prophylactic and in the pre-emptive arm) had stable disease and 1 patient treated pre-emptively had disease progression (Table 2). Four patients had detectable disease in the BM at the time of CD19CAR CTL infusion. Of these, P010, who had EBV reactivation at the time of CD19CAR

CTL infusion, showed a partial response with transient clearance of BM MRD (Figure 3a) until 4 months post CD19CAR CTL infusion despite the absence of detectable CD19CAR CTL in the blood. Two patients had stable disease for 1–2 months and one showed no response with rapid disease progression. Overall, 5 patients in this cohort have relapsed at 2 weeks—5 months post CD19CAR CTL infusion and in all cases the disease remained $CD19^+$. One patient, treated in MRD-negative remission, remains alive in complete remission 3 years after CD19CAR CTL infusion and 5 have died from progressive disease. Thus, although CD19CAR CTL alone were safe, their persistence and anti-leukemic efficacy was limited.

*Ex vivo* EBV restimulation of CD19CAR CTL

To determine whether CD19CAR CTL were present at low frequencies *in vivo*, we restimulated peripheral blood mononuclear cells obtained from a patient post CTL transfer with donor LCL *in vitro*. In ELISPOT analyses, expanded lymphocytes from samples taken 2 weeks after CTL infusion secreted IFN-γ in response to stimulation with autologous LCL, but also with $EBV^-$, $CD19^+$ leukemia cells (SupB15, REH), whereas $CD19^-$ allogeneic targets failed to induce IFN-γ responses (Supplementary Figure 2). The memory phenotypes of CD19CAR CTL from P004 prior to adoptive transfer and *ex vivo* post transfer and post restimulation were not substantially different (Supplementary Figure 3). Lymphocytes from a later time-point, 2 months after CTL infusion, failed to respond to $CD19^+$ targets even after LCL restimulation. Thus, low numbers of CD19CAR CTL undetectable by flow cytometry/qPCR are present in the peripheral blood at early time-points and restimulation with viral antigen can re-induce functional responses to $CD19^+$ leukemia cells. These results encouraged us to explore whether vaccination *in vivo* to provide signaling through the endogenous EBV-specific TCR could improve the expansion/persistence and efficacy of CD19CAR CTL.

**Table 1.** Patient characteristics

| *Pt no.* | *Age/Sex* | *Arm* | *Status at infusion* | *EBV at infusion* | *Donor* | *Cell dose* | *Transduction efficiency* | *% Lysis of SUPT1CD19/ pt PHA blasts at 20:1* | *Average copy no.* |
|---|---|---|---|---|---|---|---|---|---|
| *COHORT 1* | | | | | | | | | |
| P000 | 8/M | Prophylactic | Third CR (MRD-ve) post second SCT | No | 10/10 MUD | $4\times10^7/m^2$ | 58.9% | 50.1/1.1% | 1.60 |
| P002 | 9/F | Pre-emptive | Cytogenetic relapse post first SCT | No | MSD | $2\times10^8/m^2$ | 19.1% | 71/0.6% | 0.56 |
| P004 | 12/M | Prophylactic | Fourth molecular relapse post first SCT[a] | No | MSD | $2\times10^8/m^2$ | 32.8% | 36.3/0.1% | 0.56 |
| P009 | 2/M | Pre-emptive | Second molecular relapse post first SCT | No | 10/10 MUD | $2\times10^8/m^2$ | 34% | 58.6/2% | 0.85 |
| P010 | 11/M | Prophylactic | Third molecular relapse post second SCT | Yes | MSD | $4\times10^7/m^2$ | 26.9% | 46.7/11.4% | 0.43 |
| P013 | 10/M | Prophylactic | Third CR (MRD-ve) post second SCT | No | 10/10 MUD | $2\times10^8/m^2$ | 25.3% | 29.2/4.6% | 0.32 |
| *COHORT 2* | | | | | | | | | |
| P007 | 9/M | Pre-emptive | Second molecular relapse Post first SCT | No | MSD | $2\times10^8/m^2$ | 32.8% | 62.1/5% | 0.31 |
| P010 | 12/M | Prophylactic | Fourth molecular relapse post second SCT | No | MSD | $2\times10^8/m^2$ | 12% | 53.6/9.6% | 0.56 |
| P019 | 8/F | Prophylactic | Second CR (MRD-ve) post second SCT | No | MSD | $2\times10^8/m^2$ | 31% | 52.7/2% | 0.20 |
| P022 | 11/M | Prophylactic | Third molecular relapse post second SCT | Yes | 10/10 MUD | $2\times10^8/m^2$ | 36% | 34.9/1.1% | 0.23 |
| P025 | 9/M | Pre-emptive | Second molecular relapse post first SCT | Yes | MSD | $2\times10^8/m^2$ | 16.9% | 54.4/1.5% | 0.14 |
| P027 | 6/M | Prophylactic | Third CR (MRD-ve) post second SCT | No | 10/10 MUD | $2\times10^8/m^2$ | 20.2% | 60/2.9% | 0.26 |

Abbreviations: CR, complete remission; pt, patient; SCT, stem cell transplantation; MUD, matched unrelated donor; MSD, matched sibling donor, MRD, minimal residual disease. [a]P004 was recruited on to the prophylactic arm of the study but became unfit for second stem cell transplant and was withdrawn from the study. Patients in cohort 1 received CD19CAR CTL alone, those in cohort 2 received CD19CAR CTL with LCL vaccination. This patient was later treated off study without a second transplant.

**Table 2.** Patient outcomes

| *Pt no.* | *Toxicity* | *CD19CAR CTL detection by PCR/ duration* | *Response at 1 month/ duration* | *Relapse/interval to relapse* | *Current status/follow-up* |
|---|---|---|---|---|---|
| *COHORT 1* | | | | | |
| P000 | None | Undetectable | CCR | No | Alive in molecular CR/37 months |
| P002 | Grade 2↑ALT, Grade 4 neutropenia | Undetectable | Non-responder | CD19+BM relapse/2 weeks | Died—disease progression/1 month |
| P004 | Grade 3 febrile neutropenia | Detected/1 month | Stable disease/2 months | CD19+BM relapse/10 weeks | Died—disease progression/5 months |
| P009 | None | Undetectable | Stable disease/1 month | CD19+BM relapse/3 months | Died—disease progression/26 months |
| P010 | Grade 4 neutropenia | Undetectable | PR/4 months | CD19+BM relapse/ 5 months → retreated | Died—disease progression/12 months |
| P013 | Grade 1 bacterial infection | Detected/1 week | CCR/2 months | CD19+BM relapse/4 months | Died—disease progression/9 months |
| *COHORT 2* | | | | | |
| P007 | Grade 3 neutropenia | Detected/2 months | Non-responder | CD19+BM relapse/1 months | Died—disease progression/12 months |
| P010 | None | Detected/1 month | Non-responder | CD19+BM relapse/2 months | Died—disease progression/12 months |
| P019 | None | Detected/3 months | CCR/7 months | CD19+BM relapse/7 months | Died—disease progression/13 months |
| P022 | None | Undetectable | Stable disease/4 months | CD19+BM relapse/6 months | Alive with molecular disease/ 11 months |
| P025 | Grade 3 cholecystitis | Undetectable | CR/2 months | CD19+liver+BM relapse/3 months | Died—disease progression/13 months |
| P027 | Grade 4 neutropenia | Detected/2 months | CCR/4 months | CD19+CNS+BM relapse/4 months | Alive with molecular disease/9 months |

Abbreviations: ALT, alanine transaminase; CCR, continuing complete response; GGT, gamma glutamyl transpeptidase; NE, not evaluable; PR, partial response.

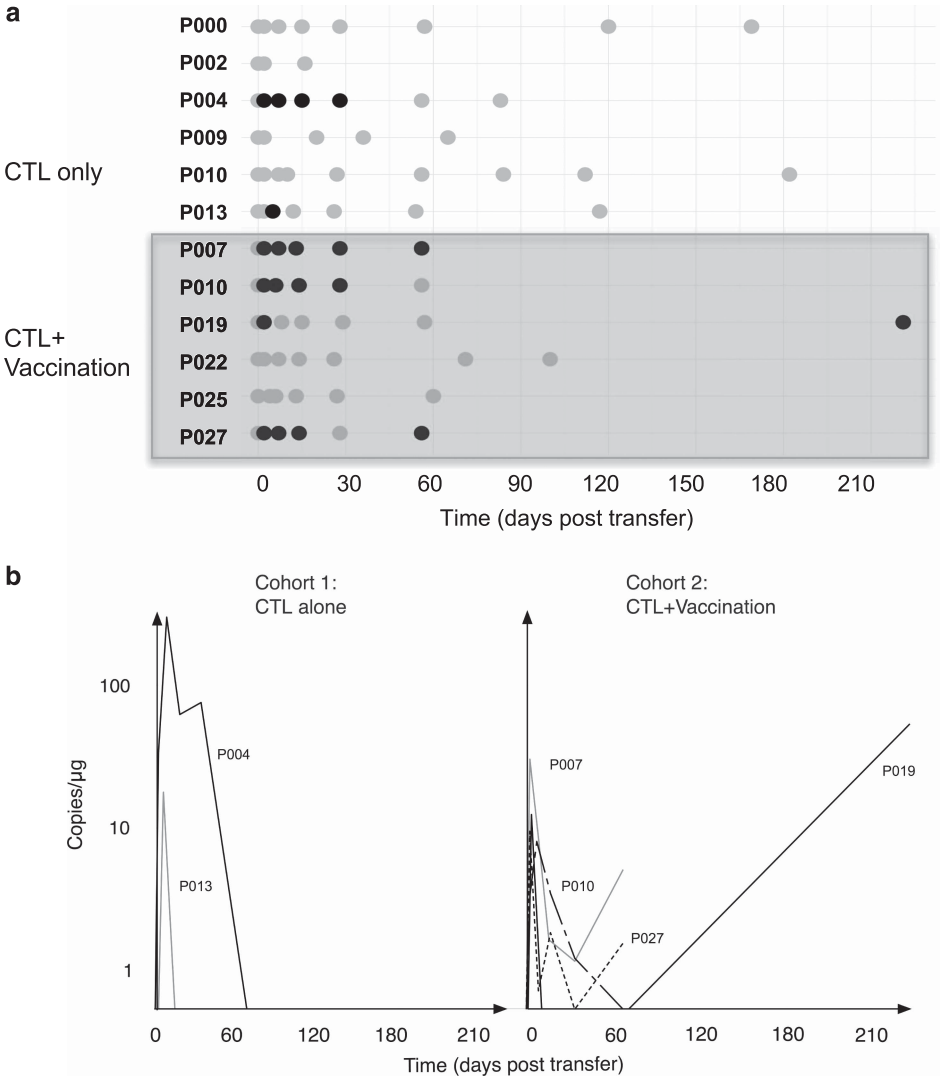


**Figure 2.** CD19CAR CTL persistence by qPCR. (**a**) CTL were detected above threshold level in 2 patients in cohort 1 (P004 and P013) and 4 patients in cohort 2 (P007, P010, P019, P027) for a maximum of 7 months, depicted by black circles. Gray circles depict assessments where CTL were not detected by qPCR. (**b**) In patients where CTL were detected, persistence and expansion are depicted as the magnitude of the qPCR signal over time.

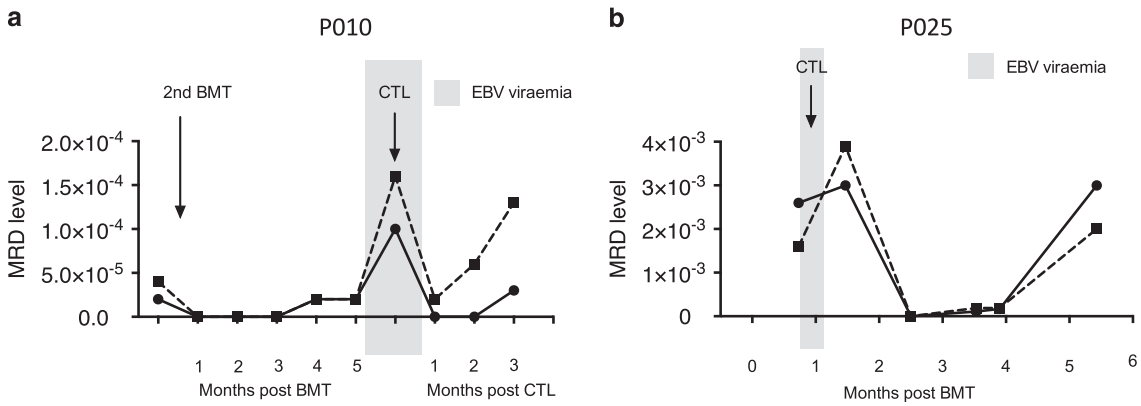


**Figure 3.** MRD responses in P010 and P025. (**a**) MRD response in P010. Bone marrow MRD levels were assessed by two markers. The MRD levels cleared transiently following the second transplant, however they rose to a maximum of $1.5 \times 10^{-4}$ at the time of CTL infusion. Following infusion of CD19CAR CTL (which was concomitantly with EBV viremia) MRD levels transiently fell on both markers, but then rose again over the following 3 months until the patient relapsed with frank disease at month 4. CD19CAR CTL were not detected in peripheral blood of this patient after CTL infusion. (**b**) MRD response in P025. MRD was assessed by two markers. This patient who had rising MRD levels post transplant became MRD-negative after CTL infusion but subsequently MRD levels rose until frank relapse at 3 months. CD19CAR CTL were not detected in peripheral blood.

Following an interim analysis by the IDMC, a planned substantial amendment was therefore made to treat a second trial cohort of patients with donor CD19CAR CTL along with subcutaneous vaccination with irradiated, donor-derived LCL 2 days before and 1 and 2 months following CTL infusion.

Cohort 2: CD19CAR CTL therapy with LCL vaccination

Four patients were treated in the prophylactic arm and 2 pre-emptively (P010 was retreated in this cohort after a transient response to CD19CAR CTL alone). No significant infusional toxicities were seen so all patients in this cohort received the planned cell dose of $2\times10^8/m^2$. Both CTL infusion and LCL vaccination were well-tolerated (Table 2). Grade 3–4 neutropenia occurred in 2 patients (likely attributable to lymphodepletion/cytoreduction and disease relapse) and 1 patient had grade 3 cholecystitis. CRS, neurotoxicity or *de novo* GVHD were not observed and likewise none of the patients developed severe inflammatory reactions or EBV viremia after LCL vaccination. Two patients (P022, P025) had low-level EBV viremia (500 and 25 000 copies/ml) at the time of CD19CAR CTL infusion. In this cohort, CTL were again undetectable in peripheral blood by flow cytometry, both with Fcγ-specific antibody and with FLAG-tagged soluble CD19 (Supplementary Figure 4), but 4 of 6 patients had detectable CD19CAR CTL in the blood by qPCR which persisted until 1–3 months post infusion (Figures 2a and b). Although absolute level of circulating CD19CAR CTL were low, CD19CAR CTL persistence was improved in the vaccination cohort. In cohort 1, 2 patients had persistence times of 5 and 28 days, the other 4 had no persistence (0 days); although in cohort 2, 4 patients had persistence times of 28, 56, 56 and 221 days and 2 had no persistence (Wilcoxon $P=0.06$, one-sided because vaccination was expected to enhance persistence). The median persistence was 0 (range: 0–28) days without vaccination compared to 56 (range: 0–221) days with vaccination. One concern is that 2 patients in cohort 1 had received lower than planned cell doses because of manufacturing issues/physician choice, and this may have contributed to the lack of detectable CAR T-cell persistence and expansion in these patients. Interestingly, persistence was not seen in the 2 vaccinated patients who had EBV viremia at the time of CD19CAR CTL infusion suggesting that endogenous low-level EBV reactivation could not adequately restimulate the CTL *in vivo* in these two patients and does not explain the enhanced persistence in cohort 2. At 1 month post CD19CAR CTL infusion, 2 patients, both in the prophylactic arm, remained in CCR, 1 patient in the pre-emptive arm obtained a *de novo* CR, and 1 patient in the prophylactic arm had stable disease and there were 2 non-responders, 1 treated pre-emptively and 1 in the prophylactic arm (Table 2). Four patients in this cohort had detectable disease at the time of CD19CAR CTL infusion. Of these, P025 achieved CR with clearance of MRD lasting 2 months (Figure 3b), P022 had stable disease for 4 months and 2 patients showed disease progression. Two patients in molecular CR at the time of CD19CAR CTL infusion remained in CR for 4 and 7 months but ultimately relapsed. All 6 patients in this cohort have relapsed at 1–7 months post CD19CAR CTL infusion and in all cases the disease remained $CD19^+$. At last follow-up, 4 patients have died from disease progression and 2 remain alive with molecular level disease following further treatment. Thus, despite the improved persistence with vaccination, CD19CAR CTL failed to mediate effective anti-leukemic responses.

Phenotype of CD19CAR CTL products

One potential reason for the lack of persistence and limited efficacy of CD19CAR CTL is senescence/exhaustion of the infused CTL after prolonged *in vitro* expansion. Flow cytometry for memory/exhaustion markers on 6 CD19CAR CTL products showed the majority of both transduced and untransduced cells were $CD8^+$ and had an effector memory $CD45RA^-CCR7^-$ phenotype (Figure 4). There was significant though variable expression of the immune-inhibitory receptors CTLA-4 and PD-1 but expression of LAG-3 and TIM-3 was low.

*In vivo* immune responses against CD19CAR CTL

An alternative explanation for the loss of CD19CAR CTL is the presence of humoral or cellular immune responses against the CAR T-cells. Human antimouse antibody (HAMA) responses against the CTL product were not identified in samples from 8 patients analyzed at 2, 6 or 12 months after CD19CAR CTL transfer. Samples from 3 patients were also analyzed for cytokine responses against the CD19CAR transgene by ELISPOT analysis. No cellular reactivity against CD19CAR was detected (Supplementary Figure 5). Thus, we have no data to suggest that host immunogenicity explains the limited expansion/persistence of CD19CAR CTL in our study.

Functional consequences of the IgG1-derived spacer

The CAR used in our study contains the extracellular IgG1-Fc CH2CH3 spacer to allow for quantification of transduction efficiencies and *in vivo* tracking by flow cytometry. This domain is known to interact with Fc receptors[14,15] that may trigger activation-induced cell death or phagocytosis by innate cells bearing Fc receptors. To investigate this, we cultured CD19CAR CTL with the monocytic cell line THP-1 and with autologous monocytes expressing Fc receptors FcRI (CD64) and FcRII (CD32) but not CD19, and with autologous NK cells that express FcRIII (CD16) but no other Fc receptors. CD19CAR CTL, but not non-transduced CTL, effectively lysed both $CD19^+$ targets and $CD19^-$ THP-1 cells (Figure 5a). CD19CAR CTL further responded to stimulation with THP-1 cells by secretion of IFN-γ and TNFα (Figure 5c). Fc receptor blockade by autologous serum (AS) prevented cytokine release in response to the $FcR^+$, $CD19^-$ targets, supporting an FcR-dependent mechanism of interaction (Figure 5b). CD19CAR CTL also upregulated the degranulation marker CD107a after coincubation with THP-1 cells or autologous monocytes (Figure 5c) and AS prevented this. In contrast, an alternative CAR with a modified, non-FcR-engaging IgG1-derived spacer domain failed to interact with $CD19^-$ targets. Despite activation of CD19CAR T-cells by THP-1 stimulators, no proliferation was observed, in contrast to after stimulation with autologous LCL (Figure 5d). Similarly CD19CAR CTL stimulated with the $EBV^-$, $CD19^+$ leukemic cell line REH also did not proliferate. Thus, FcRI and FcRII engagement on monocytes may trigger off-target activation of CD19CAR CTL, but this is insufficient to induce proliferation and potentially could result in exhaustion of the CTL *in vivo*.

## DISCUSSION

This is the first published multi-center study of CD19CAR T-cell therapy and demonstrates the feasibility of delivering this novel therapeutic approach with central manufacture and administration across multiple centers, which will be crucial for broader application of this technology. It has significant implications for the design of future studies in that the pre-emptive arm was inefficient: only 4 of 15 patients in whom CD19CAR CTL were generated received them. Adoptive transfer of donor EBV CTL transduced with a first generation CD19CAR was safe with no CRS, neurotoxicity or GVHD and the main toxicity observed was cytopenia secondary to lymphodepletion/cytoreduction +/− disease. At the time the study was designed, part of the rationale for using donor EBV CTL as effectors was to reduce the risk of GVH.[3,16] However, subsequent studies have demonstrated a low risk of GVH when bulk donor-derived T-cells from the patient post SCT are used to generate CD19CAR T cells. The grade 4–5 toxicity

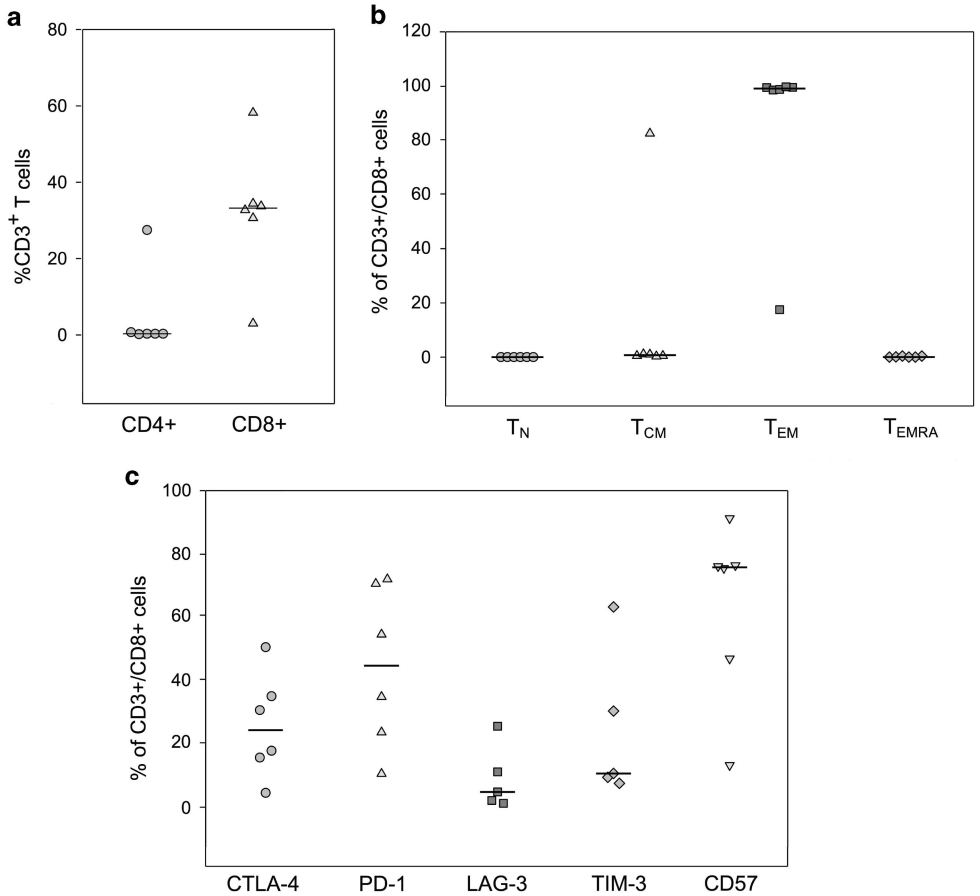


**Figure 4.** Phenotypic analysis of CD19CAR CTL products ($n = 6$). (**a**) Proportion of $CD4^+$ and $CD8^+$ $CAR^+$ cells among $CD3^+$ T cells. (**b**) Proportion of effector memory subtypes among CD19CAR CTL (Fcγ+) $CD8^+$ T cells. Naive T cells ($T_N$) were characterized by coexpression of CCR7 and CD45RA, central memory T cells ($T_{CM}$) by coexpression of CCR7 and CD45RO, $T_{EM}$ by expression of CD45RO in the absence of CCR7 and $T_{EMRA}$ by CD45RA without CCR7. (**c**) Expression of various activation and exhaustion markers among $CD8^+$ T cells. Results are summarized from 6 CTL lines infused into patients P002, P004, P007, P009, P013 and P019.

rate of 27% (3/11 patients; all grade 4 neutropenia) was within our pre-specified rate of 30%.

At 1 month post CD19CAR CTL infusion, 5 patients were in CR/CCR and 1 in PR. P010 and P025 showed partial/complete clearance of high level BM MRD. These responses are unlikely to reflect lymphodepleting chemotherapy as in both cases MRD levels were rising following lymphodepletion on the day of infusion. However, long-term efficacy was limited and 10/11 patients ultimately relapsed, all with $CD19^+$ disease. Indeed, the only long-term remission was a patient who was MRD-negative at the time of infusion and this may therefore simply reflect the effect of the second transplant. Likewise, sustained B-cell aplasia (a surrogate for the presence of an ongoing response against $CD19^+$ targets) beyond 1 month was not observed. This is likely to reflect the poor *in vivo* expansion and persistence of transferred CD19CAR CTL.

The persistence of CD19CAR T-cells is critical to durable responses and loss of detectable circulating CD19CAR T-cells is often a prelude to disease relapse in ALL.[5] Moreover, this is likely to determine whether CD19CAR T cells are ultimately used as a bridge to SCT or a stand-alone therapy. It is therefore of central importance to enhance the persistence of CD19CAR T cells. It is well established that signaling through first generation CARs is insufficient to induce CAR T-cell proliferation[17] and this is confirmed by our findings in Figure 5d. The incorporation of costimulatory molecules in second generation CARs enhances expansion and persistence of T cells clinically.[18] However, even when virus-specific CTL were engineered with a second generation CD19CAR with a CD28 costimulatory domain they showed limited expansion, persistence and efficacy,[19] suggesting that the poor persistence and expansion of CD19CAR CTL observed in cohort 1 of our study is not simply due to lack of costimulation. In this study, we investigated whether a vaccination strategy can be used to boost CAR T-cell persistence/expansion. We demonstrate for the first time that vaccination with irradiated, donor EBV LCL does indeed enhance the persistence of adoptively transferred CD19CAR CTL. However, in the absence of a costimulatory domain in the CAR, signaling through the native TCR appeared insufficient to drive expansion of CD19CAR CTL *in vivo,* resulting in limited anti-leukemic activity. The central question arising is why CD19CAR CTL failed to expand despite apparent stimulation through the endogenous EBV-specific TCR.

One potential explanation is that the prolonged *in vitro* culture of the CTL with repeated stimulation in the presence of IL-2 induces cellular senescence post infusion, resulting in a limited capacity to expand. Our CD19CAR CTL showed a predominantly effector memory phenotype with a low proportion of central memory T cells, which can proliferate and persist *in vivo* after adoptive transfer.[20] Consistent with this, Cruz *et al*.,[19] observed limited expansion and efficacy of CD19CAR transduced virus-specific T cells administered to patients with B-cell precursor ALL and CLL post allogeneic transplant,[19] compared with the other

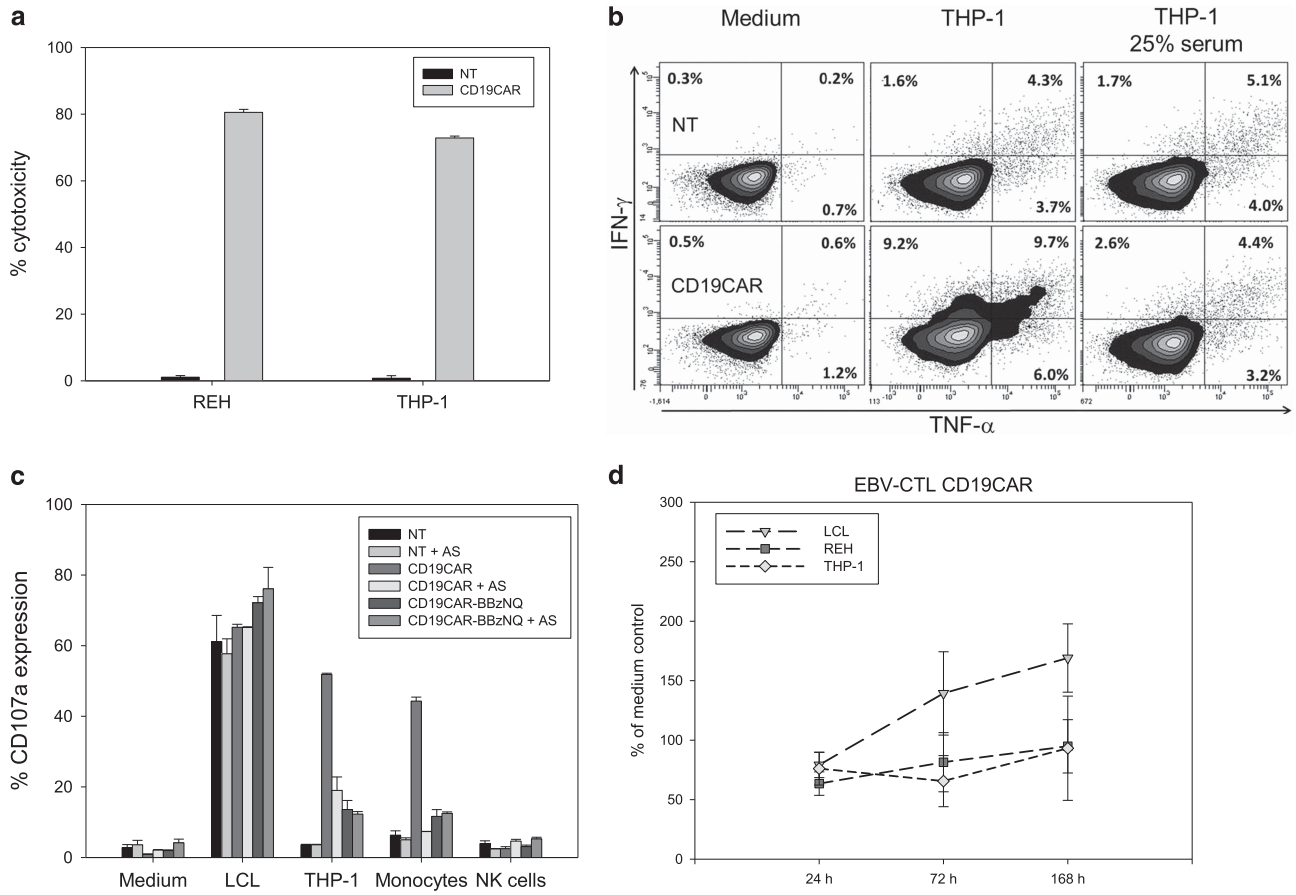


**Figure 5.** CD19CAR CTL interact with CD19-negative monocyte targets via FcRs. (**a**) CD19CAR CTL show specific cytotoxicity against $CD19^+$ REH cells and the $CD19^-$, FcγR-expressing target THP-1 in a 7AAD viability assay. (**b**) Fc receptor blockade by serum effectively prevents intracellular IFN-γ secretion by CD19CAR CTL in response to THP-1 cells. (**c**) CD107a degranulation responses of non-transduced (NT) or CD19CAR CTL, or CTL transduced with an alternative CD19-specific CAR with a modified, non Fc-engaging IgG1-derived spacer domain (CD19CAR-BBzNQ) to coincubation with LCL, THP-1 cells, autologous monocytes and autologous NK cells, in the presence and absence of AS. Representative experiment of two. (**d**) CD19CAR CTL expand in response to stimulation with autologous LCL but not $CD19^+EBV^-$ targets (REH) or THP-1 cells. Overall, $1 \times 10^5$ CD19CAR CTL were coincubated with $2.5 \times 10^4$ irradiated tumor cells or autologous EBV LCL in CTL-medium with 40 U rhIL-2 in 96-well flatbottom plates for 7 days. Proliferation of EBV CTL was analyzed after 24, 72 and 168 h by staining with 7AAD and flow cytometry. The cell count was taken from $7AAD^-$ cells within the lymphocyte gate. Shown is the mean of 3 donors.

studies with similar second generation CD19CARs utilizing peripheral blood mononuclear cells as effectors.[4,6] Although CD19CAR CTL did not show an exhausted phenotype at the time they were infused, *in vivo* analysis following transfer was not technically possible as expansion was too low for detection in the blood flow cytometrically. However, retrovirally gene-marked EBV CTL with comparable effector memory phenotypes infused to augment EBV-specific T-cell immunity post allogeneic SCT have shown prolonged persistence for up to 9 years[8] albeit at low levels. It is possible that that this discrepancy may reflect differences in antigenic stimulation through the EBV-specific TCR: in the gene marking studies CTL were infused in the context of active EBV reactivation/disease. In contrast, only a minority of our patients (3/11) had this at the time of CD19CAR CTL infusion. Although this was associated with anti-leukemic responses in two cases (Figure 3), the lack of qPCR detection of CD19CAR CTL in the three patients argues against a contribution of these low-level EBV reactivations to their persistence and *in vivo* function. Although LCL vaccination improved CD19CAR CTL persistence in cohort 2 patients, it remains possible that this antigenic stimulation does not fully recapitulate that seen with endogenous EBV reactivation. Although autologous LCL stimulation *in vitro* was highly effective in restimulating CD19CAR CTL, data on the efficacy of subcutaneous autologous LCL vaccination *in vivo* are limited: vaccination with LCL expressing mutated *Ras* elicited significantly increased responses to this tumor antigen in 6 of 7 patients with prostate cancer (Kubuschok *et al*.,[21] and Pfreundschuh, personal communication). Thus, it may be that the vaccination strategy used, while improving persistence somewhat, is inadequate to induce the proliferation of CD19CAR CTL needed for an effective anti-leukemic response.

An alternate possibility for the lack of expansion/persistence of CD19CAR CTL is related to the presence of the CD19CAR itself. In this regard, it is of note that virus-specific CTL transduced with a second generation CD19CAR in the study of Cruz *et al*.,[19] also showed more limited persistence (1–12 weeks) and expansion (undetectable by flow) than previous studies of Neo marked EBV CTL.[8,11] Although rejection through recognition of immunogenic murine and junctional components of the CAR has been observed,[7] we did not find any evidence for either cellular or antibody responses against CD19CAR CTL. Alternatively, it is possible the IgG1 extracellular spacer may have mediated clearance of the CAR T cells from peripheral blood. We found that CD19CAR CTL receive strong activation stimuli by $CD19^-$ monocyte targets expressing FcRI and FcRII receptors, inducing off-target effector responses. These data confirm that IgG-derived

extracellular spacer domains can substantially affect the functional properties of CAR T-cells and provide a potential explanation for their limited *in vivo* life-span. It is of note that other clinical studies using the Fc spacer have also shown limited expansion and persistence of CAR T-cells.[11,18] Modifications of IgG spacer domains have been developed to reduce Fc receptor binding.[14] It may therefore be prudent to use such mutated Fc or alternate (for example, CD8) spacer domains.

We aimed to investigate the *in vivo* phenotype and functional capacities of CAR T cells. However, this requires reliable identification of circulating adoptively transferred CAR T cells by flow cytometry. Despite undertaking two flow cytometric methods of detection of CAR T cells (staining with antibiodies specific for the Fcγ hinge domain as well as with FLAG-tagged soluble CD19 and secondary anti-FLAG antibodies), neither method was able to detect the low numbers of CAR T cells persisting in the patients in this study.

Despite the recent successes of clinical second generation CD19CAR T-cell trials,[4–7] optimizing their *in vivo* persistence remains a central issue. The use of a 4-1BB costimulatory domain[22] and modifying manufacturing conditions to enrich for stem cell and central memory CAR T-cells appear to improve persistence but loss of circulating CD19CAR T-cells remains the major cause of treatment failure. There is thus a need for alternative ways to enhance the persistence of CAR T cells. Boosting their *in vivo* function by vaccination may be more effective with second and third generation CAR T-cells and this could potentially be achieved either by stimulation of the endogenous TCR in effector T cells of defined specificity or by stimulation of the CAR itself with a CD19 expressing vaccine once circulating $CD19^+$ targets are eradicated.

In conclusion, this study demonstrates the potential for enhancing persistence of CAR T cells with vaccination. Further studies with improved CAR design and refined vaccination strategies are required to evaluate the full anti-leukemic potential of this strategy.

## CONFLICT OF INTEREST

MP has received research funding from Cellectis, honoraria from Amgen and Roche and owns stock/receives salary from Autolus Ltd. PJA has received research funding from Bluebird bio and honoraria from Novartis. RR is now an employee of Genentech Inc. All other authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank Profs Rob Wynn, Wendy Qian and Malcolm Brenner for oversight of the trial as the IDMC. The study was supported by the European Commission Sixth Framework Program through the CHILDHOPE - Chimaeric T-cells for the treatment of paediatric cancers project (LSHC-CT-2006-037381, specific targeted research project - STREP), the UK Leukaemia and Lymphoma Research Fund (Bloodwise), Children with Cancer UK, UK Department of Health, JP Moulton Charitable Foundation (grants to PA), Deutsche Krebshilfe, Deutsche Kinderkrebsstiftung and Löwenkinder e.V (grants to CR) and the Italian AIRC (grants to EB). MP is supported by the University College London Hospital BRC and PJA is in receipt of an NIHR Research Professorship.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)