

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

Running title: Vaccination and CD19CAR T cell therapy for ALL

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ABSTRACT

Trials with 2nd generation CD19 chimeric antigen receptors (CAR) T-cells report unprecedented responses but associated with risk of Cytokine Release Syndrome (CRS). Instead, we studied use of donor Epstein Barr virus-specific T-cells (EBV CTL) transduced with a 1st 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 ALL. Patients were eligible pre-emptively if they developed molecular relapse ($> 5 \times 10^{-4}$) post-1st SCT, or prophylactically post-2nd SCT. An initial cohort showed poor expansion/persistence. We next investigated EBV-directed vaccination to enhance expansion/persistence. 11 patients were treated. No CRS, neurotoxicity or 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. Whilst 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 feasibility of such multi-center studies and the potential for enhancing persistence with vaccination.

INTRODUCTION

Clinical trials of 2nd generation CD19 Chimeric Antigen Receptors (CAR) T-cells have shown impressive outcomes in relapsed/ refractory ALL¹⁻⁷ 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 approximately 30%, reflecting supraphysiological activation through CAR signaling, which may limit broader application. Additionally, 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 recipients⁸ without causing 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 2nd 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 1st generation GD2 CAR circulated at higher levels than non-specifically activated T-cells⁹. 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 vaccination improved their expansion/survival and 1 year relapse/disease-free survival after immunotherapy.

METHODS

Study design

This open-label phase I/II multi-center trial of CD19CAR CTL therapy in children with ALL aged ≤ 18 years was conducted across 6 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 2 arms (Figure 1A). In the **pre-emptive arm** patients in 1st or subsequent CR who were at high (>50%) risk of relapse post-SCT (Inclusion criteria-Supplemental Table 1) were monitored in the BM for emergence of MRD monthly (months 1 to 6) and then 6-weekly (months 7-12) for 1 year post-SCT. CD19CAR CTL were generated from stem cell donors prior to SCT and administered in case of MRD positivity $\geq 5 \times 10^{-4}$. In the **prophylactic arm** patients who had relapsed after 1st SCT were treated with CD19CAR CTL 60-70 days after 2nd 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 protocol¹⁰) whereas those in the prophylactic arm received conditioning with fludarabine/treosulphan/thiotepa for their 2nd transplant and serotherapy in those receiving grafts from unrelated donors. CD19CAR CTL infusion was contraindicated in patients with acute GVHD \geq 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 described¹¹ by repetitive stimulation of donor T cells with LCL except that autologous and human AB serum were used. EBV CTL were then retrovirally transduced with the CD19CAR transgene¹². Our CD19CAR comprises the heavy and light chains of the α -CD19 antibody FMC63 in frame with a human IgG1 CH2-CH3 hinge linked to the transmembrane of CD28 and the endodomain of CD3 ζ . A GALV-pseudotyped SFG retroviral vector was used to express the CD19 ζ CAR gene in EBV-CTL. Clinical grade vector supernatant was generated by EUFETS (Idar-Oberstein, Germany). EBV-CTL generation, transduction, testing and cryopreservation was performed centrally at Unité de Therapie Cellulaire et Genetique (Nantes, France). Release criteria for CD19CAR CTL included sterility, transduction efficiency ($\geq 15\%$ expression of CD19CAR by flow) and $\geq 25\%$ lysis of the CD19⁺ cell line SupB15 in ⁵¹Cr 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/m² on day -5 to -3 prior to CTL infusion (total 90 mg/m²) (Figure 1B). Patients with detectable residual disease also received cytoreduction with vincristine (1.5 mg/m² i.v., max 2 mg on Day -9) and dexamethasone (10 mg/m²/day orally, day -9 to -3) prior to infusion of cryopreserved CD19CAR CTL. Patients were treated with a target dose of 2 x 10⁸/m² thawed CD19CAR CTL split into 2 doses of 4x10⁷/m² and 1.6x10⁸/m² administered iv 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 3 doses of 5 x 10⁶ 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 London¹³ 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. PBMC on day 0 prior to CTL infusion were analyzed as controls. CTL persistence was analyzed by flow cytometry and qPCR. Flow cytometry detected the Fc γ hinge domain of the CD19 CAR using FITC-labeled goat antimouse IgG Fc γ -specific antibody (Jackson ImmunoResearch, Newmarket UK) with a sensitivity of 1:10,000 determined by spiking sorted CD19CAR CTL into PBMCs (Supplementary Figure 1). As an alternative method, we used FLAG-tagged soluble CD19 (Origene, Rockville, 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 Supplemental 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 1st 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 2nd transplant. One further patient was treated on the prophylactic arm off study prior to registry approval. One patient (P010) who had a transient response to CD19 CAR 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 x 2nd, 2 x 3rd and 2 x 4th molecular relapse) and 4 patients were in remission (1 x 2nd CR, 3 x 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 ($2 \times 10^8/m^2$ recipient BSA) 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 leukaemic 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 1st 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 $\geq 5 \times 10^{-4}$. Four patients received the target dose of $2 \times 10^8/m^2$ and 2 received only the 1st dose of $4 \times 10^7/m^2$ 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 Fc γ 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 (Figure 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 since 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 preemptive arm) had stable disease and 1 patient treated preemptively 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, while 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 PBMC 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.

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 infusion toxicities were seen so all patients in this cohort received the planned cell dose of $2 \times 10^8/m^2$. 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 Fc γ -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 (Figure 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); whilst 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 due to manufacturing issues / physician choice, and thus 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 (Fig 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 anti-mouse 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} which 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 TNF α (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 1st 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 CD19 CAR T cells. The grade 4-5 toxicity 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 ALL⁵. 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 1st generation CARs is insufficient to induce CAR T cell proliferation¹⁷ and this is confirmed by our findings in Figure 5D. The incorporation of costimulatory molecules in 2nd generation CARs enhances expansion and persistence of T cells clinically¹⁸. However, even when virus-specific CTL were engineered with a 2nd generation CD19CAR with a CD28 costimulatory domain they showed limited expansion, persistence and efficacy¹⁹, 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*

in vivo after adoptive transfer²⁰. 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 transplant¹⁹, compared to other studies with similar 2nd generation CD19CARs utilizing PBMC as effectors^{4,6}. While CD19CAR CTL did not show an exhausted phenotype at the time they were infused, *in vivo* analysis following transfer was not technically possible since 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 years⁸ albeit at low levels. It is possible 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 2 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. While 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 (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 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 2nd 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^R marked EBV CTL^{8,11}. Although rejection through recognition of immunogenic murine and junctional components of the CAR has been observed⁷, 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 binding¹⁴. It may therefore be prudent to use such mutated Fc or alternate (eg 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 2 flow cytometric methods of detection of CAR T cells (staining with antibodies specific for the Fc γ 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 2nd generation CD19CAR T-cell trials⁴⁻⁷, optimizing their *in vivo* persistence remains a central issue. The use of a 4-1BB costimulatory domain²² 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 2nd and 3rd 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.

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CONFLICT OF INTEREST DISCLOSURES

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FIGURE LEGENDS

Figure 1. Study design and treatment. A. Study design. The study had 2 arms: patients in the **pre-emptive arm** were in 1st 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 $\geq 5 \times 10^{-4}$ (in bone marrow). The **prophylactic arm** included patients with ALL relapsing after SCT who had achieved morphological remission and were candidates for a 2nd 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 2×10^8 . Vaccination for cohort 2 consisted of 3 doses of 5×10^6 irradiated (70 Gy) donor-derived LCL given on 2 days prior to CTL infusion and on days 30 and 60 post CTL infusion.

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. Grey 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 2 markers. The MRD levels cleared transiently following the second transplant, however they rose to a maximum of 1.5×10^{-4} at the time of CTL infusion. Following infusion of CD19 CAR 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 2 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.

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 (Fc γ +) 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.

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⁻, Fc γ R-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 autologous serum (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. 1×10^5 CD19CAR CTL were coincubated with 2.5×10^4 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 analysed after 24 hours, 72 hours and 168 hours 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.