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Intent to Treat Leukemia Remission By CD19CAR T Cells of Defined Formulation And Dose in Children and Young Adults

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Short Title: Performance of CAR T cells of defined formulation

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Key Points:

1. Defined composition manufacturing platform of CD19CAR T cells contributes to >90% intent to treat CR rate.

2. Uniformity of durable persistence of CAR T cells and mitigation of antigen escape are key aspects for further optimization.

Abstract

Transitioning CD19-directed CAR-T cells from early phase trials in relapsed patients to a viable therapeutic approach with predictable efficacy and low toxicity for broad application in patients with high unmet need is currently complicated by product heterogeneity resulting from transduction of undefined T cell mixtures, variability of transgene expression, and terminal differentiation of cells at the end of culture. A phase 1 trial of 45 children and young adults with relapsed or refractory B-lineage ALL was conducted using a CD19 CAR product of defined CD4/CD8 composition, uniform CAR expression, and limited effector differentiation. Products meeting all defined specifications occurred in 93% of enrolled subjects. The maximal tolerated dose (MTD) was 10^6 CAR-T cells/kg, and there were no deaths or instances of cerebral edema attributable to product toxicity. The overall intent-to-treat (ITT) minimal residual disease (MRD)-negative remission rate for this Phase I study was 89%. An MRD-negative remission rate measured among subjects who received a CAR T cell product was 93%, and 100% in the subset of patients that received flu/cy lymphodepletion. Twenty-three percent of patients developed reversible severe cytokine release syndrome (sCRS) and/or reversible severe neurotoxicity (sNT). These data demonstrate that manufacturing a defined composition CD19 CAR-T cell identifies an optimal cell dose with highly potent antitumor activity and a tolerable side effect profile in a cohort of patients with an otherwise dismal prognosis.

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INTRODUCTION

CD19-specific chimeric antigen receptor (CAR)-expressing autologous T cells administered following lymphodepleting chemotherapy can induce clinical remissions in B-lineage malignancies including refractory pediatric acute lymphoblastic leukemia (B-ALL), irrespective of disease burden and anatomic dissemination.[1-6] Obstacles to broad clinical deployment of this treatment are (1) a high failure rate in CAR-T cell manufacturing, (2) heterogeneity of anti-tumor responses, and (3) severe modality-associated toxicities in some patients.[1,7,8] While structure-function attributes of the various CD19-specific CARs have been delineated in preclinical models,[9] variations among the polyclonal transduced CAR-T cell products tested in clinical trials have limited opportunities for the systematic study of product attributes related to cellular composition, differentiation, and transgene expression that impact therapeutic potency and safety.[10] Additionally, a major barrier to the overall success of this therapy in other reported studies, has been the exclusion of research subject enrollment in up to 24% of subjects based on an in-vitro assay predicting failure to manufacture a CAR T cell product.[8] Given the high rates of remission in ALL patients, manufacturing feasibility is a significant contributor to intent to treat remission induction.

We engineered a CAR-T cell product that comprises a defined 1:1 ratio of $CD4^+/CD8^+$ CAR-T cells, selects for uniform high-level CAR expression, and limits activation-induced differentiation of $CD4^+/CD8^+$ T cells by using homeostatic cytokines for CAR-T cell expansion. Herein, we report a robust intent-to-treat (ITT) product manufacturing success rate of 100% in minimally selected heavily pre-treated patients, a 93% MRD-negative (MRD-neg) complete remission (CR) rate in treated subjects resulting in 89% overall efficacy based on the ITT population, with a tolerable side effect profile having an overall decrease in severity of CRS compared with prior studies and no cases of cerebral edema.[1,2] We report on duration of leukemic remissions, and the impact of lymphodepleting regimen and CD19 antigen burden on

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sustained CAR engraftment and durable remissions. These data establish the feasibility of this advanced manufacturing platform and support further study of this highly defined CD19 CAR-T cell product.

METHODS

Study Design and Participants

This phase I/II study (NCT02028455) for recurrent/refractory $CD19^+$ ALL in children and young adults was conducted in accordance with US Food and Drug Administration and International Conference on Harmonization Guidelines for Good Clinical Practice, the Declaration of Helsinki, and applicable institutional review board requirements. All patients or their guardians provided written informed consent.

Enrollment criteria included: aged ≥12 months and <27 years and weighing ≥10 kg. Subjects with no prior history of allogeneic HCT were required to have one of the following characteristics: second or later marrow relapse, with or without extramedullary disease; first marrow relapse at the end of the first month of reinduction, with the marrow having ≥0.01% blasts by multiparameter flow cytometry, with or without extramedullary disease; primary refractory disease, defined as having M2 or M3 marrow after ≥2 separate induction regimens; an indication for HCT but ineligible for the procedure. Among those who had undergone allogeneic HCT, patients were required to have a confirmed $CD19^+$ leukemia recurrence, defined as ≥0.01% disease and were required to be free from active graft-vs-host disease and off immunosuppressive therapy for ≥4 weeks prior to enrollment. Patients with CNS leukemic involvement were eligible for the study, provided they were asymptomatic. Patients with significant neurologic deterioration were not eligible until alternate therapies resulted in neurologic stabilization and return to baseline status. Patients had a Lansky performance status score of ≥50 or a Karnofsky score of ≥50 for patients aged ≥16 years. Patients were required to have a life expectancy of ≥ 8 weeks

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and must have recovered from the acute toxic effects of prior chemotherapy, immunotherapy, or radiotherapy. Patients were required to have adequate organ function and an absolute lymphocyte count of ≥100 cells/μL.

Clinical Lentiviral Vector and Cell Product Manufacture

The CD19CAR-T2A-EGFRt sequence (Supplemental Figure 2) and expressed proteins have been previously described[11,12]. $CD8^+$ and $CD4^+$ T cells were sequentially isolated from subject apheresis product by immunomagnetic separation using the CliniMACS™ device (Miltenyi Biotec) in positive selection mode. Selected cell fractions then either proceeded directly to stimulation as described below, or were cryopreserved in Cryostor-CS5 (BioLife Solutions) for future expansion.

Following enrichment, $180x10^6$ CD4 and CD8 selected T cells were separately stimulated with GMP Dynabeads® CD3/CD28 CTS® (Thermo Fisher) at a 1:3 ratio (T cell:bead), and transduced via spinoculation with clinical grade SCRI-19CAR-01_epHIV7 at an approximate MOI of 0.1 in X Vivo15 containing 10% FCS with 0.1mg/mL protamine sulfate (APP Pharmaceutical). CD4 cultures were supplemented with 5 ng/mL rhuIL-7 and 0.5 ng/mL rhuIL-15 (CellGenix), whereas CD8 cultures were supplemented with 0.5ng/mL rhuIL-15 and 50 U/mL rhuIL-2 (Prometheus). Cultures were then transferred to appropriately sized VueLife FEP culture bags (SaintGobain) and maintained at 37°C, 75% relative humidity, 5% CO_2. Cultures were fed every two to three days with fresh X-Vivo15 + 10% FCS, supplemented with appropriate cytokines as described above in order to keep cell density between $5x10^5$ and $2x10^6$ viable cells/mL. Approximately ten days after the lentiviral transduction, the CD3/CD28 CTS particles were removed using the Dynal DynaMag CTS® magnet. Approximately two days following bead removal, cultures received immunomagnetic positive selection for EGFRt-expressing cells using the CliniMACS device.

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Cultures were propagated until sufficient cells for testing and subject administration were generated as determined by Trypan blue exclusion, at which time cultures were harvested, washed in Isolyte (Braun) with 2% HSA, then resuspended in Cryostor-CS5 for cryopreservation in CryoMACS bags (Miltenyi Biotec). Quality control tests on freshly thawed cells included viability by Trypan blue exclusion, potency (cell surface expression of EGFRt), identity (cell surface expression of CD4 or CD8 as appropriate), average transgene copy number (WPRE qPCR), replication competent virus testing (VSV-G qPCR and formal RCL testing at the University of Indiana), residual bead count, mycoplasma (PCR), endotoxin (LAL), and sterility (USP bacterial and fungal). Typically, these release tests were completed within 7 days of product cryopreservation.

Evaluations

The primary end points of this study were to evaluate the feasibility and toxicity. Feasibility was evaluated by the ability to generate a therapeutic product after 2 attempts using a single apheresis product as starting material. Toxicity was evaluated relative to a baseline assessment taking place within 24 hours prior to the T cell infusion. The primary efficacy end point was the minimal residual disease (MRD) negative rate by bone marrow aspirate by day 63; assessments were also conducted at days 7, 21, and 42 post-infusion. Responses were graded per standard ALL criteria. Secondary end points included persistence of transferred T cells (defined as the detection of transferred T cells in the peripheral blood and bone marrow); disease response and anti-CD19 activity response (defined as a measurable effect of disease reduction and absence of CD19+ cells), and the efficacy of cetuximab to ablate EGFRt+ T cells. Isolated peripheral blood and bone marrow lymphocytes and whole CSF were stained with a viability dye and the following monoclonal anti-human antibodies: CD3, CD4, CD8, CD19, CD14 and APC-conjugated Erbitux (BD Biosciences). Cells were acquired on a LSRFortessa (BD, Franklin Lakes, NJ) and analyzed using FlowJo software (TreeStar, Ashland, OR). Immunophenotyping of surface markers on PBMC, starting and final products was performed using standard staining and flow

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cytometry techniques with combinations of the following fluorophore-conjugated anti-human monoclonal antibodies: CD3, CD8α, CD4, CD14, CD45RO, CD27, CD45RA, CCR7, CD95, PD-1, LAG-3 (BD Biosciences), TIM-3 and CD39 (Biolegend). CAR T cell expression (EGFRt) was quantified using custom-conjugated Cetuximab-APC (BD Biosciences). Cells were also stained with a live/dead viability dye (BD). Cells were acquired on an LSRFortessa (BD Biosciences) and flow cytometric analysis was performed using FlowJo software (Treestar). T cells were defined as Singlets/Lymphocytes/Live CD3+CD14-/CD4+ or CD8+.

Statistical Analysis

The maximum tolerated dose at the end of Phase 1 dose escalation was defined as the highest T cell dose with ≥6 toxicity-evaluable patients and a cumulative dose-limiting toxicity rate <34%.

Standard descriptive statistics (median/range, percent, and response rates) are reported for key variables. Directional associations between categorical variables with 3 levels (sCRS, neurotoxicity) and various predictors were evaluated via univariate proportional-odds logistic regression (POM). The significance level for these tests and the ones described further below was 0.05.

Kaplan-Meier (K-M) curves were calculated for overall survival (OS), event-free survival (EFS) and B-cell aplasia (BCA), a marker of T-cell persistence. The OS and EFS curves include data from all infused subjects. For BCA, only data from patients achieving CR were included. Pairs of curves were calculated for these endpoints, dividing patients into two groups by characteristics and variables that are either inherent to the design or known as risk and Log-rank tests were performed to assess significance.

Survival endpoints were analyzed with multivariable proportional-hazards Cox models and adjusted for dose (log-transformed) and CD19 burden, and incorporated prior HSCT (yes/no) as a risk stratum.

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For engraftment analysis, peak engraftment (as percent of all T-cells) and time-averaged engraftment ("area under the curve") from infusion through Day 63 were calculated for each patient, then grouped by risk factors as listed above, and differences tested via two-sided Wilcoxon-Mann-Whitney (when 2 groups were compared) or Kruskal-Wallis tests (when 3 or more groups were compared).

Analyses were performed using R 3.3.1 (R foundation for statistical computing, Vienna), and SAS (version 9.4; SAS Institute Inc, Cary, NC).

RESULTS

Forty-five subjects with relapsed or refractory $CD19^+$ ALL were enrolled onto the phase 1 arm of the PLAT-02 protocol with a median age of 12.3 years (range, 1.3 – 25.4), including four subjects less than three years of age (Table 1 and supplemental table 1). Twenty-eight subjects (62%) had a history of at least one prior allogeneic transplant, with a range of 121 – 1783 days from the time of most recent transplant prior to enrollment. The disease burden varied, with 22 subjects having M3 marrow and 9 subjects having active CNS involvement at time of lymphodepletion. Seven patients had previously received CD19 directed therapy: blinatumomab (n=6) and 2^{nd} generation (CD28zeta) CD19-specific CAR-T cells (n=1).The mean time between completion of blinatumomab and PLAT-02 CAR T cell infusion was 52 days (range 36 - 73) and 21 months from prior CD19CAR T cell infusion.

An objective of our study was to determine the feasibility of manufacturing and releasing patient-derived products with distinct features: (1) a defined 1:1 ratio of CAR-expressing $CD4^+/CD8^+$ subsets, (2) uniform high-level expression of CD19-specific 4-1BB:zeta CAR, and (3) homogeneity of T cell differentiation at the end of culture with enrichment of cells expressing cell surface markers associated with engraftment fitness.[13,14] In the heavily treated population of children and young adult ALL patients

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enrolled on this study, the sole eligibility requirement pertaining to manufacturing feasibility was an absolute lymphocyte count (ALC) >100 cells/µl. The average ALC at the time of apheresis was 1228 cells/µl (range, 168 – 4488) and no in vitro proliferation screen was required. All enrolled subjects had successful manufacturing of a clinical CAR T cell product and 33/45 products were manufactured from fresh apheresis material, whereas 12/45 were manufactured from cryopreserved CD4 and CD8 T cell subsets. Paired CD4/CD8 products were manufactured (Fig 1A) and released at the protocol prescribed doses from 41/45 (89%) on an ITT basis, where as an additional three patients had released products that deviated from specification (two without CD4 unit, one with a less than 1:1 CD4:CD8 dose administered). One subject (S39) had a product that failed to expand adequately and required a second apheresis which produced a CAR T cell product that was released for infusion at the prescribed 1:1 ratio. The cytokine cocktails used for manufacturing of CD8 and CD4 T cells enriched for T cells having phenotypic markers associated with engraftment fitness such as CD127, CCR7, and CD27 (Fig 1B). The mid-process immunomagnetic EGFRt selection step enriched for transgene expressing T cells (Fig 1C) at a uniform level of expression (Fig 1D). The average length of cell product manufacturing was 15 days for CD8 cell products (range, 11 – 22) and 14 days for CD4 products (range, 10 – 20). A median of 53 days transpired (range, 29 – 156) from consent to product infusion.

Detectable engraftment and expansion of CAR-T cells was observed in 42 of the 43 (98%) infused subjects, and B cell aplasia (BCA) was observed in 40 of 43 (93%). Irrespective of cell dose, all subjects (n=14) who received prescribed lymphodepletion with fludarabine and cyclophosphamide (flu/cy) achieved uniform engraftment of functional CAR-T cells as defined by detectable CAR-T cells in blood and subsequent development of BCA and MRD-neg remission. Engrafted CAR-T cells were present in peripheral blood, marrow and CSF (Fig 2A) and were functional in each of these anatomic compartments based on ablation of malignant and nonmalignant $CD19^{+}$ cells (Fig. 2B). The median time to peak engraftment in peripheral blood was 10 days (range, day 7 – 18). For the entire cohort of treated

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subjects, the magnitude (area under the curve) and peak engraftment in peripheral blood did not correlate with the infused cell dose (p=0.9; p=0.8) or disease burden at the time of product infusion (p=0.12, 0.14), but positively correlated with the total level of $CD19^{+}$ antigen load in the marrow (tumor + nonmalignant B cells >15% versus <15%; p=0.001; p=0.003) and the use of flu/cy versus cy alone as the lymphodepletion regimen (p=0.05, p=0.03)(Fig. 2C). However, the significance of flu/cy on peak engraftment is lost when accounting for CD19 antigen load. Engraftment kinetics in the bone marrow were similar to peripheral blood; however, M3 disease burden significantly correlated with the magnitude of CAR T engraftment in marrow specimens (Supplemental Fig 2A and 2B). The persistence of functional CD19 CAR-T cells was assessed by measuring the duration of BCA by flow cytometry. With a median follow-up duration of 9.6 months for the entire cohort (range of 2 to 28 months), the median expected duration of BCA was 3 months (95% CI 2.07, 6.44) (Fig 2D).

The rate of MRD-neg CR, as measured by multiparameter flow cytometry, was 89% (40/45) of all enrolled subjects, and 93% (40/43) among research participants who received a CAR T cell infusion (Table 2). All remissions occurred by day 21. There was no impact of disease burden, relapsed/refractory status, prior transplant status, or high-risk cytogenetics on achievement of MRD-neg CR. The rate of MRD-neg CR in M3 subjects was 90% (19/22) and 100% for those who receive flu/cy (14/14). Four of the six subjects who had received prior blinatumomab obtained an MRD-neg CR. Of the 3 evaluable subjects who failed to obtain a CR, two had evidence of engraftment with expansion and functional activity of the CAR-T cells (Fig S3). The ALC at the time of apheresis was lower in the three non-responders (mean 469 vs 1327, p=0.04). Of the 40 subjects who obtained an MRD-neg CR, 27 had a malignant clone identified by next-generation sequencing (NGS). Sixty-five percent (17/27) of these subjects achieved a molecular CR by day 63.

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The estimated 12-month EFS is 50.8% (95%CI 36.9, 69.9) (Fig 3A) and OS is 69.5% (95%CI 55.8, 86.5%) (Fig 3B) of infused subjects with a median time of follow up of 9.6 months. The temporal relationship between the duration of BCA, duration of remission, and occurrence of relapse is depicted in Fig 3C. Eighteen of the 40 subjects who obtained an MRD-neg CR relapsed, of which 17 were isolated to marrow and one was isolated to CNS (supplemental table 1). Seven relapses were associated with loss of cell surface detection of CD19, including one lineage switch to AML occurring one month after CAR-T cell infusion.[15] Of the 11 relapses in which leukemic cell-surface CD19 expression was preserved, the median time from T cell infusion to relapse was 5.98 months (range of 1.25-14) with a median time from loss of BCA to relapse of 3.7 months (range of 0-11). Eleven subjects received a consolidative alloHSCT and 2 have subsequently recurred, both with $CD19^+$ disease. These two subjects exhibited molecularly detectable leukemia by NGS at the time of transplant, one of whom was MRD negative by flow cytometry. Of 29 subjects who reached a CR and did not go on to a consolidative alloHSCT, 13 subjects remain in continuous CR with a median time of follow up of 12.2 months (range of 1.9-27.5 months).

In analyzing the 40 patients achieving MRD-neg remission on this study, we found that the loss of functional CAR-T cells, as measured by loss of BCA, adversely affects the risk for $CD19^+$ leukemic relapse (hazard ratio (HR): 34 [95% CI 2.1,552] p=0.01), whereas the HR for all relapses, $CD19^+$ and $CD19^-$, is 3.5 ([95% CI 1.01,11.88] p=0.04). In a multivariate analysis of factors that predict BCA duration, inclusive of dose level, disease burden, and lymphodepletion, marrow $CD19^+$ antigen load of <15% prior to lymphodepletion is a significant risk factor for early loss of BCA (HR: 2.99 [95% CI: 1·32,6·81] p=.005). The median duration of BCA for patients with a $CD19^+$ antigen load >15% was 6.4 months (95% CI of 2.6 months, upper bound not defined) vs 1.7 months for patients with a load of <15% (95% CI lower bound 1.4 months, upper bound not defined) (Fig 3D). The median duration of BCA for the 29 subjects who received lymphodepletion therapy without flu/cy was 2.1 months (95% CI 1.4 – 6.4months) versus 6.4 months (95% CI lower bound 2.5 months) for the 14 subjects that received flu/cy (p=0.15) (Fig 3E).

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Ten subjects received a second dose of CAR-T cells (supplemental table 2). Administration of a second dose of CAR-T cells in 8 subjects who had lost engraftment resulted in only 2 demonstrating evidence of re-engraftment. The 6 subjects who did not obtain re-engraftment received the less immunosuppressive lymphodepletion regimen that did not include fludarabine with the first infusion, accordingly the role of immunologic rejection cannot be discounted as an etiology of re-engraftment failure.[3,4] Two of the eight (S04, S32) were given CAR-T cells for $CD19^+$ relapse following loss of CAR-T cells with one (S32) obtaining a second MRD negative CR. Two additional subjects were given repeat infusions for ongoing (S09) or re-emergence (S37) of $CD19^+$ disease with persistent low level CAR-T cell engraftment: following re-infusion these two subjects continued to have ongoing CAR-T cell persistence without substantial re-expansion, induction of BCA, or anti-leukemic effect.

The occurrence of severe toxicities resulting in patient mortality is a barrier to broad adoption of CAR-T cell immunotherapy. No toxic deaths were reported on this trial. Dose-limiting toxicities (DLT) were observed at all dose levels and disease burdens (Table 3). Dose level 2 (1×10^6 CAR-T cells /kg) was determined to be the MTD with a DLT rate of 17% (1/6) with flu/cy and 9% (1/11) overall. No subject met criteria to receive cetuximab. The most common adverse events related to CAR-T cells were CRS and neurotoxicity (Supplemental Tables 3 and 4). The overall incidence of CRS was 93% (40/43) of subjects and the rate of protocol defined severe CRS (requirement for pressor, ionotropes or respitratory failure) was 23% (10/43). The predominant symptom that contributed to severity of CRS was hypotension requiring pressor support. No subject required intubation for respiratory failure, multiple pressors or high dose pressors. For treatment of CRS, sixteen subjects received one or more (5/16) doses of tocilizumab, and 10 subjects received corticosteroids for a median duration of 5 days (range 1-13). The use of tociluzimab and/or corticosteroids did not impact response rates or durability of remission. A dose level effect on severity of CRS was noted, with higher doses having higher rates of sCRS ($p=0.03$) (Fig 4A). A correlation between disease burden at the time of CAR-T cell infusion with

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severity of CRS was not observed (p=0.56), nor with CD19 antigen load (p=0.13), however there was a trend towards increased CRS with increased CD19 antigen load. Similarly, there was no impact of the lymphodepletion regimen on severity of CRS (p=0.63).

The overall incidence of neurotoxicity was 49% (21/43); severe neurotoxicity (any grade seizure or grade 3/4 neurotoxicity exclusive of headache) occurred in 21% (9/43). No cases of cerebral edema were encountered. There was no effect of dose level (p=0.32), disease burden (p=0.93), CD19 antigen load (p=0.23), or flu/cy lymphodepletion (p=0.32) on the occurrence of severe neurotoxicity; however, the presence of sCRS was predictive of subsequent severe neurotoxicity (p=0.01) (Fig. 4B). All subjects experiencing neurotoxicity experienced reversible symptoms and eventual return to baseline neurologic status.

Twenty-seven subjects on the trial had a prior allogeneic HSCT. One of these patients experienced recrudescence of grade 3 acute skin GVHD (supplemental figure 4). This subject (S03) was 2.3 years from prior transplant and had tapered off GVHD medications more than a year before CAR-T cell treatment. Although the infused CAR-T cells cannot be formally ruled out as direct mediators of the skin GVHD, a biopsy of involved skin revealed that 9% of $CD3^+$ T cells infiltrating the dermis were $EGFRt^+$ compared with 78% $EGFRt^+$ T cells in peripheral blood. The subject was treated with prednisone 2 mg/kg/day followed by a taper for approximately 1 month with resolution of GVHD and without untoward effect on the persistence of CAR-T cells or BCA. Functional persistence of CAR T cells was noted up until the time of CD19 negative relapse 8 months following CAR T cell infusion.

DISCUSSION

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This study evaluated treatment of children and young adults with relapsed/refractory B-ALL with CD19 CAR-T cells characterized by a defined CD4/CD8 T cell ratio, a uniform level of CAR expression, and a less differentiated phenotype. Of 43 patients treated at CAR-T cell doses ranging from 0.5 to $10x10^6$ cells/kg, 41/43 (93%) obtained MRD-neg CR, with a rate of 89% among all enrolled subjects. After flu/cy lymphodepletion, 14/14 patients experienced MRD-neg remission. Specific subgroups of subjects that may predict for increased or decreased efficacy were not identified owing to the high CR rate and trial size. In particular, there was no difference in response rates based on number of prior relapses or among low and high disease burden subjects. The estimated 12-month OS for this poor prognosis cohort of patients is 65.9% and EFS is 50.3%. A longer duration of post-remission functional persistence of CAR T cells, as inferred by ongoing BCA, correlated significantly with the durability of remission. Higher CD19 antigen load positively correlated with prolonged CAR-T cell persistence and B cell aplasia.

There is significant heterogeneity in CD19 CAR clinical trials reported to date with respect to receptor composition and product formulation.[1,2,4,7,16,17] Accordingly, early phase trials have reported a spectrum of efficacy and toxicity results. Our data show that, despite significant inter-patient heterogeneity of apheresis T cell populations from children and young adults with relapsed/refractory ALL, manufacturing utilizing cell purification, enrichment of transgene expression, and cocktails of recombinant human cytokines can reproducibly generate products that exhibit phenotypic and functional attributes associated with therapeutic potency.[13,14] This is in contrast to reported studies that require a screening in vitro proliferation assay or ALC of >500 cells/μl for enrollment, and have excluded 24% of patients enrollment in CD19 CAR trials.[8] The cell selection, homeostatic cytokines, and enrichment of transgene expressing T cells likely all contribute to the robustness of our product manufacturing approach. Given the efficacy of CD19 CAR T cell infusions in inducing leukemic remissions in select trials, the greatest intent to treat risk of therapeutic failure now resides in the feasibility of manufacturing a product for any given research participant. This manufacturing platform therefore provides a significant advantage over

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prior reported trials which excluded substantial numbers of subjects due to inability to manufacture a product or have a marked decrease in efficacy.[1,2]

No toxic deaths occurred on trial with an acceptable incidence of sCRS (23%) and severe neurotoxicity (23%).[1-3,7] The rate of sCRS increased with cell dose level escalation, however we did not find an effect of higher disease burden with increased rates of sCRS. The level of CD19 antigen burden, although not statistically significant, did trend towards higher rates of sCRS. Although the reported rate of severe CRS is 23%, the threshold definition of severe CRS used for this trial is lower than other reported comparable trials.[1,2] When applying the definition of severe CRS from other protocols, the rate of severe CRS is remarkably less, and in fact, there were no subjects meeting the definition of grade 4/severe CRS from other trials[1]. Therefore this product appears to have an improved toxicity profile with decreased severity of CRS. It has previously been shown in animal models that the defined composition product can induce remissions at lower doses[10] and we believe that the defined composition formulation of this product enables the use of lower doses of CAR T cells, while still retaining high efficacy, resulting in attenuated CRS severity.

This Phase I study investigated tolerability of formulated CAR T cell product infusions over a range of cell doses and with various lymphodepletion chemotherapy regimens. At the MTD of 10^6 cells/kg following Cy/Flu lymphodepletion we observed uniform engraftment and MRD-neg remissions in 14 of 14 research participants and enhanced durability of functional CAR T cell persistence. Our Phase I trial has revealed that a subset of patients exhibit durable remissions without consolidative alloHSCT and the durability of remission is enhanced by ongoing CAR T cell persistence. A risk factor for relapse with $CD19^+$ disease is a short duration of B cell aplasia that in turn can be predicted by the quantity of CD19 expressing B cells (malignant and non-malignant) in the bone marrow prior to CAR T cell dosing. Our observation that low quantities of $CD19^+$ B cells results in suboptimal expansion and persistence of CD19

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CAR T cells portends a future challenge when this modality is applied to patients earlier in the course of their therapy, for instance patients in second remission as a consolidative therapy that obviates prolonged chemotherapy and/or alloHSCT. Approaches to augment CD19 CAR T cell persistence independent of B cell/leukemic cell burden are warranted and are being pursued by our group by the provision of T cells transduced to express CD19 following CAR T cell infusion. Additionally, the uniform use of flu/cy lymphodepletion may also promote longer persistence of the CAR T cells allowing more comparable functional CAR T cell persistence to other CD19 CAR T cells with 4-1BB costimulation[1]. Although we did not observe a statistically significant advantage on the duration of CAR T cell persistence with flu/cy lymphodepletion, there was a trend towards improved persistence, and this has previously been shown by others to significantly enhance long term CAR T cell persistence, potentially through the reduction of T cell rejection responses to the murine scFv CAR binder domain.[3,4] For this reason, a Phase 2 arm of this protocol is now enrolling and treating patients at the MTD of 10^6 cells/kg following flu/cy lymphodepletion.

CD19 antigen escape is an established mechanism of relapse after remissions induced by CD19 CAR T cell immunotherapy.[18] In this Phase I study, relapse occurred in 45% of patients who achieved a MRD-neg CR, and CD19-negative escape variant leukemia accounted for 39% of these relapses. Targeting B cell lineage ALL with CD22-specific CAR T cells may be able to salvage a proportion of patients who suffer a CD19-neg relapse.[19] Current efforts by our group are focusing on bispecific CAR engineering and vector technologies that allow for expression of two CARs that enable dual targeting of CD19 and CD22, as the simultaneous targeting of two or more antigens is anticipated to reduce therapeutic failures from antigen escape following immunotherapy with monospecific CAR T cells.

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Authorship Contributions

RAG, JRP, MCJ designed, conducted and analyzed the study and wrote the paper, DL and AO analyzed the data and wrote the paper. MB designed and reviewed the paper. CA, KL, CS conducted the study and reviewed the paper. OF, HS designed, conducted and analyzed the study and reviewed the paper, CL, SM, and CB manufactured the products, KS designed the study and reviewed the paper, VH conducted the study, SRR designed the study and reviewed the paper.

DECLARATION OF INTERESTS

RAG, CA, CS, KL, CB, SM, OF, HS, KS, VH, CL, and AO have no personal interests to disclose.

DL is an employee of, and has an equity interest in, Juno Therapeutics, Inc.

SRR has received consulting fees and grants from, and is an inventor of patents licensed to, Juno Therapeutics, Inc., in which he has an equity interest.

MCJ has received consulting fees and grants from, and is an inventor of patents licensed to, Juno Therapeutics, Inc., in which he has an equity interest.

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Tables

Table 1. Patient Characteristics

Characteristic	N = 45
Age	12.2 (1.3 - 25.3)
Female, n (%)	22 (48.9)
Disease status, n (%)	
Primary refractory	3 (6.7)
1st relapse	15 (33.3)
2nd relapse	22 (48.9)
≥ 3rd relapse	5 (11.1)
Prior allogeneic transplant, n (%)	
0	17 (37.8)
1	24 (53.3)
2	4 (8.9)
Time since allogeneic transplant, median (range), mo	18.3 (4.0 - 58.6)
Disease status at lymphodepletion	
M3	22 (48.9)
M2	6 (13.3)
M1	8 (17.8)
MRD-negative	7 (15.6)
CNS status at lymphodepletion	
CNS1	34 (79.1)
CNS2	7 (16.3)
CNS3	2 (4.7)
ALC, median (range), cells/µL	1228 (168 - 4488)
ABC, range, cells/µL	0 - 48407

ABC, absolute blasts count; ALC, absolute lymphocyte count; CNS, central nervous system; MRD, minimal residual disease.

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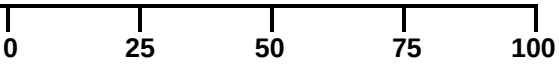
Table 2. MRD-negative CR rate by Patient Subgroup

	N	MRD-CR (%)	95% CI	MRD-negative CR rate (95% CI)
Overall	43	93	81-99	
Disease Burden				
M3	22	86	65-97	
M2	6	100	54-100	
M1	8	100	63-100	
MRD-negative	7	100	59-100	
Dose CAR-T cells/kg				
0.5×10^6	13	92	64-100	
1.0×10^6	18	89	65-99	
5.0×10^6	7	100	59-100	
10.0×10^6	5	100	48-100	
Lymphodepletion				
Cy	27	93	76-99	
Flu/cy	14	100	77-100	
Transplant status				
Pre	15	93	68-100	
Post	28	93	77-99	
Disease status				
1st relapse	14	93	66-100	
2nd relapse	22	91	71-99	
≥ 3rd relapse	5	100	48-100	
Cytogenetics				
Ph+ or Ph-like	8	100	63-100	
MLL	5	100	48-100	



CAR, chimeric antigen receptor; CR, complete remission; cy, cyclophosphamide; flu, fludarabine; MRD, minimal residual disease; Ph, Philadelphia chromosome.

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Table 3. Summary of Dose-Limiting Toxicities

Dose Level, cells/kg	Type	Rate, n/n (%)
0.5×10^6	Grade 4 encephalopathy	1/7 (14)
1.0×10^6	Grade 4 hydrocephalus	1/11 (9)
5.0×10^6	Grade 4 seizure (flu/cy); Grade 3 encephalopathy (flu/cy)	2/7 (29)
10.0×10^6	Grade 3 left ventricular dysfunction; Grade 3 seizure	2/5 (40)
Prescribed Lymphodepletion With Flu/Cy		
0.5×10^6	-	0/6
1.0×10^6	Grade 4 encephalopathy	1/6 (17)

Cy, cyclophosphamide; Flu, fludarabine.

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1.0×10^6	Grade 4 encephalopathy	1/6 (17)

Cy, cyclophosphamide; Flu, fludarabine.

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Figure Legends

Figure 1. Product manufacturing, and product phenotype. A) Manufacturing schema of PLAT-02 formulated CD19 CAR products; B) Flow cytometric phenotype of input apheresis T cells and final products; C) Frequency of transgene expressing T cells in formulated CD4 and CD8 products based on EGFRt flow cytometric enumeration; D) EGFRt transgene expression levels of formulated products based on mean fluorescent intensity

Figure 2. Magnitude and duration of functional CD19 CAR engraftment. A) Representative engraftment and detection of CD19 CAR^+/$EGFRt^+$ T cells in blood, bone marrow, and CSF 21-days following infusion; B) Representative functional activity of CD19 CAR product as measured by clearance of B cells (leukemic and normal) in blood, marrow, and CSF; C) Magnitude and duration of CAR engraftment by flow cytometric quantitation of $EGFRt^+CD3^+$ T cells; D) Durability of functional CD19 CAR engraftment in treated patients based on B cell aplasia

Figure 3. Anti-leukemia response durability. Kaplan-Meier of event-free survival (A) and overall survival (B); C) Waterfall plot of individual research subject remission duration, BCA duration, and timing of alloHSCT and/or relapse; D) Effect of CD19 burden on BCA durability; E) Effect of fludarabine as component of lymphodepletion regimen on BCA durability

Figure 4. Severity of cytokine release syndrome and neurotoxicity. A) Severity of CRS in treated subjects as a function of cell dose, disease burden, CD19 burden, and lymphodepletion regimen, B) severity of neurotoxicity based on parameters described in (A) as well as severity of CRS

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Figure 1

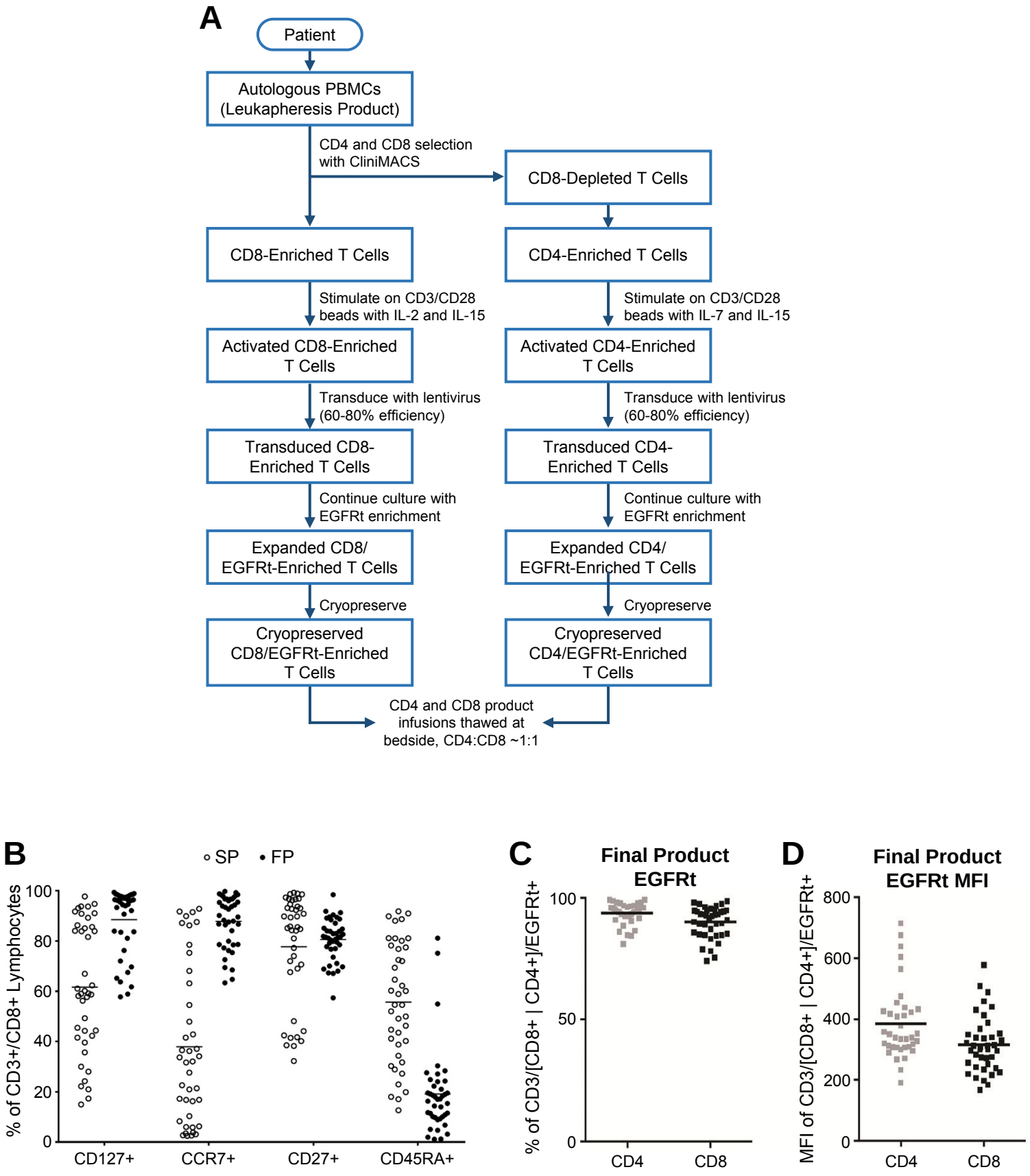

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Figure 2

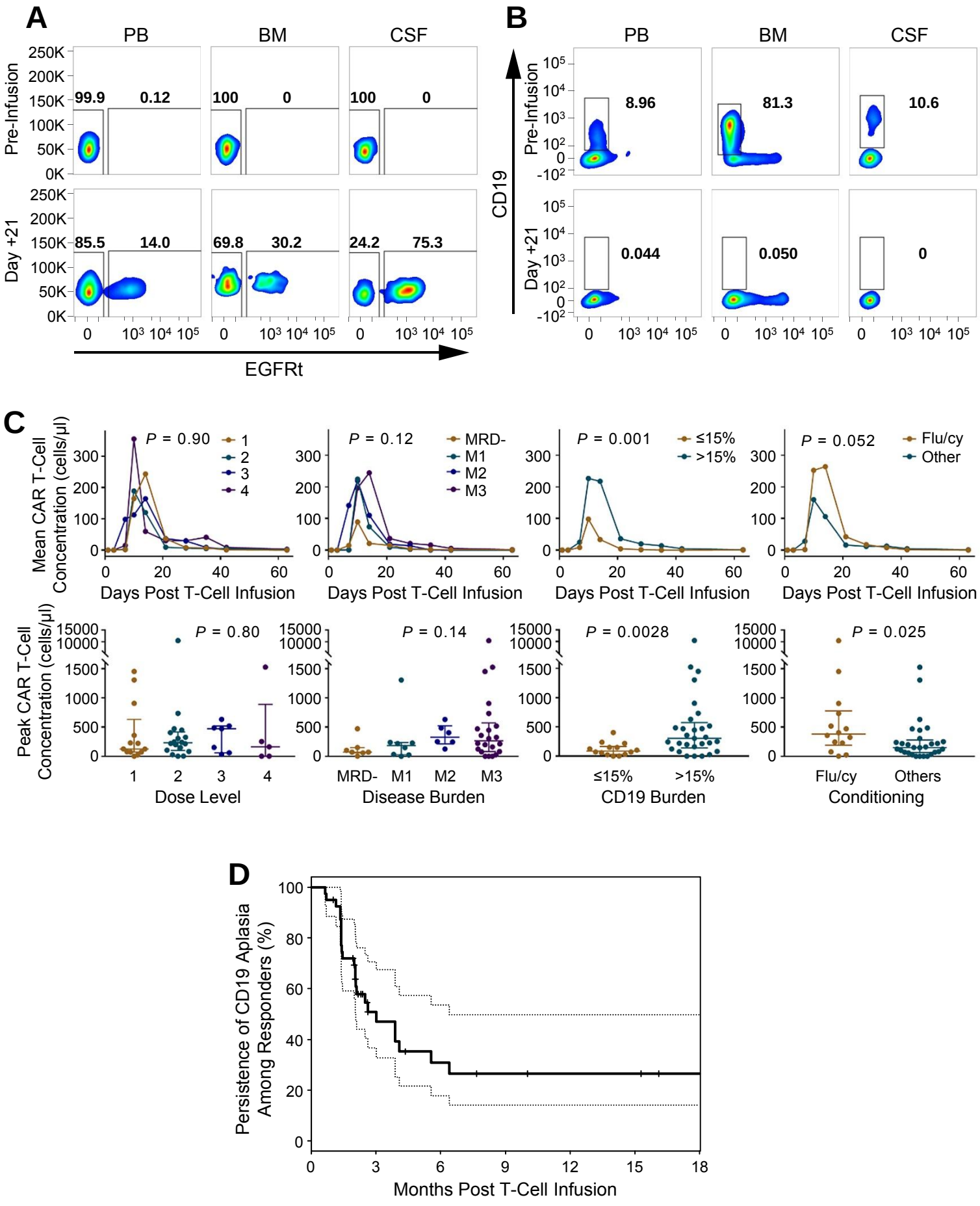

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Figure 3

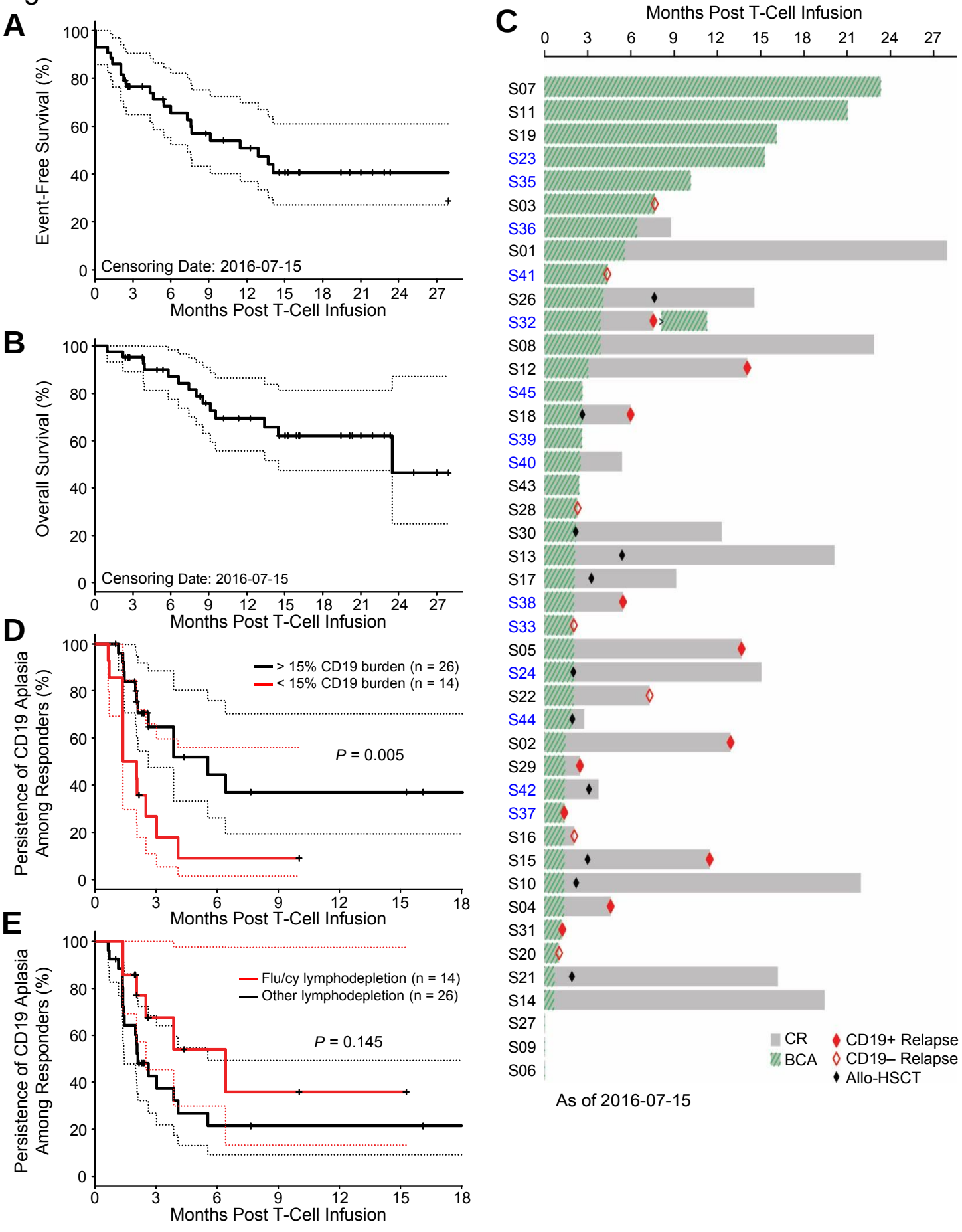

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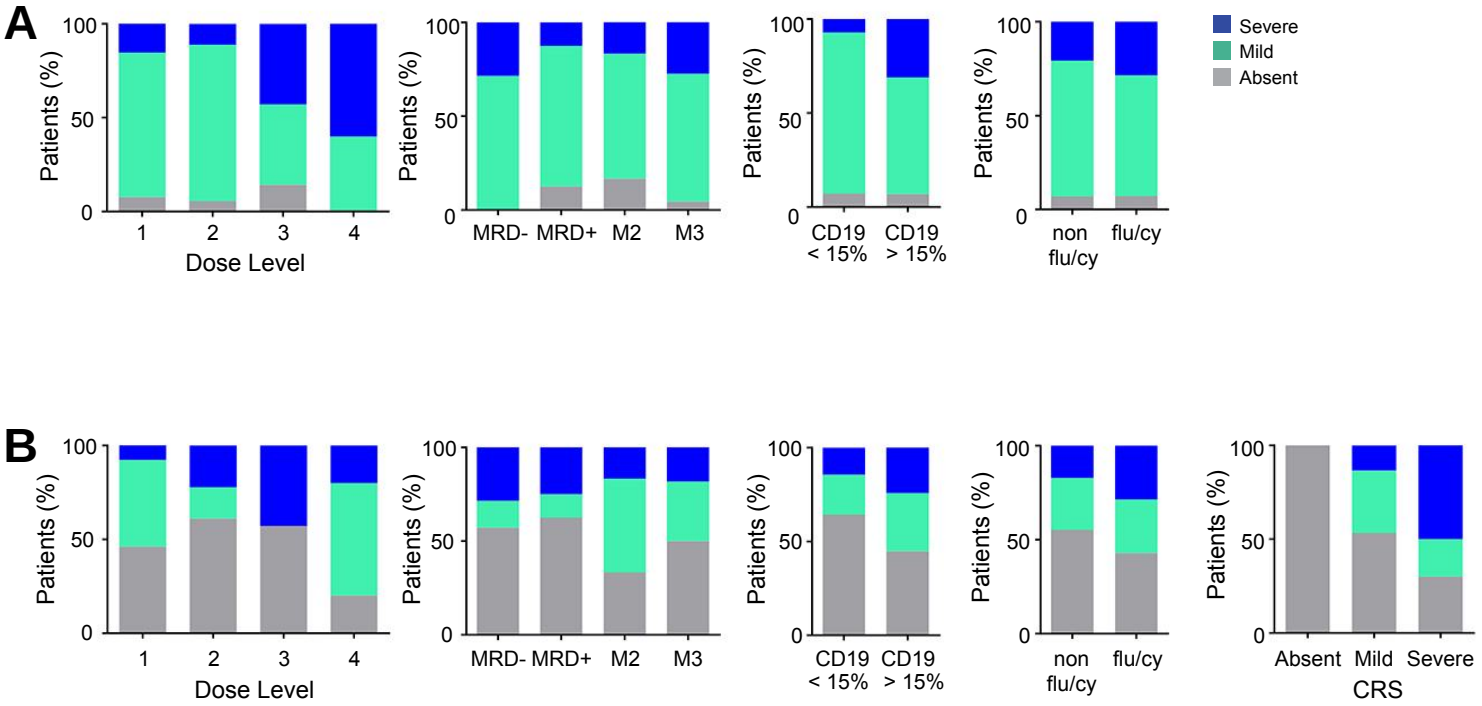
Figure 4



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Intent to treat leukemia remission by CD19CAR T cells of defined formulation and dose in children and young adults

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