Defining Optimal T Cell Characteristics for Pediatric Chimeric Antigen Receptor (CAR) T Cell Trials

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Abstract

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Background: Engineered chimeric antigen receptor (CAR) T cells have emerged as a powerful, highly personalized immunotherapy in pediatric cancer. Early phase clinical trials using CAR T cells targeting CD19 have resulted in complete response (CR) rates as high as 93% in children with relapsed and refractory acute lymphoblastic leukemia (ALL). Despite this success, there are many challenges that must be overcome before CAR T cell therapy can be used routinely in pediatric ALL or other malignancies.

Objective: To develop novel biomarkers that will identify patients at high risk for poor product expansion, treatment failure and/or toxicity with current immunotherapy protocols and that may ultimately help improve manufacturing methods to produce safer and more effective CAR T cells.

Design/Methods: Using flow cytometry, we evaluated T cell characteristics (memory phenotype, cytokine production, presence of markers of activation/exhaustion) of starting products in 43 patients enrolled and treated in a Phase I clinical trial, PLAT-02. Potential predictors of poor product expansion were assessed using the Wilcoxon Rank Sum Test.

Results: A higher percentage of cells producing cytokines and expressing PD-1 in CD4 starting products was associated with poor expansion. Poor expansion was not associated with patient toxicities or outcomes.

Discussion: Increased cytokine production and PD-1 expression suggest a more differentiated, effector-like phenotype of starting products that subsequently experience poor expansion, consistent with preclinical data, although numbers are limited.

Conclusion: Ongoing correlative biology studies will be important in future immunotherapy trials as we seek to identify biomarkers that predict product expansion, toxicities and outcomes.

Introduction

Adoptive cellular therapy has emerged as a pivotal opportunity to improve outcomes for cancer patients. Autologous T cells engineered to express a chimeric antigen receptor (CAR) are a powerful and targeted immunotherapy. Early phase clinical trials using CAR T cells targeting CD19 have resulted in complete response (CR) rates as high as 93% in patients with relapsed and refractory acute lymphoblastic leukemia (ALL).[1–4] Despite such success, there are many challenges that must be overcome before CAR T cell therapy can be integrated into standard treatment for ALL or become an effective therapy for solid tumors.

The first challenge of this highly personalized therapy is the successful engineering of a patient's T cells to become a CAR T cell product with adequate cell dose, viability and functional capacity. There is a subset of patients for whom a product is either difficult to grow or cannot be successfully produced, which may be related to heterogeneity of starting T cell populations among patients.[5] Secondly, although CR rates are exceptional, the duration of CAR T cell engraftment (measured as B cell aplasia (BCA)) is highly variable across patients and studies, ranging from weeks to years, and appears to be a key factor in achieving long-term remission.[1,6,7] Finally, cytokine release syndrome (CRS) and neurotoxicity are potentially life-threatening toxicities associated with CAR T cell therapy that could limit widespread use.[1,8] Currently, we have limited ability to predict who will experience these adverse events.[8]

Preclinical studies demonstrate that the memory phenotype of T cells used to manufacture CAR-T-cell products impacts subsequent *in vivo* expansion, antitumor activity, and cytokine production.[9–11] CAR-T-cells manufactured from less differentiated T cells (naïve and central memory) perform better and with more durable responses than CAR T cells manufactured from more differentiated T cells (effector memory and terminal effector). Given the data linking

memory phenotype to increased cytokine production and proliferation, the starting phenotype and cytokine production of the T cells may also impact the severity of toxicities including CRS and neurotoxicity. Finally, in the setting of cancer or chronic infection, T cells can enter a state termed 'exhaustion', with loss of effector functions and expression of receptors, and this state may result in poor expansion capabilities. These markers can also be expressed on activated T cells and can therefore be classified as markers of activation/exhaustion.[12,13] There is limited published data that examine the role of T cell characteristics in successful product manufacturing, patient outcomes or toxicities.

Utilizing samples from PLAT-02, a local phase I clinical trial using CD19 CAR T cells to treat pediatric patients with $CD19^+$ relapsed and refractory ALL, we evaluated: 1) the role of T cell characteristics (memory phenotype, cytokine production, markers of activation/exhaustion) on the successful expansion of CAR T cell products; and 2) the impact of poor product expansion on toxicities and outcomes.

Methods

Study Enrollment and CAR T cell Manufacturing

PLAT-02 is a phase 1, open-label, nonrandomized clinical trial at Seattle Children's Hospital. Eligible patients include males and females aged ≥12 months or <27 years and weighing ≥10 kg with relapsed or refractory $CD19^+$ ALL. Participants without a prior history of allogeneic hematopoietic stem cell transplant (HSCT) were required to meet one of the following criteria: second or later marrow relapse (with or without extramedullary disease), first relapse at the end of the first month of reinduction, with the marrow having ≥0.01% blasts by morphology and/or multiparameter flow cytometry, with or without extramedullary disease; primary

refractory disease, as defined as having M2 (5-25% blasts) or M3 (>25% blasts) marrow after at least two separate induction regimens; an indication for HSCT but ineligible for the procedure. Among those who had undergone allogeneic HSCT, patients were required to have a confirmed CD19+ leukemia recurrence, defined as ≥0.01% disease.

Eligible patients underwent standard apheresis for collection of peripheral blood mononuclear cells. Apheresis units underwent sequential positive selection for the CD4 and CD8 subsets using magnetic beads (starting product) and then stimulated with anti-CD3/CD28 beads, transduced with a lentivirus containing the CAR construct and expanded in media supplemented with cytokines.

Additional patient eligibility requirements, CAR T cell product production processes and patient treatment and monitoring have previously been described in detail.[1]

Study Endpoints

The primary endpoints of this study were to evaluate the association of poor product expansion with T cell phenotypes, as well as toxicities and outcomes. Poor product expansion was defined as any product that did not meet required cell count or viability thresholds during product manufacturing. In some cases, the product could undergo a rescue procedure in order to remove non-viable cells and a product was successfully produced. In other cases, no product was produced. If no CD4 product was produced, a patient was treated with a CD8 product alone. No patients were treated with a CD4 product alone.

Toxicity and Outcome Definitions

Cytokine release syndrome and neurotoxicity symptoms were graded according to Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. Severe cytokine release syndrome was defined as any life-threatening consequence, including requirement for vasopressor medication or ventilator support. Severe neurotoxicity was defined as any CTCAE grade seizure or CTCAE grade 3 or 4 neurotoxicity exclusive of headaches. Response criteria were graded per standard ALL criteria. B cell aplasia was defined as the absence of $CD19^+$ cells as detected by flow cytometry in patient peripheral blood and bone marrow samples following CAR T cell infusion.

Flow cytometric analysis of T cell characteristics

Starting and final CAR T cell products underwent immunophenotyping of surface markers using standard staining and flow 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). Cytokine production in response to antigen-specific stimulation was determined by co-incubation of CD4 and CD8 cells at a 1:1 ratio with K562 or K562-CD19 target cells for 18 hours in the presence of fluorophore-conjugated CD107a. SEB was used as a positive control stim. Following stimulation, intracellular staining was performed using flurophore-conjugated anti-human monoclonal antibodies for IL-2, IFN-γ, and TNF-α. All 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-Dead-/CD4+ or CD8+. T cell memory phenotypes of

starting products were defined as follows: naïve ($CD45RO^-$ $CCR7^+$), central memory ($CD45RO^+$ $CCR7^+$), effector memory ($CD45RO^+$ $CCR7^-$), and terminal effector memory ($CD45RO^-$ $CCR7^-$).

Data Analysis

Poor Product Expansion

Phenotypic characteristics of CD4 and CD8 starting products as predictors of poor product expansion were assessed using the Wilcoxon Rank Sum test. Baseline clinical characteristics were also assessed as potential predictors of poor product expansion. Logistic regression was used to assess the impact of prior transplant (yes/no) and disease status (primary refractory, 1^{st} relapse, 2^{nd} relapse, 3^{rd} or greater relapse). Wilcoxon Rank Sum test was used to assess the impact of days since transplant, absolute lymphocyte count at apheresis, and absolute blast count at apheresis.

Fisher's exact test was used to assess the predictive value of poor product expansion on a patient experiencing severe cytokine release syndrome, severe neurotoxicity and achieving complete response. Cox regression was used to assess the predictive value of poor product expansion on clinical outcomes of loss of B cell aplasia, relapse or death, or death alone. For the analyses of relapse or death and loss of B cell aplasia, the three patients who did not achieve CR were excluded.

T Cell Characteristics of Starting and Final Products and Toxicities/Outcomes

T cell characteristics of CD4 and CD8 starting and final products were assessed to determine associations with severe CRS, severe neurotoxicity and CR using Wilcoxon Rank Sum test. Cox regression was used to assess the predictive value of T cell characteristics of CD4 and CD8 starting and final products on clinical outcomes of time to loss of B cell aplasia, relapse

or death, or death alone. Patients who were treated with a CD8 product only or decreased ratio of CD4:CD8 products were excluded from CD4 analyses. For the analyses of relapse or death and loss of B cell aplasia, the three patients who did not achieve CR were excluded.

Results

A total of 45 patients were enrolled in the Phase I portion of PLAT-02. Two patients died of progressive disease prior to being treated with CAR T cells and so only the remaining 43 treated patients were included in this analysis. There was a similar distribution of ages and sex between patients with normal (n=34) and poor (n=9) product expansion (Table 1). Half of the patients in each group had experienced multiple relapses. The median absolute lymphocyte count was higher and the average leukemic burden and CD19 antigen burden were lower among those with normal versus poor expansion, though these differences were not statistically significant. Those with poor expansion tended to have received HSCT more recently (188 vs 453 days; p=0.06).

In total there were 10 patients for whom there was poor expansion of the CD4 and/or CD8 CAR T cell products (Table 2). Five patients had poor expansion of both the CD4 and CD8 products, while three patients had poor expansion of the CD4 product only, and two patients had poor expansion of the CD8 product only. One patient had poor expansion of both CD4 and CD8 CAR T cell products with no product produced so underwent repeat apheresis with subsequent successful production of both CD4 and CD8 CAR T cell products. CD4 products could not be produced for two additional patients who were treated with a CD8 product only. An insufficient dose of CD4 CAR T cells was produced for one patient who was then treated with a ratio of 30:70 CD4:CD8 CAR T cells.

Poor Product Expansion

Analysis of the starting phenotype of the CD4 products revealed no difference between those with normal and poor expansion based on memory phenotype. Further, no clinical characteristics (prior transplant, days since transplant, relapse status, absolute lymphocyte count at apheresis, absolute blast count at apheresis) were found to predict expansion of CD4 or CD8 products (data not shown). However, those with poor expansion had significantly higher percentage of cells expressing PD-1 compared to those with normal expansion (mean (SE) 36.1% (4.7) vs 17.3% (1.9); p=0.001; Figure 1). Additionally, they had a significantly higher percentage of cells producing IFNγ (6.2% (1.2) vs 3.1% (0.7); p=0.027) and IL-2 (22.4% (2.9) vs 12.0% (2.1); p=0.031), and a higher percentage of cells expressing CD107a (4.0% (1.4) vs 1.9% (0.4); p=0.034). There were no significant differences in the starting memory phenotypes, expression of activation/exhaustion markers or cytokine production of CD8 products with normal versus poor expansion.

Neither CD4 nor CD8 expansion status were associated with severe CRS (n=10) or neurotoxicity (n=9) (p>0.17 for all comparisons). However, poor CD8 expansion was associated with not achieving a complete response (n=3) (p=0.047); no association between CD4 expansion and complete response was seen (p=0.42). Finally, poor product expansion was not associated with an increased risk of loss of B cell aplasia, relapse or death, or death alone (data not shown).

T-cell Characteristic of Starting and Final Product and Toxicities/Outcomes

Higher percentages of cells expressed CD107a, a marker of T cell degranulation and killing, in the CD4 (mean (SE) 84.1% (3.4) vs 67.6% (3.3); p=0.01) and CD8 final products

(87.4% (1.8) vs 77.3% (2.2); p=0.02) of patients who experienced severe neurotoxicity (Figure 2). The percentage of cells producing IL-2 was also higher amongst those with severe neurotoxicity for both the CD4 (9.3% (2.6) vs 5.0% (0.5); p=0.08) and CD8 (2.6% (1.3) vs 0.74% (0.1); p=0.07) but the findings were not outside the limits of chance. The production of other cytokines (IFNγ, and TNFα) did not differ (data not shown). The production of cytokines in both CD4 and CD8 starting products also did not differ amongst those who experienced severe neurotoxicity, nor did memory phenotype or the expression of markers of activation/ exhaustion. The T cell characteristics of CD4 and CD8 starting and final products were not associated with patients experiencing severe CRS, duration of B cell aplasia, relapse or death, or death alone (data not shown).

Discussion

This study evaluated the role of T cell phenotypes in successful CAR T cell product expansion, treatment related toxicities and outcomes. While ten patients experienced poor product expansion of either CD4 and/or CD8 products, 40 of the 43 patients who were alive after CAR T cell manufacture had successful manufacture of both CD4 and CD8 products, and the three additional subjects were able to be treated with a decreased ratio of CD4:CD8 CAR T cells, or CD8 CAR T cells alone.

The baseline characteristics of patients were assessed to determine predictors of poor product expansion, but none were identified. However, baseline characteristics of a patient population that has received HSCT are often heterogeneous. The role of recent therapy, and its potential impact on product expansion, is also very difficult to assess. Singh et al demonstrated that patients undergoing routine chemotherapy for ALL had significant depletion of their naïve T

cell populations following standard chemotherapy regimens containing cyclophosphamide and cytarabine, resulting in less robust cell expansion.[5] However, given the wide spectrum of disease burden and relapse status at time of enrollment, patients in this study received a variety of chemotherapeutic and immunotherapeutic treatments in the month(s) preceding apheresis. It is difficult to know which agents and specific doses should be evaluated, and the length of time prior to apheresis that should be considered. While the difference in median days since last transplant between those with normal versus poor expansion approached significance (453 vs 188 days; p=0.06), it is difficult to know if this reflects an effect of transplant itself or the more intensive chemotherapy that often precedes transplant. So while we did not identify any baseline clinical characteristics as predictors of poor expansion, future studies with larger sample size may identify important predictors.

We had hypothesized that starting products with more differentiated T cell memory phenotypes would be more likely to demonstrate poor expansion based on published preclinical and clinical data.[5,9,14] While there was significant heterogeneity amongst patients in the starting memory phenotypes of starting products, there were no meaningful differences between those with normal vs poor expansion. However, those with poor CD4 product expansion did have a significantly higher percentage of cells producing the cytokines IL-2 and IFNγ, and expressing the marker of activation/exhaustion, PD-1. The increased cytokine production is consistent with a more effector-like phenotype, even if this was not observed in analysis of the memory phenotypes. Additionally, cells that experienced poor expansion had a higher percentage of cells expressing CD107a, a marker upregulated in cells undergoing degranulation and also with increased cytokine secretion.[15] The increased PD-1 expression is somewhat difficult to interpret as increased PD-1 expression can be seen on activated cells and the impact of PD-1 expression

alone is uncertain. In the future it will be helpful to try to assess the percentage of cells expressing 0, 1, 2 or 3 makers of activation or exhaustion to better understand the functional impact of these markers.

No T cell characteristics were found to predict CD8 product expansion. It is possible that this is due to limited study numbers, with fewer CD8 products having poor expansion and being available for analysis. Nonetheless, given that CD8 cells are the essential cytolytic component of any CAR T cell product, it is reassuring that CD8 products were successfully produced for all patients.

Given the high rate of CR amongst patients treated with CAR T cells, it has been challenging to identify predictors of CR on clinical trials. Poor *in vivo* expansion as well as the lymphodepletion regimen have been associated with CR in two adult CAR T cell trials.[2,16] In a mouse model, Sommermeyer et al found that CAR T cell products initiated from select CD8 central memory cells and CD4 naïve cells conferred the strongest antitumor response, with complete eradication in a subset of mice beyond four months, whereas mice treated with CAR T cell products initiated from bulk PBMCs all expired within two months.[9] Amongst the 43 treated patients on our trial, only 3 did not achieve complete response. We did not identify T-cell characteristics in the starting or final products that predicted CR. However, poor CD8 expansion was found to be associated with not achieving a complete response ($p=0.047$). Two of the three patients who did not achieve CR demonstrated *in vivo* expansion and engraftment of their CAR T cells. This could be consistent with the hypothesis that while the cells are able to demonstrate effector function, they were not capable of long-term engraftment. However, much greater numbers of patients would need to be available to fully adequately assess this relationship.

Neither CD4 nor CD8 expansion were associated with severe CRS nor neurotoxicity. To our knowledge, there are no published studies examining product expansion or starting or final product T cell characteristics and occurrence these toxicities in animal models or human subjects. Nonetheless, published data has demonstrated elevated *in vivo* cytokine levels in patients with B cell malignancies treated with CD19 CAR T cells who experience both severe CRS (IL-6, IFN-γ, IL-15, IL-8) and severe neurotoxicity (IL-6, IFN-γ, IL-15, TGB-β).[2,17,16] In our study, no patients with poor expansion of either product experienced severe CRS, and of the nine patients who experienced severe neurotoxicity, only one had poor product expansion. We therefore would have liked to investigate the cytokine production of the final products. Unfortunately, there were only two CD4 final products and two CD8 final products available for analysis amongst those that experienced poor expansion which precluded our ability to analyze these outcomes.

Independent of expansion, higher levels of CD107a in the final CD4 and CD8 CAR T cell products were associated with patient experiencing severe neurotoxicity, as well as higher percentage of cells producing IL-2. This is consistent with findings in a study of patients with non-Hodgkin's lymphoma treated with CD19-specific CAR T cells.[16] In the future, we would like to assess if these patients also had higher serum and cerebral spinal fluid levels of IL-2 following CAR T cell infusion and while experiencing symptoms of neurotoxicity and expand the number of cytokines tested.

This study was limited by the small sample size of phase I trials. However, these correlative studies will continue to be done during the Phase II portion of the clinical trial, increasing the available samples to study and our ability to detect more meaningful differences. Missing data was also a concern. Given one of the primary aims of the study was to assess the

feasibility and manufacturing of the CAR T cell products, patient apheresis and final products during Phase I were prioritized for creating CAR T cell products and treating the patient, limiting available specimens for our correlative studies. Given the relatively small number of cells required to run these correlative studies and the relative success of product expansion during Phase I, apheresis samples during Phase II will be banked prior to initiation of the expansion culture when possible so that more complete analysis of the starting products can be completed to understand potential factors contributing to poor product growth.

In the future we would like to obtain *in vivo* CAR T cell expansion data to determine if cells that expand poorly in culture also demonstrated altered expansion patterns in the patient. As mentioned above, we plan to compare peak *in vivo* cytokine levels in patient serum and cerebral spinal fluid, to see if the lack of CRS and neurotoxicity in those with poor expansion correlate with lower cytokine levels *in vivo* which would suggest possible functional differences in those products that had poor expansion. Finally, we will compare and contrast our findings of T cell characteristics with a concurrent CAR T cell trial for neuroblastoma which has demonstrated less anti-tumor efficacy to determine if there are identifiable differences in the starting or final products that might suggest a rationale for the differences in tumor response.

Conclusion:

Increased cytokine production and PD-1 expression may be useful biomarkers to predict poor CD4 CAR T cell product expansion. Although numbers were limited, data from this phase I clinical trial did not show that products with poor expansion had more treatment-related toxicities or worse outcomes. There are a growing number of clinical trials utilizing CAR T cells for a variety of malignancies. The routine use of similar correlative studies in these trials will

increase our understanding of CAR T cell therapies and the identification of biomarkers that can predict response and toxicity.

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Figures and Tables

Table 1. Clinical characteristics of patients treated on Phase I PLAT-02 by those with normal expansion versus those with poor expansion of CD4 and/or CD8 products.

	Normal Expansion (n=34)	**Poor Expansion (n=9)**	**p-value**
Age (median, range)	13.7 (1.3-23.2)	9.9 (3.1-25.4)	0.28
Sex (n, %)			0.65[+]
Female	18 (52.9)	4 (44.4)	
Male	16 (47.1)	5 (55.6)	
Disease status (n, %)			0.55[+]
Primary refractory	2 (5.9)	0 (0)	
1st relapse	12 (35.3)	2 (22.2)	
2nd relapse	17 (50)	5 (55.6)	
>=3rd relapse	3 (8.8)	2 (22.2)	
Transplant status			0.62[+]
Pre (n, %)	12 (35.3)	3 (33.3)	
Post (n, %)	22 (64.7)	6 (66.7)	
Days since Transplant (median, range)	453 (175-1783)	188 (118-1194)	0.06
ALC at Apheresis (median, range)	1107 (168-4488)	714 (350-3720)	0.25
ABC at Apheresis (median, range)	0 (0-48807)	0 (0-40920)	0.91
Leukemic burden (%)[*] (median, range)	21 (0-99)	54 (0-97)	0.42
CD19 Antigen burden (%)[*^] (median, range)	28.2 (0.8-99.2)	59.0 (1.7-98)	0.39

*n=33 for Normal Expansion cohort (uninterpretable bone marrow aspirate results for one subject)
^n=8 for Poor Expansion cohort (uninterpretable CD19 antigen burden result for one subject)
[+] p-values calculated by Fisher's Exact Test; all other p-values calculated by Wilcoxon Rank Sum Test

Table 2. PLAT-02 patients with poor expansion of CD4 and/or CD8 products, and outcome of product manufacturing.

Patient	CD4 Expansion	CD4 Product Produced?	CD8 Expansion	CD8 Product Produced?
1	Poor	Yes	Normal	Yes
2	Poor	No	Normal	Yes
3	Poor	No	Normal	Yes
4	Normal	Yes	Poor	Yes
5	Normal	Yes	Poor	Yes
6	Poor	Yes	Poor	Yes
7	Poor	Yes	Poor	Yes
8	Poor	Yes	Poor	Yes
9	Poor	No	Poor	Yes
10	Poor	No	Poor	No

Figure 1. Phenotypes of CD4 and CD8 starting products for patients treated on PLAT-02 Phase I. (NE = Normal expansion, PE = Poor expansion).

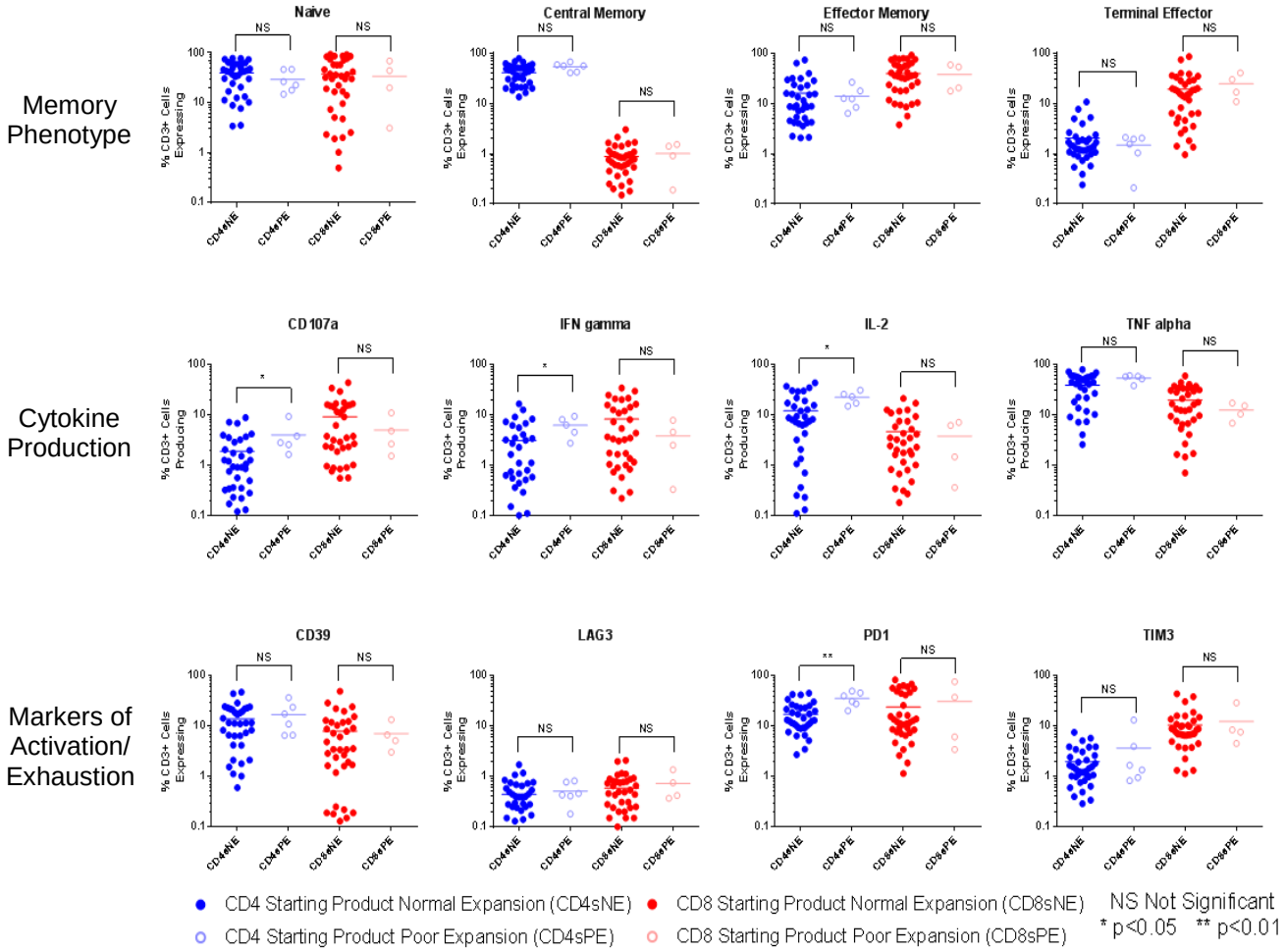

Figure 2. CD107a expression in starting and final CD4 and CD8 CAR T cell products for patients with and without severe neurotoxicity.