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Decade-Long Safety and Function of Retroviral-Modified Chimeric Antigen Receptor T-cells

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Abstract

The success of adoptive T cell gene transfer for treatment of cancer and HIV is predicated on generating a response that is both durable and safe. Here we report long term results from three clinical trials to evaluate gammaretroviral vector engineered T-cells for HIV. The vector encoded a chimeric antigen receptor (CAR) comprised of CD4 linked to the CD3-ζ signaling chain (CD4ζ). CAR T-cells were detected in 98% of samples tested for at least 11 years post-infusion at frequencies that exceed average T cell levels after most vaccine approaches. The CD4ζ transgene retained expression and function. There was no evidence of vector-induced immortalization of cells as integration site distributions showed no evidence of persistent clonal expansion or enrichment for integration sites near genes implicated in growth control or transformation. The

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CD4ζ T cells have stable levels of engraftment, with decay half-lives that exceed 16 years, in marked contrast to previous trials testing engineered T cells. These findings indicate that host immunosuppression prior to T cell transfer is not required in order to achieve long term persistence of gene-modified T cells. Further, our results emphasize the safety of T cells modified by retroviral gene transfer in clinical application, as measured in >500 patient years of follow up. Thus, previous safety issues with integrating viral vectors are hematopoietic stem cell or transgene intrinsic, and not a general feature of retroviral vectors. Engineered T cells are a promising form of synthetic biology for long term delivery of protein based therapeutics. These results provide a framework to guide the therapy of a wide spectrum of human diseases.

INTRODUCTION

Retroviral vectors have been associated with safety concerns in clinical applications (1). For example, when individuals with X-linked SCID (SCID-X1) were treated by gene transfer to restore the missing IL-2 receptor γ (IL2RG) gene to hematopoietic stem cells using gammaretroviral vectors, while 9 of 10 patients were successfully treated, 4 of the 9 developed T cell leukemia several years after gene therapy (2). Similarly, a lentiviral vector encoding β-globin flanked by insulator elements has been used to treat β-thalassemia successfully in one human subject, However, a clonal expansion was observed after integration in the HMGA2 locus, raising concerns regarding the long term safety of this approach (1). Thus, an issue is whether expansion of cells harboring vectors integrated near genes involved in growth control will inevitably result in clonal proliferation, or whether the safety concerns are cell type specific.

Adoptive transfer therapies are often further limited by a requirement for host lymphodepletion prior to T cell transfer (3). Until recently, persistence of gene modified T-cells in the absence of a strong selective advantage has been modest. However, in children with congenital immunodeficiency, persistence of gene corrected lymphocytes has been detected for at least 12 years (4). Similarly, in lymphopenic patients after hematopoietic stem cell transplantation, gene-marked EBV-specific cytotoxic T lymphocytes have been shown to persist for up to 9 years (5). The study reported here was undertaken as part of long-term follow-up for gene transfer studies using integrating vectors as mandated by the US Food and Drug Administration (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072957.htm). T-cells expressing CD4ζ become activated upon binding HIV gp120 envelope protein on infected cells (6, 7). Between 1998 and 2005 three clinical studies evaluated the CD4ζ CAR expressed in autologous CD4+ and CD8+ T-cells in subjects with active viremia (8), or in T cell reconstituted patients with chronic HIV-1 infection (clinicaltrials.gov NCT01013415 and (9)). See Supplementary Material (SM) for protocol information, including other variables that were tested across the trials including dose and dose schedule, and the effect of IL-2 administration on cell persistence.

RESULTS

CD4ζ CAR T cells have T½ >16 years

To assess the durability of gene marking, total genomic DNA (gDNA) from peripheral blood mononuclear cell (PBMC) samples from 43 subjects collected between 1 and 11 years post-infusion were analyzed by quantitative PCR (qPCR) for CD4ζ. Remarkably, stable engraftment was observed in 212 of 221 subject samples (Fig. 1A). The majority of subjects had an average CD4ζ frequency in PBMC of 0.01 to 0.1%, with some exceeding 0.1%. (Fig. 1B–D). Linear mixed effects modeling (10) was used to measure their decay rates, indicating that their disappearance half-life ($t_{1/2}$) was >16 years for the three trials (Table 1), suggesting CD4ζ modified T-cells may persist for decades. The extrapolation beyond year nine is subject to the unknown biology of whether or not decay remains linear after year 9. In contrast, persistence of gene modified T-cells has been modest in previous trials involving patients with cancer and HIV, with decay half lives of less than 30 days.

Decade long expression and function of CD4ζ CAR transgene

Gene silencing of integrating vectors is a potential limitation of retroviral gene therapy (11). To interrogate expression of CD4ζ, we isolated total RNA from 13 subjects 2–10 years post infusion, and measured transcriptional activity by RT-PCR for CD4ζ. All but two samples (the samples with lowest detectable engraftment) had measurable CD4ζ RNA (Fig. 2A) at levels which significantly correlated with DNA copies (Table S1). This indicates ongoing transcription of CD4ζ for at least 10 years after infusion of gene-modified T cells.

We then evaluated whether the CD4ζ CAR was expressed and functional. CD4ζ positive cells could not be sorted for functional testing by standard methods such as cell surface staining and cytokine release due to (1) limiting numbers of cells in annual samples, (2) the inability to phenotypically distinguish native CD4 from CD4ζ, (3) CD4 expression by natural CD8 cells upon activation (12). Therefore, we developed an assay to enrich CD4ζ cells in response to ligation of CD4ζ using artificial antigen presenting cells (Fig. 2B; and Fig. S1A). We evaluated cryopreserved PBMC samples obtained 3–7 years after infusion. In seven of nine subjects, the ratio of the CD4ζ copies after stimulation showed a 3–13 fold increase in prevalence compared to before stimulation (Fig. 2C). Thus continued expression and function of CD4ζ may contribute to prolonged survival.

Genomic and epigenetic features of retroviral insertion sites

The durable persistence of the CD4ζ T-cells provided a unique opportunity to interrogate selection for preferred gammaretroviral integration sites over time in T cells, resulting from modulation of cellular growth control genes as a result of integration-mediated events such as distal effects from the LTR enhancer (2) or disruption of gene regulation (1). We isolated vector integration sites from 11 individuals with high level marking, and from whom samples were available in sufficient quantities, using LM-PCR and Mu-mediated recovery (13–15). A total of 202,435 sequence reads and 7,222 unique integration sites were determined from the infused cell product and post-infusion PBMC (Table S2).

We first examined the global integration site pattern relative to genomic features such as gene density, gene expression, and CpG islands. For comparison, we also analyzed integration sites from SCID-X1 gene-corrected subjects (16) and from MLV infected primary CD4+ T-cells infected in culture (17). Gammaretroviruses are known to integrate in gene dense, transcriptionally active regions near gene 5′ ends and CpG islands. Typical gammaretrovirus integration patterns were found in CD4ζ subjects (Fig. S2). An analysis over ChIP-Seq data sets querying 44 forms of histone post-translational modification and DNA binding proteins in T cells showed the expected associations with features enriched near gene 5′ ends. Comparison of global integration patterns in the CD4ζ subjects before and after infusion over genomic features and ChIP-Seq maps showed no notable differences.

Dynamics of modified cells can be tracked by detecting the prevalence of cell clones across multiple timepoints using the proportion of sequence reads corresponding to an integration site as a surrogate for cell abundance. By this approach, we failed to detect signs of persistent clonal expansion in CD4ζ subjects. Instead, integration sites were mostly unique at every time point (Table S3) and sites with elevated CD4ζ marking at one time point were absent at later timepoints (Fig. 3A). Another method for estimating clonal abundance is to count the number of independent recovery events that capture the same integration site (13). Most sites were recovered from only a single starting molecule (Table S3). Of the 15 sites recovered independently more than once (15 out of 682 sites), only two of the sites were in close proximity (<50kb) to cancer-related genes, a frequency indistinguishable from pre-infusion samples. We compared whether CD4ζ integration sites were enriched near genes marked by clusters of integration sites from SCID-X1 gene therapy in stem cells (16). These represent candidates for genes involved in clonal expansion or persistence. However, we did not find evidence for enrichment (Table S4). Because gammaretroviruses integrate near gene promoters, we also compared the frequency of integration near the 5′-ends of cancer-related genes in the pre-infusion gene-modified T cell product and post-infusion samples. We failed to detect significant enrichment over time (Fig. 3B).

Retroviral gene transfer safety record in >500 patient years of observation

The stable level of engraftment with a functional transgene combined with the maturity of the clinical trials provides a unique opportunity to determine the safety and durability of gene transfer with integrating vectors. Clinical monitoring of the patients at yearly intervals has not detected any suspected or documented occurrences of hematologic disorders suggestive of retroviral genotoxicity. The clinical data set represents over 540 patient years without integration mediated toxicity, therefore, based on a Poisson distribution assumption, we are 95% confident that the true adverse event rate is less than 0.0068 per person-year, or equivalently, no more than one event in every ~147 years.

DISCUSSION

The safety of gene transfer with retroviral vectors has been difficult to establish due to a paucity of studies with persistently high levels of gene-modified cells. The persistence of gene modified T-cells has been modest in previous trials involving patients with cancer and HIV, with decay half lives of less than 30 days (18–27). Given the large number of subjects

analyzed in this study and the extent of marking, it is likely that most targets in the human genome hosted vector integration, given that T cells with more than 2×10^{11} integration events were infused in the trials and that the human genome consists of approximately 3×10^{9} base pairs. Thus the absence of adverse events and clonal expansion is unexpected given previous estimates at 10^{-6} to 10^{-8} adverse events per retrovirus insertion event (28). It is likely that mature human T cells are more resistant to insertional genotoxicity, consistent with the known resistance of mature mouse T-cells to transformation (29). In contrast, mature B cells do not appear to have this resistance to transformation (30). A potential mechanism for observations that mature T cells appear to offer "safe harbor" to integration events is that unlike B cells where homeostasis is regulated at the population level, the homeostasis of T cell mass is asserted at the clonal level by mechanisms involving intraclonal competition (31, 32). The essentially stable persistence of CD4ζ CAR T cells that we have observed is similar to the 14 year half life reported for vaccinia-specific human T cells (33). The notion of homeostatic regulation of CD4ζ T cells at the clonal level is consistent with the previous demonstration that CAR T cells continue to express their natural TCRs (6).

The mechanisms responsible for the high level persistence of CD4ζ CARs likely include the use of improved cell culture technology that promotes central memory cells (34), a non-immunogenic transgene, and signaling from the CD4ζ CAR moiety. The decay rates of CD4ζ modified T cells in the peripheral blood suggests persistence could last decades. One possible mechanism for long term persistence in this study could be repetitive CAR stimulation as a result of periodic encounters with HIV envelope (7), given that persistent replication of HIV-1 occurs in the presence of antiretroviral therapy (35). Alternatively, binding of the extracellular CD4 portion of the CD4ζ chimera with its low affinity ligand, MHC class II, might lead to signaling through the ζ chain to promote persistence (36). Both are supported findings that T cell persistence is enhanced by TCR signals to self ligands (37).

One limitation of the present study is that it is not yet possible to determine the survival rates of the CAR T cells in this study beyond 9 years, due to limited numbers of samples. The predicted decay rate is based on modeling that assumes continued linear decay rates that were observed during the initial 9 years. Another caveat is that the clinical safety that we have observed may be specific for T cells and/or CD4ζ; whether similar safety could be obtained in other cell types is unknown.

In addition to the safety features of CD4ζ CAR cells, an important clinical implication of our results is that the patients did not require conditioning with lymphoablative regimens in order to achieve stable engraftment that has been necessary with stem cell therapies and for T cell engraftment in cancer patients. The safety and long term persistence of engineered T cells provides a further rationale for cell based HIV eradication strategies (38). Finally, these findings provide a framework for the design of long term gene delivery strategies for genetic disorders and other benign conditions where chemotherapy is not feasible.

MATERIALS AND METHODS

Clinical Protocols

Each of the three CD4ζ trials had unique therapeutic protocols in anti-retroviral drug (ARV) treated HIV subjects. The Mitsuyasu study (8) was a phase II open label trial, infusing a single dose of $2–3\times10^{10}$ CD4 and CD8 T cells per subject with detectable HIV loads divided into two groups: 1) those that received 6 million units of IL2 continuously infused over 4 days beginning 4 hours prior to T cell infusion or 2) those that received no IL-2. The Deeks study (9) was a phase II clinical trial treating ARV controlled HIV subjects (levels near limits of detection) and 1×10^{10} CD4/CD8 cells infused three times 2 weeks apart. Subjects were enrolled onto a cohort of 1) those receiving CD4ζ modified T-cells or 2) those receiving only unmodified T-cells. The Aronson study (clinicaltrials.gov NCT01013415) was a randomized, three cohort trial of subjects with ARV controlled HIV infections (levels below detection). Two cohorts received infusions of $8–9\times10^{9}$ CD4ζ modified CD4/8 T-cells with or without subcutaneous injections of 1.2 MIU/m^2/day of IL-2 for 56 days. The third cohort group received IL-2 only. For each of these studies, subjects were intensely monitored and evaluated post-infusion in the first year for clinical responses, safety, and correlative effects. This report was initially undertaken as part of the FDA mandated long term follow-up for monitoring for delayed adverse events in patients receiving gene therapy using integrating vectors. Under this requirement, subjects participated in annual laboratory and physical exams up to 15 years post infusion. The collection of frozen PBMCs from the annual visits for each subject in each of the studies was compiled. The completeness of each subject's annual visit follow up profile was primarily determined by the subjects compliance with protocol-specified study visits and to adequate specimen quality.

Determination of CD4ζ copy numbers

A qPCR assay was developed to detect the amplicon formed by the CD4 and zeta chimera. gDNA for qPCR was isolated from frozen pellets of 1×10^{6} PBMCs obtained from the buffy coat of processed blood from subject annual visits using QIAamp DNA Micro Kit (Qiagene, 56304). qPCR was performed in 20μl volumes using the 384 well format on ABI HT7900. Validated CD4ζ and huGAPDH primer/probe sets were used to quantify respective copy numbers of subject samples from standard curves. Assay results were evaluated by ABI's SDS2.3, Excel and Sigma Plot software. To determine the average CD4ζ copies/1×10^{6} PBMCs, CD4ζ copy numbers were determined using four replicates of 250 ng gDNA, GAPDH copy numbers were determined using three replicates of 50 ng gDNA and then used to normalize the CD4ζ copies to 1E6 cells using the formula: 2× (CD4ζ copies/ GAPDH copies) × 1 $\times10^{6}$. Taqman qPCR Assay performance criterion: Limit of Quantification (LOQ) is ~1 copy in 7600 cells, Limit of Detection (LOD) is ~1 copy in 38,000 cells, positive control within 80% of expected value, and R^2 must be greater than 0.995.

Determination of CD4ζ decay slopes and half-life

A linear mixed effects model (10) with the time since infusion as an independent variable was used to estimate the rate of CD4ζ decay from year 1 to year 9 post-infusion. By using both random intercepts and random slopes, potential correlation among repeated

measurements over time and between-subject variability in the initial values and as well as the rate of decay was modeled. For the combined data, the difference between studies in the year 1 cell counts was adjusted by including indicator variables of individual study in the model as covariates.

RT-PCR of CD4ζ subject samples

Cryopreserved subject PBMCs were thawed and allowed to recover overnight before total RNA was isolated using QIAgene RNAeasy Plus kit (74134). The RNA was reversed transcribed using mix hexamers and evaluated by taqman analysis for the detection of CD4ζ and the reference gene huGAPDH. A resulting positive Ct value for CD4ζ then subtracted away the Ct value of the GAPDH to determine deltaCt of CD4ζ relative to GAPDH. As a control for CD4ζ DNA contamination, a reverse transcription reaction without the reverse transcriptase was done for each sample. A negative signal was found in all control samples indicating samples had no contaminating DNA. The two samples with undetectable CD4ζ RNA described in figure 2A also had transgene copy numbers below 100, suggesting that 0.01% of CD4ζ modified T-cells in PBMCs may be the limit to detect CD4ζ RNA by this RT-Taqman procedure.

Integration site recovery and analysis

Purified genomic DNA was digested with MseI, Tsp509I, or for Mu-mediated recovery (13), with BanI. Samples with limiting DNA amounts were whole-genome amplified using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) prior to digestion. PCR adapters were installed by T4 ligase or Mu Transposase. MseI and Tsp509I samples were then digested again using MscI to prevent recovery of vector sequence. Nested PCR was performed using conditions described previously (15) and primers specific to the CD4ζ vector LTR. Amplification products were purified using AmPure beads and sequenced using 454 pyrophosphate sequencing technology (39). Genomic sequences aligning within three base pairs of the LTR end and showing unique best alignments to the human genome by BLAT (hg18, version 36.1, >98% match score) were considered true integration sites. Comparisons to genomic features were carried out as described previously (40, 41) using Fisher's exact tests, Chi square, or a combination of conditional logit, regression and Bayesian model averaging. An explanation of supplementary genomic heatmaps can be found in Ocwieja et al. 2011 supplementary text "Guide to Interpreting Genomic Heat Maps Summarizing Integration Site Distributions" (42). Identification of cancer-related genes was done using a collection of cancer-related gene lists from seven different sources (http://microb230.med.upenn.edu/protocols/cancergenes.html). Integration site datasets used and oligonucleotides used for analysis are listed in Tables S5 and S6. The CD4ζ integration sites were compared to integration sites recovered from other studies that used gammaretroviral vectors in T cells (17) and hematopoietic stem cells (16).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Annual	1	2	3	4	5	6	7	8	9	10	11	Total
Detected	*20*	*25*	*33*	*31*	*28*	*25*	*24*	*13*	*8*	*4*	*1*	*212*
Tested	*20*	*25*	*35*	*33*	*29*	*26*	*24*	*15*	*9*	*4*	*1*	221

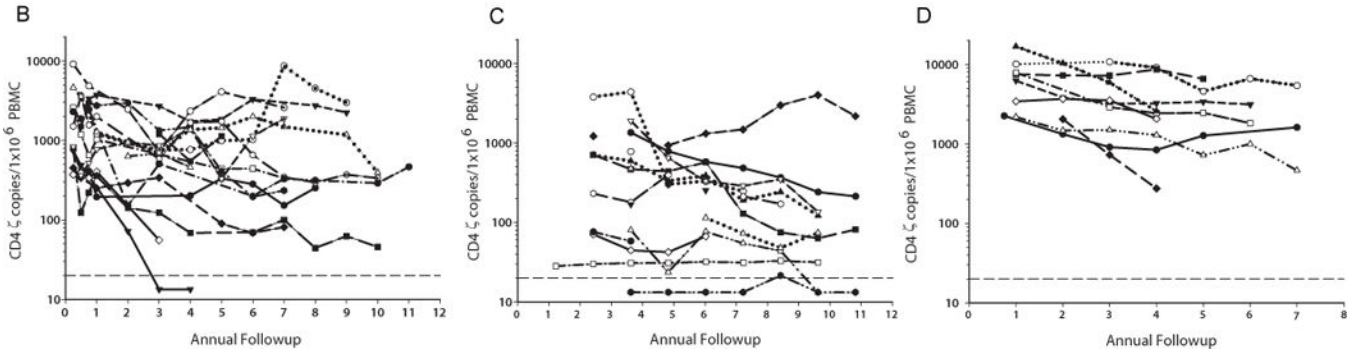

Figure 1. Persistence of CD4ζ modified CAR T-cells over 11 years post infusion

(A) Total samples tested at annual visits and the corresponding number of samples with detectable CD4-ζ. Persistence of CAR T cells for the 43 individual patients in the **(B)** Mitsuyasu (8), **(C)** Deeks (9) and **(D)** Aronson (clinicaltrials.gov NCT01013415) trials, at annual visits beginning at 1 year post infusion. The LOD for the assay is plotted as a dotted reference line.

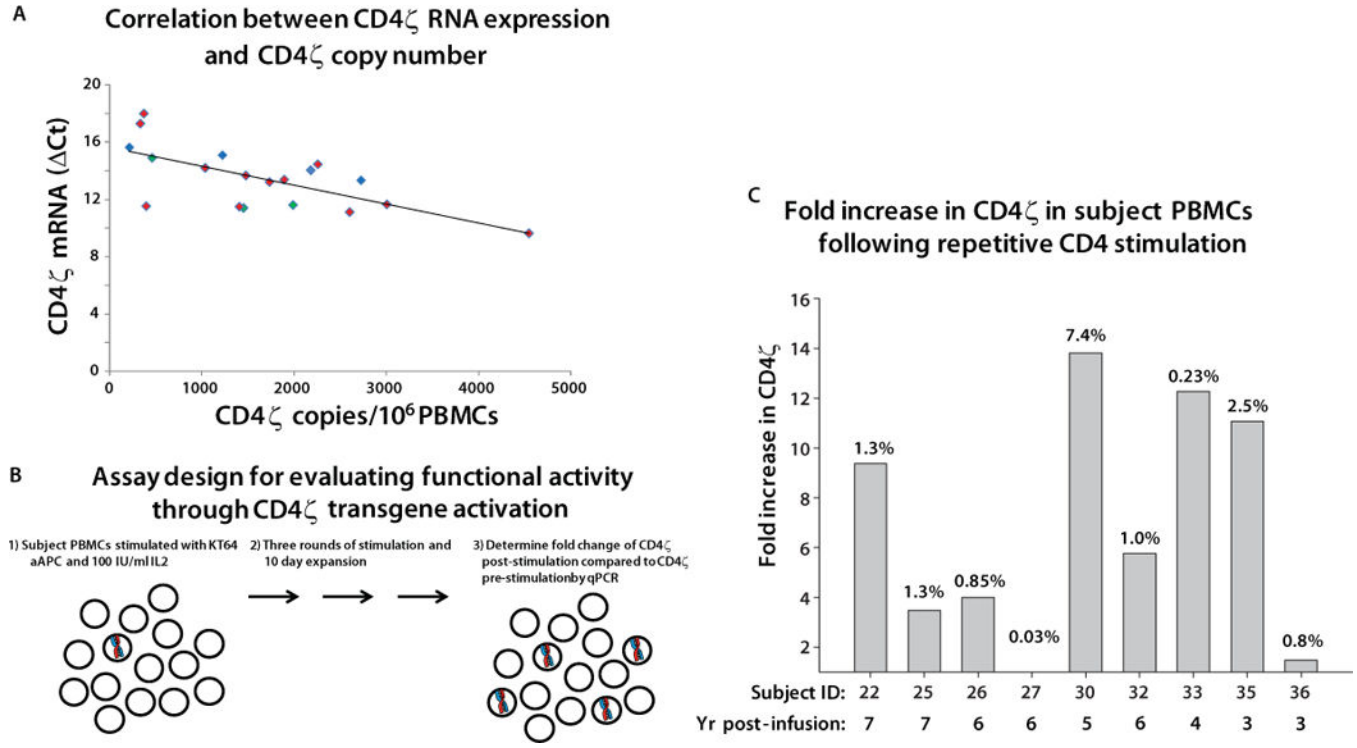

Figure 2. Transcriptional activity and CAR function in persisting cells

(A) The CD4ζ RNA level (y axis) is plotted versus the number of DNA CD4ζ DNA copies per million PBMC of each tested sample. Samples from the Deeks, Mitsuyasu and Aronson studies are plotted as red, blue and green symbols, respectively. CD4ζ RNA expression was calculated from the ΔCt values for RT-PCR of CD4ζ and GAPDH mRNA. GAPDH is expressed at a high level, so that greater expression of CD4ζ results in a smaller expression difference and so a smaller ΔCt. The values are significantly correlated by linear regression analysis (p=0.0018) testing whether rho=0 or not. No RT-controls were run in parallel and all plotted CD4ζ samples were negative, confirming the signal observed is due to RNA template. Two subjects did not have detectable CD4ζ RNA. **(B)** Design of the proliferation assay used to interrogate function of CD4-ζ CAR in T-cells. This assay was validated prior to employing as described in Fig. S1. Functionality is measured as the relative increase in the average copy number of CD4ζ cells following anti-CD4 antibody activation over percentage of CD4ζ before stimulation. **(C)** Fold-increase of CD4ζ expressing cells following three 10-day rounds of anti-CD4 mAb loaded irradiated K562 artificial antigen presenting cells expressing the high affinity Fc Receptor CD64 (KT64) and 100 IU of IL-2. CD4ζ copy numbers were evaluated from the gDNA of subject PBMCs before and after activation by qPCR analysis. The final percentage of CD4ζ in each culture is indicated by the number at the top of each bar. Each bar is designated at the bottom with the subject ID and year post-infusion of the sample.

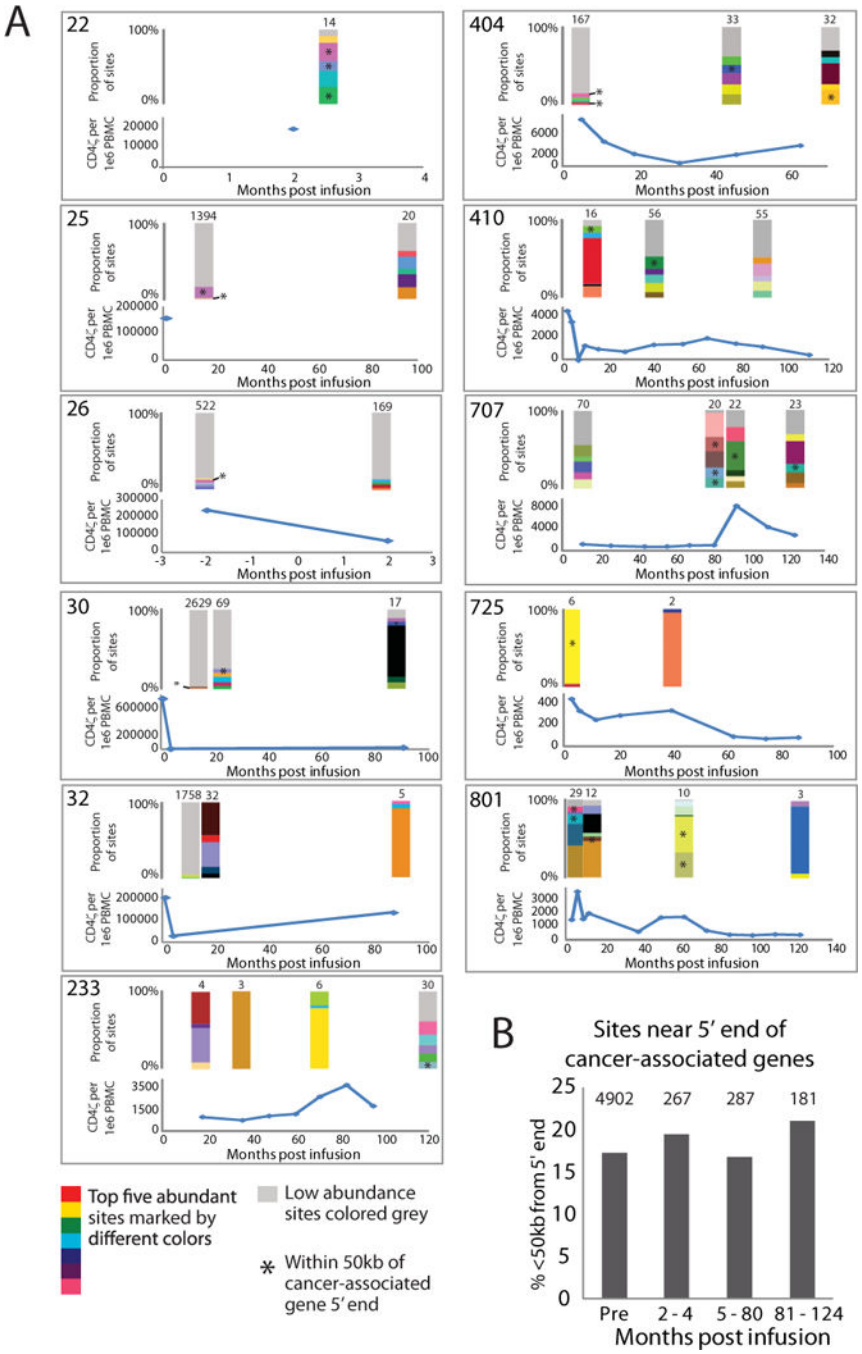

Figure 3. Integration site analysis of CD4ζ modified CAR T cells

(A) Longitudinal abundance and dynamics of CD4ζ modified T-cells. The top-left corner of each panel shows the patient number. Q-PCR measurements of total CD4ζ copy number per million PBMCs are shown longitudinally for individual subjects (blue line). The x-axis shows months post infusion, the y axis shows Q-PCR vector copy number. Stacked bar graphs are shown directly above time points where integration sites were isolated and depict the relative abundance of integration sites based on the proportion of sequence reads detected using MseI and Tsp509I. The top five abundant sites are differentially colored with

all other less abundant sites colored grey. The total number of unique sites detected at a given time point is shown above the corresponding bar graph. **(B)** Frequency of integration near cancer-associated gene 5′ ends. Integration sites were separated into four bins with one bin for preinfusion sites and three bins for post infusion sites (x-axis). The percent of sites found within 50kb from a gene 5′ end that were also within 50kb from a cancer-associated gene's 5′ end are shown (y-axis). The number of sites <50kb from a gene's 5′ end are shown at the top of each bin. No significant difference between the preinfusion and post infusion bins was found in pairwise comparisons using Fisher's exact tests.

Table 1

Decay rates and half-lives of engrafted CD4-ζ cells in subjects on the 3 clinical protocols.

Study	No. of subjects in followup	Avg no. of followup samples evaluated per patient	Decay rate (log10 copies/yr)			Half-Life (yrs post infusion)		Years to Reach LOD	
			Estimate	95% CI	P value	Estimate	95% CI	Estimate	95% CI
Deeks	17	5.4	−0.06	−0.115, −0.006	0.034	24.5	13.3, 235.9	24.2	13.1, 233.4
Mitsuyasu	17	4.9	−0.085	−0.145, −0.025	0.005	16.5	10.5, 50.1	14.2	9.2, 42.5
Aronson	9	5	−0.112	−0.200, −0.023	0.013	17.5	10.2, 80.2	21.2	12.3, 98.3
Combined	43	5.3	−0.074	−0.107, −0.041	0.001				

Limit of quantitation is $26.6/1\times10^6$ or 1.42 copies on $\log_{10}$ scale; see figure 1 for data points. Note, although the estimated half-life and time to reach limit of detection (LOD) are listed as year post infusion, they were calculated using the estimated cell count beginning at year 1 (*or year 2 for Mitsuyasu study due to limitation in sample availability in year 1) rather than the actual initial infusion cell counts. In this analysis, there was on average 25 independent patients available (minimum: 10 patients) for estimating the rate of decline at each time point through year 9.