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Association of fludarabine pharmacokinetic/dynamic biomarkers with donor chimerism in nonmyeloablative HCT recipients

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Abstract

Purpose—Fludarabine monophosphate (fludarabine) is an integral component of many reduced-intensity conditioning regimens for hematopoietic cell transplantation (HCT). Fludarabine's metabolite, 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), undergoes cellular uptake and activation to form the active cytotoxic metabolite fludarabine triphosphate (F-ara-ATP), which inhibits cellular DNA synthesis in $CD4^+$ and $CD8^+$ cells. In this study, we evaluated whether fludarabine-based pharmacologic biomarkers were associated with clinical outcomes in HCT recipients.

Methods—Participants with hematologic diseases were conditioned with fludarabine and low-dose total body irradiation (TBI) followed by allogeneic HCT and post-grafting immunosuppression. After fludarabine administration, we evaluated pharmacological biomarkers for fludarabine – F-ara-A area under the curve (AUC) and the ratio of circulating $CD4^+$ and $CD8^+$ cells ($CD4^+/CD8^+$ ratio) after fludarabine administration – in 102 patients; F-ara-ATP accumulation rate in enriched $CD4^+$ and $CD8^+$ cells was evaluated in 34 and 36 patients, respectively.

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COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of potential conflicts of interest

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Research involving Human Participants and/or Animals

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Results—Interpatient variability in the pharmacological biomarkers was high, ranging from 3.7-fold (F-ara-A AUC) to 39-fold (F-ara-ATP in $CD8^+$ cells). Circulating $CD8^+$ cells were more sensitive to fludarabine administration. A population pharmacokinetic-based sampling schedule successfully allowed for estimation of F-ara-A AUC in this outpatient population. There was poor correlation between the F-ara-AUC and the F-ara-ATP accumulation rate in $CD4^+$ (R^2=0.01) and $CD8^+$ cells (R^2=0.00). No associations were seen between the four biomarkers and clinical outcomes (day +28 donor T-cell chimerism, acute graft-versus-host disease (GVHD), neutrophil nadirs, cytomegalovirus reactivation, chronic GVHD, relapse, non-relapse mortality, or overall mortality).

Conclusions—Considerable interpatient variability exists in pharmacokinetic and fludarabine-based biomarkers, but these biomarkers are not associated with clinical outcomes in fludarabine/TBI-conditioned patients.

Keywords

Fludarabine; biomarkers; nucleoside analogs; pharmacokinetics; pharmacodynamics; hematopoietic cell transplantation

INTRODUCTION

Fludarabine monophosphate (fludarabine) is an essential component of many reduced-intensity hematopoietic cell transplantation (HCT) conditioning regimens. [1] One of the least immunosuppressive conditioning regimens is the nonmyeloablative regimen of 90 mg/m^2 fludarabine plus 2 to 4.5 Gy total body irradiation (TBI) developed by the Seattle group. [2,3] Initially, patients were conditioned with TBI only. Engraftment difficulties in TBI-only patients led to the addition of fludarabine to the conditioning regimen and engraftment rates increased. [1,2,4] In recipients of a peripheral blood stem cell graft obtained from a human leukocyte antigen (HLA) matched related donor, a prospective randomized trial revealed that fludarabine/TBI resulted in higher median T-cell and natural killer cell chimerism on day +28, higher progression-free survival, and improved overall survival compared to TBI alone. [3] Thus, fludarabine is important for enhancing the graft-versus-tumor effect by ensuring more rapid and sustained donor engraftment shortly after transplantation. Fludarabine use, however, was also associated with a higher risk of grades 3–4 acute graft-versus-host disease (GVHD), lower $CD4^+$ counts, and a higher rate of bacterial infections. [5–8] Thus, identifying an optimal fludarabine dose, one that maintains high engraftment rates, improves graft-versus-tumor responses, and minimizes the risks of acute GVHD and infections, could be of significant benefit to nonmyeloablative HCT recipients.

After administration, fludarabine is rapidly dephosphorylated by nucleotidases to 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), [9–11] which is subsequently transported into the cell. In the cell, F-ara-A is sequentially phosphorylated, resulting in the active metabolite fludarabine triphosphate (F-ara-ATP, Supplemental Figure 1).[10] F-ara-ATP inhibits ribonucleotide reductase and DNA polymerase and ultimately leads to cellular apoptosis in both actively dividing and resting cells. [10] Individual patients can have substantially different rates of F-ara-ATP accumulation, which could affect the extent of their T-cell

suppression and clinical outcomes. [12] To date, it is not feasible to reliably quantify F-ara-ATP concentrations in lymphocytes isolated from plasma samples obtained from HCT recipients after fludarabine administration. [13] Therefore, we pursued evaluating F-ara-A area under the concentration-time curve (AUC), F-ara-ATP accumulation in $CD4^+$ cells, F-ara-ATP accumulation in $CD8^+$ cells, [12] and the after fludarabine $CD4^+/CD8^+$ ratio as potential biomarkers of response to fludarabine/TBI conditioning. Both F-ara-A AUC[14] and F-ara-ATP accumulation rate in $CD4^+$ and $CD8^+$[12] cells are highly variable in HCT recipients, but the clinical relevance of this variability has yet to be examined in fludarabine/TBI conditioned patients. It is essential, however, that any pharmacokinetic/dynamic study of F-ara-A AUC in fludarabine/TBI conditioned patients use a sampling schedule that maximizes compliance for these patients, who undergo HCT in the ambulatory clinic. Therefore, we developed a F-ara-A population pharmacokinetic model and used it to identify a limited sampling schedule to maximize participant compliance. [14] Furthermore, we evaluated the novel phenotypic biomarker of *ex vivo* accumulation rate of the active metabolite F-ara-ATP in two separate cell populations: $CD4^+$ and $CD8^+$ cells. [12] Finally, we also evaluated $CD4^+$ and $CD8^+$ cell counts circulating in peripheral blood before and after fludarabine administration. Fludarabine administration in patients with chronic lymphocytic leukemia leads to a marked, prolonged reduction in circulating $CD4^+$ and $CD8^+$ cells, [15–17] but the immediate effects of its administration upon these cells has yet to be described. *In vitro* data regarding the effects of fludarabine on the proportion of $CD4^+$ vs. $CD8^+$ cells undergoing apoptosis are contradictory and suggest that $CD4^+$ cells are similarly[18] or less susceptible[19] to fludarabine than $CD8^+$ cells. The immediate effects of fludarabine upon recipients' circulating $CD4^+$ and $CD8^+$ counts in the peripheral blood may influence the $CD4^+/CD8^+$ ratio. The *in vitro* $CD4^+/CD8^+$ ratio shows an inverse relationship with the amount of apoptosis, both with and without radiation, of $CD4^+$ cells but not $CD8^+$ cells. [20] Therefore, we hypothesized that fludarabine-induced changes in the $CD4^+/CD8^+$ ratio could influence sensitivity to subsequent TBI *in vivo*. We measured circulating $CD4^+$ and $CD8^+$ counts in the peripheral blood before and immediately after fludarabine administration and evaluated possible correlations with F-ara-A AUC and intracellular F-ara-ATP.

In this study, we evaluated the association of clinical outcomes with four fludarabine-specific pharmacological biomarkers: F-ara-A area under the curve (AUC) and the ratio of circulating $CD4^+$ and $CD8^+$ cells ($CD4^+/CD8^+$ ratio) after fludarabine administration in 102 patients, and F-ara-ATP accumulation rate in enriched $CD4^+$ and $CD8^+$ cells in 34 and 36 patients, respectively. The long-range goal of this work is to determine whether overall survival could be improved by personalized dosing of fludarabine in nonmyeloablative HCT recipients. Interpatient variability in the pharmacological biomarkers was high. However, no associations were seen between the four biomarkers and clinical outcomes (day +28 donor T-cell chimerism, acute graft-versus-host disease (GVHD), neutrophil nadirs, cytomegalovirus reactivation, chronic GVHD, relapse, non-relapse mortality, or overall mortality). Thus, our results suggest a minimal impact of fludarabine-based pharmacological biomarkers (i.e., F-ara-A AUC, F-ara-ATP accumulation rate in $CD4^+$ and $CD8^+$ cells) and T-cell suppression (i.e., $CD4^+/CD8^+$ ratio) upon clinical outcomes.

MATERIALS AND METHODS

Participant characteristics

Between May 2008 to February 2012, 102 patients with hematologic diseases participated in this prospective ancillary biomarker study, which was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board (Clinicaltrials.gov identifier NCT00764829). All participants provided written informed consent prior to study procedures. Table 1 summarizes the participant characteristics. The conditioning regimen and postgrafting immunosuppression were not affected by participation in this study.

Participants received a conditioning regimen (summarized in Supplemental Figure 2) of fludarabine (30 mg/m^2/day intravenously) from day −4 to day −2 (cumulative dose 90 mg/m^2) followed by a single fraction of 2 to 4.5 Gy TBI on day 0.[2] The fludarabine dose was based on body surface area using actual body weight and was not adjusted for renal function, which is supported by our population pharmacokinetic model that revealed that body surface area (and not body weight) was a covariate for F-ara-A clearance. [14] Post-grafting immunosuppression consisted of a calcineurin inhibitor (CNI), either cyclosporine or tacrolimus, starting on Day −3 and mycophenolate mofetil (MMF), starting on Day 0 after the graft infusion. MMF at a dose of 15mg/kg, was given twice a day (Q12h) to recipients of HLA-matched related grafts and three times a day (Q8h) to recipients of unrelated grafts. In general, MMF was continued until Day +27 (related donor) or Day +40 (unrelated donor) at which time, the MMF dose was reduced by 10% weekly in the absence of GVHD. Some participants also received sirolimus in addition to MMF and a CNI, as determined by their HCT treatment protocol. [21] Donor grafts were matched for HLA-A, -B, -C, and -DRB1 by high resolution DNA typing and HLA-DQB1 by intermediate-resolution techniques, with the following exceptions: one related and two unrelated donor grafts with an antigen mismatch and nine unrelated donor grafts with an allelic mismatch. The median time of last follow-up among participants was 1.9 years (range: 0.6 – 3.8 years).

Fludarabine-based biomarkers

A full description of the fludarabine-based biomarkers is included in the Supplemental Methods.

F-ara-A pharmacokinetic sampling and quantification

The F-ara-A pharmacokinetic sampling schedules and their respective participant compliance rates are summarized in Supplemental Table 1. The F-ara-A AUC for an individual participant was determined via maximum a posteriori (MAP) Bayesian estimation, where the population prior (from the population pharmacokinetic analysis) may potentially offset the loss of individual data due to the use of the limited sampling schedule. [14] The F-ara-A pharmacokinetic sampling schedule with the highest (98%) compliance rate had samples drawn immediately at the end of the 30-minute infusion, 5 minutes after the end of the infusion and then 1.5h and 24h from the start of the infusion. The F-ara-A plasma concentrations were quantified as previously described. [22] The F-ara-A pharmacokinetic parameters and AUC from each participant were calculated as empirical

Bayes estimates using the POSTHOC option in the nonlinear mixed-effects modeling software NONMEM (version 7.2, Icon Development Solutions, Ellicott City, MD).

F-ara-ATP accumulation rate in T-cell subsets

The F-ara-ATP accumulation rate in enriched $CD4^+$ and $CD8^+$ T-cell subsets was evaluated for each participant using our previously published method with minor modifications; the F-ara-ATP sampling time was the same as in our prior analysis. [12] Prior to fludarabine administration, a peripheral blood sample (60 mL) was obtained, which subsequently underwent an enrichment and purification procedure for $CD4^+$ and $CD8^+$ cells. [12] The $CD4^+$ and $CD8^+$ T-cells were purified and subsequently underwent an *ex vivo* incubation with fludarabine. Cells were incubated with fludarabine for 4h, then washed, solubilized in 1 M perchloric acid, and frozen. After thawing, the sample was centrifuged and the supernatant was neutralized prior to F-ara-ATP quantification using the LC-MS method described previously, with modifications as described in the Supplemental Methods. [13]

Immediate suppression of circulating $CD4^+/CD^+$ ratio in the peripheral blood

The circulating $CD4^+$ and $CD8^+$ counts in the peripheral blood were assessed immediately before the first fludarabine dose and then one day after the final fludarabine dose. For the before fludarabine sample, the median time between the collection of the circulating $CD4^+$ and $CD8^+$ count sample and the start of the first fludarabine dose was 4 min (range: 0 – 10.0 h). Fludarabine was then administered once daily for three days. For the after fludarabine sample, the median time between the start of the last fludarabine dose and the collection of the circulating $CD4^+$ and $CD8^+$ count sample was 23.8 h (range: 20.4 – 43.9 h). All $CD4^+$ and $CD8^+$ data were obtained before TBI and allogeneic graft infusion. The $CD4^+$ and $CD8^+$ cell counts were quantitated in a College of American Pathologist-certified clinical laboratory using a flow cytometer.

The percent decline in $CD4^+$ or $CD8^+$ counts was calculated by subtracting the after fludarabine count from the before fludarabine count and dividing the result by the before fludarabine count. A 100% decline means that no cells were detectable after fludarabine administration. A negative percent decline indicates that the participant's cell count was greater after fludarabine administration, which occurred in three of the 102 participants. Of these three participants, one, diagnosed with non-Hodgkin's lymphoma, had increased $CD4^+$ counts and two, diagnosed with chronic lymphocytic leukemia, had increased $CD8^+$ counts after fludarabine administration.

Clinical outcomes

Day +28 donor T-cell chimerism was the primary endpoint. Additional endpoints of interest were post-HCT neutrophil nadir, cytomegalovirus (CMV) reactivation, acute GVHD, chronic GVHD, relapse, non-relapse mortality (NRM), and overall survival. Neutropenia was analyzed as a binary endpoint (odds ratio, abbreviated OR), and all others as time-to-event endpoints (hazard ratio, abbreviated HR).

Participants' peripheral blood samples were evaluated as per standard practice on day +28 after HCT, or as clinically indicated, for the percentage of donor $CD3^+$ cells present. $CD3^+$

cells were sorted by flow cytometry and polymerase chain reactions of polymorphic microsatellite regions were used to measure chimerism. [23]

Post-HCT neutropenia was only assessed through day +28 because multiple potential confounding variables (e.g., viral infection or reactivation, corticosteroid therapy) could affect the neutrophil counts subsequently. Complete blood counts with differential and assessment of absolute neutrophil count (ANC) were used to evaluate neutropenia. CMV reactivation was also evaluated as it represents a significant consequence of immunosuppressed status. CMV serological status was assessed in each participant and donor prior to HCT, and all participants underwent weekly testing to detect the CMV pp65 antigen for the first three months following HCT. Acute GVHD and chronic GVHD were graded according to established criteria. [24–26] To evaluate relapse consistently, the Kahl criteria were used to classify hematological diseases as having a low, standard, or high risk of relapse. [27] Disease relapse or disease progression was defined as disease recurrence after complete remission or progression of persistent disease.

Statistical analysis

Fludarabine-based biomarkers (i.e., F-ara-A dose 1 AUC, F-ara-ATP accumulation rate in $CD4^+$ cells, F-ara-ATP accumulation rate in $CD8^+$ cells, or after fludarabine $CD4^+/CD8^+$ ratio) were treated as fixed covariates. The effects of fludarabine-based biomarkers on HRs and ORs were expressed as the effect per doubling of each biomarker. The clinical outcomes evaluated were post-HCT neutrophil nadir (dichotomized into below or above the median ANC nadir of 165 cells/10^3 μL), Day +28 T-cell chimerism, grades 2–4 acute GVHD, grades 3–4 acute GVHD, chronic GVHD, relapse, CMV reactivation, non-relapse mortality, and overall mortality were evaluated. Cumulative incidence curves for acute GVHD and relapse were estimated using methods previously described. [28] Cox regression analysis was used to model the impact of recipient fludarabine-based biomarkers on time-to-event endpoints. Death and relapse were treated as competing risks for analysis of acute and chronic GVHD. Relapse was treated as a competing risk for the analysis of NRM. Logistic regression was used to evaluate the relationship between the fludarabine-based biomarkers and the post-transplant neutrophil nadir.

All analyses were adjusted for Kahl risk category (low, standard, high), donor-recipient gender (female to male, other), and donor type (related, unrelated). All statistical tests were two-tailed with the threshold for statistical significance set at 0.005 to adjust for multiple comparisons. P-values estimated from regression models were derived from the Wald test. Statistical analysis was performed using SAS 9.3 (Cary, NC, U.S.A.).

RESULTS

Fludarabine-based pharmacologic biomarkers

Patient characteristics are listed in Table 1 and pharmacological biomarker results in Tables 2 and 3. All biomarkers showed considerable interpatient variability (given by fold variability, maximum/minimum). There was a 3.7-fold variability in F-ara-A AUC with a mean of 19.6 μM×h (range: 9.96 – 36.4). The mean F-ara-A clearance was 5.71 L/h/m^2

(range: 2.87 – 10.63). Among participants with more than one 24h (trough) concentration, the mean within-patient coefficient of variation (standard deviation/mean) was 28% (range: 0.5 – 63%), and the mean within-patient fold variability was 1.8 (range: 1.0 – 3.7). The mean (range) accumulation rate of F-ara-ATP in $CD4^+$ cells was 4.6 pmol/10^6 cells/4h (range: 0.7 – 9.4) and in $CD8^+$ cells was 4.0 pmol/10^6 cells/4h (range: 0.3 – 11.6). There was 13-fold and 39-fold variability in intracellular F-ara-ATP accumulation rate in $CD4^+$ and $CD8^+$ cells, respectively. This variability is greater than our previous observations in HCT patients (10.5- and 12.5-fold variability in $CD4^+$ and $CD8^+$ cells, respectively).[12] Notably, there were no obvious differences in the pharmacological biomarkers between the three most common diagnoses: acute myeloid leukemia, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma (data not shown).

Immediate suppression of circulating $CD4^+/CD8^+$ ratio in the peripheral blood

At the time of study initiation, most participants had low blood counts, most likely due to the underlying disease and previous treatments. The majority of participants experienced declines in their $CD4^+$ and $CD8^+$ counts after fludarabine administration (Figure 1 and Table 3). For $CD4^+$ cells, the mean cell count before fludarabine was 0.25×10^3 cells/μL (range: 0.004 – 0.97); after fludarabine the mean $CD4^+$ count dropped to 0.08×10^3 cells/μL (range: 0 – 0.30). For $CD8^+$ cells, the mean cell count before fludarabine was 0.31×10^3 cells/μL (range: 0 – 1.4); after fludarabine, the mean $CD8^+$ count dropped to 0.05×10^3 cells/μL (range: 0 – 0.76). The mean (range) percent decline in $CD4^+$ counts was 68% (−47 – 100) and the mean (range) percent decline in $CD8^+$ counts was 82% (−20 – 100). Before fludarabine, the mean (range) $CD4^+/CD8^+$ ratio cells was 1.65 (0.09 – 15) and after fludarabine, the mean (range) of this ratio was 3.08 (0 – 11).

The percent decline of $CD4^+$ cells correlated poorly with F-ara-A AUC (Figure 2A, R^2=0.06, P=0.01) and with the F-ara-ATP accumulation rate in $CD4^+$ cells (Figure 2B, R^2=0.01, P=0.64). Similarly, the percent decline in $CD8^+$ cells had a low correlation with F-ara-A AUC (Figure 2C, R^2=0.03, P=0.07) and with the F-ara-ATP accumulation rate in $CD8^+$ cells (Figure 2D, R^2=0.03, P=0.36). F-ara-A AUC was also not correlated with the accumulation rate of F-ara-ATP in $CD4^+$ cells (Supplemental Figure 3A, R^2=0.01, P=0.63) or in $CD8^+$ cells (Supplemental Figure 3B, R^2=0.00, P=0.97). The F-ara-ATP accumulation rate in $CD4^+$ cells had a low correlation of that in $CD8^+$ cells (R^2=0.194, Supplemental Figure 3C).

Association with clinical outcomes

None of the fludarabine-based pharmacological biomarkers achieved the 0.005 threshold for a statistically significant association with clinical outcomes. The mean (range) donor chimerism on Day +28 was 83% (35 – 100%). Two participants experienced graft failure, and one participant died of relapse before engraftment could be determined. Thus, graft failure could not be evaluated as an endpoint. None of the associations between F-ara-A AUC (N=103) and the various clinical outcomes were statistically significant, with p-values ranging from 0.12 to 0.96. Similarly, clinical outcomes were not associated with F-ara-ATP accumulation rate in $CD4^+$ cells (N=36) or in $CD8^+$ cells (N=34), with p-values ranging from 0.02 to 0.95. Similarly, the after fludarabine $CD4^+/CD8^+$ (N=99) ratio was not

associated with clinical outcomes, with p-values ranging from 0.01 to 0.96. Although not statistically significant after consideration of multiple comparisons, two associations were notable. One was an inverse association between $CD4^+$ F-ara-ATP accumulation rates and chronic GVHD (p=0.02). The hazard ratio was 0.53 (range: 0.3–0.9), so the hazard of chronic GVHD decreased by 47% with each doubling of F-ara-ATP accumulation rate in $CD4^+$ cells. The other was an inverse association between the $CD4^+/CD8^+$ ratio and grades 2–4 acute GVHD (p=0.01). The hazard ratio was 0.41 (0.2–0.8), so the hazard of developing grades 2–4 acute GVHD lowered by 59% with a log increase in the $CD4^+/CD8^+$ ratio.

DISCUSSION

The key findings of this study are the striking interpatient variability in the fludarabine-based pharmacological biomarkers and the lack of a pharmacodynamic relationship between these biomarkers and clinical outcomes following the fludarabine/TBI regimen. Fludarabine is an essential component of reduced-intensity HCT conditioning regimens, the goal of which is to achieve acceptably low rates of GVHD and graft rejection and to maximize the graft-versus-tumor effect. [1] In fludarabine/TBI recipients, the level and rate of change in donor T-cell chimerism has been correlated with several clinical outcomes such as graft rejection, GVHD, disease relapse/progression (via a graft-versus-tumor effect), and progression-free survival. [29] Some of the observed associations between donor T-cell chimerism levels and subsequent clinical responses could, in part, reflect differences in each recipient's sensitivity to the conditioning regimen. Day +28 T-cell chimerism was not, however, associated with the evaluated biomarkers of F-ara-A AUC, F-ara-ATP accumulation rate in $CD4^+$ or in $CD8^+$ cells, or the $CD4^+/CD8^+$ ratio after fludarabine administration and immediately before TBI administration (Figure 3A–D).

After administration, fludarabine is dephosphorylated to F-ara-A, which is subsequently transported intracellularly and phosphorylated to F-ara-ATP, the active metabolite. Day +28 T-cell chimerism and subsequent clinical outcomes were not associated with F-ara-A AUC. Of note, this pharmacokinetic study was conducted in outpatients due to the creation of a clinic-friendly limited sampling schedule, which allowed for fewer pharmacokinetic samples from each participant, and by using the prior F-ara-A population pharmacokinetic model to predict the MAP Bayesian estimates of each individual AUC. [14] These data suggest that the pharmacodynamic relationship between F-ara-A AUC and clinical outcomes differs by conditioning regimen, since prior pharmacodynamic studies have reported an association between F-ara-A AUC and outcomes in patients receiving fludarabine-based myeloablative conditioning regimens. [30,31] The design of new fludarabine-based conditioning regimens may benefit from the use of a population pharmacodynamic model that characterizes the time course and variability of absolute lymphocyte count suppression – which affects a mixed population of T cells, B cells, and natural killer cells – by plasma F-ara-A. [32]

F-ara-ATP is the major cytotoxic metabolite of fludarabine. Unfortunately, quantification of the F-ara-ATP accumulation rate in T-cells from pharmacokinetic samples obtained from patients receiving fludarabine has not been feasible to date. We developed a novel phenotypic method to assess F-ara-ATP accumulation rate in $CD4^+$, $CD8^+$, or natural killer cells isolated from a peripheral blood sample obtained from HCT recipients. [12,33] In this

study, the average (± standard deviation) F-ara-ATP accumulation rate in $CD4^+$ cells and in $CD8^+$ cells was lower than our previous results in 34 myeloablative HCT recipients. [12] Due to the considerable interpatient variability in F-ara-ATP accumulation rate, however, there was overlap between the two cohorts. The underlying reason for this discrepancy is most likely differences in the patient population, because the same blood draw timing (i.e., before conditioning was administered), cell isolation, incubation, and F-ara-ATP quantitation methods were used. Notably, there was a low correlation between F-ara-ATP accumulation rate between $CD4^+$ and $CD8^+$ cells (R^2=0.194, Supplemental Figure 3C), which is worse than our previous findings (R^2=0.553) in myeloablative HCT recipients. [12] The clinical significance of these differences, however, is unclear since F-ara-ATP accumulation rate was not associated with clinical outcomes (Figure 3). One limitation is that F-ara-ATP accumulation rate in $CD4^+$ and $CD8^+$ cells was assessed in only 36 and 34 participants, respectively. This was, in part, due to the logistical challenges detailed in the Supplemental Methods (F-ara-ATP accumulation rate in T-cell subsets). A further improvement in analytical sensitivity (current limit of detection of 50 fmol F-ara-ATP) is also desirable since F-ara-ATP concentrations could be quantified in $CD4^+$ and $CD8^+$ cells from 80% and 76% participants, respectively, with a minimum of 1.25×10^5 cells. We suggest using F-ara-ATP accumulation rate as a biomarker for fludarabine sensitivity be revisited after these logistical and analytical barriers are overcome. The benefit of pharmacogenomic studies of genes involved in F-ara-A pharmacokinetics, using DNA from the HCT recipient, are unclear since fludarabine is mainly renally eliminated. [34,16,35] Alternatively, proteomic[36] or metabolomic[37] signatures after fludarabine administration could be worthy of investigation.

The complexity of the pharmacodynamic effects of fludarabine upon lymphocytes makes it unsurprising that there is poor correlation of F-ara-A AUC with F-ara-ATP accumulation rate (Supplemental Figure 3) and with the decline in circulating $CD4^+$ and $CD8^+$ counts (Figures 2B and 2D, respectively). After *ex vivo* exposure to pharmacologically-relevant fludarabine concentrations, apoptosis of human $CD4^+$ and $CD8^+$ cells occurs after 24h. [12] We also characterized the immediate effects of fludarabine administration on circulating $CD4^+$ and $CD8^+$ cells (Figure 1 and Table 3). *In vitro* data regarding the effects of fludarabine on the proportion of $CD4^+$ vs. $CD8^+$ cells undergoing apoptosis are contradictory and suggest that $CD4^+$ cells are similarly[18] or less susceptible[19] to fludarabine than $CD8^+$ cells. On average, our data shows $CD4^+$ cells are less susceptible to fludarabine than $CD8^+$ cells *in vivo* (Figure 1 and Table 3). We recently reported that fludarabine results in a concentration-dependent and time-dependent decrease in viable $CD4^+$ and $CD8^+$ T-cells after 24h exposure to concentrations of fludarabine between 5 and 25 μM. [12] While no apoptosis had occurred after 4h (the duration of our *ex vivo* incubation procedure to evaluate F-ara-ATP), very few viable cells remained after 48h. [12] *In vivo*, $CD8^+$ cells seem to have more short-term sensitivity after fludarabine administration, but with considerable variability (Table 3 and Figure 2). Three participants had aberrant responses of their circulating $CD4^+$ or $CD8^+$ counts in the peripheral blood after fludarabine administration. Two of these patients had chronic lymphocytic leukemia, and the other had anaplastic large cell non-Hodgkin's lymphoma. Their F-ara-A AUCs were not aberrant (3.41, 7.67, and 13.8 μM×h); none had F-ara-ATP accumulation rates available. None of

these patients rejected their graft, and all had grade 2 acute GVHD (days +27, +41, and +85).

The immediate effects of fludarabine upon recipients' circulating $CD4^+$ and $CD8^+$ cell counts may influence their $CD4^+/CD8^+$ ratios, which in turn could affect their sensitivity to TBI. [20] This issue cannot be evaluated in preclinical HCT models because human lymphocytes are more sensitive to the cytotoxicity of fludarabine than those from mice, rats, or dogs. [38] Using pooled lymphocytes from four individuals, Wilkins *et al.* observed *ex vivo* that the $CD4^+/CD8^+$ ratio has an inverse relationship with the amount of apoptosis of lymphocytes and $CD4^+$ cells, but not $CD8^+$ cells, with or without radiation. [20] In our study, the average $CD4^+/CD8^+$ ratio rose from 1.65 before fludarabine administration to 3.08 afterwards (Table 3). No association was observed, however, between $CD4^+/CD8^+$ ratios and clinical outcomes. In HCT recipients, there is increasing interest in the influence of peritransplant T-cell and neutrophil counts with clinical outcomes. In a separate analysis in 459 nonmyeloablative HCT recipients, [39] Storb *et al.* found that high lymphocyte counts immediately before HCT had significant associations with reduced risks of relapse and overall mortality, but no association with the risks of GVHD or non-relapse mortality. Further research is required to identify sources of the patient heterogeneity in peritransplant T-cell counts.

We conclude that F-ara-A AUC and circulating $CD4^+/CD8^+$ ratio are not associated with clinical outcomes in fludarabine/TBI conditioned patients. Furthermore, technological improvements (e.g., T-cells from a lower blood volume or improved analytic sensitivity for F-ara-ATP) are needed before further evaluating this potential biomarker. Future work should focus on alternative biomarkers (e.g., peritransplant T-cell counts, proteomics or metabolomics) to see if fludarabine/TBI conditioning can be improved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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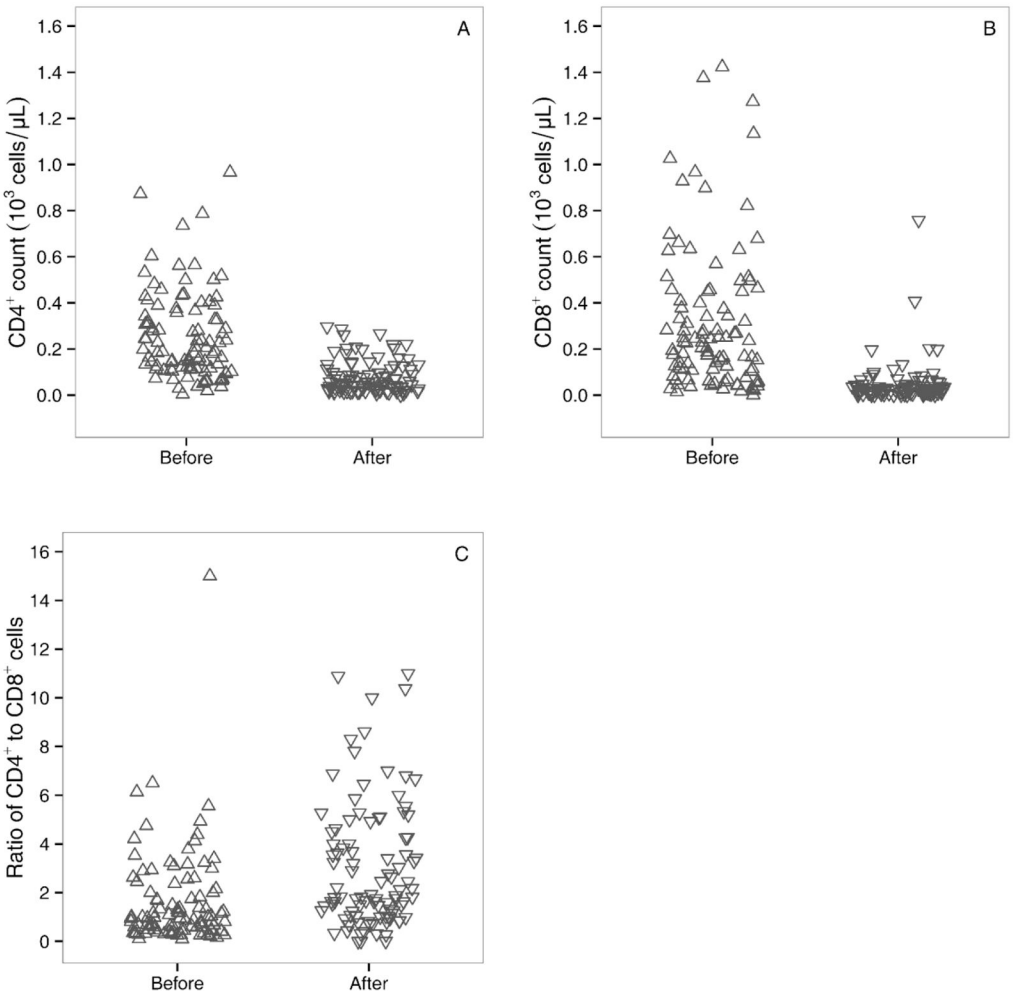

Figure 1.

Effect of fludarabine administration on circulating $CD4^+$ counts (A), circulating $CD8^+$ counts (B), and ratio of circulating $CD4^+$ to $CD8^+$ cells (C). Samples obtained within 12h of first fludarabine dose (labeled “before”) and within 24h after the last fludarabine dose (labeled “after”).

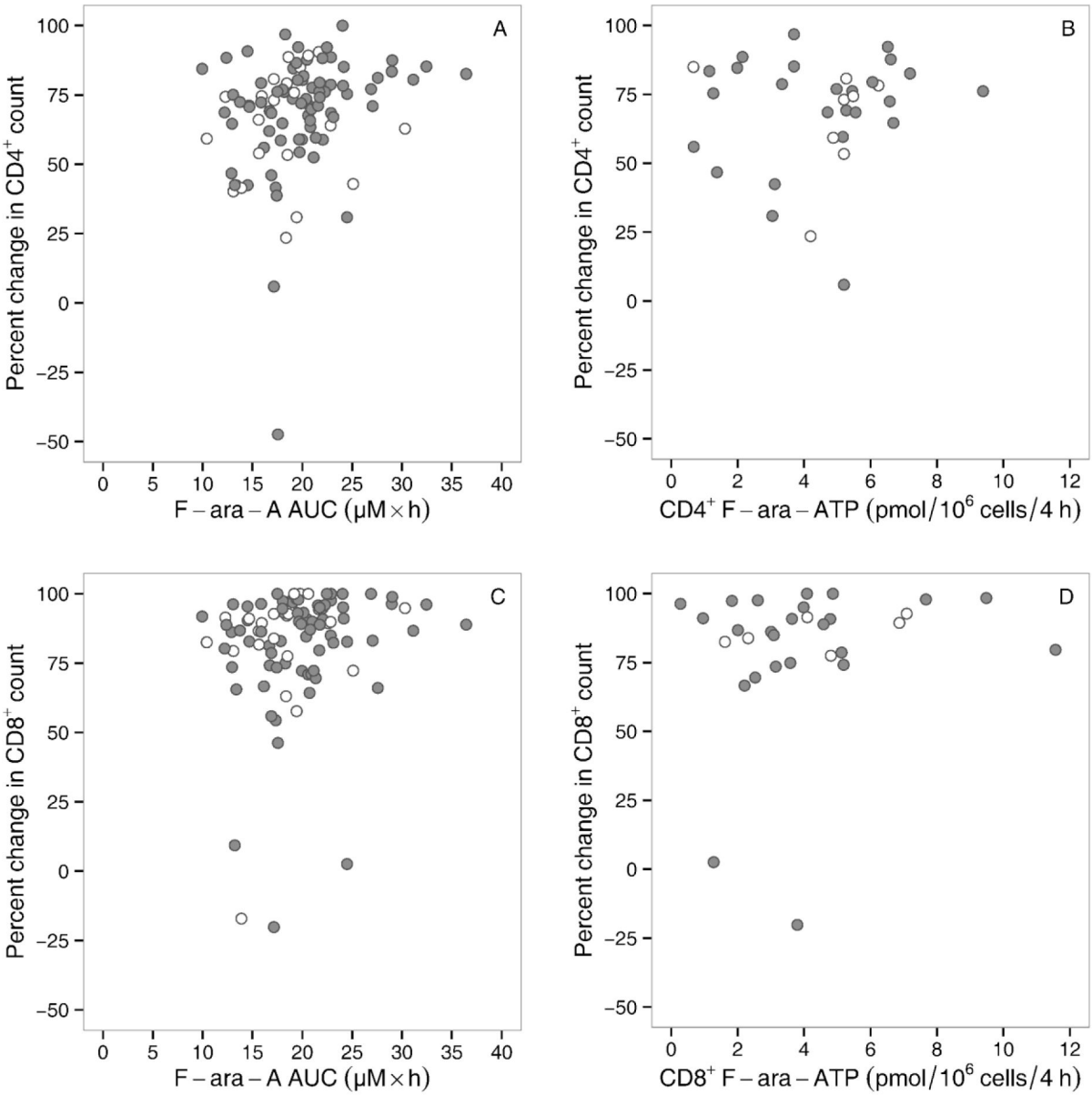

Figure 2.

Association of F-ara-A AUC or F-ara-ATP accumulation rate with lymphosuppression: Percent decline in related (white) or unrelated (grey) donor grafts in $CD4^+$ cells with F-ara-A AUC (A) and accumulated F-ara-ATP (B). Percent decline in $CD8^+$ cells with F-ara-A AUC (C) and accumulated F-ara-ATP (D).

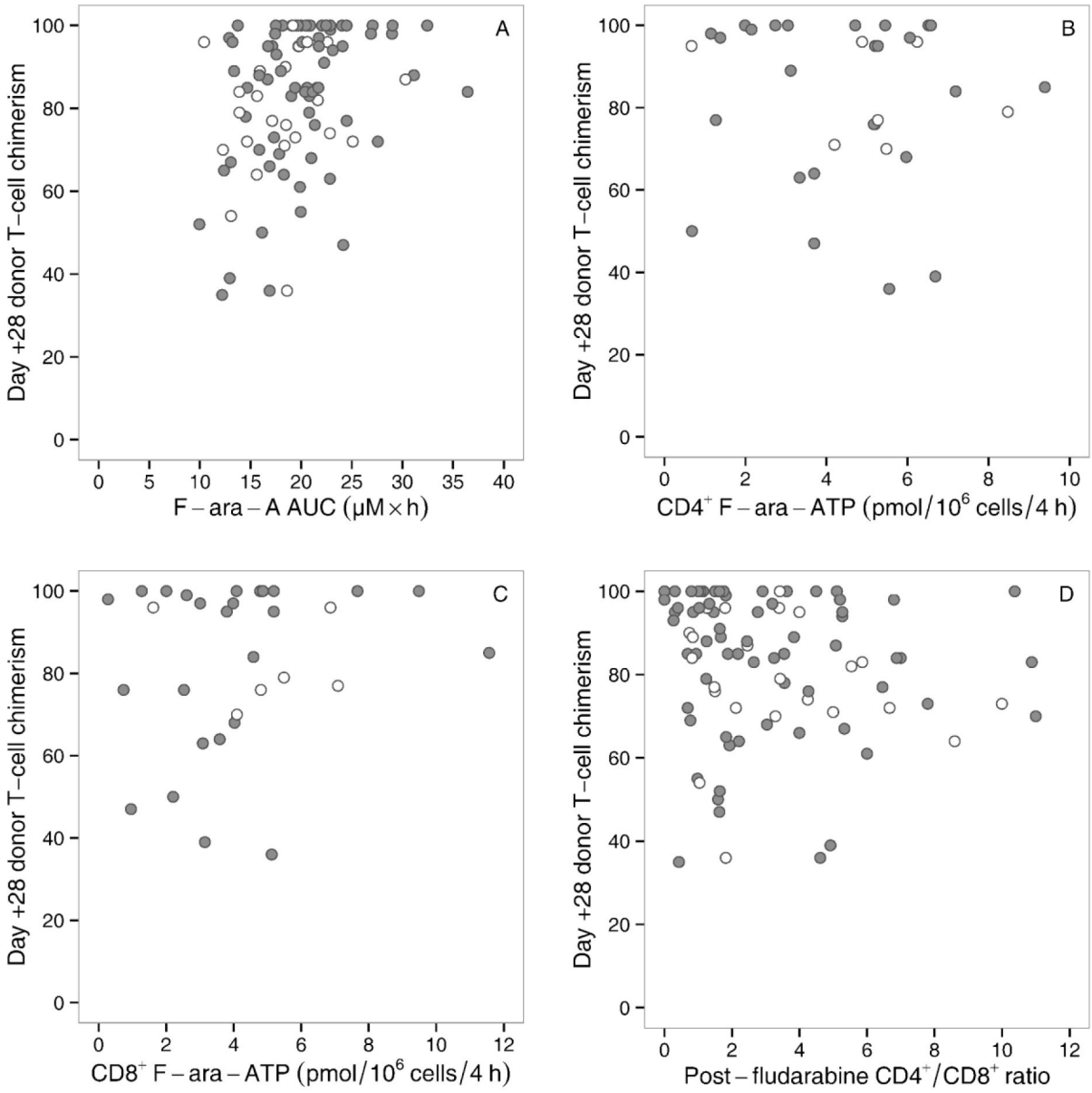

Figure 3.

Day +28 T-cell chimerism and fludarabine-related biomarkers in related (white) or unrelated (grey) donor grafts: F-ara-A AUC (A), F-ara-ATP accumulation rate in $CD4^+$ cells (B), and F-ara-ATP accumulation rate in $CD8^+$ cells (C), and fludarabine $CD4^+/CD8^+$ ratio from the day after the last dose of fludarabine (D).

Table 1

Participant characteristics[a]

	Donor Type		
	Related	**Unrelated**	**All participants**
Total number	24	78	102
Sex, female/male (% female)	9/15 (38%)	28/50 (36%)	37/65 (36%)
Actual body weight (kg)	80 (50 – 119)	83 (45 – 142)	82 (45 – 142)
HCT-CI[40]			
0	1 (4%)	8 (10%)	9 (9%)
1–2	3 (13%)	17 (22%)	20 (20%)
3–4	11 (46%)	30 (38%)	41 (40%)
≥5	9 (38%)	23 (29%)	32 (32%)
Recipients' ages, years	59 (20 – 69)	62 (28 – 75)	62 (20 – 75)
CMV positive recipient or donor	16 (67%)	51 (65%)	67 (66%)
Kahl Disease risk[27]			
Low	4 (17%)	29 (37%)	33 (32%)
Standard	16 (67%)	33 (42%)	49 (48%)
High	4 (17%)	16 (21%)	20 (20%)
Diagnosis			
Non-Hodgkin lymphoma	7 (29%)	27 (35%)	34 (33%)
Chronic lymphocytic leukemia	8 (33%)	14 (18%)	22 (22%)
Acute myelogenous leukemia	4 (17%)	11 (14%)	15 (15%)
Myelodysplastic syndrome	1 (4%)	9 (12%)	10 (10%)
Multiple myeloma	1 (4%)	8 (10%)	9 (9%)
Acute lymphocytic leukemia	1 (4%)	3 (4%)	4 (4%)
Myelofibrosis / Myeloproliferative disorder	0	3 (4%)	3 (3%)
Aplastic anemia	0	2 (3%)	2 (2%)
Hodgkin disease	2 (8%)	0	2 (2%)
Paroxysmal nocturnal hemoglobinuria	0	1 (1%)	1 (1%)
Female donor to male recipient	10 (42%)	19 (24%)	29 (28%)
Donors' ages, years	55 (23 – 73)	31 (20 – 58)	35 (20 – 73)
HLA-mismatched graft	1 (4%)	2 (3%)	3 (3%)
Conditioning regimen			
2Gy TBI + FLU ± auto	11 (46%)	38 (49%)	49 (48%)
2Gy TBI + FLU + rituximab ± auto[b]	11 (46%)	20 (26%)	31 (30%)
3Gy TBI + FLU ± rituximab[b]	2 (8%)	13 (17%)	15 (15%)
4–4.5 Gy TBI + FLU	0	7 (9%)	7 (7%)
Post-grafting immunosuppression			
MMF Q8h	1 (4%)	78 (100%)	79 (77%)
MMF Q12h	23 (96%)	0	23 (23%)
Cyclosporine+MMF±sirolimus[c]	14 (58%)	54 (69%)	68 (67%)

	Donor Type		
	Related	**Unrelated**	**All participants**
Tacrolimus+MMF±sirolimus[d]	10 (42%)	24 (31%)	34 (33%)

[a] Data shown as median (range) or as number (%).

[b] Rituximab given on days −3, +10, +24, and +38 relative to transplant;

[c] Ten participants received cyclosporine + sirolimus, one with a matched donor and nine with unrelated donors;

[d] Five participants received tacrolimus + sirolimus, all with unrelated donors.

Abbreviations: auto: autologous transplant; CMV: cytomegalovirus; FLU: fludarabine monophosphate; HCT-CI: HCT comorbidity index; HLA: human leukocyte antigen; MMF: mycophenolate mofetil; TBI: total body irradiation.

Table 2

Variability in F-ara-A AUC and F-ara-ATP accumulation rate

Biomarker	N	Mean ± SD (range)
F-ara-A AUC (μM×h) dose 1[a]	100	19.6 ± 4.80 (9.96 – 36.4)
Variability in 24hr F-ara-A concentration		
Within-patient CV	88[a]	28 ± 13% (0.5 – 63)
Within-patient maximum/minimum	88[a]	1.8 ± 0.4 (1.0 – 3.7)
F-ara-ATP accumulation rate (pmol/10^6 cells/4h)		
in $CD4^+$ cells	36	4.6 ± 2.1 (0.7 – 9.4)
in $CD8^+$ cells	34	4.0 ± 2.4 (0.3 – 11.6)

Abbreviations: AUC: Area under the concentration-time curve; CV: coefficient of variation; F-ara-A: 9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-ATP: fludarabine triphosphate.

[a]Dose 1 administered on HCT day −4.

Table 3

Immediate effects of fludarabine 90 mg/m^2 upon circulating lymphocyte counts

	Before fludarabine[a]	**After fludarabine**[b]
Fludarabine dose number	Dose 1[c]	Day after dose 3[c]
Time between lymphocyte count and fludarabine dose	4 min (0 – 10.0 h)	23.8 h (20.4 – 43.9 h)
Circulating CD4$^+$ (10^3 cells/μL)	0.25 ± 0.19 (0.004 – 0.97)	0.08 ± 0.07[d] (0 – 0.30)
% decline in circulating CD4$^+$	N/A	68 ± 21[e] (−47 – 100)
Circulating CD8$^+$ (10^3 cells/μL)	0.31 ± 0.31 (0 – 1.4)	0.05 ± 0.09[d] (0 – 0.76)
% decline in circulating CD8$^+$	N/A	82 ± 22[f] (−20 – 100)
CD4$^+$/CD8$^+$ ratio	1.65 ± 1.99 (0.09 – 15)	3.08 ± 2.59[d] (0 – 11)

Data presented as mean ± standard deviation;

[a] N=100;

[b] N=99;

[c] Doses 1, 2, 3 administered on HCT days −4, −3, −2, respectively.

[d] P<0.001 using paired t-test of before and after fludarabine data;

[e] One participant's CD4$^+$ count and

[f] two participants' CD8$^+$ counts increased after fludarabine