# Correlation of Infused $CD3^+CD8^+$ Cells on Single-Donor Dominance after Double Unit Cord Blood Transplantation

Milano F[1], Heimfeld S[1,2] ,Gooley T[1,2] ,Jinneman J[3] ,Nicoud I[1], Delaney C [1,4]

[1] Clinical Research Division, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA; [2]University of Washington (UW), Seattle, WA; [3]Seattle Center Cancer Alliance (SCCA), Seattle, Wa; [4] Department of Pediatrics, UW, Seattle, WA.

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Corresponding author:

Filippo Milano
1100 Fairview Ave. N.
Mailstop D2-100
Seattle WA 98109-1024

E-mail: fmilano@fhcrc.org
Phone: (206) 667-5925
Fax: (206) 667-5899

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**Abstract**

Single-donor dominance is observed in the majority of patients following double cord blood transplantation (dCBT), however the biological basis for this outcome is poorly understood. To investigate the possible influence of specific cell lineages on dominance, flow cytometric assessment for $CD34^+$, $CD14^+$, $CD20^+$, $CD3^-CD56^+$, $CD3^+CD56^+$ (NKT), and T-cell subsets ($CD4^+$, $CD8^+$, Memory, Naïve, and Regulatory) was performed on individual units. Subsets were calculated as infused viable cells/kg of recipient actual weight, with sixty patients included in the final analysis. Higher $CD3^+$/kg dose was statistically concordant with the dominant unit in 72% of cases (p=0.0006). Further T-cell subset analyses showed dominance was correlated more with the naive $CD8^+$ cell subset (71% concordance; p=0.009) than the naive $CD4^+$ cell subset (61% concordance; p=0.19). These data indicate that a greater total $CD3^+$/kg cell dose and in particular naïve $CD3^+CD8^+$ T-cells, may play an important role in determining single donor dominance after dCBT.

## Introduction

Cord blood transplantation (CBT) is an accepted treatment for adults and children with hematologic malignancies [1, 2]. In order to overcome the limitation in cell dose provided by a single CB graft and to more reliably achieve sustained donor engraftment, double unit CBT (dCBT) is commonly performed for adult and larger adolescent patients [3, 4]. Interestingly, in the vast majority of dCBT recipients, only one unit emerges as the source of long-term hematopoiesis [1,2,5]. However, the factors that determine which of the two units will achieve single donor dominance after dCBT remain poorly understood.

Unit parameters such as total nucleated cell (TNC) and $CD34^+$ cell doses, viability, degree of HLA-matching and order of infusion have not been uniformly associated with donor dominance [6-8]. Conversely, $CD3^+$ cell dose is emerging as a reliable predictor of single donor dominance [9-11]. Indeed, in a report from our institution the first direct evidence that only effector T-cells derived from the dominant unit produced interferon-γ in response to cells derived from the non-engrafting unit [12] was shown. More recently, we also provided strong evidence that the unit with higher CD3 chimerism at day 7 is the one more likely to be the dominant unit [13].

Herein, the association of infused CD3 cell dose, and in addition possible correlations of specific T cell subsets ($CD3^+/CD8^+$, $CD3^+/CD4^+$, naïve/memory, and regulatory) with the emergence of single unit donor dominance were further investigated. An extended immunophenotyping flow cytometry panel was performed on a sample obtained from each CB unit just prior to infusion in 72 consecutive patients undergoing dCBT for hematologic malignancies. Infused cell doses were calculated and

used to investigate whether any specific T-cell subsets were also highly correlated with single donor dominance.

## Methods

### *Patient/Donor Characteristics*

Between January 2008 and May 2011 72 patients received a dCBT at our institution. Twelve patients (17%) were excluded because they either died before obtaining informative chimerism data or had primary/secondary graft failure. As per institutional priority, patients received a CBT if they lacked an available HLA-compatible related or matched unrelated donor. All patients received unrelated donor CB grafts, which were 4/6- to 6/6-matched at HLA-A, B, and DRB1 antigens. HLA-typing was performed at the antigen level for HLA-A and B, and high resolution HLA-typing was performed for HLA-DRB1 alleles. The individual CB units were at least 3/6-HLA-A, B, and DRB1 matched to each other, and each contained a minimum of $1.5 \times 10^7$ TNC/kg. All study activities were approved by the FHCRC Institutional Review Board, and all participants provided written informed consent according to the principles of the Declaration of Helsinki.

### *Conditioning regimens and GVHD prophylaxis*

Myeloablative conditioning consisted of cyclophosphamide (Cy) 60 mg/kg intravenously daily for 2 days, total body irradiation (TBI) 1320 or 1200 cGy, and fludarabine (Flu) 40 mg/m$^2$ intravenously daily for 3 days. Eleven patients received Flu at a dose of 30 mg/m$^2$ intravenously daily for 5 days, Treosulfan (Treo) at 14 gm/m$^2$ intravenously daily for 3 days, and a single fraction of TBI 200 cGy. Reduced intensity

conditioning consisted of Flu 40 mg/$m^2$ intravenously daily for 5 days, a single dose of Cy 50 mg/kg intravenously, and a single fraction of TBI 200 or 300 cGy.

GVDH prophylaxis consisted of cyclosporine-A along with mycophenolate mofetil and emergence of GVHD was treated per treating physician discretion.

***Cell dose analysis***

All CB units were thawed and washed by centrifugation prior to resuspension in preparation for infusion. Units were infused sequentially with an interval of less than 45 minutes between each infusion. A small aliquot was removed for analysis from the final product just prior to infusion. This sample was then processed for measurement of total nucleated cells (TNC) and flow cytometric assessment of graft composition. In particular the following viable (7-AAD negative) cell subsets were measured by multicolor FACS analyses and then expressed as infused cell subset/kg of actual recipient weight: stem/progenitors ($CD34^+$), monocytes ($CD14^+$), B ($CD20^+$), NK ($CD3^-CD56^+$), NK/T ($CD3^+CD56^+$), and T-cell subsets ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD45RA^-/CD45RO^+$ (Memory) $CD45RA^+/CD45RO^-$ (Naïve), and $CD4^+CD25^+CD127^{lo}$ for Regulatory T-cells). Results were used to calculate total cell subset/kg of recipient weight for each cord blood unit.

***Chimerism analysis***

Analysis of host and cord blood unit chimerism was performed on flow cytometry sorted CD3+, CD56+, and CD33+ fractions of peripheral blood on days 7, 14, 21, 28, 56, and 80 after transplantation. Whole bone marrow chimerism was performed on days 28, 56 and 80 after transplantation. DNA chimerism analysis was performed by

amplified fragment length polymorphism (detection sensitivity 1-5%, range of accuracy ±5%) [14]. Single donor dominance was generally defined as more than 95% single unit derived cells in all fractions. In the rare case of the two CB units engrafting long-term, the unit contributing to >60% hematopoiesis was considered the dominant one. Similarly, in patients who died before day 100, the dominant unit was considered the one contributing to >60% hematopoiesis at the time of the last chimerism result.

***Statistical analysis***

If the infused dose of a cell subset was not associated with subsequent development of single-donor dominance, the expected concordance between the dominant unit and a higher or lower dose of that subset should be the random chance of 50% (e.g. a coin flip). Thus, a one-sample test of the null hypothesis that a binomial proportion is equal to 0.5 was used to test the pair-wise association of concordance of the relatively higher cell subset dose with unit dominance. Similarly, in a separate analysis, if there was no association between cell dose and unit dominance, the expected mean difference in dose between winning and losing units should be zero. Accordingly, a one-sample t-test was used to test the null hypothesis that the mean difference in cell dose between winning and losing units was zero. Wilcoxon rank sum test was used to compare graft characteristics between dominant and non-dominant units.

## Results

### *Patient and graft characteristics*

Sixty patients were included in the final analysis. Patient, transplantation and graft characteristics are summarized in **Table 1.** Forty-six (77%) and 14 (23%) received a myeloablative (MC) or non-myeloablative conditioning (NMC), respectively. Median time to neutrophil engraftment was 13 (Interquartile range [IQR] 7-18) and 25 (IQR 19-31) after NMC and MC conditioning, respectively. At day +28 post-transplant a dominant unit (as defined ≥ 60% chimerism) was observed in all recipients of MC. In particular, in all but one patient complete contribution from a single unit (as defined ≥ 95% chimerism) was seen at a median time of 14 days (IQR, 14 -21 days). The only patient with persistent contribution from both units, still present at 1 year post-transplant, received a Treosulfan based conditioning regimen and 2 HLA 6/6 units. In contrast, NMC patients initially had mixed donor-host chimerism and at day +28, single donor dominance (as defined ≥ 60% chimerism) was observed in only 4 patients. The remaining 10 patients converted to single donor dominance at a median time of 56 days (IQR 56-80 days) post-transplant, however at day +80 3 patients still had significant contribution from both units.

Among the 120 CB units given to these 60 patients, the degree of matching were as follows: HLA-A, -B, -DRB1 matched (n=15); mismatched at 1 Ag (n=44), mismatched at 2 Ag (n=61). HLA mismatch was not a critical factor in affecting dominance. Indeed, in 6 (10%) cases the unit with the better HLA matching to the recipient became the dominant unit while in 9 (15%) cases the unit with less HLA matching predominated. In the remaining cases both units had the same degree of HLA mismatching.

***Influence of $CD3^+$ cells on determining dominant unit***

No significant differences were seen in the median pre-thaw and post-wash TNC and $CD34^+$ cell doses and viability post-wash between dominant and non-dominant units (Table 2). For some patients, a sample was not pulled or insufficient material was available from each double cord blood unit for complete cell subset analysis on both CB units (Table 3). Of the 60 cohort patients, 58 had a sufficient sample to determine $CD3^+$, $CD4^+$and $CD8^+$ cell doses. In 42 (72%, p=0.0006), 41 (71%, p=0.002) and 45 (78%, p<0.0001) cases the unit containing the higher $CD3^+$, $CD4^+$ or $CD8^+$ cell dose became the dominant engrafting unit, respectively. In the subset of 14 patients who received NMC, the proportion of positive correlation was identical to the whole cohort [CD3 10/14 (71%); CD8 11/14 (78%) and CD4 10/14 (71%)]. In a smaller cohort of 38 patients (28 MC and 10 NMC) further analysis of various $CD4^+$ and $CD8^+$ subsets showed that only the naïve $CD8^+$ cell dose was highly associated with donor dominance (71%, p=0.009) while memory $CD8^+$, memory $CD4^+$ and naïve $CD4^+$ cell doses did not significantly correlate with the dominant unit (Table 3). This association of a higher ratio of infused cell dose with dominance was defined as positive concordance, and is shown graphically in Figure 1. As expected in CB products, the total $CD8^+$ cell doses were strongly correlated with the majority naïve $CD8^+$ fraction (R=0.90, Figure 2), supporting the concept that total viable CD8+ cell dose can be used as surrogate marker for naïve CD8 cells infused.

As an alternative approach to assess cell subset associations with single unit dominance, the mean difference in infused cell dose between dominant and non-

dominant units was also analyzed. This calculation also showed a statistically significant difference for $CD3^+$, $CD8^+$, $CD4^+$, naïve $CD8^+$ and naïve $CD4^+$ (Table 4).

### *Association of other cell subsets*

Among the other cell types analyzed, only higher NKT (68%, p=0.02) and $CD20^+$ (68%, p=0.02) were also statistically significantly correlated with donor dominance (Table 3). However, the mean difference between dominant and not-dominant unit was only statistically significant for NKT but not for $CD20^+$ cells (Table 4). Viability post-wash was suggestively concordant (62%, p=0.07) while no statistically significant associations with the infused dose of monocytes, NK, or regulatory T-cells were observed (Table 3 and 4).

We also found no significant associations between pre-freeze or post-wash infused dose of total cells or $CD34^+$ cells and the likelihood of being the dominant unit. Furthermore we did not find any association between the order of infusion and the probability of becoming the long-term engrafting unit (p=0.60; Table 3).

## Discussion

The absolute number of T cells infused with CB stem cell grafts is relatively lower compared with bone marrow or PBSC products. Nevertheless, in this study evidence is provided that having a higher $CD8^+$ cell content, in particular the naïve $CD3^+CD8^+$ T-cell subset, is an important predictor in determining which CB unit will ultimately predominate. Similar to other published reports [9,10], a high concordance was observed between greater $CD3^+$/kg content and establishment of single-donor dominance. However, to our knowledge, this is the first published report that infused

$CD3^+CD8^+$ and $CD3^+CD4^+$ subsets in the CB graft are significantly correlated with emergence of single-donor dominance.

Furthermore, in this study, it is the naïve $CD8^+$/kg cell dose in particular that is most highly statistically associated with unit dominance. Thus, it may be hypothesized that naïve $CD8^+$ T-cells in one CB unit, when that unit becomes exposed in vivo to the allogeneic antigens expressed by the second CB unit, will become activated. Having a relatively higher content of those naïve $CD8^+$ T-cells will give that unit an advantage in facilitating the ultimate rejection of the second CB unit. This hypothesis is supported by data from animal models and in vitro testing showing that predominantly naïve T-cells can mount an anti-allogeneic response and cause GVHD [15], while transplantation of memory $CD8^+$ and $CD4^+$ cells does not result in GVHD reactivity [16,17]. Similarly, our results suggest that higher doses of memory $CD4^+$ and $CD8^+$ subsets do not appear to influence emergence of single-unit dominance. Although it was not always possible to determine the $CD8^+$ subtypes content for each unit, the high correlation between total $CD8^+$ and naïve $CD8^+$ in cord blood products allow us to infer that it was also the higher infused dose of the naïve population in those cases which was responsible for determining the dominant unit.

The limited number of paired samples available for the naïve T-cell subset analyses did not indicate a significant statistical correlation between higher naïve CD4+ cell dose and probability of becoming the dominant unit. However when we analyzed the results using instead the mean of differences, we did also find a statistically significant higher content of naïve CD4+ in the dominant units. It has been shown that HLA-alloreactivity is derived from both naïve $CD4^+$ and $CD8^+$ subsets [18], thus this

difference in mean infused naïve CD4+ cell dose suggests a potential role for the naïve $CD4^+$ along with naïve $CD8^+$ in influencing single unit dominance.

In support of additional immune-mediated mechanisms determining single unit dominance, we also found, a higher infused content of NKT and B-cells in the ultimately dominant CB. NKT cells can exhibit potent direct killing activity, both through MHC-unrestricted and TCR-mediated mechanisms, against different cell line targets [19]. Thus, it is possible that higher NKT content might also influence the emergence of single-donor dominance specifically through HLA-mismatched immune-mediated rejection responses towards the other CB unit. Similarly, higher numbers of CD20+ cells in allogeneic grafts have been previously associated with immune reactions such as increased development of chronic GVHD and better clinical outcome [20]. These preliminary findings need to be confirmed in future studies and a clinical analysis is ongoing at our institution to better clarify the potential role played by NKT and CD20+ cells in dCBT.

In contrast to other published reports, no statistically significant associations were found with the infused dose of total cells or $CD34^+$ cells and the dominant CB unit. While viable $CD34^+$ dose has been reported as the most important factor in determining the unit that will dominate [8], this was not observed in our study. The lack of correlation reported here as compared to the study conducted by Scaravandou and colleagues may result from differences in the flow cytometric methods used to assess $CD34^+$ cell viability after post thaw/wash processing of the cord blood units.

The results presented in this study strengthen the evidence that the basis for single unit predominance is an immunological-mediated rejection mechanism between

the two cord blood units. However, besides infused cell dose there are likely to be other factors that may play a role such as specific HLA-matching interactions between the residual host immune system and the two cord blood donor, interactions between the two CB units, the host bone marrow environment, differences in homing efficiencies [21] which could also impact which unit will ultimately predominate. Larger studies that support multivariate analyses of host and graft characteristics on influencing the emergence of a dominant unit after dCBT could improve unit selection criteria. To date, unit selection is based on HLA-matching, followed by TNC and/or $CD34^+$ prefreeze measurements. Our results, along with findings from other groups [9, 10], support the concept that prefreeze CD3 and/or CD8 measurements might further improve these unit selection algorithms. Knowing the dose of $CD8^+$ might be even more critical for clinical trials that involve graft manipulation. For example, in studies of $CD34^+$ ex-vivo expansion [22] focused on just reducing the time to neutrophil engraftment, the unit with less $CD8^+$ cells could be chosen for processing since it would have a lower chance of long-term predominance.

In conclusion, in this study we have provided evidence that higher T-cell content, in particular the naïve $CD3^+CD8^+$ T-cell subset, is an important predictor of which CB unit will ultimately predominate. The data support immune-mediated mechanisms as the primary driver of single unit dominance. Having an ability to accurately predict, prior to infusion, which CB unit will ultimately become the dominant long-term engrafting unit may have practical implications for optimal unit selection. Furthermore, an improved understanding of the determinants of short-term and long-term engraftment after dCBT

also has implications for ex-vivo graft manipulations, immune reconstitution, and disease control.

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**Authorship Contributions**

F.M., S.H., and C.D. participated in the study design, data analysis and interpretation of data for the manuscript. F.M. wrote the first draft and I.B.N., J.J., S.H., T.A.G., and C.D. provided revisions and critical review of the final manuscript. T.A.G. and F.M. performed the statistical analyses.

**Conflicts of Interest**

All authors declare no conflicts of interest.

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**Table 1.** Characteristics of the 60 patients.

| ***Patient Characteristics*** | |
|---|---|
| **Patient Age, Median Years (IQR)** | 38.3 (18.6 – 51.4) |
| **Gender, No (%)** | |
| Female | 30 (50) |
| **Median Recipient Weight in Kg (IQR)** | 72.0 (59.9-79.8) |
| **Recipient CMV Serostatus, No (%)** | |
| Positive | 36 (60) |
| Negative | 24 (40) |
| **Transplant Type, No (%)** | |
| Myeloablative | 46 (77) |
| Non-myeloablative | 14 (23) |
| **GVHD Prophylaxis, No (%)** | |
| Cyclosporine/Mycophenolate | 60 (100) |
| **Disease, No (%)** | |
| ALL | 18 (30) |
| AML | 26 (44) |
| MDS | 5 (8) |
| CLL | 4 (7) |
| Other | 7 (11) |
| **Sex Match, No (%)** | |
| Match, match | 22 (37) |
| Match, mismatch | 28 (47) |
| Mismatch, mismatch | 10 (16) |
| **HLA disparity, No (%) §** | |
| 4/6 + 4/6 | 26 (43) |
| 4/6 + 5/6 | 9 (15) |
| 4/6 + 6/6 | - |
| 5/6 + 5/6 | 13 (22) |
| 5/6 + 5/6 | 8 (14) |
| 6/6 + 6/6 | 4 (6) |
| **Time to engraftment ** days, (IQR range)** | |
| Myeloablative | 25 (19-31) |
| Non-myeloablative | 13 (7-18) |

Abbreviations: IQR = Interquartile range; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; MDS = myelodysplastic/myeloproliferative diseases; CLL = chronic lymphocytic leukemia; CMV = cytomegalovirus; TNC = total nucleated cell.
§ HLA matching reflects the lowest HLA-match of the 2 units
** The day of neutrophil engraftment was defined as the first of 3 consecutive days of an absolute neutrophil count of 500/µl or greater.

**Table 2.** Characteristics of the dominant and non-dominant CB units.

| Factors | Dominant Unit (n=60) | Non-dominant (n=60) | p-value |
|---|---|---|---|
| **TNC x $10^7$/Kg †, Median (IQR)** | 2.4 (1.9 – 3.3) | 2.4 (1.9 – 3.0) | 0.18 |
| **TNC x $10^7$/Kg §, Median (IQR)** | 2.0 (1.6 – 2.6) | 1.9 (1.5 – 2.4) | 0.15 |
| **$CD34^+$ x $10^6$/Kg †, Median (IQR)** | 0.11 (0.07 – 0.15) | 0.10 (0.06 – 0.15) | 0.44 |
| **$CD34^+$ x $10^6$/Kg §, Median (IQR)** | 0.12 (0.08 – 0.19) | 0.11 (0.07 – 0.19) | 0.32 |
| **Viability, Median % (IQR)** | 74.3 (66.5 – 81) | 67.6 (63.2 – 77) | 0.07 |

Abbreviations : TNC: Total Nucleated cells; IQR : Interquartile range
† Pre-thaw values
§ Post-wash values

**Table 3.** Cell subset/total number of patients where larger infused value was in concordance with winning unit / total number of patients

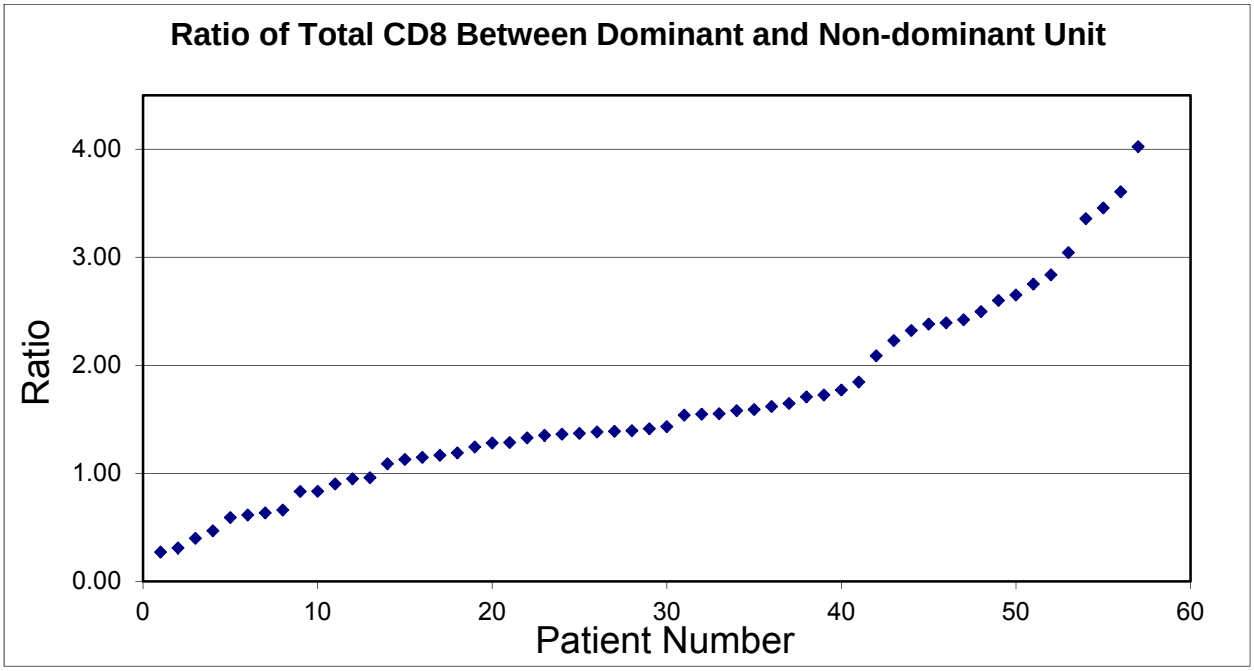
| Cell Fractions (n = # of evaluable unit pairs) | Units with positive concordance n (%,CI 95%) | P-value |
|---|---|---|
| Total CD8 (58) | 45 (78%, 67-88) | **<0.0001** |
| Total CD3 (58) | 42 (72%, 61-84) | **0.0006** |
| Naïve CD8 (38) | 27 (71%, 57-85) | **0.009** |
| Total CD4 (58) | 41 (71%, 59-82) | **0.002** |
| CD20 (41) | 28 (68% 54-83) | **0.02** |
| NKT (38) | 26 (68%, 54-83) | **0.02** |
| Viability post-wash (60) | 37 (62%, 49-74) | 0.07 |
| TNC post-wash (60) | 35 (59%, 46-71) | 0.15 |
| Naïve CD4 (38) | 23 (61%, 45-76) | 0.19 |
| Memory CD4 (38) | 23 (61%, 45-76) | 0.19 |
| $CD34^+$ post-wash (60) | 34 (57%, 44-69) | 0.30 |
| CD14 (57) | 26 (46%, 33-59) | 0.51 |
| NK (39) | 18 (46%, 31-62) | 0.63 |
| Order of infusion* | 31 (53%, 41-66) | 0.60 |
| CD34 pre-freeze (58) | 31 (53%, 41-66) | 0.60 |
| TNC pre-freeze (58) | 32 (53%, 36-70) | 0.73 |
| T-reg (34) | 18 (53%, 36-70) | 0.73 |
| Memory CD8 (38) | 19 (50% 34-66) | 1.00 |

* Unit infused as first

**Table 4**. Mean of the difference in infused viable cell dose between dominant and non-dominant units for each cell subset.

| Cell Fractions dose/Kg | Mean difference dominant and non-dominant unit (CI 95%) | P-value |
|---|---|---|
| Total CD8 x $10^5$/Kg | 5.96 (3.40 – 8.52) | **<0.0001** |
| Naïve CD8 x $10^5$/Kg | 4.16 (1.35 – 6.97) | **0.006** |
| Total CD3 x $10^6$/Kg | 1.44 (0.55 – 2.32) | **0.002** |
| Total CD4 x $10^6$/Kg | 1.05 (0.41 – 1.70) | **0.002** |
| NKT x $10^4$/Kg | 1.23 (0.28 – 2.17) | **0.02** |
| Naïve CD4 x $10^5$/Kg | 7.22 (0.54 -13.91) | **0.04** |
| Viability post-wash % | 3.72 (0.17 – 7.28) | **0.04** |
| CD20 x $10^5$/Kg | 2.15 (-0.88 – 5.18) | 0.17 |
| T-reg x $10^4$/Kg | -2.06 (-4.89 – 0.78) | 0.16 |
| TNC pre-freeze x $10^9$/Kg | 0.11 (-0.09 – 0.32) | 0.26 |
| CD34 post-wash x $10^6$/Kg | 1.16 (-1.18 – 3.51) | 0.32 |
| Memory CD8 x $10^4$/Kg | 2.04 (-3.45 – 7.52) | 0.47 |
| TNC post-wash x $10^9$/Kg | 0.001 (-0.002 – 0.004) | 0.39 |
| CD34 pre-freeze x $10^6$/Kg | 0.01 (-0.02 – 0.05) | 0.45 |
| NK x $10^5$/Kg | 0.97 (-2.71 – 4.65) | 0.61 |
| Memory CD4 x $10^4$/Kg | 4.31 (-8.95-17.56) | 0.53 |
| CD14 x $10^4$/Kg | - 2.17 (-30.82 – 26.48) | 0.88 |

**Figure 1.** Ratio between dominant and non-dominant units. A) Total $CD8^+$ B) naïve $CD8^+$.



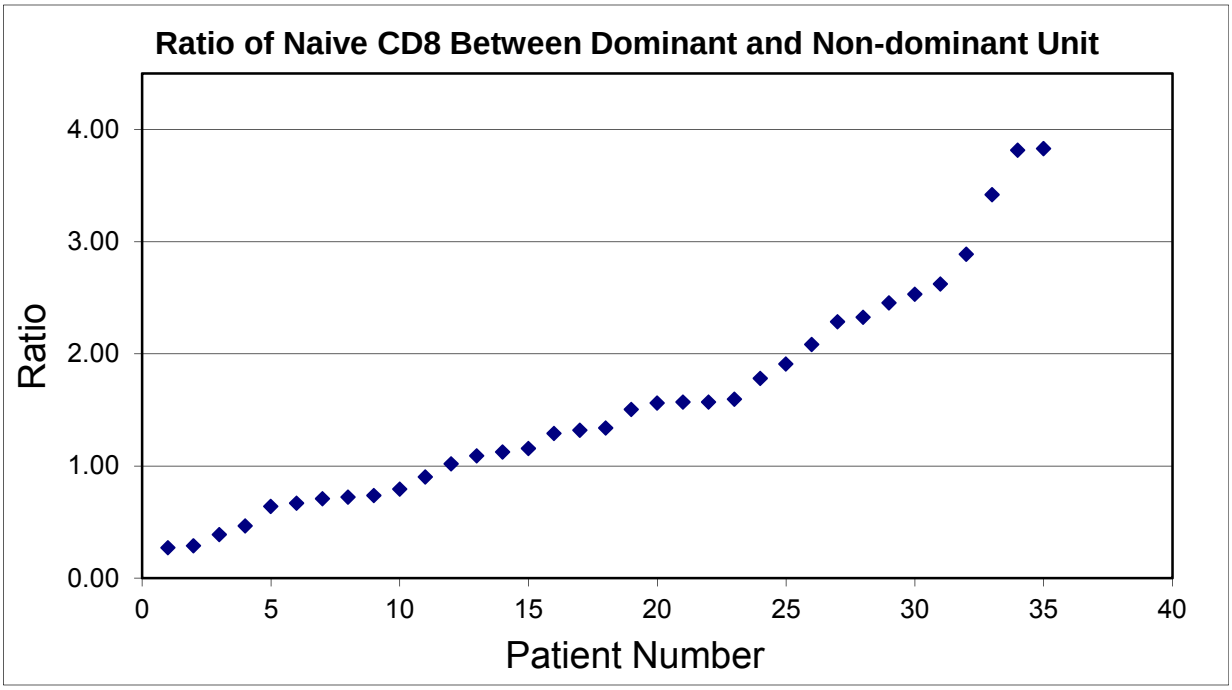



**Figure 2.** Correlation between total $CD8^+$ and naïve $CD8^+$ cell dose in the units with evaluable samples (n=38) (R=0.90).