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Research paper

Autograft immune content and survival in non-Hodgkin's lymphoma: A post hoc analysis

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ABSTRACT

The infusion of autograft absolute lymphocyte and monocyte counts affect survival in patients undergoing autologous peripheral hematopoietic stem cell transplantation (APHSCT). However, the specific autograft immune effector cells affecting survival post-APHSCT are unknown. Thus, we performed an ad hoc analysis from our published double-blind, randomized phase III clinical trial in non-Hodgkin's lymphoma (NHL) patients, looking at the infused autograft immune effector cells and their relationship with clinical outcomes post-APHSCT.

Between December 2007 and October 2010, we performed a double-blind phase III randomized study registered with ClinicalTrials.gov, number NCT00566228. A total of 111 patients finished the trial and apheresis collection samples were analyzed for immune effector cells. Overall survival (OS) and progression-free survival (PFS) were calculated from the date of APHSCT.

With a median follow-up of 82.8 months (range: 2.1–122.3 months), we identified by univariate analysis that the autograft numbers of macrophage type 1 (M 1), macrophage type 2 (M 2), dendritic cell type 1 (DC 1), dendritic cell type 2 (DC 2), myeloid-derived suppressor cells (MDSC), CD4 + PD-1-, CD4 + PD-1 +, CD8 + PD-1-, CD8 + PD-1 +, lymphocyte to monocyte ratio (A-LMR), NKp30, and KIR2DL2, were predictors for OS and PFS. Multivariate analysis revealed that A-LMR, MDSC, NKp30, KIR2DL2 and lactate dehydrogenase were independent predictors for OS. Independent predictors for PFS identified by multivariate analysis included DC1, MDSC, NKp30, CD4 + PD-1- and M 2.

Our findings indicate that the number of specific infused autograft immune effector cells affect survival ; thus providing a platform to develop an immunocompetent autograft with direct impact on clinical outcomes in NHL post-APHSCT.

1. Introduction

The discovery that day 15 absolute lymphocyte count recovery (ALC-15) [1–6], day 15 absolute monocyte recovery (AMC-15) [7], infusion of both autograft absolute lymphocyte count (A-ALC) [8–10] and autograft absolute monocyte count (A-AMC) [11], infused autograft lymphocyte/monocyte ratio (A-LMR) [11] directly affect clinical outcomes post-autologous peripheral blood hematopoietic stem cell transplantation (APHSCT), are reshaping our understanding of the APHSCT as an adoptive immunotherapeutic strategy rather than just a method to deliver high-dose chemotherapy for the treatment of hematologic malignancies. Our group performed a randomized phase III clinical trial [12] manipulating the apheresis machine Amicus settings [13] with the hope to increase the collection and infusion A-ALC to improve clinical outcomes post-APHSCT. Patients were randomized to the standard Amicus setting versus the modified Amicus setting. Patients that collected and infused an A-ALC $\geq 0.5 \times 10^9$ cells/kilogram at either Amicus setting (standard versus modified) was associated with better survival. Even though, the modification of the Amicus machine did not increase the number of A-ALC, there were other confounding factors such as the number of leuko-apheresis that affected the collection of A-ALC in our phase III trial; thus, suggesting that methods to enhance the collection and infusion of A-ALC, such as the number of apheresis collections, mobilization agents to increase not only CD34 but also immune effector cells in the peripheral blood (i.e, lymphocytes) prior to apheresis collection and continue to improve the apheresis machines for collection of immune effector cells, would improve clinical outcomes post-APHSCT.

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However, ALC-15, AMC-15, A-ALC, A-AMC, and A-LMR do not provide information regarding immune activating and suppressor effector cells affecting survival post-APHSCT. Therefore, we performed an ad hoc analysis of the autograft immune effector cells collected and infused to non-Hodgkin's Lymphoma (NHL) patients that participated in our Phase III clinical trial [12], in an attempt to identify the autologous immune effector cells impacting clinical outcomes post-APHSCT.

2. Materials and methods

2.1. Patients

A total of 111 patients out of 121 that finished our double-blind, randomized phase III clinical trial were eligible to participate in the study. Patients in the study were 18 years of age or older with NHL. Patients were excluded if they required bone marrow harvest to collect stem cells, did not achieve chemo-sensitivity as defined by either partial response or complete response prior to APHSCT, or participated in any APHSCT study not using the standard BEAM conditioning (Carmustine, Etoposide, Cytarabine, and Melphalan) regimen.

Approval for the study was obtained from the Mayo Clinic institutional review board and was in accordance with federal regulations and the Declaration of Helsinki. Written informed consent was obtained from all patients.

2.2. End point

The primary endpoint was to assess the impact of infused autograft immune effector cells on overall survival (OS) and progression-free survival (PFS) post-APHSCT. Hereafter, disease status was assessed following the guidelines from the International Harmonization Project on Lymphoma [14].

2.3. Prognostic factors

The prognostic factors tested in this study included: international prognostic index (IPI) at diagnosis (≥ 2), age at diagnosis (≥ 60 versus < 60), lactate dehydrogenase at diagnosis ($>$ normal), ECOG (Eastern Cooperative Oncology Group) performance status at diagnosis (> 1 versus ≤ 1), extra-nodal sites at diagnosis (> 1 versus ≤ 1), stage at diagnosis (III/IV versus I/II), histologies (diffuse large B-cell lymphoma versus others), disease status before APHSCT (complete response versus partial response), infused CD34+ cell count, the use of Plerixafor, A-LMR, CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; dendritic cell type 1 (DC 1); dendritic cell type 2 (DC 2); macrophage type 1 (M 1); macrophage type 2 (M 2); myeloid-derived suppressor cells (MDSC); activating NKp30 and inhibitory KIR2DL2 natural killer cells.

2.4. Peripheral blood stem cell (autograft) collection

This double-blind randomized phase III clinical trial was designed to determine whether the progression-free survival (PFS) was significantly increased in patients with stem cells collected using a modification to a standard Fenwal Amicus setting where mononuclear cells offset = 1.5 and red blood cells = 6.0 compared with mononuclear cell offset = 1.5 and red blood cells = 5.0.

Mobilization of stem cells was performed by using 10 μg/kg of granulocyte colony-stimulating factor (G-CSF) daily for 5–7 consecutive days alone or in combination with 0.24 mg/kg of Plerixafor for up to 4 consecutive days by subcutaneous injection. After 4 days of G-CSF treatment, if the peripheral blood CD34+ count was 10 cells/μl, stem cell collection was started. If the peripheral blood CD34+ count was less than 10 cells/μl, Plerixafor was added that evening and collections started the next day.

2.5. Conditioning regimen

All patients received BEAM [BCNU (300 mg/m^2)] on day -6, Etoposide (100 mg/m^2) twice daily from days -5 to -2, ARA-C [Cytarabine (100 mg/m^2)] twice daily from days -5 to -2, and Melphalan (140 mg/m^2) on day -1.

2.6. Autograft immune effector cells analysis

Flow cytometry analysis was used to analyze the immune effector cell content from frozen autograft mononuclear cells ($0.5–1.0 \times 10^6$ cells/mL) from each apheresis collection sample. Absolute number of the following collected and infused immune effector cells were analyzed: CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2; MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells.

The infused A-ALC for each apheresis unit collection was calculated as follows: A-ALC = % collection lymphocytes x (absolute white blood cells count (WBC)/kg) [14,15].

The infused A-AMC for each apheresis unit collection was calculated as follows: A-AMC = % collection monocytes x (absolute white blood cells count (WBC)/kg) [20].

For the analysis of the autograft lymphocyte subset analysis, patients' blood samples for each apheresis collection were collected and studied by flow cytometric analysis (see Flow Cytometry analysis in the appendix). The autograft LMR was calculated by dividing A-ALC by A-AMC (A-ALC/A-AMC).

The absolute numbers of autograft CD4 + PD-1-;CD4 + PD-1 +;CD8 + PD-1-;CD8 + PD-1 +; NKp30; and KIR2DL2 were calculated by multiplying the autograft percentage of CD4 + PD-1-;CD4 + PD-1 +;CD8 + PD-1-;CD8 + PD-1 +; NKp30; and KIR2DL2 times A-ALC.

The absolute numbers of autograft DC 1; DC 2; M 1;M 2; and MDSC were calculated by multiplying the autograft percentage of DC 1; DC 2; M 1; M 2; and MDSC times A-AMC.

2.7. Statistical analysis

OS was defined as the date of infusion to the date of death from any cause. PFS was defined as the time from the date of infusion to disease progression or death due to any cause. OS and PFS were analyzed using the approached of Kaplan and Meier [15]. Differences between survival curves were tested for statistical significance using the two-tailed log-rank test. The cox proportional hazard model [16] was used for the univariate and multivariate analysis to evaluate the variable tested for prognostic significance for OS and PFS. A stepwise forward regression analysis was performed to identify the most significant prognostic variables in the univariate analysis to study in the multivariate analysis. The choice of cut-off values for CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2; MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells was based on their utility as a marker for the clinically relevant binary outcome of death/survival using the receiver operating characteristics curves (ROC) and area under the curve (AUC). The binary clinical outcome (death/survival) was established at 5 years after the APHSCT. Patients were classified as "alive/censored" when follow-up was greater than 5 years and "death/uncensored" for patients known to have died before this time point [17]. A K-fold cross-validation with K values of 10 was performed to validate the results of CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2;MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells cutoff values obtained by the ROC and AUC curves. Randomly chosen subsets containing 90% of the cohort were used for training and the remaining 10% were left for testing. The cross-validation process was then repeated 10 times. Based on this analysis, cross-validation AUC by the ROC was produced, representing the discriminating accuracy of CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2; MDSC; activating NKp30 and

inhibitory KIR2DL2 natural killer cells for the binary clinical outcomes of death/survival. The LMR cut-off of 1.0 was based on our previous publication [11].

Chi-square tests and Fisher exact tests were used to determine relationships between categorical variables as appropriate. The Wilcoxon rank test was used to determine associations between continuous variables and categorical and nonparametric tests were used to evaluate associations for continuous variables. All *P*-values represented were 2-sided and statistical significance was declared at $P < 0.05$.

3. Role of the funding source

The funders of the phase III clinical trial [12] had no role in this study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

4. Results

4.1. Patient characteristics

The median age at the time of APHSCT was 57 years (range: 20–74 years). The distribution of additional baseline-characteristics for the cohort is presented in Table 1. The median follow-up from APHSCT was 82.8 months (range: 2.1–122.3 months) and for the living patients (N = 65), (94.8 months, range: 53·4–122·3 months). The day 100 transplant-related mortality was 1·8% (2/111). One patient died of acute respiratory distress syndrome and one patient died of septic shock. Forty patients died due to relapse or progression of lymphoma, two patients died of myocardial infarction, one patient of acute leukemia, and one patient of astrocytoma. Sixty-two patients experienced relapse or progression of lymphoma.

4.2. Cutoff values for autograft immune effector cells for survival analysis

To identify which autograft immune effector cells were relevant for survival analysis, we performed a univariate analysis for OS and PFS, using each autograft immune effector cell as a continuous variable (see Table 1 in the appendix). CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2; MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells were predictors for OS and PFS, as continuous variables in the univariate analysis. ROC curves and AUC (see Fig. 1 in the appendix) were used to determine the optimal cutoff values for CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2; MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells based on their utility as markers for the clinical binary outcome of death/survival. Table 2 in the appendix shows the ROC/AUC values including the sensitivity/specificity, as well as the internal validation AUC values obtained from the *k*-fold cross-validation with $k = 10$.

4.3. Predictors and survival cures for OS and PFS

Using the new cutoff values for CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; M 1; M 2 ; MDSC; activating NKp30 and inhibitory KIR2DL2 natural killer cells, we tested in the univariate analysis for OS and PFS with the other prognostic factors described in the prognostic factors section under Methods (Tables A1 and A2).

The following prognostic factors were significant in the univariate analysis for OS: female; LDH; extra-nodal disease; IPI; CD4 + PD-1; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; A-LMR: M 1; M 2; MDSC; KIR2DL2; and NKp30 (see Table 2). The following prognostic factors were significant in the univariate analysis for PFS: Stage; extra-nodal-disease; IPI; complete response prior to APHSCT; CD4 + PD-1-; CD4 + PD-1 +; CD8 + PD-1-; CD8 + PD-1 +; DC 1; DC 2; A-LMR; M 1; M 2; MDSC; KIR2DL2; and NKp30 (see Table 2).

Due to the multiple significant variables in the univariate analysis and the number of events for both OS and PFS, we performed a stepwise forward regression analysis for OS (see Table A3 in the Appendix A) and PFS (see Table A4 in the appendix) to identify the most significant

Table 1
Patient Characteristics.

Characteristics	Median (range)	N (%)
Age at diagnosis, years	55 (20–73)	
Age, years		
> 60		33 (30%)
≤60		78 (70%)
Gender		
Female		31 (28%)
Male		80 (72%)
LDH (range: 122–222 U/L)	211 (106–3364)	
LDH (U/L)		
Abnormal		53 (48%)
Normal		58 (52%)
Extranodal disease		
0		51 (46%)
1		52 (47%)
2		8 (7%)
Stage		
I		7 (6%)
II		11 (10%)
III		27 (24%)
IV		66 (60%)
Performance status		
0		28 (25%)
1		71 (64%)
2		12 (11%)
International prognostic index		
0		15 (13%)
1		34 (31%)
2		32 (29%)
3		25 (22%)
4		5 (5%)
Histology		
Diffuse large B cell		51 (45%)
Mantle cell		25 (22%
Follicular		16 (16%)
Other		19 (17%)
Status prior to transplantation		
Complete Remission		63 (57%)
Partial Remission		48 (43%)
Plerixafor		
No		67 (60%)
Yes		44 (40%)
CD34 infusion x 10^6/kilogram	5.15 (2.02–11.37)	
Autograft immune effector cells		
A-ALC x 10^9cells/kg	0.46 (0.1–2.16)	
A-AMC x 10^9cells/kg	0.61 (0.04–1.97)	
A-LMR ratio	0.76 (0.17–4.13)	
CD4 + PD-1- x 10^9cells/kg	0.12 (0.0046–0.265)	
CD4 + PD-1 + x 10^9cells/kg	0.06 (0.007–0.2)	
CD8 + PD-1- x 10^9cells/kg	0.15 (0.016–0.87)	
CD8 + PD-1 + x 10^9cells/kg	0.02 (0.0007–0.136)	
KIR2D2L x 10^9cells/kg	0.05 (0.002–0.39)	
NKp30 × 10^9cells/kg	0.09 (0.009–0.48)	
DC 1 × 10^9cells/kg	0.13 (0.007–0.46)	
DC 2 × 10^9cells/kg	0.1 (0.01–0.45)	
DR + CD80 + Lin- (APC) x 10^9cells/kg	0.016 (0.0008–0.33)	
DR + CD86 + Lin- (APC) x 10^9cells/kg	0.14 (0.02–0.5)	
M 1 × 10^9cells/kg	0.16 (0.01–0.51)	
M 2 × 10^9cells/kg	0.03 (0.002–0.07)	
MDSC x 10^9cells/kg	0.2 (0.02–0.6)	

Abbreviation: A-ALCautograft absolute lymphocyte count; A-AMCautograft absolute monocyte count; A-LMRautograft lymphocyte to monocyte ratio; DC 1dendritic cell type 1; DC 2dendritic cell type 2; LDHlactate dehydrogenase; M 1macrophage type 1; M 2macrophage type 2; and MDSCmyeloid derived suppressor cells.

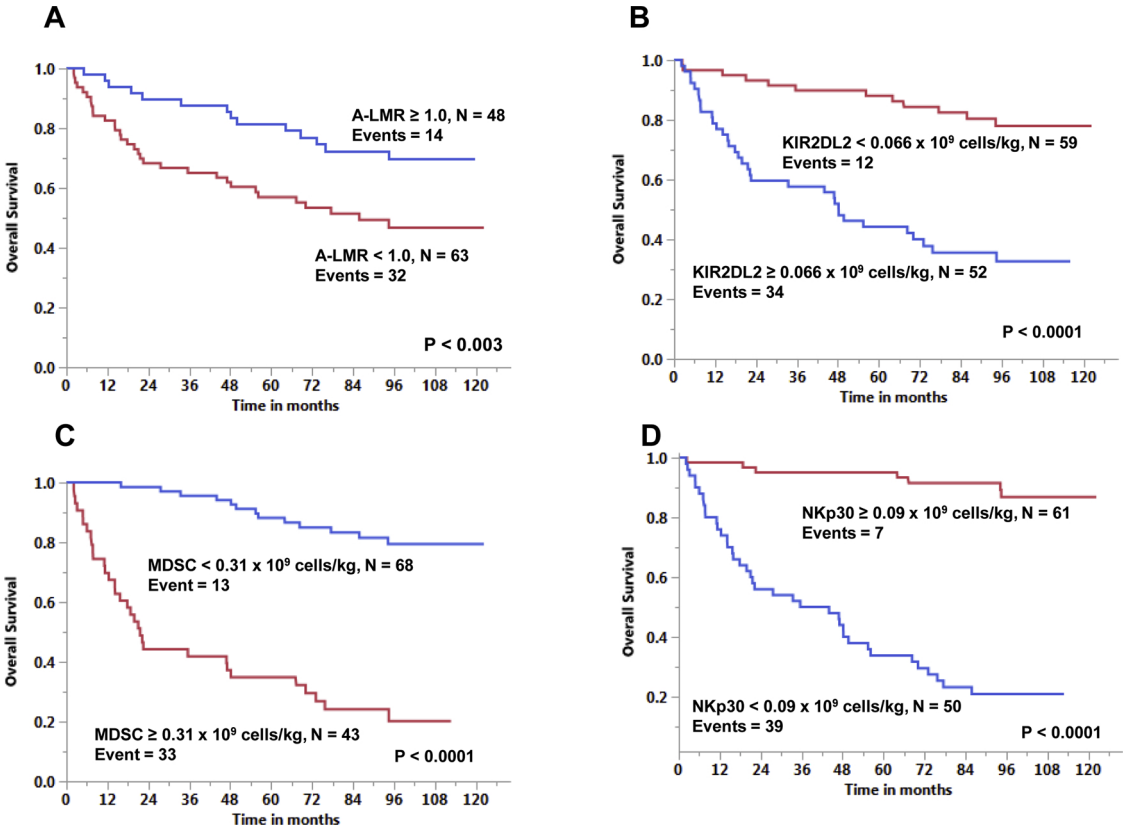

Fig. 1. (A) Overall survival for (A) autograft lymphocyte to monocyte ratio (A-LMR), (B) KIR2DL2, (C) myeloid-derived suppressor cells (MDSC), and (D) NKp30.

prognostic variables in the univariate analysis to study in the multivariate analysis. From the stepwise forward regression analysis the following variables were significant for OS: CD4 + PD-1 +; KIR2DL2; LDH; A-LMR; MDSC; and NKp30. From the stepwise forward regression analysis the following variables were significant for PFS: CD4 + PD-1-; CD4 + PD-1 +; complete remission prior to ASCT; DC 1; A-LMR; M 1; M 2: MDSC; and NKp30. The multivariate analysis showed the following variables to be independent predictors for OS: LDH; A-LMR; KIR2DL2; MDSC; and NKp30 (see Table 3). The multivariate analysis identified the following variables to be independent predictors for PFS: CD4 + PD-1-; DC 1; M 2; MDSC; and NKp30 (see Table 3).

Fig. 1 depicts the OS Kaplan and Meier curves for A-LMR, KIR2DL2,

Table 2
Univariate analysis for overall survival and progression-free survival.

Variables	Overall Survival HR 95%CI P	Progression-Free Survival HR 95%CI P
Age, years > 60 years	1.672 0.978–2.987 0.09	1.159 0.686–1.918 0.6
Female	1.916 1.031–3.452 < 0.04	1.510 0.868–2.540 0.1
LDH abnormal > 222 U/L	2.174 1.211–4.005 < 0.009	1.527 0.921–2.531 0.1
Stage III/IV	1.408 0.644–3.701 0.4	2.250 1.050–5.848 < 0.04
Extranodal-disease > 1	4.926 1.984–10.570 < 0.001	3.269 1.345–6.785 < 0.01
Performance status > 1	1.341 0.510–2.934 0.5	1.175 0.487–2.412 0.7
International Prognostic Index > 2	2.523 1.376–4.579 < 0.003	1.865 1.086–3.115 < 0.02
Partial remission vs complete remission at transplantation	1.624 0.907–2.923 0.1	2.259 1.364–3.780 < 0.002
Other histologies vs Diffuse Large B-cell Lymphoma	1.742 0.975–3.160 0.06	1.006 0.611–1.672 0.9
Autograft		
CD4 + PD-1- < 0.13×10^9 cells/kilogram	2.064 1.146–3.838 < 0.02	2.916 1.736–5.046 < 0.0001
CD4 + PD-1 + ≥ 0.09×10^9 cells/kilogram	1.899 1.060–3.403 < 0.03	2.073 1.254–3.428 < 0.005
CD8 + PD-1- < 0.04×10^9 cells/kilogram	3.924 1.703–11.372 < 0.0006	3.771 1.898–8.596 < 0.0001
CD8 + PD-1 + ≥ 0.05×10^9 cells/kilogram	3.444 1.773–7.343 < 0.0002	3.459 1.977–6.414 < 0.0001
DC1 < 0.12×10^9 cells/kilogram	2.711 1.504–5.043 < 0.0009	2.830 1.705–4.790 < 0.0001
DC2 ≥ 0.13×10^9 cells/kilogram	3.173 1.758–5.908 < 0.0001	2.716 1.638–4.567 < 0.0001
A-LMR < 1.0	2.140 1.084–4.224 < 0.003	2.036 1.149–3.606 < 0.01
M1 < 0.13×10^9 cells/kilogram	2.683 1.497–4.904 < 0.0009	2.557 1.548–4.248 < 0.0003
M2 ≥ 0.03×10^9 cells/kilogram	2.293 1.247–4.441 < 0.007	2.720 1.593–4.843 < 0.0002
Myeloid-derived suppressor cells ≥ 0.31×10^9 cells/kilogram	7.922 4.227–15.744 < 0.0001	7.453 4.353–13.134 < 0.0001
KIR2DL2 ≥ 0.066×10^9 cells/kilogram	4.843 2.567–9.790 < 0.0001	2.578 1.553–4.365 < 0.0002
NKp30 < 0.09×10^9 cells/kilogram	12.778 6.013–35.516 < 0.0001	6.491 3.737–11.734 < 0.0001

Abbreviation: A-LMR = autograft lymphocyte to monocyte ratio; DC 1 = dendritic cell type 1; DC 2 = dendritic cell type 2; LDH = lactate dehydrogenase; M 1 = macrophage type 1; and M 2= macrophage type 2.

Table 3
Multivariate analysis for overall survival and progression-free survival.

Variables	Overall Survival HR 95%CI P	Progression-Free Survival HR 95%CI P
Partial remission vs complete remission at transplantation		1.586 0.896–2.850 0.1
LDH (U/L) abnormal	2.484 1.348–4.705 < 0.003	
CD4 + PD-1- < 0.13 × 10^9 cells/kilogram		2.162 1.254–3.792 < 0.03
CD4 + PD-1 + ≥0.09 × 10^9 cells/kilogram	1.381 0.739–2.597 0.3	1.478 0.827–2.630 0.1
DC1 < 0.12 × 10^9 cells/kilogram		2.162 1.254–3.792 < 0.005
A-LMR < 1.0	1.570 1.016–2.425 < 0.04	1.126 0.609–2.083 0.7
M1 < 0.13 × 10^9 cells/kilogram		1.324 0.735–2.668 0.3
M2 ≥ 0.03 × 10^9 cells/kilogram		1.996 1.069–3.889 < 0.03
Myeloid-derived suppressor cells ≥ 0.31 × 10^9 cells/kilogram	4.186 1.968–9.215 < 0.0002	3.422 1.809–6.531 < 0.0002
KIR2DL2 ≥ 0.066 × 10^9 cells/kilogram	2.550 1.236–5.600 < 0.01	
NKp30 < 0.09 × 10^9 cells/kilogram	11.047 4.817–28.975 < 0.0001	3.422 1.868–6.490 < 0.0001

Abbreviation: A-LMR = autograft lymphocyte to monocyte ratio; DC 1 = dendritic cell type 1; M 1 = macrophage type 1; and M 2 = macrophage type 2.

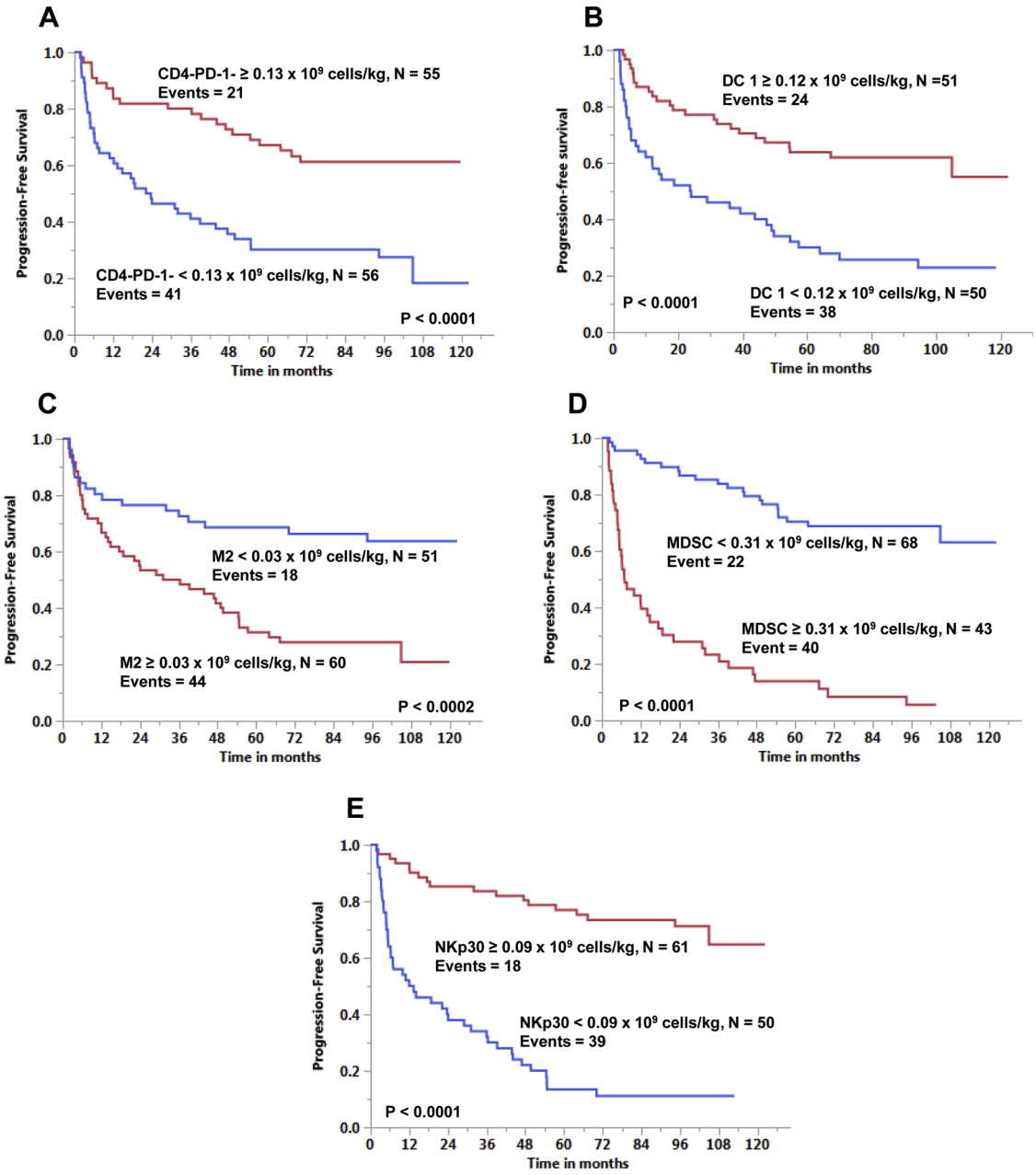

Fig. 2. (A) Progression-free survival for (A) CD4 + PD-1-, (B) Dendritic cell type 1 (DC 1), (C) macrophage type 2 (M 2), (D) myeloid-derived suppressor cells (MDSC), and (E) NKp30.

MDSC, and NKp30. Fig. 2 depicts the PFS Kaplan and Meier curves for CD4 + PD-1-, DC 1, M 2, MDSC, and NKp30. Table A5 in the appendix contains the median times and 5 years-rates for OS and PFS with 95% confidence intervals of the variables shown in Figs. 1 and 2.

5. Discussion

A limitation of the prognostic factors A-ALC and A-AMC is their lack of identifying the specific immune effector cells affecting clinical outcomes post-APHSCT. Thus, we set up to perform a more comprehensive analysis of collected and infused autograft immune effector cells and their relationship with clinical outcomes in NHL patients treated with APHSCT. From the A-ALC standpoint we analyzed the PD-1 status of CD4 and CD8 T-cells, as well as the inhibitory KIRD2L2 and activator NKp30 NK cells. From the A-AMC standpoint, we analyzed DC 1 and DC 2; M 1 and M 2; and MDSC.

Our study identified that immunosuppressive immune effectors affecting OS included KIR2DL2 and MDSC and for PFS included M 2 and MDSC. To our knowledge, we are the first reporting the negative survival impact of infusing high doses of type 2-polarized macrophages (M 2), which has been associated with negative survival in cancer patients [18]. In similar fashion, we are reporting for the first time that the infusion of high doses of MDSC worsens the clinical outcomes post-APHSCT. Possible immunosuppressive mechanisms of MDSCs include the production of immunosuppressive cytokine; disruption of the major histocompatibility complex class 1 receptor causing T-cells to become unresponsive to antigen-specific; Fas-FasL interaction leading to T-cell apoptosis; induction of regulatory T-cells; and inhibition of natural killer (NK) cells function/proliferation [19]. NKp30 was the only activating immune effector cell associated with OS and NKp30 and DC 1 were predictors for PFS. Our study confirmed a previous report that DC 1 is important for clinical outcomes in APHSCT [20]. We are reporting that the infusion of high numbers of NKp30 cells improved survival post-APHSCT.

In regard to immune checkpoints, higher numbers of infused CD4 + PD-1- were associated with better PFS. This suggests that the infusion of non-activated/non-exhausted T cells is important to the PFS of NHL patients treated with APHSCT. Our study confirmed that A-LMR still an independent predictor for OS.

A major limitation of this study is that it is a *post hoc* analysis of our randomized phase III trial [12]. Thus; further studies are warranted to support our findings. Another limitation of the study was a selected analysis of autograft immune effectors cells due to the constraint allowed by our Institutional Review Board on the amount of blood volume obtained from each apheresis sample to minimize the risk of engraftment failure. Thus a priority ranking of the specific immune effector cells was developed. MDSC, M 1, M 2, and immune checkpoint PD-1 content in the autograft of NHL patients undergoing APHSCT has not previously been studied. The negative impact on survival by the infusion of MDSC in the autologous stem cell transplantation setting is a new finding in contrast to reports of decreased graft-versus-host disease and improved relapse-free survival by infusing MDSC in the allogeneic stem cell transplantation setting [21]. Dendritic cells were selected to confirm a prior report that showed that dendritic cells were predictors of survival in multiple myeloma patients undergoing APHSCT [28]. KIR2DL2 and NKp30 were selected as there have been reports in autologous stem cell transplantation in regard to activation of these two NK cells KIR [22]. However, the authors acknowledged that other autograft immune effector cells not tested in this study could contribute to the clinical outcomes of patients undergoing APHSCT. Our group believes that the immune-parameters reported in this study should be also analyzed in the post-APHSCT setting. We reported using the lymphocyte count (as a surrogate marker of host immunity) that patients post-APHSCT developing lymphopenia was associated with risk of relapse [23]. Furthermore, the combination of lymphopenia in conjunction with neutrophilia and/or monocytosis (as possible surrogate markers of inflammation/tumor microenviroment) was also correlated with risk of relapse (data not published) post-APHSCT. Thus, our hypothesis is that the infusion of an immune-competent autograft needs to be sustained post-APHSCT. Our group is in the process of studying the immune parameters reported in this study, plus the cytokine profile of these patients in the post-APHSCT setting.

In conclusion, our study provides new information to build upon our understanding of how complete and functional host immunity has direct impact on the survival of NHL patients undergoing APHSCT. Furthermore, our study provides a platform for the development of strategies to minimize the infusion of autograft immunosuppressive cells such as MDSC, M 2, and KIR2DL2 NK cells and strategies to enhance the mobilization and collection of immune activator cells such DC 1, NKp30 NK cells, and immune checkpoint CD4 + PD-1-. However, further independent studies are warranted to confirm our findings.

Contributors

LFP: study design; data interpretation; drafting, critical revision, and final approval version of the manuscript; DJI: drafting, critical revision, and final approval version of the manuscript; SMA: drafting, critical revision, and final approval version of the manuscript; INM: drafting, critical revision, and final approval version of the manuscript; PBJ: drafting, critical revision, and final approval version of the manuscript; JCV: drafting, critical revision, and final approval version of the manuscript; and

SNM: data interpretation; drafting, critical revision, and final approval version of the manuscript.

Declaration of interests

The authors have no competing interests.

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Appendix A

Flow cytometry analysis

Pretreatment, post-treatment, and apheresis patient samples were isolated by ficoll gradient and viably frozen. Cell aliquots were thawed and analyzed by multi-parametric flow cytometry to determine the frequency of dendritic cells (DC), macrophages, antigen presenting cells (APC), myeloid derived suppressor cells (MDSC), natural killer cells (NK cells) and PD-1 positive T-helper and cytotoxic T-cells. All antibodies were purchased from BD Bioscience (Franklin Lakes, NJ) and are as follows: FITC anti-human CD14, CD3, CD4, CD19, CD4, and CD158b, PE anti-human CD197, CD80, CD86, CD11b, PD-1, APC anti-human CD206, CD11c, CD123, CD8, CD56 and PE-Cy5.5 anti-human HLA-DR, and CD16. For DC, MDSC, and APC, cells were first gated on lineage negative cells (CD3, CD14 and CD19). NK cells were gated on CD16 and CD56 positive cells. Cells types were defined as shown in Table A1. Cells were stained for 30 min at 4 °C in FACS buffer (0.5% BSA and 0.1% sodium azide in PBS), washed 3 times in FACS buffer, and run on a Guava 8 H T flow cytometer (Millipore, Burlington, MA). Data analysis was performed using InCyte software

(Millipore, Burlington, MA).

Cell Type	Markers
Dendritic Cell 1	Lineage-, HLA-DR+, CD11c+
Dendritic Cell 2	Lineage-, HLA-DR+, CD123+
Macrophage 1	CD14+, CD197+
Macrophage 2	CD14+, CD206+
MDSC	Lineage-, HLa-DRlo, CD11b+
NK cells	CD16+, CD56+, CD337+, CD158b+
NK cells	CD16+, CD56+, CD337+, CD158b-
T-cells	CD4+, PD-1+
T-cells	CD8+, PD-1+

Table A1
Univariate analysis for overall survival and progression-free survival.

Continuous Variables	Overall Survival HR 95%CI P	Progression-Free Survival HR 95%CI P
CD4+PD-1-	0.319 0.107–0.929 < 0.04	0.245 0.098–0.603 < 0.002
CD4+PD-1+	2.379 1.096–5.497 < 0.04	2.939 1.415–6.020 < 0.004
CD8+PD-1-	0.175 0.042–0.587 < 0.004	0.109 0.031–0.325 < 0.0001
CD8+PD-1+	0.135 0.042–0.399 < 0.0006	0.141 1.254–3.792 < 0.0001
CD158b + CD337- (KIR2DL2)	12.492 5.046–29.564 < 0.0001	6.604 2.919–14.091 < 0.0001
CD158b-CD337+ (NKp30)	0.033 0.012–0.495 < 0.0001	0.032 0.004–0.226 < 0.0001
DC1	0.155 0.032–0.648 < 0.02	0.080 0.020–0.293 < 0.0001
DC2	9.690 3.290–26.876 < 0.0001	6.728 2.656–16.075 < 0.0001
DR + CD80+Lin-(APC)	0.242 0.154–2.504 0.3	0.228 0.004–1.854 0.2
DR + CD86+Lni-(APC)	1.124 0.257–4.209 0.9	0.679 0.180–2.248 0.5
M1	0.162 0.045–0.513 < 0.002	0.089 0.028–0.252 < 0.0001
M2	3.666 1.428–9.412 < 0.007	3.688 1.641–8.267 < 0.0001
MDSC	93.758 25.46–365.1 < 0.0001	42.44 16.47–109.05 < 0.0001

Abbreviations: APC = antigen presenting cells; DC 1= dendritic cell type 1; DC 2= dendritic cell type 2; M 1 = macrophage type 1; M 2 = macrophage type 2; and MDSC = myeloid derived suppressor cells.

Table A2
Receiver operating characteristic (ROC), area under the curve (AUC), sensitivity/specificity, and K-fold cross-validation AUC for the autograft immune effector cells.

Variables	ROC/AUC	Sensitivity/Specificity	P	K-Fold Cross Validation AUC
CD4+ PD-1- $< 0.13 \times 10^9$ cells/kilogram	0.63	68%/69%	< 0.02	0.72
CD4+ PD-1+ $\geq 0.09 \times 10^9$ cells/kilogram	0.62	47%/78%	< 0.02	0.65
CD8+ PD-1- $< 0.04 \times 10^9$ cells/kilogram	0.63	89%/45%	< 0.01	0.66
CD8+PD-1+ $\geq 0.05 \times 10^9$ cells/kilogram	0.69	78%/55%	< 0.0007	0.72
DC 1 $< 0.12 \times 10^9$ cells/kilogram	0.65	63%/68%	< 0.02	0.71
DC 2 $\geq 0.13 \times 10^9$ cells/kilogram	0.70	61%/76%	< 0.006	0.73
KIR2DL2 $\geq 0.066 \times 10^9$ cells/kilogram	0.79	74%/73%	< 0.0001	0.79
M 1 $< 0.13 \times 10^9$ cells/kilogram	0.68	59%/74%	< 0.003	0.70
M 2 $\geq 0.03 \times 10^9$ cells/kilogram	0.64	57%/72%	< 0.01	0.65
MDSC $\geq 0.31 \times 10^9$ cells/kilogram	0.77	71%/84%	< 0.0001	0.80
NKp30 $< 0.09 \times 10^9$ cells/kilogram	0.86	85%/85%	< 0.0001	0.85

Abbreviations: DC 1= dendritic cell type 1; DC 2= dendritic cell type 2; M 1 = macrophage type 1; M 2 = macrophage type 2; and MDSC = myeloid derived suppressor cells.

Table A3
Forward Stepwise Regression for Overall Survival.

Rank	Parameter	R^2	P-value
1	Myeloid-derived suppressor cells	0.3949	< 0.0001
2	NKp30	0.5664	< 0.0001
3	KIR2DL2	0.6033	< 0.002
4	LDH	0.6226	< 0.02
5	CD4+PD-1+	0.6389	< 0.03
6	A-LMR	0.6411	< 0.04
7	CD8+PD-1+	0.6457	0.2
8	CD8+PD-1-	0.6502	0.3
9	Extranodal Disease	0.6522	0.4
10	CD4+PD-1-	0.6536	0.5
11	M1	0.6547	0.6

(*continued on next page*)

Table A3 (*continued*)

Rank	Parameter	R^2	P-value
12	Gender	0.6549	0.8
13	M2	0.6551	0.9
14	CD8 + PD-1-	0.6551	0.9
15	International Prognostic Index	0.6511	0.9
16	DC2	0.6511	0.9

Abbreviations: A-LMR = autograft lymphocyte to monocyte ratio; DC 1 = dendritic cell type 1; DC 2 = dendritic cell type 2; LDH = lactate dehydrogenase; M 1 = macrophage type 1; and M 2 = macrophage type 2.

Table A4
Forward Stepwise Regression for Progression-Free Survival.

Rank	Parameter	R^2	P-value
1	Myeloid-derived suppressor cells	0.4092	< 0.0001
2	NKp30	0.5728	< 0.0001
3	CD4 + PD-1-	0.6115	< 0.002
4	DC1	0.6266	< 0.04
5	Partial remission vs complete remission at transplantation	0.6427	< 0.03
6	CD4 + PD-1 +	0.6563	< 0.04
7	M1	0.6697	< 0.04
8	M2	0.6833	< 0.04
9	A-LMR	0.6852	< 0.04
10	Stage	0.6895	0.2
11	KIR2DL2	0.6955	0.2
12	CD8 + PD-1 +	0.6969	0.5
13	CD8 + PD-1-	0.6975	0.7
14	International Prognostic Index	0.6975	0.9

Abbreviations: A-LMR = autograft lymphocyte to monocyte ratio; DC 1 = dendritic cell type 1; DC 2 = dendritic cell type 2; LDH = lactate dehydrogenase; M 1 = macrophage type 1; and M 2 = macrophage type 2.

Table A5
Median times, 5 years rates and 95% confidence intervals (95%CI) for overall and progression-free survival Kaplan and Meier curves.

Variables	Median time (months)	5-years rates (95% CI)	P-values
Overall Survival			
A-LMR $\geq$ 1.0	Not reached	64% (48%–77%)	< 0.003
A-LMR < 1.0	47.1	39% (29%–51%)	
KIR2DL2 < 0.066×10^9 cells/kilogram	Not reached	88% (77%–94%)	< 0.0001
KIR2DL2 $\geq 0.066 \times 10^9$ cells/kilogram	48.21	44% (31%–58%)	
Myeloid-derived suppressor cells < 0.31×10^9 cells/kilogram	Not reached	88% (78%–94%)	< 0.0001
Myeloid-derived suppressor cells $\geq 0.31 \times 10^9$ cells/kilogram	21.63	33% (22%–50%)	
NKp30 $\geq 0.09 \times 10^9$ cells/kilogram	Not reached	95% (86%–98%)	< 0.0001
NKp30 < 0.09×10^9 cells/kilogram	39.80	34% (20%–47%)	
Progression-free survival			
CD4 + PD-1- $\geq 0.13 \times 10^9$ cells/kilogram	Not reached	67% (54%–78%)	< 0.0001
CD4 + PD-1- < 0.13×10^9 cells/kilogram	23.00	30% (20%–43%)	
DC1 $\geq 0.12 \times 10^9$ cells/kilogram	Not reached	64% (51%–75%)	< 0.0001
DC1 < 0.12×10^9 cells/kilogram	23.90	30% (19%–44%)	
Myeloid-derived suppressor cells < 0.31×10^9 cells/kilogram	Not reached	70% (58%–80%)	< 0.0001
Myeloid-derived suppressor cells $\geq 0.31 \times 10^9$ cells/kilogram	7.1	19% (6%–28%)	
M2 < 0.03×10^9 cells/kilogram	Not reached	69% (55%–80%)	< 0.0002
M2 $\geq 0.03 \times 10^9$ cells/kilogram	33.75	31% (21%–44%)	
NKp30 $\geq 0.09 \times 10^9$ cells/kilogram	Not reached	77 %(65%–86%)	< 0.0001
NKp30 < 0.09×10^9 cells/kilogram	12.67	13% (6%–26%)	

Abbreviations: DC 1 = dendritic cell type 1; and M 2 = macrophage type 2.

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