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Immune profiling in Diffuse Large B-Cell Lymphoma and Mantle Cell Lymphoma Patients treated with Autologous Hematopoietic Cell Transplant

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Abstract

This is the first longitudinal study of immune profiles and autologous hematopoietic cell transplant (AHCT) survival in B-cell non-Hodgkin lymphoma (B-NHL) patients and the effect of plerixafor mobilization on immune reconstitution in this population. A comprehensive immunophenotyping panel was performed in 104 consecutive adult B-NHL patients (58% diffuse large B-cell, 42% mantle cell) who received AHCT (1/2008-11/2014), at a median of 28 days pre-AHCT (N=104) and Day+100 (N=83) post-AHCT. Median follow-up post-AHCT was 61 months (range: 8-120 months). Compared to patients mobilized with filgrastim and plerixafor, patients mobilized with filgrastim alone had a higher proportion of CD4+ naïve (p=0.006) and CD8+ central memory T-cells (p=0.006) pre-AHCT. For patients transplanted in complete remission (CR), a higher proportion of CD8+ effector memory T-cells pre-AHCT was associated with worse progression-free survival (PFS; p<0.01) and overall survival (OS; p<0.01). A higher ratio of CD8:CD4+ central memory T-cells pre-AHCT was associated with worse PFS (p<0.0001) and OS (p=0.0034). This same ratio measured post-AHCT among patients in CR on Day+100 was associated with worse

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and OS (p=0.008) but not PFS (p=not significant). These immune subsets are complementary biomarkers which identify patients transplanted in CR who have poor survival prognoses and may warrant further clinical interventions.

Keywords

Immune reconstitution; non-Hodgkin lymphoma; survival; memory T-cells

Introduction

Autologous hematopoietic cell transplant (AHCT) plays an important role in the treatment of B-cell non-Hodgkin lymphoma (B-NHL), including diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL), both in the upfront and relapsed/refractory settings [1]. In addition to patient and disease-related factors such as age [2], Charlson comorbidity index [3], disease status pre-AHCT [4], international prognostic index (IPI) score at AHCT [5] and high-dose regimen [6], composition of the infused cell product (dendritic cell content [7] and lymphocyte to monocyte ratio [8]) and immune reconstitution [9] after AHCT have recently emerged as independent predictors of AHCT survival in B-NHL.

The majority of these prognostic factors were evaluated before the introduction of plerixafor for hematopoietic cell mobilization and should be re-examined to determine if they retain their relevance in the plerixafor era. Specifically, plerixafor-mobilized grafts have different characteristics compared with those obtained with filgrastim (also known as granulocyte colony-stimulating factor; G-CSF) alone [10, 11]. For example, contrary to a previous study conducted in patients mobilized with filgrastim alone [12], patients mobilized with plerixafor/filgrastim demonstrated an association between CD34+ stem cell dose and better long-term platelet recovery after AHCT, but this did not correlate with time to initial neutrophil or platelet engraftment, long-term neutrophil or hemoglobin recovery, or overall survival (OS) [13].

We report the first study of peripheral blood immune cell subsets in patients before and after AHCT with recovery patterns and AHCT survival in B-NHL patients mobilized with or without plerixafor. We hypothesized that a more detailed analysis of peripheral blood sub-populations beyond absolute lymphocyte count (ALC) would identify phenotypes associated with shorter survival after AHCT. Sub-populations of T-cells, natural killer cells, B-cells, and dendritic cells of B-NHL patients prior to and at Day +100 AHCT were examined.

This study aimed to 1) investigate whether previously described patient and disease related factors retain their association with survival in patients mobilized with plerixafor-containing regimens, 2) compare immunological recovery and survival in patients mobilized with filgrastim alone vs filgrastim and plerixafor, and 3) identify patterns of immune recovery associated with progression-free and overall survival to identify patients with poor prognosis who may benefit from alternative or additional therapies.

Methods

Patient population

This is a retrospective cohort comprised of 104 consecutive adult (≥18 years) B-NHL patients (DLBCL 58%, MCL 42%) who received AHCT at Roswell Park Comprehensive Cancer Center between 1/2008 and 11/2014 (Supplemental Figure 1). Approval for the review of these records was obtained from the Roswell Park Institutional Review Board and research was performed in accordance with the Declaration of Helsinki.

Patients' therapy was clinically determined by age and comorbidities. Specifically, patients <60 years received VCB (n=48; etoposide 2.4 g/m^2 continuous infusion over 30 hours starting on Day −8, cyclophosphamide 1800 mg/m^2 daily for 4 doses starting on Day −7, carmustine 600 mg/m^2 on Day −3) [14]. Patients ≥60 years or those <60 years who had a high HCT-CI score received BuCy (n=56; busulfan 0.8 mg/kg (pharmacokinetically dosed to a target level of 750 ng/ml steady state level) every 6 hours for 16 doses starting on Day −7 and cyclophosphamide 60 mg/kg daily for 2 doses starting on Day −3).

Mobilization and collection of hematopoietic cells

Upfront plerixafor-based mobilization was instituted in all B-NHL patients in April of 2010 in order to improve hematopoietic cell mobilization and collection efficiency. Therefore, mobilization regimens in B-NHL patients included either 1) filgrastim 10 μg/kg/day alone (prior to 4/2010) or 2) filgrastim 10 μg/kg/day for at least 4 days followed by plerixafor 0.24 mg/kg/daily administered on the day prior to start of mobilization (after 4/2010).

Filgrastim was given daily until the white blood cell count reached 8000×10^9/L at which time apheresis commenced. Filgrastim +/− plerixafor were continued during daily apheresis collection per standard protocol until the prescribed dose of CD34+ cells/kg was obtained. All patients underwent a minimum of 2 days of hematopoietic cell collection and received unmanipulated, peripheral blood derived hematopoietic cells for AHCT.

Immune profiles were tested pre- and post-AHCT on Day +100 to assess for consistency, potential effect of AHCT on immune profile and to determine the most relevant time point which is associated with PFS/OS.

Flow cytometric analysis

A comprehensive immunophenotyping panel was performed blinded to treatment and outcome at a median of 28 days pre-AHCT (N=104) and at Day +100 post-AHCT (N=83), via methods previously described [14]. Blood for flow cytometric studies was drawn into heparinized tubes and processed by the laboratory within 24 hours of collection. Briefly, blood was washed 3 times with flow cytometry buffer (0.5% BSA, 0.04 g/L Na_2EDTA and 0.1% sodium azide in PBS pH 7.2) and immunophenotyped using a stain-and-then-lyse technique. Data acquisition was performed on a FACSCanto II flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) controlled daily using cytometer, setup and tracking beads. For the peripheral blood phenotyping, 50 000 events were acquired for each of the determinations. Data were analyzed using WinList version 6 (Verity Software House,

Topsham, ME). The comprehensive flow cytometry strategy to identify the different lymphoid subsets is shown in Table 1.

Statistical analysis

Univariate analyses of patient and clinical characteristics were conducted using Pearson $\chi 2$ test or Fisher's exact text for categorical variables, and Wilcoxon two-sample test or Kruskal-Wallis one-way test for continuous variables, as appropriate. All box-and-whisker plots can be interpreted as the median dividing the box in half with the lower edge of the box as the 25^{th} percentile and the upper edge of the box as the 75^{th} percentile. Any dots outside the minimum and maximum (the "whiskers") are considered outliers. OS was calculated from the date of AHCT (Day 0, infusion of hematopoietic cells) to the date of death due to any cause with patients censored at last follow-up. Progression-free survival (PFS) was calculated from the date of AHCT to the date of first disease progression/relapse or death due to any cause; patients were censored at the last follow-up in the absence of disease progression/relapse. Early ALC recovery was defined as >500 lymphocytes/μL (by peripheral blood differential count) by Day +15 post-AHCT. Cell marker categories were pre-defined as >25%, 25-75%, and <75% of the absolute count for each cell type; these categories were then collapsed based on data frequencies. Analyses on immune cell markers pre-AHCT were conducted among patients who were in CR at the time of transplant (n=76), whereas post-AHCT analyses were conducted among patients who were in CR at Day +100 (n=71). Kaplan-Meier survival curves were constructed and significance was tested using the log-rank statistic with results shown as p-values. Multiple comparisons were considered in the setting of a small sample size, with $p<0.01$ set as the statistical significance threshold. All statistical analyses were 2-sided and performed using SAS v9.4 (SAS Institute, Cary, CA) and analytic figures were created using SPSS v21.0 (SPSS Inc., Chicago, IL) and R v3.3.2 (R Core Team, Vienna, Austria). Sensitivity analyses were conducted to assess if differences existed between DLBCL and MCL pre- and post-AHCT; since no substantial differences existed, all analyses were conducted using the full dataset.

Results

Clinical characteristics and correlation with survival in all B-NHL patients

Baseline characteristics of all 104 patients pre-AHCT and the 76 patients in complete remission (CR) are reported in Table 2. Median follow-up was 61 months (range: 8-120 months) with no patients lost to follow-up. In all 104 patients, 2 patients died of infection (both before 1-year post-AHCT), 4 patients died of second cancers (1 AML, 2 MDS, 1 lung cancer, all diagnosed between 2-3 years post-AHCT) and 30 patients died of disease relapse (71% of all deaths). For patients in CR pre-AHCT, the median CD34+ cell dose infused was 4.79×10^6/kg (range: $1.64\text{-}197.5\times10^6$/kg), and median ALC dose infused was 0.81×10^9/kg (range: $0\text{-}2.48\times10^9$/kg). Patients who received plerixafor were more likely to be in CR pre-AHCT ($p<0.01$; Table 2), which is probably a consequence of the sequential nature of the two cohorts and better patient selection for transplant.

Most MCL patients were transplanted in CR1 (77%). Of the DLBCL patients, 25% were transplanted in CR1 (due to high risk features including double or triple hit, 2 inductions to

achieve CR1, transformed from low grade NHL) and 35% in CR2, with 40% relapsed or refractory at time of AHCT. At Day +100 post-AHCT, 91% of patients had maintained CR while 9% had relapsed disease. Of the 76 CR patients, 4 patients died before 1 year (3 of disease, 1 of infection), and 18 died between 1 and 5 years after AHCT (11 of disease, 4 of a second cancer, 3 of other causes). There were no substantial baseline differences between all patients and those patients in CR; therefore, because of a significant difference in OS by disease status pre-AHCT (p=0.005; Supplemental Figure 2), the subset of 76 patients in CR at time of AHCT are reported for the pre-AHCT for the remainder of the results. Post-AHCT results are reported among those patients who were in CR at Day +100 (n=71).

Effect of plerixafor on immune recovery

Baseline immunophenotyping data was available in all patients, while Day +100 immunophenotyping data was available in 82% (n=62/76) of patients in CR pre-AHCT. Time to platelet and neutrophil recovery was not associated with plerixafor use (data not shown). In comparison to patients mobilized with plerixafor/filgrastim, patients mobilized with filgrastim alone had similar proportions of immune cells (Supplemental Figure 3), and PFS and OS did not significantly differ by plerixafor use (Supplemental Figure 4), NHL histology, age, Karnofsky performance status, body mass index, or high-dose regimen.

Relative numbers of immune cells and correlation with survival

PFS and OS were significantly associated with specific immune cell populations. Prior to AHCT, higher levels of CD8+ EM T-cells (>75%; Figure 1) conveyed worse PFS (p=0.0088) and OS (p=0.023) than those at lower levels (≤75%), although this was not statistically significant for OS. This association did not persist when CD8+ EM T-cells were measured for patients in CR at Day +100 post-AHCT (Figure 2; PFS: p=not significant (NS), OS: p=0.052) nor did it exist if a different threshold of CD8+ EM T-cells was used (≥25% versus <25%; PFS: p=NS; OS: p=0.056; Supplemental Figure 5). There was no significant difference in PFS or OS between patients who had a higher proportion of CD8+CM T-cells or CD4+ EM T-cells pre-AHCT or at Day+100 post-AHCT in comparison to those that had a lower proportion (≤75%) (Supplemental Figure 6 and 7, respectively).

In these B-NHL patients who had received prior B-cell depleting therapy (primarily rituximab), total CD19+ and CD20+ B-cells were near absent when measured at both the pre-AHCT and Day+100 AHCT timepoints. Therefore B-cell subsets could not be analyzed in relation to any outcomes. NK cells and dendritic cells (defined in Table 1) were not associated with any outcomes when measured at either pre-AHCT or Day+100 post-AHCT (data not shown). No difference in survival existed by chemotherapy type (DLBCL: RDHAC/P vs R/OICE vs other; MCL: RCHOP vs RHyperCVAD vs other) nor was there a difference in reported immune cell phenotypes by last chemotherapy regimen pre-AHCT (data not shown). Additionally, there was no difference in survival by the number of lines of therapy (1-2 vs ≥3 lines) among DLBCL patients, and MCL patients had no variability in the number of lines of therapy (91% received 1 line of therapy).

Relative Ratios of Immune cells and correlation with survival

Relative proportions of immune cell subsets measured pre-AHCT were also prognostic of PFS and OS (Figure 3). A higher ratio of CD8:CD4+ CM T-cells was significantly associated with worse PFS ($p<0.0001$) and OS ($p=0.0034$), with similar results for a higher ratio of CD8:CD4+ EM T-cells (PFS: $p=0.028$, OS: $p=0.014$). No difference was seen for the ratio of CD8+ EM:CD4+Tregs (PFS: p=NS, OS: p=NS).

Similar results were found for the relative ratios of immune cell subsets measured for patients in CR at Day +100 post-AHCT (Figure 4). Among patients who were in CR at Day +100, a lower ratio of CD8:CD4+ CM T-cells did not have a statistically significantly different PFS (p=NS) but had a significantly better OS ($p=0.0080$). Despite a similar rate of progression, these patients were less likely to die from their disease than those with a higher CD8:CD4 CM T-cell ratio (Figure 4a).The relative proportions of CD8:CD4+ EM T-cells (Figure 4B) and CD8+ EM:CD4+Tregs (Figure 4C) measured at Day +100 post-AHCT were not associated with PFS or OS.

Discussion

This is the first study to investigate patterns of immune cell subsets before and after AHCT which may be associated with survival in patients with DLBCL and MCL. We report that previously published patient and disease related prognostic factors are not validated in this cohort of patients mobilized with plerixafor-containing regimens, with the sole exception of disease status (in CR) at time of AHCT. Hence, our immunophenotype analyses focused on patients in CR at time of AHCT and found several new prognostic biomarkers of long term PFS and OS (Supplemental Figure 8).

While we confirm that patients transplanted in CR have longer PFS and OS [4], we did not find confirm prior reports of significant associations with OS or PFS for age [2], IPI [5], or high dose regimen [6]. It is unknown if this is due to better patient selection, use of plerixafor for mobilization or lack of statistical power. It is worth noting that although patients in the plerixafor group were older, there were no differences in CR, PFS, or OS between the two mobilization groups. Additionally, there was no difference in immune cell subsets pre-AHCT by mobilization agent. These immune cell subsets were associated with survival independent of the mobilization agents assessed.

The immune system has been an area of interest for understanding control of B-NHL and other malignancies. In the setting of B-NHL patients uniformly treated with B-cell depleting therapy (confirmed by near absent numbers of B cells in the samples tested, data not shown), the focus of this study was on the impact of various T-cell, Natural Killer cell and dendritic cell subsets on anti-tumor responses. While we did not find any significant impact of NK or dendritic cells on PFS/OS, we do report novel associations of T cell subsets and ratios which convey poor prognosis in patients in CR at time of AHCT. Our results demonstrating higher CD8+ CM T-cells with worse survival after AHCT contrasts with a prior report in allogeneic HCT patients indicating specificity of T-cell subsets by treatment [15, 16]. Interestingly, the similar results between pre- and post-AHCT suggest either time point can be measured as a biomarker for long-term disease control and survival. The primary effect could be 1) patient

biology or 2) patient response to treatment, although we did not find a difference by therapy, which may be explained by the temporary effects of cyclophosphamide on T-cells in comparison to the more prolonged effects of rituximab on B-cells. Additionally, our previous study using the same immunophenotype panel after AHCT for multiple myeloma found higher γδ T-cells were associated with improved PFS and OS with no significant findings for the T-cell subsets reported in our study of B-NHL [15]. Taken together, this growing literature supports the specificity of T-cell subsets by underlying malignancy and treatment which advocates for study designs focused on single disease/treatment combinations. Alternatively, the specific impact of CD8+ CM T-cells on survival may be related to differential antigen thresholds for recall proliferation between naïve and memory CD8+ T-cells which varies by disease [17].

Of the patients who achieved a CR at Day+100, those with a lower ratio of CD8:CD4+ CM T-cells did not have at significantly different rate of relapse from those who had a higher ratio, but they were able to be salvaged and had a better overall survival. A higher ratio of CD8:4+ CM T-cells will have a poorer prognosis and possibly can be targeted for adjuvant strategies for relapse (consolidation/maintenance). Conversely, patients with a lower ratio of CD8:4+ CM T-cells do well suggesting this is a useful biomarker after outcome. Our finding that a higher ratio of CD8:CD4+ CM T-cells was associated with worse survival is in contrast to prior reports in solid tumor patients where a higher ratio was associated with a favorable prognosis [18, 19]. This also seems counter-intuitive to what is known about antitumor immunity. However, T-cell exhaustion, which was not measured by our immunophenotyping panel, is a likely explanation [20]. In the setting of chronic viral infection, higher numbers of CD8+ T-cells that have lost their effector functionality is associated with CD4+ T-cell deficiency [21]. Similarly, studies of adoptive T-cell transfer to generate anti-tumor responses have discovered a role for T-cell exhaustion leading to disease relapses after an initial favorable response [26, 27].

This is the first longitudinal study of the impact of immune profiles on AHCT survival in B-NHL patients in the plerixafor era. In otherwise favorable risk patients transplanted in CR, we were able to distinguish novel immune cell biomarkers to define patients with poor prognosis. Additional studies are needed to further classify these immune profiles into clinically actionable subgroups in order to improve the success of AHCT for B-NHL and to investigate the relevance of these findings in the setting of chimeric antigen receptor T-cell therapy for B-NHL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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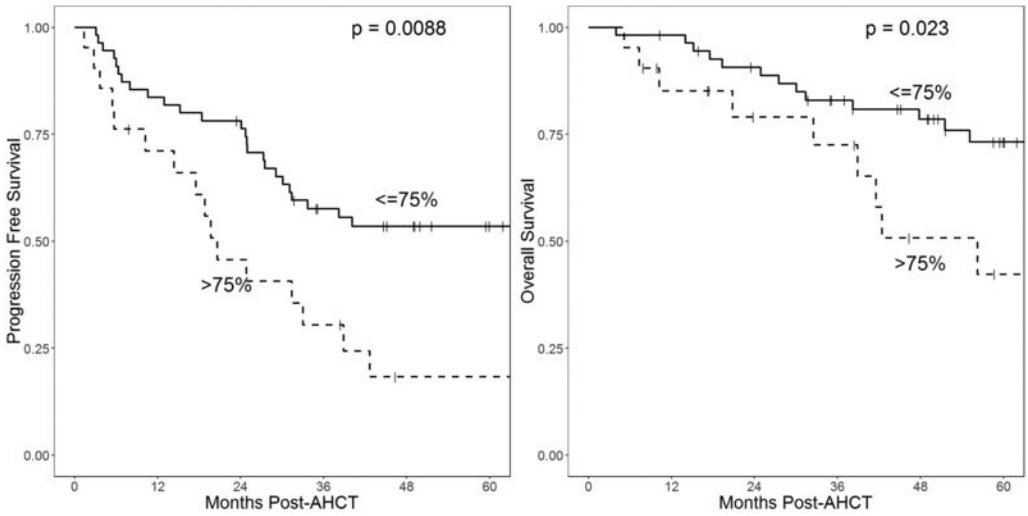

Figure 1.

Proportion of CD8+ EM T-cells measured **before** AHCT in B-NHL patients in CR at time of AHCT (n=76) and the effect on A) PFS and B) OS. Higher proportions of CD8+ EM T-cells pre-AHCT were associated with worse PFS and OS, although this was not statistically significant for OS.

Abbreviations: AHCT: autologous hematopoietic cell transplant, B-NHL: B-cell non-Hodgkin lymphoma, CR: complete remission, EM: effector memory, PFS: progression-free survival, OS: overall survival.

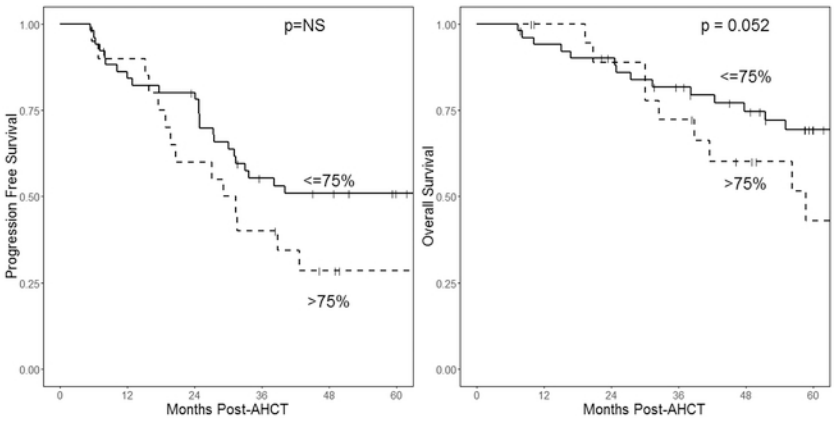

Figure 2.

Proportion of CD8+ EM T-cells measured at **Day +100 after** AHCT in B-NHL patients in CR at Day +100 (n=76) and the effect on A) PFS and B) OS. Higher proportions of CD8+ EM T-cells at Day +100 post-AHCT were not associated with worse PFS or OS.

Abbreviations: AHCT: autologous hematopoietic cell transplant, B-NHL: B-cell non-Hodgkin lymphoma, CR: complete remission, EM: effector memory, PFS: progression-free survival, OS: overall survival.

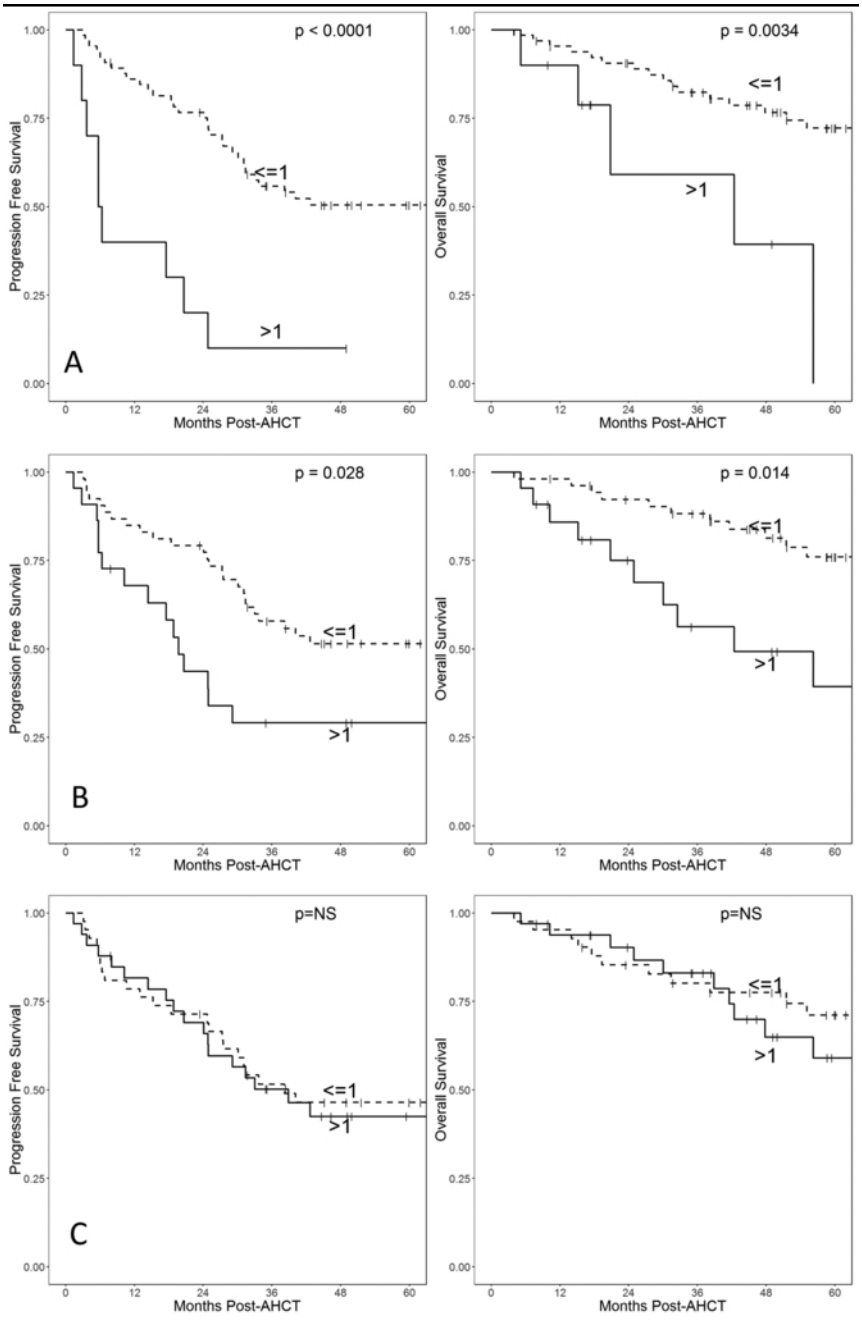

Figure 3.

Progression-free and overall survival of B-NHL patients in CR at time of AHCT (n= 76) by the following relative ratios of immune cells measured **before** AHCT A) CD8:CD4+ CM cells, B) CD8:CD4+ EM cells, C) CD8+ EM:CD4+T-regs. A higher ratio of CD8:CD4+ CM cells was associated with worse PFS and OS. Additionally, a higher ratio of CD8:CD4+ EM cells was associated with worse OS. No difference in PFS or OS was seen for CD8+ EM:CD4+ T-regs.

Abbreviations: AHCT: autologous hematopoietic cell transplant, B-NHL: B-cell non-Hodgkin lymphoma, CM: central memory, CR: complete remission, EM: effector memory, T-regs: T regulatory cells, PFS: progression-free survival, OS: overall survival.

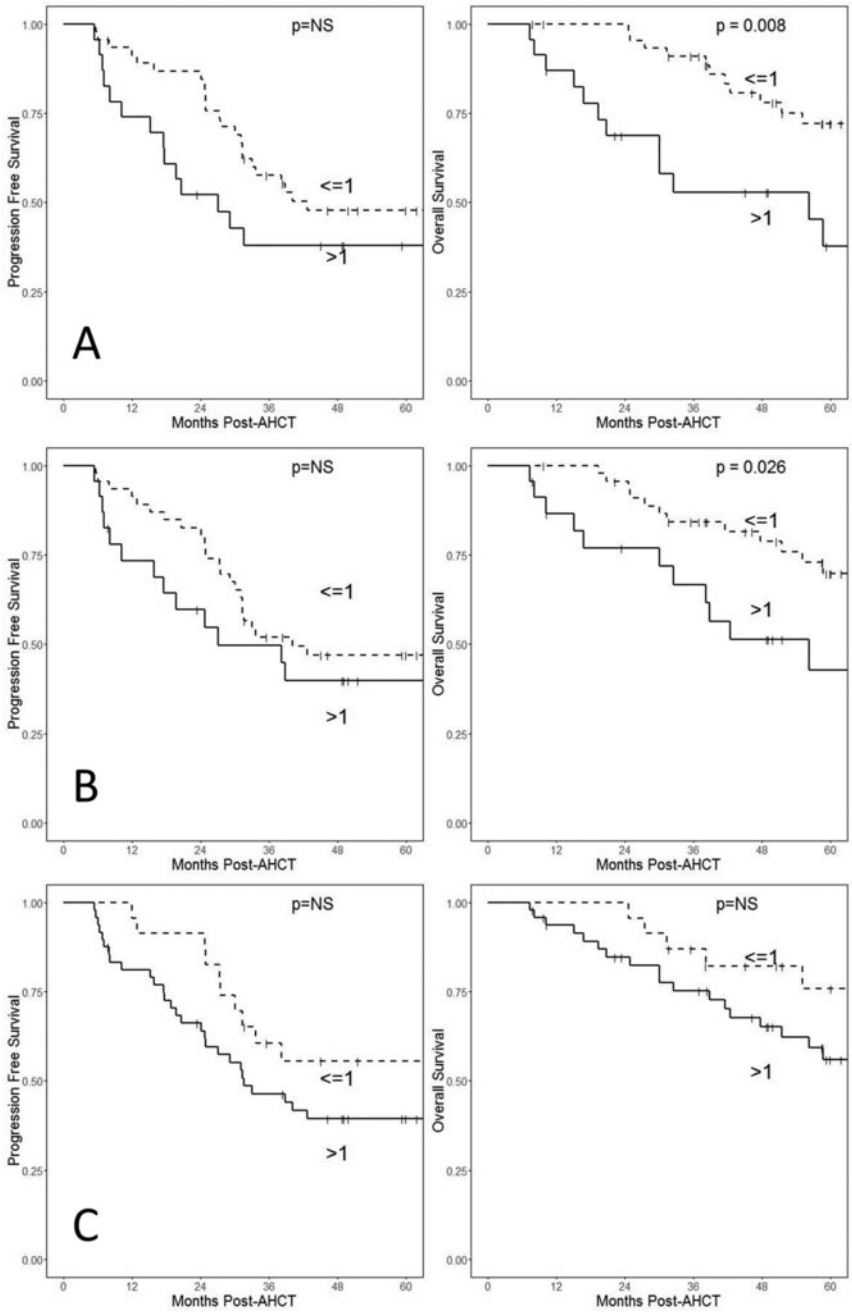

Figure 4.

Progression-free and overall survival of B-NHL patients in CR at Day +100 (n= 71) by the following relative ratios of immune cells measured at **Day +100 after** AHCT A) CD8:CD4+ CM cells, B) CD8:CD4+ EM cells, C) CD8+ EM:CD4+ T-regs. A higher ratio of CD8:CD4+ CM cells was associated with worse PFS and OS. No difference in PFS or OS was seen for CD8:CD4+EM cells or CD8+ EM:CD4+ T-regs.

Abbreviations: AHCT: autologous hematopoietic cell transplant, B-NHL: B-cell non-Hodgkin lymphoma, CM: central memory, CR: complete remission, EM: effector memory, T-regs: T regulatory cells, PFS: progression-free survival, OS: overall survival.

Table 1.

Immune cells measured in the Immunophenotyping Panel.

Cell type		
T-cell markers		
	CD3+ Total	CD3+
	CD4+ Total	CD4+
	CD8 +Total	CD8+
T-cell subsets		
	CD3+CD4+	CD3+CD4+
	CD4+ Naïve	CD3+ CD4+ CD45RA+ CD45RO− CD27+
	CD4+ Central Memory	CD3+ CD4+ CD45RA− CD45RO+ CD27+
	CD4+ Effector Memory	CD3+ CD4+ CD45RA− CD45RO+ CD27−
	CD4+ Recent Thymic Emigrants	CD3+ CD4+ CD45RA+ CD31+ CD45RO−
	Tregs bright (CD4+)	CD3+ CD4+ CD25 (br)
	Tregs dim (CD4+)	CD3+CD4+CD25+CD127 (d)
	Tregs DR+ (CD4+)	CD3+CD4+CD25+CD127(d)HLADr+
	CD3+CD8+	CD3+CD8+
	CD8+ Naïve	CD3+ CD8+ CD45RA+ CD45RO− CD27+
	CD8+ Primed (Effector)	CD3+CD8+ CD45RA+ CD45RO− CD27−
	CD8+ Central Memory	CD3+ CD8+ CD45RA− CD45RO+ CD27+
	CD8+ Effector Memory	CD3+ CD8+ CD45RA− CD45RO+ CD27−
	T-gamma/delta cells	CD3+ γδ+
B-cell markers		
	CD19+ Total	CD19+
	CD20+ Total	CD20+
	CD19+ Naïve	CD19+ CD27−
	Naïve (Bm 1)	CD19+ CD38− IgD+ CD27− CD20+/−
	Naïve (Bm 2)	CD19+ CD38+ IgD+ CD27− CD20+/−
	CD 19+ Memory	CD19+ CD27+
	Memory Pre Switch	CD19+ CD38+/− IgD+ CD27+ CD20+/−
	Memory Post Switch	CD19+ CD38+/− IgD− CD27+ CD20+/−
NK cells		
	Total NK Cells	CD56+ CD16+
Dendritic cells		
	Myeloid CD11c+	HLADr+ CD123+/− Dump− CD11c+
	Plasmacytoid CD123+	HLADr+ CD123+ Dump− CD11c−

Abbreviations: CD: cluster of differentiation, NK: natural killer.

Table 2.

Patient demographics.

Patient characteristics	All Patients (n=104)	Patients in CR pre-AHCT (n=76)
Age at AHCT (years)		
Median (range)	59 (24-77)	59 (35-77)
<60	52%	53%
≥60	48%	47%
Male	71%	71%
KPS (≥ 80)	67%	66%
NHL histology		
DLBCL	58%	53%
MCL	42%	47%
MIPI risk group		
Low	50%	50%
Intermediate	36%	39%
High	14%	11%
IPI risk group *		
Low/ low-intermediate	60%	58%
High-intermediate/ high	40%	42%
Median number of prior regimens	2 (1-6)	2 (1-5)
Mobilization regimen		
Plerixafor+ filgrastim	69%	79%
Filgrastim alone	31%	21%
High-dose regimen		
BuCy	54%	54%
CBV	46%	46%
Median number of days of collection	2 (2-15)	2 (2-15)
Disease status pre-AHCT/post-AHCT		
CR/CR	66%	
CR/not CR	8%	
Not CR/CR	16%	
Not CR/not CR	11%	

Abbreviations: AHCT: autologous hematopoietic cell transplant, CR: complete remission, filgrastim: granulocyte-colony stimulating factor, CBV: cyclophosphamide+ BCNU+ etoposide, BuCy: busulfan+ cyclophosphamide, DLBCL: diffuse large B-cell lymphoma, MCL: mantle cell lymphoma, IPI: international prognostic index, MIPI: mantle cell lymphoma IPI.

* 2 patients did not have risk group status.