Research Article

Interleukin-15 Affects Patient Survival through Natural Killer Cell Recovery after Autologous Hematopoietic Stem Cell Transplantation for Non-Hodgkin Lymphomas

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Received 3 June 2009; Revised 18 January 2010; Accepted 11 February 2010

1. Introduction

Day 15 absolute lymphocyte count (ALC-15) after autologous peripheral blood hematopoietic stem cell transplantation (APH SCT) has been shown to be a significant predictor for survival in multiple hematologic malignancies [1–9] as well as solid tumors [10–12]. Natural killer cells at day 15 (NK-15) have been identified as the key lymphocyte subset in the ALC-15 that has a direct impact on survival post-APH SCT [13]. In addition, the lymphocytes collected at the same time with stem cells and infused to the patients have a direct impact on ALC-15 and NK-15 post-APH SCT, suggesting that the autograft can be viewed not only as the means to achieve hematologic engraftment by the infusion of stem cells but also as an adoptive immunotherapeutic maneuver by infusion of an autograft absolute lymphocyte count (A-ALC) affecting immune recovery and survival post-APH SCT [14–16]. Adoptive cellular therapy depends on the ability to optimally select the desired antigenic specificity immune effector cells and then induce cellular proliferation while preserving the effector function, engraftment, and homing abilities of the lymphocytes [17]. The infusion of A-ALC will provide the desired antigenic immune effector cells, but what factors to induce cellular proliferation while preserving the effector function, engraftment and homing abilities of the lymphocytes to provide an effective adoptive cellular therapy in APH SCT are currently not well defined. Therefore, we set out to investigate the cytokine milieu at day 15 post-APH SCT to assess which cytokines affect NK-15 and the superior survival mediated by a faster NK-15 recovery post-APH SCT. To achieve this goal the following endpoints were evaluated in the study: (1) to assess a correlation between cytokines and NK-15 post-APH SCT, (2) to assess if interleukin-15 (IL-15) affects survival post-APH SCT and through which immune effector cell, and (3) to identify a source of IL-15 production at day 15 post-APH SCT.
2. Material and Methods

2.1. Patient Population. Patients in the study were the same patient group enrolled in prospective study from February 2002 to February 2007 to assess the role of ALC-15 and NK-15 on survival post-APHSCT [13]. For this study 27 normal controls donated blood samples to assess cytokines levels. All patients signed written, informed consent to participate in the study. Approval of the study was obtained from the Mayo Clinic institutional review board and was in accordance with U.S. federal regulations and the Declaration of Helsinki.

2.2. A-ALC, ALC-15, Monocytes, and NK-15. Autograft absolute lymphocyte count (A-ALC) was determined as previously reported: % collection lymphocytes × absolute autograft white blood cell count/kilogram (kg) [18]. Autograft monocyte count (A-mono) was determined as follows: % collection monocytes × absolute autograft white blood cell count/kg. ALC-15 and monocytes at day 15 (mono-15) were determined from the differential white blood cell count obtained at day 15 post-APHSCT. NK-15, defined as CD16+/CD56+/CD3−, was according to manufacture’s recommendations. Stained cells were then analyzed using flow cytometry (FACS callibur, Becton-Dickson, CA). Cells were analyzed for the percentage of cells expressing said antigens as well as average quantity of antigen expression [13].

2.3. Cytokine Assay. Cytokines were measured using the Bio-rad (Hercules, CA) human 27-plex system. Briefly, diluted human plasma was incubated for 30′ at room temperature with washed beads. The beads are coated with antibodies to the cytokines of interest. After incubation with patient plasma the beads were washed and incubated for 30′ with biotinylated 20 antibodies, followed by incubation with PE-conjugated streptavidin. Quantization of cytokines was performed on the Luminex 200 system (Austin, TX). The cytokines tested included Interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-G), interferon-gamma-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins 1a and 1b (MIP-1a and MIP-1b), platelet-derived growth factor (PDGF), RANTES, tumor-necrosis-factor-gamma (TNF-G), and vascular endothelial growth factor (VEGF).

2.4. Prognostic Factors. Prognostic factors for post-APHSCT OS and PFS evaluated in this study included cytokines, ALC-15, NK-15, mono-15, international prognostic index (IPI) factors, infused CD34+, and disease status at transplant.

2.5. Peripheral Blood Stem Cell Collection. All patients received G-CSF (10 μg/kg) daily for 5 to 7 consecutive days by subcutaneous injection. Once their peripheral blood CD34+ cell count was ≥10 cells/μL, patients began daily apheresis until they achieved a target of 5 × 10⁶ CD34+ cells/kg. A minimum target of 2 × 10⁶ CD34+ cells/kg was required for the patient to be considered for transplantation. Patients were assigned to the Baxter Amicus (Baxter, healthcare Corp., Deerfield, IL), Fenwal CS 3000 Plus (Baxter) or COBE Spectra (Gambro BCT, Lakewood, CO) based on instrument availability on the day of collection. Instrument setting used for the collection procedures has previously been described (ref). The number of apheresis collections was dependent upon collection of an adequate number of CD34+ cells to achieve hematologic engraftment post-APHSCT.

2.6. Conditioning Regimen. All patients received Carmustine (BCNU) 300 mg/m² on day-6; etoposide 100 mg·m⁻² twice a day on days-5, -4, -3, and -2; and melphalan 140 mg/m² on day-1 (BEAM). All patients received growth factor support starting on day +6 with sargramostim 500 μg daily until evidence of neutrophilic engraftment defined as an absolute neutrophil count (ANC) ≥ 500 cells/μg on 3 consecutive days.

2.7. Response and Survival. Response criteria were based on the guidelines from the non-Hodgkin lymphoma (NHL) international Workshop [19]. OS was measured from the date of transplant to the date of death, or last follow-up. PFS was defined as the time from transplant to the time of progression, relapse, death, or last follow-up.

2.8. Statistical Analysis. OS and PFS were analyzed using the approach of Kaplan and Meier [20]. Differences between survival curves were tested for statistical significance using the 2-tailed log-rank test. The Cox proportional hazard model [21] was used for the univariate and multivariate analysis to evaluate cytokines, NK-15, ALC-15, and mono-15 as a prognostic factor for post-APHSCT OS and PFS times.

In addition to the evaluation of cytokines (specifically IL-15) and its prognostic significance for OS and PFS, its utility as a marker of NK-15 was also assessed. The choice of optimal cutoff of IL-15 was based on its utility as a marker of NK-15 using Receiver operating characteristics (ROCs) curves and area under the curve (AUC) as well as its prognostic value for post-APHSCT OS and PFS. Prediction of NK-15 was explored further in logistic regression models, univariately assessing continuous and dichotomized values of A-ALC, cytokines, and mono-15. χ²-tests were used to determine relationships between categorical variables. The Wilcoxon-rank test was used to determine associations between continuous variable and categories, and Spearman correlation coefficients were used to evaluate associations for continuous variables. Owing to multiple factors tested in the univariate analysis, multiple comparison corrections using Bonferroni correction procedure were applied declaring statistical significance at P < .002 (α = 0.05/n, n = 27 cytokines).

The cut-off of ALC-15 ≥ 500 cells/μL, A-ALC ≥ 0.5 × 10⁹ lymphocytes/kg, and NK-15 ≥ 80 cells/μL was based on data from our previous studies [13–15].
| Characteristics                          | n (%)   | Median | Range       |
|-----------------------------------------|---------|--------|-------------|
| Age at transplant, years                | 50 (100%) | 57.5   | (23–73)     |
| Sex                                     |         |        |             |
| Female                                  | 17 (34%) |        |             |
| Male                                    | 33 (66%) |        |             |
| Lymphoma type                           |         |        |             |
| DLBCL                                   | 34 (68%) |        |             |
| Mantle cell                             | 10 (20%) |        |             |
| T-cell                                  | 5 (10%)  |        |             |
| Follicular                               | 1 (2%)   |        |             |

**Prognostic factors at diagnosis**

| Extra nodal sites                      |         |        |             |
|----------------------------------------|---------|--------|-------------|
| 0                                      | 33 (66%) |        |             |
| 1                                      | 15 (30%) |        |             |
| 2                                      | 2 (4%)   |        |             |
| LDH (U/L)                              | 50 (100%) | 209    | (150–1515)  |

| Performance status                     |         |        |             |
|----------------------------------------|---------|--------|-------------|
| 0                                      | 31 (62%) |        |             |
| 1                                      | 17 (34%) |        |             |
| 2                                      | 2 (4%)   |        |             |

| Stage                                  |         |        |             |
|----------------------------------------|---------|--------|-------------|
| I                                      | 6 (12%)  |        |             |
| II                                     | 5 (10%)  |        |             |
| III                                    | 8 (16%)  |        |             |
| IV                                     | 31 (62%) |        |             |

**IPI score**

| Age at transplant, years               |         |        |             |
|----------------------------------------|---------|--------|-------------|
| ≥60                                     | 23 (46%) |        |             |
| <60                                     | 27 (56%) |        |             |
| LDH                                    |         |        |             |
| Normal                                 | 21 (42%) |        |             |
| Abnormal                               | 29 (58%) |        |             |
| Extra nodal sites                      |         |        |             |
| ≥2                                     | 2 (4%)   |        |             |
| <2                                     | 48 (96%) |        |             |
| Performance status                     |         |        |             |
| ≥2                                     | 2 (4%)   |        |             |
| <2                                     | 48 (96%) |        |             |
| Stage                                  |         |        |             |
| I/II                                   | 11 (22%) |        |             |
| III/IV                                 | 39 (78%) |        |             |
| Infused CD34 cells × 10⁶/kg            | 50 (100%) | 4.68   | (2.03–8.56) |
| Infused A-ALC × 10⁹ lymphocytes/kg     | 50 (100%) | 0.55   | (0.08–1.5)  |
| ALC-15 cells/µL                        | 50 (100%) | 670    | (2.0–4,500) |
| NK-15 cells/µL                         | 50 (100%) | 103    | (10–999)    |
| Mono-15 cells/µL                       | 50 (100%) | 860    | (2.0–4,500) |
| Infused A-mono × 10⁹ monocytes/kg      | 50 (100%) | 0.58   | (0.08–1.5)  |

A-ALC: autograft, absolute lymphocyte count; ALC-15: absolute lymphocyte count at day 15 post transplant; A-mono: autograft absolute monocyte count; DLBCL: diffuse large B-cell lymphoma; IPI: International prognostic index; LDH: lactate dehydrogenase; Mono-15: absolute monocyte count at day 15 post transplant; NK-15: absolute natural killer cell count at day 15 post transplant.
| Cytokines   | Normal                | Pretransplant            | Day 15 post transplant | P-value Normal versus Pretransplant | P-value Normal versus Day 15 post transplant | P-value Pre versus Day 15 post transplant |
|-------------|-----------------------|--------------------------|------------------------|-------------------------------------|-----------------------------------------------|------------------------------------------|
| IL-1b       | 3.59 (2.32–5.55)      | 4.72 (3.48–7.79)         | 2.99 (1.3–8.11)        | .5                                  | .8                                            | .1                                       |
| IL-1ra      | 120 (90.59–287.77)    | 235.56 (228.95–662.87)   | 283.33 (142.52–434.73) | <.0001                             | <.0001                                        | .01                                      |
| IL-2        | 0 (0–56.12)           | 4.24 (0–23.87)           | 0 (0–7.89)             | .01                                 | .7                                            | <.0004                                   |
| IL-4        | 3.09 (2.09–3.99)      | 3.95 (3.33–5.43)         | 2.12 (1.25–3.63)       | .6                                  | .7                                            | .02                                      |
| IL-5        | 0.92 (0.69–1.67)      | 2.82 (1.79–3.96)         | 4.3 (2.93–13.75)       | <.0001                             | <.0001                                        | .01                                      |
| IL-6        | 0 (0–2.14)            | 11.78 (5.6–21.05)        | 24.48                  | <.0001                             | <.0001                                        | .01                                      |
| IL-7        | 24.64 (13.59–39.08)   | 21.99 (16.85–39.95)      | 15.66                  | .04                                 | .007                                          | .02                                      |
| IL-8        | 2.57 (0.35–7.71)      | 30.89 (19.91–97.72)      | 36.67 (27.02–71.78)    | <.0001                             | <.0001                                        | .2                                       |
| IL-9        | 4.09 (0–68.28)        | 40.18 (4.93–91.22)       | 27.58 (8.29–64)        | .04                                 | .04                                           | .003                                     |
| IL-10       | 1.97 (1.24–3.84)      | 4.91 (3.91–8.16)         | 8.94 (5.91–13.61)      | .01                                 | <.0001                                        | <.0002                                   |
| IL-12p70    | 6.7 (2.52–16.35)      | 15.27 (7.29–29.68)       | 7.61 (4.45–12.93)      | <.0006                             | .2                                            | <.0004                                   |
| IL-13       | 2.93 (1.33–4.17)      | 2.49 (1.71–5.26)         | 1.46 (0.56–2.31)       | .02                                 | .5                                            | <.0002                                   |
| IL-15       | 0 (0–0)               | 8.75 (0.07–110.5)        | 76.5 (5.4–219.22)      | <.0001                             | <.0001                                        | <.0001                                   |
| IL-17       | 10.62 (0–30.66)       | 18.43 (4.33–49.92)       | 7.08 (0–17.14)         | .01                                 | .2                                            | <.0001                                   |
| Eotaxin     | 49.39 (41.43–76.41)   | 68.18 (42.26–104.04)     | 73.76 (44.96–108.87)   | .02                                 | <.0001                                        | .6                                       |
| FGF         | 0 (0–0)               | 0 (0–84.64)              | 0 (0–13.07)            | .7                                  | .8                                            | .7                                       |
| G-CSF       | 989 (385–1243)        | 83.08 (60.01–124.8)      | 67.48 (45.74–93.55)    | <.0001                             | <.0001                                        | <.0001                                   |
| GM-CSF      | 161.74 (32.6–1142.8)  | 80.99 (41.13–173.59)     | 63.15 (25.4–164.6)     | .1                                  | <.0001                                        | <.0002                                   |
| IFN-G       | 142.49 (87.71–264.05) | 125.06 (78.04–193.52)    | 65.65 (28.81–86.42)    | .03                                 | <.0009                                        | <.0001                                   |
| IP-10       | 245.22 (150.9–326.4)  | 915.6 (585.98–1783.83)   | 1440.33 (102.2–3067.40)| <.0001                             | <.0001                                        | <.0001                                   |
| MCP-1       | 41.3 (0–90.25)        | 86.34 (33.83–146.48)     | 120.43 (54.46–209.55)  | .02                                 | <.0001                                        | .08                                      |
| MIP-1a      | 7.4 (4.58–10.52)      | 12.9 (8.62–31.7)         | 8.56 (5.35–1363)       | <.001                               | .7                                            | <.0001                                   |
| MIP-1b      | 51.23 (21.15–106.98)  | 97.36 (57.01–256.38)     | 104.34 (63.59–179.76)  | <.002                               | <.0001                                        | .6                                       |
| PDGF        | 1260.24 (805.22–2289.82)| 2071.16 (1060.34–3485.72)| 423.71 (112.94–697.84)| <.0001                             | <.0001                                        | <.0001                                   |
| RANTES      | 2650.98 (1584.55–4500)| 2600 (2600–3000)         | 2302.37 (297.92–1072.83)| .7                                  | .1                                            | <.001                                    |
| TNF-G       | 0.74 (0–188.99)       | 70.48 (52.82–132.48)     | 32.06 (23.37–82.85)    | .02                                 | .07                                           | <.0001                                   |
| VEGF        | 23.13 (12.55–54.42)   | 77.38 (28.42–104.34)     | 36.52 (10.28–91.85)    | <.0007                              | <.0002                                        | <.0001                                   |

Interleukin (IL)-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-G), interferon-gamma-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory proteins 1a and 1b (MIP-1a and MIP-1b), platelet-derived growth factor (PDGF), RANTES, tumor-necrosis-factor-gamma (TNF-G), and vascular endothelial growth factor (VEGF).
3. Results

3.1. Patient Characteristics. The median age at the time of transplant for this cohort of 50 NHL patients was 57.7 years (range: 23–73). Baseline characteristics for the patients are described on Table 1. No patients were lost to follow-up. The updated median follow-up for the cohort was 25.2 months (range: 6–80.8 months). By the time of this analysis 25 patients (50%) had evidence of relapse and 18 patients (36%) had died. The transplant-related mortality for the cohort was only 2% (1 of 50). One patient died of an intracranial bleed, the rest because of progressive lymphoma. No patient received fludarabine-based regimens prior to APHSCT.

Table 2 shows the cytokine levels comparison between normal volunteers and the cohort of patients before APHSCT and at day 15 post-APHSCT. The following cytokines showed increased levels at day 15 post-APHSCT compared with normal volunteers and before APHSCT: IL-5, IL-6, IL-8, IL-10, IL-15, IP-10, MCP-1, and MIP-1b. No sargramostim was administered on day 15 post-APHSCT during research blood collection for the study as all patients engrafted their neutrophils prior to day 15 post-APHSCT.

3.2. IL-15 and NK-15. We previously reported that NK-15 is the key lymphocyte subset at day 15 affecting survival post-APHSCT. Thus we set out to investigate which cytokine affects NK-15 recovery post-APHSCT. Table 3 shows the cytokine univariate analysis for correlation with NK-15. IL-15 and fibroblast growth factor (FGF) were strongly correlated with NK-15. We also published that A-ALC affected NK-15 recovery. In logistic regression analysis, both A-ALC ($P < .001$) and IL-15 ($P < .002$) retained their ability to affect NK-15 recovery post-APHSCT. IL-15 was found to be both a strong predictor for area under the curve (AUC = 0.87, $P < .002$) and strongly correlated with ($r_s = 0.7$, $P < .0001$) NK-15. Figure 1 shows the scatter plot for IL-15 and NK-15. Only 11 patients had IL-15 level higher than 500 pg/mL. To rule out the possibility that these 11 patients made skewed the data resulting in the strong correlation between IL-15 and NK-15, we truncated and analyzed the data for the 39 patients that had IL-15 levels between 0 and 500 pg/mL. A strong positive correlation was still observed between IL-15 and NK-15 (Spearman correlation rho factor, $r_s = 0.6, P < .0002$).
between IL-15 and NK-15, we truncated and analyzed the data for the 39 patients that had IL-15 levels between 0 and 500 pg/mL. We still observed a strong correlation between IL-15 and NK-15 ($r_s = 0.6, P < .0002$). If we dichotomized the NK-15 patients with an NK-15 ≥ 80 cells/μL had higher levels of IL-15 compared with those who did not (median IL-15 of 155 pg/mL versus 8.89 pg/mL, $P < .002$) (Figure 2).

3.3. Cytokines and Survival Post-APHSCT. Table 4 shows the cytokines univariate analysis for survival post-APHSCT. The cytokines were evaluated as continuous variables. IL-15 at day 15 post-APHSCT was the only cytokine that was statistically significant for OS. IL-15 and PDGF were statistically significant for PFS. In the multivariate analysis for PFS, IL-15 was the only cytokine that remained statistically significant (IL-15: HR = 0.950; 95% CI = 0.824–0.978, $P < .002$; PDGF: HR = 1.020; 95% CI = 1.010–1.058, $P = .02$).

A cut-off of 76.5 pg/mL for IL-15 was supported by being the median value of the data as well as it yielded the greatest differential in survival based on $\chi^2$ values analyzed at different cutoff points (between the 25th and 75th quartiles) from the log-rank tests. Patients with an IL-15 ≥ 76.5 pg/mL ($n = 25$) experienced superior OS (Figure 3(a)) and PFS (Figure 3(b)) compared with those who did not ($n = 25$); median OS; not reached versus 19.2 months, 3 years OS rates of 79% versus 47%, $P < .002$; and median PFS; not reached versus 6.8 months, 3 years PFS rates of 64% versus 31%, $P < .002$, respectively.

In order to minimize uncontrolled factors that could have affected IL-15 levels post-APHSCT, we analyzed patients characteristics based on IL-15 ≥ 76.5 pg/mL versus an IL-15 < 76.5 pg/mL (Table 5). We also analyzed if the pretransplant IL-15 levels were associated with day 15 post-APHSCT IL-15 levels. We found a positive correlation between pre- and post-APHSCT IL-15 levels ($r = 0.6, P < .0001$).

To assess the role on survival of NK-15 and IL-15, we categorized the patients into 4 groups: group I was patients with an NK-15 ≥ 80 cells/μL and IL-15 ≥ 80 cells/μL; group II was NK-15 ≥ 80 cells/μL and IL-15 < 76.5 pg/mL; group III was NK-15 < 80 cells/μL and IL-15 ≥ 76.5 pg/mL; group IV was NK-15 < 80 cells/μL and IL-15 < 76.5 pg/mL. We observed worsening PFS in patients with progressing low NK-15 and IL-15 levels compared with patients with high NK-15 and IL-15 levels (3 years PFS rates for group I was 68%; group II was 49%; group III was 33%; group IV was 0%) (Figure 4). In the multivariate analysis comparing NK-15 and IL-15 for OS and PFS, NK-15 truncated IL-15 (OS; HR = 0.32, $P = .02$; and PFS: HR = 0.33, $P = .01$).

### Table 3: Univariate analysis of the correlation between cytokines and NK-15.

| Cytokines   | Spearman (rho) correlation | P-value |
|-------------|----------------------------|---------|
| IL-1b       | 0.17                       | .2      |
| IL-1ra      | 0.09                       | .5      |
| IL-2        | 0.06                       | .7      |
| IL-4        | 0.2                        | .1      |
| IL-5        | 0.2                        | .2      |
| IL-6        | 0.1                        | .3      |
| IL-7        | 0.06                       | .6      |
| IL-8        | 0.2                        | .1      |
| IL-9        | 0.2                        | .1      |
| IL-10       | −0.2                       | .3      |
| IL-12p70    | 0.1                        | .4      |
| IL-13       | 0.1                        | .5      |
| IL-15       | 0.7                        | <.0001  |
| IL-17       | 0.1                        | .4      |
| Eotaxin     | 0.1                        | .3      |
| FGF         | 0.6                        | <.0001  |
| G-GSF       | 0.1                        | .3      |
| GM-CSF      | 0.1                        | .4      |
| IFN-G       | 0.2                        | .2      |
| IP-10       | 0.1                        | .4      |
| MCP-1       | 0.06                       | .7      |
| MIP-1a      | 0.2                        | .2      |
| MIP-1b      | 0.2                        | .2      |
| PDGF        | 0.2                        | .1      |
| RANTES      | 0.06                       | .7      |
| TNF-α       | 0.3                        | .06     |
| VEGF        | 0.2                        | .2      |

Interleukin (IL)-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-GSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-G), interferon-gamma-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins 1a and 1b (MIP-1a and MIP-1b), platelet-derived growth factor (PDGF), RANTES, tumor-necrosis-factor-gamma (TNF-G), and vascular endothelial growth factor (VEGF).
## Table 4: Univariate analysis for overall and progression-free survival.

| Cytokines          | Overall survival P-value | Progression-free survival P-value |
|--------------------|--------------------------|----------------------------------|
| IL-1b              | .08                      | .1                               |
| IL-1ra             | .2                       | .4                               |
| IL-2               | .09                      | .1                               |
| IL-4               | .03                      | .02                              |
| IL-5               | .8                       | .3                               |
| IL-6               | .07                      | .08                              |
| IL-7               | .06                      | .06                              |
| IL-8               | .01                      | .04                              |
| IL-9               | .1                       | .2                               |
| IL-10              | .02                      | .04                              |
| IL-12p70           | .06                      | .06                              |
| IL-13              | .06                      | .06                              |
| IL-15              | <.0001                   | <.001                            |
| IL-17              | .09                      | .1                               |
| Eotaxin            | .4                       | .02                              |
| FGF                | .2                       | .3                               |
| G-CSF              | .06                      | .06                              |
| GM-CSF             | .06                      | .06                              |
| IFN-G              | .04                      | .06                              |
| IP-10              | .4                       | .5                               |
| MCP-1              | .1                       | .2                               |
| MIP-1a             | .008                     | .008                             |
| MIP-1b             | .07                      | .1                               |
| PDGF               | .004                     | <.002                            |
| RANTES             | .6                       | .7                               |
| TNF-α              | .007                     | .006                             |
| VEGF               | .4                       | .3                               |
| Age ≥ 60          | .16                      | .06                              |
| A-ALC              | .01                      | .004                             |
| ALC-15             | <.0004                   | <.0001                           |
| CD34               | .3                       | .8                               |
| Disease status at transplant: complete remission | .11 | .1 |
| Extra nodal sites ≥ 2 | .46 | .8 |
| LDH; abnormal      | .3                       | .2                               |
| Mono-15            | <.001                    | <.002                            |
| NK-15              | <.0002                   | <.0002                           |
| PS > 1             | .24                      | .81                              |
| Stage III/IV       | .2                       | .28                              |

Interleukin (IL)-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-G), interferon-gamma-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins 1a and 1b (MIP-1a and MIP-1b), platelet-derived growth factor (PDGF), RANTES, tumor-necrosis-factor-gamma (TNF-G), vascular endothelial growth factor (VEGF), autograft absolute lymphocyte count (A-ALC), absolute lymphocyte count at day 15 post transplant (ALC-15), lactate dehydrogenase (LDH), absolute monocyte count at day 15 post transplant (Mono-15), natural killer cells at day 15 post transplant (NK-15), and performance status (PS).

### 3.3.1. Source of IL-15 at Day 15 Post-APHSCT (Monocytes/Macrophages).

The monocytic/macrophage system has been reported as a source of IL-15. Thus, we set out to investigate that the monocytic count at day 15 (mono-15) post-APHSCT affects IL-15 levels. We identified a good correlation between IL-15 and mono-15 ($r_s = 0.8, P < .0001$) (Figure 5). We also identified a good correlation between NK-15 and mono-15 ($r_s = 0.54, P < .0001$). However, in logistic regression analysis, IL-15 and A-ALC remained statistical significant as sources of NK-15 and truncated mono-15 ($P = .1$) (Table 6), suggesting that the mono-15 ability to affect NK-15 is mediated by the production of IL-15. Mono-15 was also associated with OS ($P < .001$) and PFS ($P < .002$) (Figures 6(a) and 6(b)). However, in multivariate
with an IL-15 cell transplantation. However, to our knowledge, recovery and survival post-APHSCT. At day 15 post-APHSCT to assess its interaction with NK-15 published that NK cells at two weeks post-APHSCT are homeostasis of IL-2/IL-15R. IL-15 has been reported to support this is the first study looking at the role of IL-15 on NK-15 recovery and survival post-APHSCT. IL-15 was the only cytokine at day 15 post-APHSCT that was associated with survival post-transplant. IL-15 has been reported to support the homeostasis of IL-2/IL-15Rβ expressing memory CD8+

The median OS was not reached in the group of patients with an IL-15 ≥ 31%, respectively (P < 0.002). (a) Overall survival (OS) of patients with an interleukin-15 (IL-15) ≥ 76.5 pg/mL versus patients with an IL-15 < 76.5 pg/mL. The median OS was not reached in the group of patients with an IL-15 ≥ 76.5 pg/mL and 19.2 months with the group of patients with an IL-15, 76.5 pg/mL. The OS rates at 3 years were 79% and 47%, respectively (P < 0.002). (b) Progression-free survival (PFS) of patients with an interleukin-15 (IL-15) ≥ 76.5 pg/mL versus patients with an IL-15 < 76.5 pg/mL. The median PFS was not reached in the group of patients with an IL-15 ≥ 76.5 pg/mL and 6.8 months with the group of patients with an IL-15, 76.5 pg/mL. The PFS rates at 3 years were 64% and 31%, respectively (P < 0.002).

Figure 3: (a) Overall survival (OS) of patients with an interleukin-15 (IL-15) ≥ 76.5 pg/mL versus patients with an IL-15 < 76.5 pg/mL. The median OS was not reached in the group of patients with an IL-15 ≥ 76.5 pg/mL and 19.2 months with the group of patients with an IL-15, 76.5 pg/mL. The OS rates at 3 years were 79% and 47%, respectively (P < 0.002). (b) Progression-free survival (PFS) of patients with an interleukin-15 (IL-15) ≥ 76.5 pg/mL versus patients with an IL-15 < 76.5 pg/mL. The median PFS was not reached in the group of patients with an IL-15 ≥ 76.5 pg/mL and 6.8 months with the group of patients with an IL-15, 76.5 pg/mL. The PFS rates at 3 years were 64% and 31%, respectively (P < 0.002).

Analysis, NK-15 remained the only statistical prognostic factor for survival compared with mono-15 (OS, P = 0.9; PFS, P = 0.9), or ALC-15 (OS, P = 0.8; PFS, P = 0.2) (Table 7). To assess the source of mono-15 recovery, we analyzed the number of A-mono infused and found a good correlation between A-mono and mono-15 (r = 0.3, P < 0.001). In addition, we identified a positive correlation between A-mono and NK-15 (r = 0.4, P < 0.008) and between A-mono and IL-15 at day 15 post-APHSCT (r = 0.3, P < 0.04).

4. Discussion

ALC-15 ≥ 500 cells/μL is a prognostic factor for survival post-APHSCT. NK-15 is the key lymphocyte subset of ALC-15 contributing to the superior clinical outcomes that observed post-APHSCT. ALC-15 and NK-15 recovery depends on the infused A-ALC, suggesting the new concept that the autograft used to infuse stem cells in APHSCT can be used also as an adoptive immunotherapeutic maneuver to enhance immune recovery, translating into better clinical outcomes post-APHSCT. However, for an adoptive cellular therapy to be effective, it requires cellular proliferation while preserving the effector function. We have previously published that NK cells at two weeks post-APHSCT are functionally active [22]. Thus, we studied the cytokine milieu at day 15 post-APHSCT to assess its interaction with NK-15 recovery and survival post-APHSCT.

Our cytokine profile at day 15 post-APHSCT is similar to what has been previously reported in autologous stem cell transplantation. [23, 24]. However, to our knowledge this is the first study looking at the role of IL-15 on NK-15 recovery and survival post-APHSCT. IL-15 was the only cytokine at day 15 post-APHSCT that was associated with survival post-transplant. IL-15 has been reported to support the homeostasis of IL-2/IL-15Rβ expressing memory CD8+

T cells and NK cells via transpresentation of IL-15 by IL-15Rx expressing cells [25, 26]. We previously reported that NK-15 is the key lymphocyte subset of ALC-15 affecting survival post-APHSCT [13]. Since IL-15 has been associated with NK cells homeostasis and differentiation, we set out to identify if there was an association between IL-15 and NK-15 post-APHSCT. We identified a strong correlation between IL-15 and NK-15 as a continuous or dichotomized variable. Our finding is similar to what has been reported
Table 5: Patients baseline characteristics based on IL-15 ≥ 76.5 pg/mL versus IL-15 < 76.5 pg/mL.

| Characteristics                        | IL-15 < 76.5 pg/mL (n = 25) | IL-15 ≥ 76.5 pg/mL (n = 25) | P-value |
|----------------------------------------|-----------------------------|-----------------------------|---------|
| Age at transplant, years: median (range) | 62 (44–73)                  | 54 (23–71)                  | .02     |
| Gender                                 |                             |                             | .2      |
| Female                                 | 6                           | 11                          |         |
| Male                                   | 19                          | 14                          |         |
| Lymphoma type                          |                             |                             | .7      |
| DLBCL                                  | 17                          | 17                          |         |
| Mantle cell                            | 5                           | 5                           |         |
| T-cell                                 | 3                           | 2                           |         |
| Follicular                              | 0                           | 1                           |         |
| Prognostic factors at diagnosis        |                             |                             |         |
| Extranodal disease                     |                             |                             | .2      |
| 0                                      | 15                          | 17                          |         |
| 1                                      | 8                           | 7                           |         |
| 2                                      | 2                           | 0                           |         |
| LDH (U/L), median (range)              | 222 (150–550)               | 200 (170–1515)              | .5      |
| Performance status                     |                             |                             | .1      |
| 0                                      | 12                          | 19                          |         |
| 1                                      | 12                          | 5                           |         |
| 2                                      | 1                           | 1                           |         |
| Stage                                  |                             |                             | .3      |
| I                                      | 2                           | 4                           |         |
| II                                     | 1                           | 4                           |         |
| III                                    | 4                           | 4                           |         |
| IV                                     | 18                          | 13                          |         |
| IPI score                              |                             |                             | .1      |
| Age at transplant, years               |                             |                             |         |
| ≥60                                    | 15                          | 8                           |         |
| <60                                    | 10                          | 17                          |         |
| Extranodal sites                       |                             |                             | .5      |
| ≥2                                    | 2                           | 0                           |         |
| <2                                    | 23                          | 25                          |         |
| LDH (U/L)                              |                             |                             | .3      |
| Abnormal                               | 13                          | 8                           |         |
| Normal                                 | 12                          | 17                          |         |
| Performance status                     |                             |                             | .9      |
| ≥2                                    | 1                           | 1                           |         |
| <2                                    | 24                          | 24                          |         |
| Stage                                  |                             |                             | .2      |
| I/II                                   | 3                           | 8                           |         |
| III/IV                                 | 22                          | 17                          |         |
| IPI index                              |                             |                             | .03     |
| 0                                      | 1                           | 4                           |         |
| 1                                      | 4                           | 11                          |         |
| 2                                      | 15                          | 7                           |         |
| 3                                      | 5                           | 3                           |         |
| Disease status prior to transplant     |                             |                             | .5      |
| CR                                     | 6                           | 9                           |         |
| PR                                     | 19                          | 16                          |         |
Table 5: Continued.

| Characteristics                                      | IL-15 < 76.5 pg/mL (n = 25) | IL-15 ≥ 76.5 pg/mL (n = 25) | P-value |
|------------------------------------------------------|-----------------------------|-----------------------------|---------|
| Infused CD34 cells × 10^6/kg; median (range)         | 5.17 (2.2–8.56)             | 4.4 (2.03–8.1)              | .2      |
| ALC-15 cells/µL; median (range)                      | 0.65 (0.02–1.91)            | 0.68 (0.1–4.5)              | .2      |
| ALC-15 cells/µL                                      |                             |                             | .2      |
| ≥500                                                 | 15                          | 20                          |         |
| <500                                                 | 10                          | 5                           |         |
| Infused A-ALC × 10^9 lymphocytes/kg; median (range)   | 0.55 (0.02–1.66)            | 0.63 (0.14–1.66)            | .1      |
| Infused A-ALC                                        |                             |                             | .2      |
| ≥0.5 × 10^9 lymphocytes/kg                           | 14                          | 19                          |         |
| <0.5 × 10^9 lymphocytes/kg                           | 11                          | 6                           |         |
| Infused A-mono × 10^9 monocytes/kg; median (range)    | 0.57 (0.06–1.5)             | 0.65 (0.26–1.5)             | .3      |
| Mono-15 cells/µL; median (range)                     | 0.85 (0.02–2)               | 0.86 (0.06–4.5)             | .8      |
| NK-15 cells/µL; median (range)                       | 98 (10–744)                 | 130 (12–999)                | .8      |

A-ALC: autograft, absolute lymphocyte count; ALC-15: absolute lymphocyte count at day 15 post transplant; A-mono: autograft absolute monocyte count; CR: complete remission; DLBCL: diffuse large B-cell lymphoma; IPI: International prognostic index; LDH: lactate dehydrogenase; Mono-15: absolute monocyte count at day 15 post transplant; PR: partial remission; NK-15: absolute natural killer cell count at day 15 post transplant.

Figure 5: Scatterplot comparing IL-15 and mono-15 at day 15 post-APHSCT. Strong positive correlation was identified between IL-15 and mono-15 post-APHSCT (Spearman correlation rho factor, $r = 0.8$, $P < .0001$).

Due to the apparent role of IL-15 on NK-15 recovery, we set out to investigate possible sources of IL-15 at day 15 post-APHSCT. Monocytes have been implicated as a source of IL-15 [28]. Thus, we studied the monocyte recovery at day 15 (mono-15) post-APHSCT, as a surrogate marker of IL-15 production, and its correlation with IL-15 levels. We identified a strong correlation between mono-15 and IL-15 at day 15 post-APHSCT. We also found an association between mono-15 and NK-15. However, a multivariate analysis showed IL-15 and not mono-15 as the factor affecting NK-15 recovery, suggesting that the effect

Table 6: Logistic regression analysis of factors affecting NK-15 recovery post-APHSCT.

| Factors                  | Estimate | Standard Error | $\chi^2$ | P-value |
|--------------------------|----------|----------------|----------|---------|
| A-ALC                    | 20.6     | 79.9           | 18.2     | <.0001  |
| IL-15 at day 15 post-APHSCT | 19.9     | 85.6           | 8.3      | .004    |
| Mono-15                  | 1.32     | 0.75           | 1.7      | .1      |

A-ALC: autograft, absolute lymphocyte count; APHSCT: autologous peripheral blood hematopoietic stem cell transplantation; IL-15: interleukin-15; Mono-15: absolute monocyte count at day 15 post transplant; $\chi^2$: chi square.

Table 7: Multivariate analysis for overall and progression-free survival.

| Factors                  | Overall survival | Progression-free survival |
|--------------------------|------------------|---------------------------|
| RR                       | 95% CI           | P-value                   | RR             | 95% CI          | P-value                   |
| ALC-15                   | 0.656±0.628–2.657 | .8                        | 0.628±0.152–2.107 | .2               |
| Mono-15                 | 1.125±0.316–3.317 | .9                        | 1.060±0.374–2.637 | .9               |
| NK-15                   | 0.176±0.162–0.797 | .006                      | 0.188±0.057–0.593 | .002             |

ALC-15: absolute lymphocyte count at day 15 post transplant; Mono-15: absolute monocyte count at day 15 post transplant; and NK-15: natural killer cells at day 15 post transplant.

in allogeneic stem cell transplantation, where faster NK cell recovery early post allogeneic stem cell transplantation is associated with elevated IL-15 levels [27]. Despite the small number of patients, patients with higher NK-15 and IL-15 levels experienced better PFS compared with patients with progressing lower NK-15 and IL-15 levels. Multivariate analysis showed that NK-15 truncated IL-15 levels, suggesting that the possibility that the survival benefit observed by IL-15 is mediated by enhanced NK cell recovery post-APHSCT. Another factor associated with NK-15 and previously reported was A-ALC. A-ALC was an independent factor affecting NK-15 recovery when compared with IL-15, suggesting that A-ALC, as an adoptive cellular therapy, provides a direct influx of immune effector cells (i.e., NK cells), while IL-15 helps to sustain the proliferation of NK cells.
on NK-15 recovery by mono-15 is most likely mediated by the production of IL-15 by mono-15. Another possible source of IL-15 is from dendritic cells [28]. Recently, it has been reported that the infusion of autograft dendritic cells as well as their recovery post transplant affects survival in lymphoma patients treated with APHSCT [29]. It is reasonable to hypothesize that the survival benefit from the infusion and recovery of dendritic cells post-APHSCT could also be related due to the production of IL-15 by dendritic cells activating NK cells. However, it is important to mention other factors that could affect the IL-15 levels during the peri-transplant period including (1) depletion of the lymphoid populations that normally consumed circulating IL-15 due to the conditioning regimens [27], or (2) response to the inflammatory environment triggered by the conditioning regimens [30, 31].

A limitation of the study is that even though we identified prospectively a correlation between IL-15 and NK-15 leading to better survival post-APHSCT, it does not prove causation. This limitation is based on the fact that this is a prospective correlitive study which might not have controlled for factors that could have affect IL-15 levels post-APHSCT such as (1) prior treatments before APHSCT and fludarabine-based regimens that could have affected A-ALC collection, ALC-15 recovery (i.e., NK-15), as well as mono-15 recovery post-APHSCT; and (2) the use of sargramostim affecting cytokine production. However, patient’s baseline characteristics were overall balanced regardless if IL-15 levels were equal/above or below 76.5 pg/mL. The positive correlation between pre- and post-APHSCT IL-15 levels suggests that the pretransplant IL-15 levels might help to anticipate prognosis after APHSCT. We hope that our ongoing Phase III randomized double-blind trial assessing if higher infusion of A-ALC translates into faster immune recovery and thus better survival post-APHSCT will address the relationship between IL-15 and NK cells as ancillary immunologic studies will be performed on this trial. In this trial none of the patients will receive sargamostim post-APHSCT. The premise of this phase III study is based on the fact that we have shown that by manipulating the mononuclear cell band during the centrifugation process of the apheresis machine, we can collect higher numbers of naïve T-cells and NK cells with direct impact on immune recovery post-APHSCT [32]. In this study, patients will be randomized to collect stem cells with the standard apheresis machine settings versus the modified apheresis machine settings, aiming at collecting more immune effector cells. Similarly, the results of the current study suggest that enriching the autograft with monocytes might also have a direct impact on NK-15 recovery and survival post-APHSCT. This hypothesis will be tested in the phase III study.

Based on the results of this study it appears that NK-15 recovery (the key lymphocyte subset of ALC-15 affecting survival post-APHSCT) depends on the adoptive infusion from A-ALC and the homeostatic/proliferative effect of IL-15. Monocytes/dendritic cells appear to mediate an NK cell supportive “homeostatic environment” potentially facilitating NK cells engraftment mediated by IL-15 following-APHSCT, ultimately leading to a therapeutic
autologous graft-versus-tumor effect (Figure 7). Evaluation of this hypothesis is currently underway on our Phase III study (Study ID: CDR0000577897, MAYO-MC0681).

In summary, this is the first study showing the role IL-15 on survival post-APHSCT most likely by supporting NK cells engraftment. In addition, this study also shows monocytes as possible source of IL-15, suggesting that the monocyte count can be used as a surrogate marker to assess the cytokine milieu (specifically IL-15) post-APHSCT. Further studies are warranted to understand the role of cytokines on survival post-APHSCT to develop more effective immunotherapeutic treatments in the APHSCT setting.

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