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Analysis

Innate Immune Recovery Predicts CD4$^+$ T Cell Reconstitution after Hematopoietic Cell Transplantation

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A B S T R A C T

Innate immune cells are the first to recover after allogeneic hematopoietic cell transplantation (HCT). Nevertheless, reports of innate immune cell recovery and their relation to adaptive recovery after HCT are largely lacking. Especially predicting CD4$^+$ T cell reconstitution is of clinical interest, because this parameter directly associates with survival chances after HCT. We evaluated whether innate recovery relates to CD4$^+$ T cell reconstitution probability and investigated differences between innate recovery after cord blood transplantation (CBT) and bone marrow transplantation (BMT). We developed a multivariate, combined nonlinear mixed-effects model for monocytes, neutrophils, and natural killer (NK) cell recovery after transplantation. A total of 205 patients undergoing a first HCT (76 BMT, 129 CBT) between 2007 and 2016 were included. The median age was 7.3 years (range, .16 to 23). Innate recovery was highly associated with CD4$^+$ T cell reconstitution probability ($P < .001$) in multivariate analysis correcting for covariates. Monocyte ($P < .001$), neutrophil ($P < .001$), and NK cell ($P < .001$) recovery reached higher levels during the first 200 days after CBT compared with BMT. The higher innate recovery after CBT may be explained by increased proliferation capacity (measured by Ki-67 expression) of innate cells in CB grafts compared with BM grafts ($P = .041$) and of innate cells in vivo after CBT compared with BMT ($P = .048$). At an individual level, patients with increased innate recovery after either CBT or BMT had received grafts with higher proliferating innate cells (CB; $P = .004$, BM; $P = .01$, respectively). Our findings implicate the use of early innate immune monitoring to predict the chance of CD4$^+$ T cell reconstitution after HCT, with respect to higher innate recovery after CBT compared with BMT.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative treatment option for pediatric patients suffering from life-threatening refractory malignancies or nonmalignant diseases. HCT in children is performed mostly using bone marrow (BM) or cord blood (CB) grafts. After HCT the immune system needs to rebuild, a process referred to as immune reconstitution (IR). In addition to early donor neutrophil engraftment, robust T cell reconstitution is essential for disease control and infection control. Poor or delayed T cell reconstitution relates to increased morbidity and mortality [1-8]. We previously reported that survival is significantly higher in patients with early CD4$^+$ T cell reconstitution compared with patients with delayed reconstitution [7,9,10]. It is therefore essential to have predictable CD4$^+$ T cell reconstitution and thus to identify factors that influence T cell reconstitution after HCT.

Although recovery of T cells to reference levels may take several months to even years, recovery of innate immune cells, such as neutrophils, monocytes, and natural killer (NK) cells, only takes weeks to a few months [11]. Neutrophils are the first cells to arise directly from the graft after transplantation and have therefore been adopted as an early measure for successful engraftment [1,11-13]. NK cells and monocytes also recover within weeks after HCT [11]. Because innate recovery generally precedes adaptive recovery [11], it might influence and/or predict CD4$^+$ T cell reconstitution and thereby (indirectly) affect outcome after HCT. Studies evaluating the dynamics of innate recovery in relation to adaptive IR after HCT are, however, lacking.

The aim of this study was to evaluate innate immune cell recovery in relation to adaptive IR after BM transplantation (BMT) and CB transplantation (CBT) in a pediatric cohort. We

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applied an immune-modeling strategy using nonlinear mixed-effects modeling to evaluate whether innate immune cell recovery relates to $CD4^+$ T cell reconstitution and to assess differences in innate immune cell recovery between CBT and BMT.

METHODS

Study Design and Patients

In this study we included pediatric patients receiving an allogeneic HCT between January 2007 and December 2016 at the University Medical Centre in Utrecht, The Netherlands. Only patients with successful engraftment of their first HCT who did not need a second HCT were included. Patients receiving serotherapy other than antithymocyte globulin (ATG; Thymoglobulin, Genzyme, Cambridge, MA) were excluded. There were no limitations regarding age, indication, or conditioning. Only patients receiving T cell–repleted donors were included. Patients were enrolled, and data were collected and registered prospectively, only after written informed consent had been given in accordance with the Helsinki Declaration. The study was approved by the local ethical committee (trial numbers 05-143 and 11-063k).

Procedures

Patients received either chemotherapy-based regimens (busulfan and fludarabine for benign disorders or clofarabine, fludarabine, and busulfan for malignant indications) or total body irradiation-based (busulfan intravenous), ATG, fludarabine, clofarabine, and total body irradiation, depending on diagnosis. Fludarabine-phosphate was given daily from days −5 to −2 relative to transplantation at a cumulative dose of 160 mg/m^2. Busulfan was given intravenously, targeted to a myeloablative cumulative exposure of 90 mg/hr/L or 30 mg/hr/L for Fanconi anemia patients (expressed as area under the curve). ATG was added in the unrelated donor HCT setting and given in 4 consecutive days from day −9 (10 mg/kg < 30 kg, 7.5 mg/kg > 30 kg). Graft-versus-host disease prophylaxis consisted of cyclosporine A combined with methotrexate 10 mg/m^2 at days +1, +3, and +6 in BM recipients and cyclosporine A combined with prednisone 1 mg/kg/day in CB recipients until day +28, which was tapered in 2 weeks. Target trough levels for cyclosporine A were 200 to 350 mg/L. All patients were prophylactically treated with acyclovir and received partial gut decontamination with ciprofloxacin and fluconazole and *Pneumocystis jiroveci* pneumonia prophylaxis using cotrimoxazol. All CBT patients received 10 mg/kg granulocyte colony-stimulating factor (G-CSF; Neupogen, Amgen, Breda, NL) from day +7 after HCT until neutrophils were above 2000 cells/μL. Patients were treated in high-efficiency, particle-free, air-filtered, positive-pressure isolation rooms.

Outcomes

In the first part of this study we associated innate recovery with $CD4^+$ T cell reconstitution probability after HCT. Innate recovery was a model-derived parameter described in detail in Statistics, below. In short, it was a latent variable built up from over-time monocyte, neutrophil, and NK cell counts in the blood of patients after HCT. For this, time-dependent dynamics of all subsets were assumed to be similar and directly related to the underlying latent variable. Low or high innate recovery was defined as having reached a maximum recovery below (<100%) or above (>100%) the standard innate cell reference value (100%), respectively. This reference value was defined as the median observed cell count observed (adjusted per cell type) after reaching full recovery (50 to 200 days after transplantation). $CD4^+$ T cell reconstitution was defined as having $>50 \times 10^6$ $CD4^+$ T cells/L blood in 2 consecutive measurements within the first 100 days after transplantation, because this cut-off was found to be the best predictor for outcomes [7]. In the second part we aimed to study the differences in innate recovery over the first 200 days between CBT and BMT. Additionally, we evaluated whether the differences found could be explained biologically.

Immune Monitoring

Absolute numbers of neutrophils and monocytes were measured in EDTA-treated whole blood at least 3 times per week during the admission period of 3 to 5 weeks after HCT, until a count of $.4 \times 10^9$ cells/L was reached using a Sapphire Coulter Counter (Abbott, Illinois, U.S.A.). From then on cell levels were measured at least every other week up to 12 weeks post-HCT and monthly thereafter up to 6 months, followed by every 3 months until 1 year, and twice in the second year after HCT with additional analyses of absolute NK cells ($CD3^-CD16^+CD56^+$) and $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) using TruCOUNT tubes (BD Biosciences, Erembodegem, Belgium)/Sapphire (Abbott Illinois, U.S.A.). For more in-depth evaluation of monocyte and NK cell subsets after transplantation, biobanked samples taken from 15 CBT and 19 BMT recipients, at weeks 2, 4, 6, 8, 16, and 24, were thawed and stained for flow cytometry. Monocyte subsets were gated as $CD3^-$ cells; $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate), and $CD14^+CD16^{++}$ (nonclassical). For NK cell subset evaluation, $CD3^-$ cells were gated as $CD56^{++}CD16^-$ (naïve), $CD56^+CD16^+$ (transitional), and CD56dimCD16$^+$ (effector). Samples for neutrophil subset evaluation were not available. Antibodies used for in-depth immune monitoring were CD3-AF700 (clone UCHT1; Biolegend, London, UK; BD Bioscience, Breda, NL; eBioscience Thermo Fisher Scientific Inc, Waltham, MA USA), CD56-PE-Cy7 (clone NCAM16.2; BD Bioscience), CD16-BV510 (clone 3G8; BD Horizon), and CD14-eFluor780 (clone 61D3; eBioscience).

For assessment of proliferation of cells within grafts, 6 CB grafts and 6 BM grafts were thawed and stained with extracellular CD3-AF700 (clone UCHT; Biolegend) and CD14-APC-eFluor780 and intracellular Ki-67-PerCP-Cy5.5 (clone B56; BD Bioscience) after permeabilization using the eBioscience kit (eBioscience; Thermo Fisher Scientific Inc. Waltham, MA USA). For assessment of proliferation of monocytes after transplantation, samples from 15 CBT and 19 BMT recipients were examined for intracellular Ki-67 expression in $CD3^-CD14^+$ cells at weeks 2, 4, 6, 8, 16, and 24 after HCT. Samples were measured on a LSR Fortessa (BD Bioscience). Results of innate cell proliferation in grafts were independent from innate cell proliferation after transplantation.

Statistics

Duration of the follow-up was defined as the time from HCT to the last assessment for surviving patients or death. Actual cell counts of monocytes, neutrophils, and NK cells in patients during the first 200 days after HCT were evaluated using nonlinear mixed-effects modeling (with NONMEM v.7.3) [14]. Models were parameterized in terms of fixed effects (population mean) and 2 levels of random effects: per subject (interindividual variability) and per measurement (residual variability). A logistic growth model was used as a structural model. Discrimination between nested models was based on the likelihood ratio test. The difference in objective function values between 2 hierarchical models follows a chi-square distribution, with n degrees of freedom for n parameters added. Covariates that were tested for inclusion in the models were age at transplantation (continuous), diagnosis (malignant/benign), graft-versus-host disease status (time-dependent: less than or at least grade II), year of transplantation (continuous), and post-transplantation area under the curve of ATG.

Because of the high correlation between the recovery of the different innate subsets (ie, neutrophils, monocytes, and NK cells), we developed a combined model for these 3 innate cell types describing the full dynamics of these cells after transplantation. For this, innate recovery was modeled as a latent variable, which was related to the different cell types using scaling to this latent variable, inclusion of relevant covariates, and inclusion of different residual errors for each subtype. A predefined reference value for innate recovery could not be derived from literature. Therefore, we assessed at which time cell counts stabilized for each individual patient, implying that the recovery is at its maximum. This time was set to where 95% of patients reached an observed (donor-derived) cell count above their model-estimated maximum. The variability of this maximum was the main independent determinant in the study. We chose the median value for all patients as the threshold between good and bad maximal recovery. Subsequently, the difference in innate recovery for patients with an early or late $CD4^+$ T cell reconstitution (adaptive IR) was tested as a covariate in the innate recovery model. To test the predictive value of innate recovery on recovery of the adaptive system, individual Bayesian estimates for innate recovery were generated using the POSTHOC option of NONMEM. These parameters were tested as predictors in an IR parametric time-to-event (TTE) model. The TTE model was constructed by choosing the optimal baseline hazard function and finding predictors for the hazard of IR. The baseline hazard resulting in the lowest Akaike information criterion was regarded as optimal. Predictors, including the individual estimates from the innate recovery model, were chosen by stepwise forward addition and backward deletion, with a significance level of .05. Continuous covariates were evaluated with a linear relationship and as a reduced cubic spline with 3, 4, and 5 degrees of freedom. The optimal relationship in the univariate setting was kept throughout the TTE model-building process.

To evaluate the influence of cell source on innate recovery, cell counts were modeled per innate cell subset. Covariates, other than cell source, found to be significant in the innate recovery model were a priori included in these models for each subset.

R version 3.3 [15] was used to make LOESS-regression curves for visualization of innate cell recovery in different patient groups and for the building of the TTE models of IR and to illustrate and analyze differences in Ki-67 expression in $CD3^-CD14^+$ cells within CB and BMgrafts. We used Graphpad v.6 to perform 2-tailed paired t-tests to evaluate subsets and CD3-$CD14^+$ Ki-67 expression differences after CBT or BMT.

RESULTS

Between 2007 and 2016 data from 312 allogeneic transplants was available. To optimize the cohort for data availability and homology 33 patients were excluded for unsuccessful engraftment of their first transplant, 7 were excluded for receiving serotherapy other than Thymoglobulin (ie, Alemtuzumab or ATG Fresenius), and 66 were excluded for lacking IR

data for 1 of the analyzed subsets. Thus, 205 patients were included with a median age of 7.3 years (range, .16 to 23) at time of HCT. In this cohort 129 patients received CB and 76 received BM. Median time to follow-up was 1412 days. In total, 3785 neutrophil samples (median, 17; range, 3 to 58 samples per patient) were available. For monocytes and NK cells these numbers were 5887 (median, 25; range, 3 to 84) and 1844 (median, 9; range, 0 to 22), respectively. Patient characteristics and differences between transplantation groups are summarized in Table 1.

Innate Immune Cell Recovery in Relation to CD4⁺ T Cell Reconstitution Probability

We evaluated the association between recovery of innate immune cells and CD4⁺ T cell reconstitution probability in both directions (Figure 1). In Figure 1A a percentage of <100% indicates a decreased innate recovery and >100% an increased innate recovery compared with the predicted innate cell reference value (which is 100%). In our model each innate immune cell subset contributed to the model, thereby retaining valuable information on innate IR. The magnitude of contribution to the model per subset was assessed by the residual error per subset. Monocyte recovery associated with the lowest residual error (65%) compared with neutrophils (75%) and NK cells (75%), indicating a strong contribution to the innate recovery model. In this model we found that patients who had a successful CD4⁺ T cell recovery also reached higher innate cell counts of >100% of the predicted innate cell reference value, which was significantly different from patients who had delayed CD4⁺ T cell recovery ($P < .001$, Figure 1A). The CD4⁺ T cell reconstitution probability over time was best characterized by a log-logistic distribution. During the forward inclusion and backward deletion procedure it was found that age at transplantation, year of transplantation, and post-transplantation ATG exposure were independent predictors for the CD4⁺ T cell reconstitution probability. In this multivariate setting (correcting for these covariates), we found that reaching a higher innate immune cell recovery strongly related to CD4⁺ T cell reconstitution (continuous, $P < .001$; Figure 1B). Stem cell source did not have a significant impact on CD4⁺ T cell probability in this cohort, after correcting for the above-mentioned covariates. In addition, cumulative incidence of CD4⁺ T cell reconstitution was increased in patients reaching innate cell counts above the previously defined standard reference value. Thus, our model indicates that patients with either monocytes $> .89 \times 10^9$/L and/or neutrophils $>4.2 \times 10^9$/L and/or NK cells $>.34 \times 10^9$/L within the first 50 days after HCT have higher chance of having successful CD4⁺ T cell reconstitution.

Innate Immune Cell Recovery According to Cell Source

Differences in innate immune cell recovery after BMT and CBT were observed. The recovery of monocytes (Figure 2A), neutrophils (Figure 2B), and NK cells (Figure 2C) is similar during the first ~30 days after either BMT or CBT. After this first month the numbers of monocytes ($P < .001$; .99 after CBT versus $.55 \times 10^9$/L after BMT), neutrophils ($P < .001$; 4.05 after CBT versus 3.05×10^9/L after BMT), and NK cells ($P < .001$; .36 after CBT versus $.24 \times 10^9$/L after BMT) were significantly higher in CBT compared with BMT recipients. This difference in innate recovery between BMT and CBT diminished by day 200 after transplantation. Interestingly, in our pediatric cohort CBT was also related to a higher chance of having successful CD4⁺ T cell reconstitution compared with BMT ($P = .018$) when residual ATG exposure after transplantation was low (<10 active ATG × day/mL) or absent [16]. Nevertheless, the higher innate recovery after CBT only increases the chance of having successful CD4⁺ T cell reconstitution ($>50 \times 10^6$ cells/L blood within 100 days after transplantation) by 3.0%, compared with BMT, when correcting for all relevant covariates (age at transplantation, year of transplantation, and post-transplantation ATG exposure).

Because of these differences we performed additional in-depth immune monitoring in 15 CBT and 19 BMT recipients. We evaluated which subsets within monocytes (Figure 3A) and NK cells (Figure 3B) were most prominent during recovery. Data from neutrophil subsets were not available. Classical

Table 1
Patient Characteristics

	Total(N = 205)	BM Group(n = 76)	CB group(n = 129)	*P* (BM vs. CB)
Male sex	125 (61)	46 (61)	79 (61)	1
Median age at transplant, yr (range)	7.3 (.16-23)	11 (.45-20)	4.9 (.16-23)	<.001*
From related donor	52 (25)	49 (64)	2 (1.55)	<.001*
Diagnosis				<.001*
Autoimmune disease	2 (.98)	0 (0)	2 (1.6)	
BM failure	22 (11)	14 (18)	8 (6.2)	
Immune deficiency	37 (18)	7 (9.2)	30 (23)	
Malignancy	105 (51)	50 (66)	55 (43)	
Metabolic/inborn	39 (19)	5 (6.6)	34 (26)	
Conditioning				.0021†
Busulfan based	168 (82)	53 (70)	115 (89)	
Total body irradiation based	18 (8.8)	9 (12)	9 (7)	
Fludarabine				
Cyclophosphamide	13 (6.3)	9 (12)	4 (3.1)	
Other	6 (2.9)	5 (6.6)	1 (.78)	
Serotherapy				<.001*
No serotherapy	64 (31)	40 (53)	24 (19)	
Thymoglobulin	140 (68)	35 (46)	105 (81)	
Year of transplantation				.77
2007-2011	64 (31)	25 (33)	39 (30)	
2011-2014	67 (33)	26 (34)	41 (32)	
2014-2016	74 (36)	25 (33)	49 (38)	
Median follow-up, days	1412	1412	1433	.64

Values are n (%) unless otherwise defined.
Statistically significant P < .05: *<.001, †<.01.

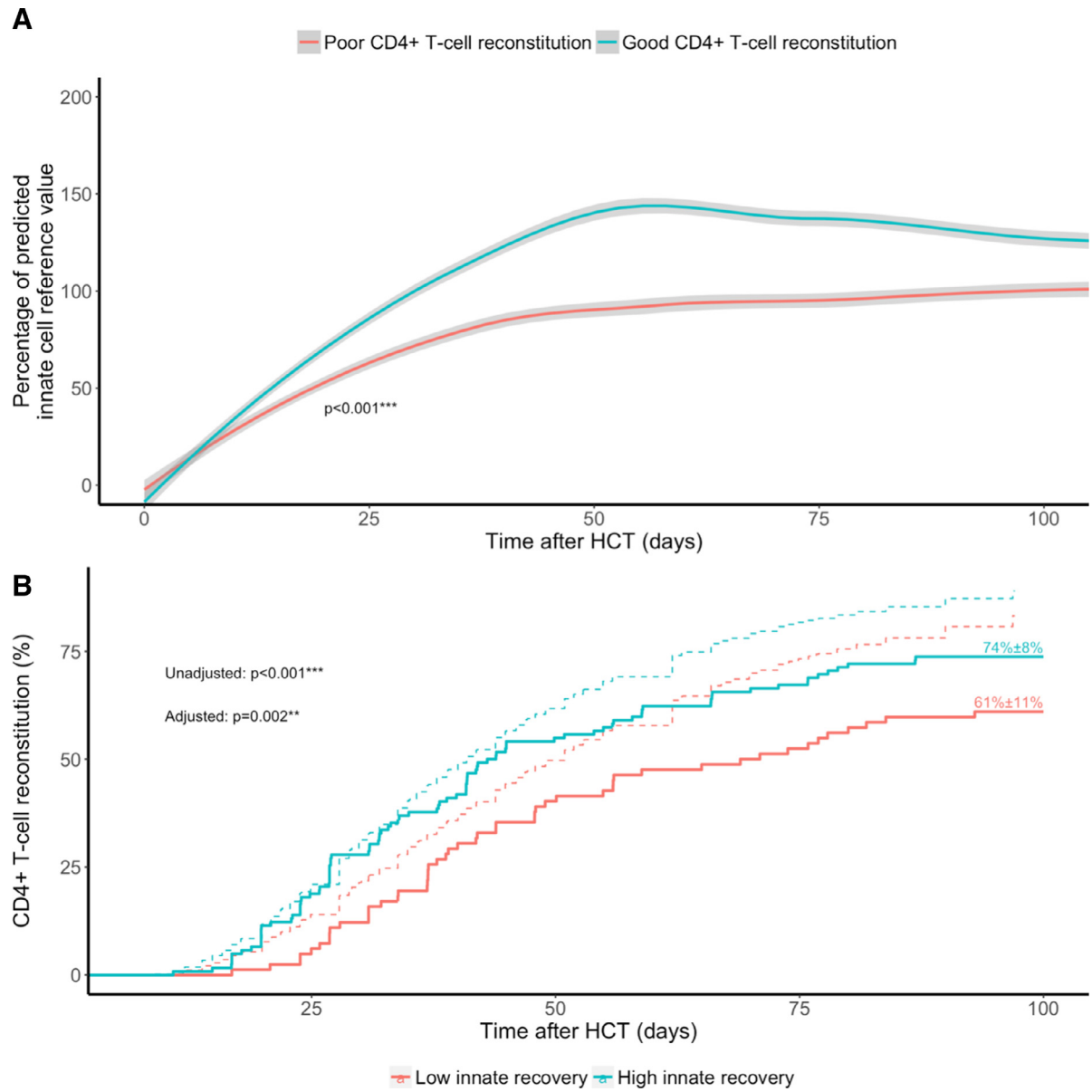

Figure 1. Innate recovery in relation to $CD4^+$ T cell reconstitution. (A) The percentage of observed innate cell counts relative to the population predicted reference value in patients who did not have successful $CD4^+$ T cell reconstitution (*red lines*) have different dynamics compared with patients who did have successful $CD4^+$ T cell reconstitution (*blue lines*). A percentage of <100% indicates a decreased innate recovery and >100% an increased innate recovery compared with the predicted innate cell reference value (which is 100%). *Gray areas* represent 95% confidence intervals of LOESS-regression curves and *P* values are derived from backward deletion of adaptive IR implemented in the innate cell model as a discrete variable (low/high). Successful $CD4^+$ T cell recovery was defined as having $>50 \times 10^6$ $CD4^+$ T cells/L blood, in 2 consecutive measurements, within 100 days after HCT. Innate recovery was defined as neutrophil, monocyte, and NK cell reconstitution. (B) Cumulative incidence curves depict higher probability of $CD4^+$ T cell reconstitution in patients with higher innate recovery ($P < .001$). *Dashed lines* reflect the model predictions.

monocytes ($CD14^{++}CD16^-$) predominated (±70% to 80%) within 2 months and 4 to 6 months after CBT and BMT, over intermediate ($CD14^{++}CD16^+$) and nonclassical monocytes ($CD14^+CD16^{++}$). Although absolute monocyte numbers differed after CBT and BMT, no differences were observed in the percentages of monocyte subsets. NK cells within the first 2 months after both CBT and BMT mostly consisted of naïve ($CD56^{++}CD16^-$; ±30% to 40%) and effector NK cells ($CD56dimCD16^+$; ±50% to 60%), with only low percentages of transitional NK cells ($CD56^+CD16^+$) present. Four to 6 months after both CBT and BMT most NK cell recovery consisted of the effector subset (±70% to 80%).

Innate IR Relates to Graft Proliferation Potency

We further investigated what biologic explanation could be found for the higher monocyte level after CBT. Differences between proliferation potential of BM monocytes and CB monocytes would provide a possible explanation for the difference in recovery. These data were not available for neutrophils and NK cells. Therefore, we investigated differences in Ki-67 expression between $CD3^-CD14^+$ cells ex vivo during the first 6 months in 15 CBT and 19 BMT recipients (Figure 4A) and $CD3^-CD14^+$ cells in 6 CB and 6 BM grafts (Figure 4B). Data from grafts and samples after transplantation were not related. We found a higher percentage of Ki-67 in CB graft $CD3^-CD14^+$ cells

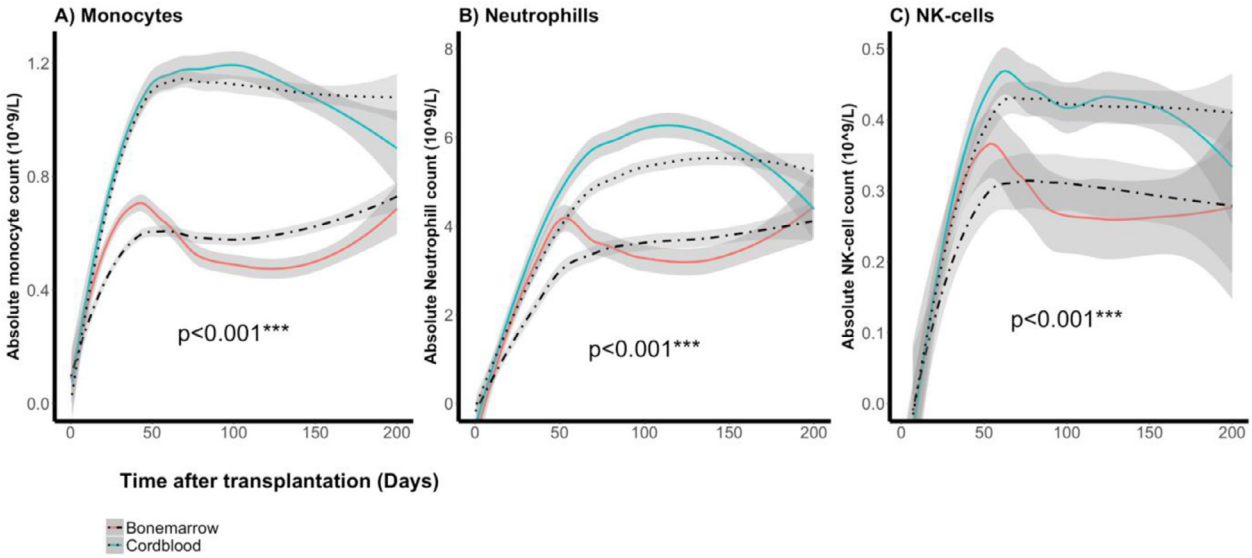

Figure 2. Recovery of innate immune cells after HCT with CB or BM. Innate subset recovery after transplantation with BM (*red lines*, n = 82) or CB (*blue lines*, n = 131); *gray areas* represent 95% confidence intervals of LOESS-regression curves. Model predictions for recovery after BMT (*intermitted line*) and after CBT (*dotted line*) are included. (A) Absolute monocyte counts ($\times 10^9$ monocytes/L blood) after CBT and BMT. (B) Absolute neutrophil counts ($\times 10^9$ monocytes/L blood) in CB and BM recipients. (C) Absolute NK cell counts ($\times 10^9$ monocytes/L blood) after BMT and CBT.

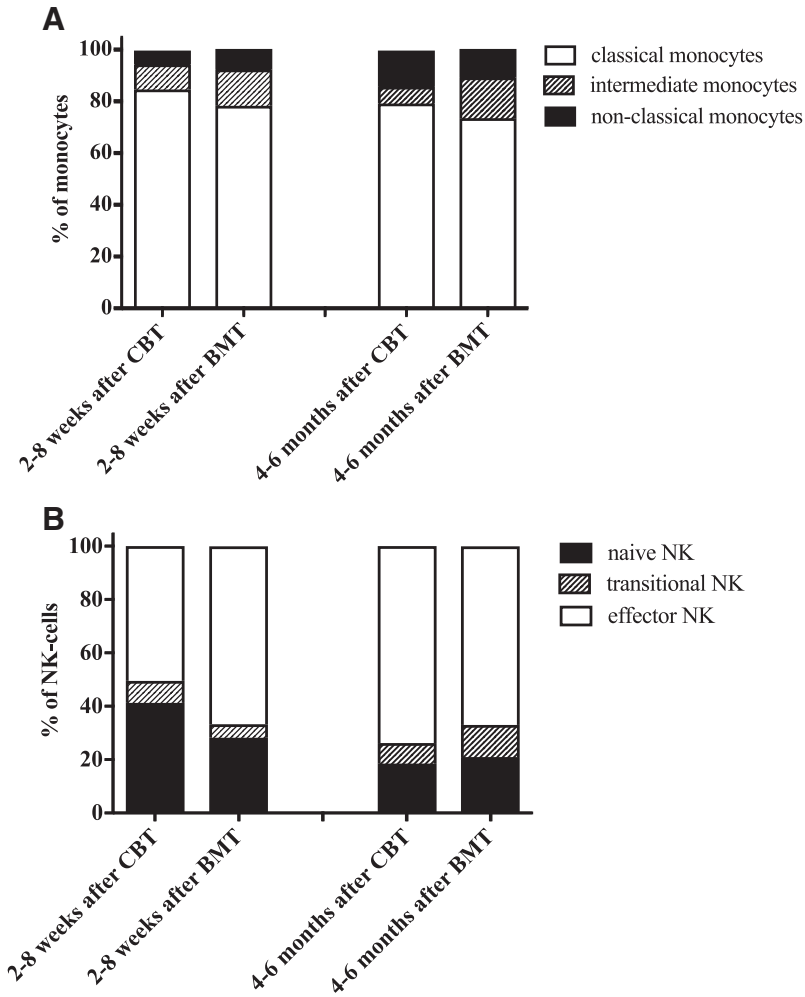

Figure 3. Recovery of innate cell subsets after HCT with CB or BM. (A) Monocyte subsets after CBT (n = 15) and BMT (n = 19), as percentages of total monocytes. (B) NK cell subsets after CBT (n = 15) and BMT (n = 19), as percentages of total NK cells.

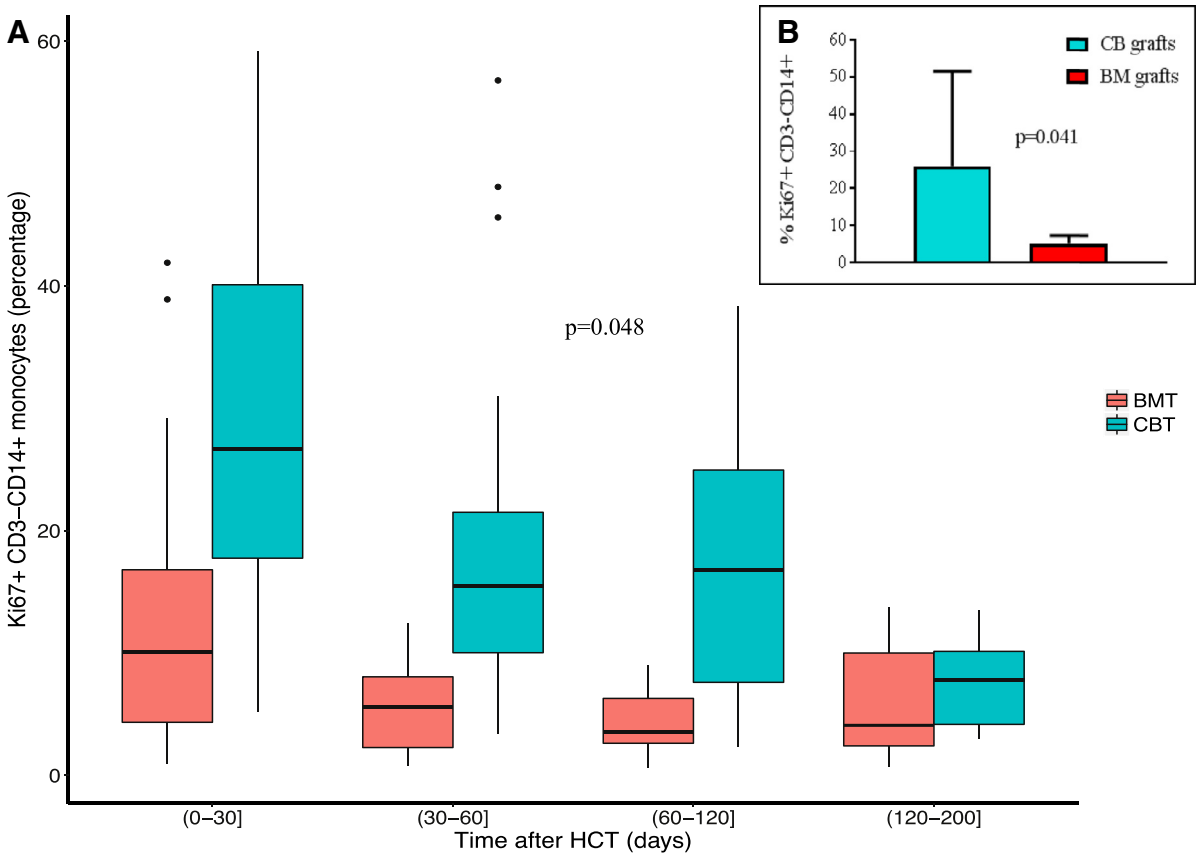

Figure 4. Proliferation of innate cells after transplantation with and in grafts of CB and BM. (A) The percentage of Ki-67$^+$ CD3-CD14$^+$ cells in patients at 2, 4, 6, 8, 16, and 24 weeks after CBT (*blue*, n = 15) was higher than after BMT (*red*, n = 19) (P = .048), as analyzed by linear-mixed effects modeling. (B) Percentage of Ki-67$^+$ CD3-CD14$^+$ cells within CB grafts (*blue*, n = 6) was statistically higher than in BM grafts (*red*, n = 6) (P = .041), as analyzed with nonparametric Mann-Whitney t-test.

compared with BM grafts (P = .041). An enhanced expression of Ki-67 in CD3$^-$CD14$^+$ cells was also found in blood of patients after CBT compared with BMT (P = .003). During the first 6 months after transplantation proliferation decreased over time, whereas higher percentages of Ki-67 remained in CB-derived CD3$^-$CD14$^+$ cells compared with BM. Interestingly, patients who received CB (Figure 5A; P = .004) or BM grafts (Figure 5B; P = .01) with higher innate Ki-67$^+$ expression had higher innate recovery (above median) during the first 200 days after CBT or BMT, and vice versa. These results indicate that enhanced innate recovery after CBT and BMT is most probably due to the enhanced proliferation potential of graft-innate cells.

DISCUSSION

This study is the first to both analyze innate recovery in context of adaptive reconstitution and compare innate recovery after CBT and BMT. We showed that superior innate immune cell recovery predicts higher probability of CD4$^+$ T cell reconstitution after pediatric HCT. This appeared to be cell source dependent, because we also observed higher levels of monocytes, neutrophils, and NK cells during the first 200 days after CBT compared with BMT. This emphasizes the potency of monitoring early innate immune cell recovery after HCT for early prediction of CD4$^+$ T cell reconstitution probability, which is highly related to better survival chances after HCT [1-9].

The association between innate immune cell levels and CD4$^+$ T cell reconstitution might be a result from enhanced graft activity. This hypothesis is supported by the relation we found between innate immune cell recovery and proliferation potential in graft cells. Grafts containing more proliferating cells resulted in more robust innate recovery, which might indicate a more optimal graft condition. The generally higher CB graft cell proliferation capacity could also explain why CD4$^+$ T cell reconstitution is faster after CBT compared with BMT in patients with no/low serotherapy exposure after transplantation [16]. Innate cell recovery might thus represent a healthy niche for homeostatic peripheral expansion mediated T cell reconstitution. Moreover, innate immune cells may also directly stimulate homeostatic peripheral expansion of T cells via TCR stimulation and/or cytokine production [17-20]. Therefore, further research is needed to assess what mechanisms exactly explain the relation between the recovery of innate immune cells and early CD4$^+$ T cell reconstitution after HCT.

Our finding that monocytes recover to higher levels after CBT compared with BMT has been reported before [21], but no other studies so far have described this difference for neutrophil and NK cell recovery. We found a biologically relevant explanation for the strikingly higher monocyte levels after CBT compared with BMT. The number of proliferating monocytes within CB grafts was significantly higher than in BM grafts. After transplantation, monocyte proliferation remained higher at least during the first 6 months after CBT compared with BMT. In addition, we found a relation between the proliferation potential in both CB and BM graft cells and innate recovery after transplantation in the respective individual patients. Of note, all CBT patients in our cohort were treated with G-CSF (from day +7 until neutrophils reach $>2000/\mu$L) as standard of care to enhance neutrophil levels, whereas BMT recipients were not [16]. This might also affect the recovery of both

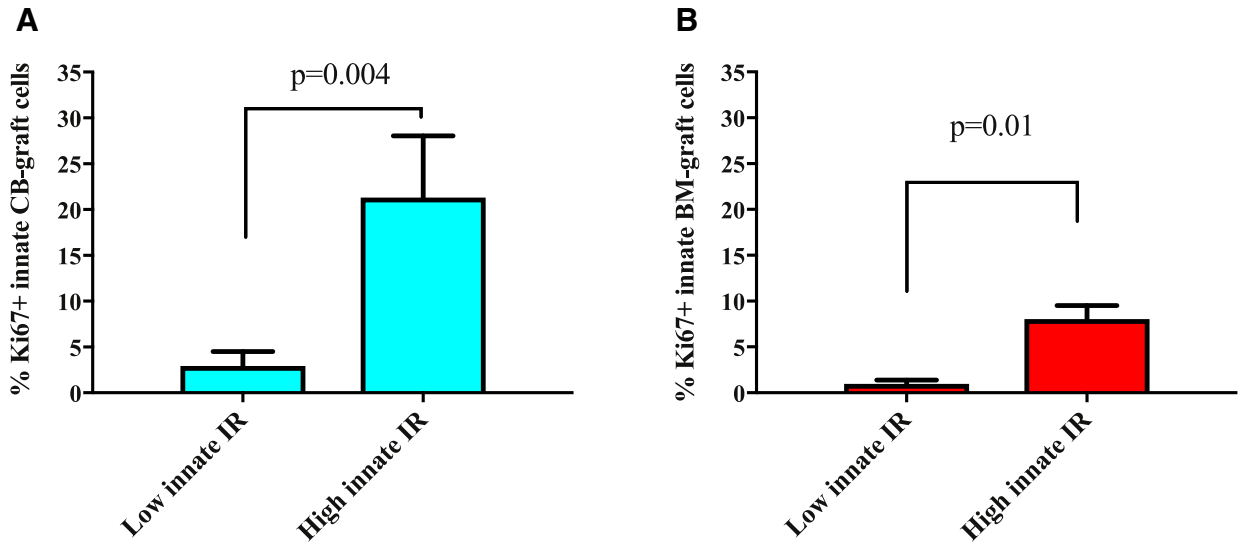

Figure 5. Innate IR relates to proliferation rate in CB and BM graft innate cells. Percentages of Ki-67$^+$ CD3$^-$CD14$^+$ cells within 6 CB (A) and 6 BM grafts (B). Groups are made based on low innate IR (below median; *left bars*, n = 3 CB and n = 3 BM) and high innate IR (above median; *right bars*, n = 3 CB and n = 3 BM), relative to graft source. The percentage of Ki-67$^+$ innate cells was higher in CB (P = .004) and BM grafts (P = .01) in patients with high innate IR compared with patients with low innate IR, as analyzed with 2-tailed paired t-tests.

neutrophils and monocytes, because monocytes have functional G-CSF receptors as well [22]. However, it is unclear if and to what extent G-CSF treatment after CBT would affect long-term monocyte recovery as it is given only for on average 2 to 2.5 weeks, and higher monocyte proliferation is already evident within CB grafts. Altogether, the observations of higher proliferation in CB graft innate cells and after CBT provide a possible explanation for the higher innate levels observed in CBT patients.

The higher innate immune cell recovery after CBT compared with BMT can be related to higher normal levels of innate cells in the blood of healthy newborns compared with healthy adults [23,24]. For instance, monocyte levels in blood of healthy adults are between 0 and .8 $\times$ 10^9/L, whereas we measured around .55 $\times$ 10^9/L after BMT [23]. For healthy newborns the reference range is .4 to 3.1 $\times$ 10^9/L [23], and levels of monocytes after CBT reached around .99 $\times$ 10^9/L. In addition, normal neutrophil levels are between 1.8 and 7.7 $\times$ 10^9/L in adults; we observed 3.05 $\times$ 10^9/L after BMT and between 6 and 26 $\times$ 10^9/L in newborns; after CBT we measured 4.05 $\times$ 10^9/L neutrophils [23]. Also for NK cells, recovery after CBT (.36 $\times$ 10^9/L) fits best within the range of normal NK cell counts in newborns of .3 to 1.7 $\times$ 10^9/L, whereas after BMT (.24 $\times$ 10^9/L) the levels meet the reference range in adults of .1 to .4 $\times$ 10^9/L [24]. CBT therefore seems to recapitulate fetal ontogeny in terms of IR, as described by others [25].

Because IR data consist of values over time, it demands overtime evaluation to be able to optimally relate IR to outcomes after HCT. We here show that immune modeling is feasible and potentially provides a more accurate and unbiased approach compared with dichotomous evaluations as presented by others [26,27]. Ultimately, more insights into the mechanisms and variables that influence immune cell reconstitution after HCT could lead to a model that is able to predict IR and clinical outcomes per patient, based on individual data. Our findings suggest importance of monitoring early innate immune cell recovery to predict CD4$^+$ T cell reconstitution later on and show differences in innate recovery after CBT and BMT in pediatric patients. Finally, these findings are of use to achieve more predictable IR to enhance outcome in future HCT recipients.

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