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## ORIGINAL PAPER

# Age-related immune cell dynamics influence outcomes after allogeneic haematopoietic cell transplantation

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**Summary**

An efficient immunological reconstitution construes the pillar for the success of allogeneic haematopoietic cell transplantation (HCT) in haematological disorders. Factors influencing post-transplant immune recovery have been largely investigated across multiple cohorts issuing heterogeneous results. Differences in outcomes in adult and paediatric populations suggest an age-related contribution to post-transplant immune reconstitution; however, it is unclear how recipient and donor age may affect the dynamics of single immune cells. Here, we retrospectively collected and analysed immunological data of 174 patients (58 children and 116 adults) consecutively transplanted for haematological disorders in our centre. We show that trajectories of specific immune cells were strictly dependent on recipient age and pretransplant virus exposure, with the strongest effect seen on T CD4+ and B-cell counterparts, while donor age and transplant platforms had a minimal impact. This mirrored different kinetics of immune reconstitution in adult and paediatric patients, with major divergences in immune cell composition in late post-transplant phases, featuring better survival, relapse-free survival and cumulative incidence of pathogen-specific infections in younger patients. Altogether, these findings underpin the importance of recipient age on post-transplant immune cell recovery and define the basic dynamics of the immune reconstitution in paediatric and adult populations as a benchmark for future studies.

**KEY WORDS**

adaptive immunity, allogeneic haematopoietic cell transplantation in children and adults, cytomegalovirus, Epstein-Barr virus, post-transplant immune reconstitution

**INTRODUCTION**

Allogeneic haematopoietic stem cell transplantation (HCT) holds a pivotal role in the treatment algorithm of haematological disorders.<sup>1-3</sup> Its therapeutic index relies on the replacement of the aberrant haematopoiesis and the

reconstruction of a graft-derived immune system to eradicate the underlying haemopathies. While innate immune reconstitution parallels haematopoietic cell recovery in early post-transplant phases and is a relatively fast process, adaptive immune reconstitution requires the accomplishment of a complex network of events, characterized by both

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thymic-independent expansion of T cells infused with the graft and thymus-dependent expansion of naive T cells derived from donor stem cells.<sup>4–6</sup> The recovery of both these components may depend on many pre- and post-transplant factors and can define the patterns of alloreactivity, influencing clinical outcomes. Underlying disease, pretransplant treatments, conditioning regimens, immunosuppressive drugs, graft source, immunogenetic background, recipient and donor age and comorbidities have been variously associated with the characteristics of the post-transplant immune recovery and with the specificity and the strength of alloreactive immune responses. These multiple factors are at the basis of disease control, anti-infectious competence and tolerance to host antigens, finally defining the success of the procedure.<sup>7–17</sup>

In this view, the laboratory monitoring of lymphocytic subpopulations is crucial for the follow-up of transplanted patients since it should drive therapeutic decisions (for instance, immunosuppressive drug tapering, vaccination programme start, or infectious prophylaxis withdrawal) and may inform on the risk of transplant-related morbidity or mortality. However, lack of systematic studies, with high variability among cohorts and monitoring strategies, renders most of immune reconstitution analyses only partially resolute in terms of incremental knowledge of the field, particularly in the paediatric setting. Moreover, to the best of our knowledge, age-related patterns of recovery if extrapolated by real-life clinical practice have never been ascertained or characterized in combined paediatric-adult cohorts.

Here, starting from complete immunological and clinical data collected in our centre, we studied the dynamic and the composition of post-transplant immune repertoire in a large cohort of patients with haematological disorders and informed on the quantitative and qualitative differences of paediatric and adult immune reconstitutions, defining their impact on clinical outcomes.

## PATIENTS AND METHODS

### Study cohort and data collection

This is a retrospective, non-interventional, study conducted at our institution aiming at investigating the differences in post-transplant immune reconstitution in paediatric and adult patients, and the association between immune cell dynamics and clinical outcomes (Figure S1). All patients and patients' parents/guardians provided written informed consent for the aims of this specific research, and the study protocol was registered by our Institutional Review Board (IRB) under the number 2021PI086.

All patients receiving a first allogeneic HCT for a haematological disorder from a matched sibling donor (MSD) and a matched (MUD) or a mismatched unrelated donor (MMUD), with complete immuno-monitoring data, and consenting to participate in this research were included in

this study. The review of medical records was approved by the institutional ethical committee under the legacy of the Helsinki Declaration of 1975, revised in 2008.<sup>18</sup>

All patients were regularly followed up from January 2015 to December 2020 (or death). Alive patients were censored on 31 December 2021. Pertinent clinical data including gender, age, diagnosis, comorbidities, donor, graft source, human leukocyte antigen (HLA) matching, conditioning regimen, graft versus host disease (GvHD) prophylaxis, acute and chronic GvHD, infectious complications, relapse and immune reconstitution of T, B, NK cell populations were collected. Clinical variable collection was based on the European Group of Blood and Marrow Transplantation (EBMT) registry through the central data processing system 'Project Manager Internet Server' (ProMISe). Details concerning study design, transplant platforms and clinical event selection are reported in the Appendix S1.

### Immunological monitoring

All patients were routinely monitored for their immunological recovery at day +30, day +90, +6 months and +12 months post-HCT (Figures S1 and S2).

Immunological monitoring comported a flow-based immunophenotyping, characterizing all classical lymphocyte subpopulations (for total T cells, CD4+, CD8+, NK and B cells) together with protein electrophoresis and immunoglobulin (Ig) G, M, A class dosages. All the analyses were performed on fresh peripheral blood samples and plasma.

### Statistical analysis

Median values and ranges were used for continuous variables and percentages for categorical variables. For all relevant comparisons, comparative analyses between continuous parameters were performed with the Wilcoxon rank-sum test at 95% confident interval (CI), after ascertaining the absence of a normal distribution. The Fisher exact test or chi-square was applied for independent group comparisons.

Probabilities of survival for overall survival (OS) and event-free survival (EFS) were calculated using Kaplan–Meier estimates, with differences between the curves based on log-rank tests. OS was defined as the time from transplantation to death for any cause. EFS was defined as the time from transplantation to death or relapse of the underlying haematological disease.

For non-malignant disorders, relapse was considered in the presence of loss of donor chimerism and reappearance of underlying disease features (i.e. bone marrow failure or hemoglobinopathy). In the case of non-event, observations were censored at the time of last follow-up.

Cumulative incidence functions (CIF) of acute and chronic GvHD and relapse were calculated in a competing

risk setting, where death was considered the competing event.

Linear regression models were applied to assess the correlation between age and lymphocyte counts, and residual analysis was performed for each model to ascertain that all requirements for linearity were satisfied.

Generalized linear models were constructed to evaluate the associations between the immune reconstitution and the other predictors at baseline. Results were expressed as odd ratio, 95% CI and  $p$ -value. All statistical tests were two-sided, and a  $p < 0.05$  was considered statistically significant.

Statistical analyses were performed with R-Project 4.2 (R Foundation for Statistical Computing) software packages.

## RESULTS

### Patient characteristics

In total, 174 patients receiving an allogeneic transplantation at our institution for a haematological disorder were involved in this study (Table 1, Figure S1), including 58 children and 116 adults. Median follow-up of the whole population was 32 months (Interquartile range [IQR]: 19.38–43.42 months). Median age was 59 years (range 18–74) in the adult population and 7 years (range 0.8–18) in the paediatric group. Disease distribution across the whole population reflected the epidemiology of transplant indications with acute lymphoblastic leukaemia (ALL) and non-malignant conditions being more frequent in paediatric group versus a predominant enrichment of acute myeloid leukaemia (AML) and myelodysplastic syndromes (MDS) in adults.<sup>19,20</sup> In terms of graft type, bone marrow was the principal source in paediatric procedures while peripheral stem cells were mostly used in adult transplants. Accordingly, conditioning regimen intensity was dependent on age, with myeloablative conditioning preferred over reduced-intensity platforms in children and young adults. The distribution of related and unrelated matched donors was similar in both groups, but paediatric patients received mismatched donor transplants more frequently than adults.

Almost all adults received anti-thymoglobulin versus 20% of paediatric patients transplanted without T-cell depletion.

### Outcome description and differences in paediatric and adult patients

Despite lack of formal comparisons, outcomes of paediatric transplanted cohorts are historically superior to those of adult patients in most disease settings.<sup>21,22</sup> Here, we start from the assumption that this difference may depend on an orchestra of recipient-related factors, among which the increased immune system's plasticity plays a fundamental role, beyond disease ontogeny and transplant platforms.

Since the degree of this divergence in outcomes has not been properly ascertained in real-life settings, here we first analysed the time-related event distributions in our cohort to understand the patterns of survival, disease relapse, infections and GvHD outcomes in paediatric vs adult patients (Table 2). Median follow-up was similar in both groups: 30.7 months (IQR 18–41.8) for adults and 34.3 (IQR 20.3–50.4) months for children,  $p = 0.1$ . OS, CIF of relapse, infections and acute and chronic GvHD of the whole cohort are reported in Figures S3 and S4. Children displayed a superior OS and EFS, an inferior rate of chronic GvHD, although they presented higher acute GvHD cumulative incidences (Figure 1A–D). No differences were found in terms of CMV and EBV reactivations, as well as severe bacterial infections. Both groups had also similar relapse rates; however, median time to relapse was longer in paediatric (337, IQR 156–607) versus adult patients (139, IQR 73–261),  $p = 0.03$ . Differences in survival among children and adults were evident also when adjusting for disease type, donor, graft source and conditioning (Table S1) with no other factor than age group impacting outcomes. Overall, 54 of 119 patients with EBV reactivation received pre-emptive rituximab treatment ( $N = 37/84 = 44\%$ —adults and  $N = 18/34 = 53\%$ —children), at a median time of 106 days (IQR: 42–196). Of them, two adults and two paediatric patients developed a post-transplant lymphoproliferative disorder.

### Recipient age patterns influence immune reconstitution

We then explored the different kinetics of immune reconstitution in the two groups.

First of all, when analysing the linear relationship between reconstitution of immune cells at 12-months post-HCT and recipient age, we observed a strong negative correlation with CD4+ T cells (Adj.  $R^2 = 0.392$ ,  $p = 5.7e-15$ ) and in minor part with B cells (Adj.  $R^2 = 0.055$ ,  $p = 0.0049$ ), while no impact of age was seen on CD8 and NK cells (Figure 2A). The same trend but with less strong correlations was seen with these immune subsets and donor age (Figure 2B).

We therefore studied the dynamics of these immune subsets across multiple post-transplant time points in paediatric and adult groups. While no differences were seen in early post-transplant phases for any of the cell subsets, in later phases, children recovered T-cell numbers better than adults, with an especially faster and more efficient reconstitution observed on CD4+ T cells (at 12-months CD4+ T cells reached a median of 0.52 G/L [IQR: 0.42–0.79] in paediatric vs 0.19 G/L [IQR: 0.13–0.29] in adult groups,  $p = 1.6e-13$ , Figure 3A–F).

The impact on age mirrored different kinetics of immune reconstitution in adult and paediatric subjects. Therefore, while early after HCT immune cell composition was similar in the two groups, with NK cells representing the largest component, followed by CD8+ T and CD4+ T cells, and

**TABLE 1** Patient and transplant characteristics.

Variables	Adults ( <i>n</i> = 116), median/ <i>N</i> (IQR/%)	Children ( <i>n</i> = 58), median/ <i>N</i> (IQR/%)	All ( <i>n</i> = 174), median/ <i>N</i> (IQR/%)	<i>p</i> -value
Age	59 (51.5–64)	7 (4–12)	50 (12–61)	<0.00001
Sex				
Female	43 (38)	23 (39)	67 (38)	ns
Male	73 (62)	35 (62)	108 (62)	
Disease				
AML	58 (50)	12 (21)	70 (40)	<0.00001
ALL	14 (13)	30 (50)	44 (25)	
Lymphoma	8 (7)	1 (3)	10 (6)	
MDS	16 (14)	0	16 (9)	
MF and MPN	19 (16)	0	19 (11)	
Other <sup>a</sup>	0	15 (26)	15 (9)	
Cytogenetic				
Favourable	2 (2)	0	2 (1)	0.01701
Normal	49 (42)	13 (23)	62 (36)	
<3 abnormalities	30 (26)	30 (51)	60 (35)	
>3 abnormalities	20 (17)	10 (18)	30 (17)	
Missing	15 (13)	5 (8)	20 (11)	
Disease status (only for malignant)				
1 RC	40 (35)	17 (30)	57 (34)	0.012495
>1RC	22 (19)	21 (40)	43 (26)	
Not RC	54 (46)	16 (30)	70 (40)	
DRI				
1	4 (4)	4 (9)	8 (5)	0.017325
2	80 (70)	38 (84)	118 (74)	
3	29 (24)	3 (7)	32 (19)	
4	3 (2)	0	3 (2)	
Donor type				
MRD	26 (22)	15 (26)	41 (24)	0.000451
MUD (10/10)	90 (78)	36 (62)	126 (72)	
MMUD (9/10)	0	7 (12)	7 (4)	
Donor age	31 (24–43)	24.5 (18–34)	29 (22–40)	0.000695
Donor sex				
Female	24 (21)	17 (28)	41 (24)	ns
Male	92 (79)	40 (72)	132 (76)	
Mismatch sex				
Yes	47 (41)	22 (38)	69 (40)	ns
No	69 (59)	35 (62)	104 (60)	
Stem cell source				
BM	2 (2)	48 (83)	50 (29)	<0.00001
PBSC	114 (98)	10 (17)	124 (71)	
CMV risk				
Low	38 (32)	15 (28)	53 (31)	ns
Intermediate	48 (42)	27 (47)	75 (43)	
High	30 (26)	15 (25)	45 (26)	
EBV risk				
Low	1 (0.1)	5 (9)	6 (3)	ns
Intermediate	61 (87)	38 (70)	99 (83)	
High	14 (12.9)	11 (21)	25 (14)	

(Continues)

TABLE 1 (Continued)

Variables	Adults (n = 116), median/N (IQR/%)	Children (n = 58), median/N (IQR/%)	All (n = 174), median/N (IQR/%)	p-value
Toxoplasmosis risk				
Low	42 (37)	38 (70)	80 (47)	0.00007
Intermediate	30 (25)	8 (17)	38 (23)	
High	44 (38)	7 (13)	51 (30)	
Conditioning regimen				
MAC	46 (40)	48 (83)	94 (54)	<0.00001
RIC	53 (45)	10 (17)	63 (36)	
NM	17 (15)	0	17 (10)	
T-depletion (ATG)				
Yes	115 (99)	45 (78)	160 (92)	<0.00001
No	1 (1)	13 (22)	14 (8)	
Immunosuppressive treatment				
CI only	16 (15)	8 (14)	24 (15)	<0.00001
CI + MMF	86 (73)	7 (12)	93 (52)	
CI + MTX	14 (12)	43 (74)	57 (33)	
Graft richness				
TNC	5.11 (3.3–7.3)	6.61 (5.04–9.94)	5.8 (4.05–7.8)	<0.00001
CD34 cells	5.9 (4.06–6.04)	6 (5.9–6.01)	6 (4.8–6.02)	ns

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; ATG, anti-thymoglobulin; BM, bone marrow; CI, calcineurin inhibitor; DRI, Disease Risk Index; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MF, myelofibrosis; MMF, mycophenolate mofetil; MMUD, mismatched unrelated donor; MPN, myeloproliferative neoplasms; MRD, matched related donor; MTX, methotrexate; MUD, matched unrelated donor; NMA, non-myeloablative; PBSC, peripheral blood stem cell; RIC, reduced-intensity conditioning; TNC, total nucleated cells.

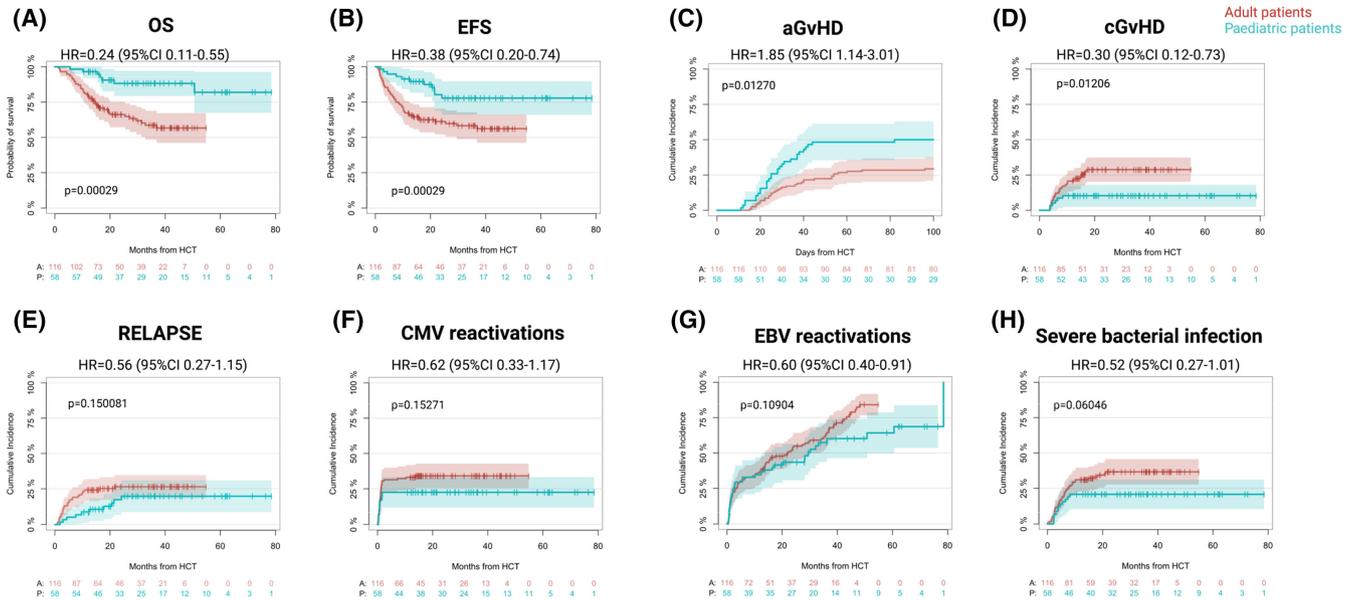
<sup>a</sup>Other diseases included Juvenile myelomonocytic leukaemia (N = 3), bone marrow failure disorders (N = 9) and haemoglobinopathies (N = 3).

TABLE 2 Post-HCT outcomes.

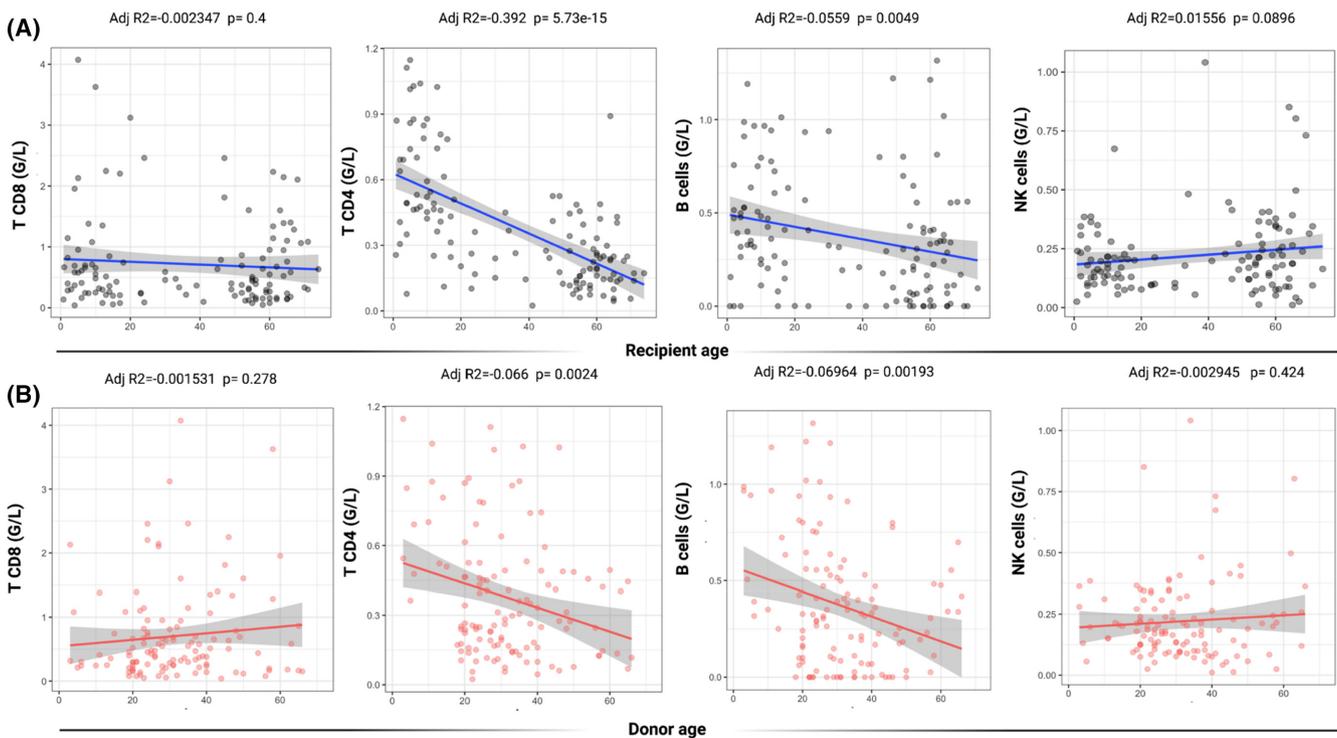
Outcomes	All, N/median (IQR/95% CI)	Adults, N/median/ percentage (IQR/95% CI)	Paediatric, N/median/ percentage (IQR/95% CI)	p-value
Median time follow-up	32.64 (IQR: 19.38–43.42)	30.79 (IQR: 18.20–41.87)	34.34 (IQR: 20.35–50.48)	0.1369
3y OS	68.7 (61.4–76.7)	58.7 (0.49–0.69)	88.2 (0.79–0.97)	0.0002
Median time acute GvHD II-IV	30 (IQR: 22–42)	30 (IQR: 21–51)	29 (IQR: 23–38)	0.3479
Median time to relapse	146 (IQR: 85–307)	139 (IQR: 73–261)	337 (IQR: 156–607)	0.0381
100-day CIF of acute GvHD II-IV	36.4 (29.3–43.5)	29.3 (21.3–37.8)	50.8 (36.4–62.1)	0.0078
100-day CIF of acute GvHD III-IV	16.1 (11.1–22)	14.7 (8.9–21.7)	19 (10.1–30)	0.7171
1y CIF of chronic GvHD	17.2 (12–23.2)	20.7 (13.8–28.5)	10.3 (4.2–19.8)	0.0121
3y CIF of chronic GvHD	28.8 (20.6–37.5)	28.8 (20.6–37.5)	10.3 (4.2–19.8)	
3y EFS	64.4 (54.2–72.4)	58.3 (49.5–68.6)	77 (66.9–90.6)	0.0002
1y CIF of relapse	19 (13.9–25.6)	24.1 (16.8–32.3)	8.6 (3.1–17.6)	0.1512
3y CIF of relapse	24.7 (18.3–34.6)	26.2 (18.4–34.6)	17.3 (8.3–29)	
Median time CMV infection	26 (18–42)	31 (19–41)	20 (13–43)	0.4489
1y CI CMV reactivation	28.4 (21.8–35.3)	33.2 (24.7–41.9)	22.5 (12.7–34)	0.1527
Median time EBV infection	39 (25–81)	39 (26–92)	50 (28–79)	0.7317
1y CI EBV infection	31.6 (24.8–38.6)	37.1 (28.3–45.8)	34.5 (22.5–46.8)	0.1091
Median time severe bacterial infection	138 (78–225)	117 (72–173)	159 (80–242)	0.2879
1y CI of severe bacterial infection	28.3 (21.7–35.2)	32.1 (23.6–40.8)	20.8 (11.4–32.1)	0.0453
Median time of fungal infection	229 (151–452)	233 (166–477)	5 (5–5) <sup>a</sup>	NA
1y CIF fungal infection	6.9 (3.8–11.3)	9.5 (5–15.7)	1.7 (0.1–8.2)	0.0089

Abbreviations: CI, confident interval; CIF, cumulative incidence function; CMV, cytomegalovirus; EBV, Epstein–Barr virus; GvHD, graft-versus-host disease; IQR, interquartile range; N, number; y, year.

<sup>a</sup>Only one event.



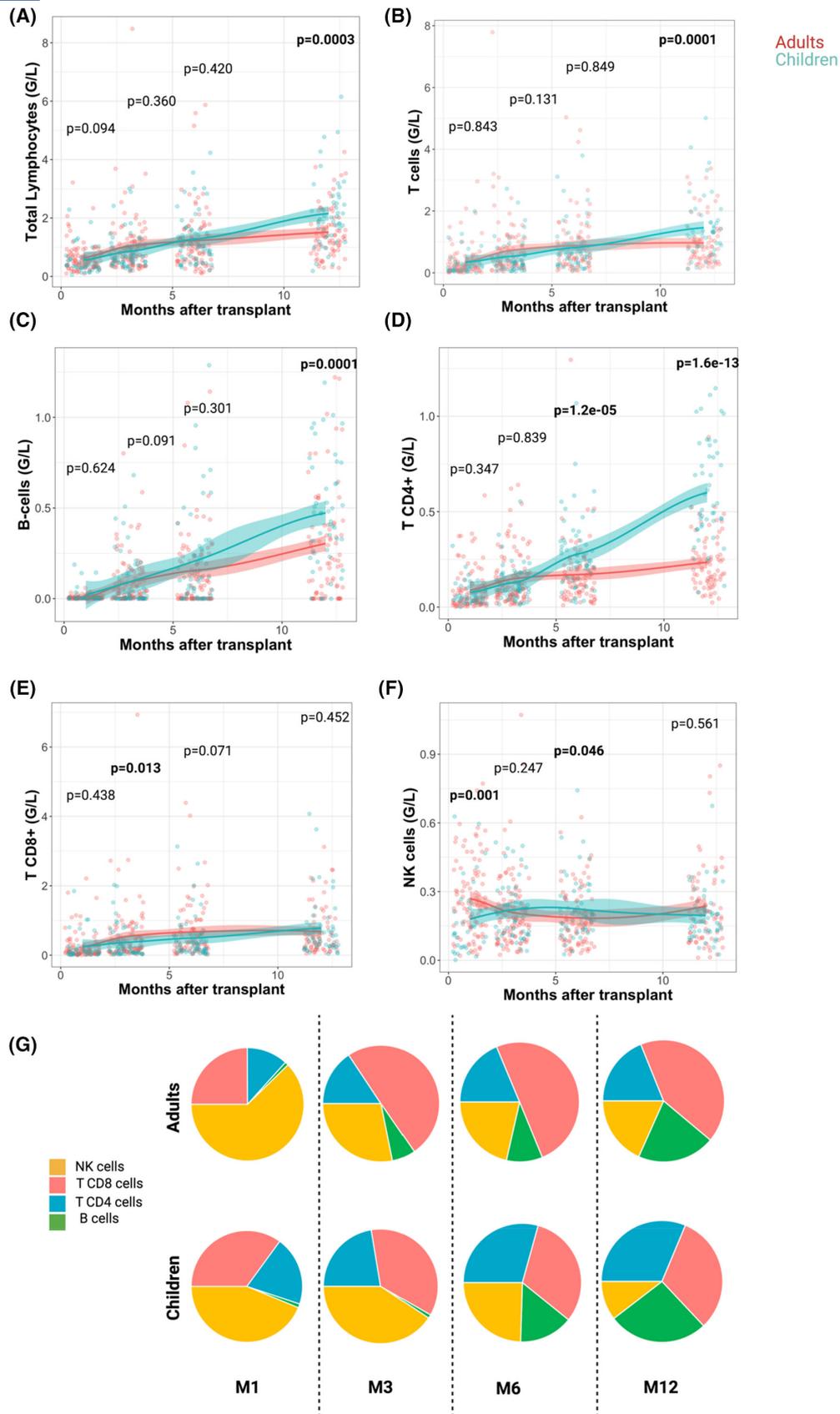
**FIGURE 1** Outcomes in adult and paediatric populations. Kaplan–Meier estimates of (A) overall survival; (B) event-free survival; cumulative incidence function of (C) acute GvHD; (D) chronic GvH; (E) relapse; (F) CMV reactivation; (G) EBV reactivation; (H) severe bacterial infections. *p*-values were calculated as log-rank test (survival) and Gray’s test (cumulative incidence function).



**FIGURE 2** Correlation between age and immune cell subsets at 12 months (M12) post-transplant. Linear regression analysis of each lymphoid cell subpopulation and recipient (A) and donor (B) age. Each graph represents a scatter plot in which dots indicates absolute values at 12 months for each cell subset (expressed in G/L) while the position on the *x*-axis indicates recipient or donor age. Goodness-of-fit of each linear model is reported as adjusted *R*<sup>2</sup> and *F* statistics.

minimal B-cell contribution, in later phases we assisted to a faster expansion of CD4+ T-cell arm in children, whereas CD8+ T cells remained the major contributors of the immune recovery processes in adults still one year after transplant

(Figure 3G). B-cell reconstitution was also faster in children, with a better immunoglobulin-level recuperation (measured on IgM to avoid bias of prophylactic IgG infusions) at later phases after transplant.

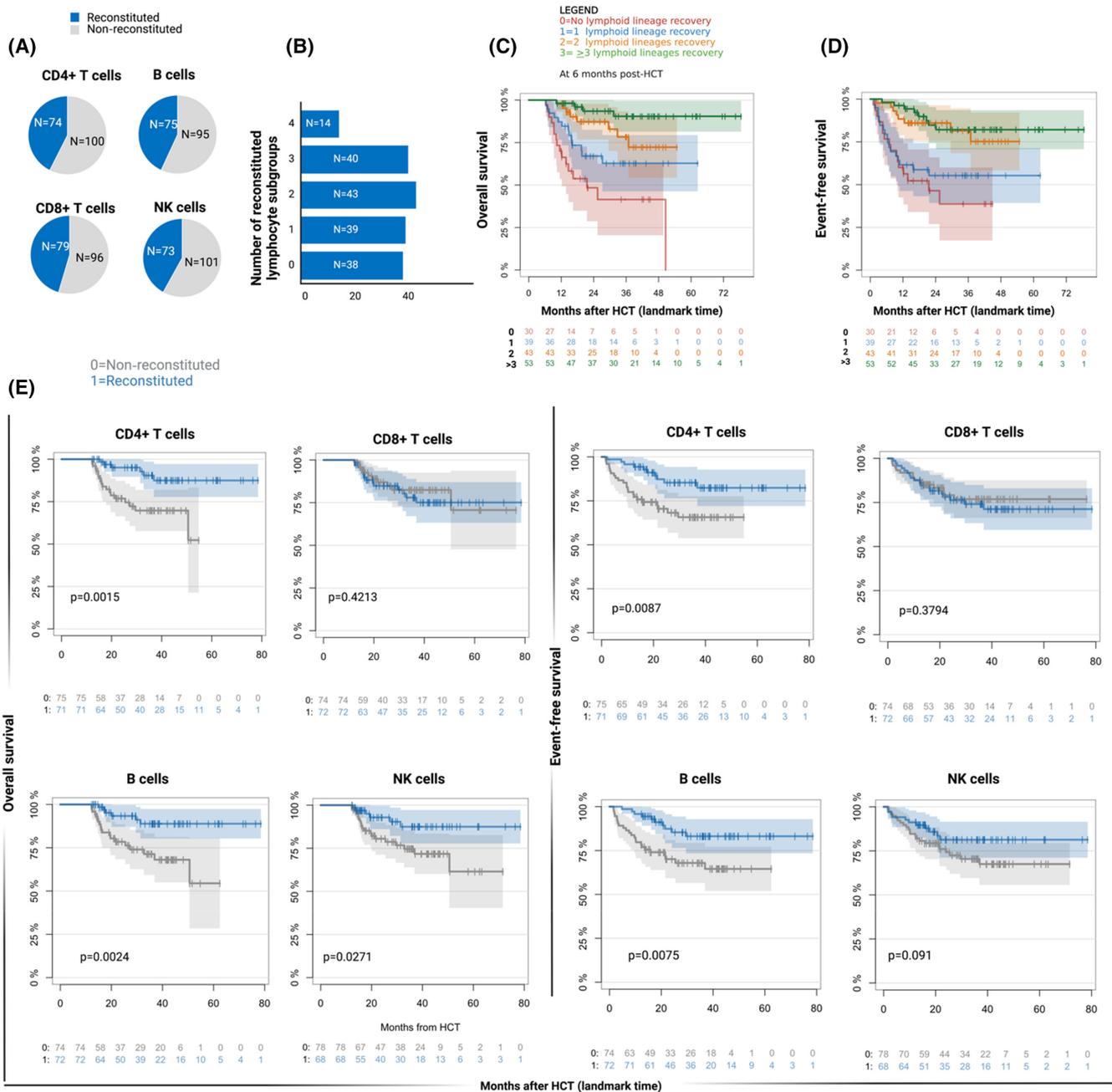


**FIGURE 3** Comparisons of immune reconstitution in adult and paediatric patients. (A–F) Distribution of immune subsets absolute counts across post-transplant periods for adult (pink) and paediatric (light blue) groups. The lines indicate the projection of the smoothed conditional means and their relative confident intervals.  $p$ -value are calculated using the Wilcoxon rank-sum test comparing the absolute value distributions of the two groups for each time point. (G) Pie charts showing the distribution of T, B and NK subsets for each time point in adult and paediatric populations.

## Immunological recovery patterns and outcome prediction

We then sought to understand how immune reconstitution impacted survival and which pretransplant factors could influence post-transplant immune cell reconstitution. Therefore, we built a series of multivariate general logistic regression models including clinically relevant variables at baseline to track their independent role in late immune recovery. For each cell subgroup, we categorized patients

in good and poor ‘reconstitutors’ using as thresholds the 50th percentile of the absolute numbers of each cell type at 12 months or, in absence of this parameter, the 75th percentile at 6 months (Table S2). This way we could identify  $N=74$ , 79, 75 and 73 patients restoring CD4, CD8, B- and NK-cell subpopulations, respectively (Figure 4A). Of them, only 14 patients had a complete 4-lineage reconstitution, whereas 40 reconstituted 3 cell subtypes, 43 recovered 2 lines, 39 restored only one cell type and 38 had a poor immune reconstitution without any lymphoid recovery (Figure 4B). When



**FIGURE 4** Impact of immune reconstitution on outcomes. (A) Pie charts showing the distribution of good and poor reconstitutors in each lymphoid subset. (B) Bar chart indicating the patterns of immune reconstitution and the number of lines reconstituted at 1-year post-transplant. (C, D) Probability of overall and event-free survival according to the number of immune reconstituted lines. (E) Probability of overall and event-free survival according to the type of immune reconstitution.

we analysed with univariate Kaplan–Meier estimates survival outcomes of patients with  $\geq 6$  follow-up (landmark time at 6 months:  $N = 165$ ), those who had reconstitution of at least 3 lymphoid cell subsets after 6 months had the better OS (HR = 0.08 [95% CI 0.02–0.24],  $p < 0.0001$ ) and EFS (HR = 0.18 [95% CI 0.08–0.44],  $p = 0.0001$ ) than other groups (Figure 4C,D).

Nonetheless, in this analysis, the only fact of having at least recovered one cell subset configured an improvement in terms of survival probabilities as compared to the complete absence of reconstitution (OS: HR = 0.46 [95% CI 0.22–0.96],  $p = 0.04$ ; Figure 4C).

A breakdown of this effect on single immune lineage constituents showed that both CD4+ T-cell and B-cell recovery were major contributors to the improved probabilities of survival (OS and EFS), with NK cells impacting only on OS (Figure 4E), while CD8+ T-cell reconstitution did not influence survival outcomes.

As well, cause-specific cox regression models of chronic GVHD and relapse capturing the events after 6 months of follow-up showed the impact of a full immunological recovery on the probability of each late event (Figure S5A,B and Table S3). While no impact on late CMV reactivations, the probability of EBV infections was lower in case of reconstitution of more than 2 lymphocyte lineages (Figure S5C,D, Table S3). Similarly, with the same landmark features, the risk of late bacterial infectious events showed a decrease directly proportional to the number of recovered lymphocyte subsets (Figure S5E, Table S3).

### Factors impacting single lineage immune reconstitution

Multivariate analysis of factors at baseline influencing post-transplant recovery of single lymphoid lineages showed that recipient age had a negative impact on CD4+ T-cell reconstitution (odds ratio, OR: 0.96, 95% CI 0.93–0.99,  $p = 0.023$ , Figure 5A), while CD8+ T-cell recovery strongly correlated with recipient CMV-positive serological status (OR: 4.41, 95% CI 1.98–10.37,  $p < 0.0001$ ). Interestingly, peripheral blood stem cells (PBSC) were independently associated with poor T-cytotoxic reconstitution (OR: 0.09, 95% CI 0.02–0.39,  $p = 0.002$  Figure 5B). Higher recipient age and donor-positive EBV serostatus were instead negative predictors of B-cell recovery (OR: 0.31, 95% CI 0.11–0.84,  $p = 0.025$ , Figure 5C), while none of the studied factors was found to influence NK-cell restoration (Figure 5D). Nonetheless, conditioning regimen, T-cell depletion, disease type, donor age and graft product cellularity were not found to influence the recovery of any of the lymphoid cell types in these multivariate models, pointing out the presence of other recipient factors at the basis of late post-transplant immune reconstitution.

Focusing on the effect of virus reactivation, while number of CD8 lymphocytes at day +30 did not influence the probability of CMV reactivation within 1-year post-transplant (landmark time captured at 1 month), intriguingly, patients

who reactivated CMV during the first post-HCT year showed a higher number of CD8+ T cells at 1 year,  $p < 0.0001$  (Figure S6A,B), likely mirroring the effect of an oligoclonal antigen-related T-cell expansion. Similarly, B-cell recovery at 1 month did not influence the probability of EBV reactivation within the first year and prolonged B-cell impairment was seen regardless of EBV and Rituximab use (Figure S6C–E). Nevertheless, patients reactivating EBV and receiving anti-CD20 therapies presented lower IgM levels at 12 months (Figure S6F).

Not surprisingly, when we investigated the impact of alloreactive events on T and B reconstitution, we observed that patients with prior acute grade II–IV GvHD displayed worse CD4+ ( $p = 0.00451$ ) and B-cell recovery ( $p = 2.7e-06$ ) at 3 months. As well, in case of chronic moderate or severe GvHD occurrence within the first-year post-transplant, 12-month CD4+ and B-cell counts were dramatically lower than those of patients not experiencing this event ( $p = 0.00962$  and  $p = 0.00083$ , respectively). Also, patients with early relapses showed inferior T CD4 recuperation ( $p = 0.01813$ ) at 1 year. Importantly, none of these events seem to impact T CD8+ recovery (Figure S7A–C).

## DISCUSSION

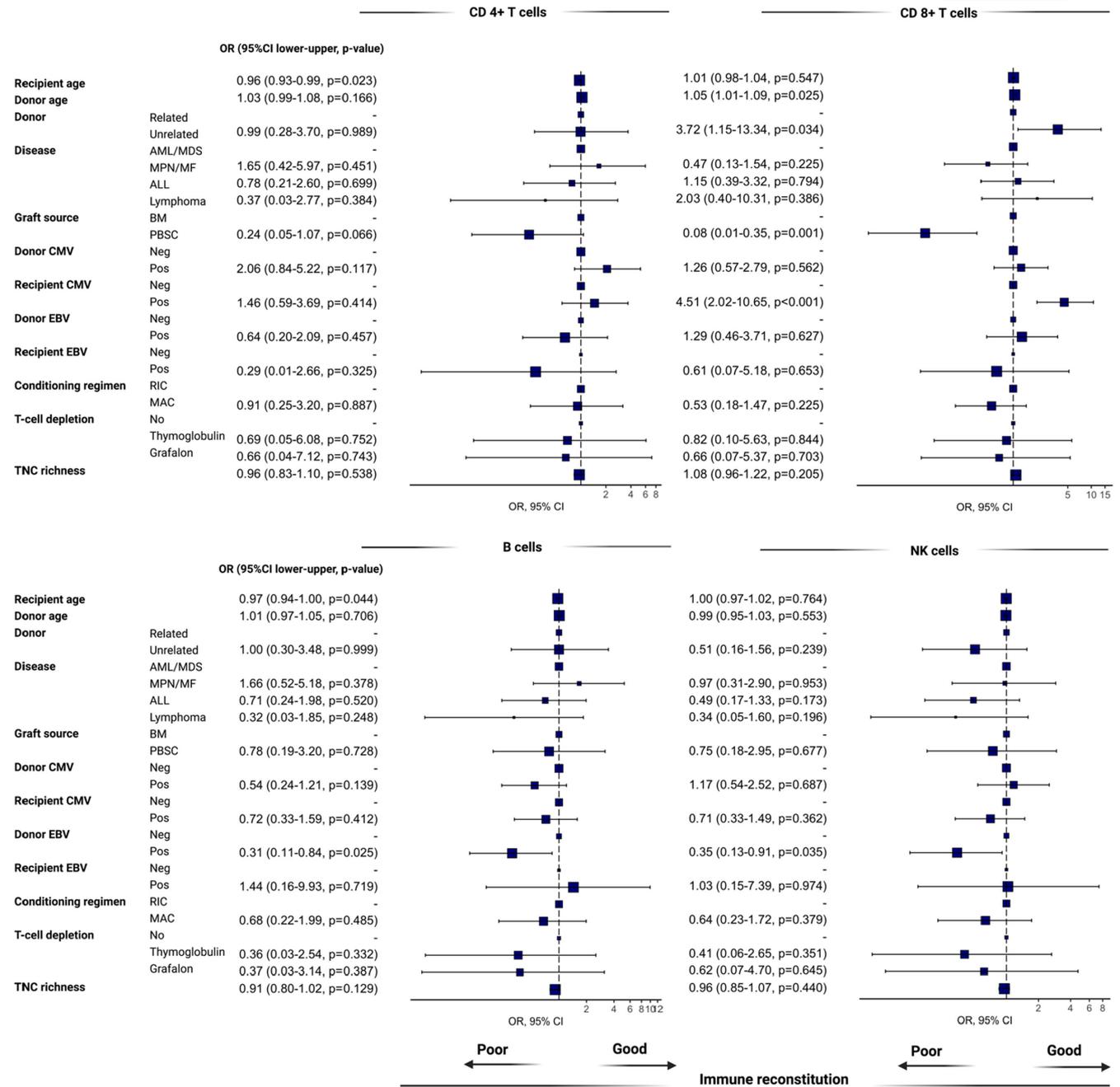
A competent immune system ensures tumour control, immune tolerance and anti-infectious responses. Failure to achieve a complete immune reconstitution is considered a risk factor unbalancing the success of allogeneic transplant procedures, losing disease control, increasing the risk of GvHD and impeding a successful pathogen clearance.

However, the complexity of such a multistep process is far from being completely elucidated and the impact of immune cell dynamics on post-transplant outcomes, as well as the factors predisposing to better immunological recovery remain poorly investigated.

Here, by deploying a longitudinal flow-based assessment of immune cells and deconvoluting granular clinical information of a combined adult and paediatric cohort of transplanted patients, we were able to reconstruct the dynamics and distribution of the principal immune cells intervening in this cross-talk.

We identified age-related patterns in single immune cell subsets, revealing a more important weight for recipient than for donor age especially for post-transplant CD4+ T-cell recovery.

This behaviour was confirmed in multiple univariate and multivariate statistical models, indicating that, despite underlying disease, graft source and transplant platforms, late T helper reconstitution may be mostly dependent on recipient thymic functionality, less efficient in middle age and older patients.<sup>23,24</sup> It is not a case that paediatric patients display a clear superiority in clinical outcomes. To the best of our knowledge, this is the first study formally comparing outcomes and immune reconstitution data in children versus adults in a transplant cohort. Despite intrinsic differences in



**FIGURE 5** Multivariate logistic regression models evaluating the impact of baseline patient and transplant characteristics on immune cell subsets. ALL, acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; BM, bone marrow; CI, confident interval; MAC, myeloablative conditioning; MDS, myelodysplastic syndromes; MF, myelofibrosis; MPN, myeloproliferative neoplasms; OR, odd ratio; PBSC, peripheral blood stem cell; RIC, reduced-intensity conditioning; TNC, total nucleated cells.

disease distribution and transplant platforms between adult and paediatric cohorts, reflecting different epidemiological and clinical landscapes of patients undergoing allo-HCT, with children receiving in general more intensive conditioning regimens, less rich stem cell grafts (BM in most of cases) and less T-depleting procedures, we were able to ascertain several key immunological aspects: paediatric patients reconstitute faster and more competent CD4+ T- and B-cell compartments than adults, while no age-related differences are seen in CD8+ T- and NK-cell subsets. This age-related

pattern is not relevant in adults, indicating that the advantage acquired on capacity and speed in immune reconstitution is meaningful in the first part of life.

The key role of CD4+ T-cell control has been shown in many disease settings as part of anti-tumour surveillance programmes<sup>25</sup> able at the same time to modulate immune tolerance,<sup>26</sup> and it is possible that these processes actively operate also in post-transplant setting. In line with these observations, one of the major immune escape mechanisms described as a driver of leukaemia relapses is the genomic

or transcriptomic loss of class II HLA molecules, decreasing antigen presentation to CD4<sup>+</sup> T-cell specificities.<sup>14,27,28</sup> Accordingly, CD4<sup>+</sup> T-cell count was a strong predictor of OS and EFS and was strongly impacted by dysfunctional alloreactive events including acute and chronic GvHD and disease recurrence.

Intriguingly CD8<sup>+</sup> T-cell compartment was found to be dependent on recipient CMV serostatus. This particular aspect may rely on the activation of T-cytotoxic cells against specific viral antigens leading to a dysfunctional expansion of anti-CMV specificities. As a consequence, we observed that patients with higher CD8<sup>+</sup> T-cell counts at 12 months were those prior diagnosed with a significant CMV DNAemia. We and others previously demonstrated the impact of CMV serostatus on the diversity of post-transplant T-cell receptor (TCR) repertoire,<sup>17,29</sup> with oligoclonal expansion of more CD8<sup>+</sup> T-cell clonotypic specificities often dominating the immune responses. This polarization may be seen also in healthy subjects and contribute together with immunogenetic factors to encode TCR repertoire diversity.<sup>30</sup> Accordingly, the improvement of CD8<sup>+</sup> T-cell counts did not show in our analysis any impact on survival outcomes, possibly because of this strong CMV bias on cytotoxic T-cell counts rendering this immune subset inapt to be considered as a parameter of immune recovery, unless characterized via other molecular platforms, able to identify specificity of single T-cell clones. To corroborate this finding, we did not observe any modification of this compartment after GvHD or relapse occurrence.

Interestingly, the impact of graft source was only seen in multivariate analysis for CD8<sup>+</sup> cell recovery, with, surprisingly, bone marrow associated with the highest CD8<sup>+</sup> post-transplant counts.

If it is true that this aspect is in contrast with historical (mainly pre-2000) literature, we need to acknowledge the huge changes in practices over the last 20 years (i.e. enrichment in fludarabine-based regimens, less aggressive radiotherapy protocols, more frequent use of in vivo T-cell depletion by ATG, more fine-tuned procedures of HSC harvest and donor selection, etc.), which could have contributed to similar divergences in our study as compared to older transplanted cohorts. Nevertheless, the lack of impact on T-cell depletion by ATG on late T-cell reconstitution is in line with previous literature showing that ATG mostly exerts its effect within the first months post-transplant, with a strong depletion of naïve T cells, compensated by a fast expansion of memory T cells.<sup>31,32</sup>

B cell and Ig M recoveries also exhibited an age-related pattern and were faster in children and young adults. It is intriguing to note that a negative predictor of humoral responses was donor EBV serostatus, and accordingly, patients reactivating EBV showed a worse trend in B cell and IgM repertoire, regardless of previous anti-CD20 exposure. This observation is in line with the effect of B-cell depleting therapies shown in other disease settings.<sup>33-36</sup>

Besides the differences in age, we showed the impact of immune recovery on clinical outcomes, demonstrating a

drop in survival when none of the immune subsets recovered within the first post-HCT year. The interesting aspect is that among all the lymphoid lineages, CD4<sup>+</sup> T and B cells were those mostly impacting survival probabilities. This aspect supports the major role of T helper and humoral responses in controlling disease recurrence and alloreactive complications after HCT, restraining again the role of CD8<sup>+</sup> T-cell specificities.

It is undoubtful that full immune reconstitution data, with lymphoid population characterization, should be implemented in clinical practice for routine follow-up; however, lack of solid and systematic evidence of the relationship with clinical factors and outcomes preclude the release of general recommendations from international consortia.

Altogether, these results enlighten new clinical-biological aspects of post-HCT immune recovery, underlining the impact of recipient age and pretransplant virus exposure on adaptive immune reconstitution, and support the utility of routine immuno-monitoring in both adult and paediatric post-HCT settings.

#### AUTHOR CONTRIBUTIONS

AJL, SP, CP and MTR designed the study; collected, analysed and interpreted the data; and wrote the manuscript. SP performed statistical analysis and data visualization; AC, ABN, GRG, MDA and CK were involved in patient management and sample collection; PF, PC and CS gave important intellectual inputs on the analysis and edit the manuscript; MDC and TAM performed flow-based immune monitoring.

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#### CONFLICT OF INTEREST STATEMENT

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### DATA AVAILABILITY STATEMENT

All the data that support the findings of this study are available within the supplementary files, and flow-based data and patient-level information used for the statistical analyses have been deposited in the following repository under restricted access: [10.5281/zenodo.7781262](https://doi.org/10.5281/zenodo.7781262). All the researchers willing to grant access to this dataset can address a request to Dr. S. Pagliuca ([s.pagliuca@chru-nancy.fr](mailto:s.pagliuca@chru-nancy.fr)).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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