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ARTICLE

Innate lymphoid cell recovery and occurrence of GvHD after hematopoietic stem cell transplantation

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Abstract

Lymphocytes are essential for microbial immunity, tumor surveillance, and tissue homeostasis. However, the *in vivo* development and function of helper-like innate lymphoid cells (ILCs) in humans remain much less well understood than those of T, B, and NK cells. We monitored hematopoietic stem cell transplantation (HSCT) to determine the kinetics of ILC development in both children and adults. It was found that, unlike NK cells, helper-like ILCs recovered slowly, mirroring the pattern observed for T cells, with normalization achieved at 1 year. The type of graft and the proportion of $CD34^+$ cells in the graft did not significantly affect ILC reconstitution. As HSCT is often complicated by acute or chronic graft-versus-host disease (GVHD), the potential role of ILC subsets in maintaining tissue integrity in these conditions was also analyzed. It was found that GVHD was associated with lower levels of activated and gut-homing $NKp44^+$ ILCP, consistent with a non-redundant role of this ILC subset in preventing this life-threatening disorder in lymphopenic conditions.

KEYWORDS
GVHD, HSCT, innate lymphoid cells

1 | INTRODUCTION

Innate lymphoid cells (ILCs) are the most recently discovered family of lymphocytes. They are characterized by a lack of rearranged Ag-specific receptors.[1,2] ILCs comprise NK cells, and three other main subsets of cells known as "helper-like" ILCs, because their diversity mirrors that of helper T cells.[1] ILC1 produces IFN-γ and TNF-α, ILC2 produces IL-4, IL-5, IL-13, and amphiregulin (AREG), and ILC3 secretes IL-22 or IL-17.[3,4] These cells are essentially tissue-resident and are involved in maintaining tissue homeostasis and repair,[5] but small numbers are found in the bloodstream.[6–8] In healthy individuals, 0.01–0.1% of circulating lymphocytes express a Lineage $(Lin)^-CD127^+$ ILC phenotype, and children have more ILCs than adults.[6] Cytokine genes are not transcribed in these cells in the peripheral blood of healthy individuals, consistent with an immature or naive status of these circulating ILCs.[9]

The reconstitution kinetics of human T cells, B cells, and NK cells after HSCT are well established.[10] However, much less is known about ILC reconstitution in humans. One study in adults reported that ILC recovery after HSCT was slow under myeloablative conditions in 51 patients with acute myeloid leukemia (AML).[11] In a previous study, we described ILC reconstitution in a small cohort of

Summary sentence: Slow recovery of circulating ILCP and reduced number of activated ILCP in patients with GVHD after HSCT.,

Abbreviations: aGVHD, acute graft-versus-host disease; AML, acute myeloid leukemia; AREG, amphiregulin; BM, bone marrow transplant; cGVHD, chronic graft-versus-host-disease; CHILP, common helper-like ILC progenitor; CILP, common innate lymphoid precursor; GVHD, graft versus host disease; HSCT, hematopoietic stem cell transplantation; ILC, innate lymphoid cells; ILCP, innate lymphoid cell progenitor; MPB, mobilized peripheral blood; SCID, severe combined immunodeficiency; UCB, umbilical cord blood.

12 children with severe combined immunodeficiency (SCID) or hemopathies undergoing HSCT in myeloablative conditions.[6] In both studies, information about the precise kinetics of ILC reconstitution was limited, as the monitoring of circulating ILCs was restricted to the first 12 weeks after HSCT for the adult cohort or was performed years after HSCT for the pediatric cohort.

Allogeneic HSCT is a key treatment for many blood cancers and nonmalignant blood cell disorders, such as sickle cell anemia, and its use is steadily increasing.[12,13] This treatment can be remarkable effective but is also associated with significant morbidity and mortality. In particular, it is often complicated by GVHD,[14] which may be acute (aGVHD) or chronic (cGVHD), the distinction being based on a threshold of 100 days post-graft.[15] The diagnosis of aGVHD is usually based on the presence of one or several of the following signs: a macular rash, an increase in bilirubin levels, and gastrointestinal tract involvement.[14] By contrast, cGVHD is more polymorphic, with the involvement of multiple organs, often resulting in permanent dysfunction.[16]

In this study, we investigated the kinetics of ILC recovery in adults and children after allogeneic HSCT for the curative treatment of a broad range of diseases. We also aimed to assess the correlation between the reconstitution of different ILC subsets and the occurrence of GVHD.

2 | MATERIALS AND METHODS

2.1 | Patients

The retrospective study included 91 adult patients from the CRYOSTEM cohort (Table 1). The CRYOSTEM cohort is a collection of biological samples from more than 5000 patients and 2000 donors initiated in 2011 to accelerate research into complications of HSCT. Samples were collected from all patients before allogeneic HSCT (R0). For patients subsequently presenting acute GVHD, samples were collected at diagnosis (A1) and 30 days later (A2). For patients presenting chronic GVHD, samples were collected at diagnosis (C1) and 90 days later (C2). For patients without GVHD, samples were collected 90 days (S1) and 365 days after HSCT (S2). Total blood counts for each patient were supplied by the CRYOSTEM project.

We performed a prospective study of ILC reconstitution after HSCT in 58 patients in collaboration with the pediatric oncohematology unit of Timone Hospital in Marseille. All patients undergoing HSCT in the unit between April 2015 and May 2017 were eligible. In total, 56 patients were included in this cohort. We included 27 of these patients in the reconstitution analysis, based on the availability of samples for at least day 45 and month 12 or month 18 (Table 2). We included 41 patients in the analysis of aGVHD occurrence according to ILC levels, based on the availability of a sample for day 45. Samples were collected before HSCT, and at day 0 (D0), D45, D90, 6 months (M6), M12, and M18 after HSCT (Table 3). Consent was obtained from the parents for the use of leftover blood from routine sampling for research purposes.

This study was performed in accordance both with French legal requirements (Art. L. 1243-1 and Art. L. 1245-2 of the Code de la Santé Publique) and legal requirements of CRYOSTEM (doi: 10.25718/cryostem-collection/2018).

We established normal ranges with data from 30 healthy adults and 27 healthy children. These healthy controls were undergoing routine blood testing before minor elective surgery. They had no current diseases or infections, and the analyses were performed on the blood left over after the routine tests had been performed, with consent from the patients or their families for this use of leftover blood for research purposes. These patients were the ones used in our previous publication.[6]

2.2 | GVHD definition and classification

We classified and graded aGVHD according to the 1994 consensus definition (Przepiorka et al., 1995). A cutoff point of 100 days was used to distinguish between aGVHD and cGVHD.[15]

2.3 | Samples

For the pediatric cohort, blood samples were collected into EDTA. Peripheral blood mononuclear cells (PBMCs) for the pediatric prospective cohort were isolated by density gradient centrifugation on lymphocyte separation medium (Eurobio). These cells were washed twice in RPMI-HEPES (Gibco) and maintained in complete RPMI medium (Gibco) containing 10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamine. Samples were processed immediately after collection. PBMCs from the CRYOSTEM cohort were stored in liquid nitrogen before processing. No significant changes in ILC distribution and numeration were observed when fresh and thawed PBMC were compared (Supplemental Fig. 1).

2.4 | Flow cytometry assay

Lymphocyte counts were obtained with BD Multitest (Becton Dickinson, San Diego, CA) containing anti-CD19 APC (clone SJ25C1, IgG1 kappa), anti-CD3 FITC (clone SK7, IgG1 kappa), anti-CD4 PE-Cy7 (clone SK3, IgG1 kappa), anti-CD8 APC-Cy7 (clone SK1, IgG1 kappa), anti- CD16 PE (clone B73.1, IgG1 kappa), anti-CD56 PE (clone NCAM16.2, IgG2b kappa), and anti- CD45 PerCP (clone 2D1, IgG1 kappa) antibodies. All reagents are listed in Supplemental Table 1.

ILC1 were defined as Lin-CD127$^+$ CD117$^-$ CRTH2$^-$. ILC2 were defined as Lin-CD127$^+$ CRTH2$^+$. ILCP were defined as Lin-CD127$^+$ CD117$^+$ CRTH2$^-$ and could express or not NKp46. NKp44$^+$ ILCP were defined as Lin-CD127$^+$ CD117$^+$ CRTH2$^-$ NKp46$^+$ NKp44$^+$. Activation markers, such as CD25 and CD69, and homing markers (CCR6 and CCR10) were also studied.

The samples were acquired on a LSR FORTESSA X20 cytometer from Becton Dickinson and analyzed with FlowJo software.

TABLE 1 Characteristics of the patients of the CRYOSTEM retrospective adult cohort ($n = 91$)

Variable	No-GVHD $n = 33$*	aGVHD $n = 33$	cGVHD $n = 25$
Age at HSCT			
Median age, y (range)	56 (16.26-69.71)	51.9 (18.06-66.27)	50.7 (21.55-69.05)
GVHD onset after HSCT			
Median days, y (range)		NA	129 (90-315)
Diagnosis, n (%)			
Acute leukemia	16 (48.5%)	9 (27.3%)	12 (48%)
MDS/MPN	5 (15.2%)	8 (24.2%)	2 (8%)
Chronic leukemia	3 (9.1%)	4 (12.1%)	1 (4%)
Lymphoma	6 (18.2%)	7 (21.2%)	7 (28%)
Plasma cell disorders	1 (3.0%)	3 (9.1%)	3 (12%)
Bone marrow failure	–	1 (3.0%)	–
Solid tumors	–	1 (3.0%)	–
Graft type, *n* (%)			
Bone marrow	5 (15.2%)	–	2 (8%)
Cord blood	–	3 (9.1%)	–
Peripheral blood	26 (78.8%)	30 (90.9%)	23 (92%)
Donor type, *n* (%)			
Unrelated	14 (42.4%)	6 (18.2%)	6 (24%)
Identical sibling	12 (36.4%)	13 (39.4%)	16 (64%)
Matched unrelated	3 (9.1%)	7 (21.2%)	1 (4%)
Mismatched relative	–	3 (9.1%)	–
Mismatched unrelated	2 (6.1%)	4 (12.1%)	2 (8%)
Conditioning regimen, *n* (%)			
Myeloablative	13 (39.4%)	13 (39.4%)	11 (44%)
Non myeloablative	18 (54.6%)	20 (60.6%)	14 (56%)
Engraftment, *n* (%)			
Yes	31 (93.9%)	33 (100%)	25 (100%)
No	–	–	–
Relapse, *n* (%)			
Yes	5 (15.2%)	7 (21.2%)	4 (16%)
No	26 (78.8%)	26 (78.8%)	21 (84%)
Death, *n* (%)			
Dead	2 (6.1%)	15 (45.5%)	6 (24%)
Alive	29 (87.9%)	18 (54.5%)	19 (76%)
GVHD prophylaxis, *n* (%)			
CSA	5 (15.5%)	3 (9.1%)	3 (12%)
CSA + Cyclophosphamide	1 (3.0%)	1 (3.0%)	–
CSA + MTX	17 (51.5%)	7 (21.2%)	11 (44%)
CSA + MMF	7 (21.2%)	19 (57.6%)	11 (44%)
MTX + Tacrolimus	1 (3.0%)	–	–
CSA + ATG	–	1 (3.0%)	–
MTX + ATG	–	1 (3.0%)	–

CSA, cyclosporine A; MMF, mycophenolate mofetil; MTX, methotrexate; CS, corticosteroids; ATG, anti-thymoglobulin antibody; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; * Clinical data available for 31 patients, NA: not available.

TABLE 2 Characteristics of the patients from the prospective pediatric cohort for the analysis of ILC reconstitution ($n = 27$)

Variable	Patients $N = 27$
Age at HSCT	
Median age, y (range)	8,38 (0,3-18)
Gender, *n* (%)	
Female	11 (40.7%)
Male	16 (59.3%)
Donor type, n (%)	
Matched related	15 (55.6%)
Matched unrelated	7 (25.9%)
Mismatched related	1 (3.7%)
Mismatched unrelated	4 (14.8%)
Stem cell source, *n* (%)	
Bone marrow	20 (74.1%)
Cord blood	–
Peripheral blood	6 (22.2%)
Bone marrow + Peripheral blood	1 (3.7%)
Myeloablative treatment, *n* (%)	
Yes	22 (81.5%)
No	5 (18.5%)
aGVHD onset after HSCT	
Median days, y (range)	25 (11-45)
GVHD *n* (%)	
No	15 (55.6%)
Acute GVHD only	7 (25.9%)
Chronic GVHD only	1 (3.7%)
Acute and Chronic GVHD	4 (14.8%)
GVHD prophylaxis, *n* (%)	
CSA	9 (33.3%)
CSA+MMF	9 (33.3%)
CSA+MTX	7 (25.9%)
CSA+CS	1 (3.7%)
Cyclophosphamide/Endoxan+CSA+MMF	1 (3.7%)
Malignant disease, *n* (%)	
Yes	17 (63.0%)
No	10 (37.0%)
Diagnosis, *n* (%)	
Acute leukemia	15 (55.6%)
Lymphoma	1 (3.7%)
Thalassemia	3 (11.1%)
Fanconi anemia	1 (3.7%)
Medullar aplasia	1 (3.7%)
Sever aplastic anemia	2 (7.4%)
Blackfan Diamond syndrome	1 (3.7%)
Mucopolysaccharidosis	1 (3.7%)
Myeolodysplasia	1 (3.7%)
Immunodeficiency	1 (3.7%)

GVHD, graft-versus-host disease; CSA, cyclosporine A; MMF, mycophenolate mofetil; MTX, methotrexate; CS, corticosteroids.

2.5 | Statistical analysis

Statistical analyses were performed with Prism 7 software (GraphPad Software, San Diego, CA). Kruskal-Wallis tests were performed for comparisons of more than two independent samples, Mann–Whitney tests were performed for comparisons of 2 independent samples; Wilcoxon tests were performed for comparisons of 2 paired samples, Spearman's rank correlation tests were performed for correlation analyses. The *t*-test comparison for Figure 4C was performed with MeV Software. *P*-values ≤ 0.05 were considered significant. All box plots show medians with the 10th and 90th percentiles

3 | RESULTS AND DISCUSSION

3.1 | Helper-like ILC reconstitution after HSCT mirrors that of T cells

ILC ontogeny is a rapidly progressing field in which knowledge remains incomplete. In the mouse, the earliest progenitors of ILCs are common lymphoid precursor-derived cells known as common innate lymphoid precursors (CILPs), which cannot produce T and B cells. CILPs differentiate into common helper-like ILC progenitors (CHILPs), which can give rise to all helper-like ILCs but not NK cells. Finally, CHILPs give rise to ILC progenitors (ILCPs), which generate helper-like ILC1, ILC2 and ILC3, and appear to retain the potential to generate NK cells, as recently reported.[17–26] ILC development in humans is less well characterized. Circulating progenitors resembling mouse CILPs have been identified in the fetal liver, blood, cord blood, and secondary lymphoid organs.[9,27] A multiple-wave model for ILC development is emerging.[28] The first wave is the seeding of peripheral tissues with ILC precursors during development. In the second wave, which extends from just before birth to weaning, the ILC precursors expand locally and complete their development, acquiring lineage-specific features and tissue-specific phenotypes.[29] These cells form local niches, in which they are sustained by local division, differentiation from local precursors, and various degrees of repopulation from bone marrow or cells from other tissues redifferentiating in stress conditions during adulthood.[28] This model is reminiscent of that for tissue-resident Mϕs, which includes the dissemination of primitive yolk sac-derived Mϕs, fetal liver monocyte dissemination, and Mϕs originating from bone marrow hematopoietic stem cells.[30–33] HSCT provided us with a remarkable opportunity to study ILC development *in vivo* in humans.

TABLE 3 Characteristics of the patients of the prospective pediatric cohort in which the occurrence of aGVHD according to helper-like ILC levels was investigated ($n = 41$)

Variable	aGVHD $N = 15$	No GVHD $N = 26$
Age at HSCT		
Median age, y (range)	9.2 (1-16)	6.25 (0-18)
Gender, *n* (%)		
Female	4 (26.7%)	14 (53.8%)
Male	11 (73.3%)	12 (46.2%)
Donor type, *n* (%)		
Matched related	6(40%)	11 (42.3%)
Matched unrelated	4 (26.7%)	5 (19.2%)
Mismatched related	1 (6.7%)	2 (7.7%)
Mismatched unrelated	4 (26.7%)	4 (15.4%)
Stem cell source, *n* (%)		
Bone marrow	9 (60%)	19 (73.1%)
Cord blood	1 (6.7%)	–
Peripheral blood	5 (33.3%)	6 (23.1%)
Bone marrow + Peripheral blood	–	1 (3.8%)
Myeloablative treatment, *n* (%)		
Yes	13 (86.7%)	22 (84.6%)
No	2 (13.3%)	4 (15.4%)
aGVHD onset after HSCT		
Median days, y (range)	27 (11-52)	
aGVHD grade, *n* (%)		
Grade 1	10 (66.7%)	–
Grade 2	1 (6.7%)	–
Grade 3	1 (6.7%)	–
Grade 4	2 (13.3%)	–
Grade ¿	1 (6.7%)	–
Affected organs by aGVHD, *n* (%)		
Skin	11 (73.3%)	–
Liver	1 (6.7%)	–
Skin+Liver	1 (6.7%)	–
Skin+Gut	1 (6.7%)	–
Skin+Liver+Gut	1 (6.7%)	–
GVHD prophylaxis, *n* (%)		
CSA	4 (26.7%)	9 (34.6%)
CSA+MMF	6 (40%)	6 (23.1%)
CSA+MTX	4 (26.7%)	7 (26.9%)
CSA+CS	–	2 (7.7%)
Cyclophosphamide/Endoxan+CSA	1 (6.7%)	1 (3.8%)
CS+ CD3 depletion of the graft	–	1 (3.8%)
Malignant disease, *n* (%)		
Yes	10 (66.7%)	12 (46.2%)
No	5 (33.3%)	14 (53.8%)

(Continues)

TABLE 3 (Continued)

Variable	aGVHD *N* = 15	No GVHD *N* = 26
Diagnosis, *n* (%)		
Acute leukemia	8 (53.3%)	11 (42.3%)
Chronic leukemia	1 (6.7%)	–
Lymphoma	1 (6.7%)	–
Myeloproliferative syndrome	–	1 (3.8%)
Immunodeficiency	3 (20%)	5 (19.2%)
Thalassemia	1 (6.7%)	2 (7.7%)
Fanconi anemia	1 (6.7%)	–
Medullar aplasia	–	1 (3.8%)
Severe aplastic anemia	–	3 (11.5%)
Diamond-Blackfan anemia	–	1 (3.8%)

CSA, cyclosporine A; MMF, mycophenolate mofetil; MTX, methotrexate; CS, corticosteroids.

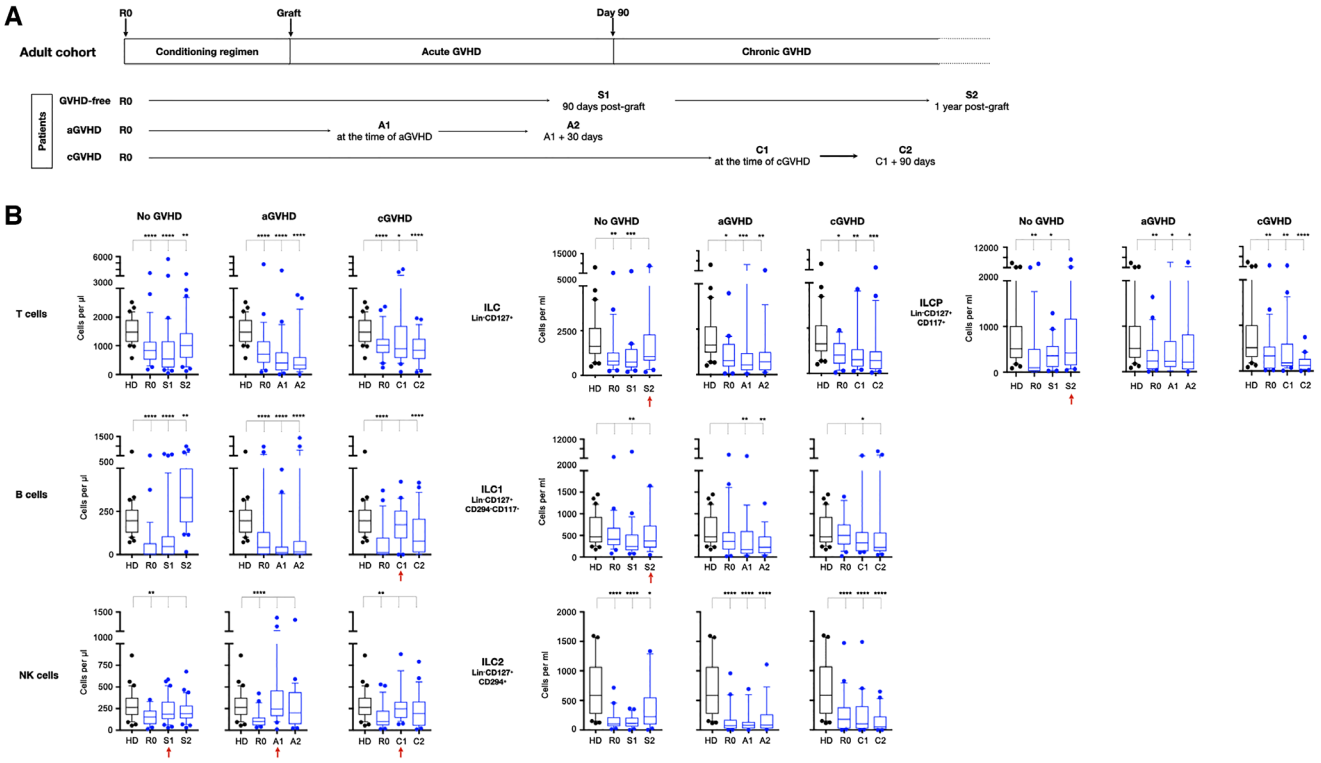

FIGURE 1 Lymphocyte lineage recovery after HSCT in adults. (A) Timeline for the CRYOSTEM cohort. (B) Kinetics of lymphocyte recovery. Black boxes represent healthy individuals. The red arrow represents the normalization timepoint for each cell type. Comparisons were made with healthy controls (in the no GVHD group: $n = 22$ at R0, $n = 22$ at S1, $n = 20$ at S2; in the aGVHD group: $n = 20$ at R0, $n = 18$ at A1, $n = 16$ at A2; in the cGVHD group: $n = 21$ at R0, $n = 20$ at C1, $n = 20$ at C2, for healthy donors $n = 30$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).
HD = healthy donors

For this study of ILC reconstitution after HSCT in adult patients, we obtained samples from 91 adult patients from the CRYOSTEM cohort: 33 without GVHD, 33 with aGVHD, and 25 with cGVHD (**Table 1**). In all groups, blood samples were taken before the conditioning regimen and HSCT (R0 timepoint) (Figure 1A). In the GVHD-free group, blood samples were taken 90 days (S1) after HSCT and 1 year after HSCT (S2). In the aGVHD group, blood samples were taken at aGVHD diagnosis (A1) and 30 days later (A2). In the cGVHD group, blood samples were

taken at cGVHD diagnosis (C1) and 90 days later (C2). At R0, ILC1 levels in adult patients were similar to those in healthy controls, whereas the frequencies of T cells, B cells, NK cells, ILC2, and ILCP before HSCT were significantly below the normal range in all groups (Figure 1B). Hematological diseases are indeed often associated with lymphopenia, and a similar phenomenon has already been reported for T and B cells, in particular.[34]

We assessed lymphocyte reconstitution in the no-GVHD group. T-cell levels did not reach the lower limit of the normal range within one year, B-cell levels increased to above the normal range at 1 year, and NK cell levels reached the normal range by day 90, falling slightly, but significantly, by one year. For helper-like ILCs, we found that ILC1 and ILCP levels reached the normal range by one year, whereas ILC2 levels were just below the normal range at this time point.

In the aGVHD group, T- and B-lymphocyte levels remained significantly below the normal range at both timepoints, whereas NK cell levels were already in the normal range by A1, remaining within this range at A2, consistent with previous findings.[35] The levels of all helper-like ILC subtypes were significantly lower than those in control subjects at both A1 and A2. In the cGVHD group, T-lymphocyte levels were significantly lower than those in healthy subjects, both at cGVHD diagnosis and 90 days later. B-cell levels had already reached the normal range by C1, but this increase was not maintained at C2. NK cell levels were in the normal range at both time points, as expected.[35] The levels of all helper-like ILC subtypes were significantly below the normal range at both time points.

Early normalization of NK cell levels is a well-known phenomenon, occurring about 30 days after HSCT.[35] Our results are concordant with this, as NK cells had normalized at aGVHD diagnosis, which occurred roughly 30 days after HSCT. Our finding of an abnormally high B-cell frequency 12 months after HSCT is also consistent with published observations,[36,37] as is the late normalization of T-cell levels, at 12 months, in adults.[38] The only other study to monitor ILC reconstitution after HSCT to date concerned a total of 51 patients, 49 of whom had AML.[11] In this previous study, the authors first obtained longitudinal determinations of total circulating helper-like ILC levels after allogeneic HSCT in six of the 49 patients with AML. These determinations highlighted the incompleteness of total helper-like ILC reconstitution at 6 months, the time point corresponding to the end of follow-up. For confirmation of these results, the authors then studied circulating ILCs in a larger cohort of 40 of the 49 AML patients. They investigated the levels of all helper-like ILC subsets before the conditioning regimen and HSCT, and at 12 weeks after HSCT (median of 84 days after allogeneic HSCT, range: day 56-day 112). ILC2, ILC1, and NKp44$^+$ILCP counts were very low. Our findings are consistent with these results and add additional information, in that they highlight a recovery time of one year for ILC1 and ILCP, and of more than 1 year for ILC2. Furthermore, as our patients suffered from a broad spectrum of diseases, our data extend findings for ILC reconstitution beyond AML-related HSCT.

We also investigated ILC reconstitution after HSCT in children. We studied lymphoid lineage reconstitution over a period of 18 months in a population of 27 pediatric patients (**Table 2**). In this population, NK cell levels normalized by 45 days after HSCT, T-cell levels normalized within 6 months and B-cell levels normalized within 12 months. ILC reconstitution was slow. ILC1 were the first helper-like ILC to recover, 6 months after HSCT, with the recovery of ILC2 and ILCP not observed until 12 months after HSCT (Figure 2). For ILC2, two distinct subsets have been defined based on differential expression of CD117 and distinct plasticity.[39] In our 2 cohorts, both subsets had similar reconstitution kinetics although CD117$^-$ ILC2 were largely predominant (Supplemental Figure 2). Along the same line, NKp46$^+$ and NKp46$^-$ ILCP harbored similar kinetics of reconstitution but NKp46$^+$ ILCP were largely predominant in the pediatric cohort (Figure 2).

ILC levels have been assessed after HSCT in children in only 1 previous study focusing on ILC recovery in patients with SCID, but ILC levels were not monitored closely after HSCT, and the data were often obtained years after transplantation.[6] The cohort studied here is thus the first cohort of pediatric patients suffering from a broad spectrum of malignant and non-malignant diseases for which ILC monitoring for 18 months after allogeneic HSCT has been reported. Our findings for children were consistent with those for adults, with low frequencies of T cells, B cells, ILC2, and ILCP recorded before HSCT. T-cell levels have already been shown to normalize more rapidly in children than in adults after HSCT.[38] We show here that, in both cohorts and regardless of the clinical conditions presented by the patients, the pattern of helper-like ILC recovery mirrors that of T cells, rather than that of cytotoxic ILCs (i.e., NK cells).

3.2 | Helper-like ILC reconstitution is not influenced by the proportion of CD34$^+$cells in the graft or graft origin

After establishing the timelines for ILC reconstitution in children and adults, we investigated whether these kinetics were influenced by the origin of the graft, as no comparisons of helper-like ILC reconstitution with grafts of different origins have ever been performed. In adults, we compared helper-like ILC reconstitution in our GVHD-free group at the three available timepoints between patients undergoing mobilized peripheral blood CD34$^+$ grafts (MPB) and patients undergoing bone marrow grafts (BM). No difference was found between these two groups at any of the timepoints (before allograft, 90 days later, and 1 year later). However, this finding should be interpreted with caution because there were only 2 patients in the BM HSCT group (Figure 3A). In children, we compared helper-like ILC reconstitution 45 days, 90 days, and 6 months after transplantation between children undergoing umbilical cord blood (UCB) and BM HSCT. We found no significant different in ILC reconstitution at any of these timepoints (Figure 3B). We also compared ILC levels on day 90 between adult patients undergoing MPB HSCT and children undergoing UCB HSCT. We found a significant difference only for the ILC2 subset, for which levels were lower in adults than in children ($P = 0.0415$). However, this difference cannot definitely be attributed to the difference in graft source between these two groups of patients, because ILC2 levels also are lower in healthy adults than in healthy children.[6] We thus did not detect an influence of graft origin on helper-like ILC reconstitution but this point needs to

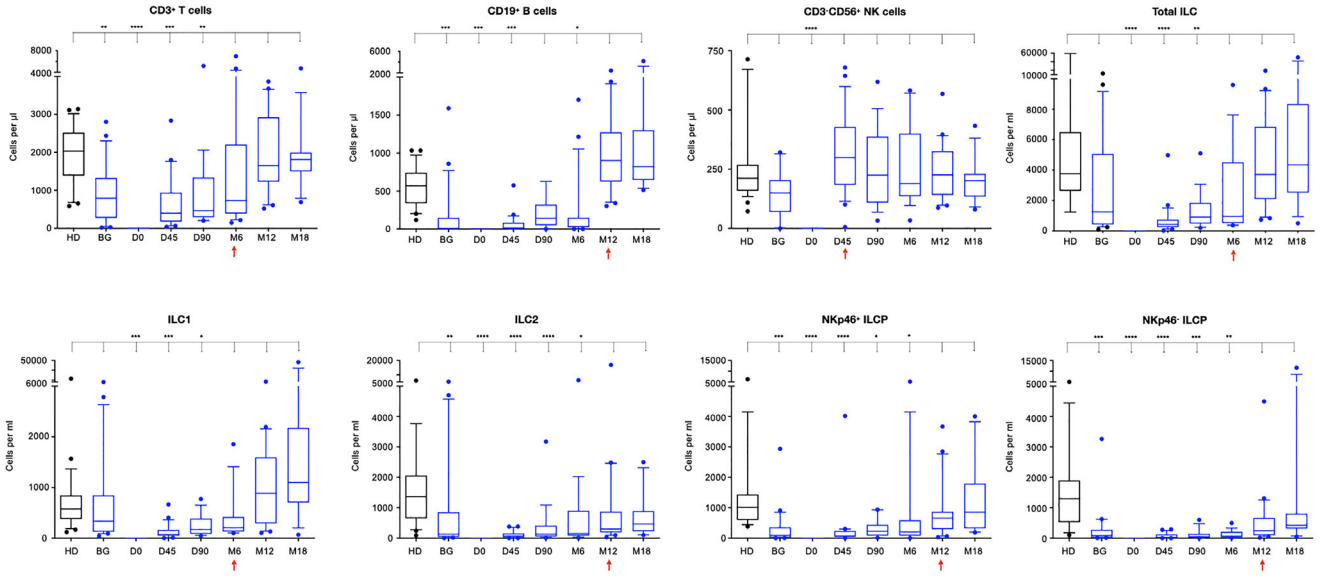

FIGURE 2 Lymphocyte lineage recovery after HSCT in children. The red arrow represents the normalization timepoint for each cell type. Comparisons were made with healthy controls. ($n = 27$ for healthy donors, $n = 22$ for BG, D0, D45, $n = 18$ for D90, $n = 21$ for M6, $n = 20$ for M12, $n = 14$ for M18; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). HD, healthy donors (black boxes); BG, before graft; D0, day 0; D45, day 45; D90, day 90; M6, month 6; M12, month 12; M18, month 18

be confirm in a larger cohort. Indeed, this result contrasts with published *in vitro* data showing that the speed of ILC reconstitution, and the ILC levels reached, were highest for UCB, followed by MPB and then BM.[40] We investigated the causes of this discrepancy, by testing the hypothesis that the proportion of $CD34^+$ cells in the graft might influence helper-like ILC reconstitution. We assessed the possible correlation between $CD34^+$ cell levels and the numbers of helper-like ILCs of the various subsets in our pediatric cohort. We found no correlation at either of the time points considered, for any of the helper-like ILC subtypes (Figure 3C). Finally, as ILC reconstitution was observed in all our patients, we considered the minimal dose of $CD34^+$ cells received by patients. The lowest dose was 0.5×10^5 $CD34^+$/kg for UCB grafts, but 21×10^5 $CD34^+$/kg for BM grafts. These numbers can therefore be considered the minimal dose of $CD34^+$cells for grafting in humans to ensure helper-like ILC reconstitution. It is obvious that other factors such as the type of disease or conditioning regimen have an impact on ILCP reconstitution after HSCT. However, one can consider a strength of the manuscript, the fact that our results are valid across various HSCT indications. In addition, the size of the cohort did not allow us to stratify the patients according to their disease conditions prior to HSCT, as it will compromise the strength of the subsequent statistical analysis.

3.3 | Low activated ILCP and $NKp44^+$ ILCP frequency is associated with GVHD occurrence and severity

We then investigated the possible association of ILC counts with the occurrence of both aGVHD and cGVHD. Indeed, a previous study reported lower counts of various ILC subsets ($CD69^+$ ILC1, $CD69^+$ ILC2, and $CD69^+$ $NKp44^+$ILCP) in patients with aGVHD.[11] An association between expression of the gut homing marker $\alpha4\beta7$ on $NKp44^+$ILCP, and gut aGVHD occurrence was also detected.[11] No data are available regarding the association of ILC counts with the occurrence of cGVHD.

We thus used our cohort of patients to investigate the possible association of activation markers on ILCP with the occurrence of cGVHD. As cGVHD, by definition, occurs at least 100 days after HSCT, we considered a comparison of the S1 and S2 (90 days and 1 year after HSCT in the no-GVHD group, respectively) and C1 and C2 (at the time of cGVHD and 90 days after cGVHD diagnosis, respectively) samples to be the most relevant. The S2 versus C2 analysis revealed that lower numbers of $NKp44^+$ ILCP, $CD25^+$ ILCP, and $CCR6^+$ ILCP are associated with increased occurrence of cGVHD (Figure 4A). Helper-like ILC lymphopenia in the cGVHD group might theoretically have been due to the effects of cGVHD treatments, as sampling occurred after cGVHD diagnosis. However, no such phenomenon was observed in the aGVHD group, despite the shorter time interval between the aGVHD diagnosis sample and the subsequent sample, and the similarity of treatments for aGVHD and cGVHD. We thus concluded that this lymphopenia was not induced by cGVHD treatment. These results pave the way for the monitoring of circulating helper-like ILCs at a time point before 100 days to predict the occurrence of cGVHD and to stratify for the risk of this condition in patients. The optimal timepoint for such testing will be determined in future studies.

Our observations concerning $NKp44^+$ ILCP, $CD69^+$, and $CCR6^+$ILCP are consistent with previous data.[11] Indeed, ILC3 are known to produce IL-22, and donor-derived IL-22 was reported to have deleterious effects in a mouse model of skin GVHD with lesion

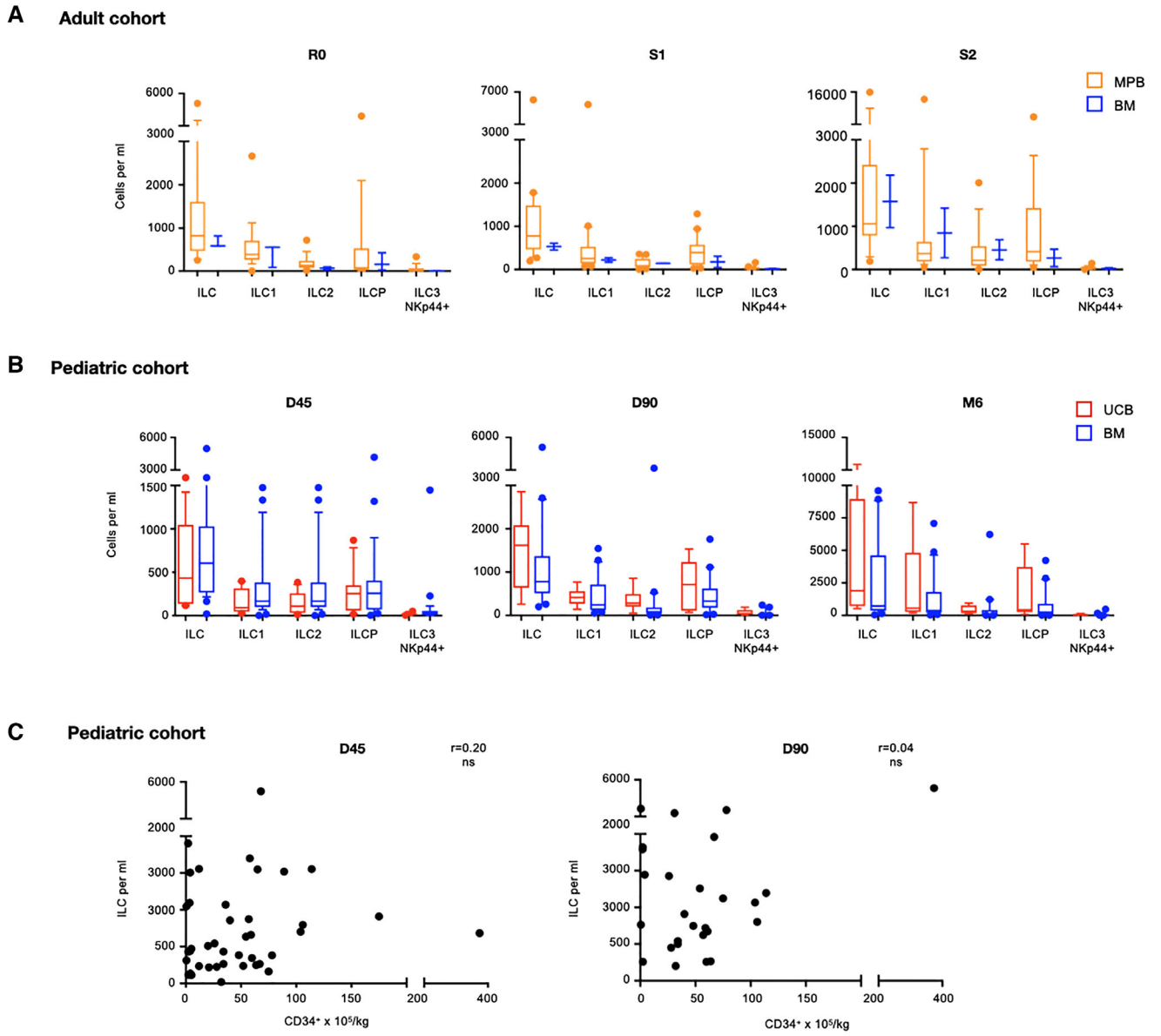

FIGURE 3 Influence of graft type and CD34$^+$ levels on helper-like ILC recovery. (A) Comparison of helper-like ILC subtype frequencies on R0 (before the conditioning regimen), S1 (90 days post-graft), and S2 (1-year post-graft), between peripheral blood (MPB) ($n = 19$ at R0, $n = 20$ at S1, $n = 18$ at S2) and bone marrow (BM) transplants ($n = 3$ at R0, $n = 2$ at S1 and S2) in the GVHD-free group of adults (P = ns for each subtype, for all time points). (B) Comparison of helper-like ILC subtype frequencies between umbilical cord blood (UCB) ($n = 11$) and bone marrow (BM) ($n = 28$) transplants in the pediatric population at D45, D90, and M6 (p = ns for each subtype, for all time points). (C) Spearman's rank correlation between CD34$^+$ levels and total helper-like ILC frequencies in the pediatric population ($n = 41$ for D45, $n = 27$ for D90; P = ns at both time points). UCB, umbilical cord blood; MPB, mobilized peripheral blood; BM, bone marrow; D45, day 45; D90, day 90; M6 month 6

exacerbation.[41] High serum IL-22 concentrations have also been reported in patients suffering from skin cGVHD.[41] This observation highlights the importance and complexity of IL-22 secretion by ILC3, NKT, CD4$^+$, and $\gamma\delta$ T cells, which can be beneficial or harmful, depending on as yet unidentified factors. If this trend is confirmed, the quantification of specific ILC subtypes before HSCT and the conditioning regimen could be used as a marker of the risk of GVHD occurrence.

For aGVHD, we compared absolute counts of all cell types on day 45 between 41 pediatric patients with and without aGVHD (**Table 3**). Counts of T cells, B cells, NK cells, and ILC1 were similar between the two groups of patients, whereas ILC2 and ILCP levels were significantly lower in patients with aGVHD (Figure 4B). Multivariate analysis revealed a significant association of aGVHD with the levels of CD4$^+$ T cells, ILC2, ILCP, CD25$^+$ ILCs, CD25$^+$ ILCP, CCR6$^+$ILCP, CCR10$^+$ ILCP, and NKp44$^+$ ILCP (Figure 4C). The association of aGVHD with low levels of certain ILCP subtypes is consistent with previous findings for adults. NKp44$^+$ ILCP are absent from the blood of healthy individuals.[42] These cells are another important source of IL-22,[43] which has been shown to protect intestinal stem cells in the context

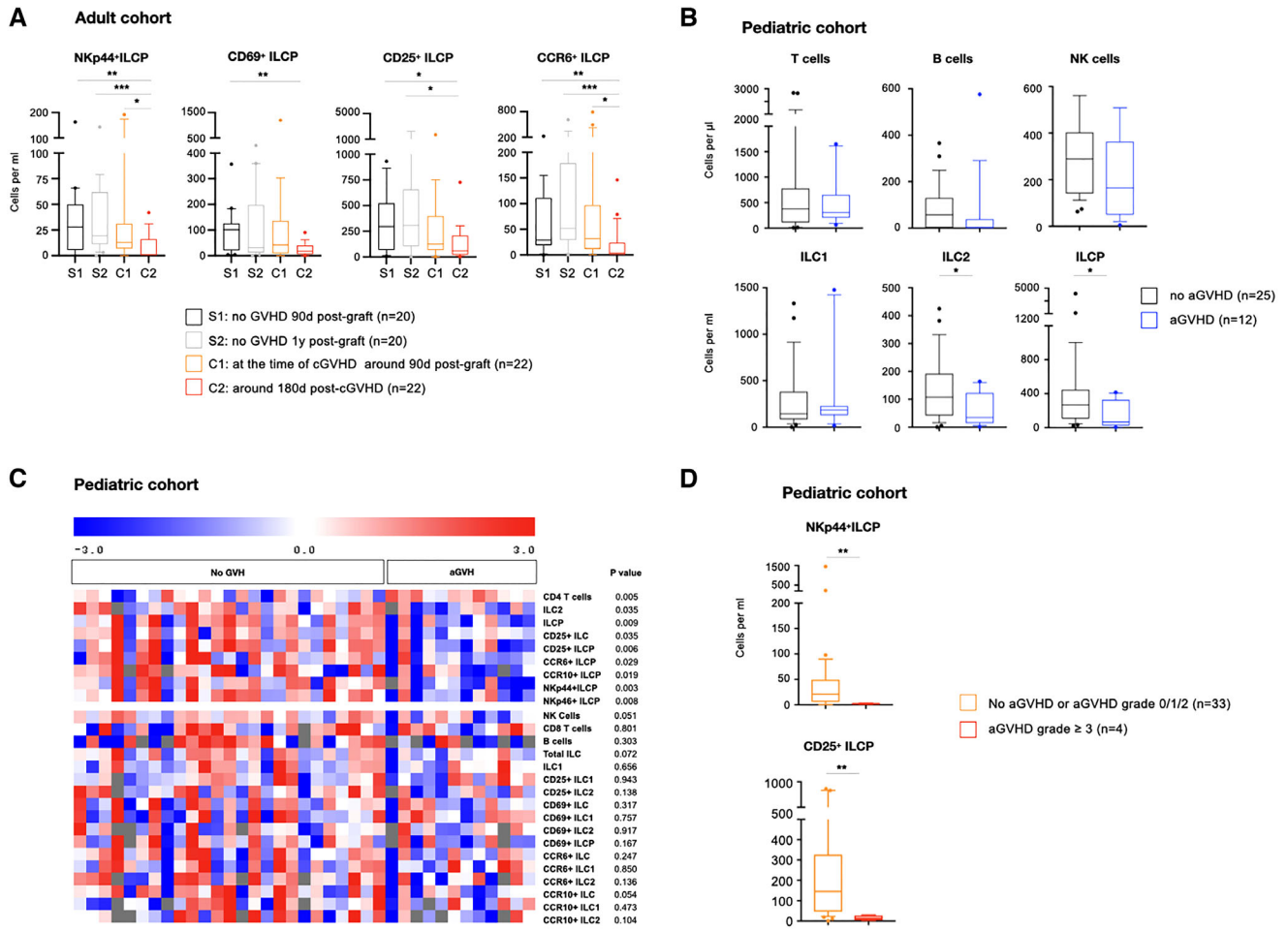

FIGURE 4 GVHD and lymphocyte subsets before and after HSCT. (A) Comparison of NKp44$^+$ ILCP, CD69$^+$ ILCP, CD25$^+$ ILCP, and CCR6$^+$ ILCP between samples from the no-GVHD group and the cGVHD group in blue, for the adult population. (B) Absolute counts of ILC1, ILC2, ILCP, T cells, B cells, and NK cells; comparison between patients with aGVHD in blue and GVHD-free pediatric patients in black on day 45. (C) Heatmap of differences in the levels of the lymphocyte subsets between the aGVHD and no-GVHD groups ($n = 41$). (D) Absolute counts, at day 45, of NKp44$^+$ ILCP and CD25$^+$ ILCP in patients with grade 0/I/II aGVHD in orange and those with grade III/IV aGVHD in red. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$)

of GVHD in a mouse model.[44,45] The finding of an association between aGVDH occurrence and ILC2 levels is interesting. ILC2 produce type 2 cytokines, such as IL-4 and IL-5, the role of which in GVHD remains unclear. However, there is some evidence from mouse studies to suggest that type 2 cytokines, especially IL-4, protect against GVHD.[46,47] ILC2 have also been shown to be a source of amphiregulin (AREG) under various conditions.[48,49] AREG plays a crucial role in tissue repair and homeostasis.[50] The transplantation of ILC2 into the gut of mice with intestinal GVHD was recently shown to be associated with a better outcome of GVHD, demonstrating the importance of ILC2 in gut homeostasis and function in the context of GVHD.[51] However, ILC2 are present in extremely small numbers in the gut at a steady state in humans.[52,53]

In accordance with the consensus classification,[54] we then investigated the possible link between the clinical severity of aGVHD and helper-like ILC subtype levels. We compared patients with no GVHD or with mild or moderate aGVHD (grade 0/I/II) with patients with severe or life-threatening aGVHD (grade III/IV). The distinction between grade 0/I/II and grade III/IV aGVHD was made in accordance with clinicians, who considered this separation to be the most clinically relevant. The small size of the cohort did not allow to draw firm conclusions but our data suggest a trend toward significantly lower levels of NKp44$^+$ ILCP and CD25$^+$ ILCP in patients with aGVHD at grades ≥ III (Figure 4D).

We previously reported that, in a cohort of patients with severe combined immunodeficiency who had undergone HSCT, helper-like ILC deficiency was not associated with any particular susceptibility to disease, with follow-up extending from 7 years to 39 years after HSCT, when T- and B-cell immunity was intact. These data led us to suggest that helper-like ILCs might be redundant for protective immunity in humans in conditions of modern hygiene and medicine.[6] The findings reported here show that, shortly after HSCT, helper-like ILCs may help to protect against GVHD. GVHD occurs within days or months of HSCT, when the immunity mediated by T and B cells is not optimal,

suggesting that helper-like ILCs may have a protective role in such conditions, even if present at a low frequency. Consistent with this possibility, helper-like ILCs have two crucial features for epithelium protection against GVHD: tissue residency and a rapid response to activation. It is also possible that tissue damage associated with GVHD patients may attract these activated ILCP into tissues as it has been proposed for ILC depletion in the context of smoking.[55] These findings thus provide support for a non-redundant role of helper-like ILCs under lymphopenic conditions in humans.

In conclusion, we have shown that helper-like ILC reconstitution after HSCT is slow, following a time course similar to that for T-cell reconstitution, based on data from the two largest cohorts studied to date. Helper-like ILC reconstitution was not affected by graft origin or the proportion of $CD34^+$ cells in the graft. As long as a decrease in specific circulating helper-like ILC subtypes is associated with the occurrence of GVHD, monitoring of helper-like ILCs before HSCT and after reconstitution could become a useful predictive marker for stratifying the patient's risk of cGVHD.

AUTHORSHIP

F.V. and E.V. conceived the project and designed the experiments. C.P., N.B., E.G.M., and A.C.A. performed the experiments and analyzed the data. G.L., A.C., and C.F. analyzed the data. B.V., L.B., G.M., V.B., and C.G. were the clinicians associated with the study. G.L., F.V., and E.V. analyzed the data and wrote the manuscript.

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DISCLOSURE

E.V. is an employee of Innate Pharma. The other authors have no conflict of interest.

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SUPPORTING INFORMATION

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