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Presence of innate lymphoid cells in allogeneic hematopoietic grafts correlates with reduced graft-versus-host disease



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ABSTRACT

Background: Allogeneic hematopoietic cell transplantation (HCT) can be devastating when graft-versus-host disease (GvHD) develops. GvHD is characterized by mucosal inflammation due to breaching of epithelial barriers. Innate lymphoid cells (ILCs) are immune modulatory cells that are important in the maintenance of epithelial barriers, via their production of interleukin (IL)-22 and their T cell suppressive properties. After chemo- and radiotherapy, ILCs are depleted, and recovery after remission-induction therapy and after allogeneic HCT is slow and incomplete in a significant number of patients, which is associated with an increased risk to develop acute GvHD. **Objective:** To investigate whether the presence of mature ILCs within G-CSF–mobilized HCT grafts is correlated with the development of acute GvHD after allogeneic HCT.

Study Design: We analyzed ILCs in a cohort of 36 patients who received allogeneic HCT for a hematologic malignancy, by flow-cytometric immune-phenotyping of prospectively collected, cryopreserved peripheral blood mononuclear cells (PBMCs) and donor-derived HCT grafts collected for the same patients. Biased analysis, with ILCs defined as CD3[−]lineage[−]CD45⁺CD127⁺CD161⁺ lymphocytes, was performed using FlowJo version 10 software. Unbiased analysis was done using FlowSOM, which uses a self-organizing map (SOM) with a minimal spanning tree (MST) to define and visualize different clusters present in the samples.

Results: Remission-induction therapy significantly depleted ILCs from the blood, and patients who had a relatively low percentage of ILCs before allogeneic HCT were significantly more prone to develop acute GvHD, confirming previous findings in a separate cohort. Allogeneic HCT grafts, which were all obtained from the blood of G-CSF–mobilized healthy donors, contained ILCs at a frequency very similar to the peripheral blood of healthy individuals. The ILC subset composition was also comparable to that of the blood of healthy individuals, with the exception of NKp44⁺ ILC3s, which were significantly more abundant in HCT grafts. The relative ILC content of the graft tended to correlate with ILC reconstitution after allogeneic HCT, suggesting that peripheral expansion of transplanted mature ILCs may contribute to early ILC reconstitution after allogeneic HCT. Patients who received a relatively ILC-poor HCT graft had a significantly increased risk to develop acute GvHD, compared with patients who received relatively ILC-rich allogeneic HCT grafts. Unbiased phenotypic analysis with the FlowSOM algorithm confirmed that allogeneic HCT grafts of patients who developed acute GvHD contained a lower frequency of ILCs that clustered in NKp44⁺ ILC3 signature groups.

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Conclusion: The presence of ILCs in allogeneic HCT grafts is associated with a reduced risk to develop acute GvHD. These data suggest that enhancement of ILC reconstitution of ILC3s in particular, for example via adoptive transfer of ILCs, may prevent acute GvHD and has the potential to improve outcome of allogeneic HCT recipients.

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Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative treatment for hematologic malignancies, as well as non-malignant blood disorders such as sickle cell disease. Unfortunately, allogeneic HCT is often complicated by acute and chronic graft-versus-host disease (GvHD), leading to significant morbidity and mortality. Acute GvHD is caused by aberrant allogeneic immune responses to barrier tissue that is damaged by pretransplantation conditioning [1–3]. Tissue damage leads to the release of damage-associated molecular patterns (DAMPs) and translocation of microbial products such as lipopolysaccharide (LPS) and other pathogen-associated molecular patterns (PAMPs). The release of PAMPs and DAMPs induces activation of host antigen-presenting cells (APCs) and innate immune cells and enhances production of pro-inflammatory cytokines, ultimately leading to the activation of donor lymphocytes present in the graft. Subsequent proliferation, differentiation and migration of activated donor lymphocytes finally leads to target tissue destruction and the development of acute GvHD [2,4,5].

ILCs are a family of innate cells with a lymphoid morphology that play an important role during homeostasis to maintain the integrity of the epithelial lining and during the early immune response in mucosal tissues when the epithelial barrier is breached. ILC subsets produce cytokines similar to those produced by polarized T helper cells but lack an antigen-specific receptor. Despite their relatively low cell numbers, ILCs rapidly respond to local changes in the cytokine and lipid milieu, enabling them to promptly act in the early phase of the immune response in defense against pathogens. In addition to NK cells and lymphoid tissue-inducer (LTi) cells, the ILC franchise includes ILC1, ILC2 and ILC3 cells [6,7]. Each subset is characterized by a specific cytokine-secreting profile and is dependent on specific transcription factors for its development. ILC1s are pro-inflammatory IFN γ -producing cells that have been implicated in the pathophysiology of Crohn's disease [8,9]. ILC2s and ILC3s, conversely, both exhibit tissue-protective and -repair functions. ILC2s have been shown to be important in restoring tissue homeostasis in the lungs of mice after viral infection [10]. ILC3s can be grouped as NKp44⁺ and NKp44⁻ and are present in the gut, where they maintain gut homeostasis through activation of the aryl hydrocarbon receptor, production of IL-22 and inhibition of pathological CD4⁺ T cell responses against commensal gut bacteria [11–13].

In the past decade, we and others have provided evidence that ILCs also play an important role in acute GvHD pathophysiology [14–16]. We found that patients who had relatively high proportions of activated ILCs before allogeneic HCT had a lower incidence of mucositis and GvHD after allogeneic HCT than patients with lower proportions of activated ILCs [16]. In mice, cotransplantation of ILC2s reduced the incidence of severe lower gastrointestinal tract acute GvHD, and treatment with ILC2s after transplantation significantly improved intestinal barrier function [14]. Furthermore, the production of IL-22 by ILC3s has been shown to protect intestinal stem cells and reduce severity of acute GvHD and GvHD-related mortality in mice [15]. In addition to preventing tissue damage and supporting tissue repair, ILCs may prevent GvHD by suppressing the proliferation of alloreactive T cells. We demonstrated that ecto-enzyme-expressing ILC3s can metabolize pro-inflammatory extracellular ATP into adenosine, which has immunosuppressive properties, and that the frequency of these ecto-

enzyme-expressing ILC3s is reduced in sigmoid biopsies of acute GvHD patients compared with control biopsies [17]. In addition, we have shown that ILCs are lost in patients upon induction and conditioning chemotherapy and that the recovery of ILCs after allogeneic HCT generally takes >6 months. Together, these observations raised the hypothesis that higher numbers of ILCs present in allogeneic HCT grafts may protect allogeneic HCT recipients against tissue damage, alloreactive T cell activation and, consequently, acute GvHD after transplantation. We addressed this hypothesis by investigating the ILC subset composition of allogeneic HCT grafts and comparing the incidence of acute GvHD in recipients of relatively ILC-poor and relatively ILC-rich HCT grafts. We found that NKp44⁺ ILC3s are relatively enriched in G-CSF-mobilized allografts compared with the peripheral blood of healthy nonmobilized donors. Furthermore, we found that patients who received a graft that was relatively rich in ILCs were less prone to develop acute GvHD, and that the grafts they received contained higher proportions of NKp44⁺ ILC3s. Although confirmation in larger cohorts is needed, these data suggest that donor-derived ILCs may help mitigate acute GvHD and that the outcome of allogeneic HCT recipients might be improved in the future by providing patients with grafts enriched for ILCs.

Methods

Study participants and clinical protocols

Study protocols were approved by the institutional Medical Ethics Committee of the Amsterdam UMC, Academic Medical Center, Amsterdam, The Netherlands. All 36 participants and five randomly selected healthy donors (volunteers of Sanquin Blood Supply Foundation) signed informed consent in accordance with the Declaration of Helsinki. Characteristics of healthy volunteers and patients are described in Tables 1 and 2. For 18 of 36 patients, sufficient peripheral blood mononuclear cells (PBMCs) were collected during allogeneic HCT conditioning (i.e. in the week before allogeneic HCT (range: day –7 to 0), at 1 month (range: 25 to 48 days) and 3 months (range: day 85 to 113) after allogeneic HCT had been viably cryopreserved and were available for analysis (Table 1). From 31 of 36 patients, an aliquot of the G-CSF-mobilized peripheral blood HCT graft was also available for analysis. Characteristics of these 31 patients and their respective graft donors are also shown in Table 1. HSCs were mobilized with G-CSF (5 μ g/kg) twice daily starting in the evening of day –5 before allogeneic HCT. HSC donation typically took place on the day of transplantation. Unmanipulated, lymphocyte-replete grafts were infused freshly, within 48 h after collection. Patients were categorized into the no acute GvHD group (all patients who did not develop acute GvHD within 100 days after allogeneic HCT) and the acute GvHD group (GvHD of liver, skin and/or intestine occurring within 100 days after allogeneic HCT, and thus excluding late-onset acute GvHD). Severity of acute GvHD was graded using the classic Glucksberg–Seattle criteria (GSC). The majority of the 36 patients participated in the HOVON 96 trial. In that trial, shorter or longer duration of cyclosporine/mycophenolic acid GvHD prophylaxis was prospectively compared (NL2128 at www.trialregister.nl). Incidence of acute GvHD was similar in both arms [18]. The duration of cyclosporine/mycophenolic acid GvHD prophylaxis for patients who were not part of this trial was comparable to the longer-duration arm. As

Table 1
Overall and figure-specific characteristics of patients, healthy donors and graft donors.

All patients (n = 36)	Value	Longitudinal analyses (Figure 1)			Graft analyses (Figures 2 and 3)		
		Acute GvHD (n = 9)	No acute GvHD (n = 9)*	Healthy donors (n = 5)	Acute GvHD (n = 15)	No acute GvHD (n = 16)	Graft donors (n = 31)
Age (y)	60 (25–70)	57.5 (26–68)	63.5 (31–68)	58 (56–68)	58 (26–70)	60.5 (25–69)	38 (22–65)
Sex, M/F (% M)	16/20 (44)	6/3 (67)	2/7 (22)	3/2 (60)	9/6 (60)	7/9 (44)	21/10 (68)
Death	12 (33)	4 (44)	3 (33)		6 (40)	6 (38)	
Diagnosis							
AML	25 (69)	6 (67)	5 (56)		11 (73)	13 (81)	
AUL	1 (3)	1 (11)					
CLL	3 (8)	1 (11)	1 (11)		2 (13)	-	
MDS	1 (3)					1 (6)	
MH	3 (8)		2 (22)		1 (7)	1 (6)	
MM	1 (3)		1 (11)				
NHL	2 (6)	1 (11)			1 (7)	1 (6)	
Conditioning							
MA	5 (14)	2 (22)			3 (20)	2 (13)	
RIST	31 (86)	7 (78)	9 (100)		12 (80)	14 (88)	
Donor							
MUD	26 (72)	5 (56)	7 (78)		10 (67)	11 (69)	21 (68)
Sib	10 (28)	4 (44)	2 (22)		5 (33)	5 (31)	10 (32)
Grade of acute GvHD							
0	19 (53)		9 (100)			16 (100)	
1	1 (3)	1 (11)			1 (7)		
2	9 (25)	2 (22)			8 (53)		
3	7 (19)	6 (67)			6 (40)		
4							
Diagnosis of acute GvHD after allogeneic HCT (days)		39 (19–92)	NA	NA	30 (9–92)	NA	NA

Data are expressed as median (range) or n (%) unless indicated otherwise. AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MH, morbus Hodgkin; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; MA, myeloablative conditioning; RIST, reduced-intensity stem-cell transplantation conditioning; MUD, matched-unrelated donor; Sib, sibling donor.

* Of three patients in this group, only baseline samples were measured.

Table 2
Characteristics of study participants.

	Acute GvHD (n = 17)	No acute GvHD (n = 20*)
Age (y)	58.5 (26–70)	61 (25–69)
Sex, n M/F (% M)	9/8 (53)	8/12 (40)
Death	6 (35)	7 (35)
Diagnosis		
ALL		1 (5)
AML	12 (71)	13 (65)
AUL	1 (6)	
CLL	2 (12)	1 (5)
MDS	-	1 (5)
MH	1 (6)	2 (10)
MM		1 (5)
NHL	1 (6)	1 (5)
Conditioning		
MA	3 (18)	2 (10)
RIST	14 (82)	18 (90)
Donor		
MUD	12 (71)	15 (75)
Sib	5 (29)	5 (25)
Grade of acute GvHD		
0		20 (100)
1	2 (12)	
2	8 (47)	
3	7 (41)	
4		
Diagnosis of acute GvHD after allogeneic HCT (days)	48 (9–92)	NA

Data are expressed as median (range) or n (%) unless indicated otherwise. AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MH, morbus Hodgkin; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; MA, myeloablative conditioning; RIST, reduced-intensity stem-cell transplantation conditioning; MUD, matched-unrelated donor; Sib, sibling donor.

ILCs represent a small lymphocyte subset, in a number of cases the yield of viable PBMCs after thawing of cryopreserved samples was too low to reliably evaluate ILC content. Missing samples are indicated in the text and figure legends.

Flow cytometry

The following antibodies were used for staining: BUV496-conjugated anti-CD3 (BD, clone UCHT1), BV421-conjugated anti-CD127 (BD, clone HIL-7R-M21), BV510-conjugated CD161 (BD, clone DX12), BV605-conjugated CD69 (BD, clone FN50), FITC-conjugated CD1a (eBioscience, clone HI149), FITC-conjugated CD14 (Sanquin, clone CLB-mon/1), FITC-conjugated CD19 (Sanquin, clone CLB-B4/1), FITC-conjugated CD34 (BioLegend, clone 581), FITC-conjugated CD123 (eBioscience, clone 6H6), FITC-conjugated BDCA2 (CD303) (Miltenyi, clone AC144), FITC-conjugated FcεR1 (eBioscience, clone AER37), FITC-conjugated CD94 (eBioscience, clone DX22), FITC-conjugated TCRα (BioLegend, clone IP26), FITC-conjugated TCRγδ (BioLegend, clone B1), PerCP-Cy5.5-conjugated CD294 (BD, clone BM16), Pe-Cy7-conjugated CD117 (BD, clone 104D2), APC-conjugated CD336 (NKp44) (eBioscience, clone 44-189), AF700-conjugated CD45 (Sanquin, clone CLB-T200) and near-infrared fluorescent LIVE/DEAD fixable dead cell stain kit (Invitrogen). Antibodies were titrated on healthy donor (HD) PBMCs or tonsil from routine tonsillectomies (in the case of NKp44, since circulating cells do not express NKp44 in healthy individuals). ILCs were defined as live CD3⁺Lin⁻CD45⁺CD127⁺CD161⁺ lymphocytes. Baseline and follow-up samples were thawed at 37°C by adding RPMI (Gibco) containing 17% FCS (Bodinco) and 5% penicillin/streptomycin (Sigma-Aldrich). Cells were incubated with 3650 U DNase/ml + 5 mM MgCl₂ at 37°C for 15 min. Grafts were thawed in 0.9% NaCl (Braun) without DNase. Cell counts and viability were determined with trypan blue and a Countess (Invitrogen). For baseline and follow-up samples,

a maximum of 5×10^6 PBMCs, and for grafts, a maximum of 20×10^6 PBMCs, were stained. Cells were first stained with near-infrared live/dead dye (Invitrogen) at room temperature in the dark for 30 min. After washing, cells were stained with antibodies diluted in PBS/0.5% BSA/0.05% NaN_3 at room temperature in the dark for 30 min. Samples were measured on an LSRFortessa cytometer (BD Biosciences) with a maximum flow rate of 7000 events/s and a threshold of 4000 to ensure the reliable detection of low-event populations.

Manual and automated analysis

Manual data analysis was performed using FlowJo version 10 software. For automated data analysis, pre-analysis data quality control was performed using the FlowAI package [19]. The signal acquisition and dynamic range parameters were used to select high-quality events. ILCs, defined as lineage-negative CD127^+ cells, were then selected by manual gating and used as input for automated data analysis with the FlowSOM algorithm [20], which creates a self-organizing map (SOM) with a minimal spanning tree (MST) to define and visualize cell populations present. Four markers (CD117 [cKIT], CRTH2 [CD294], CD161 and NKp44) were used to generate 100 clusters and 4 to 10 meta-clusters. The optimal number of meta-clusters was determined based on 2D scatterplots of the four phenotype markers as 6, as this number provided the best separation of different cell populations based on the 4 markers (Figure S1). Other parameters were kept as default. To test stability, the analyses were repeated at least three times with different seeds. One representative seed was used for reporting of the data.

Statistical analyses

Data are shown as median \pm interquartile range. GraphPad Prism software was used to determine statistical significance. Differences between healthy donors and patients or grafts were calculated using a two-tailed Mann–Whitney U test. Differences between survival curves were calculated using a Gehan–Breslow–Wilcoxon test, and correlations were calculated by simple linear regression. Differences between proportion of cells in clusters and meta-clusters from the FlowSOM analysis were calculated using a two-tailed Mann–Whitney U test. P values ≤ 0.05 were considered statistically significant.

Results

Patient ILC frequency is associated with risk of acute GvHD

We studied changes in the composition of the peripheral blood ILC compartment longitudinally in the 18 of 36 patients with a hematologic malignancy, from whom PBMCs were available before and after receiving an allogeneic HCT, and in 5 healthy donors (Table 1). Patient samples were collected prospectively in the week before allogeneic HCT (pre-HCT), and 1 and 3 months after allogeneic HCT. Previously we demonstrated that pre-HCT remission-induction chemotherapy depletes ILCs from the circulation, and that a relative deficiency in activated ILCs before allogeneic HCT is associated with an increased risk to develop acute GvHD [16]. In this second and independent cohort, we confirmed that ILC frequencies in patients pre-HCT were significantly lower than in healthy individuals ($P = 0.029$) (Figure 1A), and the ILCs that had reconstituted after remission-induction chemotherapy (before conditioning therapy and allogeneic HCT) consistently displayed a more activated, CD69^+ phenotype compared with healthy individuals ($P = 0.0002$) (Figure 1B). The subset composition of the ILC pool pre-HCT was also different from the healthy ILC compartment. While the frequencies of CD117^+ (c-kit) $^-$ CRTH2^- ILC1s and CRTH2^- CD117^+ NKp44^+ ILC3s were similar to those in healthy individuals, CRTH2^+ ILC2s constituted a

significantly lower frequency and CD117^+ NKp44^- ILC3s a significantly higher frequency ($P = 0.0012$ and $P = 0.0072$, respectively) (Figure 1C). This observation is in line with our previous finding that, in particular, ILC2s reconstitute very slowly after remission-induction chemotherapy [16]. Patients who had a relatively low percentage of ILCs before allogeneic HCT were significantly more prone to develop acute GvHD, defined as clinically significant (grade 1 to 4) GvHD of liver, skin or intestine occurring within 100 days after allogeneic HCT and thus excluding late-onset acute GvHD ($P = 0.0139$) (Figure 1D). With this second cohort, we thus confirmed our previous data showing that having relatively higher proportions of ILCs immediately before allogeneic HCT is associated with a lower incidence of acute GvHD [16].

ILCs in allogeneic HCT grafts and ILC reconstitution

Swift ILC recovery after remission-induction therapy thus seems beneficial; however, the mechanisms of ILC recovery after chemotherapy-induced ILC depletion remain to be elucidated. After allogeneic HCT, reconstituting donor lymphoid cells originate from the G-CSF–mobilized HCT graft. We therefore questioned whether allogeneic HCT grafts also contain ILCs. The frequency of total ILCs in unmanipulated, allogeneic HCT grafts was very similar to the peripheral blood of healthy individuals (Figure 2A). The proportion of NKp44^+ ILC3s in mobilized grafts, however, was significantly more abundant compared with non-mobilized blood of healthy individuals, which normally does not contain NKp44^+ ILC3s ($P = 0.0225$) (Figure 2B). The proportion of the other ILC subsets was comparable in both mobilized blood and blood of healthy individuals. Previously it has been demonstrated that T cell reconstitution correlates with the number of T cells infused with the graft [21]. We therefore analyzed the correlation between ILC reconstitution and the proportion of ILCs in the graft in patients from whom enough ILCs could be measured and from whom total CD45^+ measurements were available ($n = 11$). Although not significant in this small group of patients, we observed a trend toward a positive correlation between ILC content of the graft and ILC reconstitution after allogeneic HCT ($P = 0.06$) (Figure 2C), suggesting that expansion of transplanted mature ILCs may contribute to early ILC reconstitution after allogeneic HCT. Also, when considering the absolute numbers of ILCs in the blood of patients after allogeneic HCT, we observed lower ILC numbers in patients who developed acute GvHD compared with patients who did not ($P = 0.0283$ 1 month after allogeneic HCT and $P = 0.0556$ 3 months after HCT) (Figure 2D).

These data demonstrate that G-CSF–mobilized peripheral blood allografts contain ILCs and are enriched for NKp44^+ ILC3s and that ILC graft content tends to correlate with ILC reconstitution, which was best in patients who did not develop acute GvHD.

ILC composition of the graft is associated with the occurrence of acute GvHD

Next, we investigated whether the presence of ILCs in the graft correlated with the development of acute GvHD. We categorized patients into 2 groups based on whether the proportion of ILCs in the graft was above or below the median proportion of ILCs in the graft. Patients who received a relatively ILC-poor allogeneic HCT graft (%ILC $<$ median) had a higher risk of developing acute GvHD than patients who received a relatively ILC-rich allogeneic HCT graft (%ILC $>$ median) ($P = 0.0422$) (Figure 3A). Although group sizes were limited, a similar increased risk was seen when assessing only the patients with low ILCs before allogeneic HCT ($P = 0.0339$) (Figure 3B). Low ILCs before allogeneic HCT predisposes for acute GvHD (Figure 1D) [16], but when these patients received an allograft with relatively high ILC content, this predisposition for acute GvHD was neutralized.

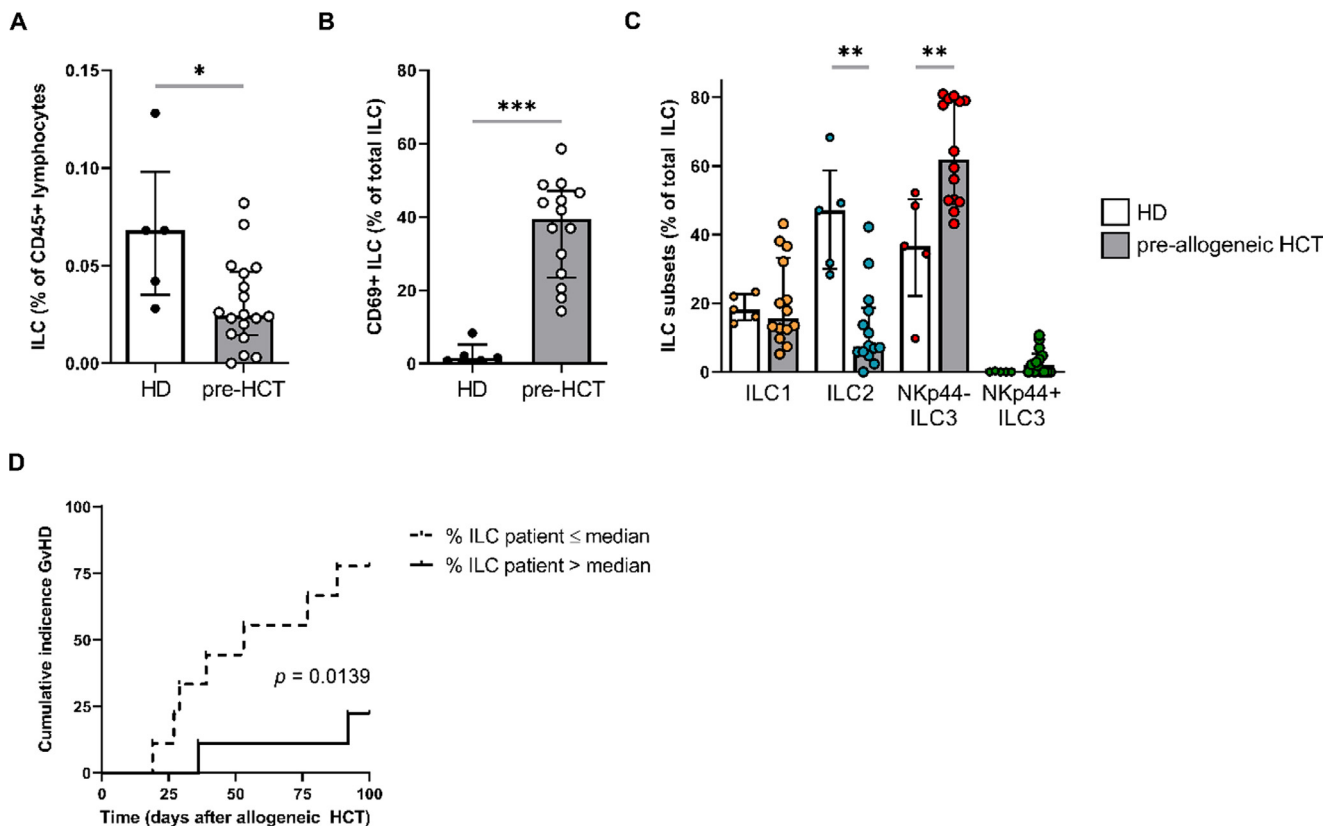


Figure 1. Low ILC frequencies in allogeneic HCT patients before transplantation were associated with an increased risk of acute GvHD. (A) ILCs as percentage of CD45⁺ lymphocytes for healthy donors (HD) (n = 5) and allogeneic HCT patients (n = 18). Samples were obtained before allogeneic HCT and after remission-induction therapy. (B) The proportion of activated (CD69⁺) ILCs in allogeneic HCT recipients (n = 14) and HD (n = 5). (C) The percentage of ILC1, ILC2, NKp44⁻ ILC3 and NKp44⁺ ILC3 of ILCs in HD (white bars) (n = 5) and allogeneic HCT patients obtained before allogeneic HCT (gray bars) (n = 14). (D) Kaplan-Meier curve of the first 100 days after allogeneic HCT showing the cumulative incidence of acute GvHD in patients with a low (n = 9) (at or below the median; dotted line) and high (n = 9) (above the median; black line) percentage of ILCs before allogeneic HCT. Data in A, B and C are shown as median with interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001. For B and C, a minimum of 25 events within the CD161⁺ ILC gate was necessary to determine the ILC percentages; 4 patients did not meet this criterion. (Color version of figure is available online.)

To better understand which ILC subset contributed to this better outcome, we analyzed the composition of the ILC population of the grafts in more detail, using the automated, unsupervised and unbiased phenotypic analysis method FlowSOM [20]. Briefly, the FlowSOM algorithm builds a self-organizing map from flow cytometry data and generates an MST with meta-clusters based on expressed cell markers. Analyses of meta-clusters demonstrated that allogeneic HCT grafts of patients who developed acute GvHD tended to have fewer ILCs that clustered in meta-clusters 1 and 2 ($P = 0.0667$ and $P = 0.0529$, respectively) (Figure 3C and 3D). The lineage⁻CD45⁺CD127⁺ cells in meta-cluster 2 were characterized by the expression of CD117 and NKp44 and some CD161 but no CRTH2 and can thus be defined as having an NKp44⁺ ILC3 signature. Meta-cluster 1 cells also expressed CD117 and some CD161 but expressed less NKp44 than the meta-cluster 2 cells and can thus be defined as NKp44⁻ ILC3 signature cells. Significant differences between patients were present when examining single clusters falling into meta-clusters 1 and 2. Representative graphs of a single patient with acute GvHD and a single patient without acute GvHD are shown in Figure S2. Allogeneic HCT grafts of patients who developed acute GvHD contained fewer ILCs that clustered in clusters 1, 7, 16, 17 and 31 than those of patients who did not develop acute GvHD ($P = 0.0074$, $P = 0.0102$, $P = 0.0102$, $P = 0.0030$ and $P = 0.0321$, respectively) (Figure 3D). Other clusters from meta-clusters 1 and 2 showed similar trends, albeit not significant (for example, cluster 6). The ILC1 and ILC2 subsets fell into meta-clusters 3 to 6. No significant differences were seen in any of these meta-clusters or in the clusters contained within them. Taken together, these data show

that patients with allogeneic HCT grafts containing more ILCs—and in particular ILC3s—have a reduced risk to develop acute GvHD.

Discussion

Acute GvHD is caused by alloreactive immune responses against damaged tissues of the host, and measures to prevent GvHD are typically directed at suppression of alloimmune responses. This approach is not airtight, however, and it significantly heightens the risk of opportunistic infections. An alternative approach to reduce the incidence and severity of acute GvHD could be to prevent tissue damage or enhance tissue repair mechanisms [2,4,5]. ILCs are innate effector cells that have tissue-protective properties and are capable of supporting tissue repair [10,14,15,22]. As such, it is of interest to study these cells in the context of acute GvHD. We have shown previously that ILCs are depleted by remission-induction therapy and that the presence of ILCs (both patient-derived before transplantation and donor-derived after transplantation) expressing activation markers (CD69) and proliferation markers (Ki67), as well as markers for homing to the skin (CCR10 and CLA) and the intestines ($\alpha 4\beta 7$ and CCR7), are associated with a lower incidence of acute GvHD [16]. Patients with acute GvHD of the intestine had reduced numbers of ILCs present in inflamed tissues compared with non-inflamed colon [17]. These observations raised the question whether ILCs in the allogeneic HCT graft could contribute to ILC reconstitution and subsequent protection against tissue damage and acute GvHD. Previous studies have shown that other cell types such as regulatory T cells [23] and

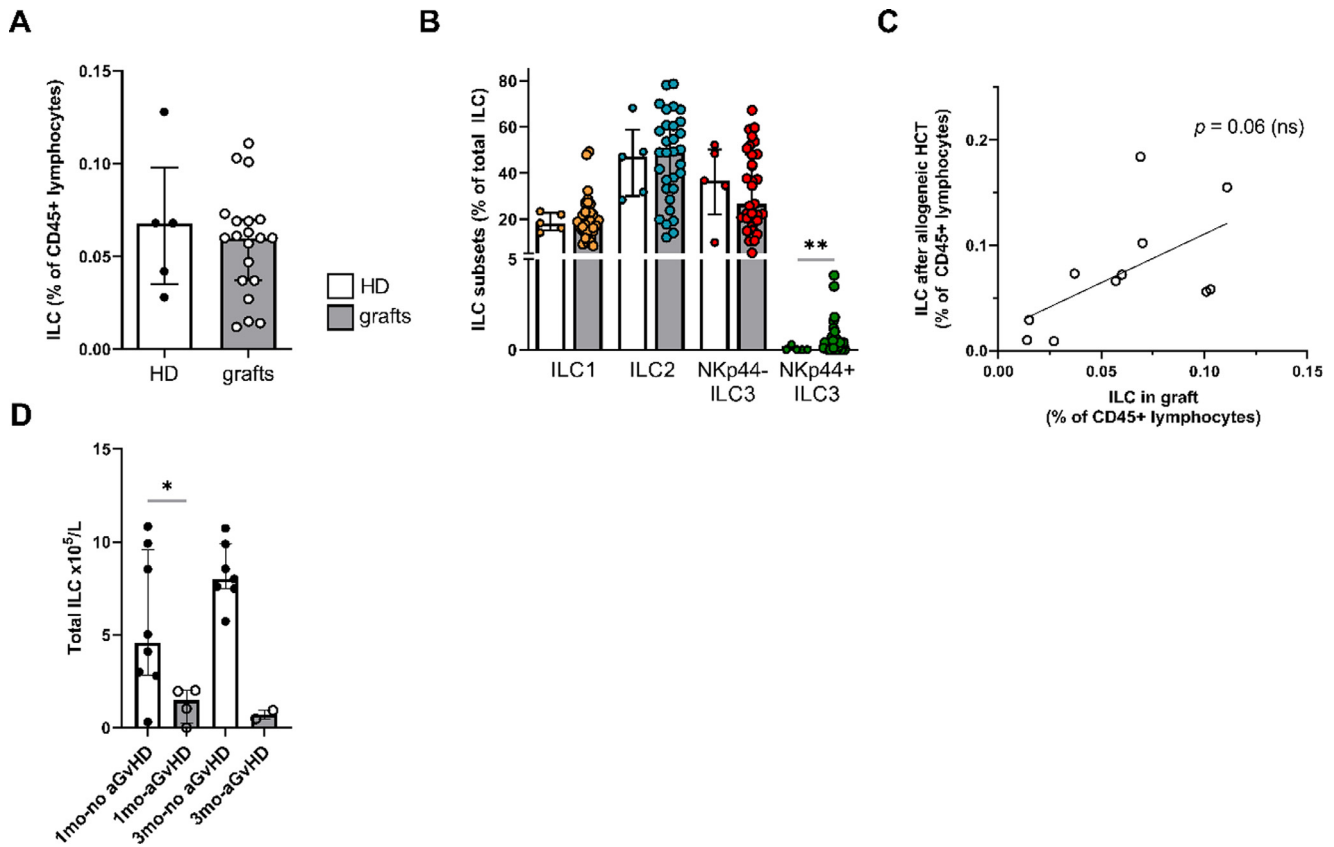


Figure 2. ILCs are present in the graft and tend to associate with ILC reconstitution. (A) ILCs as a percentage of CD45⁺ lymphocytes for HD (white bars) (n = 5) and graft samples (n = 20) (gray bars). Eleven of 31 graft samples were excluded because CD45⁺ expression was not measured. (B) Percentage of ILC1, ILC2, NKp44⁻ ILC3s and NKp44⁺ ILC3s of ILCs for HD (white bars) (n = 5) and graft samples (gray bars) (n = 31). (C) Correlation between the percentage of ILCs in the graft versus the proportion of ILCs measured in the blood of the corresponding recipient (n = 11) after allogeneic HCT ($r^2 = 0.34$, $P = 0.06$). (D) Number of circulating ILCs in patients with (gray bars) and without (white bars) acute GvHD measured at 1 month (n = 8 for no aGvHD and n = 4 for aGvHD) and 3 months (n = 7 for no aGvHD and n = 2 for aGvHD) after allogeneic HCT. Data in A and B are shown as median with interquartile range. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

monocytic myeloid-derived suppressor cells [24] present in allogeneic HCT grafts may be able to provide protection against acute GvHD. In the present study, we examined the composition of ILC subsets in allogeneic HCT grafts, studied the reconstitution of ILCs in patients who received these grafts and correlated the results to the incidence of acute GvHD.

First, we confirmed in this independent cohort of 36 allogeneic HCT patients that the percentage of ILCs in the blood of allogeneic HCT patients was significantly reduced compared with blood of healthy individuals, and that patients with a relatively higher frequency of ILCs immediately before allogeneic HCT seemed significantly less likely to develop acute GvHD than patients with a relatively lower frequency of ILCs before allogeneic HCT. These results verify previous findings of our group and others that ILC reconstitution in blood after remission-induction chemotherapy is slow, that reconstitution of ILC3s is generally faster than that of ILC2s and that having more ILCs before allogeneic HCT is likely protective for acute GvHD [16,25]. Although the group of healthy donors included in our study was relatively small (n = 5), they closely match the findings in healthy donors in our previous study [16]. The observed clinical benefit of having relatively more ILCs before allogeneic HCT could be related to their tissue-protective and -reparative properties, limiting tissue damage and immune activation caused by radio- and chemotherapy. It is of note, however, that the present analyses were performed in blood only. While we have demonstrated that tissue ILCs are diminished in patients with acute GvHD [17], it will be imperative for future studies to validate these findings in tissue biopsies from patients before allogeneic HCT.

Recovery of the damaged ILC compartment is of importance, but the mechanisms that control ILC reconstitution remain to be determined. Early reconstitution of T lymphocytes after allogeneic HCT relies heavily on the presence of T cells in the allograft [21]. We therefore questioned whether ILCs are present in allogeneic HCT grafts and whether the presence of ILCs in allogeneic HCT grafts impacts ILC reconstitution and development of acute GvHD after transplantation. Total ILC frequencies in the allogeneic HCT grafts were very similar to those found in healthy donors; however, the frequency of NKp44⁺ ILC3s was significantly increased in the grafts. It is possible that G-CSF mobilization before obtaining HCT grafts leads to an increase in proliferation of these NKp44⁺ ILC3s and/or mobilization of these cells from lymphoid and nonlymphoid tissues. It cannot be excluded that ILC reconstitution also depends on differentiation of CD34⁺ HSCs present in the graft. Previous research that argues against this possibility showed that G-CSF inhibited recovery of ILCs upon culture of CD34⁺ HSCs *in vitro* and that recovery of ILCs in CD34⁺ cultures from mobilized peripheral blood was lower compared with CD34⁺ cultures from bone marrow and cord blood. Whereas this suggests that the source of HSCs may play a role in ILC reconstitution after allogeneic HCT, it may also demonstrate that ILC recovery after allogeneic HCT is more likely to derive from expansion of mature ILCs present in the graft than from CD34⁺ HSCs present in the graft [26]. As NKp44⁺ ILC3s have demonstrated importance in epithelial tissue homeostasis and repair of damaged tissues, the increased frequency of NKp44⁺ ILC3s in the grafts may be beneficial for patients. The percentage of ILCs in the graft tended to positively correlate with the percentage of ILCs after HCT, suggesting that mature ILCs present in

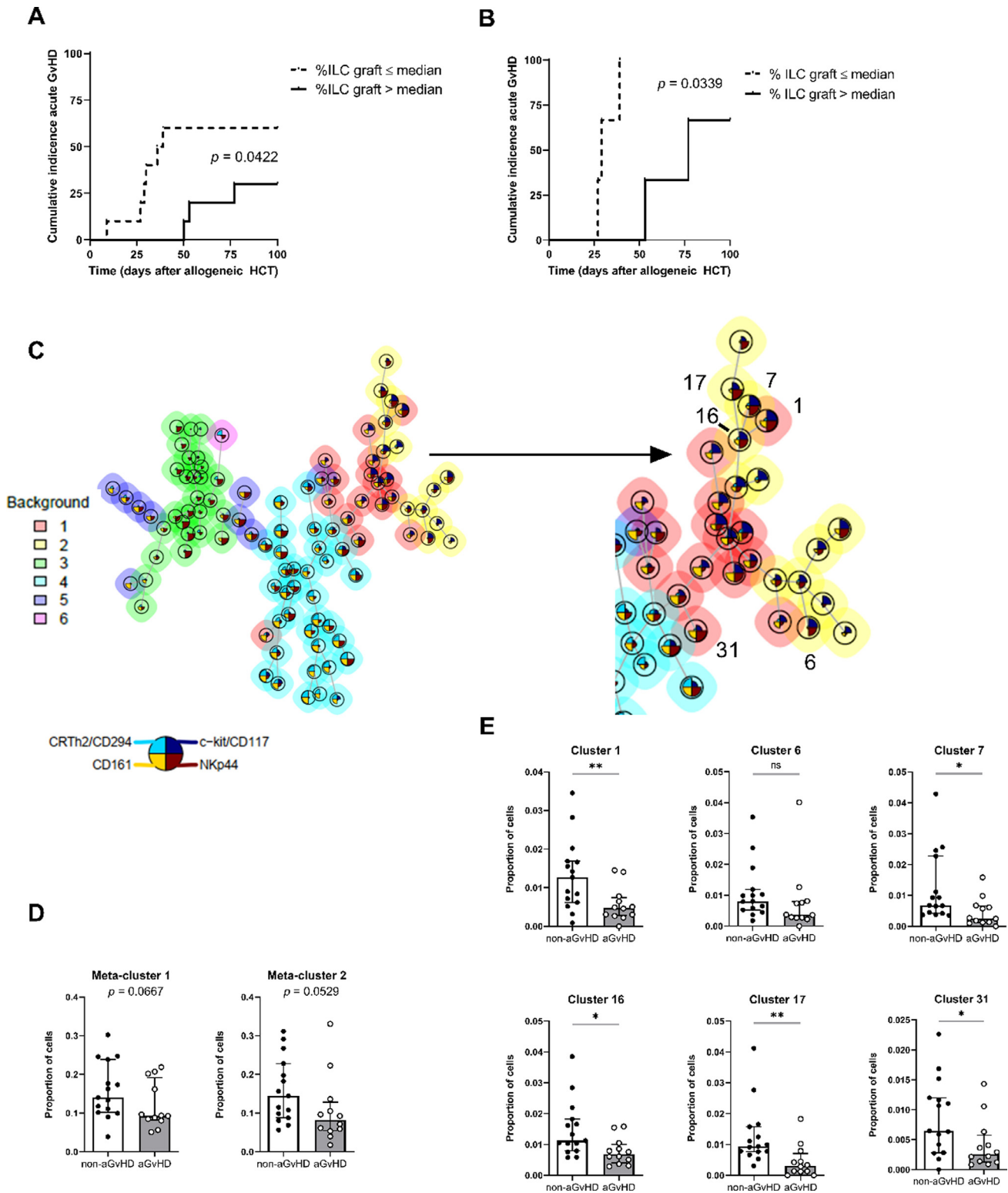


Figure 3. ILC composition of the HCT graft associates with acute GvHD. **(A)** Kaplan–Meier curve showing the cumulative incidence of acute GvHD in patients who received a graft with a low (below the median) (dotted line) ($n = 10$) or a high (above the median) (black line) ($n = 10$) percentage of ILCs. Eleven of 31 graft samples were excluded because CD45⁺ expression was not available. **(B)** Kaplan–Meier curve showing the cumulative incidence of acute GvHD in patients with a low percentage of ILCs (below the median) who received a graft with a low (below the median) (dotted line) ($n = 3$) or a high (above the median) (black line) ($n = 3$) percentage of ILCs. **(C)** MST generated by FlowSOM from allogeneic HCT graft samples. Of the 31 graft samples, 27 met all pre-analysis quality control requirements and were included for analysis by FlowSOM. Background colors indicate the different meta-clusters, and the star plots indicate the median marker expression per cluster. Individual clusters of interest are labeled with numbers in the partial tree on the right. **(D)** Proportion of cells in allogeneic HCT graft samples from non-GvHD (white bars, $n = 15$) and acute GvHD (gray bars, $n = 12$) patients falling into meta-clusters 1 and 2. **(E)** Proportion of cells of allogeneic HCT graft samples from non-GvHD (white bars, $n = 15$) and acute GvHD (gray bars, $n = 12$) patients falling into clusters 1, 6, 7, 16, 17 and 31. Data in D and E are shown as median with interquartile range. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

the graft before transplantation are able to expand after allogeneic HCT and contribute to reconstitution of ILCs. This is an important finding, because it suggests that manipulating the ILC content of grafts before allogeneic HCT may provide a way of improving ILC reconstitution in patients after transplantation, possibly leading to a reduction in the incidence of mucositis and acute GvHD.

Although increased ILC content of the graft tended to correlate with better ILC reconstitution after transplantation, the question remained whether a higher frequency of ILCs in the graft leads to a better outcome for patients. To investigate this, we compared the cumulative incidence of acute GvHD of patients who received a graft with relatively low ILC numbers and patients who received a graft with relatively high ILC numbers. Interestingly, patients who received a graft with a relatively high number of ILCs seemed significantly less likely to develop acute GvHD, further supporting the hypothesis that ILCs can elicit protection against GvHD. To determine which ILC subsets may be important for the prevention of GvHD, we used an automated and unsupervised analysis method, FlowSOM, to analyze the composition of ILC subsets in the grafts. With the use of this unbiased method, we found that patients who developed acute GvHD tended to have fewer NKp44⁺ and NKp44⁻ ILC3s. Release of IL-23 and IL-1 β upon tissue damage induces NKp44⁺ and NKp44⁻ ILC3s to produce IL-22, the cytokine that induces epithelial tissue repair via its interaction with IL-22 receptor-expressing epithelial stem cells in the gut [15,27,28]. Moreover, IL-1 β induces ecto-enzyme expression by NKp44⁺ and NKp44⁻ ILC3s, which enables them to neutralize extracellular ATP that is released by damaged tissues into adenosine, which has anti-inflammatory capacities [17]. ILC3s, in addition, have been demonstrated to suppress commensal bacteria-reactive T cells in the gut via MHCII-mediated interaction [11]. Together, these data suggest that ILC3s in particular play an important tissue-protective and anti-inflammatory role in transplant recipient tissues, and that allografted ILC3s may provide protection against the development of acute GvHD.

A weakness of this study is the relatively small size of our cohort; it will be important to confirm our findings in a larger, independent cohort. On the other hand, all patients analyzed in the current study were included in the HOVON 96 trial, and as such, received similar treatment with respect to HCT conditioning, GvHD prophylaxis and acute GvHD therapy. Furthermore, our extensive analysis of the data using a biased and an unbiased approach provides valuable insights into the importance of ILCs present in allogeneic HCT grafts. Our data suggest that damage induced before and during transplantation may be mitigated by recipient ILCs before allogeneic HCT and donor-derived ILCs early after allogeneic HCT. Prevention and repair of damage will reduce the amount of danger signals and consequently limit allogeneic immune activation and acute GvHD.

In summary, these data verify in a second, independent cohort of allogeneic HCT patients our previous findings that ILCs are depleted with remission-induction chemotherapy and the importance of having ILCs present both before and after allogeneic HCT. We show that recipients of relatively ILC-rich allogeneic HCT grafts are less prone to develop acute GvHD, and that ILC3s in particular seem to be pivotal for the protection against GvHD. As such, future HCT may be improved by pre-expanding ILC3s in vitro and providing patients with grafts enriched for ILC3s.

Contribution

A.K., V.v.H. and M.W.V. designed and performed the research, analyzed the data and wrote the paper. N.W. performed the research and helped write the paper. A.W.T., S.v.G., S.S.Z. and B.B. helped analyze the data, provided critical input and helped write the paper. C.V. and M.D.H. designed the research, analyzed the data and wrote the paper.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.jcyt.2021.10.011](https://doi.org/10.1016/j.jcyt.2021.10.011).

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