CXCR4-Related Increase of Circulating Human Lymphoid Progenitors after Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

Immune recovery after profound lymphopenia is a major challenge in many clinical situations, such as allogeneic hematopoietic stem cell transplantation (allo-HSCT). Recovery depends, in a first step, on hematopoietic lymphoid progenitors production in the bone marrow (BM). In this study, we characterized CD34+Lin−CD10+ lymphoid progenitors in the peripheral blood of allo-HSCT patients. Our data demonstrate a strong recovery of this population 3 months after transplantation. This rebound was abolished in patients who developed acute graft-versus-host disease (aGVHD). A similar recovery profile was found for both CD24+ and CD24− progenitor subpopulations. CD34+lin−CD10+CD24− lymphoid progenitors sorted from allo-HSCT patients preserved their T cell potential according to in vitro T-cell differentiation assay and the expression profile of 22 genes involved in T-cell differentiation and homing. CD34+lin−CD10+CD24− cells from patients without aGVHD had reduced CXCR4 gene expression, consistent with an enhanced egress from the BM. CCR7 gene expression was reduced in patients after allo-HSCT, as were its ligands CCL21 and CCL19. This reduction was particularly marked in patients with aGVHD, suggesting a possible impact on thymic homing. Thus, the data presented here identify this population as an important early step in T cell reconstitution in humans and so, an important target when seeking to enhance immune reconstitution.

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Introduction

In many clinical situations, such as intensive chemotherapy, acquired immunodeficiencies and allogeneic hematopoietic stem cell transplantation (allo-HSCT), immune recovery is a major challenge. Allo-HSCT is used to treat malignant and nonmalignant blood diseases, and remains the most widely used regenerative therapy. Accordingly, the profound and long-lasting immunodeficiency following this treatment leads to an increased risk of infection or relapse. Complete rescue of immune competence is closely linked to the recovery of de novo T-cell production in the thymus. This is a slow process depending on many factors, including patient age, disease status, source and composition of graft, type of conditioning, whether the donor is related or unrelated, human leukocyte antigen (HLA) mismatches, and the occurrence of graft-versus-host disease (GVHD) [1].

The thymus is the primary lymphoid organ responsible for T lymphocyte generation throughout life, its role is essential for post-lymphopenia reconstitution [2–4]. Two murine models have recently challenged the view that thymus function is exclusively dependent on the supply of bone marrow (BM)-derived progenitors [5,6]. However, these studies involved animals with a defective functional progenitor supply. In human clinical situations, apart from with congenital T-cell immunodeficiencies, lymphoid progenitors are available. Thus, in most settings, T-cell immune reconstitution relies on the generation of lymphoid progenitors in the BM, their homing to the thymus for T-cell commitment and intrathymic differentiation. Despite increasing knowledge of the hierarchy of hematopoietic lineage determination in humans [7], no study has yet characterized lymphoid progenitors in the context of immune reconstitution in humans. Six et al. [8] characterized two progenitor populations: CD34+Lin−CD10+CD24− cells, with a very low myeloid potential capable of giving rise to B, T and NK lymphocytes; and CD34+Lin−CD10+CD24− cells, which are already committed to the B-cell lineage. The CD24− population may represent thymus-
seeding progenitors (TSP). It is found in cord blood (CB), BM, thymus and, importantly, it is detectable in peripheral blood throughout life. These cells express low levels of CD38 and may correspond to the multipotential progenitor population (MLP) described by Doulatov et al. [9]. MLPs are defined by their potential to give rise to B cells, T cells and NK cells. In most cases, they also retain some potential towards the granulomonocytic and dendritic cell lineages. More recently, data from gene expression profiles and functional analysis have shown that, in human BM, CD10<sup>+</sup>CD24<sup>+</sup>CD62L<sup>+</sup>CD38<sup>+</sup> progenitors are developmental intermediates between the multipotent CD34<sup>+</sup>Lin<sup>+</sup>CD38<sup>-</sup> population and the lymphoid progenitor, or CD34<sup>+</sup>Lin<sup>-</sup>CD10<sup>+</sup> population [10]. However, this study did not distinguish between CD34<sup>+</sup>Lin<sup>-</sup>CD10<sup>+</sup>CD24<sup>+</sup> progenitors and the more prevalent B-cell-committed CD24<sup>+</sup> population. The relationship between CD10<sup>-</sup>CD62L<sup>-</sup> and TSP remains to be elucidated.

In allo-HSCT, T-cell reconstitution in patients is impaired during acute GVHD (aGVHD). This disease could target the BM niche and hamper hematopoietic progenitor generation, TSP homing to the thymus, and even thymic function itself. In experimental murine models, aGVHD has been linked to both a reduction in thymic cellularity and destruction of the thymic architecture [11–13]. In the thymus, stromal and epithelial cells produce growth factors locally, as well as cytokines essential for thymocyte survival such as interleukin (IL)-7, stem cell factor (SCF), and Flt3L; or chemokines involved in T-cell precursor homing such as C-C chemokine ligand (CCL) 19, CCL21 and CCL25. In addition to thymic GVHD, murine models revealed the existence of an early BM GVHD, where the osteoblastic hematopoietic niche is targeted [14]. Therefore, aGVHD could reduce the number of multipotent precursor cells periodically released from the BM and migrating to the thymus. Indeed, Zlotoff et al. [15] showed that, in mice, a limited supply of hematopoietic progenitors to the thymus may impair T-cell recovery after allo-HSCT. In humans, we have shown that aGVHD transiently impairs thymic output in young patients after allo-HSCT [16]. aGVHD affected T-cell differentiation prior to TCR β-chain recombination, as shown by a decrease in T-cell receptor (TCR) β-chain rearrangement circles (TREC) in peripheral blood. T-cell progenitors have, as yet, to be characterized during T-cell recovery after allo-HSCT, and the consequences of aGVHD examined. This could help to better define which steps should be targeted to optimize T-cell immune reconstitution in immuno compromised patients.

This article presents a snapshot analysis of cells between the time when they leave the BM and when the thymus is seeded. For the first time in humans and despite their very small number, we have characterized the recovery of circulating lymphoid progenitors, particularly the CD34<sup>+</sup>Lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>+</sup> population, in patients after allo-HSCT. We show that their quantitative recovery at 3 months post-transplant is affected by aGVHD and associated with downregulation of CXCR4 expression.

**Patients and Methods**

**Patient characteristics**

The study population consisted of 107 patients who received a non–T-cell–depleted allogeneic HSCT at the Bone Marrow Transplantation Unit, Hôpital Saint-Louis (Paris, France) between February 2002 and August 2012. Details on patients, donors and graft characteristics, as well as on transplantation outcome, are reported in Table 1. Acute GVHD was diagnosed and graded according to published criteria [17]. Nine of the 39 patients with grade 0-I aGVHD and 43 of the 48 patients with grade II–IV aGVHD were treated with corticosteroids. No significant difference was found between patient groups for sex ratio, GVH (0, I vs. II, III, IV), age, PB/BM, RIC (reduced intensity conditioning)/MAC (myeloablative conditioning) and diagnosis before allo-HSCT. Almost all patient were full donor chimera at month 3 as assessed by microsatellite analysis and only 1 patient had cells less than 92% of donor origin. Seventy-nine out of 107 patients were monitored for immune reconstitution by lymphocyte phenotyping and sjTREC content. Because of the small number of progenitor cells, different groups of patients were studied in the subsequent experiments: 39 for flow cytometry analysis, 22 for PCR analysis, 6 in OP9–ALI co-culture and 40 to quantify plasma analytes. Peripheral blood samples (up to 25 mL) were collected at 3 months after transplant. Peripheral blood mononuclear cells (PBMCs) were separated on lymphocyte separation medium and either sorted immediately or frozen in Fetal Calf Serum (FCS). 10% DMSO for phenotypic analysis. As control, peripheral blood samples from healthy donors (HD) (n = 46) and cord blood (n = 9) were obtained from the Hôpital Saint-Louis Transfusion Center and Cord Blood Bank, respectively. These samples were treated according to the same protocols as patient samples. No significant difference was found between patients and HD in terms of age and sex ratio. The investigation was approved by the Committee on Medical Ethics of the Hôpital Saint-Louis, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

**Flow cytometry analysis**

All antibodies were from BD Biosciences, unless otherwise indicated. PBMCs were thawed and at least 2<sup>10<sup>6</sup></sup> cells were stained with monoclonal mouse anti-human CD34–allophycocyanin (APC; 8G12), monoclonal mouse anti-human CD24–fluorescein isothiocyanate (FITC; ML5) and monoclonal mouse anti-human CD10–phycoerythrin cyanine 7 (PE-Cy7; HI10a). The lineage (Lin) PE-conjugated antibody cocktail contained antibodies against CD2 (RPA-2.10), CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD13 (WM15), CD14 (M5E2), CD15 (H98), CD16 (3G6), CD19 (HB19), CD20 (2H7), CD33 (WM53), CD35 (B159), and CD235a (GA-R2). Cells were also stained with LIVE/DEAD Fixable Dead Cell Stain (aqua-fluorescent reactive dye, Invitrogen). Absolute numbers of total lymphocytes were determined using BD TruCOUNT Tubes. For immune reconstitution analysis, PBMCs were thawed and stained with CD4-peridinin chlorophyll protein (PerCP), CD45RA–PE, CD19–PE, CD62L–FITC (Beckman Coulter) and CD27 (Pharmingen). Stained cells were analyzed on a FACS Canto II running with FACS DIVA software (BD Biosciences). Absolute counts of total lymphocytes were assessed by microsatellite analysis and only 1 patient had cells less than 92% of donor origin. Seventy-nine out of 107 patients were monitored for immune reconstitution by lymphocyte phenotyping and sjTREC content. Because of the small number of progenitor cells, different groups of patients were studied in the subsequent experiments: 39 for flow cytometry analysis, 22 for PCR analysis, 6 in OP9–ALI co-culture and 40 to quantify plasma analytes. Peripheral blood mononuclear cells (PBMCs) were separated on lymphocyte separation medium and either sorted immediately or frozen in Fetal Calf Serum (FCS). 10% DMSO for phenotypic analysis. As control, peripheral blood samples from healthy donors (HD) (n = 46) and cord blood (n = 9) were obtained from the Hôpital Saint-Louis Transfusion Center and Cord Blood Bank, respectively. These samples were treated according to the same protocols as patient samples. No significant difference was found between patients and HD in terms of age and sex ratio. The investigation was approved by the Committee on Medical Ethics of the Hôpital Saint-Louis, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

**sjTREC quantification**

PBMCs were separated on lymphocyte separation medium (Eurobio, Les Ulis, France), and 3 to 10<sup>10</sup> cells were lysed and stored in TriReagent (Molecular Research Center, Cincinnati, OH). RNA and genomic DNA were then extracted from samples in line with manufacturer’s instructions. sjTREC were quantified by real-time quantitative PCR (ABI PRISM7700; Applied Biosystems, Foster City, CA) as described [16]. Values were normalized for the genomic copy number, based on albumin gene quantification. Data were expressed per 150 000 PBMC, or the total number of TREC per cubic millimeter of blood was

**DEAD Fixable Dead Cell Stain (aqua-fluorescent reactive dye, Invitrogen)**
Table 1. BMT pairs, disease, and transplant characteristics.

| CHARACTERISTICS | Gr 0-I | Gr II-IV |
|-----------------|--------|----------|
| RECIPIENTS      | n = 59 | n = 48   |
| Male/Female     | 40/19  | 27/21    |
| Median age, y (range) | 36 (6–66) | 40 (7–66) |
| Positive CMV Serology | 35 (59%) | 27 (56%) |
| Underlying diagnosis |
| - Chronic Leukemia | 3 (5.1%) | 6 (12.5%) |
| - Acute Leukemia | 25 (42.4%) | 25 (52.1%) |
| - Other malignant hemopathies | 11 (18.6%) | 6 (12.5%) |
| - Myelodysplasia | 5 (8.5%) | 7 (14.6%) |
| - Aplasia | 15 (25.4%) | 4 (8.3%) |

| DONORS |
|--------|
| Male/Female | 30/29 | 25/23 |
| Median age, y (range) | 33 (5–65) | 36 (21–66) |
| Female donor to male recipient | 17 (29%) | 13 (27%) |
| Positive CMV Serology | 31 (53%) | 30 (62.5%) |

| TRANSPLANTATION |
|-----------------|
| Donor |
| - SIB | 49 (83%) | 37 (77%) |
| - MUD | 10 (17%) | 11 (23%) |
| Source of cells |
| - PB | 35 (59%) | 27 (56%) |
| - BM | 24 (41%) | 21 (44%) |
| Conditioning |
| - Nonmyeloablative | 25 (42%) | 16 (33%) |
| - TBI-based | 17 (29%) | 23 (48%) |
| - Busulfan-based | 24 (41%) | 19 (40%) |

| GVHD Prophylaxis |
|------------------|
| - CSA | 1 (2%) | 0 (0%) |
| - CSA+MTX | 29 (49%) | 28 (58%) |
| - CSA+MMF | 15 (25%) | 14 (29%) |
| - CSA+Others | 2 (3%) | 1 (2%) |
| - Tacrolimus | 1 (2%) | 1 (2%) |

| Acute GVHD grades: |
|-------------------|
| - 0 | 46 (78%) |
| - 1 | 13 (22%) |
| - 2 | 35 (73%) |
| - 3 | 11 (23%) |
| - 4 | 2 (4%) |

No significant difference was found between these two groups for the characteristics listed. BMT: bone marrow transplantation; SIB: HLA-matched sibling, MUD: HLA-matched unrelated donor; PB: peripheral blood; BM: bone marrow; TBI: Total body irradiation; CSA: cyclosporin A; MTX: Methotrexate; MMF: Mycophenolate mofetil; GVHD: Graft-versus-host disease.

Calculated using the absolute cell counts determined using the TruCOUNT system.

Cell sorting

EDTA blood tubes were collected, and PBMCs were separated on lymphocyte separation medium. CD34<sup>+</sup> cells were enriched using CD34 MicroBead Kit (MACS, Miltenyi Biotec) before staining for flow cytometry analysis as described above. The CD34<sup>+</sup> fraction was counted and frozen in FCS, 10% DMSO or lyed in TriReagent. The stained CD34<sup>+</sup> fraction was sorted on a FACS ARIA (BD Biosciences) to isolate CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells and CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells and CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells were collected in 1.5 ml tubes containing 60 μL of RLT Buffer for multiplex PCR analysis or CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells were sorted and seeded at 1, 5, 10, 20, 40 and 100 cells on OP9-hDelta1-coated 96-well plates for limiting dilution assay.

**In vitro T-cell differentiation limiting dilution assay**

T lymphoid potential was tested by plating candidate progenitors on OP9 stromal cells. The stromal cells were transduced with a replication-defective retrovirus containing a double cassette expressing the human Notch ligand Delta1 (hDelta1) and GFP [8]. All cocultures were performed in complete medium (αMEM (Gibco, Invitrogen) supplemented with 20% FCS HyClone (ThermoSCIENTIFIC), 1% penicillin, 1% streptomycin, 5 ng/ml Fli3L, 10 ng/ml SCF and 2 ng/ml IL-7 [all from R&D Systems]). After 3 weeks, wells containing CD5<sup>+</sup>CD7<sup>+</sup> cells were identified by flow cytometry. Data are presented as the percentage of negative wells against the Log<sub>10</sub> of the initial number of cells plated per well. The proportion of lymphoid precursors was calculated by linear regression based on a Poisson distribution. Data were analyzed for statistical significance using a non-parametric Mann-Whitney test. For ethical and methodological reasons, patients with severe aGVHD could not be tested. Patient blood samples measured 30–35 mL, whereas HD provided 100 mL. The high volume of blood from healthy donors permitted us to sort them despite the small amount of blood. In patients without aGVHD, the higher frequency of these cells permitted us to sort them despite the small amount of blood. In patient with severe aGVHD their lower frequency associated with small amount of blood could not allow us to sort enough CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells for the assay.

**Quantitative real-time PCR**

After cell sorting, up to 400 CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> and CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> were lyed in TriReagent. Samples were used to extract RNA according to manufacturer’s instructions. RNA was reverse transcribed (with random hexamers) and amplified (with specific primer, Table S1) using a GeneAmp RNA PCR Kit (Applied Biosystems). For this amplification, the genes studied (with specific primer, Table S1) using a GeneAmp RNA PCR Kit (Applied Biosystems). The same primer couple was used for multiplex PCR analysis or CD34<sup>+</sup>lin<sup>-</sup>CD10<sup>-</sup>CD24<sup>-</sup> cells were tested by plating candidate progenitors on OP9-hDelta1-coated 96-well plates for limiting dilution assay.

**Quantification of cytokine and chemokine concentration**

CCL25 concentration was determined with a human CCL25/TECK DuoSet ELISA (R&D Systems). IL-7 concentration was determined.
Results

Circulating lymphoid progenitor rebound after allo-HSCT in humans is decreased by aGVHD

Naïve T-cell reconstitution was analyzed in a cohort of 79 patients (see Patients and Methods and Table 1) using flow cytometry to track CD4+CD45RA-CD62L+ thymic output was measured by quantitative PCR for 3TREC. As we reported previously [16], patients with grade II to IV aGVHD showed a decreased reconstitution at 3 and 6 months after allo-HSCT. We also observed a delay in naive CD19+CD27+ B-cell reconstitution with aGVHD (Fig. 1A). Based on these observations, month 3 was chosen as the best time point at which to assess the CD34+CD10+CD24- population after allo-HSCT.

Circulating progenitors were quantified by flow cytometry in a subgroup of 39 allo-HSCT patients, in 19 age-matched healthy donors (HD), and in 8 cord blood (CB) samples (Fig. S1). Median percentage and absolute number of CD34+ cells (Data not shown). Lymphoid progenitors characterized as CD34+Lin−CD10+ cells could be detected throughout the postnatal period [8]. They have a very limited myeloid potential when cultured on MS5 stromal cells or in methylcellulose colony formation assay (data not shown). The percentage of CD34+Lin−CD10+ cells within the CD34+Lin− population was not significantly different between CB, HD or allo-HSCT patients (p = 0.014 and p = 0.014 respectively, Fig. 1G). This correlation factorizes the total variability in gene expression. This analysis was displayed using Principal Component Analysis (PCA) which efficiently discriminated between CD34+Lin−CD10+CD24- cells at 3 months and total CD19+ cells, as well as naïve CD19+CD27- B cells at 6 months after allo-HSCT (p = 0.022 and p = 0.034, Table 2). Also, the grafted CD34+ cells dose is predictive of survival, posttransplant morbidity, and rate of hematologic recovery after allo-HSCT [18,19]. Accordingly, in our study, there was a significant correlation between the number of CD34+ cells in the graft and the number of CD34+Lin−CD10+CD24- cells in peripheral blood at 3 months. This correlation was not seen in presence of severe aGVHD (Fig. 1F), suggesting that severe alloimmune BM reactions affect the supply of lymphoid progenitors to the periphery. In addition, another correlation was found between absolute numbers of B-cell-committed CD34+Lin−CD10+CD24- progenitors at 3 months and total CD19+ cells, as well as naïve CD19+CD27- B cells at 6 months after allo-HSCT (p = 0.014 and p = 0.014 respectively, Fig. 1G). This correlation was also only significant in the absence of aGVHD. Overall, at 3 months after allo-HSCT, we observed that the rebound of circulating lymphoid progenitors was abrogated in patients presenting severe aGVHD.

CD34+Lin−CD10+CD24− progenitors display functional features of T-cell progenitors after allo-HSCT

The next step was to ensure that this increased circulating progenitor population detected after transplantation was truly T cell committed. To this aim, CD34+Lin−CD10+CD24- HD and patients without aGVHD were sorted and cultivated on the OP9-DL1 stromal cell line in the presence of Flt3L, IL-7 and SCF to check their T-cell differentiation potential [20]. CD34+Lin−CD10+CD24- cells from patients with severe aGVHD could not be assessed in this assay due to the small volume of blood (<25 ml) available for ethical reasons. In both HD and patients, FACs sorted cells effectively gave rise to T-cells in this system. Using limiting dilution, we found a similar frequency of T-progenitor present between HD (mean = 1/19.3; n = 5) and patients without aGVHD (mean = 1/12.4; n = 6) (Fig. 2A). In conclusion, the rebound of circulating CD34+Lin−CD10+CD24- cells at 3 months after HSCT was accompanied by a sustained T-cell potential.

The gene expression profile was then assessed in two multiplex assays. Genes implicated in progenitor T cell differentiation, 22 genes in total, were screened using a quantitative gene expression method on very limited cell numbers [21]. The 22 genes analyzed were: the chemokine receptors CCR7 and CCR9; CXCR4, a chemokine receptor which plays a role in progenitor egress from the BM; CD44, ITGα4 and PSGL1, adhesion molecules particularly involved in adhesion between progenitor and thymic cells [22]; the cytokine receptors IL7Rα and αK1; actors of the Notch pathway, Notch1 and HES1; several transcription factors preferentially found in stem cells (LMO2, cmyb), progenitors of B cells (EBF1), T-cells (GATA3, RORC, IKAROS) or erythroid cells (GATA1) and finally some markers of the T lineage such as CD3ε, CD4, CD8, CD3δ and RAG1. Cells from 22 patients and 11 HD were isolated by FACs, then the real-time PCR gene expression median value for CD34+Lin−CD10+ cells was compared to that for CD34+Lin−CD10- cells as a control of non-lymphoid primed progenitors, and to that for CD34+ cells (Table S2). Global gene expression was displayed using Principal Component Analysis (PCA) which factorizes the total variability in gene expression. This analysis efficiently discriminated between CD34+Lin−CD10+CD24- and the two other cell subpopulations (Fig. 2B). According to PCA,
CD34+CD10−CD24− cells from HD and patients with or without aGVHD clustered together (Fig. 2C). Thus, both in vitro differentiation on OP9-DL1 stromal cells and gene expression profiling showed the CD34+Lin2CD10+CD24− population have an effective T-cell potential in the context of allo-HSCT.

CXCR4 and CXCL12 expression profiles are consistent with progenitor egress from the bone marrow and are altered in aGVHD.

To get a further mechanistic insight into the CD34+Lin2CD10−CD24− increase, we examined the expression levels of genes involved in progenitor mobilization and homing, and the levels of chemokines in the plasma. We first studied the CXCR4/CXCL12 axis which plays an important role in retaining HSC and progenitors in the BM [23,24]. Our results for patients without aGVHD showed a significant reduction in CXCR4 mRNA expression levels in CD34+Lin2CD10−CD24− cells. Plasma CXCL12 concentration was also reduced in all patients. This is consistent with disruption of CXCL12/CXCR4 signaling and release of lymphoid progenitors into the periphery (Fig. 3A). Patients with severe aGVHD have significantly lower plasma CXCL12 levels than patients without aGVHD (Fig. 3A) due to a reduced production or a higher destruction rate of the chemokine.

Thymic homing and T-cell commitment could be altered after allo-HSCT, especially with severe aGVHD

Several molecules have been shown to play a role in thymus colonization in mice. These include CCR7 and its ligand (CCL19/21), CCR9 and its ligand (CCL25) [25,26], and PSGL1 [27]. In humans, the mechanisms of thymus colonization have been less studied, but similar chemokines may be involved, at least during the fetal and perinatal periods [8,10,28]. In our study, whereas PSGL1 expression was not modified (Data not shown), CCR7 and CCR9 mRNA expression was reduced after allo-HSCT, even in the absence of aGVHD. The plasma concentration of the CCR9 ligand, CCL25, was not modified. Conversely, CCL19 and CCL21 concentrations were significantly reduced in patients after allo-HSCT. CCL19 was further reduced in patients presenting severe aGVHD (Fig. 3B).

The majority of T-cell commitment markers (CD3ε, IKAROS, RAG1) were not modified by allo-HSCT or GVHD. IL7 serum concentration was reported as maximum right after the transplant conditioning regimen and IL7 level at days +7, +14 [29] or +30...
has been associated with aGVHD incidence. However, in this study at day +90, GATA-3 and IL-7Rα mRNA expression, together with IL7 plasma concentrations, were significantly reduced in all patients compared to HD. These changes were independent of aGVHD status (Fig 3C).

Discussion

The initial report of a rebound in thymic activity after intensive chemotherapy in children [31] has been extended to other settings in adults including autologous [32] and allogeneic [3] HSCT and appears to be crucial in the regeneration of peripheral T-cell populations. Thymic-dependent T-cell reconstitution is a slow process after allo-HSCT in humans which can be evaluated phenotypically, by TREC quantification and TCR diversity analysis [4]. Immune recovery is further impeded in humans during the aGVHD immune reaction [1]. Whereas the impact of aGVHD on thymic function has been documented [16], there is no data yet on the characteristics of lymphoid progenitors in the condition of human allo-HSCT and aGVHD.

Our findings show in humans that immune reconstitution needs to be considered in a global perspective integrating progenitor supply by the bone marrow and, for T cells, their competence in thymic homing and T-cell differentiation. It seems striking to us [30] has been associated with aGVHD incidence. However, in this study at day +90, GATA-3 and IL-7Rα mRNA expression, together with IL7 plasma concentrations, were significantly reduced in all patients compared to HD. These changes were independent of aGVHD status (Fig 3C).

Table 2. Univariate and multivariate analysis of factors influencing CD34+Lin CD10+ and TSP recovery after HSCT.

| Factors          | Median Nb/blood mm3 (Range) | Univariate analysis p | Multivariate analysis p |
|------------------|----------------------------|-----------------------|-------------------------|
| CD34+Lin CD10+   | N=18                      | 0.055 (0–0.362)       | 0.014 (0–0.194)         |
| CD34+Lin CD10+   | N=21                      | 0.144 (0–0.194)       | 0.025 (0–0.154)         |
| CD34+Lin CD10+   | N=19                      | 0.334                 | 0.032 (0–0.236)         |
| CD34+Lin CD10+   | N=20                      | 0.334                 | 0.032 (0–0.236)         |
| CD34+Lin CD10+   | N=19                      | 0.334                 | 0.032 (0–0.236)         |
| CD34+Lin CD10+   | N=20                      | 0.334                 | 0.032 (0–0.236)         |

Statistical significance was tested using a non-parametric Mann-Whitney test for univariate data and using ANOVA for multivariate analysis.

Figure 2. CD34+Lin CD10+CD24- progenitors display functional features of T-cell progenitors after allo-HSCT. (A) Limiting dilution assay analysis on OP9-DL1 with CD34+Lin CD10+CD24- cells sorted from peripheral blood of healthy donors (HD) and at day 100 post allo-HSCT in patients without aGVHD, or with grade I aGVHD (Gr 0-1). NS: Not Significant (Mann-Whitney). (B) Principal Component Analysis (PCA) of CD34+Lin CD10+CD24- (circles), CD34+Lin CD10- cells (squares) and CD34- cells (triangles) from 11 HD and 21 allo-HSCT patients using a panel of 22 genes (see Material & Methods). (C) PCA of CD34+Lin CD10+CD24- from healthy donors (HD, open circles) or patients with (green filled circles) or without severe aGVHD (red filled circles).

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that the circulating progenitors rebound we detected 3 months after transplantation was concomitant to the reduced CXCR4 expression in these cells and to a decreased CXCL12 plasmatic concentration. CXCL12, or stromal cell-derived factor 1a (SDF-1a), is constitutively produced at high levels in the BM by stromal cells where it maintains HSC in a quiescent state. It is also required for their retention in the BM [23,24]. Disruption of the interaction between CXCL12 and its receptor, CXCR4, results in the mobilization of cells from the BM and their release into the blood. CXCR4 antagonists, in particular AMD3100, have been used to mobilize HSCs for HSCT in humans [33]. Disruption of CXCL12/CXCR4 signaling is also a key step in HSC mobilization by G-CSF [34,35]. This led us to suspect a causative role of CXCR4/CXCL12 in the lymphoid progenitors rebound during the profound lymphopenia observed after allo-HSCT. Whether this could apply to other situations of lymphopenia and myelosuppression, during intensive chemotherapy for instance, will deserve further studies. In the BM, CXCL12 is produced by osteoblast precursors and CXCL12-abundant reticular cells (CAR), a stromal perivascular population involved in HSC and B lymphoid progenitor maintenance [29]. Some recent evidence in mice indicates that early lymphoid progenitors occupy a distinct endosteal niche not shared by HSC [36,37]. Acute GVHD may alter this microenvironment and the capacity to produce CXCL12. In line with this, in a murine model, osteoblasts were found to be the main target of aGVHD in BM [14]. CXCL12 degradation could also explain the reduced levels of this chemokine in the periphery, especially with severe aGVHD, as CXCL12 is extremely sensitive to proteolysis [35]. We believe that, in the absence of aGVHD, low CXCL12 levels favor proliferation and egress of committed progenitors from the BM as part of a regenerative mechanism to correct for lymphopenia. This hypothesis is supported by murine models of myelosuppression [24]. In patients presenting aGVHD, due to stromal injury and possibly also to therapies such as corticosteroids, a further decrease of CXCL12, together with disruption of the BM niche, could alter this process [38]. However, patient with grade I aGVHD that don’t receive corticosteroid, had already less progenitor cells than patients without aGVHD (Fig. S2), arguing for a direct effect of the disease.

A second finding was the potential limitations in thymus seeding properties of CD34+Lin−CD10−CD24− cells gene expression and plasma concentrations of chemokines and cytokines are associated with alterations to BM egress, thymic homing and T-cell commitment. (A) Relative CD34Lin−CD10−CD24− mRNA expression by quantitative RT-PCR of CXCR4 and plasma concentration of CXCL12. (B) RT-PCR quantification of CCR7, CCR9 and plasmatic dosage of their respective ligands CCL19, CCL21 and CCL25. (C) RT-PCR quantification of IL7RA and GATA3 and plasmatic dosage of IL-7 in healthy donors (HD, white boxes/circles), patients with grade II-IV aGVHD (Gr II-IV, black boxes/circles), patients with grade 0-I aGVHD (Gr 0-1, grey boxes/circles). *p<0.05, **p<0.01, ***p<0.001, NS: Not Significant (Mann-Whitney). doi:10.1371/journal.pone.0091492.g003

Figure 3. Allo-HSCT and aGVHD effects on CD34Lin−CD10−CD24− cells gene expression and plasma concentrations of chemokines and cytokines are associated with alterations to BM egress, thymic homing and T-cell commitment.
in vivo -generated T-cell progenitors [44]. However, these approaches may also be hampered by an altered thymic environment when used in clinical settings.

In total, we show in humans a physiological rebound of circulating lymphoid progenitors after allo-HSCT. This rebound is impaired by aGVHD supporting BM stromal injury as the initial mechanism impacting immune recovery. In terms of therapeutic consequences, attempts should be made to protect the BM stromal microenvironment, and especially the endosteal niches where early lymphoid progenitors are found [36,37]. Radiation-resistant myeloid cells, by limiting ROS production, may play a protective role in this zone under stress condition [43]. Finally, Cellular Therapy approaches have been developed to bypass the delivery of lymphoid progenitors from the BM by using adoptive transfer of in vitro-generated T-cell progenitors [44]. However, these approaches may also be hampered by an altered thymic environment when used in clinical settings.

References

1. Krenger W, Blazar BR, Holland GA (2011) Thymic T-cell development in allogeneic stem cell transplantation. Blood 117: 6760–6766.
2. Clave E, Rocha V, Talvensaari K, Busson M, Douay C, et al. (2005) Prognostic value of pretransplantation host thymic function in HLA-identical sibling hematopoietic stem cell transplantation. Blood 105: 2608–2613.
3. Roux E, Dumont-Girard F, Stastnikov B, Sigrist CA, Helg C, et al. (2000) Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood 96: 2299–2303.
4. Talvensaari K, Clave E, Douay C, Rabian G, Garderet L, et al. (2002) A broad T-cell repertoire diversity and an efficient thymic function indicate a favorable long-term immune reconstitution after cord blood stem cell transplantation. Blood 99: 1458–1464.
5. Martins VC, Ruggiero E, Schlenner SM, Madan V, Schmidt M, et al. (2012) Thymus-autonomous T-cell development in the absence of progenitor import. The Journal of experimental medicine 209: 1409–1417.
6. Peaudecerf L, Lemos S, Galgano A, Krem G, Vansever F, et al. (2012) Thymocytes may persist and differentiate without any input from bone marrow progenitors. The Journal of experimental medicine 209: 1401–1408.
7. Doulasov S, Notta F, Laurenti E, Dick JE. (2012) Hematopoiesis: a human perspective. Cell stem cell 10: 120–136.
8. Six EM, Bonhomme D, Monteiro M, Beldjord K, Jurkowski M, et al. (2007) A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. The Journal of experimental medicine 204: 3085–3093.
9. Doulasov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, et al. (2010) Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nature immunology 11: 585–593.
10. Kohn LA, Hao Q-L, Sasidharan C, Ge S, et al. (2012) Lymphoid priming in human bone marrow begins before expression of CD10 with upregulation of L-selectin. Nature immunology 13: 963–971.
11. Hauri-Hohl MM, Keller MP, Gill J, Hafen K, Pachaltko E, et al. (2007) Donor T-cell alloreactivity against host thymic epithelium limits T-cell development after bone marrow transplantation. Blood 109: 4090–4091.
12. Seemayer TA, Lapp WS, Bolande RP (1977) Thymic involution in murine graft-versus-host disease. American journal of pathology 88: 119–134.
13. Krenger W, Blazar BR, Holland GA (2010) Thymic T-cell development in allogeneic stem cell transplantation. Blood 117: 6760–6766.
14. Haddad R, Guimiot F, Six E, Jourquin F, Setterblad N, et al. (2006) Dynamics of hematopoietic progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood 80: 3961–3980.
15. Schmitt TM, Zúñiga-Pflücker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17: 749–756.
16. Mavroidis D, Read E, Cotterill Fox, Courier D, Mollodren J, et al. (1996) CD34+ cell dose predicts survival, posttransplant morbidity, and rate of hematologic recovery after allogeneic marrow transplants for hematologic malignancies. Blood 88: 3223–3229.
17. Zlotoff DA, Zhang SL, De Obaldia ME, Hess PR, Todd SP, et al. (2011) Absolute numbers of lymphoid progenitors correlate with aGVHD severity. (PDF)
18. Mavroidis D, Read E, Cotterill Fox, Courier D, Mollodren J, et al. (1996) CD34+ cell dose predicts survival, posttransplant morbidity, and rate of hematologic recovery after allogeneic marrow transplants for hematologic malignancies. Blood 88: 3223–3229.
19. Weaver CH, Hazeldon B, Birch R, Palmer P, Allen G, et al. (1995) An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood 80: 3961–3980.
20. Schmitt TM, Zúñiga-Pflücker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17: 749–756.
21. Rossi FMV, Corbel SY, Merzaban JS, Carlow DA, Gossens K, et al. (2005) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25: 937–948.
22. Teng Y-S, Li H, Kang Y-L, Chen W-C, Cheng W-C, et al. (2011) Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myeloablation. Blood 117: 429–439.
23. Sugiyama T, Kohara H, Noda M, Nagasawa T (2006) Maintenance of the hematopoietic stem cell pool by CXCL12/CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25: 937–948.
24. Tzeng Y-S, Li H, Kang Y-L, Chen W-C, Cheng W-C, et al. (2011) Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myeloablation. Blood 117: 429–439.
25. Zlotoff DA, Sambandam A, Logan TD, Bell J, Schwartz BA, et al. (2010) CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. Blood 115: 1906–1912.
26. Zlotoff DA, Sambandam A, Logan TD, Bell J, Schwartz BA, et al. (2010) CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. Blood 115: 1906–1912.
27. Boyce WR, Willerford D, Lyster DJ, Kremm E, Forster R, et al. (2010) CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. Blood 115: 1906–1912.
28. Haddad R, Guimiot F, Six E, Jourquin F, Setterblad N, et al. (2006) Dynamics of hematopoietic colonizing cells during human development. Immunity 24: 217–230.
29. Dean RM, Fry T, Mackall C, Steinberg SM, Hakim F, et al. (2008) Association of serum interleukin-7 levels with the development of acute graft-versus-host disease. J Clin Oncol 26: 5733–5741.
30. Thiart S, Lalabette M, Trauet J, Couture VS, de Berranger E, et al. (2011) Plasma levels of IL-7 and IL-15 after reduced intensity conditioned allo-SCT and relationship to acute GVHD. Bone Marrow Transplantation 46: 1374–1381.
31. Mackall CL, Fleisher TA, Brown MR, Andrich MP, Chen CC, et al. (1995) Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. The New England journal of medicine 332: 143–149.
32. Hakim FT, Memon SA, Cepeda R, Jones EC, Chow CK, et al. (2005) Age-dependent incidence, time course, and consequences of thymic renewal in adults. The Journal of clinical investigation 115: 930–939.
33. Dar A, Schajnovitz A, Lapid K, Kalinkovich A, Itkin T, et al. (2011) Rapid mobilization of hematopoietic progenitors by AMD3100 and catecholamines is mediated by CXCR4-dependent SDF-1 release from bone marrow stromal cells. Leukemia 25: 1286–1296.
34. Rettig MP, Anastas G, DiPersio JF (2012) Mobilization of hematopoietic stem and progenitor cells using inhibitors of CXCR4 and VLA-4. Leukemia 26: 34–53.
35. Lévesque J-P, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. The Journal of clinical investigation 111: 187–196.
36. Ding L, Morrison SJ (2013) Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495: 231–235.
37. Greenbaum A, Hsu Y-MS, Day RB, Schuettelpelz LG, Christopher MJ, et al. (2015) CXCL12 in early mesenchymal progenitors is required for hematopoietic stem-cell maintenance. Nature 495: 227–230.
38. Kollet O, Dar A, Shvietel S, Kalinkovich A, Lapid K, et al. (2006) Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nature medicine 12: 657–664.
39. Misliitz A, Pabst O, Hinzgen G, Ohol I, Kremmer E, et al. (2004) Thymic T cell development and progenitor localization depend on CCR7. The Journal of experimental medicine 200: 481–491.
40. Tichelli A, Gratwohl A (2008) Vascular endothelium as ‘novel’ target of graft-versus-host disease. Best practice & research Clinical haematology 21: 139–146.
41. Hooya T, Kursha T, Moriyachi T, Cummings D, Maillard I, et al. (2009) GATA-3 is required for early T lineage progenitor development. The Journal of experimental medicine 206: 2987–3000.
42. Mackall CL, Fry TJ, Gress RE (2011) Harnessing the biology of IL-7 for therapeutic application. Nature reviews Immunology 11: 330–342.
43. Ludin A, Itkin T, Gur-Cohen S, Mildner A, Shezen E, et al. (2012) Monocytes-macrophages that express α-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. Nature immunology 13: 1072–1082.
44. Reimann C, Six E, Del-Cortivo L, Schario A, Appourchaux K, et al. (2012) Human T-lymphoid progenitors generated in a feeder-cell-free Delta-like-4 culture system promote T-cell reconstitution in NOD/SCID/γc(-/-) mice. Stem cells (Dayton, Ohio) 30: 1771–1780.