

DNA Barcoding of Human Stem and Progenitor Cells Reveals Differences in Clonal Dynamics of B and T lymphoid Progeny

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Abstract

Proper immune reconstitution after hematopoietic stem cell (HSC) transplantation requires *de novo* T cell development in the thymus to ensure a broad and diverse T cell receptor (TCR) repertoire. The clonal relationship between HSCs and T lymphocytes has long been unclear, especially for human T cells. Previous work showed severe clonal restriction in the thymus. Using a cellular barcoding system, which allows quantitative tracking of the progeny of individual stem cells, we here show that clonal restriction is less severe in B cells and that B cells and T cells often arise from different stem cell clones. Finally, when using total CD34⁺ progenitor cells rather than stem cells, there is a significant contribution from non-stem cell clones to the T cell pool. Thus, relatively short-lived T cell progenitors can provide significant contribution to long living T lymphocytes.

Keywords: Cellular barcoding; HSC; NSG mice; Xeno transplantation; B cell development; T cell development

Introduction

Hematopoietic stem cell transplantation (HSCT) has been highly successful in the treatment of malignant diseases such as various types of leukemia and myelodysplastic syndromes but also for blood-borne, non-malignant diseases (hemoglobinopathies, sickle cell anemia, immunodeficiencies).

While often curative, three problems are commonly associated with HSCT: (1) low hematological reconstitution as witnessed by low numbers of neutrophils and platelets due to low HSCs numbers in the graft, (2) poor immune reconstitution mostly due to poor T lymphocyte recovery and (3) Graft versus host disease. Low stem cell numbers in the transplant lead to graft failure, but in the case that enough cells are present to ensure a timely neutrophil recovery occurs, a partial immunodeficiency might still be introduced due to the lack of thymus repopulating cells. This problem of proper T cell reconstitution has gained much more interest in recent years, as it is a significant cause of morbidity, often from common viral infections.

As patients experience slower T cell reconstitution after umbilical cord blood (UCB) HSCT, they are at risk for infections and virus reactivation. Mortality related to infections after UCB HSCT can be as high as 30-45% shortly after transplantation [1,2] and up to 11% of treatment related mortality is caused by infection. Of transplant related mortality, cause of infectious death are estimated at 36% for bacteria, 31% for viruses, 28% for fungi, and 5% for parasites [3].

Thus, T cell reconstitution, the process starting from T cell progenitors in the bone marrow that in a multistage developmental process result in self-restricted CD4 and CD8 T cells is critical because failure to generate new T cells results in acute infection and late complications caused by immunodeficiency.

An accompanying complication of limited T cell repopulation is that reactivation of cytomegalo virus (CMV) or Epstein-Barr virus (EBV) will drive post-transplant T cell reconstitution and result in defects in the underlying TRB repertoire [4]. In addition, presence of naive T cells in the transplant can lead to homeostatic proliferation of these cells, resulting in a limited immunological repertoire as defined by TCR complexity. Depletion of T cells from the transplant removes the first wave of repopulation from naive cells in the UCB HSCT but this results in slower T cell reconstitution [5] albeit with a broader T cell repertoire than after bone marrow (BM) HSCT. In general, slower T cell reconstitution is observed after UCB HSCT [6]. Compared to other sources of stem cells, although higher donor chimerism is obtained after UCB HSCT.

A relationship between proper hematological reconstitution and proper immunological reconstitution has often been assumed, as transplantation of more stem cells should in principle also lead to presence of more T cell progenitors that can seed the thymus. However, attempts to improve immune reconstitution by transplanting larger cell doses have been mostly unsuccessful underscoring the need for better insight into the clonal relationship between HSCs and T lineage cells. To gain a better understanding into the number of HSCs seeding the thymus and giving rise to peripheral T cells, we made use of a technique called lentiviral cellular barcoding. We marked individual human CD34⁺ progenitor cells or highly purified human HSCs [7] and transplanted these cells using an immune deficient mouse model that allows for human T cell development. While in mice clonal hematopoietic reconstitution has been studied in great detail, the study of human T cell repopulation has not yet reached this level of understanding, mostly because the study of human hematopoiesis is more cumbersome. Virus insertion sites have been used as markers for clonality, which allowed repopulation studies in mice [8,9] and rhesus monkeys [10,11] and severe combined immunodeficiency (SCID) patients that underwent gene therapy [12]. The studies using virus integration sites had several disadvantages concerning recovery and

quantification the individual marked cells, but provided early insight into the dynamics of clonal repopulation.

DNA barcoding improves quantification of clones

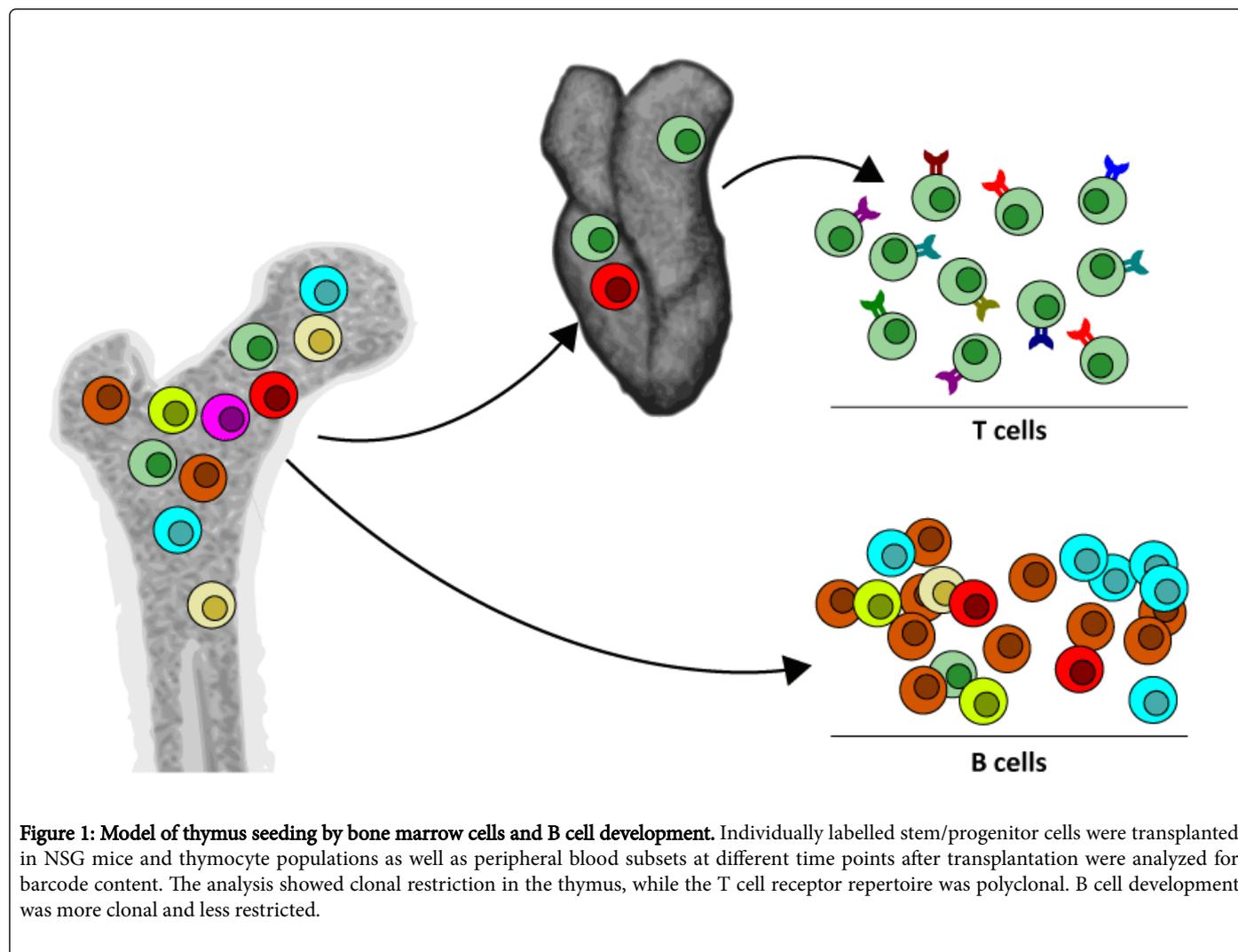
Understanding the hematopoietic clonality of T cell development is also important when T cell development from HSC must be closely monitored, which for example is the case in gene therapy of monogenic hematopoietic diseases. Early trials using gene modified CD34⁺ cells, although quite effective, showed that a fraction of the patients developed T cell leukemias as a result of the viral insertion [13-16]. Retrospective analysis in patient samples, using deep sequencing technology revealed that the insertion associated with the T cell deregulation was difficult to retrieve from earlier samples. Sensitivity of virus integration site determination increased with newly developed methods [17-21] but many of these techniques have not been rigorously tested for reproducibility and method relying on PCR amplification of the insertion site fragments (LM-PCR, LAM-PCR and nsLAM-PCR) suffer from quantification issues [19].

The use of DNA barcodes in the viral vectors further enhanced quantification and allowed large numbers of HSC clones to be traced over time in mice [22,23], NOD/SCID/IL2Rg^{-/-} (NSG)

xenotransplantation [24] and rhesus macaques [25], but these studies focused on hematopoietic reconstitution without considering the development of T cells.

Clonal analysis of hematopoietic reconstitution

Using xenografting of DNA barcoded cord blood CD34⁺ cells and HSC, we were able to demonstrate gated entry into the thymus using human cells and showed progressive selection of hematopoietic clones that were able to generate a diverse TCR repertoire from single hematopoietic progenitors [26]. Analysis of phenotypically defined human HSC transduced with a lentivirus carrying a defined set of DNA barcodes and transplanted into NSG mice, gave new insights into the clonal composition of T cell development in the thymus (Figure 1). Even in samples with a very limited clonal diversity, where one or two HSC clones make up most of the thymic CD4⁺CD8⁺ double positive (DP) and CD4 or CD8 single positive (SP) compartments, a diverse spectrum of TRB, TRG and TRD rearrangements was found. Thus, a diverse TCR repertoire can be derived from a small number of HSC clones. Therefore clonality at the HSC level (hematological clonality) and a fully diverse TCR repertoire (immunological clonality) are independently regulated.



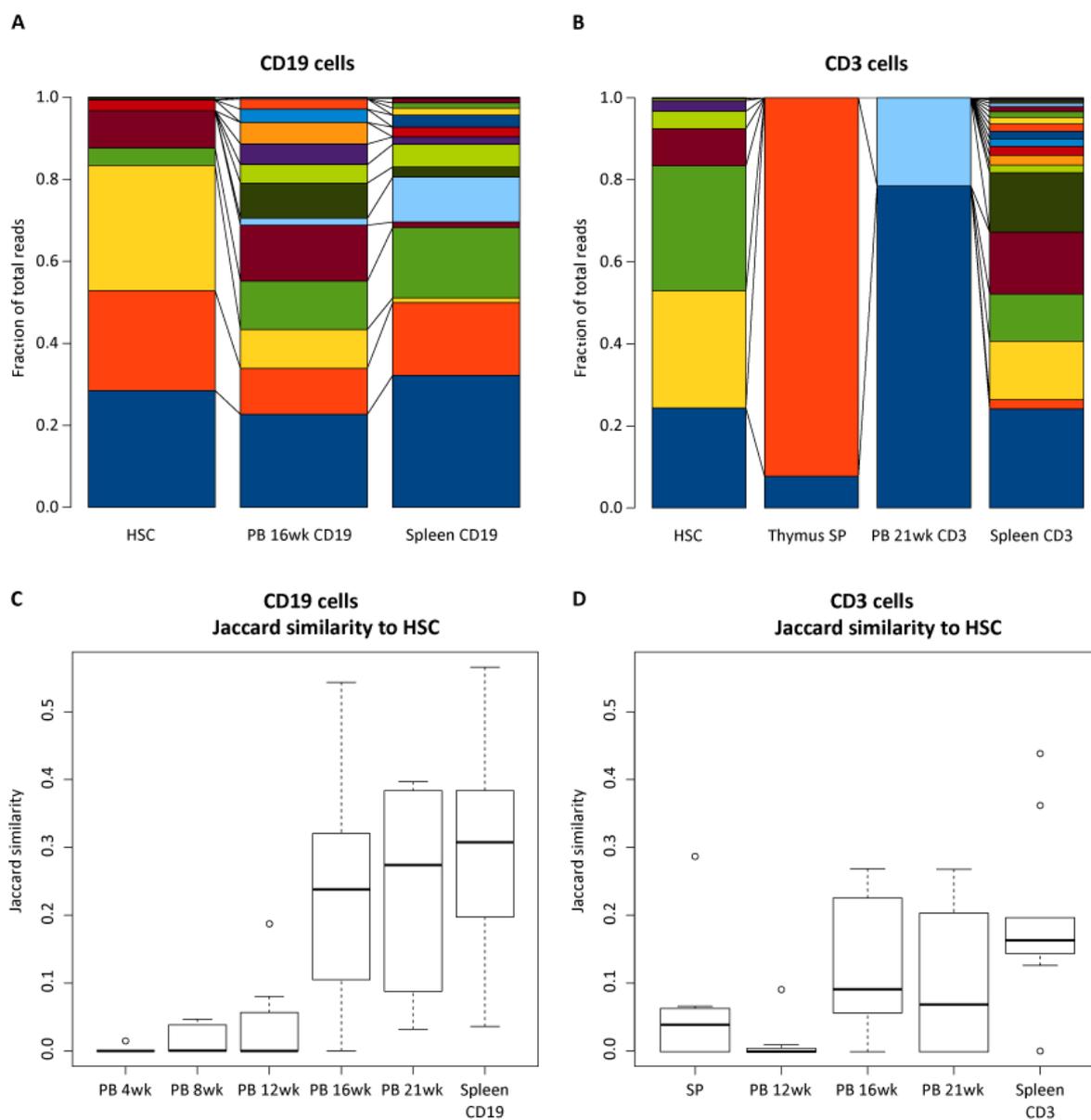


Figure 2: Different clonal contribution in T vs B cell clones. Data from 9 different NSG mice transplanted with barcoded purified human HSCs. The individual clones are represented by different colors, with the full bar representing 100% of that population. **(A)** For B cells, HSC, peripheral blood at 16 wk post-transplant and spleen 16 weeks post-transplant are shown for a representative animal. **(B)** For T cells, HSC, single positive thymocytes, peripheral blood and spleen are shown at 21 weeks. The Jaccard index was calculated between HSC and the indicated samples obtained in 9 mice in the CD19 **(C)** or CD3 **(D)** sorted samples. The diversity in B cells is greater and arising from different clones compared to T cells

Clonal restriction during T cell development

From studies in mice the critical factors governing T cell development have been identified. First, entry into thymus is regulated by chemokines that attract progenitors to the thymus. The presence of blood borne precursors of T cells was demonstrated already 25 years ago in mice [27]. The import of these precursors, which are contained in the multipotent progenitor (MPP) population in the murine BM, is

regulated by CCR9 [28], CCR7 [29-32] and CXCR4 [33], suggesting that a redundant system for homing of T cell progenitors is in place. The amount peripheral T cells are carefully controlled and T cell numbers are maintained by both peripheral expansion and production of new T cells in the thymus. In the thymus, T cells progenitors are allowed entry from the bone marrow in a process that is controlled by P-selectin on thymic stroma and P-selectin glycoprotein ligand-1 (CD162 / PSGL-1) on the T cell progenitors [34] and the expression of

P-selectin is controlled by sphingosine-1-phosphate (S1P) concentration [35], which functions as the gatekeeper for entry into the (early thymocyte progenitor) ETP niches, the number of which is estimated to be around 160 per mouse [36]. Fucosyltransferase 4 and 7 double knockout cells, which fail to produce PSGL-1, correspondingly are insensitive to P-selectin signals and fail to home to the thymus [37].

Second, intrathymic events select for certain clones, through mechanisms that are not well understood but involve clonal fitness, as was shown by Rodewald and coworkers for preleukemic clones in the thymus. These investigators have proposed cell competition as a mechanism underlying selection for cellular fitness during T cell development [38]. It would provide a possible explanation for the further clonal restriction at CD4⁺CD8⁻CD34⁺CD1a⁺ (DN3), DP and SP stages that we observe in many mice after the initial thymic entry restriction. We have therefore proposed that the progeny from different HSC clones have inherently different capacity to pass through various developmental checkpoints in the thymus, for instance based on metabolic activity.

Third, control mechanisms from the periphery, i.e. the numbers of T cells feedback to the thymus. In mice, the import of thymus seeding cells is governed by S1P levels in the peripheral blood, which in turn are governed by the pool of T cells in the periphery [35].

Differences in B versus T lymphocyte stem cell clonality

In the experiments where we transplanted phenotypically defined human HSC or CD34⁺ cells into irradiated NSG mice [26], we observed that the clonal development of B cells in the spleen and peripheral blood followed the clonality in the bone marrow (Figure 2A). T cells clonality was remarkably different, and showed clonal skewing upon progression through the thymus. PB and splenic CD3 cells, harvested at the same time, showed increased number of T clones, (Figure 2B) suggesting that the T cell clones, as determined by barcode, rather than by TCR repertoire, had been seeded to the thymus and had already undergone selection before accumulating in spleen and PB. Analysis of the similarity measures of the clonal content demonstrated that similarity between HSC and B cell samples at later time points is larger than the similarity between HSC and T cell samples (Figures 2C and 2D). This phenomenon seems to demonstrate the gated thymus seeding behavior that has previously been described in mice. Our studies underpinned the molecular understanding of thymus seeding by showing the clonal dynamics of thymus seeding of human cells.

Strategies to improve T cell repopulation

Our data suggests that increase in cell dose is probably not an effective strategy for improving T cells recovery, since T cell development is limited by the thymic environment [26], yet our increased understanding of the molecular and clonal processes that lead to T cell repopulation provoke suggestions of how to enhance T cell repopulation. Several strategies focusing on function and proliferation of thymic epithelium rather than transplanted cell dose have been suggested [39], for example using keratinocyte growth factor (KGF) [40-42], IL7 [43], FMS-like tyrosine kinase 3 ligand (FLT3L) [44,45], growth hormone [46], thyrotropin [47], or employing androgen depletion which results in improved T cell reconstitution [48-50], possibly due to the removing the sex steroid inhibition of Notch signalling through Dll4 on the cortical thymic endothelium cells (cTECs) [51].

In vivo cytokine treatments should however be approached with caution at the risk steering homeostatic peripheral expansion rather than thymopoiesis with use of IL7 [52] increased graft versus host disease [53] and the risk of antibody formation against the cytokine used [43].

Since S1P levels in mice are controlling the gate keeping process for seeding the ETP niches, it seems likely that S1PR agonists, such as FTY720 or SEW2871 could be used to similar effect, yet these compounds have mostly been used to control thymus egress [54,55] and lymphoid cell trafficking [56].

Other strategies to improve T cell expansion are aimed at improving the thymus function, such as intrathymic injection of multipotent HSPC in mice [57] or pretreatment of the thymus with *in vitro* generated T cell progenitors in a xenotransplantation setting [58]. In this context it is important to note that such strategies are not only applicable after HSCT, but can also be used to stimulate rediversification of TCR repertoire in cases of autoimmune disease, for example using autologous HSCT [59].

Origin of bone marrow derived T cell progenitors

A comparison of transplanted cell and the calculated frequency of 1/250 HSC per CD34⁺ cell, showed that the CD34 transplantation had repopulated T cells from approximately twice the number of clones than were in the initial HSC population. In HSC transplanted samples the number of clones and transplanted HSC were roughly equal, providing evidence for the existence of T cell progenitors in the cord blood. This phenomenon demonstrates that the presence of thymus seeding cells in BM CD34⁺ that was suggested by Fisher is an important fraction of the CD34⁺ sample when compared to the true HSC [7].

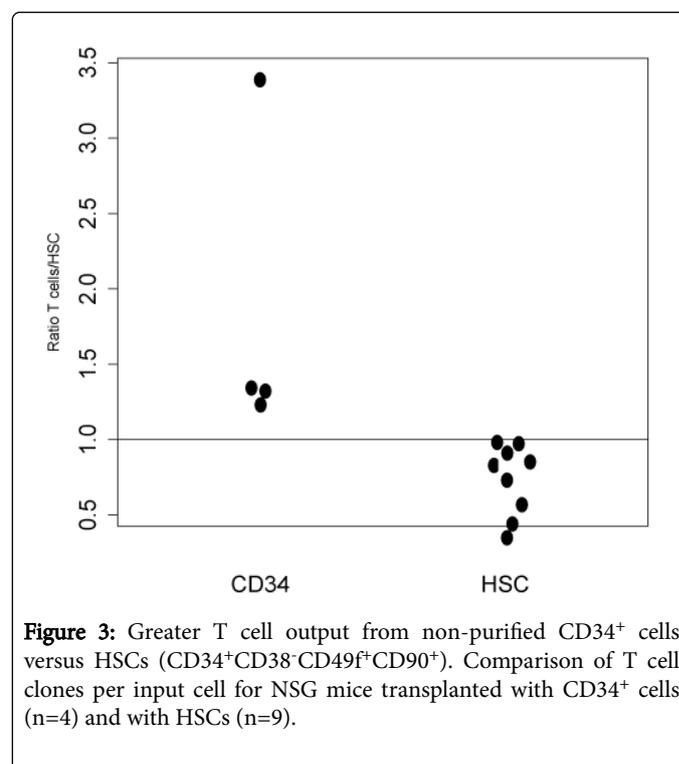


Figure 3: Greater T cell output from non-purified CD34⁺ cells versus HSCs (CD34⁺CD38⁻CD49f⁺CD90⁺). Comparison of T cell clones per input cell for NSG mice transplanted with CD34⁺ cells (n=4) and with HSCs (n=9).

Putative T cell progenitors have been isolated and expanded from cord blood cells previously with the aim to increase the number of cells that can functionally seed the thymus [58,60-64]. In our T cell reconstitution experiments with barcoded human HSC, our data demonstrated that we observed T cell reconstitution as progeny from these barcoded stem cells by these cells. Based on the phenotype of the human HSC [7], a transplant of 150,000 CD34⁺ should contain ~625 HSC per mouse, and considering transduction efficiency of 14% HD, 88 transduced clones per mouse are expected. In several other experiments, we used different numbers of total CD34⁺ cells, which contain more differentiated cells besides HSCs. The number of observed clones in the CD34 transplanted mice was 160 ± 92 clones per mouse for HD CD34 (mean ± standard deviation), while from the mice that received 1000 HSC with a transduction efficiency of ~10% (resulting in 100 transduced cells) a slightly lower number of 81 ± 14 clones per mouse was retrieved (Figure 3). This suggests that in mice transplanted with CD34⁺ cells, even at relative late time points of 16-21 weeks after transplantation, cells derived from non-HSC progenitors contribute a significant (Mann-Whitney p=0.007) proportion to the repopulation compared to phenotypically defined HSC. This is consistent with a notion put forward by Fisher and co-workers [65] that patients transplanted with CD34⁺ cells repopulate in part from progenitors.

Final Remarks

Data presented elsewhere [26] and here confirm the notion that proper T cell reconstruction with a highly diverse repertoire is difficult to achieve and increasing the transplanted cell dose does not necessarily lead to improved T cell reconstitution. There also seems to be a fundamental difference in the clonal B lineage output versus T cell lineage output, which is much more clonally restricted and often resulting from different stem cell clones than the B cells. Therefore, immune incompetence after HSCT is not related to the transplantation of limited numbers of HSC but to intrathymic events. Efforts aimed at improving function of the thymic epithelium are key in improving immunological immune reconstitution after stem cell transplantation. Finally, when using total CD34⁺ progenitor cells rather than stem cells, there is a significant contribution from non-stem cell clones to the peripheral T cell pool. Thus, relatively short-lived T cell progenitors can provide significant contribution to long living T lymphocytes, which is important to note when using gene therapy to cure T cell deficiencies. Highly purified stem cells, while useful from the point of view of transplanting lower numbers of gene modified cells in the patient, may inherently provide less robust T cell reconstitution.

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