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Mini-Review

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Space-time considerations for hematopoietic stem cell transplantation

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The mammalian blood system contains a multitude of distinct mature cell lineages adapted to serving diverse functional roles. Mutations that abrogate the development or function of one or more of these lineages can lead to profound adverse consequences, such as immunodeficiency, autoimmunity, or anemia. Replacement of hematopoietic stem cells (HSC) that carry such mutations with HSC from a healthy donor can reverse such disorders, but because the risks associated with the procedure are often more serious than the blood disorders themselves, bone marrow transplantation is generally not used to treat a number of relatively common inherited blood diseases. Aside from a number of other problems, risks associated with cytoreductive treatments that create "space" for donor HSC, and the slow kinetics with which immune competence is restored following transplantation hamper progress. This review will focus on how recent studies using experimental model systems may direct future efforts to implement routine use of HSC transplantation to cure inherited blood disorders.

Key words: Common lymphoid progenitor · Common myeloid progenitor · Hematopoietic stem cell · Immune reconstitution · Transplantation

Introduction

Lifelong blood homeostasis is primarily the responsibility of hematopoietic stem cells (HSC), which can self-renew for life while retaining the capacity to differentiate into distinct mature lineages. The numbers of HSC and the decisions to undergo self renewal or differentiation are generally thought to be regulated by specific HSC-supportive niches [1], which may be agents of clonal selection and/or direct regulators of cell fate through their actions; yet deleterious cell-intrinsic mutations in HSC, as they are passed along to downstream progenitors and mature cells, can cause immunodeficiency, anemia, various types of autoimmunity, and increase the chance of leukemia and lymphoma. In these settings, the replacement of the mutant HSC with genetically normal HSC is an important clinical goal.

Fortunately, HSC possess several unique properties that have allowed them, in the context of bone marrow transplantation, to become the only type of stem cell in routine clinical use. First, although most HSC normally reside in the extravascular space in the bone marrow, they have the remarkable ability to home back to their specialized niches in the bone marrow after injection into venous circulation. This ability to re-circulate appears to be linked to the normal properties of HSC, since they constitutively migrate between the bone marrow, blood, lymph, and extramedullary organs under physiological conditions [2, 3]. Thus, HSC can be injected intravenously rather than orthotopically into the bone marrow of recipients, making their clinical use relatively straightforward. Second, by enhancing the circulatory properties of HSC using pharmacological agents such as granulocyte colony stimulating factor or AMD3100 [4, 5], physicians now routinely obtain HSC from peripheral blood of healthy donors rather than from bone marrow, a more invasive procedure. Finally, HSC express a unique combination of cell surface markers that enable their purification. Although rigorous purification of HSC prior to clinical transplantation is only rarely performed [6], HSC can be isolated to near homogeneity as evidenced by single-cell transplants with murine HSC and ten-cell xenotransplants using human HSC [7, 8].

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Nevertheless, a number of clinical barriers remain which prevent the routine use of bone marrow transplantation for the treatment of blood diseases, particularly inherited disorders. Some of the most serious issues are graft-*versus*-host disease (GVHD), balancing the side effects of cytotoxic pre-conditioning regimens against the chance of graft failure, and delayed immune reconstitution following transplantation. Because several excellent recent reviews exist on GVHD [9, 10], this review will focus on the latter two issues and on potential solutions offered by recent experimental studies, particularly as they relate to non-malignant disorders.

Niche space: A limitation to HSC engraftment?

The concept of the need for "space" for transplanted hematopoietic cells has been controversial. Early experiments by Micklem and colleagues [11] suggested that free space or empty niches were a limiting factor to the productive engraftment of bone marrow cells into unconditioned recipients. Later studies by Brecher, Micklem and colleagues [12], however, concluded that space was not an important factor to donor bone marrow engraftment and that specialized sites to allow for the proliferation of donor cells likely did not exist. Similar subsequent studies by several other groups concluded that transplanted bone marrow (containing HSC) could readily displace endogenous HSC in unirradiated recipients [13–15]. These studies were particularly important because HSC are the only cells capable of populating the entire blood system for life, and the replacement of genetically defective HSC with normal HSC in the absence of conditioning would represent a significant clinical advance.

Other studies, however, came to very different conclusions. Gambel and colleagues [16] found that donor marrow cells failed to engraft in unconditioned recipients, but could be stably transplanted into mice that had been pretreated with a depleting antibody against the major histocompatibility complex class I (MHC class I), a molecule that is highly expressed on enriched hematopoietic progenitors [17]. While the anti-MHC class I antibody could have facilitated engraftment at least in part through the ablation of the host immune response, studies by our group have found only very low levels of engraftment of purified donor HSC in unconditioned mice even when the hosts are genetically incapable of rejecting the graft [18, 19]. Severe combined immunodeficiency (SCID) patients represent unique clinical cases in which marrow transplants are often performed without pre-conditioning of the host, since they are incapable of rejecting the donor cells [20]. When these patients are not conditioned prior to receiving the transplant, donor HSC chimerism is less than 1% [21].

The studies that concluded that empty HSC niche space is not a limitation to transplantation all used unfractionated bone marrow transplants to reach their conclusions. At late time points following transplantation, total donor chimerism was measured in various tissues to estimate HSC chimerism in these studies. However, we have found that total hematopoietic chimerism, and in certain cases even progenitor cell chimerism, correlates poorly with and overestimates bone marrow HSC chimerism [19, 22]. Peripheral blood neutrophil chimerism appears to correlate well with HSC chimerism following transplantation of purified HSC into unconditioned immunodeficient hosts [18, 19], but not after unfractionated marrow transplantation into wild-type mice (D.B., unpublished observations). The experimental and clinical studies that transplanted highly or partially purified HSC into unconditioned immunodeficient recipients found little evidence for high levels of sustained neutrophil chimerism or endogenous bone marrow HSC displacement [18, 19, 21, 23]. Taken together, these studies suggest that in the absence of conditioning, donor HSC engraftment is limited by the occupancy of appropriate niches in both humans and mice.

Conditioning regimens: How little is too little – how much is too much?

For patients suffering from inherited blood disorders, cytoreductive pre-conditioning regimens are applied for two main purposes: to create space for the transplanted donor cells as described above, and to ablate the immune response of the host to prevent rejection of the graft. The determination of the appropriate types of drugs and doses to accomplish these goals is not at all trivial. A unique clinical setting that has shed some light on this issue is the treatment of SCID, in which, as mentioned above, patients are incapable of rejecting the graft [20]. Thus, the analysis of these patients allows for separation of the immunosuppressive effects of conditioning regimens from the myeloablative effects.

Prior to marrow transplantation, SCID patients are either left unconditioned or are treated with reduced-intensity or fully myeloablative conditioning regimens, depending on the specific clinical parameters and the institution performing the transplant [24]. A recent study by Cavazzana-Calvo and colleagues [23] demonstrated that both unconditioned and reduced-intensity-conditioned patients generally failed to show sustained donor neutrophil chimerism when analyzed at least 10 years after transplantation, implying that few donor HSC had engrafted at the time of transplantation [25]. Consistent with this, patients showed very low levels of new T cell production by 10 years after transplantation [23, 26]. In contrast, the neutrophils of patients that received full myeloablative conditioning regimens were exclusively of donor origin and new T cell production was sustained. These data imply that reduced-intensity conditioning regimens may be insufficient to create space for transplanted HSC and that fully myeloablative regimens must be employed. Fully myeloablative approaches using irradiation or DNA-alkylating agents, however, are associated with serious side effects due to their lack of cellular target specificity and the risk of oncogenic DNA damage [27]. Thus, the risks associated with these regimens discourage the use of HSC transplantation for the treatment of disorders that are not immediately fatal.

Some exciting recent clinical advances have come through depleting or inhibitory monoclonal antibodies due to the specificity and the lack of DNA damage that accompanies their use. Thus, antibody-based approaches for host HSC removal prior to transplantation could be attractive alternatives to the regimens currently in use. Yoshihara and colleagues [28] recently demonstrated that when coupled with 5-fluoruracil, *in vivo* administration of a specific antibody against c-mpl prior to donor HSC transplantation resulted in increased chimerism over recipients conditioned with 5-fluorouracil alone. Our group [19] recently demonstrated that administration of an anti-c-kit antibody to immunodeficient mice prior to transplantation was sufficient to remove host HSC and allow extremely high levels of donor HSC engraftment in the absence of any additional conditioning, although future studies will be required to translate these findings into immunocompetent animals. These types of more specific approaches may facilitate the creation of HSC niche space while avoiding the DNA damage and other lasting side effects associated with the current fully myeloablative approaches. Thus, coupling highly specific reagents that create HSC niche space with highly specific immunodepleting antibodies and/or immunosuppressive agents that prevent rejection might increase the safety of conditioning regimens without reducing efficacy.

Delayed immune reconstitution: Causes and solutions

Following allogeneic marrow transplantation, patients are highly susceptible to opportunistic infections due to the immunosuppressive effects of GVHD and the conditioning regimen [29]. GVHD can be prevented by the exclusion of donor T cells from the graft [30], but the conditioning regimens will invariably lead to at least transient immunosuppression due to the elimination of mature lymphocytes and, in some treatments, HSC and progenitors. Although donor HSC can eventually repopulate all mature blood lineages for life, the kinetics with which they do so after transplantation leaves an early period of immunological deficiency.

One strategy to enhance the kinetics with which mature blood cells are regenerated is through the co-transplantation of progenitor cells (alongside HSC) that have fewer developmental steps to take than do HSC before reaching maturity. Moreover, unlike transplanted allogeneic mature T cells, lymphoid progenitors transferred at developmental stages prior to antigen receptor rearrangement and negative selection cannot cause GVHD [31–34]. Below we summarize the markers and functional properties of myeloid and lymphoid progenitors that have shown promise when used in conjunction with HSC for rapid immune reconstitution following cytotoxic conditioning. Although many other downstream progenitors and differentiation pathways have also been extensively characterized, in this review we will focus on the progenitors and lineages that have specifically been implicated in mediating protection against pathogens following cytoreductive treatments.

Purification and use of myeloid progenitors

Common myeloid progenitors (CMP) were originally identified as cells in the mouse bone marrow that express c-kit and CD34 and low levels of FcγRII/III, but lack expression of Sca-1 and a panel of markers associated with lineage commitment [35]. This c-kit$^+$IL-7Rα$^-$ population was found to generate mature cells of the myeloid, but not the lymphoid lineage [35]. Recent studies found that the Sca-1$^-$ mouse CMP population was fairly heterogeneous, containing multiple progenitors restricted to either the megakaryocyte/erythroid or granulocyte/monocyte fates [36–39], and that the true CMP express high levels of the transcription factor GATA-1, and low but positive levels of Sca-1 [40]. In humans, CMP can be isolated as cells that lack expression of lineage commitment markers and CD45RA, but do express CD34, CD38, and low levels of IL-3Rα [41]. At least 15% of these cells have the clonal ability to generate both granulocyte/macrophage colonies and megakaryocyte/erythroid colonies. Moreover, these cells can rapidly differentiate into IL-3Rα-lacking megakaryocyte/erythroid-restricted or CD45RA$^+$ granulocyte/macrophage-restricted progenitors.

In a mouse model of allogeneic bone marrow transplantation, BitMansour and colleagues [42, 43] found that transplantation of CMP alongside HSC, but not of HSC alone, led to the protection of mice against lethal *Aspergillus* and *Pseudomonas* infections induced at early time points after lethal irradiation. Importantly, the donor CMP need not be MHC-matched to the host to mediate protection [44], and thus could be relatively straightforward to obtain for clinical purposes. These proof-of-principle experiments demonstrated the potential utility of progenitor cell transplantation to augment HSC reconstitution.

Phenotype and pre-clinical use of lymphoid progenitors

T cells are the master orchestrators of the adaptive immune response, mediating both humoral and cellular immunity to intracellular and extracellular pathogens. Conditioning regimens that circumvent graft rejection will necessarily impair host T cell function. Therefore, rapid, host-tolerant T cell reconstitution following bone marrow transplantation is extremely desirable. T cells develop in the thymus throughout life where they become self-MHC-restricted and self-tolerant; however, the thymus lacks long-term self-renewing progenitors and must be continually seeded by cells from the bone marrow that home to the thymus through blood circulation [45]. The identity of the bone marrow progenitor immediately upstream of the earliest thymus-resident T cell progenitor has remained controversial. An early candidate arose with the identification of the murine common lymphoid progenitor (CLP), which retains the ability to generate the T cell, B cell, dendritic cell (DC), and natural killer (NK) cell lineages, but can no longer differentiate into myeloid cells [46]. Murine CLP lack expression of a panel of mature lineage markers and have the surface phenotype IL-7Rα$^+$c-kit^{lo}Sca-1^{lo}.

Initial characterization indicated that mouse CLP were able to generate significant numbers of splenic T cells in sublethally irradiated recipients 2 wk after intravenous injection. In contrast, it took over 3 wk before comparable T cell chimerism was seen following transplantation of an equivalent number of HSC (c-kit^{hi}lin^{-}Sca-1^{+}). This delay in T cell generation was mirrored in the thymus where generation of donor HSC-derived CD4^{+}CD8^{+} (double-positive) thymocyte development also lagged by over 1 wk relative to CLP-derived thymocytes [46]. These data suggest that CLP are a good candidate for rapid T cell reconstitution from a bone marrow progenitor. In support of this hypothesis, Arber and colleagues [34] demonstrated that following irradiation, transplantation of CLP alongside HSC, but not HSC alone, led to the rapid protection of mice against lethal doses of murine cytomegalovirus; both MHC-matched and -mismatched CLP provided protection. As an alternative strategy, Zakrzewski and colleagues [47] demonstrated that T cell progenitors derived from HSC could be expanded *ex vivo* using OP9-DL1 stromal cells. These cells, when transplanted, underwent proper negative selection and did not mediate GVHD, yet could maintain protection against *Listeria monocytogenes* infections.

Recently, the efficiency and relevance of CLP as T progenitors have been called into question [48–53]. Allman and colleagues [48] found that the most immature mouse thymocytes (early thymic progenitors, ETP) were phenotypically more similar to the upstream multipotent progenitors (MPP) than to CLP for the expression of a number of cell surface proteins. Furthermore, since phenotypic CLP are not present in Ikaros$^{-/-}$ mice, which retain nearly normal numbers of ETP, the authors argued that CLP may not be important thymic progenitors [48]. These data were used to suggest that a cell developmentally more proximal to HSC, such as MPP, may be the true bone marrow thymic T cell progenitor.

There are important caveats to consider for each of these arguments before excluding CLP as thymic progenitors. First, it has been demonstrated that the c-kit and Sca-1 levels of CLP can be rapidly altered when CLP are cultured on the stromal line OP9-DL1, which supports thymic development [54]. Therefore, the reported differences in surface phenotypes between cells in the bone marrow and those in the thymus cannot be used to draw precursor-progeny relationships. Indeed, Schwarz and colleagues [51] demonstrated that when injected intrathymically, CLP increased expression of c-kit and gave rise to phenotypic ETP, a result confirmed in our own studies [55]. Second, examination of the kinetics of mouse thymocyte development between intrathymically injected ETP, CLP, and MPP clearly showed a high degree of similarity in the kinetics of early development of double-positive thymocytes between ETP and CLP. However, MPP lagged behind by a couple of days [48]; thus, it is difficult to justify using these data to argue that ETP resemble MPP more closely than CLP developmentally. Finally, Ikaros-null mice lack phenotypic CLP, but they also lack phenotypic MPP, since they lack Flk2 expression. An Ikaros-EGFP knock-in reporter revealed the presence of would-be Flk2^{+} MPP, though these cells were functionally impaired in their ability to generate lymphocytes [38]. Thus, both MPP and CLP may be phenotypically and/or functionally deficient in Ikaros-null mice, even though ETP are present in Ikaros$^{-/-}$ thymuses. These findings do not argue against either MPP or CLP as a physiological thymic progenitor under normal conditions, but may instead reflect flexibility in input populations that can contribute to thymopoiesis under duress.

Several groups have argued that the larger absolute number of thymic progeny downstream of MPP injection demonstrates that MPP rather than CLP are the major contributor to thymopoiesis [49–51]; however, it should be noted that MPP can give rise to CLP, and this precursor-progeny relationship opens the possibility that MPP promote thymopoiesis only through a CLP intermediate. Thus, the larger readout downstream from MPP may be reflective of their ability to give rise to multiple CLP. Consistent with this model, CLP produce an earlier wave of thymopoiesis than do MPP [46, 51, 56]. It should be noted that several groups altered the original method of CLP isolation by selecting for cells that express high levels of AA4.1/CD93 but lack Sca-1 expression [57]. Recent studies from our laboratory have shown that this method preferentially selects for B lineage-committed progenitors rather than CLP, and that the use of Flk2 expression in conjunction with the original markers is a far superior method for the isolation of highly purified CLP [55]. These Flk2^{+} CLP yield over 50% thymic chimerism 3 wk after transfer into sublethally irradiated recipients, underscoring their potency as T cell precursors [55].

Very recently, two prominent papers have suggested that the majority of ETP retain myeloid potential at a clonal level, thus concluding that a lymphoid-committed progenitor cannot give rise to a physiologically relevant number of ETP [52, 53]. That ETP isolated in bulk yield low-level myelopoiesis has previously been reported, and is not contentious [48, 58]. This low-level myeloid potential could be due to a rare subpopulation of ETP, and would not be inconsistent with the majority of thymopoiesis arising from CLP. On the other hand, if nearly all ETP retain myeloid potential, then they cannot arise from an irreversibly committed lymphoid progenitor, such as the CLP. Only a clonal assay can distinguish between these two possibilities.

In the study by Wada *et al.* [53], very-low-level myeloid potential was detected in bulk ETP cells, consistent with a rare progenitor maintaining myeloid potential. Furthermore, a clonal *in vitro* assay for myeloid potential of ETP revealed only a small percentage of wells with both myeloid and T lineage potential. Myeloid potential was determined by the presence of Mac-1^{+} cells; when further assayed for additional markers, nearly all of the Mac-1^{+} cells in these cultures co-expressed CD11c, a known marker of DC [53]. No definitive assays were performed to rule out the possibility that the "myeloid" cells in the clonal assays were DC. Importantly, it has previously been demonstrated that CLP can produce DC [59]. Regardless of the identity of the Mac-1^{+} cells, if only a minority of the ETP population possesses clonal T and myeloid potential, it remains possible that the majority of thymopoiesis arises from CLP.

In contrast to Wada *et al.* [53], Bell and Bhandoola [52] conclude that 87% of ETP have both T and myeloid potential using a slightly altered clonal assay. Again, myeloid potential was

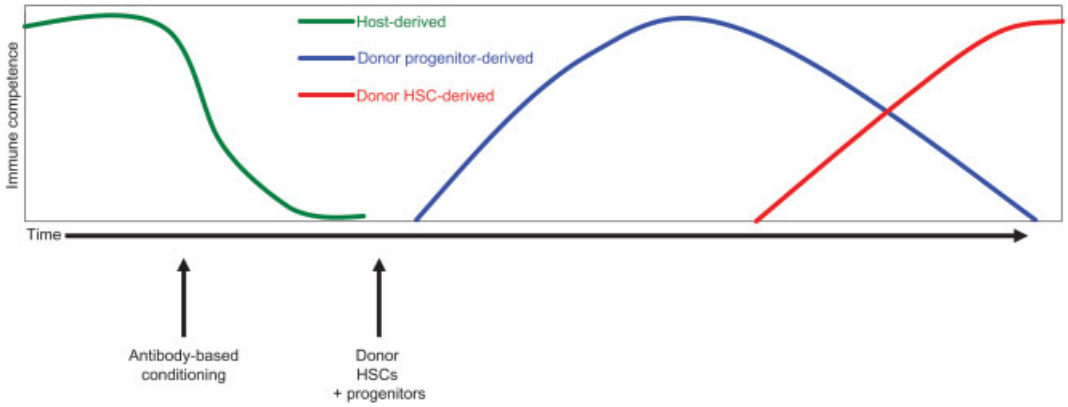

Figure 1. Idealized model for HSC transplantation. Following antibody-based ablation of host HSC and mature lymphocytes (green), transplantation of progenitor cells (blue) and HSC (red) from a donor would mediate rapid and sustained hematopoietic reconstitution.

determined by the expression of Mac-1, but it was not stated whether cells in this clonal assay expressed CD11c . Therefore, the possibility remains that many of the wells had T and DC potential instead, which does not rule out CLP as major thymocyte progenitors. Additionally, ETP have clearly been shown to have very minor myeloid potential in comparison to MPP using bulk *in vivo* and *in vitro* assays, such as colony forming assays on methycellulose (for example [48, 60–62]).

This discrepancy with the clonal assay obtained by Wada *et al.* [53] further raises the questions as to whether the readout obtained was truly myeloid and whether it accurately represented ETP lineage potential *in vivo*. An important but omitted control in these clonal assays would have been the inclusion of CLP, which have no detectable myeloid potential *in vivo*. Thus, the identity of the bone marrow progenitor that is responsible for the majority of thymopoiesis remains contentious and will require additional experiments to reach a conclusive result. However, the rapid reconstitution of T cells downstream of CLP has been experimentally documented as noted above, making it a useful progenitor in a transplantation setting irrespective of the above controversy.

In humans, CLP were first found within the bone marrow as cells that express CD34, CD38, CD45RA, and CD10, but lack expression of a number of lineage commitment markers [64]. Studies by our group [41] found that these cells also express IL-7Rα transcripts. These cells, when assayed in xenogeneic transplants or in limiting-dilution cultures, gave rise to T cells, B cells, NK cells, and DC, but not to myeloid cells. Other studies found that in the cord blood, only the $CD10^+CD7^+$ population contains CLP, while the $CD10^+CD7^-$ population contains myeloerythroid potential [64]. Later studies, however, found that the majority of the T cell and NK cell potential is contained within the $CD10^-CD7^+$ population in the cord blood, while $CD10^+CD7^-$ cells are mainly B lineage-committed [65].

A recent study has shown that the majority of $CD34^+CD10^+$ cells in cord blood as well as adult bone marrow express CD24, which is a marker of B lineage-committed progenitors, probably resolving these previous discrepancies. They also demonstrate that CD7 is not expressed on the $CD34^+CD10^+$ population in adult bone marrow. Importantly, the $CD34^+CD10^+CD24^-$ progenitors contained true CLP in that they could generate B cells, T cells, NK cells, and DC, but possessed little myeloid potential. Furthermore, $CD34^+CD10^+$ cells with similar potentials could be isolated from both adult blood and thymus, demonstrating that a lymphoid-committed progenitor likely transits from the bone marrow to the blood to the thymus to give rise to thymopoiesis in humans [66]. Given that a lymphoid-committed progenitor appears to seed the thymus in humans, it would be highly surprising if this extremely important developmental step was not conserved in mice.

Concluding remarks

The field of bone marrow transplantation has made remarkable strides since the first clinical attempts by Thomas and colleagues [67]. Improved methods for HLA typing and maintenance of donor registries, advancements in T cell depletion methods to minimize GVHD, new broad-spectrum antimicrobial drugs, and better conditioning regimens have brought us to a point where ~50 000 bone marrow transplants are now performed annually [68]. Nevertheless, there is still considerable room for improvement at all stages of the process as it is currently performed. In an ideal setting, highly specific conditioning drugs, such as antibody reagents, would be used to eliminate host HSC and graft-reactive lymphocytes prior to transplantation. Transplanted progenitor cells would then mediate rapid immunity without GVHD prior to lasting donor HSC-derived multilineage reconstitution (Fig. 1). Translational studies using model systems can point us in the right direction on how to achieve these goals and, consequently, the safer use of HSC transplantation and replacement for a broad range of clinical purposes.

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Abbreviations: **CLP:** common lymphoid progenitor · **CMP:** common myeloid progenitor · **ETP:** early thymic progenitor · **GVHD:** graft-*versus*-host disease · **MPP:** multipotent progenitor

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