Targeting the Spleen as an Alternative Site for Hematopoiesis

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Problems and Paradigms

Targeting spleen as an alternative site for hematopoiesis

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Abstract

Bone marrow is the main site for hematopoiesis in adults. It acts as a niche for hematopoietic stem cells (HSC) and contains non-hematopoietic cells that contribute to stem cell dormancy, quiescence, self-renewal and differentiation. HSC also exist in resting spleen of several species, although their contribution to hematopoiesis under steady-state conditions is unknown. Spleen can however can undergo extramedullary hematopoiesis (EMH) triggered by physiological stress or disease. With loss of bone marrow niches on aging and disease, spleen as an alternative tissue site for hematopoiesis is an important consideration for future therapy particularly during HSC transplantation.

In terms of harnessing spleen as a site for hematopoiesis, here we consider the remarkable regenerative capacity of spleen with a view to forming additional or ectopic spleen tissue through cell engraftment. Studies in mice indicate potential for such grafts to support the influx of hematopoietic cells leading to development of normal spleen architecture. An important goal will be the formation of functional ectopic spleen tissue as an aid to hematopoietic recovery following clinical treatments that impact bone marrow. For example, expansion or replacement of niches could be considered where myeloablation ahead of HSC transplantation compromises treatment outcomes.

1. A role for spleen during HSC transplantation

Increasing evidence now reveals that spleen contains hematopoietic stem cells (HSC), supports extramedullary hematopoiesis (EMH), and is also capable of regeneration. These findings have general interest in that spleen, long considered to have a main role in red blood cell turnover, can now be considered for its therapeutic potential as an alternative site for hematopoiesis. The hypothesis considered here is that spleen can function as a backup tissue for hematopoiesis during HSC transplantation. In murine models, it is already known that HSC enter spleen directly on transplantation [1]. For example, specific inhibitors or immunomodulatory drugs can be used to direct more HSC and hematopoietic progenitors into spleen, a process that may provide an early burst of differentiation of myeloid cells. This could boost immune recovery, allow time for HSC to establish niches in bone marrow and so amplify the total hematopoietic output over time. Engraftment of splenic tissue or cells following myeloablation or irradiation could be used to replace HSC niches damaged through treatment or disease. This proposal therefore has direct translation in the area of cellular therapy and in improving HSC transplantation outcomes.

2. Hematopoiesis in adults

Bone marrow becomes the primary site for hematopoiesis in the murine adult following colonisation with HSC at day 17 in the embryo [2]. The hematopoietic system is then

maintained throughout life by differentiation of HSC and embodies a hierarchical structure of development giving rise to progeny and mature blood cells. In the murine model, the lineage$^-$ Sca-1$^+$c-Kit$^+$ compartment of bone marrow contains all stem cell activity. This reflects a heterogeneous subset comprising functionally distinct cells, including the long-term (LT-) and short-term (ST-) repopulating HSC [3] and the multipotent progenitors (MPP) [4]. LT-HSC are rare, self-renewing cells, also described as homeostatic or dormant [5]. HSC subsets can now be more fully defined through expression of the SLAM markers CD150, CD41 and CD48 [6]. Through stepwise gain of CD34, CD48 and CD135 expression, along with loss of CD150 expression, MPP develop from LT-HSC and ST-HSC as four lineage-committed subpopulations, namely MPP1, MPP2, MPP3, and MPP4 [5]. MPP1 derive initially from ST-HSC, and then produce the three remaining MPP subsets, each functionally distinct and lacking self-renewal potential. These then generate the lineage-committed progenitors known as the common myeloid progenitor, the common lymphoid progenitor, and the megakaryocyte-erythroid progenitor; further development of progenitors results in progressive loss of differentiation capacity and self-renewal potential [7]. These progenitor cells eventually give rise to all mature blood cells.

3. Reconstitution studies confirm heterogeneity amongst HSC

Hematopoietic reconstitution remains the gold standard test for the differentiative potential of hematopoietic stem and progenitor subsets. In murine models, these experiments have demonstrated subtle differences in donor cell output and kinetics between multiple subsets [8]. The test determines the ability of a stem cell to reconstitute blood cell formation after transplantation into a host pre-conditioned through irradiation or myeloablation. These treatments deplete endogenous HSC from their niche so allowing donor stem cell engraftment [9]. In this setting, LT-HSC can sustain long-term hematopoietic cell production with full reconstitution by 16 weeks in the mouse model [7]. The self-renewal capacity of LT-HSC is confirmed through secondary transfer, which also allows full long-term reconstitution. In contrast, ST-HSC display short-term multi-lineage reconstitution, but cannot sustain serial reconstitution. Finally, MPP give only very brief multi-lineage hematopoietic reconstitution in primary recipients because they possess no self-renewal potential [10].

Despite their ability to sustain long-term reconstitution, LT-HSC are very inefficient at reconstitution of the hematopoietic system while rapid reconstitution appears to be the realm of ST-HSC [7]. The ST-HSC subset contains two functionally distinct sub-populations differing in Flt3 expression [3]. The Flt3$^-$ subset is capable of rapidly reconstituting myelopoiesis, rescuing myeloablated mice, and generating the second Flt3$^+$ subset which is primarily responsible for rapid lymphoid reconstitution. In these studies, ST-HSC produce three independent myeloid-biased MPP subsets (MPP1, MPP2, MPP3), followed by the lymphoid-biased MPP4 subset. Myeloid-biased MPP are vital for maintaining steady-state hematopoiesis and for myeloid output in regenerative conditions. MPP2, MPP3 and MPP4

only exhibit short-term myeloid reconstitution for up to a month [11]. These reonstitution studies identify both the heterogeneity and distinct differentiative potential of the hematopoietic stem/progenitor population.

4. Spleen supports hematopoiesis as well as immunity

It is well known that spleen plays a major role in both T and B cell immunity. It also maintains a unique population of B-1a B cells which function in early innate immunity against encapsulated bacteria [12]. The central role of spleen in erythropoiesis and the recycling of aged red blood cells has also been well documented [13]. The spleen first assumes a role in hematopoiesis in the embryo [14], but during adult life appears to adopt the role of a hematopoietic organ under physiological stress such as pregnancy or infection, or during an immune response [15] [16]. When bone marrow is compromised through disease or treatment, spleen can also take the place of bone marrow as a hematopoietic organ [17]. The current perception in the field has been that spleen functions only as an emergency hematopoietic organ with efflux of HSC from bone marrow in response to physiological stress [18] [19] [20] [21]. HSC have been isolated from murine spleen and shown to be multipotent, giving rise to all hematopoietic cell types following transplantation into an irradiated mouse [22] (Figure 1).

Hematopoiesis occurring outside the bone marrow is referred to as extramedullary hematopoiesis (EMH). HSC in adult murine spleen are rare under normal physiological conditions but are mobilised from bone marrow into spleen and other sites in response to stress. Questions remain as to whether spleen makes a contribution to hematopoiesis under steady-state or resting conditions, and the range of blood cells produced underthose different states. Myeloablation with cyclophosphamide, or cyclophosphamide/granulocyte colony stimulating factor (G-CSF) can be used to mobilise HSC out of bone marrow and into peripheral blood and spleen leading to EMH [23] [24]. HSC are then found located around the sinusoids in the red pulp region of murine spleen, raising the possibility of perisinusoidal niches for HSC [6]. Similar results were obtained upon EMH induced through blood loss or pregnancy [25]. This was associated with increased numbers of HSC and progenitors and increased numbers of stromal cells forming the niche [25].

These recent studies now emphasise both the important contribution that spleen makes to hematopoiesis and the uniqueness of spleen as a hematopoietic niche. It will also be important to consider the role that spleen plays in hematopoiesis during HSC transplantation in humans. Studies seeking to demonstrate the presence of HSC in human spleen are few but have been limited by the availability of normal tissue [26] [27] [28]. While human spleen shows minor structural differences from mouse spleen [29], it has the same functional immune capacity and likely the same stem cell populations. The difficulty of finding a rare HSC population in human spleen should not be taken as a negative finding. In our view, there is no convincing data excluding HSC from human spleen.

5. Spleen contains perivascular niches for HSC

The dynamic role of spleen in provisioning emergency hematopoiesis appears to rely on the rapid expansion of perivascular reticular cells which support HSC [25, 30]. One possibility yet to be investigated is that mesenchymal stem and progenitor cells reside in spleen and quickly differentiate to expand stromal cells which form the niche. The recent study by Inra et al. provided convincing evidence that spleen under stress conditions can function as a niche for HSC through expansion of stromal cells producing stem cell factor (SCF). Stromal cells include both endothelial cells and PDGFRb$^+$ mesenchymal cells resembling the perivascular reticular cells described in bone marrow [25]. That study also determined that a subset of SCF-producing PDGFRb$^+$ perivascular reticular cells in spleen red pulp was the main source of CXCL12, a chemokine essential for hematopoiesis [31]. Tcf21-expressing PDGFRb$^+$ stromal cells are unique to spleen and not bone marrow, and found to produce both SCF and CXCL12 [25]. Both endothelial cells and Tcf21-expressing PDGFRb$^+$ stroma together form the niche that supports HSC development in spleen and the expression of EMH [25]. The known cellular components of HSC niches in spleen is shown in Figure 2.

Mobilisation of human stem cells out of bone marrow through G-CSF treatment involves not only migration of HSC into peripheral blood, but also the movement of *Hox11 (Tlx1)*-expressing mesenchymal cells from spleen into blood [32]. It has long been known, that Hox11 (or Tlx1) expressed by splenic stromal cells is an essential transcription factor for spleen organogenesis [33]. Overexpression of Tlx1 is sufficient to induce EMH in the absence of any other physiological stressor [30]. When *Tlx1* conditional knockout mice were investigated it was concluded that *Tlx1*-expressing stromal cells are required for recruitment of HSC into spleen following EMH, and that those cells are a component of HSC niches in spleen [30]. Tlx1-expressing cells also resemble bone marrow niche elements through expression of PDGFRa/b, CD51, CD105 and production of CXCL12 and SCF [30]. An unanswered question is whether mobilisation treatments (like G-CSF) impact splenic niches as well as bone marrow niches such that spleen contributes to the supply of HSC into peripheral blood.

6. Comparison of HSC niches in spleen and bone marrow

HSC niches have been identified in both endosteal and perivascular sites in bone marrow, and osteoblasts, endothelial cells and mesenchymal stromal cells are considered to be important cellular components. The majority of HSC reside adjacent to sinusoidal blood vessels, a location considered to be a main stem cell niche or microenvironment [6]. Because spleen supports EMH, it follows that it must maintain or develop functional niches to support and expand hematopoiesis. There are no osteoblasts in spleen, and hence HSC niches in spleen portend to be distinct in comparison with bone marrow. Recent studies have identified a role

for both endothelial and mesenchymal cells in formation of HSC niches supporting EMH [25] [30].

A wealth of evidence now supports the essential role of perivascular reticular cells in bone marrow HSC niches. These stromal cells comprise a dichotomy of periarteriolar and perisinusoidal reticular cells [34] [35] as the main source of SCF for HSC proliferation and of CXCL12 for HSC maintenance [36] [37]. Several subsets are known such that heterogeneity exists with overlapping subsets of more primitive nestin$^+$ cells [38], Lepr-expressing cells [37] and CXCL12-abundant reticular cells [31]. In human bone marrow, perivascular cells were first aligned with pericytes surrounding vascular endothelium and reflecting mesenchymal progenitors [39]. Our own recent studies show that murine splenic stromal lines that support *in vitro* hematopoiesis resemble perivascular reticular cells described in bone marrow [40] [41]. Similarly, CD146$^+$ perivascular cells from human bone marrow also support multi-lineage hematopoeisis and maintain hematopoietic progenitors in co-cultures [42]. Murine spleen stromal lines which resemble perivascular reticular cells [43] [44] have been shown to readily support myelopoiesis from overlaid LT-HSC [41], providing a sufficient *in vitro* niche for hematopoiesis. Recently it was also shown that these same stromal cell lines support hematopoietic development *in vivo* through production of an ectopic niche (O'Neill et al, unpublished data). Extramedullary hematopoiesis in spleen has been associated with the sinusoidal-rich red pulp region, based on evidence that HSC mobilised out of bone marrow enter spleen and localise in red pulp, and that mature myeloid cells are also localised in the red pulp region [25] [6] (Figure 2).

7. Splenic stromal cells reflect a sufficient niche for *in vitro* hematopoiesis

The presence of HSC niches in spleen has been of interest since we discovered that long-term spleen cultures can support hematopoiesis with continuous production of distinct myeloid dendritic-like cells which were termed 'L-DC' [45] [46] with transient production of several known myeloid cell types [47] [48] [49]. Hematopoietic progenitors were shown to be maintained in long-term cultures through gene expression analysis [44] [50], and the ability to give long-term hematopoietic reconstitution upon adoptive transfer into irradiated mice [43]. Stromal lines derived from long-term spleen cultures were then shown to reflect variants with different hematopoietic support capacity [51]. When some splenic stromal lines were overlaid with hematopoietic progenitors from bone marrow or spleen they supported long-term restricted *in vitro* myelopoiesis [52]. Cells produced were identified as myelomonocytic cells, conventional dendritic-like cells and the dendritic-like 'L-DC' [48] [52]. We recently identified an *in vivo* counterpart to 'L-DC' [53], lending physiological relevance to *in vitro* studies. Here we propose that spleen like bone marrow must contain perisinusoidal niches that reflect a heterogeneous subset of cells, perhaps with distinct functional roles in hematopoiesis under steady-state and stress conditions.

Splenic stromal lines were aligned with perivascular reticular cells in bone marrow through expression of CXCL12, SCF and mesenchymal markers like CD140a/b, CD90, CD105 and CD51 [54] [55] [40] [56]. Production of Angiopoetin-1, TGFB1, TGFB3 and CXCL12 is consistent with potential to support HSC maintenance and quiescence. Expression of high levels of M-CSF, MIF and CCL8 is consistent with the production of factors that support myeloid cell development and migration. Gene expression studies revealed genes relating to capacity to support early hematopoiesis with upregulation of molecules of the WNT pathway including WNT5a, SRP2 and RSPO, as well as other known regulators of HSC including ALDH1, VCAM1 and SVEP1 [40]. Stromal cell lines were also shown to have osteogenic differentiative capacity, suggesting the presence of mesenchymal osteoprogenitors in spleen which support *in vitro* hematopoiesis (O'Neill et al, unpublished data). Indeed, these stromal cell lines reflect perivascular reticular cells which provide a niche for maintenance of HSC in steady-state spleen. Furthermore, restricted myelopoiesis leading to development of only L-DC raises the possibility that the spleen microenvironment supports production of tissue-specific antigen presenting cells. Their location in spleen red pulp could reflect a specific role in monitoring blood-borne antigens and interacting with migrating lymphoid cells.

8. Impact of myeloablative therapy on HSC niches

Myeloablative high-dose chemotherapy is used ahead of HSC transplantation for blood disorders or leukemia and to treat congenital immunodeficiency disorders [57]. Transplantation of HSC into myeloablated patients represents highly effective therapy for immune system restoration. The differentiative potential of HSC is such that as few as 100 cells can provide full reconstitution in a myeloablated mouse [58]. However, HSC transplantation in humans is associated with a high incidence of morbidity and mortality, complications like graft-versus-host disease and graft failure. A significant concern is mortality due to infection developing between myeloablative therapy, HSC engraftment and reconstitution. Myeloablative treatments like chemotherapy or irradiation commonly lead to bone marrow aplasia and so immunocompromise the patient [59] [60]. Acceleration of immune recovery and hematopoietic reconstitution should reduce mortality and morbidity associated with early post-transplant infection.

Irradiation permanently damages bone marrow stromal cells, particularly endothelial cells in the sinusoids [59], and this impacts the maintenance and support of HSC [61] . The success of hematopoietic reconstitution is thought to be closely linked to the recovery of the bone marrow stromal microenvironment. This has been directly demonstrated through transplantation of bone marrow endothelial cells which can augment hematopoiesis following HSC transplantation [62]. Co-infusion of bone marrow endothelial cells and whole bone marrow cells into myeloablated recipients gives long-term, multi-lineage engraftment and increased survival [63].

The effect of irradiation on splenic niches for HSC is not well documented. However, as a secondary lymphoid organ, the spleen undergoes continuous remodelling of the stromal microenvironment to facilitate immune responses [64]. Chronic inflammatory conditions can promote stromal cell activation and fibrosis [65]. Spleen also has remarkable regenerative capacity, and Castagnaro et al. have identified multipotent mesenchymal precursors and lymphoid tissue organiser cells that support injury-induced regeneration of spleen [66]. Indeed, the regenerative capacity of spleen could contribute to tissue repair and recovery of HSC niches following irradiation damage.

9. Protocols used in human HSC transplantation

In the clinical setting, bone marrow was first used as a source of HSC [67], although alternative sources now include mobilised peripheral blood and umbilical cord blood. HSC can be differentiated *in vitro* from induced pluripotent stem cells and will represent a future cell source suitable for therapy [68]. The use of mobilising agents to extravasate HSC from the bone marrow into the peripheral blood for collection by leukapheresis has proven far less invasive than bone marrow extraction from pelvic bone. Use of mobilised peripheral blood for HSC transplantation is comparable with bone marrow in terms of patient survival, incidence of relapse, disease-free survival, transplant-related mortality, incidence of graft-versus-host disease and time to engraftment [69]. For these reasons, mobilised peripheral blood is now the primary source of allogeneic and autologous HSC for transplantation [70].

Mobilised peripheral blood produces rapid, stable long-term engraftment, such that the success and rate of engraftment with autologous transplantation can be correlated with the number of $CD34^+$ cells transplanted [71]. The best method for peripheral blood mobilisation uses G-CSF to mobilise $CD34^+$ cells from bone marrow for leukapheresis [72]. Other mobilising agents like GM-CSF have been compared with G-CSF, and the yield of $CD34^+$ cells was found to be similar [73]. However, GM-CSF gave earlier neutrophil and platelet recovery, hence providing benefit in terms of rapid recovery with less need for blood and platelet transfusions, and fewer infections during the period of myeloablation. Plerixafor (or AMD3100) is also used to mobilise HSC from bone marrow. As a highly specific antagonist of CXCR4 expressed by HSC, it interrupts CXCR4 binding to CXCL12 expressed on bone marrow stromal cells [74].

Immune reconstitution following allogeneic HSC transplantation occurs in several phases [75]. An early phase of rapid myeloid cell reconstitution occurs within 20-30 days, while lymphoid cell reconstitution is delayed by up to a year [76]. Patients experience an 'aplastic phase' of severe neutropenia until neutrophil progeny appear. During the first 100 days, patients are susceptible to virus reactivation and diseases related to slow reconstitution of NK cells and T cells [77]. Following autologous HSC transplantation, an absolute neutrophil count of $\geq 0.5\times10^9$ cells/L for 3 consecutive days is commonly defined as the time to myeloid cell recovery and establishment of innate immunity [78]. Growth factors such as G-CSF and GM-CSF are routinely

administered to accelerate hematopoietic recovery by boosting myeloid cell development and recovery of neutrophils [79]. Infection risk increases steeply with low neutrophil count, particularly in the absence of antibiotic therapy. The optimal cell dose for transplantation is 2-5×10^6 mobilised $CD34^+$ cells/kg body weight which is the threshold number of cells associated with rapid and sustained reconstitution at 4 weeks post-transplantation [80].

While technology has improved so that it is now possible to isolate highly pure populations of human $CD34^+CD38^-CD45RA^-CD90^+$ HSC from mobilised peripheral blood [81], purified primitive HSC take a significantly longer time to engraft and reconstitute the hematopoietic system. The use of CD34 as a delineating marker for preparation of stem cells for transplantation must be considered carefully because it is expressed by other hematopoietic progenitors including the MPP, common myeloid progenitor, B cell/natural killer cell progenitor and the granulocyte-macrophage progenitor [82]. The $CD34^+$ fraction of mobilised blood therefore represents a mixture of HSC and other progenitors. Further enrichment of HSC from the $CD34^+$ population can be achieved using additional markers. For example, Vose et al. evaluated high-dose chemotherapy followed by transplantation of highly purified $CD34^+CD90^+$ HSC into patients with non-Hodgkin's lymphoma and mantle cell lymphoma [83]. Purification of HSC from mobilised blood also provided the opportunity to eliminate tumour cells [84]. Rapid neutrophil and platelet engraftment was achieved in the majority of patients, although infection remained a concern with 14 of the 20 patients reporting significant infections post-transplantation [83].

10. Spleen involvement in HSC transplantation

The mouse model has provided convincing evidence that spleen contributes to hematopoiesis following HSC transplantation (Figure 1). In fact, spleen is the most frequent site for engraftment following HSC transplantation and over one third of HSC enter that organ [1]. Transplantation-induced EMH leads to the development of spleen colonies as macroscopic nodules on the spleen surface. These derive from a single parent cell or colony forming unit (CFU) and contain a mixture of mature cells of myeloid and erythroid lineage [85]. CFU arise from different HSC and progenitors including CFU-GEMM (granulocyte-erythrocyte-monocyte-megakaryocyte), CFU-L (lymphocyte) and CFU-E (erythrocyte). After transplantation, bone marrow c-Kit^+ and c-Kit^{lo} progenitors form CFU in spleen on days 8-10 and days 12-14, reflecting clonal development of different progenitors in comparison with LT-HSC, which form late CFU after 16-20 days [86]. When daughter cells of spleen CFU are serially transplanted into secondary recipients they generate multi-lineage colonies confirming the maintenance of self-renewing HSC in spleen colonies [87].

The contribution of spleen to HSC transplantation and hematopoietic rescue in humans remains contentious although several findings are relevant. Spleen size in patients with myelofibrosis has been found to have a negative effect on the engraftment efficiency of $CD34^+$

cells following transplantation [88]. HSC engraftment is also delayed in patients with splenomegaly [89] and in children with hypersplenism [90]. Early pooling of $CD34^+$ HSC in spleen, coupled with a defect in bone marrow homing, has been shown to reduce the engraftment of HSC into bone marrow following transplantation and may be the cause of graft failure [88] [91]. Reduction in spleen size is thought to improve HSC homing to bone marrow, and partial or total splenectomy can improve the rate of HSC engraftment [92]. Splenectomy can lead to a slight increase in neutrophil engraftment in bone marrow [93]. Successful engraftment of HSC depends on the availability of niches in bone marrow and spleen at the time of HSC transplantation: therefore, engraftment can be improved by increasing the number of available niches. Specifically directing HSC into spleen as an extramedullary niche could enhance transplantation outcomes for patients [94].

11. Harnessing EMH in spleen to improve HSC transplantation outcomes

We propose targeting spleen as a site for EMH in order to accelerate early immune cell reconstitution and to improve hematopoietic recovery following myeloablation and HSC transplantation. While most clinical HSC transplantations use unseparated bone marrow [67], or mobilised peripheral blood [95] [69], the direction in the field has been to isolate and transplant more purified HSC populations [96]. Transplantation of highly purified $CD34^+$ HSC grafts has been successful, although is still associated with significant infection post-transplantation [97] [98] [83]. The hypothesis presented here is based on the premise that early myelopoiesis in spleen could be protective following myeloablation for HSC transplantation, and so improve long-term hematopoietic recovery. There are several approaches that could be effective.

a) Induction of early myelopoiesis in spleen

The first approach is to select hematopoietic progenitors or stem cell subsets for transplantation that target spleen and so accelerate early transient myelopoiesis expecting this to be protective until full hematopoiesis is established in bone marrow. Evidence of early spleen CFU following transplantation confirms that some bone marrow stem/progenitor cells do preferentially enter spleen [86]. Multipotent progenitors downstream of HSC can provide rapid, short-term hematopoietic reconstitution as early as 2 weeks following transplantation in an irradiated recipient [7]. We propose that transplantation of MPP in addition to purified HSC may provide early and more effective hematopoietic reconstitution. It will however be necessary to describe those progenitors and to assess their hematopoietic potential, their contribution to myelopoiesis, and any protective effect they may contribute to early recovery. Transplantations involving mice could involve defined subsets of MPP or ST-HSC that enter spleen ahead of LT-HSC colonisation of bone marrow niches. The homing of MPP into spleen after transplantation is expected since spleen is the most frequent site of initial engraftment following transplantation [1]. If spleen supports MPP engraftment and differentiation

following transplantation, this approach could be used to accelerate early hematopoietic recovery.

b) Increased HSC engraftment in spleen

Another approach is to use specific drug treatment to direct transplanted HSC into spleen and give rapid differentiation of myeloid cells. Several drugs are already in clinical use that can directly mobilise HSC into spleen. Plerixafor (AMD3100) is a highly specific, reversible antagonist of the CXCR4 receptor expressed by HSC. Although Plerixafor is commonly used as a mobilising agent for peripheral blood HSC [99], post-transplant administration in mice can have beneficial effects on hematopoietic reconstitution [94]. Treatment is associated with a significant increase in HSC engraftment in spleen, and later restoration of bone marrow density and cellularity. Treatment also leads to increased CFU in spleen, improved recipient survival, and enhanced reconstitution of all blood cell lineages. Whether enhanced migration of HSC to spleen results in short-term myelopoiesis is not known. To determine the effect of post-transplant Plerixafor administration on recovery following HSC transplantation, LT-HSC could be transferred, followed by 'rescue' with host bone marrow cells or MPP, and then treatment with Plerixafor. Effectiveness of the protocol would be indicated by early myelopoiesis evident through the appearance of monocytes in peripheral blood and of colonies in spleen within 2 weeks after transplantation. The important role of spleen could be assessed by investigating splenectomised mice in which all HSC home to bone marrow. In terms of redirecting HSC into spleen, anti-VLA antibody adsorption to HSC is another possible treatment option to significantly reduce homing of HSC into bone marrow with increased circulation of HSC and their uptake by spleen [85].

c) Expansion of HSC niches in spleen or ectopic sites

A final more speculative approach is to repair, expand or replace stromal niches in spleen or other ectopic site, following irradiation or myeloablation. This could accelerate early hematopoietic events in spleen that are protective. This more progressive approach relies on knowledge of spleen organogenesis and HSC niche formation. The impact of irradiation or myeloablation on splenic niches is not well understood in terms of expansion or loss of particular subsets of stroma, and whether repair can be accelerated through intervention. Inra et al. recently showed that EMH induced by several different stressors leads to expansion of perisinusoidal niches in the red pulp region, and that these niches were retained following return of the animal to normal physiological state [25]. This suggests plasticity amongst splenic stromal cells, a property conducive to repair and expansion of HSC niches following structural damage through irradiation or myeloablation. Spleen structural damage would need to be assessed in terms of subsets of cells affected and possibilities for repair. For example, loss of sinusoids in the red pulp region could be correctable through introduction of endothelial cells, and evidence exists to support that possibility [100]. Other spleen stromal cells or purified

subsets of perisinusoidal reticular cells or their progenitors could also be used to repair specific niches (Figure 3). Until recently it was not possible to identify stromal subsets with certainty, or to analyse cell populations at the single cell level. It is now possible to perform stem cell isolation, single cell analysis, and to use *in vitro* differentiation protocols to identify the specific cell types that constitute stromal niches. Already we have embarked on analysis of splenic stromal subsets in mouse [101] and human (Petvises et al. unpublished data) and have also developed protocols for engraftment of splenic stromal cells to form an ectopic or artificial niche (O'Neill et al. unpublished data) (Figure 3).

The spleen has innate regenerative capacity and is readily amenable to engraftment for tissue regeneration [102] [103] [104] [105] [106]. Successful grafting of tissue fragments leads to development of tissue with the structural identity of spleen, including red and white pulp formation with evidence of full hematopoietic reconstitution [103]. Engraftment of stromal fractions isolated by enrichment based on cell surface markers led to the identification of a spleen organiser cell as an endothelial-like CD31+MAdCAM-1+ cell [102]. However, formation of ectopic tissue was also found to be dependent on the presence of mesenchymal PDGFRb+ cells [102]. Stromal cell lines derived from long-term cultures of spleen which support *in vitro* hematopoiesis have also been grafted under the kidney capsule and shown to accumulate HSC and myeloid cells from the host (O'Neill et al. unpublished data). Engraftment of highly purified splenic stromal subsets or stromal cell progenitors should however give greater insight into the specific stromal cell types needed for formation of ectopic niches. The possibility that they could be sourced from readily available tissues or blood, would open new opportunities for therapy to improve HSC uptake into spleen during transplantation.

12. **Conclusions and outlook**

A history of work from this lab has shown that spleen contributes to hematopoiesis. As a highly regenerative organ, it offers potential for reengineering niches to increase hematopoietic cell production. For example, if unique stromal cells could be isolated which expand HSC *in vitro*, or provided as an ectopic niche *in vivo* for the same purpose, then the potential exists to enhance hematopoiesis during HSC transplantation. Regeneration or expansion of these niches could represent future therapy for patients undergoing myeloablative treatment, involution of lymphoid tissue with ageing, or HSC transplantation.

The field of tissue engineering is developing rapidly, offering benefits for disease and the ageing population. The lack of understanding of spleen as a hematopoietic organ has limited clinical application to date. Information on the role of spleen in hematopoiesis has been lacking in the literature, probably because of a lack of tools and the inability to study rare cell types with certainty. The hematopoiesis field has been built upon the mouse model, and so analysis of mice is well justified in terms of informing the field ahead of human studies. The analysis of spleen as an alternate or ectopic site for hematopoiesis, and the development of

immunotherapies targeting HSC niches in spleen, could open new avenues for treatment of patients undergoing HSC transplantation who have become immunocompromised through bone marrow decline, cancer or diseases related to ageing.

Abbreviations

CFU, colony forming unit; EMH, extramedullary hematopoiesis; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HSC, hematopoietic stem cell; LT-HSC, long-term-HSC; SCF, stem cell factor; ST-HSC, short-term HSC.

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Conflict of interest

The authors declare no conflict of interest.

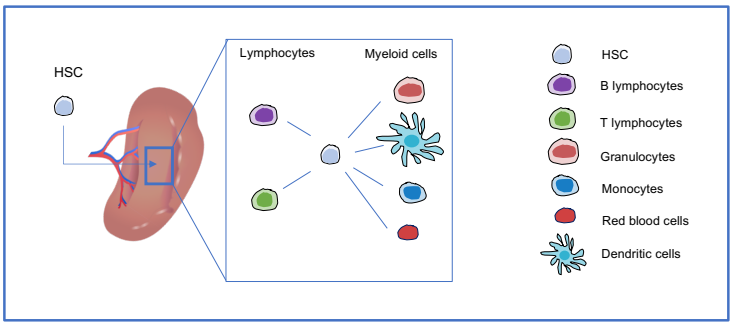

Figure 1. Spleen as a backup tissue for hematopoiesis during HSC transplantation. It is already known that HSC enter spleen directly on transplantation. Spleen also supports development of a range of blood cells including monocytes, dendritic cells, granulocytes, red blood cells and T and B lymphocytes.

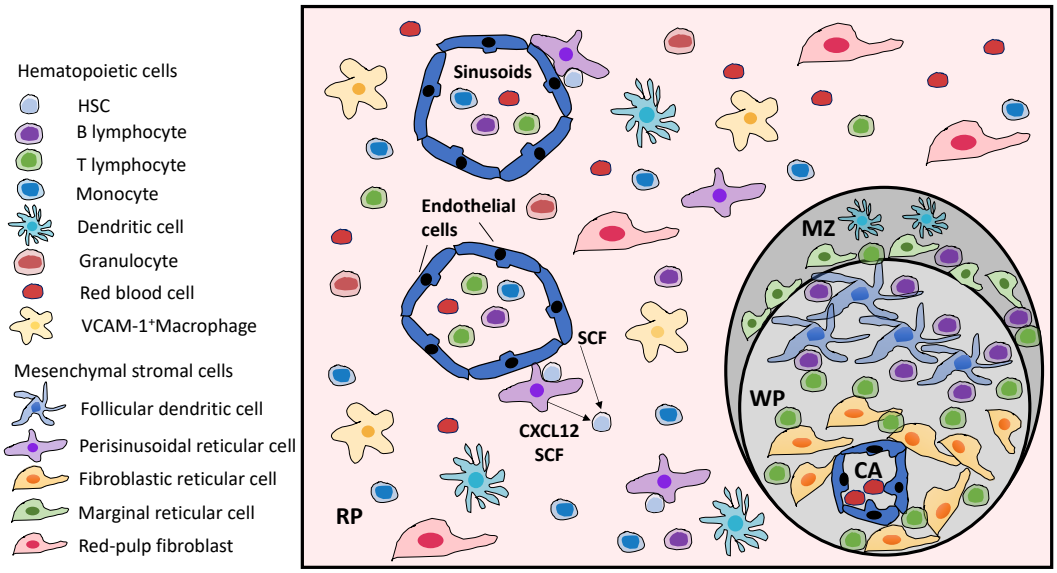

Figure 2. Location of HSC niches within the red pulp of spleen. HSC are localised in red pulp (RP) in association with the CXCL12-producing perisinusoidal reticular cells and in close proximity with endothelial cells forming sinusoids. The sinusoidal network dominates the red pulp region, which filters many red blood cells. It also contains many monocytes, as well as lymphocytes, macrophages and dendritic cells. The white pulp region (WP) contains organised follicles of B cells supported by follicular dendritic cells, while the central arteriole (CA) is surrounded by T cells supported by fibroblastic reticular cells. The marginal zone (MZ) surrounds the white pulp and is home to specific subsets of dendritic cells and marginal reticular cells.

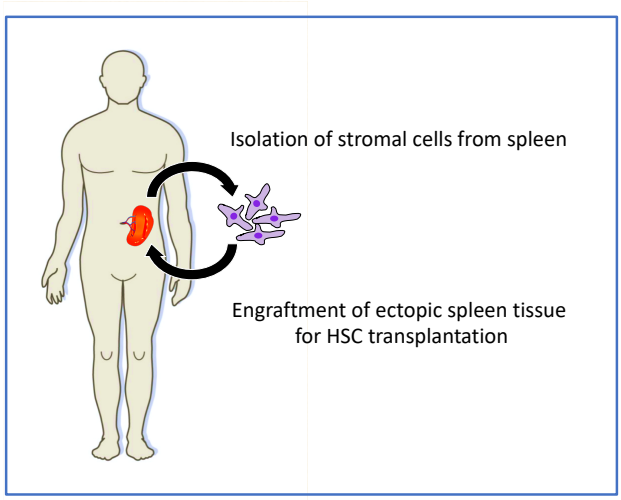

Figure 3. Formation of ectopic spleen tissue to increase hematopoietic cell production. Unique spleen stromal cells could be isolated from spleen or even blood before myeloablation, and then grafted as an ectopic niche to enhance hematopoiesis following HSC transplantation.

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