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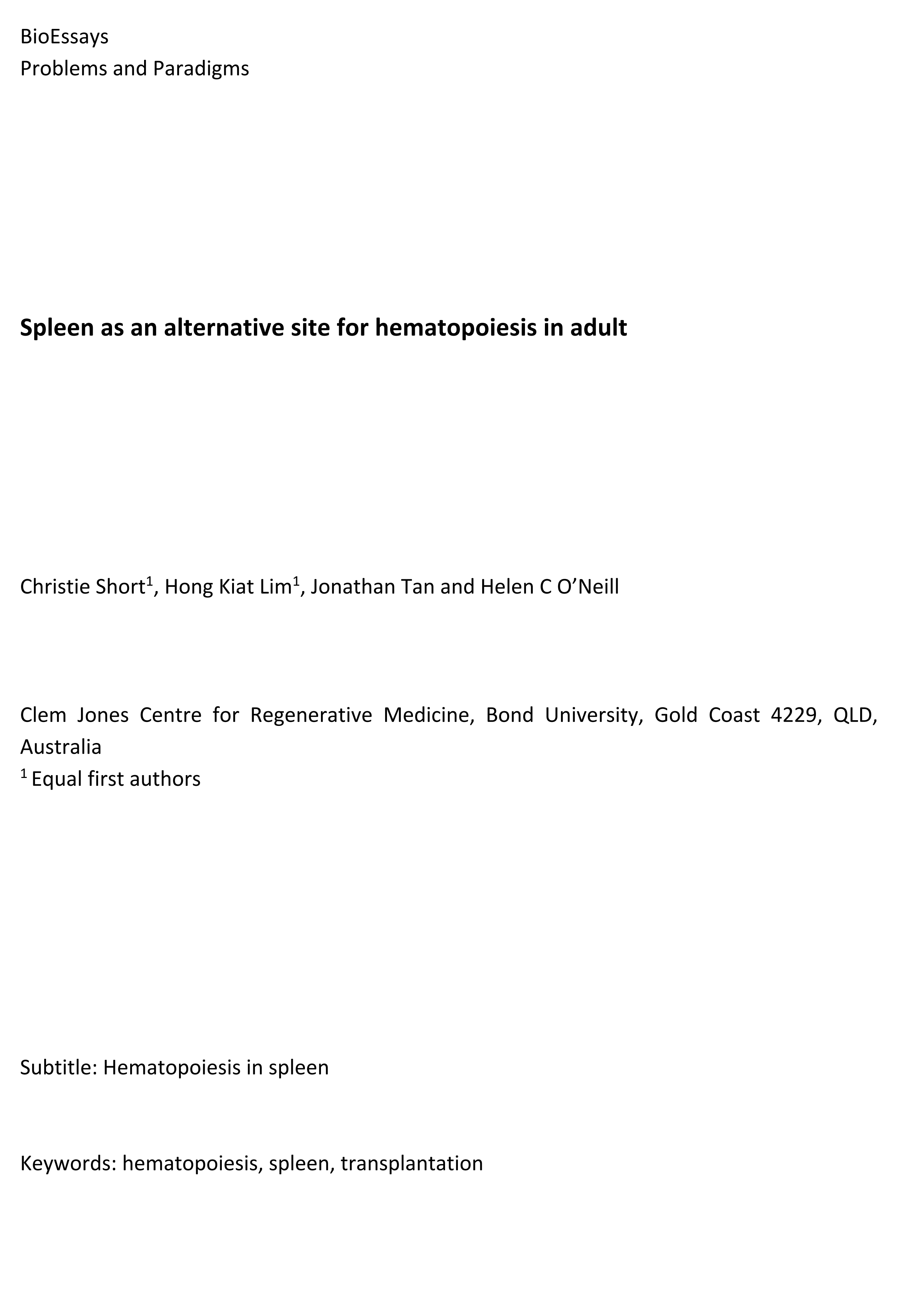

BioEssays
Problems and Paradigms

Spleen as an alternative site for hematopoiesis in adult

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Abstract

Hematopoiesis occurs throughout life and involves formation of blood cells from hematopoietic stem cells (HSC). The main site for hematopoiesis is bone marrow and the HSC niche comprises non-hematopoietic cells which contribute to stem cell dormancy, quiescence, self-renewal and differentiation. HSC have been identified in resting spleen of several species, although their contribution to hematopoiesis under steady-state conditions is unknown. Spleen also undergoes 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.

Spleen has remarkable ability to regenerate and can form ectopic tissue following engraftment. Studies in mice indicate potential for grafts to support influx of hematopoietic cells, with adoption of normal cellular architecture. An important goal could be formation of functional ectopic tissue to aid recovery following treatment for diseases which impact bone marrow. Expansion or replacement of niches could be considered where myeloablation for HSC transplantation compromises treatment outcomes. Immunotherapies which target spleen as an alternative hematopoietic site for improvement in recovery following HSC transplantation, could provide new treatment opportunities for patients with cancer and diseases of bone marrow.

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]. Inhibitors or antibodies have been shown to direct more HSC and hematopoietic progenitors into spleen [1, 2] which 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. This proposal therefore has direct translation in the area of cellular therapy and in improving HSC transplantation outcomes.

2. Hematopoietic stem cells in adults

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Colonisation of bone marrow with HSC occurs from day 17 in the mouse embryo and bone marrow then remains the primary site for hematopoiesis in the adult [3]. The hematopoietic system is then maintained throughout life by differentiation of HSC, with 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 [4]. LT-HSC are rare, self-renewing cells, also referred to as homeostatic or dormant [5]. HSC subsets can now be more fully defined through expression of the SLAM markers CD150, CD41 and CD48 [6], which then give rise to ST-HSC and multipotent progenitors (MPP) that also share the lineage$^-$Sca-1$^+$c-Kit$^+$ phenotype [7]. Through stepwise gain of CD34, CD48 and CD135 expression, along with loss of CD150 expression, MPP then develop 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, with further development resulting in progressive loss of differentiation capacity and self-renewal potential [8]. These three progenitor cells eventually give rise to all mature blood cells.

Hematopoietic reconstitution studies in murine models remain the gold standard test for verification of the differentiative potential of hematopoietic stem and progenitor subsets. These tests describe the ability of a stem cell to reconstitute blood cell formation following transplantation into a host pre-conditioned through irradiation or myeloablation, procedures which deplete endogenous HSC from their niches allowing donor stem cell engraftment [9]. In this setting, LT-HSC are capable of sustaining long-term hematopoietic cell production and full reconstitution evident by 16 weeks in the mouse [8]. 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, since they possess no self-renewal potential [10]. Hematopoietic reconstitution experiments have demonstrated subtle differences in donor cell output and kinetics between multiple subsets of HSC and MPP [11].

Despite the 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 [8]. The CD34$^+$ population of ST-HSC contains two functionally distinct sub-populations differing in Flt3 expression [4]. 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. ST-HSC then 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

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hematopoiesis, and for myeloid output in regenerative conditions. MPP2, MPP3 and MPP4 only exhibit short-term myeloid reconstitution ability of up to a month [12]. These studies identify both the heterogeneity and distinct differentiative potential of the HSC population.

3. Extramedullary hematopoiesis

Spleen plays a main role in immunity and its role in erythropoiesis and recycling of aged red blood cells has been well documented [13]. During fetal life, the spleen and liver assume a major role in hematopoiesis [14]. Throughout adult life, the spleen appears to adopt the role of a hematopoietic organ only under stress or pathological states, or during an immune response [15] [16]. It also takes the place of bone marrow as a hematopoietic organ during bone marrow pathogenesis [17]. The current perception in the field has been that spleen functions as an emergency hematopoietic organ, with efflux of HSC from bone marrow in response to physiological stress, pregnancy or infection [18] [19] [20]. HSC isolated from spleen care multipotent and when transplanted into an irradiated host mouse can give rise to all lineages of hematopoietic cells [21] (Figure 1).

Hematopoiesis occurring outside the medullary of bone marrow is referred to as extramedullary. Evidence that HSC migrate into spleen during EMH raises questions about the extent of hematopoiesis and the range of blood cells produced in humans undergoing physiological stress such as pregnancy or disease. The contribution of HSC in adult spleen to hematopoiesis occurring under steady-state versus physiological stress is unknown. HSC in adult murine spleen are rare under normal physiological conditions but are thought to be mobilised from bone marrow into spleen and other sites in response to stress. Myeloablation with cyclophosphamide and subsequent treatment with G-CSF can be used to mobilise HSC out of bone marrow and into peripheral blood and spleen, leading to EMH [22] [23]. HSC were found to be 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 myeloablative cyclophosphamide/G-CSF treatment blood loss or pregnancy [24]. This was associated with increased numbers of HSC and progenitors, and increased numbers of stromal cells forming the niche [24]. This study provided convincing evidence that spleen under stress conditions can function as a niche for HSC, through expansion of cells producing stem cell factor (SCF) produced by both endothelial cells, and by PDGFRb$^+$ stromal cells resembling the perivascular reticular cells described in bone marrow. 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 factor known to be essential for hematopoiesis in mouse spleen [24]. Tcf21-expressing PDGFRb$^+$ stromal cells were identified as unique to spleen and not bone marrow and found to produce SCF and CXCL12. Both endothelial cells and Tcf21-expressing PDGFRb$^+$ stroma together form the niche which supports HSC development in spleen and the expression of EMH [24]. A model for HSC niches in red pulp pf spleen is shown in Figure 2.

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An unanswered question relates to whether mobilisation treatments impact splenic niches such that spleen contributes to the supply of stem cells into peripheral blood. One report claims that G-CSF treatment in humans involves not only mobilisation of $CD34^+$ HSC from bone marrow into peripheral blood, but also the movement of *Hox11*-expressing cells from spleen into blood [25]. It has long been known, that Hox11 (or Tlx1) is an essential transcription factor for spleen organogenesis [26]. When *Tlx1* conditional knockdown mice were made, those studies showed that *Tlx1*-expressing stromal cells are a component of the spleen HSC niche, and are required for recruitment of HSC into spleen following EMH [27]. Overexpression of Tlx1 was found to be sufficient to induce EMH in the absence of any other physiological stressor [27]. Tlx1-expressing cells were also shown to resemble bone marrow niche elements, through expression of PDGFRa/b, CD51, CD105 and production of CXCL12 and SCF [27].

These recent studies emphasise both the important contribution that spleen makes to hematopoiesis, and the uniqueness of spleen as a hematopoietic niche. It is now necessary to consider the role that spleen plays in hematopoiesis in general, and in any study involving HSC transplantation in humans. Studies seeking to demonstrate the presence of HSC in human spleen are few [28] [29] [30] and have been limited by the availability of normal tissue. While human spleen shows minor structural differences from mouse spleen [31], they have the same functional immune capacity, and likely the same stem cell populations. The difficulty of finding the 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.

One could argue that the dynamic role of spleen in provisioning emergency hematopoiesis relies on the rapid expansion of rare hematopoietic supporting stromal cells[24, 27]. One hypothesis would be that rare mesenchymal stem and progenitor cells reside in the spleen and quickly differentiate to expand stromal cell niches. Since most studies rely on models involving induced EMH, it is possible that hematopoietic supporting cell types induced under physiological stress might differ from those naturally occurring in steady-state spleen. Only comprehensive investigation of stromal cells and their subsets will provide that information.

4. Niches for Hematopoietic Stem Cells

Both endosteal and perivascular sites have been identified as HSC niches in bone marrow, with osteoblasts, endothelial cells and mesenchymal stromal cells considered to be important contributors. The majority of HSC reside adjacent to sinusoidal blood vessels which is considered to be a main stem cell niche or microenvironment [6]. Since spleen supports extramedullary hematopoiesis, it follows that it must maintain or develop functional niches to support and expand hematopoiesis. Since there are no osteoblasts in spleen, HSC niches in spleen portend to be distinct when compared with bone marrow. Recent studies have

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identified an important role for both endothelial and mesenchymal cells as niches for HSC supporting development of EMH [24] [27].

A wealth of evidence now identifies an essential role for mesenchymal cells in bone marrow HSC niches. These stromal cells comprise a dichotomy of periarteriolar and perivascular reticular cells [32] [33], as the main source of SCF for HSC proliferation and of CXCL12 for HSC maintenance [34] [35]. Several subsets are known such that heterogeneity exists with overlapping subsets of more primitive nestin^{+} cells [36], Lepr-expressing cells [35] and CXCL12-abundant reticular cells [37]. In human bone marrow, perivascular cells were first aligned with pericytes surrounding vascular endothelium and reflecting mesenchymal progenitors [38]. Our own recent studies show that mouse splenic stromal lines which support *in vitro* hematopoiesis resemble perivascular reticular cells [39] [40]. CD146^{+} perivascular cells from human bone marrow support multi-lineage hematopoeisis and also maintain hematopoietic progenitors in co-cultures [41]. This has now been shown for murine stromal lines isolated from spleen [42] [43] which readily support myelopoiesis *in vitro* from overlaid LT-HSC [40]. It would appear that perivascular reticular cells provide a sufficient *in vitro* niche for hematopoiesis. Recently it was also shown that the same stromal cell lines can support hematopoietic development *in vivo* through production of an ectopic niche (Submitted ms).

Perivascular reticular cells in humans and mice reflect mesenchymal stem/progenitor cells located in close proximity with endothelial cells associated with the vasculature. Evidence that spleen contains HSC in the vicinity of perisinusoidal niches in red pulp following induction of extramedullary hematopoiesis [24], raises the question of whether spleen contains HSC niches in both the resting and stressed states, and what role these plays in hematopoiesis across the lifespan of the animal. Since the perivascular cells in bone marrow constitute the HSC niche surround the sinusoids, it is possible that a similar cell type exists in steady-state spleen, within the red pulp region which is dense with sinusoids. Extramedullary hematopoiesis in spleen occurs in the sinusoidal-rich red pulp region, based on evidence that mobilised HSC entering spleen from bone marrow localise in red pulp, and that mature myeloid cells are restricted to the red pulp region [24] [6] (Figure 2).

We have considered the role of splenic stroma in hematopoiesis since it was discovered that long-term spleen cultures can support hematopoiesis with continuous production of a distinct subset of myeloid dendritic-like cells [44] [45], as well as transient production of several myeloid cell types arising from precursors [46] [47] [48]. Hematopoietic progenitors are also maintained in long-term cultures, evident through gene expression [43] [49], and through ability to transfer long-term reconstitution upon adoptive transfer of cells into irradiated host mice [42]. Stromal lines derived from long-term cultures of spleen have been shown to reflect variants with different hematopoietic support capacity [50]. *In vitro* studies favour a role for spleen specifically in myelopoiesis, since splenic stromal lines overlaid with hematopoietic progenitors, from bone marrow or spleen, support long-term restricted *in vitro* myelopoiesis

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which is restricted to production of only several cell types [51]. These include myelomonocytic cells, conventional dendritic-like cells, and a novel dendritic-like 'L-DC' [47] [51]. We recently identified an *in vivo* counterpart to 'L-DC' [52], lending physiological relevance to *in vitro* hematopoiesis studies and the existence of a spleen-specific antigen presenting cell. These studies reveal a broader capacity of stroma to support myelopoiesis, with evidence that different stroma can have distinct hematopoietic support capacity. Here we propose that spleen like bone marrow must contain a number of perisinusoidal and perivascular reticular cells which are heterogeneous, with subsets perhaps having distinct functional roles in hematopoiesis in steady-state versus 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 [53] [54] [39] (Lim et al, accepted ms). 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 which support myeloid cell development and migration. Gene expression studies have 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 [39]. Also consistent with this is the osteogenic capacity of 5G3 cells, suggesting the presence of mesenchymal osteoprogenitors in spleen with ability to support *in vitro* hematopoiesis (Submitted ms). Perivascular reticular cells present in the red pulp of spleen could provide a niche for maintenance of HSC in the steady-state. Furthermore, restricted myelopoiesis leading to development of only L-DC raises the possibility that the spleen microenvironment may support the production of tissue-specific antigen presenting cells. Their location in red pulp could reflect a specific role in monitoring blood-borne antigens and interacting with migrating lymphoid cells.

HSC niches in bone marrow are impacted by conditioning regimes like irradiation given ahead of HSC transplantation. Bone marrow stromal cells suffer permanent damage from irradiation, which impairs the maintenance and support of HSC [55]. Irradiation used as a conditioning regime ahead of HSC transplantation damages bone marrow and abolishes HSC niches, in particular endothelial cells in the sinusoids [56]. However, the interaction of surviving or transplanted HSC with bone marrow niche cells does rapidly reconstitute hematopoiesis, rescuing the host from complications associated with long-term bone marrow suppression [9]. Therefore, the success of hematopoietic reconstitution is thought to be closely linked with 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 [57], and through co-infusion of bone marrow endothelial cells together with whole bone marrow cells into myeloablated recipients, giving in long-term, multi-lineage engraftment, and increased survival [58].

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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 [59]. Chronic inflammatory conditions can also promote uncontrolled stromal cell activation and fibrosis [60]. Furthermore, Castagnaro et al (2013) identified multipotent mesenchymal precursors and lymphoid tissue organiser cells that support injury-induced regeneration of spleen, and these cells may take part in tissue repair upon irradiation damage [61].

5. Potential for spleen as an alternative site for hematopoiesis

As with irradiation, little is understood about the impact of myeloablation on HSC niches and how this ultimately affects HSC transplantation success and recovery. HSC transplantations are given to patients undergoing myeloablative high-dose chemotherapy for blood disorders or leukemia and to treat congenital immunodeficiency disorders like severe immune deficiency [62]. Chemotherapy or irradiation treatment commonly leads to bone marrow aplasia [56] and immunocompromises the patient [63]. The differentiative potential of HSC is such that as few as 100 cells can provide full reconstitution in a myeloablated mouse model [64]. Therefore, transplantation of HSC into myeloablated patients should represent highly effective therapy for immune system restoration [65]. However, transplantation is associated with a high incidence of morbidity and mortality. Complications include graft-versus-host disease and graft failure, which are also linked to availability of a suitable donor. Another significant concern is mortality due to infection developing between myeloablative irradiation, HSC engraftment and reconstitution. Acceleration of immune recovery and hematopoietic reconstitution should reduce mortality and morbidity associated with early post-transplant infections.

In the clinical setting, bone marrow was first used as a source of HSC [66], 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 [67]. 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 [68]. For these reasons, mobilised peripheral blood is now the primary source of allogeneic and autologous HSC for transplantation [69].

Mobilised peripheral blood has produced rapid, stable long-term engraftment, such that the success and rate of engraftment in autologous transplantation can be correlated with the number of $CD34^+$ cells transplanted [70]. The best method for peripheral blood mobilisation uses G-CSF to mobilise $CD34^+$ cells from bone marrow for leukapheresis [71]. Other mobilising

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agents like GM-CSF have been compared with G-CSF, and the yield of $CD34^+$ cells was found to be similar [72]. However, GM-CSF provided earlier neutrophil and platelet recovery which provides a benefit in terms of rapid recovery with less need for blood and platelet transfusions, and fewer infections during the period of myeloablation. AMD3100 is also used to mobilise HSC from bone marrow. It is a highly specific antagonist of CXCR4 expressed by HSC and interrupts HSC binding to CXCL12 expressed on bone marrow stromal cells [73].

Immune reconstitution following HSC transplantation occurs in several phases [74]. An early phase of rapid myeloid cell reconstitution occurs within 20-30 days following allogeneic HSC transplantation, with lymphoid cell reconstitution delayed by up to a year [75]. Following transplantation, patients experience an 'aplastic phase' of severe neutropenia, until neutrophil progeny cells appear. During the first 100 days following transplantation, patients are susceptible to virus reactivation, as well as diseases due to slow reconstitution of NK cells and T cells [76]. Following autologous HSC transplantation, an absolute neutrophil count of $\geq 0.5 \times 10^9$ cells/L for 3 consecutive days is commonly defined as the time to myeloid cell recovery and establishment of innate immunity [77]. Growth factors such as G-CSF and GM-CSF are routinely administered to boost myeloid cell development after autologous transplantation and to accelerate hematopoietic recovery until neutrophil count is achieved [78]. Infection risk increases steeply with low neutrophil count, particularly for patients receiving no antibiotic therapy. The optimal cell dose is 2-5 x 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 [79].

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 [80], 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 since it is expressed by other hematopoietic progenitors including the MPP, common myeloid progenitor, megakaryocyte-erythroid progenitor, B cell/natural killer cell progenitor and the granulocyte-macrophage progenitor [81]. 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. Vose et al. (2001) [82] evaluated high-dose chemotherapy followed by transplantation of highly purified $CD34^+CD90^+$ HSC into patients with non-Hodgkin's lymphoma and mantle cell lymphoma. In these patients, purification of HSC from mobilised blood also provided the opportunity to eliminate tumour cells [83]. Rapid neutrophil and platelet engraftment was successful in the majority of patients, although infection remained a concern with 14 of the 20 patients reporting significant infections post-transplantation [82].

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

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engraftment following HSC transplantation, with over one third of HSC entering that organ [1]. Transplantation-induced EMH leads to 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 [84]. CFU arise from different HSC and progenitors including CFU-granulocyte-erythrocyte-monocyte-megakaryocyte (GEMM), CFU-lymphocyte (L) and CFU-erythrocyte (E). After transplantation, bone marrow c-Kit$^+$ and c-Kit^{lo} progenitors form CFU in spleen on days 8-10 and 12-14, reflecting clonal development of different progenitors, compared with LT-HSC which form late CFU after 16-20 days [85]. 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 [86].

In contrast to the mouse model, the role of human spleen in HSC transplantation and hematopoietic rescue in humans remains contentious. Spleen size has been found to have a negative effect on the engraftment efficiency of CD34$^+$ cells following transplantation into patients with myelofibrosis [87]. HSC engraftment is delayed in patients with splenomegaly [88] and in children with hypersplenism [89]. Early pooling of CD34$^+$ HSC in spleen, coupled with a defect in bone marrow homing, can reduce the engraftment of HSC into bone marrow following transplantation and may even cause graft failure [87] [90]. Reduction in spleen size is thought to improve bone marrow homing and partial or total splenectomy can improve the rate of HSC engraftment [91]. Splenectomy has been shown to lead to a slight increase in neutrophil engraftment in bone marrow [92]. Since successful engraftment of HSC depends on the availability of niches in bone marrow and spleen at the time of HSC transplantation, 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 [93].

6. Improving niche recovery following HSC transplantation

There is a recognised need to improve hematopoietic recovery following high-dose irradiation and stem cell transplantation. Here we propose targeting spleen as a site for EMH following irradiation and HSC transplantation, in order to accelerate early immune cell reconstitution and to reduce mortality associated with post-transplant susceptibility to infection. While the majority of clinical HSC transplantations use unseparated bone marrow [66], or mobilised peripheral blood [2] [68], the direction in the field has been to isolate and transplant more purified HSC populations [23]. Transplantation of highly purified CD34$^+$ HSC grafts has been successful, although is still associated with significant infection post-transplantation [94] [95] [82].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 levels at which this could be achieved.

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The first approach here is to select hematopoietic progenitors or stem cell subsets which target spleen and so accelerate early transient myelopoiesis expecting this to be protective until full hematopoiesis is established in bone marrow. Another approach is to use specific drug treatment to direct transplanted HSC into spleen and give rapid differentiation of myeloid cells. A final more speculative approach is to repair, expand or replace stromal niches in spleen, e.g. following irradiation or myeloablation, in order to accelerate early hematopoietic events in spleen which are protective. These different approaches could be applied to the mouse model, with a view to translation into methodologies for use in human HSC transplantation.

A first possibility is that transplantation of MPP in addition to purified HSC may provide early and more effective hematopoietic reconstitution in graft recipients, so reducing the period over which the patient is immunocompromised. Evidence of early spleen CFU following transplantation confirms that some bone marrow stem/progenitor cells do preferentially enter spleen [85]. Multipotent progenitors downstream of HSC can provide rapid, short-term hematopoietic reconstitution as early as 2 weeks following transplantation in an irradiated recipient [8]. It is, however, 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 could involve defined subsets of MPP or ST-HSC which enter spleen with a view to establishing early myelopoiesis to aid recovery, ahead of LT-HSC colonisation of bone marrow niches, and eventually full hematopoietic development. The role of spleen in supporting MPP differentiation is still unclear, but the homing of MPP into spleen after transplantation is expected since spleen is the most frequent site of initial engraftment following transplantation [1]. Hematopoietic cell differentiation from MPP to give myeloid cells might occur across 1 to 4 weeks following transplantation. Evidence that spleen supports MPP engraftment and differentiation following transplantation could open new therapeutic options around acceleration of early hematopoietic recovery.

Several drugs are already in clinical use which can directly mobilise HSC into spleen. AMD3100 is a highly specific, reversible antagonist of the CXCR4 receptor expressed by HSC. Interaction of AMD3100 with CXCR4 interrupts binding to CXCL12 in the bone marrow niche [96]. Hence, AMD3100 is commonly used as a mobilising agent for peripheral blood HSC collection. However, post-transplant administration of AMD3100 in mice can have beneficial effects on hematopoietic reconstitution [93]. Treatment is associated with a significant increase in HSC homing and engraftment to spleen, with later restoration of bone marrow density and cellularity. Treatment also leads to increased CFU appearing in spleen, improved recipient survival, and enhanced reconstitution of all blood cell lineages. A question not yet addressed is whether enhanced homing of HSC to spleen results in short-term myelopoiesis. To determine the effect of post-transplant AMD3100 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 AMD3100. Effectiveness of the protocol could be

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indicated by early myelopoiesis evident in peripheral blood and the presence of colonies in spleen within 2 weeks after transplantation. The important role of spleen in this process could be assessed by investigating splenectomised mice in which all HSC home to bone marrow. In terms of redirecting HSC into spleen, anti-VLA adsorption to HSC is another possible treatment option which has been shown to significantly reduce homing of HSC into bone marrow post-transplantation and is accompanied by increased circulating HSC and their subsequent uptake by spleen [84].

A final approach to enhancing recovery following myeloablation with HSC transplantation is to repair, expand or replace splenic niches for hematopoiesis. This is the most progressive approach and relies on knowledge of splenic organogenesis and HSC niche formation. The impact of irradiation or myeloablation on splenic niches is also 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 (2015) [24] recently showed that EMH induced by several different stressors leads to expansion of perisinusoidal niches in the red pulp region which were retained following return of the animal to normal physiological state. This type of evidence points to plasticity amongst splenic stromal cells, a property in line with strategies to directly repair and expand HSC niches following irradiation or myeloablation. Such an approach would require that initially spleen structural damage 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 approached through introduction of endothelial cells, and some evidence has been reported which supports that possibility [97]. 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 which constitute stromal niches. A significant advancement would be engraftment of splenic stromal cells to form an ectopic or artificial niche (Figure 3).

The spleen has innate regenerative capacity and is readily amenable to engraftment for tissue regeneration [98] [99] [100] [101] [102]. Successful grafting of tissue fragments led to development of tissue with the structural identity of spleen, including red and white pulp formation and evidence of full hematopoietic reconstitution. Engraftment of stromal fractions isolated by enrichment on the basis of cell surface markers led to the identification of a spleen organiser cell as an endothelial-like CD31+MAdCAM-1+ cell. However, formation of ectopic tissue was also found to be dependent on the presence of mesenchymal PDGFRb+ cells. Stromal cell lines derived from long-term cultures of spleen and shown to support *in vitro* hematopoiesis have also been engrafted under the kidney capsule and shown to accumulate HSC and myeloid cells from the host (Submitted ms). 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

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sourced from readily available tissues or blood, would open new opportunities for therapy to improve HSC uptake into spleen during transplantation.

7. Conclusion

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 and used to 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, open new avenues for treatment of patients who have become immunocompromised through bone marrow decline, cancer or disease related to ageing.

Abbreviations

CFU, colony forming unit; EMH, extramedullary hematopoiesis; HSC, hematopoietic stem cell; LT-HSC, long-term-HSC; ST-HSC, short-term HSC.

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Conflict of interest

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The authors declare no conflict of interest.

Figure legends

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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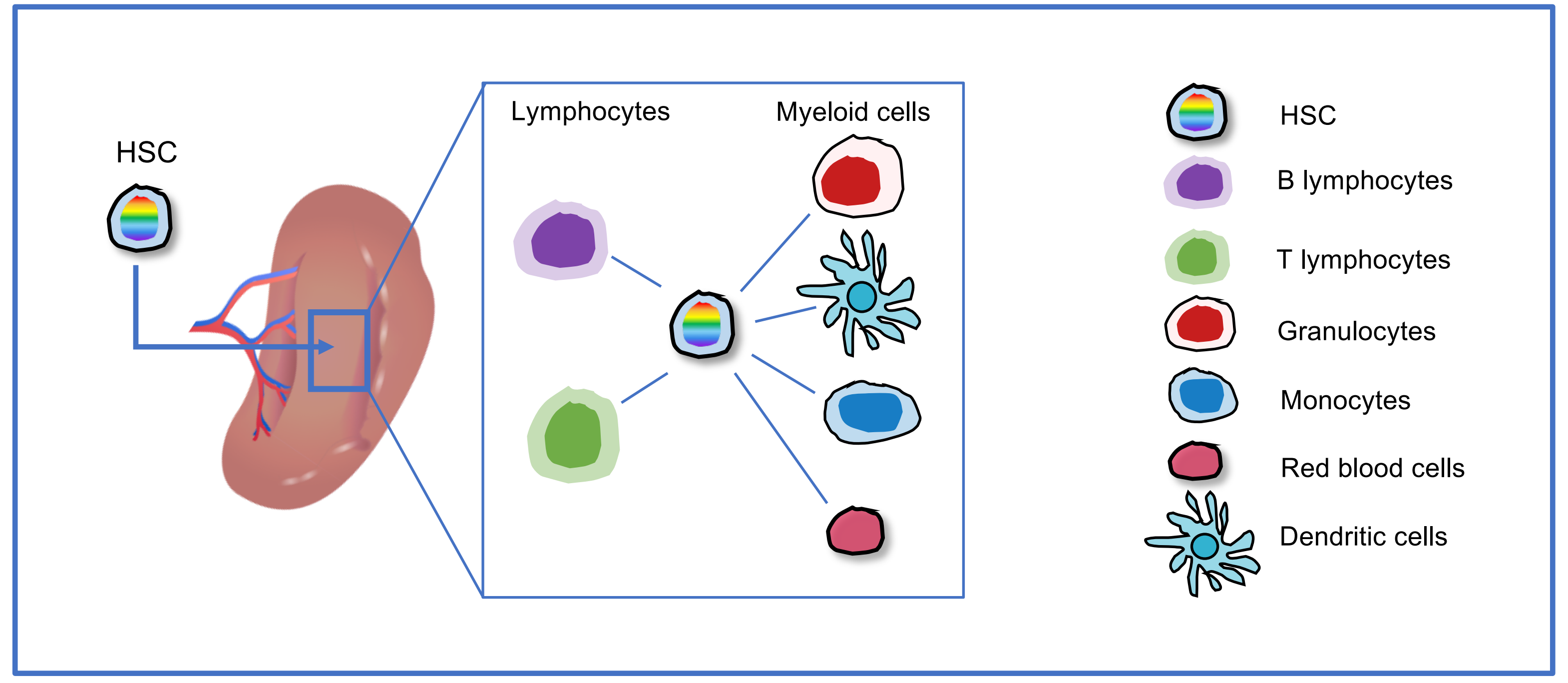

Fig 1

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Hematopoietic cells

HSC

B lymphocyte

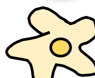
T lymphocyte

Monocyte

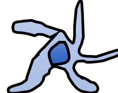
Dendritic cell

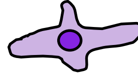
Granulocyte

Red blood cell

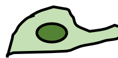
 VCAM-1^{+}Macrophage

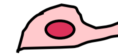
Mesenchymal stromal cells

 Follicular dendritic cell

 Perisinusoidal reticular cell

Fibroblastic reticular cell

 Marginal reticular cell

 Red-pulp fibroblast

Sinusoids

Endothelial cells

SCF

CXCL12
SCF

RP

MZ

WP

CA

Fig 2

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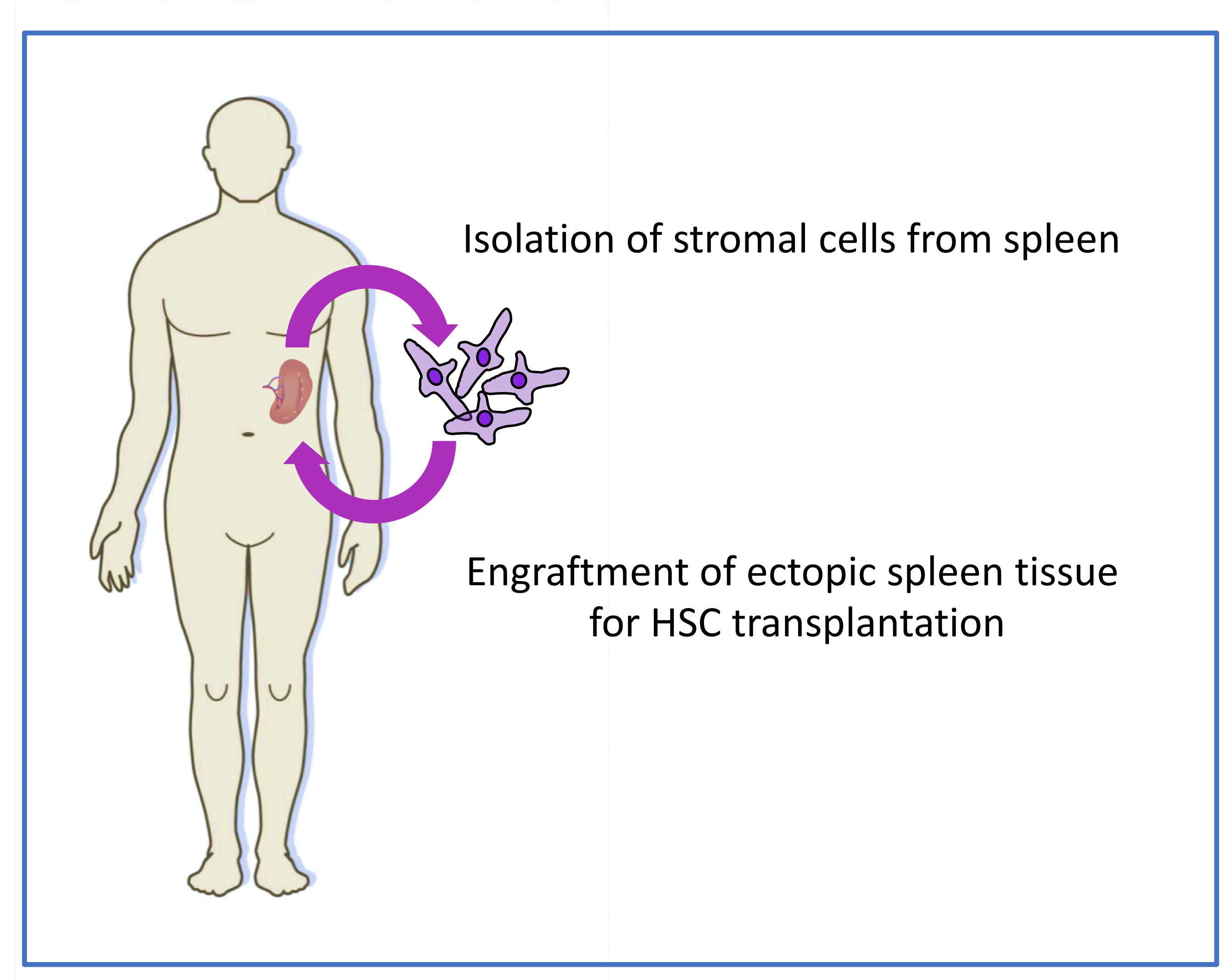

Fig 3

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