Splenic hematopoietic stem cells display a pre-activated phenotype

Emilie Coppin¹, Jonathan Florentin¹, Sathish Babu Vasamsetti¹, Anagha Arunkumar¹, John Sembrat^{1,2}, Mauricio Rojas^{1,2}, and Partha Dutta¹

¹Division of Cardiology, Department of Medicine, Vascular Medicine Institute, University of Pittsburgh Medical Center, BST 1720.1, 200 Lothrop Street, Pittsburgh, PA 15213, USA

²Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, PA 15261, USA

Abstract

Splenic hematopoiesis is crucial to the pathogenesis of diseases including myocardial infarction and atherosclerosis. The spleen acts as a reservoir of myeloid cells, which are quickly expelled out in response to acute inflammation. In contrast to the well-defined bone marrow hematopoiesis, the cellular and molecular components sustaining splenic hematopoiesis are poorly understood. Surprisingly, we found that, unlike quiescent bone marrow hematopoietic stem cells (HSC), most of splenic HSC are in the G1 phase in C57BL/6 mice. Moreover, splenic HSC were enriched for genes involved in G0-G1 transition and expressed lower levels of genes responsible for G1-S transition. These data indicate that, at steady state, splenic HSC are pre-activated, which may expedite their cell cycle entry in emergency conditions. Consistently, in the acute phase of septic shock induced by LPS injection, splenic HSC entered the S-G2-M phase, whereas bone marrow HSC did not. Mobilization and transplantation experiments displayed that bone marrow HSC, once in the spleen, acquired cell cycle status similar to splenic HSC, strongly suggesting that the splenic microenvironment plays an important role in HSC pre-activation. In addition, we found that myeloid translocation gene 16 (*Mtg16*) deficiency in C57BL/6 mice resulted in significantly increased S-G2-M entry of splenic but not bone marrow HSC, suggesting that *Mtg16* is an intrinsic negative regulator of G1-S transition in splenic HSC. Altogether, this study demonstrates that compared to bone marrow, splenic HSC are in a pre-activated state, which is driven by extracellular signals provided by splenic microenvironment and HSC intrinsic factor *Mtg16*.

Keywords

Hematology; HSC; spleen

Correspondence: Partha Dutta, Division of Cardiology, Department of Medicine, Vascular Medicine Institute, University of Pittsburgh Medical Center, BST 1720.1, 200 Lothrop Street, Pittsburgh, PA 15213, USA. duttapa@pitt.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

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INTRODUCTION

The spleen is considered as a secondary hematopoietic organ, which has long been assumed to mainly contribute to erythropoiesis.¹ However, during late embryonic life and at birth, the spleen is one of the major sites of hematopoiesis. The presence of hematopoietic stem cells (HSC) in adult spleen at steady state has been described albeit less numerous than in bone marrow.² O'Neil's group reported that the reconstitution ability of splenic HSC is similar to that of bone marrow HSC, demonstrating that splenic HSC have equivalent functional HSC.^{1,3} Extramedullary hematopoiesis in the spleen is crucial to pathogenesis of different diseases in humans, such as atherosclerosis and acute myocardial infarction.^{4,5} Metabolic activity of splenocytes determined by 18F-fluorodeoxyglucose-positron emission tomography imaging in patients with acute coronary syndrome was strongly correlated with inflammation in atherosclerotic plaques and predicted future cardiovascular events.⁶ Despite the role of the spleen in hematopoiesis in stress,⁷⁻¹⁰ the molecular and environmental cues of splenic hematopoiesis are not known.

Tissue microenvironments regulate HSC proliferation and differentiation.^{11,12} Kiel and colleagues described that splenic HSC were associated with red pulp sinusoidal endothelial cells.¹³ More recently, Morrison's group showed that specific deletion of endothelial cells expressing *Scf* and *Tcf21*⁺ perivascular stromal cells expressing *Cxcl12* reduced splenic HSC numbers, strongly suggesting their role in splenic HSC maintenance.¹⁴ Other splenic cell types are potentially part of splenic HSC niche. Indeed, we recently reported that splenic macrophages retain splenic HSC via VCAM-1.¹⁵ The mechanisms of splenic HSC proliferation and differentiation in emergency conditions are not well studied. Besides extracellular signals delivered by microenvironment, splenic HSC activation status may also be driven by cell intrinsic factors. Indeed, the transcription factor c-Myc is an intrinsic regulator of HSC quiescence in the bone marrow. c-Myc overexpression has been related to decreased expression of N-cadherin, leading to HSC proliferation and differentiation.¹⁶ Cell intrinsic factors governing splenic HSC function are yet to be discovered.

The goals of this study are as follows: (1) to determine the differences between bone marrow and splenic HSC in cell cycle activity and their ability to respond to "emergency" conditions, (2) to study the impact of the microenvironment in splenic HSC activation and (3) to delineate the importance of cell intrinsic factors governing the pre-activation state of splenic HSC. We found that, during the acute phase of septic shock, splenic HSC entered the cell cycle, while bone marrow HSC were still in the G0 phase of the cell cycle, indicating pre-activated state of splenic HSC at steady state. Congruently, most of the splenic HSC were in the G1 phase of the cell cycle at steady state. In addition, they expressed higher levels of genes involved in G0-G1 transition than bone marrow HSC. Splenic HSC expressed declined levels of genes involved in G1-S transition and increased levels of genes encoding proteins responsible for G1 cell arrest. These data indicate that steady-state splenic HSC are in the G1 phase of the cell cycle and are poised to be rapidly activated in response to danger signals. We also found that bone marrow HSC, when mobilized to the spleen after G-CSF-mediated mobilization or bone marrow reconstitution, acquired pre-activated phenotype similar to native splenic HSC, indicating the role of splenic microenvironment in

HSC activation. Finally, we found that myeloid transcription gene 16 (*Mtg16*) was specifically involved in G1 arrest of splenic HSC in the steady state.

RESULTS

Sterile inflammation activates splenic hematopoiesis

To investigate splenic hematopoiesis, we induced acute inflammation by coronary ligation in a mouse model of myocardial infarction (MI). It has been reported that MI induces a strong inflammation characterized by expansion of systemic myeloid cell content.⁸ Figure 1a displays the gating strategy of splenic hematopoietic stem and progenitor cells (HSPC) ($\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$) population that includes HSC and the multipotent progenitors (MPP). Analysis of spleens 7 days after MI displayed a twofold increase in spleen weight, indicating splenic hematopoiesis (Figure 1b). The absolute number of total splenic myeloid cells (CD11b^+) and monocytes ($\text{CD11b}^+ \text{CD115}^+$) significantly increased in the spleens of mice with MI compared to sham surgery (myeloid cells: sham $10.3 \pm 1.6 \times 10^5$ and MI $19.8 \pm 2.6 \times 10^5$; monocytes: sham $93.8 \pm 1.5 \times 10^5$ and MI $18 \pm 2.31 \times 10^5$) (Figure 1b). Analysis of splenic hematopoietic progenitor cells revealed 3.2- and 15.3-fold increase in absolute HSPC and HSC numbers, respectively, after MI compared to sham-operated mice (Figure 1b). Next, we investigated the splenic hematopoiesis in chronic inflammatory disease condition. To this end, we enumerated inflammatory myeloid cells and HSPC in high-fat diet-induced type 2 diabetes mouse model. Diabetic mice had larger spleens, and 2.5-, 3.9- and 1.7-fold increase in monocyte, HSC and HSPC numbers, respectively, compared to age and sex-matched nondiabetic mice (Figure 1c). In addition, we found a significant increase in splenic myeloid cell, neutrophil, monocyte and $\text{Ly6C}^{\text{high}}$ inflammatory monocyte numbers in diabetic mice (Supplementary figure 1). These results demonstrated that both acute and chronic sterile inflammation drive splenic hematopoiesis. The increased splenic hematopoiesis in disease may be due to increased proliferation and differentiation of splenic HSC. To test this possibility, we performed BrdU pulse chase experiment to assess proliferation of splenic HSPC in diabetic and nondiabetic mice. BrdU, a synthetic nucleoside, is incorporated into *de novo* synthesized DNA of proliferating cells. After 3 weeks of pulse with BrdU, most of the HSC are labeled with the synthetic nucleoside. After the pulse phase, cells that undergo proliferation dilute BrdU progressively. Thus, the cells retaining BrdU are quiescent. Our results showed a significant decrease in percentage of HSPC and HSC retaining BrdU in diabetic mice (Figure 1d) (HSPC: diabetes 7.1% and nondiabetes 12.84%; HSC diabetes 15.79% and nondiabetes 26.7%), attesting the increased proliferation of splenic HSPC in diabetic mice. Altogether, these data revealed increased activation and proliferation of splenic HSPCs, leading to increased production of myeloid cells in response to sterile inflammation.

Splenic HSC are phenotypically different

Next, we compared the expression of cell surface markers specific for HSC between splenic and bone marrow HSC. Flow cytometry analysis of extracellular markers shows a significant decrease in c-Kit, Sca-1, CD34 and CD150 markers in splenic HSC compared to bone marrow HSC at steady state (Supplementary figure 2). No change has been noted in CD48 expression. In a mouse model of type 2 diabetes, which is a chronic inflammatory condition,

the expression of c-Kit, Sca-1, CD150 and CD48 was also significantly decreased in splenic HSC compared to bone marrow HSC. Moreover, the expression of Sca-1 and CD150 in the bone marrow or splenic HSC was significantly diminished in diabetic mice. These data reveal a slight but significant phenotypic difference between splenic and bone marrow HSC.

Splenic and bone marrow HSPC cell cycle patterns are distinct

The rapid induction of splenic hematopoiesis in MI and type 2 diabetes suggests that the progenies originate from splenic HSC *in situ*. We hypothesize that splenic HSC are in a pre-activated state and can quickly respond to an inflammatory insult. To test this hypothesis, we investigated HSPC cell cycle at steady state in the bone marrow and the spleen. Splenic HSPC and HSC were significantly less quiescent (G0 phase) than bone marrow HSPC (Figure 2a). In mice, the frequency of splenic HSPC in the G1 phase was significantly higher than the bone marrow HSPC (Figure 2a). In fact, about 71.1% of splenic HSPC were in the G1 phase versus only 26.4% for bone marrow HSPC. However, the percentage of HSPC in the S-G2-M phases (proliferating cells) in both of the organs was similar. We observed a similar phenotype in human splenic HSPC (Figure 2b). Human spleen had significantly decreased frequency of HSPC in the G0 phase (bone marrow $71.4 \pm 3.6\%$ and spleen $53.7 \pm 2.6\%$) and increased frequency of HSPC in the G1 phase (bone marrow $17.2 \pm 2.3\%$ and spleen $32.9 \pm 1.0\%$) and unchanged frequencies of cells in the S-G2-M phases (bone marrow $8.7 \pm 2.2\%$ and spleen $9.8 \pm 1.7\%$). These data indicate that at steady state, splenic HSPC are less quiescent than bone marrow HSPC and are arrested in the G1 phase of the cell cycle. To investigate the molecular actors that regulate the cell cycle of splenic HSPC, we sorted bone marrow and splenic HSC and MPP, and investigated expression of genes involved in cell cycle progression (Figure 2c–e). Genes encoding the activators of cell cycle progression from G0 to G1, such as *Cdk3*, *Cdk4*, *Cdk6* and *Ccnd1*, were enriched in HSC and MPP isolated from the spleen compared to bone marrow. The mRNA expression of *Cdkn3*, an inhibitor of G0-G1 transition, was significantly less in both HSC and MPP in the spleen. These data indicate that splenic HSPC are more prone to enter the cell cycle compared to bone marrow HSPC. Although the frequency of splenic HSC in the G1 phase was higher compared to bone marrow HSC, both of these organs had similar proportion of proliferating (in S-G2-M) HSC, indicating that there is reduced G1-S transition in splenic HSC. Consistently, the level of *Ccne2*, a facilitator of G1-S transition, was diminished and the level of *Inca-1*, an inhibitor of G1-S transition, was increased in splenic HSC compared to bone marrow HSC (Figure 2d). We next determined expression levels of genes, such as *Gpr132*, *Gadd45a* and *Slfn-1*, which are involved in cell cycle arrest. We found that these genes were upregulated in splenic HSC (Figure 2e). These results suggest that splenic HSC are arrested in the G1 phase of the cell cycle at steady state.

Splenic HSC respond to “danger” faster than bone marrow HSC

The data discussed above indicate a more activated state of splenic HSC compared to their bone marrow counterpart at steady state, suggesting that splenic HSC respond to inflammatory conditions faster than bone marrow HSC. To test this, we challenged mice with high dose of the Toll-like receptor 4 ligand LPS to induce septic shock. One hour after LPS injection, we observed augmented splenic HSC proliferation (Figure 3a). In contrast, bone marrow HSPC and HSC cell cycle was not affected at this time (Figure 3b). Frequency

of splenic HSC in the G0 phase was significantly decreased (HSPC: LPS $12.3 \pm 0.6\%$ and PBS $18.7 \pm 1.7\%$; HSC: LPS $23.7 \pm 1.0\%$ and PBS $34 \pm 3.2\%$), whereas the proportion of splenic HSC in the S-G2-M phases significantly augmented (HSPC: LPS $25.2 \pm 1.0\%$ and PBS $13.2 \pm 2.2\%$; HSC: LPS $16.4 \pm 3.2\%$ and PBS $5.0 \pm 1.3\%$). Spleen and bone marrow HSC and HSPC numbers were not affected (Supplementary figure 3), indicating that these cells just started proliferating in response to LPS treatment. These results were in line with the steady-state cell cycle profile of HSC and strongly suggest that splenic HSC are pre-activated compared to bone marrow, allowing them to proceed to the S-G2-M phase faster.

Splenic microenvironment affects splenic HSPC phenotype

One of the questions raised from these observations is why splenic HSPC are inclined to enter the cell cycle and quickly proliferate compared to bone marrow HSPC. Bone marrow microenvironment is known to consist of distinct well-characterized niches which can provide signals to HSC to maintain them in a protective quiescent state.^{17–22} However, splenic HSC microenvironment has not yet been extensively studied, and is probably very different from bone marrow HSC niche because spleen lacks osteoblastic cells, which play a major role in bone marrow HSC quiescence.²¹ We hypothesize that the pre-activated phenotype of splenic HSC is due to differential instructive signals emanated from splenic microenvironment. To determine the role of splenic microenvironment on HSC, we induced HSC mobilization from the bone marrow to the spleen by G-CSF injection (Figure 4a). We observed 85-fold increase in splenic HSC numbers on the day of G-CSF injection and a 65-fold increase in splenic HSC 6 days after the mobilization compared to PBS-injected control mice, demonstrating migration of mobilized bone marrow hematopoietic stem cells into the spleen. Investigation of cell cycle status of the HSPC and HSC recruited to the spleen 6 days after G-CSF treatment revealed a cell cycle phenotype significantly different from bone marrow HSPC and HSC 6 days after their mobilization to the spleen (Figure 4b). The numbers of quiescent HSPC and HSC were significantly decreased (HSPC in G0: bone marrow $75.1 \pm 3.4\%$ and G-CSF-treated spleen $47.3 \pm 4.1\%$; HSC in G0: bone marrow $82.7 \pm 5\%$ and G-CSF-treated spleen $46 \pm 6\%$) and the frequency of cells in the G1 phase was significantly increased (HSPC in G1: bone marrow $18.2 \pm 2.8\%$ and G-CSF-treated spleen $48.4 \pm 3.4\%$; HSC in G1: bone marrow $12.2 \pm 5\%$ and G-CSF-treated spleen $52 \pm 2\%$), suggesting that HSPC from bone marrow origin changed their cell cycle phenotype once they were recruited into the spleen. To confirm these results, we engrafted bone marrow CD45.2⁺ HSPC into sublethally irradiated CD45.1⁺ recipient mice. After complete hematopoietic reconstitution, we studied the cell cycle phenotype of splenic CD45.2⁺ and CD45.1⁺ HSPC compared to bone marrow HSPC (Figure 4c). The cell cycle profile of donor bone marrow-derived CD45.2⁺ HSC in the spleen was similar to that of CD45.1⁺ host-derived splenic HSC, but significantly distinct from bone marrow HSC. Similar to CD45.1⁺ native splenic HSC, donor-derived CD45.2⁺ splenic HSC are significantly less quiescent compared to bone marrow HSC (CD45.2⁺ HSPC in G0: bone marrow $54.3 \pm 4.8\%$ and spleen $22.3 \pm 5.1\%$; CD45.2⁺ HSC in G0: bone marrow $69.4 \pm 7.2\%$ and spleen $29.9 \pm 7.7\%$). These data demonstrate that HSPC of bone marrow origin, after relocalization into the spleen, adopt the pre-activated phenotype. Next, we investigated whether splenic HSC, after being relocalized to the bone marrow, adopt a quiescent phenotype. To this end, we transplanted splenic CD45.1⁺ HSPC into lethally irradiated CD45.2⁺ recipient mice. We

ascertained the cell cycle phenotype of donor-derived bone marrow HSPC and HSC compared to native bone marrow and splenic HSPC and HSC (Figure 4d). We observed that splenic HSC and HSPC adopt a quiescent phenotype after relocalization into the bone marrow. Collectively, these data strongly suggest that splenic microenvironment is responsible for the pre-activated state of splenic HSC.

Mtg16 regulates G1-S transition in splenic HSPC

Our results showed the involvement of splenic microenvironment in the pre-activated cell cycle state of splenic HSC. However, we cannot exclude the possibility that a cell intrinsic regulatory mechanism could contribute to G1 arrest phenotype of splenic HSC. The inability of splenic HSC to enter the S-G2-M phase of the cell cycle could involve distinct cell intrinsic mechanism. Chyla *et al.* describe that myeloid translocation gene 16 (*Mtg16*) deficiency resulted in splenomegaly with increased myeloid cell generation in the spleen indicating the role of *Mtg16* in splenic HSC proliferation.²³ To investigate whether *Mtg16* regulates the entry of splenic HSC arrested in the G1 phase into the S-G2-M phase of the cell cycle, we performed cell cycle analysis. We observed that the frequency of splenic HSC of *Mtg16*^{-/-} mice in the G1 phase was significantly decreased, while the percentage of splenic HSC in the S-G2-M phase was significantly increased at steady state (HSC in G1 phase: wt 52.1 ± 3.9% and *Mtg16*^{-/-} 32.1 ± 2.6%; HSC in S-G2-M phase: wt 7 ± 1.5% and *Mtg16*^{-/-} 12.7 ± 0.9%) (Figure 5a). In contrast, the cell cycle of bone marrow HSPC was not affected in *Mtg16*-deficient mice (Figure 5b). Moreover, total myeloid cell contents were significantly increased in spleen and peripheral blood, but not bone marrow *Mtg16*-deficient mice compared to wild-type mice (Supplementary figure 4), suggesting the involvement of *Mtg16* in the S-G2-M entry of splenic HSC and splenic myelopoiesis.

Regulation of cell cycle entry of HSPC by *Mtg16* can be due to either cell intrinsic effect or the microenvironment or both. To determine whether the regulation of HSPC proliferation by *Mtg16* is cell intrinsic, we sorted splenic and bone marrow HSC from wild-type and *Mtg16*-deficient mice and assessed *in vitro* time to first division (Figure 5c). Indeed, *Mtg16*-deficient HSC proceeded faster to the first division than wild-type HSC (bone marrow – logEC50 wild type: 63.06; *Mtg16*^{-/-}: 49.05 h and spleen – logEC50 wild type: 55.55; *Mtg16*^{-/-}: 47.61 h).

Mtg16 is essential for HSC maintenance

To further delineate the importance of *Mtg16* in HSC function and phenotype, we interrogated the role of *Mtg16* in HSC maintenance. For this purpose, we transplanted bone marrow *Mtg16*-deficient HSC into lethally irradiated recipients. Surprisingly, more than 75% of the mice engrafted with the *Mtg16*^{-/-} HSC died within 30 days after the graft (Figure 5d). In contrast, about all of the recipient mice transplanted with wild-type HSC survived up to 30 days after the transplantation. These data suggest that *Mtg16* is crucial for HSC maintenance.

Mtg16 deficiency increases myelopoiesis and inflammation in sterile injury

To assess whether *Mtg16* expression increases in inflammatory conditions, we induced myocardial infarction in wild-type mice. Despite the fact that *Mtg16* expression was

significantly increased in splenic HSC compared to bone marrow HSC, myocardial infarction did not affect *Mtg16* expression in bone marrow and splenic HSC compared to sham-operated control mice (Supplementary figure 5a). As discussed above, *Mtg16* blocks the S-G2-M entry of splenic HSC in the steady state. Based on these data, we hypothesize that *Mtg16*-deficient mice will have exaggerated inflammation after a sterile injury. To test this, we determined HSC numbers and myelopoiesis in *Mtg16*-deficient mice after myocardial infarction. Two days following myocardial infarction, spleens of *Mtg16*-deficient mice with MI displayed significant increase in myeloid cell, neutrophil, Ly-6C^{high} inflammatory monocyte and HSPC numbers compared to sham-operated control (Supplementary figure 5b). In contrast, we did not observe an increase in the numbers of these cells in the bone marrow (Supplementary figure 5c).

DISCUSSION

Spleen is the major site of extramedullary hematopoiesis and possesses its own *in situ* HSC. HSC in the spleen have self-renewal and repopulating potential similar to that of the bone marrow.²⁴ Under physiological conditions bone marrow HSC are the main source of hematopoiesis.²⁵ However, the spleen plays a crucial role in “emergency hematopoiesis”.^{4–10,14} In fact, the spleen generates about 50% of myeloid cells in atherosclerosis.²⁶ In concord with this, our results demonstrated the increase in splenic hematopoiesis in acute and chronic sterile inflammation. In the course of understanding the mechanisms of splenic hematopoiesis, we surprisingly observed a distinct pattern of cell cycle activity of splenic HSC compared to bone marrow HSC. Steady-state splenic HSC were significantly less quiescent and the majority of them were in the G1 phase of the cell cycle. Splenic HSC were enriched for cyclins and cyclin-dependent kinases that are involved in G0-G1 transition. However, they had reduced expression of genes that mediate S-G2-M cell cycle entry and were enriched for *Slfn-1*, *Gpr132* and *Gadd45a* genes that code for proteins reported to be involved in cell cycle arrest.^{27–31} These data suggest that splenic HSC are in a “pre-activated state”, which provide them with the potential to proliferate and generate mature cells faster in case of emergency. Congruently, only 1 h after LPS challenge, splenic HSC left the G1 phase and entered the S-G2-M phases, whereas bone marrow HSC did not exit G0 quiescence phase. Our experimental results demonstrate that the spleen harbors a “ready to use” pool of HSC, which unleash emergency hematopoiesis. This is in line with the finding that muscle stem cells “alerted” by a tissue injury have better muscle regenerative ability than “non-altered” stem cells.³²

Bone marrow microenvironments provide distinct signals that determine HSC proliferation and differentiation. Our experiments of bone marrow HSC mobilization and adoptive transfer revealed that bone marrow HSC display a pre-activated phenotype after they relocate into the spleen, indicating that the splenic microenvironment is responsible for the pre-activated phenotype of splenic HSC. The splenic stroma is composed of a mixed population of vascular and lymphatic endothelial cells, marginal reticular cells, fibroblasts, macrophages, follicular dendritic cells and red pulp fibroblastic cells.³³ Splenic HSC reside close to endothelial vascular cells,¹³ suggesting their role in HSC maintenance. Interestingly, splenic HSC have been located near to megakaryocytes-like cells, which express SDF-1, OPN, N-Cadherin and b-Catenin, which are also secreted by bone marrow osteoblasts,³⁴

suggesting the role of megakaryocytes-like cells in splenic HSC function. We have recently reported that splenic VCAM-1-expressing macrophages are essential for splenic HSC retention,¹⁵ suggesting that VCAM-1⁺ macrophages are a component of splenic HSC microenvironment. Bone marrow osteoblasts are essential for HSC niche establishment;^{21,22,35–38} however, HSC are well known to develop in absence of osteoblasts in the embryo, and in adult spleen and liver. In fact, Morrison's group showed that SCF expression in endothelial cell and CXCL12 expression in stromal cells are important for splenic HSC maintenance.¹⁴ The difference in niche components of the bone marrow and spleen may cause a distinct cell cycle status of HSC.

As discussed above, splenic HSC are in G1 “alert” phase at steady state. However, they did not enter the S-G2-M phases of the cell cycle in absence of any external triggers. We found that *Mtg16* is responsible for the G1 arrest of splenic HSC. *Mtg16*-deficient mice had increased proportions of splenic HSC in the S-G2-M phases of the cell cycle. In contrast, the percentage of bone marrow HSC in the S-G-M phases did not alter. These data suggest that *Mtg16* is a cell intrinsic regulator, retaining splenic HSC in the G1 phase. Consistent to this and in line with previous publications,^{23,39} we observed myelopoiesis in the spleen but not bone marrow of *Mtg16*^{-/-} mice, faster first division in *Mtg16*-deficient HSC, and increased myelopoiesis in spleens but not bone marrow of *Mtg16*-deficient mice compared to wild-type counterparts after myocardial infarction. How *Mtg16* mediate splenic HSC proliferation is not clear. *Mtg16* is a transcriptional corepressor involved in cell fate determination via the Notch signaling pathway.⁴⁰ The Notch pathway has been described to be involved in many developmental processes, including HSC fate decision toward lymphoid lineage.^{41,42} The Notch signaling has also been demonstrated to instigate G1 to S cell cycle progression by increasing the expression of *ccnd3*, *cdk4* and *cdk6*.⁴³ We hypothesize that *Mtg16* impedes S-G2-M progression of splenic HSC, by negative regulation of the Notch pathway and Notch cell cycle related genes expression. Further studies are warranted to test this hypothesis.

We observed that transplantation of *Mtg16*-deficient HSC drastically decreased survival of recipient mice indicating a crucial role of *Mtg16* in HSC maintenance. It is well known that after transplantation, HSC seeds primarily the spleen where they are able to form multilineage colonies at day 12 after engraftment⁴⁴ and then repopulate the bone marrow. Reduced survival of recipient mice transplanted with *Mtg16*^{-/-} HSC suggests that HSC cannot engraft and repopulate in the spleen in the absence of *Mtg16*. As splenic hematopoiesis determines the pathogenesis of diseases,^{7,9,15,45–47} understanding the mechanisms by which splenic HSC maintain their “alert” phenotype will be important to impede deleterious myelopoiesis in different diseases such as atherosclerosis.

METHODS

Animals, diabetes induction and myocardial infarction surgery

Male C57BL/6 wild-type mice (10–12 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). *Mtg16*^{-/-} mice were obtained from Scott Hierbert's group (Department of Biochemistry, School of Medicine, Vanderbilt University). To induce type 2 diabetes, mice were fed for 16 weeks with high-fat diet (62% kcal, Harlan Teklad [Fredrick, MD]). Myocardial infarction was induced in intubated mice ventilated with isoflurane 2%.

Thoracotomy was performed in the fourth left intercostal space, then the left coronary artery was ligated with a monofilament nylon 8–0 suture and the thorax was closed with a 8–0 suture.

Organ harvesting and flow cytometry

Mouse blood, spleens and bone marrow (femurs and tibias) were harvested. Bone marrow was processed by grinding with a pestle and mortar. Cells from the bone marrow and spleens were passed through 40-µm filters, washed in FACS buffer (PBS containing BSA 5%) and stained with monoclonal antibodies. Mouse cell stainings were performed to analyze myeloid and progenitor cells (Supplementary table 1). Myeloid cells and monocytes were, respectively, defined as CD19[−] CD11b⁺ and CD19[−] Ly6G[−] CD11b⁺ CD115⁺. HSPC were identified as Lin[−] c-Kit⁺ Sca-1⁺, GMP as Lin[−] c-Kit⁺ Sca-1[−] CD16/32⁺ CD34⁺, MPP as Lin[−] c-Kit⁺ Sca-1[−] CD150[−] CD48⁺ and HSC as Lin[−] c-Kit⁺ Sca-1[−] CD150⁺ CD48[−]. Human cell stainings were performed to analyze progenitor cells (Supplementary table 2). Human HSPC population was identified as Lin[−] CD34⁺ CD38[−] CD45RA[−] CD90⁺. For Mtg16 staining, splenic and bone marrow HSC (Lin[−] c-Kit⁺ Sca-1[−] CD150⁺ CD48[−]) were permeabilized (eBioscience FoxP3 permeabilization kit, San Diego, CA), incubated with anti CD16/32 Fc block antibody and then stained with mouse anti-Mtg16 (gift from Dr Scott Hiebert) or mouse isotype control.

Data acquisition and analysis were performed using Fortessa Flow Cytometer (Becton Dickinson, Downers Grove, IL) and FlowJo software (Tree Star, Ashland, OR).

Adoptive transfer

Five hundred CD45.2⁺ HSC (Lin[−] c-Kit⁺ Sca-1[−] CD150⁺ CD48[−]) sorted from bone marrow or spleen were injected *i.v.* into CD45.1⁺ sublethally irradiated (5 Gy) mice. Analyses were performed 16 weeks after engraftment. For the survival experiment, lineage negative cells isolated from bone marrow *Mtg16*-deficient or wild-type mice were engrafted in lethally irradiated (9 Gy) C57BL/6 mice.

Cell cycle analysis

Cell cycle analysis was performed *in vitro* with Ki67 and prodidium iodide staining. For the *in vivo* BrdU pulse chase experiment, 100 µg of BrdU was injected once a day for 3 weeks *i.p.* and followed by 4 weeks of chase.

LPS challenge and G-CSF mobilization

For the LPS challenge experiment, mice were injected *i.p.* with either 100 µg of LPS or PBS 1 h prior to sacrificing and subsequent spleen and bone marrow harvesting. For G-CSF mobilization, mice were injected *i.p.* with either PBS or G-CSF (Filgastrim) 125 µg kg^{−1} twice a day for 4 days. Mice were sacrificed, and spleen and bone marrow were harvested at day 0 and day 6 after the injection.

Cell sorting and RT-PCR

HSC and MPP cells were sorted using an ARIA II SORP cell sorter (Becton Dickinson). Sorted MPP and HSC were collected in RNA extraction buffer. Total RNA was extracted

using a Arcturus™ PicoPure™ RNA isolation kit (Applied Biosystems, Foster City, CA, USA) and cDNA was prepared from 100 ng of mRNA using the high-capacity RNA to cDNA synthesis kit (Applied Biosystems). Quantitative RT-PCR was performed using Taqman primers (Applied Biosystems). Results were expressed as Ct values normalized to the housekeeping gene *Gapdh*.

Single HSC *in vitro* culture

HSC (Lin⁻ c-Kit⁺ Sca-1⁻ CD150⁺ CD48⁻) isolated from bone marrow of *Mtg16*^{-/-} or wild-type mice were sorted and plated in 96-wells plates (1 cell per well) in SFEM media supplemented with 20 ng mL⁻¹ SCF (STEM cell technologies) and Penicillin/Streptomycin. They were cultured and counted twice a day for 7 days.

Statistics

Data were represented as mean ± s.e.m. Statistical significance between groups was performed using the Prism *t*-test or ANOVA according to the data set. Results were considered as statistically significant when *P* < 0.05.

Study approval

All animal experiments were conducted following NIH guidelines under protocols approved by the Institutional Animal care and Use Committee of the University of Pittsburgh. All experimental procedures involving the use of human samples included the relevant receipt of written informed consent from family members of deceased patients before tissue collection for the study. Cause of death was head trauma. This study was approved by the Institutional Review Board of the University of Pittsburgh No. REN16070123/PRO11070366 as well as the Institutional Review Board of the University of Pittsburgh No. REN17030011/IRB0306040. Ethical approval for this study and informed consent conformed to the standards of the Declaration of Helsinki. The procurement of organs from deceased patients was approved by the Committee for Oversight of Research and Clinical Training Involving Decedents (CORID No. 724).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Myocardial infarction and diabetes increase splenic myelopoiesis. **(a)** Flow cytometric gating strategy of splenic progenitors: HSPC (Lin⁻, c-Kit⁺, Sca-1⁺), multipotent progenitors (Lin⁻, c-Kit⁺, Sca-1⁺, CD48⁺) and HSC (Lin⁻, c-Kit⁺, Sca-1⁺, CD150⁺, CD48⁻). Spleen weight, and myeloid cell, monocyte, HSPC and HSC absolute numbers after MI compared to sham-operated control, **(b)** ($n = 4-8$ per group; data representative of two experiments) and in high-fat diet-induced type 2 diabetic mice compared to nondiabetic mice, **(c)** ($n = 7-16$ per group; data were pooled from two independent experiments). **(d)** The schematic diagram depicts the experimental design. Representative flow cytometric plots and bar graphs show percentages of HSPC and HSC retaining BrdU after 4 weeks of chase. ($n = 8-12$ per group; data were pooled from two independent experiments). Mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 2.

Splenic HSPCs are pre-activated at steady state. **(a)** Cell cycle profiles of splenic and bone marrow HSPC and HSC at steady state in mice **(a)** ($n = 9$ per group, representative of two independent experiments) and human **(b)** ($n = 1$ per group, experimental triplicate). mRNA expression of genes involved in G0 to G1 transition **(c)**, G1 to S transition **(d)** and cell cycle arrest **(e)** in FACS-sorted bone marrow and splenic HSC and multipotent progenitors. ($n = 6-11$ per group; data were pooled from two to six independent experiments). Mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3.

Splenic but not bone marrow HSC enter the S-G2-M phase in the acute phase of septic shock. Septic shock was induced by LPS injection. Representative flow cytometric plots (left) and bar graphs (right) showing percentages of HSPC and HSC in the spleen **(a)** and bone marrow **(b)** 1 h after LPS challenge. ($n = 5$ per group; representative of two independent experiments). Mean $\pm$ s.e.m. * $P < 0.05$.

Figure 4.

Spleen microenvironment is responsible for pre-activated phenotype of splenic HSC. **(a)** Flow cytometric plots and histograms showing HSPC, HSC and GMP numbers in PBS- and G-CSF-treated mice at day 0 and day 6 post-treatment. ($n = 5-10$ per group; results representative of one experiment). **(b)** Flow cytometric plots and bar graphs depicting percentages of splenic and bone marrow HSPC and HSC in the G0, G1 and S-G2-M phases at 6 days after either PBS or G-CSF treatment ($n = 5$ per group; results representative of one experiment). **(c)** Top: schema of the HSC transplantation experiment. Bottom: percentages of recipient and donor-derived splenic and bone marrow HSPC and HSC in the G0, G1 and S-G2-M phases. ($n = 3-4$ per group; results representative of one experiment). **(d)** Proportions of bone marrow, splenic from nonengrafted mice and donor-derived splenic HSPC and HSC in different cell cycle phases in lethally irradiated mice transplanted with splenic HSPC. Mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 5.

Splenic HSC proliferation is regulated by Mtg16. Representative flow cytometric plots (left) and bar graphs (right) showing percentages of HSPC and HSC in the spleen (a) and bone marrow (b) of wild-type and *Mtg16*^{-/-} mice. (*n* = 9–10 per group; data are pooled from two independent experiments). (c) Cumulative first-division kinetics of bone marrow and splenic HSC (*n* = 96 per group for bone marrow and 48 per group for splenic HSC). Arrowheads represent time to first division as estimated from sigmoid fit (*t*_{FirstDiv} = logEC50). (d) Survival curve of lethally irradiated mice transplanted with either wild-type or *Mtg16*^{-/-} HSC. Mean ± s.e.m. **P* < 0.05, ****P* < 0.001.