TUMOR BURDEN INDUCES A SELF-AMPLIFYING LOOP OF MYELOPOIESIS THAT IS MEDIATED BY NF-κB-KIT LIAGND SIGNALING

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ABSTRACT OF THE DISSERTATION

Tumor Burden Induces a Self-Amplifying Loop of Myelopoiesis That Is Mediated by NFκB-Kit Ligand Signaling

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Cancer cells induce an inflammatory microenvironment which consists of inflammatory cells, cytokines and chemokines. In the meanwhile, malignant tumors are associated with hematological abnormalities that disrupt homeostasis of hematopoiesis. Therefore how tumor burden influence hematopoiesis through inflammatory cytokines is studied in this thesis.

Firstly, IFNγ was found to play a major role in lineage^{low}Sca-1^{+}C-kit^{+} (LSK) cell expansion by activating the expression of Sca-1 in lineage^{low}Sca-1^{-}C-kit^{+} cells in vivo and in vitro. This process was dependent on IFNγR1 signaling and the STAT1 pathway. The IFNγ-induced LSK cells had a higher proliferation potential

than control LSK cells. Moreover, the IFNγ-induced hematopoiesis was more biased toward the differentiation of myeloid lineages. Therefore, our findings demonstrated a novel role of IFNγ in activating hematopoietic progenitor cells and provide a new insight into the clinical application of interferon.

Secondly, in tumor-bearing mice, with the decline of hematopoiesis in bone marrow, spleens are greatly enlarged and harbor a greatly expanded population of hematopoietic progenitor cells (HPCs). While such HPCs can differentiate into both myeloid and lymphoid lineages when transplanted into tumor-free hosts, they preferentially give rise to myeloid lineages including macrophages/monocytes in tumor-bearing hosts. We further showed that macrophages/monocytes, derivatives of HPCs, are essential for the expansion of HPCs in spleen. Thus, HPCs and myeloid cells form a positive feedback loop in sustaining splenic myelopoiesis during tumorigenesis. This self-amplifying loop of HPCs and myeloid cells depends on an NFκB-Kit signaling cascade. Tumor-stimulated inflammatory factors are essential for the increased production of Kit ligands by macrophages/monocytes that drives splenic myelopoiesis. Targeting this HPC-myeloid loop may have potential in impeding tumor progression in cancer therapy.

In conclusion, we found that the inflammatory cytokine IFNγ was demonstrated to

enhance the expansion of hematopoietic stem cells (LSK cells) in vitro and in vivo, which provides important insights into studying the interaction between inflammation and hematopoiesis. Moreover, via mouse tumor models, we revealed the cellular and molecular basis for tumor-induced hematopoiesis and how such abnormal hematopoiesis influenced tumor progression. The information is important to understand the relationship between cancer and hematopoiesis, lending novel hope for cancer therapy.

ACKNOWLDGEMENT

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I would like to thank my parents for their endless love. Without their support, this work would not have been possible to carry out. I'd also like to thank my brother, aunt and uncle, for their encouragement to me, as well as taking care of my parents while I am abroad.

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DEDICATION

To my beloved family members

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CHAPTER I Introduction

1. Hematopoiesis

Hematopoiesis is the process of formation of blood and immune cells. All cellular blood components are derived from hematopoietic stem cells (HSCs), which are multipotent stem cells that give rise to daughter HSCs and all blood cell lineages. In a healthy adult, to maintain the steady state of the body, approximately 10^{11}–10^{12} fresh blood cells are generated daily. As shown in Figure 1, HSCs have the capability to differentiate into both myeloid (dendritic cells, macrophages, granulocytes, erythrocytes, and platelets) and lymphoid lineages (T-cell, B-cell and NK cell). Therefore, HSCs are entirely responsible for the development and maintenance of the whole blood system.

During embryonic development, hematopoiesis occurs in different anatomical locations at different stages. In mice, during the embryogenesis, the yolk sac can support the generation of primitive hematopoietic cells (Haar and Ackerman, 1971). Later on, colonization of fetal liver by yolk sac cells begins on day 10 or day 11, and by day 12 the fetal liver becomes the major site of hematopoiesis. Fetal liver HSCs eventually migrate to the bone marrow on day 16-17 (Morrison et al., 1995). After birth, bone marrow becomes the dominate organ of hematopoietic cell development (Clapp et al., 1995). In addition, spleen is considered to be a

primary site of extramedullary hematopoiesis (EMH) in mice (Khaldoyanidi et al., 2003; Kondo et al., 2003; O'Malley et al., 2005). In human, although spleen does not normally function as a site of hematopoiesis, it can serve as a site of extramedullary hematopoiesis under some conditions, such as hematopoietic neoplasms, nonhematopoietic tumors and some disorders of the spleen and circulation systems (Barbui et al., 1987; Farhi and Ashfaq, 1996; O'Malley et al., 2005). EMH often occurs in response to bone marrow failure (WYATT and SOMMERS, 1950). The major sites of EMH, spleens are usually enlarged and harbor a higher proportion of hematopoietic progenitor cells (Walkley et al., 2007b; Walkley et al., 2007a). Tumor-bearing mice characteristically show EMH (Young et al., 1987; Ilkovitch and Lopez, 2009).

HSCs are critical for a healthy body since they are the origin of all the specialized blood cell types in the hematopoietic system. But there are many differences between the human and mice hematopoietic cell markers for the commonly accepted types of haematopoietic stem cells. Usually, human HSC are recognized as cell have the following phonotype: CD34+, CD59+, Thy1/CD90+, CD38lo/-, C-kit/CD117+, lin-. But Mouse HSC are CD34lo/-, Sca-1+, Thy1.1+/lo, CD38+, C-kit+, Lin-. In mice, all HSCs possess the lineage marker Lin-, Sca-1+, and C-kit+. According to their surface markers and their self-renewal capacity, HSCs are further divided into distinct subpopulations. Reciprocal expression of

the markers Thy-1 and Flk-2 is observed in long-term (LT)-HSCs and short-term (ST)-HSCs, respectively. Recently, CD150 is used as a cell surface marker to distinguish LT-HSCs and ST-HSCs. LT-HSCs have a high self-renewal potential, whereas ST-HSCs have limited self-renewal potential and further give rise to (Kondo et al., 2003)(Kondo et al., 2003)(Kondo et al., 2003)(Kondo et al., 2003)(Kondo et al., 2003)(Kondo et al., 2003)multipotent progenitor cells (MPPs), which are Lin-, Sca-1+, C-kit+, Flk-2+, CD34+, and CD135+. LT-HSCs, ST-HSCs and MPPs, are known as LSK (Lin-Sca-1+ C-Kit+) cells.

Hematopoietic progenitor cells (HPCs) consist of LSK cells and committed progenitors. The distribution, frequency and the absolute number of HPCs can be changed when the body is under the condition of injury, stress and cancer.

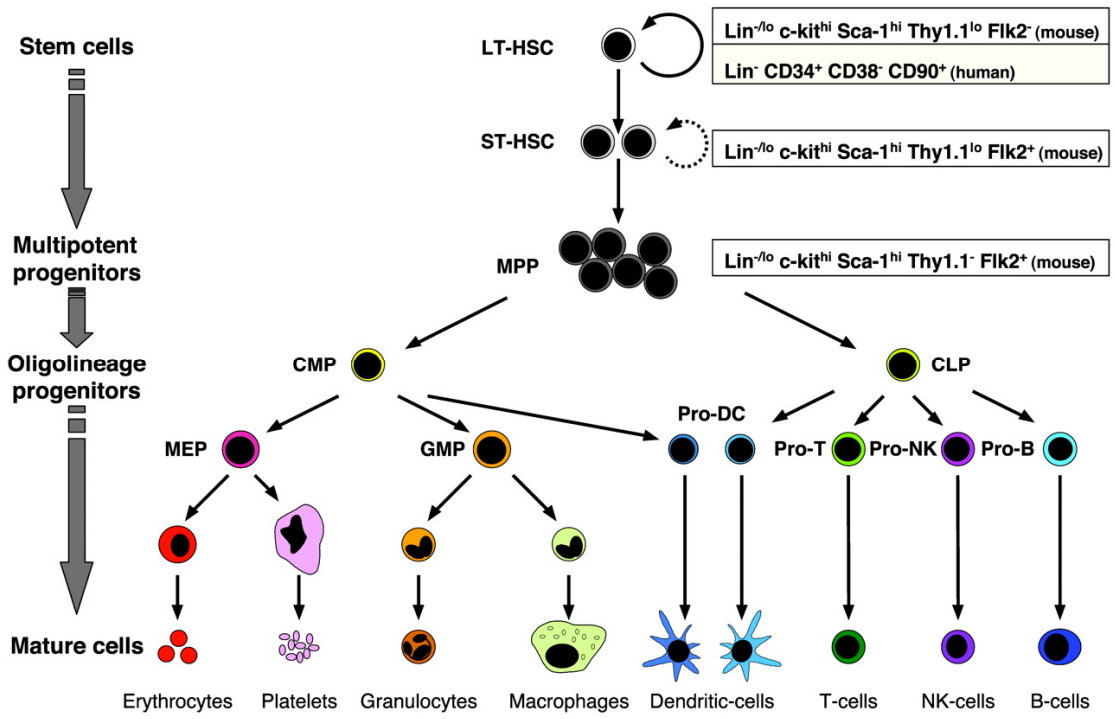

Figure 1. Hematopoietic and progenitor cell lineages. HSCs can be divided into LT-HSCs, highly self-renewing cells that reconstitute an animal for its entire life span, or ST-HSCs, which reconstitute the animal for a limited period. ST-HSCs differentiate into multipotent progenetors (MPPs), which usually do not self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny through functionally irreversible maturation steps. The common lymphoid progenitors (CLPs) give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells. The common myeloid progenitors (CMPs) give rise to GMPs, which then differentiate into monocytes/macrophages and granulocytes, and to megakaryotic/erythroid progenitors (MEP), which produce megakaryocytes/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells.

2. Hematopoietic Progenitor Cell Expansion

It is generally believed that the expansion and self-renewal of HPC cells are maintained by the signaling molecules and the local tissue microenvironments, or niches. Adult HPCs mainly reside in bone marrow and therefore bone marrow constitutes the HPC niches in normal healthy adults (Calvi et al., 2003; Zhang et al., 2003). Bone marrow contains many different hematopoietic and nonhematopoietic cells. Among them, the stromal cells, including osteoblasts, osteoclasts, fibroblasts, and mesenchymal progenitors, are believed to be the primary niche cells that support the maintenance of HPCs via direct cell-cell contact, or by secreting factors that act at a distance (Adams and Scadden, 2006; Zhang et al., 2003). In the secondary hematopoietic organs, such as spleen, HPCs are primarily observed around sinusoids, although the niche cell types and function were unknown (Kiel et al., 2005).

It becomes clearer that the HPC niches regulate the resident progenitor cells using various extrinsic and intrinsic signaling pathways: the niche cells act on HPCs through cell-cell contact or soluble factors (extrinsic pathways), which stimulates the transcriptional signaling pathways in HPCs that are responsible for the self-renewal and expansion (intrinsic pathways) (Zon, 2008). The most studied intrinsic pathways include Bmi-1, homeobox gene pathway, Wnt pathway

and Notch pathway, and the extrinsic pathways are mainly mediated by some hematopoietic cytokines.

Besides intrinsic pathways, hematopoietic cytokines are also capable of stimulating the proliferation of hematopoietic progenitor cells at various stages. Many of these cytokines are produced by local stromal cells in bone marrow microenvironment. For example, mouse HSCs can be induced to undergo self renewal upon exposure to IL-3, IL-6 and stem cell factor (SCF) (Bodine et al., 1989; Bodine et al., 1992). Meanwhile, human HSCs are stimulated to proliferate by fms-related tyrosine kinase 3 (FLT3) and thrombopoietin (Thpo) (Petzer et al., 1996). Vascular endothelial growth factor (V-EGF), fibroblast growth factor 1 (FGF-1), and angiopoietin (ANG) were also reported to promote HSC self-renewal in vitro or in vivo (de Haan et al., 2003; Gerber et al., 2002; Zhang et al., 2006). Interestingly, a recent report indicated that insulin-like growth factor binding protein 2 (IGFBP2) secreted by a tumorigenic cell line 293-T supported ex vivo expansion of mouse HSCs (Zhang et al., 2008).

It was recently demonstrated that, in response to some systemic infection and inflammatory diseases, HSCs have the potential to proliferate, which is likely to be responsible for replenishment of the effector immune cells. IFNγ is a crucial

pro-inflammatory cytokine in host defense, linking adaptive immunity with innate immunity. It is mainly produced by type 1 helper T (Th1) cells and natural killer (NK) cells, but exerts its function through acting on the innate immune cells such as dendritic cells and macrophages. Therefore, IFNγ plays a central role in both lymphoid and myeloid lineage cells in the hematopoietic system. Recently, IFNγ was found to stimulate HSCs in vitro and in vivo, through binding its receptor IFNγR1 and initiating their downstream STAT1 pathway(Baldridge et al., 2010; Zhao et al., 2010). Interestingly, two recent studies indicated that the type I interferons could also transiently stimulate the proliferation of hematopoietic stem cells, although the long-term stimulation would result in stem cell exhaustion(Essers et al., 2009; Sato et al., 2009). IFNα/β and IFNγ activate different signaling pathways. IFNα/β binds to the type I IFN receptor and activates both STAT1 and STAT2. Such inflammatory factors also include tumor necrosis factor-α (TNFα) and Toll-like receptors, which act similarly to promote transcriptional changes, mainly through NF-κB pathway (Baldridge et al., 2011; King and Goodell, 2011).

3. Role of Hematopoietic Progenitor Cells in Tumor Development

Injury, stress and other conditions are able to change the frequency as well as the absolute number of stem cells, progenitor cells and different types of mature blood

cells. For instance, hematopoietic stem cells and progenitor cells (HSPCs) expand in bacterial infection model(Zhang et al., 2008; Takizawa et al., 2011). In the bone marrow transplantation surgery, the donor of bone marrow are administrated with G-CSF, then their HSPCs may undergo expansion and are mobilized to peripheral circulation. The tumor development challenges also can drastically change the dynamics of hematopoiesis. Many hematological abnormalities were documented in cancer patients, such as anemia (Spivak, 1994) and a shift to myelopoiesis (Wilcox, 2010; Spivak, 1994). Leukocytosis in cancer patients is correlated with poor prognosis (Shoenfeld et al., 1986; Donskov and von der, 2006; Mandrekar et al., 2006; Michael et al., 2006; Schmidt et al., 2007). Hematological abnormalities were well documented in experimental mice. Increased leukocytosis, especially in the spleen, is characteristic of tumor-bearing mice. Such extramedullary hematopoiesis (EMH) in spleen and other lymphoid organs is frequently associated with hematopoietic and non-hematopoietic disorders in human (O'Malley et al., 2005). However, it is still unclear what roles extramedullary hematopoiesis plays in the disease process, and how hematopoiesis is regulated by the expansion and self-renewal of HPCs.

The EMH is always associated with great expansion of some specified myeloid cells such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). MDSCs are a heterogeneous population of cells, which

hold the potential to promote tumor growth through immunosuppression and angiogenesis. MDSCs are comprised of two major subsets: granulocytic MDSCs and monocytic MDSCs. Granulocytic MDSCs are phenotypically CD11b+Ly6G+, wherease monocytic MDSCs are CD11b+Ly6C+. In most tumour models, granulocytic MDSCs contain 70-80% of the whole MDSCs and monocytic MDSCs contain 20-30%. Tumor-asscoaited macrophages are differentiated from monocytes and migrated to the tumour by stimulation with some chemotactic factors. They induce angiogenesis by production of factors such as vascular endothelial growth factor (VEGF), angiopoietin 1 (ANG1) and ANG2. Additioanlly, TAM exert their tumor promoting effect through breakdowning the matrix.

While these findings underscore the importance of hematopoietic progenitor cells and myeloid cells in tumorigenesis and other pathological conditions, many questions remain. Are those myeloid cells associated with cancer derived from a population of progenitors that are committed to myeloid lineage? What are responsible for the accumulation/expansion of the progenitor cells in EMH during tumorigenesis? What are the signaling pathways invovled? Using mouse transplantation tumors as a model system, we determined the genesis of the cancer-associated hematopioetic progenitor cells and characterized the relationship between myeloid cells and HSPCs in the spleen of tumor-bearing mice. We showed that HPCs, which are greatly expanded in the spleens of

tumor-bearing mice, primarily give rise to myeloid lineages, including monocyte/macrophages, in tumor-bearing hosts. The monocytic derivatives of HPCs, in return, are responsible for the expansion of HPCs in spleen. Thus, HPCs and their myeloid derivatives form a self-amplifying loop in driving splenic myelopoiesis.

CHAPTER II IFNγ Induces Expansion of Lin^{-}Sca-1^{+}C-kit^{+} Cells

ABSTRACT

The balance between Th1 and Th2 cells is critical for homeostasis of the immune system. Th1 cells can also regulate hematopoietic progenitor cell homeostasis by production of oncostatin M. Here we show that Th1 cell products, but not those of Th2 cells, caused a rapid expansion of lineage^{low}Sca-1^{+}C-kit^{+} (LSK) cells *in vivo* and *in vitro*. Among Th1 cytokines, IFNγ was found to play a major role in this expansion by activating the expression of Sca-1 in lineage^{low}Sca-1^{-}C-kit^{+} cells. This process was dependent on IFNγR1 signaling and the STAT1 pathway. Furthermore, those IFNγ-induced LSK cells had a higher proliferation potential than control LSK cells. In addition, while the overall production of colony forming units (CFUs) in bone marrow was decreased after IFNγ treatment, the sorted LSK cells could give rise to a higher yield of CFUs. Finally, the IFNγ-induced hematopoiesis was more biased toward the differentiation of myeloid lineages. Therefore, our findings demonstrated a novel role of IFNγ in activating hematopoietic progenitor cells and provide a new insight into the clinical application of interferon.

Introduction

Helper T (Th) cells, an important T cell sub-type in adaptive immunity, function by activating other immune cells such as B cells, cytotoxic T (Tc) cells, and antigen-presenting cells (APCs), and by secretion of specific cytokines. Th cells develop into two distinct effector populations-Th1 cells and Th2 cells. Although the recently characterized new helper T cell subsets, such as Th17 cells(Bettelli et al., 2006) and Th9 cells(Veldhoen et al., 2008), have further advanced our understanding of the helper T cells, the Th1/Th2 balance is still the paradigm for studying the adaptive immune system, especially in autoimmune disease research. Although Th1/Th2 balance is widely recognized for its role in the regulation of homeostasis and the function of mature lymphoid/myeloid lineage cells, it probably also plays a role in maintaining the homeostasis of hematopoietic stem cells and progenitor cells. For example, IFNγ, the Th1 cells-defining cytokine, has been reported to suppress hematopoiesis *in vitro* and *in vivo*. *In vitro*, the addition of exogenous IFNγ significantly inhibited myeloid and erythroid colony formation, in both murine and human systems(Maciejewski et al., 1995; Raefsky et al., 1985; Zoumbos et al., 1984; Zoumbos et al., 1985), and this suppressive effect was partially mediated through interferon regulatory factor-1(Sato et al., 1995). Furthermore, IFNγ-transgenic mice and various other models have shown that IFNγ over-expression leads to severe B-cell lineage reduction, T-cell lineage alteration, deficiency of NK cell development, decrease of colony formation of

myeloid progenitor cells, as well as the apoptosis of hematopoietic stem cells (Selleri et al., 1996; Shimozato et al., 2002; Young et al., 1997). However, some reports showed an opposite effect of IFNγ on hematopoiesis. It was reported that impaired Th1 development, due to the absence of STAT4, is associated with a great reduction in the absolute number of hematopoietic progenitor cells(Broxmeyer et al., 2002). In addition, IFNγ was observed to promote the expansion of the earliest $CD34^+$ hematopoietic precursors(Brugger et al., 1993), and it was capable of stimulating the early stage of myelopoiesis in the presence of some growth factors(Caux et al., 1992). Therefore, the effect of IFNγ probably differs depending on the stages and lineages of hematopoiesis.

In this study, we demonstrated that Th1 cell product-IFNγ indeed suppressed the colony forming capabilities of bone marrow cells; however, it can stimulate a significant expansion of a primitive $lineage^{low}Sca\text{-}1^+C\text{-}kit^+Flk\text{-}2^{low}Thy1.1^{low}$ hematopoietic stem/progenitor cells, through the acquisition of Sca-1 on $lineage^{low}Sca\text{-}1^-C\text{-}kit^+$ progenitor cells. We believe that this expansion of LSK cells may compensate for the loss of hematopoietic progenitors caused by IFNγ-exposure during infection and autoimmune disorders.

Materials and Methods

Animals.

C57BL/6 and *IFNγ-R1*$^{-/-}$ mice (*Ifngr1*^{tm1Agt}) were from Jackson Laboratory (Bar Harbor, ME). *Stat1*$^{-/-}$ mice in 129 background were from Taconic Farm (Germantown, NY). Animals were matched for age and gender in each experiment. All procedures were approved by the Institutional Animal Care and Use Committee.

Cell sorting.

A MoFlo XDP Cell Sorter (Beckman Coulter, Inc., Fullerton, CA) was used to isolate the lineage^{low} Sca-1^{+}C-kit^{+} and lineage^{low} Sca-1^{-}C-kit^{+} cells.

Colony forming unit (CFU) assays.

CFU assays were performed using MethoCult GF M3434 medium, according to the protocol from the manufacturer (STEMCELL Technologies, Seattle, WA).

Th1/Th2 conditioned medium

Th1 and Th2 cells were prepared as described previously(Devadas et al., 2006). The supernatant was collected from the 24h-anti-CD3 reactivated T cell blast (1X106 cells/ml) cultures.

Statistical Analysis

Significance was assessed by unpaired two-tailed Student's t test. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

Results

To test whether and how Th1/Th2 cell products affect the expansion of hematopoietic progenitor cells, we firstly subjected T cells to Th1 or Th2 polarization *in vitro*, and injected the Th1/Th2 conditioned medium into mice to mimic both Th1 and Th2 biased immune status *in vivo*, with undifferentiated Th0 cells as a control. When we evaluated the long-term hematopoietic stem cells in bone marrow, which are enriched in lineage^{low}Sca-1^{+}C-kit^{+} Flk-2^{low}Thy1.1^{low} (LSK) cells in control mice, we observed, to our surprise, that application of Th1 cell-conditioned medium, but not that of Th2 cells, led to a dramatic expansion of the LSK population, though at the expense of lineage^{low}Sca-1^{-}C-kit^{+} progenitor cells, *in vivo* and *in vitro* (**Figure 2**).

Th1 cells characteristically produce IFNγ while Th2 cells mainly produce IL-4, IL-5, and IL-10. This cytokine production pattern was confirmed by our cytokine multiplex assay (**Figure 3A**). Therefore, we speculated that IFNγ might play a role in stimulating the expansion of the LSK cells. To test this possibility, the Th1 conditioned medium was co-injected with neutralizing antibody to IFNγ. As shown in Figure 1, the stimulating effect of Th1 conditioned medium on LSK expansion *in vivo* was completely eliminated by anti-IFNγ, indicating a critical role of IFNγ in this process. LSK expansion *in vitro* was similarly blocked by the addition of anti-IFNγ (**Figure 2**).

To investigate if IFNγ can act alone, we injected rmIFNγ into the mice intraperitoneally, or added IFNγ into bone marrow cell cultures *in vitro*. The results showed that IFNγ alone had the same effect as the Th1 conditioned medium in inducing the LSK cell expansion. This effect was completely absent in *IFNγ R1*-deficient mice *in vivo* or bone marrow cell culture *in vitro* (**Figure 3B** and **3C**). Since one of the major signaling molecules of IFNγ is STAT-1, we also tested the effect of IFNγ on *Stat-1* deficient mice. As expected, the effect of IFNγ on LSK expansion was greatly, though not completely, reduced in *Stat1*-deficient mice or bone marrow cells, indicating that the LSK cell expansion stimulated by IFNγ is mediated through the IFN□R1-STAT1 signaling pathway (**Figure 3B** and **3C**). Moreover, other inflammatory cytokines including TNFα, IL-1β, IL-6, GM-CSF, and TGF□ failed to stimulate the LSK cell expansion (**Figure 4**), which is in contrast to a recent report about the role of TNFα and IL-6 in LSK cell expansion in a model of bacteremia(Zhang et al., 2008b). However, consistent with other two recent reports(Essers et al., 2009; Sato et al., 2009), type I IFNs, IFNα and IFNβ were able to significantly stimulate LSK cell expansion, *in vitro* and *in vivo* (**Figure 4 and data not shown**).

Next, we determined the origin of the LSK cells induced by IFNγ. The flow cytometry patterns (**Figure 3B**) suggested that the LSK cells might be derived from the conversion of lineage^{low}Sca-1^{-}C-kit^{+} cells. To test this, the

lineage^{low}Sca-1^{-}C-kit^{+} cells were sorted from the whole bone marrow cells from control C57BL/6 mice, and treated with IFNγ *in vitro* for 2 days. As indicated in **Figure 5A**, IFNγ treatment completely converted the lineage^{low}Sca-1^{-}C-kit^{+} cells to LSK cells. Interestingly, many of the *in vitro* converted LSK cells formed colonies (**Figure 5B**), indicating their strong proliferation potential.

To further characterize the proliferation potentials of LSK cells that are induced by IFNγ, we sorted LSK cells from both control and IFNγ-administrated mice and compared their DNA contents. We found that the LSK cells from IFNγ-treated mice had a greater proportion of cells in S+G2/M phases, indicating their more active status of proliferation (**Figure 6A**). Colony forming unit assay indicated that IFNγ treatment led to a reduction in the number of progenitor cells in bone marrow, which might be due to the egression of the progenitor cells to peripheral systems such as spleen (**Figure 6B**). However, we found that the LSK cells derived from IFNγ-administrated mice were able to yield more CFUs, when compared to control lineage^{low}Sca-1^{-}C-kit^{+} cells and control LSK cells (**Figure 6C**). Thus, the effect of IFNγ on bone marrow hematopoiesis is two-fold. Furthermore, we found that the LSK cells from IFNγ-treated mice had a greater potential to develop into common myeloid progenitors (CMPs) *in vitro* than control LSK cells (**Figure 7A**). Moreover, CD11b^{+}Gr-1^{+} myeloid cells were more commonly observed in the bone marrow cell cultures from IFNγ-treated mice. CD19^{+} B cells, on the other hand,

were less represented (**Figure 7B**). Together these results indicate that IFNγ-treatment can stimulate a differentiation program that favors the development of myeloid lineages.

In summary, we showed that under an acute Th1 inflammatory condition, the Th1 product IFNγ can stimulate the conversion of lineage^{low}Sca-1^{-}C-kit^{+} cells to LSK cells, resulting in the virtual expansion of LSK cells. IFNγ-induced LSK cells possess an enhanced proliferative capacity and are more likely to differentiate into myeloid lineages. The rapid emergence of these LSK cells possibly serves to compensate for the IFNγ-induced depletion of progenitor cells. Our findings on IFNγ, the type II interferon, are comparable to two recent studies on IFNα a type I interferon(Essers et al., 2009; Sato et al., 2009). Interestingly, another recent report showed that a cytokine/JAK/STAT pathway initiated from enterocytes is critical for intestinal stem cell division and differentiation in Drosophila(Jiang et al., 2009). Collectively, these findings all indicate that by initiating a cytokine signaling pathway, terminally differentiated cells，such as T cells, can act on stem cells or progenitor cells in a feedback mechanism to maintain cellular homeostasis and tissue function.

FIGURES AND LEGNEDS

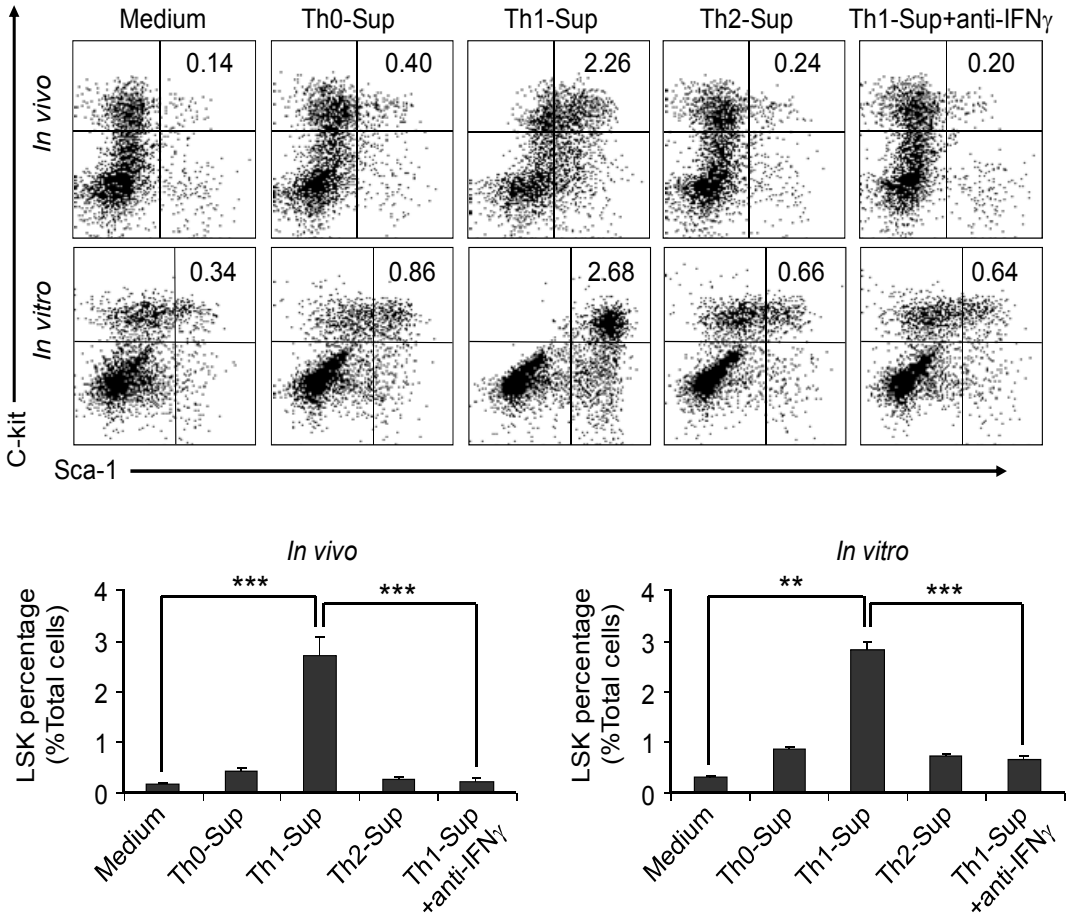

Figure 2. Th1, but not Th2, conditioned medium stimulated the expansion of LSK cells *in vivo* and *in vitro*. *In vivo*, 0.5 ml of conditioned medium produced by the same number of each T cell type was injected into C57BL/6 mice intraperitoneally daily for 2 days. *In vitro*, bone marrow cells from C57BL/6 mice were plated in 24-well plates at a concentration of 5 X 10^6 cells/ml, and were then treated with T cell conditioned medium (50% of the total volume) for 2 days. Anti-IFNγ was added at 50 μg/ml. The percentage of LSK cells in the total cells was assayed by flow cytometry (gated on $lineage^{low}$ $Flk\text{-}2^{low}$ $Thy1.1^{low}$ cells) and the number indicated the ratio in the total cells. The representative flow cytometry

patterns are shown (top) and the statistical data are means ± SD of three independent experiments (bottom). **, $p<0.01$ and ***, $p<0.001$.

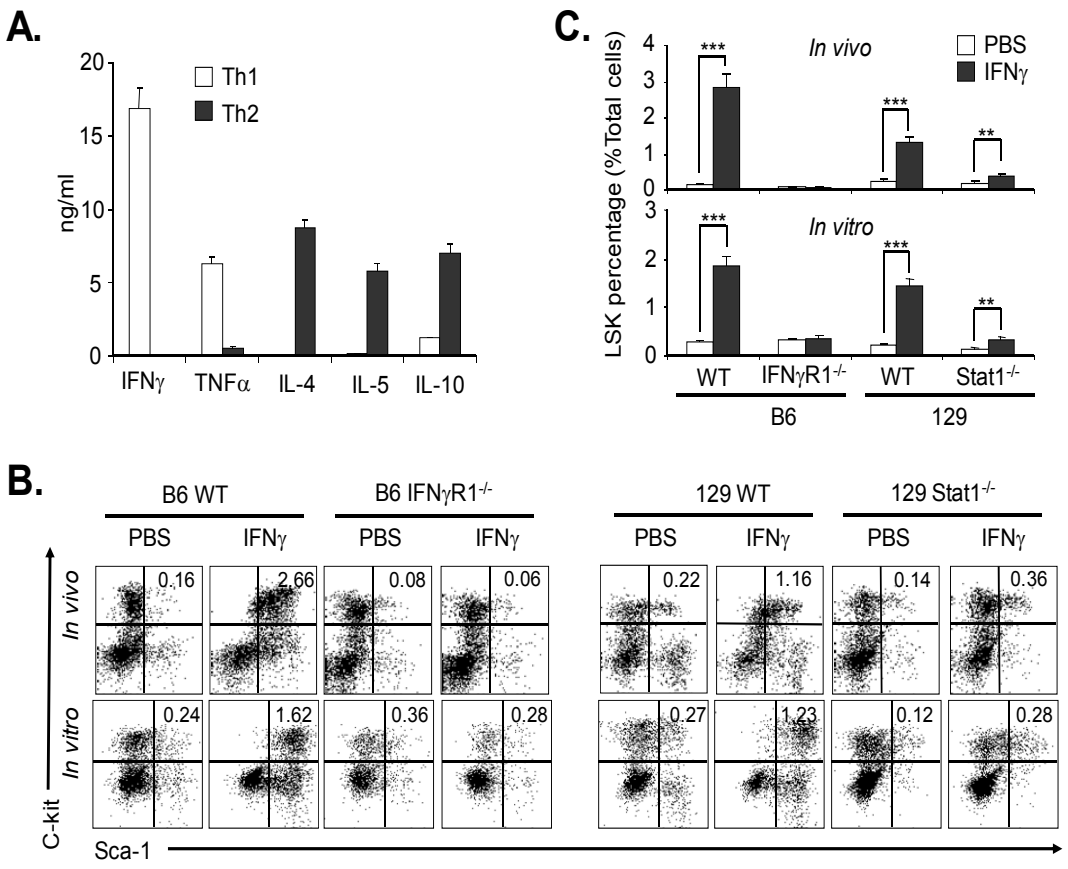

Figure 3. The IFNγ-induced LSK cell expansion is dependent on IFNγR1 and STAT1 signaling *in vivo* and *in vitro*.

(**A**). Cytokine production pattern in Th1/Th2 cells. Th1/Th2 cells were differentiated as previously reported. After 2d-resting in IL-2 containing medium, they were reactivated with plate-bound anti-CD3 for 24 hours, at a concentration of $1X10^6$ cells/ml. The cytokine production in the supernatants was assayed by multiplexed bead array immunoassay using Luminex Technology. Data shown are means ± SD of three independent experiments.

(**B**). Using the same protocol as in **Figure 2**, rmIFNγ was administrated at a doses of 1μg/mouse per day for two days, or added at a final concentration of 20 ng/ml, *in vivo* and *in vitro*, respectively. The representative flow cytometry patterns are

shown. .Statistical data are means ± SD of three independent experiments (**C**). **, $p<0.01$ and ***, $p<0.001$.

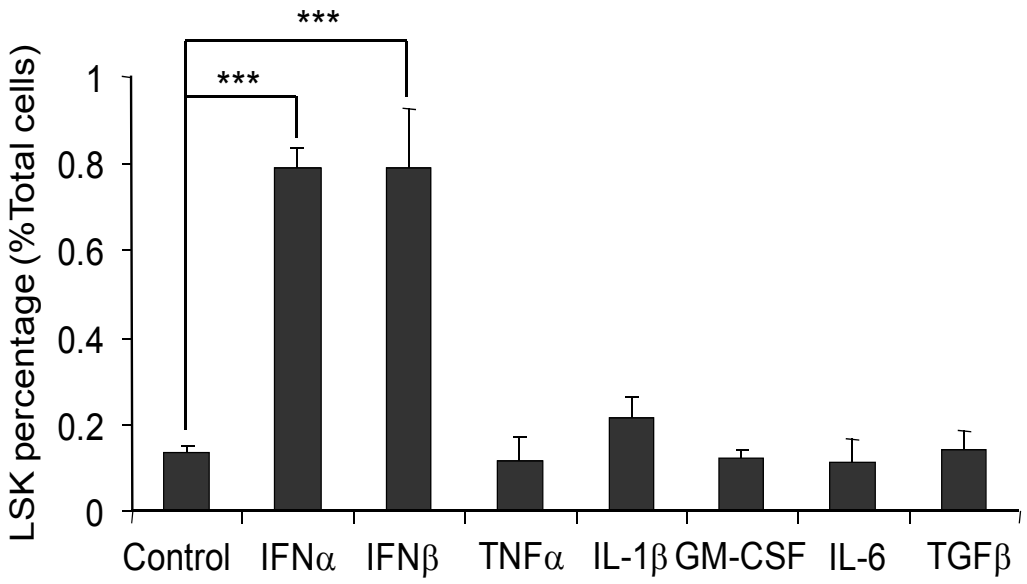

Figure 4. IFNα and IFNβ had a similar effect as IFNγ. Bone marrow cells from C57BL/6 mice were plated in 24-well plates at a concentration of $5X10^6$ cells/ml. Cytokines were then added to the cultures. 48 hours later, the LSK percentage in the total cells was determined by flow cytometry. The concentration of each cytokine: IFNα, and IFNβ: 500U/ml; TNFα, IL-1β, GM-CSF, and IL-6: 20ng/ml; TGFβ: 5ng/ml. Data shown are means ± SD of three independent experiments. ***, $p<0.001$.

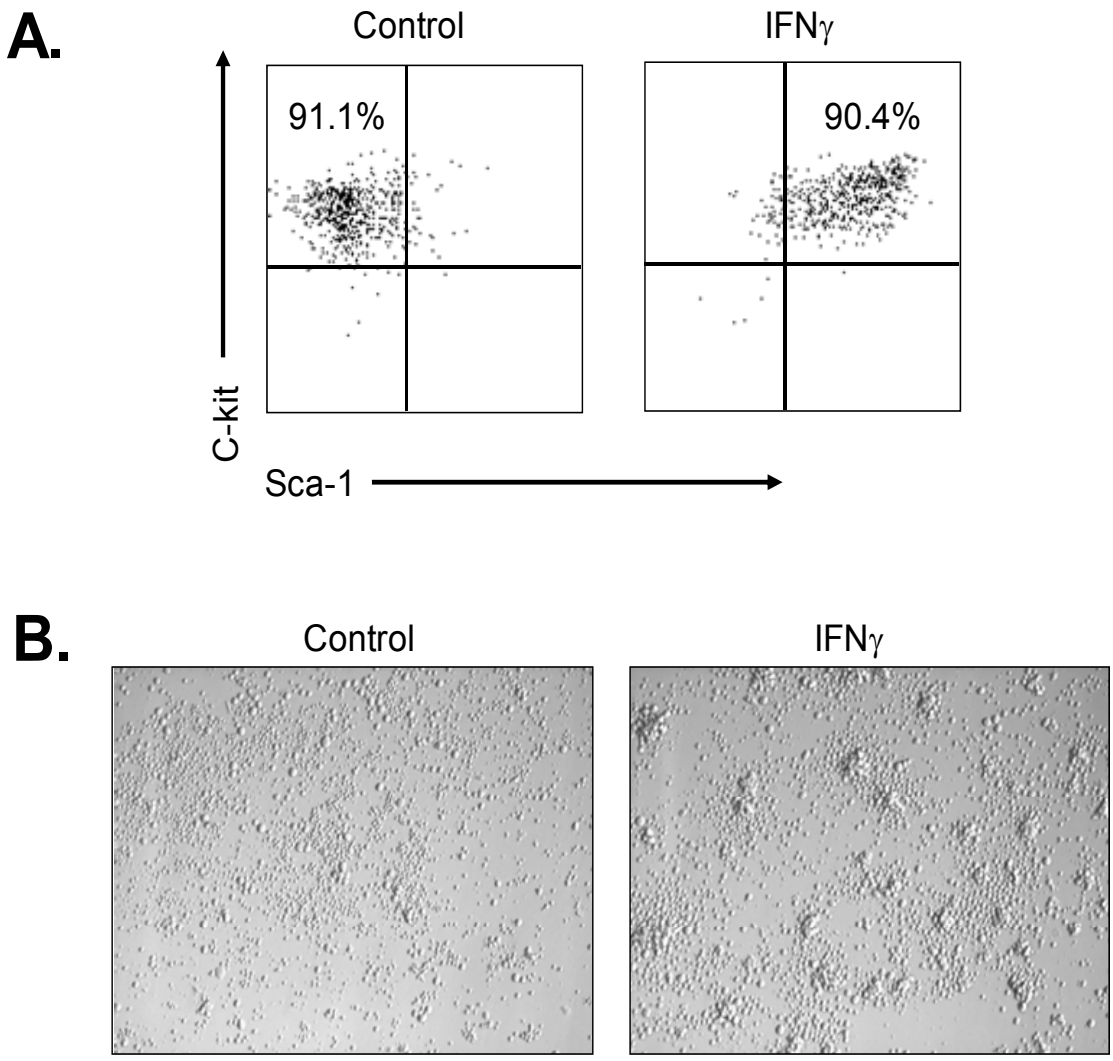

Figure 5. The IFNγ-induced LSK cells were converted from Lineage^{low}Sca-1^{-}C-kit^{+} cells. (**A**). Lineage^{low}Sca-1^{-}C-kit^{+} cells, sorted from C57BL/6 bone marrow cells, were cultured *in vitro* in the absence or presence of 20 ng/ml of rmIFNγ. Two days later, the percentage of LSK cells was assayed by flow cytometry. (**B**). Representative photographs of LSK cells in (**A**) were taken with light microscopy (magnification: 100 X). Data are representative of three independent experiments.

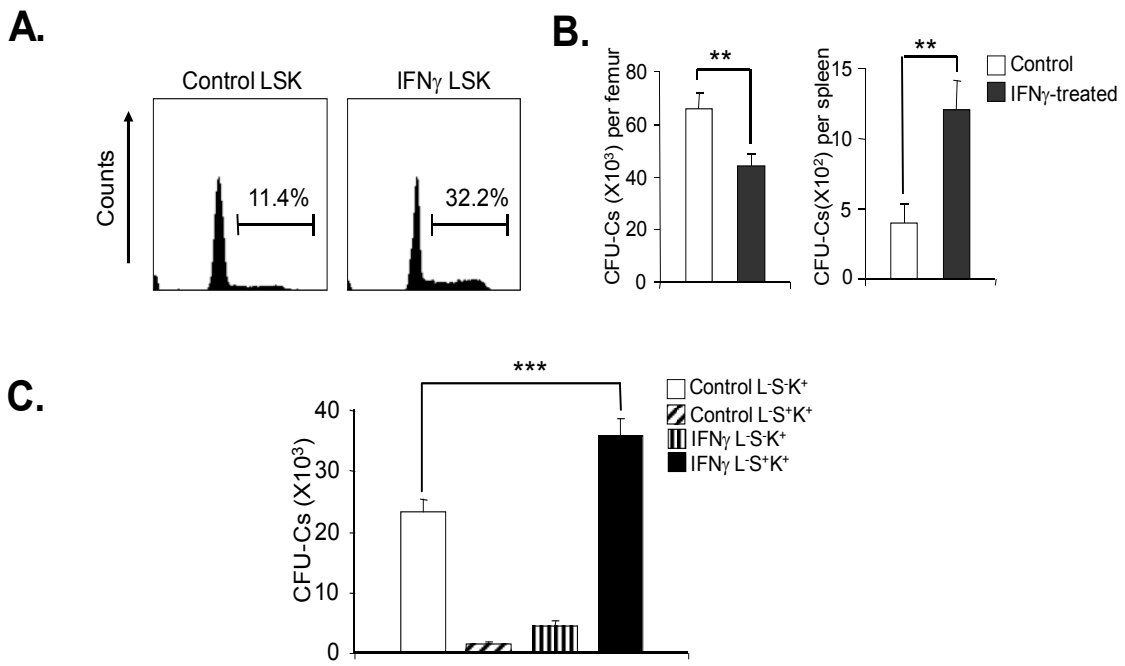

Figure 6. LSK cells induced by IFNγ had a greater proliferation potential. (**A**). LSK cells, sorted from C57BL/6 or IFNγ-administrated mice (1μg/mouse per day for 2 days), were analyzed for DNA contents by flow cytometry. The numbers indicated the percentages of cells in S+G2/M stages. (**B**). 2 X 10^4 bone marrow cells, or 1X10^6 splenic cells from control or IFNγ-administrated mice (1μg/mouse per day for 2 days) were subjected to colony forming unit assays. The total CFUs from femur and spleen were then calculated. (**C**). Lineage^{low}Sca-1$^+$C-kit$^+$ ($L^-S^+K^+$) cells and lineage^{low}Sca-1$^-$C-kit$^+$ ($L^-S^-K^+$) cells, sorted from control or IFNγ-administrated mice (1μg/mouse per day for 2 days), were subjected to CFU assays and the total CFUs in each group were then calculated. Data in (**A**) are representative of three independent experiments. Data in (**B**) and (**C**) are means ± SD, representative of three independent experiments. **, p<0.01 and ***, p<0.001.

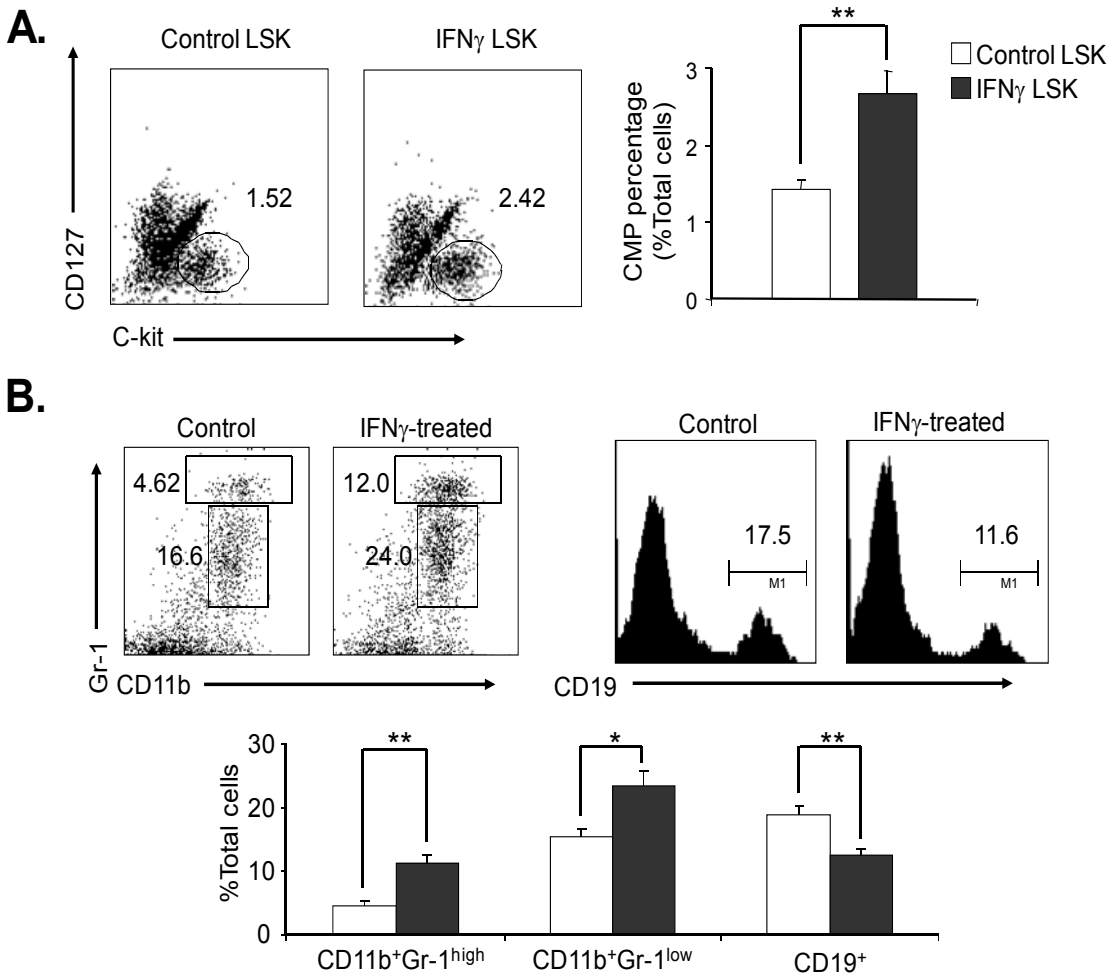

Figure 7. IFNγ treatment led to a preferential proliferation of myeloid cells. (**A**). The sorted LSK cells from control or IFNγ-administrated mice (1μg/mouse per day for 2 days) were cultured in MethoCult GF M3434 medium for 12 days. The percentage of CMP (Lineage^{low} Sca-1^{-}C-kit^{+}CD127^{-} population) was assayed by flow cytometry. (**B**) Bone marrow cells, from control C57BL/6 or IFNγ-treated mice (1μg/mouse per day for 2 days), were cultured in T-25 flask at a concentration of 2X10^{6} cells/ml *in vitro* for 6 days. The percentage of CD11b^{+}Gr-1^{+} myeloid cells and CD19^{+} B cells in the final cultures were tested by flow cytometry. The representative flow cytometry patterns are shown and the statistical data are

means ± SD which are representative of two independent experiments. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

CHAPTER III Tumor Burden Induces a Self-amplifying Loop of Hematopoiesis in Spleen

Abstract

Malignant tumors are frequently associated with hematological abnormalities that disrupt homeostasis. A shift to myelopoiesis, in particular, is conducive to tumor progression as a constant influx of myelomonocytic cells to tumor sites is required for angiogenesis and stroma remodeling of the tumors. In tumor-bearing mice, with the decline of hematopoiesis in bone marrow, spleens are greatly enlarged and harbor a greatly expanded population of hematopoietic progenitor cells (HPCs). While such HPCs can differentiate into both myeloid and lymphoid lineages when transplanted into tumor-free hosts, they preferentially give rise to myeloid lineages including macrophages/monocytes in tumor-bearing hosts. We further showed that macrophages/monocytes, derivatives of HPCs, are essential for the expansion of HPCs in spleen. Thus, HPCs and myeloid cells form a positive feedback loop in sustaining splenic myelopoiesis during tumorigenesis. This self-amplifying loop of HPCs and myeloid cells depends on an NFκB-Kit signaling cascade. Tumor-stimulated inflammatory factors are essential for the increased production of Kit ligands by macrophages/monocytes that drives splenic myelopoiesis. Targeting this HPC-myeloid loop may have potential in impeding tumor progression in cancer therapy.

Introduction

Hematopoietic stem cells (HSCs) give rise to all the blood cells that are required for oxygen transport, tissue homeostasis and immune defense. Lying between HSCs and the mature blood cells is a hierarchy of multipotent progenitors and committed precursors. Under steady physiological state, HSCs mainly reside in bone marrow and the absolute numbers and the frequencies of HSCs and their derivatives are relatively stable. However, in cases of injury, stress and other conditions, the distribution, frequency as well as the absolute number of stem cells, progenitor cells and different types of mature blood cells can change dramatically. For example, bacterial infection has been reported to greatly expand the hematopoietic stem cell and progenitor cell (HSPC) population in vivo(Zhang et al., 2008; Takizawa et al., 2011). In response to hematopoietic stimuli such as G-CSF, type I and type II interferon, HSPCs are mobilized and may undergo expansion (Essers et al., 2009; Sato et al., 2009; Zhao et al., 2010; Baldridge et al., 2010; Christopher et al., 2011). While acute and exogenous challenges can drastically change the dynamics of hematopoiesis, tumor development represents a special condition the host hematopoietic system also responds to. Many hematological abnormalities were documented in cancer patients, such as anemia (Spivak, 1994) and a shift to myelopoiesis (Wilcox, 2010; Spivak, 1994). Leukocytosis in cancer patients is correlated with poor prognosis (Shoenfeld et al., 1986; Donskov and von der, 2006; Mandrekar et al., 2006; Michael et al., 2006;

Schmidt et al., 2007). Hematological abnormalities were also well documented in experimental mice. Increased leukocytosis, especially in the spleen, is characteristic of tumor-bearing mice (Young et al., 1987). A particular set of myeloid cells that are characterized by presence of surface markers CD11b and Gr-1 are consistently found to be increased in mice bearing solid tumors, most notably in spleens (Kusmartsev and Gabrilovich, 2002; Bronte et al., 2000; Melani et al., 2003; Yang et al., 2004). Increased production of those cells was also observed in human patients (Almand et al., 2001; az-Montero et al., 2009; Hoechst et al., 2009). The elevation of those myeloid cells was not just a side product of cancer development, it was demonstrated to contribute to tumor progression by exerting immunosuppressive activity (Sica and Bronte, 2007; Youn et al., 2008; Gabrilovich et al., 2007; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Movahedi et al., 2008; Tu et al., 2008; Abe et al., 2009), thus also called MDSCs for myeloid-derived suppressor cells, by promoting tumor angiogenesis and metastasis (Yang et al., 2004; Yang et al., 2008; Hiratsuka et al., 2006; Hiratsuka et al., 2008; Murdoch et al., 2008).

Extramedullary hematopoiesis (EMH), the formation of hematopoietic tissues outside of bone marrow, often occurs in response to bone marrow failure (WYATT and SOMMERS, 1950). As the major sites of EMH, spleens are usually enlarged and harbor a higher proportion of hematopoietic progenitor cells (Walkley et al.,

2007b; Walkley et al., 2007a). Tumor-bearing mice characterstically show EMH (Young et al., 1987; Ilkovitch and Lopez, 2009). Spleens are recently shown to be the source of tumor-associated macrophages and neutrophils in tumor-bearing mice (Cortez-Retamozo et al., 2012), as well as that of the monocytes that infiltrate atherosclerotic lesions (Robbins et al., 2012) and myocardial infarction (Leuschner et al., 2012).

While these findings underscore the importance of myeloid cells in tumorigenesis and other pathological conditions, many questions remain. Are those myeloid cells associated with cancer derived from a population of progenitors that are committed to myeloid lineage? What are responsible for the accumulation/expansion of the progenitor cells in EMH during tumorigenesis? What are the signaling pathways invovled? Using mouse transplantation tumors as a model system, we determined the genesis of the cancer-associated hematopioetic progenitor cells and characterized the relationship between myeloid cells and HSPCs in the spleen of tumor-bearing mice. We showed that HPCs, which are greatly expanded in the spleens of tumor-bearing mice, primarily give rise to myeloid lineages, including monocyte/macrophages, in tumor-bearing hosts. The monocytic derivatives of HPCs, in return, are responsible for the expansion of HPCs in spleen. Thus, HPCs and their myeloid derivatives form a self-amplifying loop in driving splenic myelopoiesis.

Materials and Methods

Animals. C57BL/6, *p53*+/-, *Gld/gld*, GFP-transgenic, CD11b-DTR and C-kit$^{+/-}$ mice were obtained from the Jackson Laboratory. *Mlh1*$^{-/-}$ mice were as described previously(Shao et al., 2004). Mice were housed in a specific pathogen-free colony in the Vivarium of Rutgers University. The animal protocols for the experiments described in this manuscript were approved by the Institutional Animal Care and Use Committee of Rutgers University. The mice were matched for age and gender in each experiment.

Antibodies and Reagents. Fluorochrome-conjugated antibodies against CD45.1, C-kit, Sca-1, CD11b, Ly6G, Ly6C, F4/80, CD11c, CD206, IL-7R□, IgM, B220, CD115, CD274, CD124, CD80 were purchased from eBioscience (La Jolla, CA) or BD Biosceince (San Diego, CA). The biotin-conjugated mouse lineage panel was from BD Biosceince (San Diego, CA). Antibodies against F4/80 and C-kit for immunohistochemical analysis were obtained from Abcam (Cambridge, MA). Ly6G neutralizing antibody was obtained from Biolegend (San Diego, CA). Diphtheria toxin and anti-SCF were purchased from Sigma (St. Louis, MO). Bay 11-7082 and Sunitinib were from EMD Chemicals (Billerica, MA) and Biovision (Milpitas, CA), respectively. Mouse lineage cell depletion kit and mouse MDSC isolation kit were from Miltenyi Biotec (Auburn, CA). Mouse SCF ELISA kit was from R & D systems (Minneapolis, MN).

Syngeneic tumor transplantation. EL4 cells or LLC1 cells ($1X10^6$ cells per mouse) were subcutaneously administered on day 0. Twelve days later, the mice were sacrificed for tumor weight measurement. Various tissues were collected for single cell preparation for flow cytometry analysis. For the treatment with C-kit inhibitor, sunitinib (50mg/kg in 0.5% methyl cellulose) was administered i.p daily from day 5-11 post tumor cell inoculation. The vehicle (0.5% methyl cellulose) was set as a control. For the treatment with NF□B inhibitor, Bay 11-7082 (10mg/kg in 1% DMSO-containing 0.5% methyl cellulose) was i.p injected on days 3, 5, 8 and 10 post tumor cell inoculation. The vehicle (1% DMSO-containing 0.5% methyl cellulose) was set as a control. For neutralization of SCF, anti-SCF (100□g per mouse) was i.p injected on days 5, 7, 9 and 11post tumor cell inoculation. Each experimental group included at least four mice. All experiments were replicated at least three times.

Depeltion of $CD11b^+Ly6G^+$ granulocytic MDSCs and macrophages/monocytes. To deplete $CD11b^+Ly6G^+$ granulocytic MDSCs, Ly6G neutralizing Ab 1A8 (150 μg per mouse) was administered i.p on days 4, 7 and 10 post-EL4 inoculation. To deplete macrophages/monocytes, diphtheria toxin (DT, 5 ng/g) or buffer (1% BSA in sterile water) was administered i.p. on day 4, 7 and 10 post-EL4 inoculation. The depletion by each treatment on different myeloid cell populations was verified by flow cytometry.

Immunofluorescence staining and flow cytometry. For cell surface marker analysis, cells were suspended in staining buffer (PBS, 2% FBS) at a concentration of 2 x 10^6 cells/ml and 100 μl of suspension was incubated with fluorescently-labeled antibodies for 30 min on ice. Cells were washed twice with staining buffer. Fluorescence intensity was measured by flow cytometry (FACSCalibur, BD Immunocytometry, San Jose, CA).

Colony forming unit (CFU) assays. CFU assays were performed using MethoCult GF M3434 medium, according to the protocol from the manufacturer (STEMCELL Technologies, Seattle, WA).

Real-time PCR. Total RNA was isolated from cell pellets or minced tissues using an RNeasy Mini Kit. Genomic DNA was removed using the RNase-Free DNase Set for DNA digestion during RNA purification. First-strand cDNA synthesis was performed using Sensiscript RT Kit with random hexamer primers (all kits from Qiagen, Valencia, CA). Levels of mRNA of genes of interest were quantitated by real-time PCR (ABI 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA) using SYBR Green Master Mix (Applied Biosystems). Thermocycling included an initial incubation at 50°C for 2 min, then 95°C for 10 min, followed by a 2-step PCR program of 95°C for 15 sec and 60°C for 60 sec, for a total of 40 cycles. The total amount of mRNA was normalized among samples

according to endogenous β-actin mRNA.

Histological analysis. Spleens from every experimental group were undergone hematoxylin/eosin (H&E) staining to display the splenic cell distribution. For immunohistochemical staining of F4/80 and C-kit, spleens were washed thoroughly in PBS and fixed in 10% PBS-buffered formalin. Tissues were embedded in paraffin, and 5 to 6 μm sections were cut, deparaffinized, and stained with indicated Abs according to the protocol provided by the manufacturer.

Statistical Analysis. Statistical significance was assessed by unpaired two-tailed Student's t test: *$p<0.05$; **$p<0.01$, ***$p<0.001$.

Results

Tumor burden induces the expansion of hematopoietic progenitor/stem cells in spleens of murine tumor models

Mice bearing lymphoma characteristically manifest splenomegaly (**Figure 8A**). In addition to having increased cellularity (**Figure 8B**), the enlarged spleens are generally disorganized in their structure, with indistinguishable demarcation between red pulps and white pulps (**Figure 8C**). This phenomenon applies to primary lymphomas as well as transplanted lymphoma models. To determine whether the enlarged spleens in those conditions serve as the sites of extramedullary hematopoiesis (EMH), we first quantified the populations of hematopoietic progenitor cells (HPCs), which are marked by their C-kit^{+}Lin^{-} phenotype, in various hematopoietic sites in mice bearing transplanted EL-4 lymphoma cells. We found that the C-kit^{+}Lin^{-} HPCs were greatly expanded in spleens, but not in bone marrow (BM) and peripheral blood (PB) (**Figure 9**). This expansion of HPCs also occurs in spleens of genetically mutated mice such as *p53*$^{+/-}$, *Mlh1*$^{-/-}$ and *gld* mice bearing spontaneously developed lymphomas (**Figure 10**). The frequency of HPCs in bone marrow, on the other hand, generally declined (**Figure 9** and **Fingure10**).

The increase in the frequency of HPCs in spleen, when combined with increased splenic cellularity, suggested that spleens function as the sites of EMH. This

notion was supported by in vitro CFU-C assay. The number of CFU-Cs in spleens was increased about 4 fold in tumor-bearing mice over that in control mice (**Figure 11A**). Importantly, the HPCs isolated from spleens of lymphoma-bearing mice were equally capable of rescuing lethally-irradiated mice and exhibited a differentiation potential similar to that of control HPCs isolated from normal mice (**Figure 12**). The number of CFU-Cs in BM, on the other hand, was significantly decreased (**Figure 11B**).

We further analyzed the changes in the population of more primitive $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ (LSK) cells, and in the populations of more differentiated common lymphoid progenitor (CLP, $Lin^{-}IL\text{-}7R\alpha^{+}Sca1^{low}C\text{-}kit^{low}$) and common myeloid progenitor (CMP, $Lin^{-}Sca\text{-}1^{-}C\text{-}kit^{+}IL\text{-}7R\alpha^{-}$) cells. Like HPCs, the LSK cells were also greatly expanded in the spleens of mice bearing lymphoma (**Figure 13**, EL4 as an example). While CLPs only showed a very mild expansion (**Figure 14**, EL4 as an example), the population of CMPs dramatically expanded in spleen of lymphoma-bearing mice (**Figure 15**, EL4 as an example).

Changes in the frequency of HPCs in various hematopoietic organs (**Figure 9**, EL4 as an example) clearly showed that hematopoiesis was not universally increased in tumor-bearing mice. There arises the question whether the expansion of progenitor cells in spleens was due to an increased emigration of

progenitor cells from BM. Alternatively, the surge in HPCs could result from in situ expansion of local progenitor cells in spleen. We therefore determined the frequency of HPCs in PB of tumor-bearing mice at different time points post tumor transplantation. However, there was no significant increase in the frequency of HPCs at all time points tested (**Figure 9** and **Figure 16**), suggesting that the expansion of HPCs in spleens was probably not due to an increased influx of HPCs from bone marrow.

HPC differentiation is biased toward myeloid lineages in the presence of tumor

When transplanted into recipient mice, HPCs could significantly enhance the growth of transplanted (s.c.) EL-4 tumors (**Figure 17**), suggesting that the expansion of HPCs serves to promote tumor progression. To gain insight into how HPCs contribute to tumor progression, we determined the fate of HPCs in tumor-free and tumor-bearing hosts, respectively. The donor HPCs were also prepared from tumor-free (control HPCs) and tumor-bearing donors (expanded HPCs), respectively (**Figure 18**). We used CD45.1 mice as donors of HPCs and CD45.2 mice as recipients and tracked the differentiation of infused HPCs in recipients. We observed that the percentage of CD45.1-expressing cells was more than 4-fold higher in PB of tumor-bearing recipients than in that of tumor-free mice (**Figure 19A and 19B**). In the spleen, the contribution by

donor-derived CD45.1 cells was 10-fold higher in tumor-bearing recipients than in tumor-free mice (**Figure 19B**). Bone marrow of tumor-bearing mice also harbors a higher proportion of donor-derived cells than that of tumor-free mice. These results suggest that the tumor-bearing mice were more receptive to the colonization by the donor HPCs. Importantly, the higher capacity to accommodate donor HPCs exhibited by tumor-bearing mice was not restricted to HPCs isolated from tumor-bearing mice. HPCs from tumor-free donors were similarly more tolerated in tumor-bearing mice than in normal mice (**Figure 19B**). Thus, it is the tumor-bearing status that enlarged the extent to which the donor cells, including their derivatives, contributed to the pools of hematopoietic cells.

We tested whether tumor-bearing status allowed more engraftment of donor HPCs by tracking transplanted GFP-HPCs in tumor-free and tumor-bearing recipients, respectively. Two days after iv infusion, there was a significant increase in the percentage of GFP-positive cells in tumor-bearing recipients compared to tumor-free recipients. This only occurred in spleen, but not PB and BM, indicating that the increase in engraftment by donor HPCs is tissue-dependent (**Figure 20A**-flow data and **Figure 20B**-statastical analysis). Therefore, spleens in tumor-bearing mice allowed more engraftment of the donor HPCs than those in tumor-free mice. However, it is also possible that donor HPCs undergo more active cell division and/or differentiation into the mature cells than

the host HPCs in tumor-bearing mice, thus contributing a larger than expected share to the pool of mature cells.

Furthermore, we examined the distribution of the derivative subsets of donor HPCs in the recipients. Strikingly, HPCs in the spleens of tumor-bearing mice primarily gave rise to myeloid lineages, such as $CD11b^+Ly6G^+$, $CD11b^+Ly6C^+$ and $F4/80^+$ cells (**Figure 21A**-flow cytometry data and **Figure 21B**-statistical analysis), in tumor-bearing recipients. The population of IgM^-B220^+ and IgM^+B220^+ lymphoid cells, on the other hand, was reduced more than 6-fold when compared to tumor-free control (**Figure 21A**-flow cytometry data and **Figure 21B**-statistical analysis). These results indicate that tumor-bearing status drives the preferential differentiation of HPCs toward myeloid lineages. Importantly, when transplanted into tumor-free hosts, HPCs from tumor-bearing donors could undergo a normal differentiation program and were capable of forming the lymphoid lineages (**Figure 21A** and **21B**), suggesting that HPCs in tumor-bearing mice are not intrinsically committed toward myeloid differentiation. Therefore, HPCs usually have a high degree of plasticity in their differentiation potential and may take cues from their environment in choosing their route of differentiation.

Because the population of HPCs is usually expanded in spleen in tumor-bearing mice, it is possible that property of splenic HPCs demonstrated above is restricted

to the HPCs in spleen. To rule out this, we prepared HPCs from bone marrow of healthy mice and repeated the same experiments as with splenic HPCs. As shown in **Figure 22A** and **22B**, BM-derived HPCs had a similar pattern as splenic HPCs in differentiation bias toward myeloid lineages in tumor-bearing mice.

The preferential differentiation of HPCs toward myeloid lineages in tumor-bearing mice may explain why HPCs could promote tumor progression, since cells of myeloid lineages, such $CD11b^+Gr\text{-}1^+$ MDSCs (including $CD11b^+Ly6G^+$ granulocytic MDSCs and $CD11b^+Ly6C^+$ monocytic MDSCs) and $F4/80^+$ macrophages, have been well documented to contribute to tumor-related immunosuppression and angiogenesis(Gabrilovich and Nagaraj, 2009; Murdoch et al., 2008; Qian and Pollard, 2010). We next asked whether the $CD11b^+Gr\text{-}1^+$ cells (putative MDSCs) derived from donor splenic HPCs were similar to the MDSCs directly isolated from spleens of EL4-bearing mice. We compared the phenotypes and immunosuppression potential between the MDSCs of different origins and found that donor HPC-derived $CD11b^+Gr\text{-}1^+$ cells are phenotypically and functionally indistinguishable from MDSCs (**Figure 23A and 23B**).

Macrophages/monocytes are required for the expansion of HPCs

Analysis of the immune cells in spleen showed that the populations of $CD11b^+Gr\text{-}1^+$ MDSCs (both $CD11b^+Ly6G^+$ granulocytic MDSCs and

CD11b$^+$Ly6C$^+$ monocytic MDSCs) and F4/80$^+$ macrophages are expanded to the greatest extent in tumor-bearing mice (**Figure 24**-flow cytometry data and **Figure 25**-F4/80 immunohistological staining). These results were not surprising because, as shown above, tumor drives HPCs to preferentially differentiate toward myeloid lineages, especially the subsets of CD11b$^+$Gr-1$^+$ MDSCs and F4/80$^+$ macrophages. The surge of HPCs in spleen of tumor-bearing mice and the preferential differentiation of HPCs toward myeloid cells prompted us to speculate that the HPCs and myeloid cells might form a positive feedback loop in driving the splenic myelopoiesis in supporting tumor progression.

We first tested the role of CD11b$^+$Ly6G$^+$ granulocytic MDSCs in the expansion of HPCs. By applying anti-Gr-1 neutralizing Ab 1A8, the CD11b$^+$Ly6G$^+$ cells were efficiently removed in spleen, but the CD11b$^+$Ly6C$^+$ and F4/80$^+$ cells remained unaltered (**Figure 26**). The same depletion effect was also achieved in PB and tumor tissues (**data not shown**). However, such depletion did not alter the HPC and CMP frequency (**Figure 27**). Moreover, the high cellularity and distortion of structure in spleen of tumor-bearing mice were not affected by the depletion of granulocytic MDSCs (**data not shown**). These results suggest that while greatly expanded, CD11b$^+$Ly6G$^+$ MDSCs are not directly involved in promoting the expansion of HPCs.

We then tested the role of macrophages/monocytes by using CD11b-DTR mice, in which the macrophages and monocytes can be depleted by ip injection of diphtheria toxin (DT)(Duffield et al., 2005). Indeed, administration of DT effectively removed F4/80^{+} macrophages and CD11b^{+}Ly6C^{+} monocytic MDSCs in spleen and PB (**Figure 28 and Figure 29**). Interestingly, DT was ineffective in reducing the percentage of the CD11b^{+}Ly6G^{+} cells (**Figure 28 and Figure 29**), consistent with a previous report (Qian et al., 2009). The depletion of macrophages/monocytes remarkably suppressed the tumor-induced splenic hematopoiesis, as shown by the reduction in spleen size (**Figure 30A**), the decrease in total spleen cell number (**Figure 30B**), and restoration of normal spleen structure (**Figure 30C**). Furthermore, the frequency and total cell number of HPCs and CMPs were greatly reduced by macrophage/monocyte depletion (**Figure 31A-B and Figure 32**). Importantly, the number of CFUs was reduced more than 5-fold by macrophage depletion (**Figure 31C**). Consistent with the expectation of HPCs playing a tumor-promoting role, suppression of splenic hematopoiesis in these CD11b-DTR mice was associated with significant inhibition of tumor progression (**Figure 33**).

We performed immunohistological staining of F4/80 and C-kit, marking macrophages and HPCs, respectively, to gain insight into their possible physical association in situ. We observed that the C-kit expressing cells are distributed in

the same regions as the macrophages (**Figure 34**), lending support to the idea that macrophages may form niche-like structures in promoting the expansion of HPCs in situ. The details of the possible interaction between those two types of cells remain to be elucidated.

Taken together, our results indicate that HPCs were induced to undergo preferential differentiation into myeloid cells in the presence of tumor, and that certain subsets of HPC derivatives in turn contribute to the further expansion of HPCs in the spleen, thus forming a self-amplifying loop of myelopoiesis.

Splenic macrophages/monocytes produce more Kit ligands

Stem cell factor (SCF, c-kit ligand) promotes the engraftment and expansion of HPCs (Toksoz et al., 1992; Okumura et al., 1996; Driessen et al., 2003). It is also required for tumor-induced expansion of MDSCs (Pan et al., 2008). Moreover, SCF was reported to be a key factor in destroying the BM environment and BM hematopoiesis in leukemic mice (Colmone et al., 2008). We therefore followed the kinetics of SCF expression in tumor-bearing mice for 13 days after tumor transplantation. SCF expression in spleen was found to reach a peak at day 7, the middle point of tumor growth observation period, and then to descend. At day 13, it was even lower than at initiation (**Figure 35**). Such SCF expression pattern was only observed in spleen. The SCF level in sera did not change at all time points

tested (**data not shown**). This spleen-specific change in SCF expression correlates with the enhanced splenic hematopoiesis in tumor-bearing mice.

We next determined whether the macrophages/monocytes are the source of SCF production. Macrophages/monocytes can be separated from other splenic cells by their adherence to culture substratum. The adherent stromal cells in such cultures (**Figure 36A**) were indeed mostly F4/80$^+$ and CD11b$^+$ macrophage-like cells (**Figure 37**). When floating cells and adherent cells are respectively measured for SCF expression, the expression of SCF was only detected in the adherent CD11b$^+$F4/80$^+$ cells (**Figure 36B**), suggesting that macrophages/monocytes may contribute to the increased production of SCF in vivo. Furthermore, and importantly, in tumor-bearing CD11b-DTR mice, depletion of macrophages/monocytes by DT administration completely abrogated the upregulation of SCF in spleens in vivo (**Figure 38**). These results demonstrate that macrophages/monocytes are responsible for the increased production of SCF in spleens of tumor-bearing mice.

Inhibition of c-kit signaling disrupts the self-amplifying loop of HPCs and myeloid cells

Tyrosine kinase C-kit is the receptor for SCF and C-kit-SCF signaling have been reported essential for hematopoiesis. We therefore speculated that inhibition of

c-kit signaling may disrupt the HPC-myeloid cell loop. Indeed, C-kit is one of the most up-regulated molecules in spleen (**Figure 9A**). To test whether C-kit signaling pathway is essential for splenic hematopoiesis, we firstly treated tumor-bearing mice with C-kit inhibitor sunitinib. Remarkably, sunitinib treatment completely restored the size, cellularity and the structure of spleen to their normal status (**Figure 39A-C**). As expected, HPC-macrophage loop collapsed, with the percentages of HPCs, CMPs, F4/80^{+} and CD11b^{+}Ly6C^{+} cells being reduced to their normal levels (**Figure 40A-C and Figure 41A-C**). However, the percentage of CD11b^{+}Ly6G^{+} granulocytic MDSCs in spleen was significantly increased (**Figure 41D**), further indicating that granulocytic MDSCs were not directly involved in splenic hematopoiesis. As a consequence of sunitinib treatment, the number of CFU-Cs in spleens of tumor-bearing mice was reduced more than 3-fold, and the tumor progression was significantly suppressed (**Figure 40D and 40E**).

Considering potential off target effects of sunitinib, we further tested the role of C-kit signaling pathway in splenic hematopoiesis by performing the same experiments on C-kit$^{+/-}$ mice and by applying neutralizing Ab against SCF to tumor-bearing mice. The number of CFUs per spleen and the splenic HPC frequency in tumor-bearing C-kit$^{+/-}$ mice were significantly reduced when compared to wild-type mice (**Figure 42 and Figure 43**). Similarly, the HPC and

CMP frequency and the total number of CFUs were significantly reduced in spleens of tumor-bearing mice treated with anti-SCF (**Figure 44 and Figure 45**).

NFκB pathway is critical for HPC-myeloid cell expansion loop.

Cancer cells are known to be capable of inducing an inflammatory microenvironment, consisting of inflammatory cells, cytokines and chemokines, that promotes the progression of cancer (Mantovani et al., 2008; Grivennikov et al., 2010). We therefore speculated that the upregulation of C-kit signaling pathway and splenic hematopoiesis that are associated with tumor burden might have been the consequence of the tumor-induced inflammatory response. We determined the expression profile of 20 cytokines/chemokines in tumor-bearing mice sera at different time points after tumor transplantation. Among them, the inflammatory cytokines IFNγ, TNFα, IL-1α, IL-1β,IL-6 and CCL-2 were found to be significantly upregulated, especially between 6-9 days after tumor inoculation (**Figure 46**). We previously showed that IFNγ can stimulate the expansion of HSPCs in vivo and drives their differentiation toward myeloid lineage(Zhao et al., 2010). We therefore tested whether IFNγ signaling was required for splenic myelopoiesis by inoculating EL-4 cells into syngeneic B6 IFNγR1$^{-/-}$ mice and analyzed the profiles of the hematopoietic cells. However, the IFNγR1$^{-/-}$ mice showed no difference from the wild type mice (data not shown), suggesting that IFNγ signaling was not essential for tumor-induced splenic myelopoiesis. We also

conducted similar experiments with B6 TNFα$^{-/-}$ mice, splenic myelopoiesis was again similar to that in wild type (data not shown).

Of particular note is that many of upregulated cytokines act upstream or downstream of NF-κB signaling pathway, as would be expected of the current assertion that NF-κB acts as a key mediator of cancer-related inflammation (Grivennikov and Karin, 2010). We next tested whether inhibition of NFκB could attenuate the splenic hematopoiesis in tumor-bearing mice. Interestingly, application of NFκB inhibitor Bay 11-7082, significantly reversed the tumor-induced hematopoiesis in spleen. The spleen size and cellularity were reduced to normal level (**Figure 47A and 47B**) and spleen structure was partially restored in tumor-bearing mice treated with Bay 11-7082 (**Figure 47C**). Consistently, the frequencies of HPCs and CMPs, as well as the total number of CFUs, were significantly reduced in spleens of those mice (**Figure 48A-C**). Lineage analysis by flow cytometry showed that Bay 11-7082 only significantly reduced the frequency of F4/80^{+} macrophages (**Figure 48D**), but not that of CD11b^{+}Ly6G^{+} cells (**Figure 48E**). These results further demonstrated that the expansion of HPCs in spleen is more closely associated with F/80^{+} macrophage-like cells than with Ly6G^{+} MDSCs. It is unclear why the populations of Ly6G^{+}MDSCs and F4/80^{+} macrophages were differentially affected by the inhibition of NFkB. Moreover, Bay 11-7082 also significantly inhibited tumor

growth (**Figure 48F**).

The data shown above indicated that NFκB signaling plays a critical role in the HPC-myeloid cell expansion loop. We speculated that NFκB might act upstream of C-kit signaling in inducing EMH. We next tested whether the upregulation of SCF was mediated by NFκB. Indeed, the in vivo SCF up-regulation in spleens of tumor-bearing mice could be greatly reduced by administration of Bay 11-7082, at both mRNA and protein levels, suggesting that NFκB is required for SCF expression (**Figure 49**). In vitro, cultured whole splenic cells from tumor-bearing mice were found to produce SCF in an NFκB-dependent manner (**Figure 50**). As expected, the adherent stromal cells in such cultures (**Figure 36A**), which are mostly F4/80^{+} and CD11b^{+} macrophage-like cells (**Figure 37**), also produce SCF in an NFκB-dependent manner (**Figure 51**). Together, these results implicate NFκB signaling as the driving force in tumor-induced splenic myelopoiesis.

The HPC-myeloid cell expansion loop is not restricted to hematological malignancy

Splenomegaly is characteristic of lymphoma. It is possible that splenic hematopoiesis and the mechanism whereby it is formed in lymphoma model do not apply to tumors of other tissue type. We therefore employed a carcinoma mouse model, by transplantation of mouse LLC1 lung cancer line, to test whether

the findings made with lymphoma are only restricted to lymphoma model. Examination of the fates of donor HPCs in LLC1-bearing host showed that, as in lymphoma model, LLC1-bearing mice were also more accommodating to the donor HPCs than control recipients, as reflected by a higher frequency of donor-derived CD45.1+ cells (**Figure 52A**) and by their biased differentiation toward myeloid lineage (**Figure 52B**).

We demonstrated in the lymphoma model that macrophages/monocytes are required for splenic hematopoiesis, as described above. Similarly, depletion of macrophages in LLC1-bearing CD11b-DTR mice by DT remarkably attenuated tumor-induced splenic hematopoiesis, leading to normal spleen size (**Figure 53A),** restoration of spleen structure **(Figure 53B)** and decrease in the frequency of HPCs (**Figure 53C**). Furthermore, inhibition of C-kit and NFκB signaling pathways greatly reduced splenic hematopoiesis in LLC1-bearing mice, again implicating the role of these pathways in HPC-myeloid cell expansion loop (**Figure 54 and Figure 55**). These results suggest that splenic hematopoiesis associated with tumorigenesis probably operate by a similar mechanism in different types of tumors.

Figures and Legends

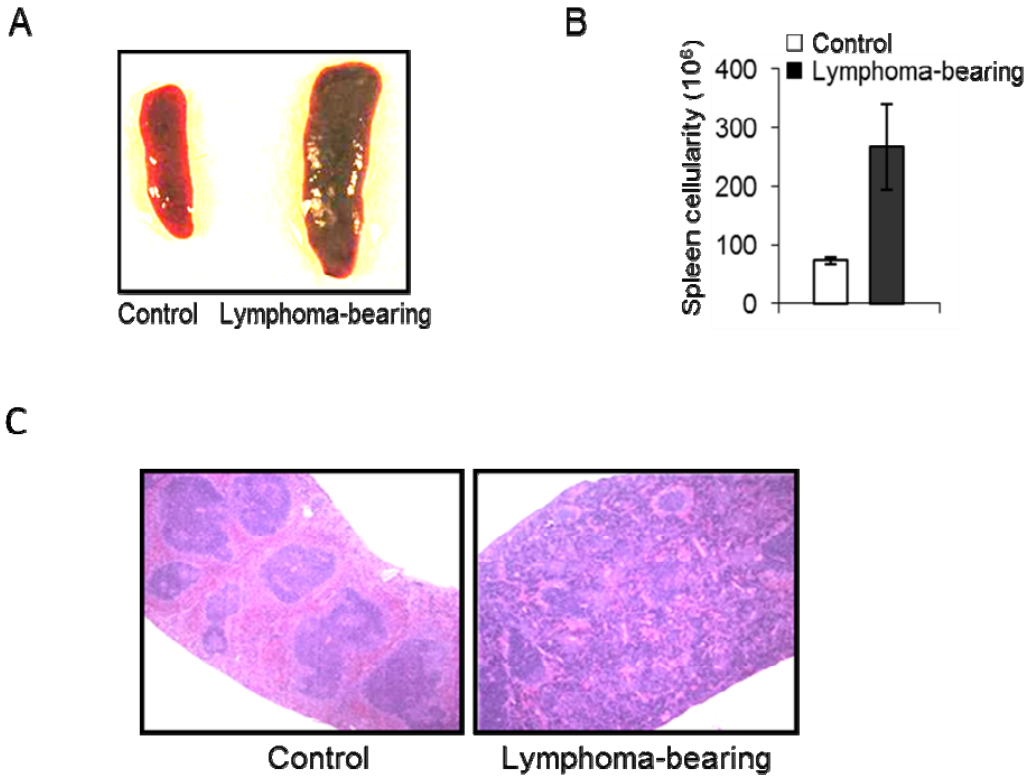

Figure 8. Lymphoma burden induces spleen enlargement

(A). Lymphoma-bearing p53$^{+/-}$ mice exhibit enlarged spleen

(B). Tumor burden increases spleen cellularity (total splenic leukocyte number)

(C) Distorted spleen histology by H&E staining.

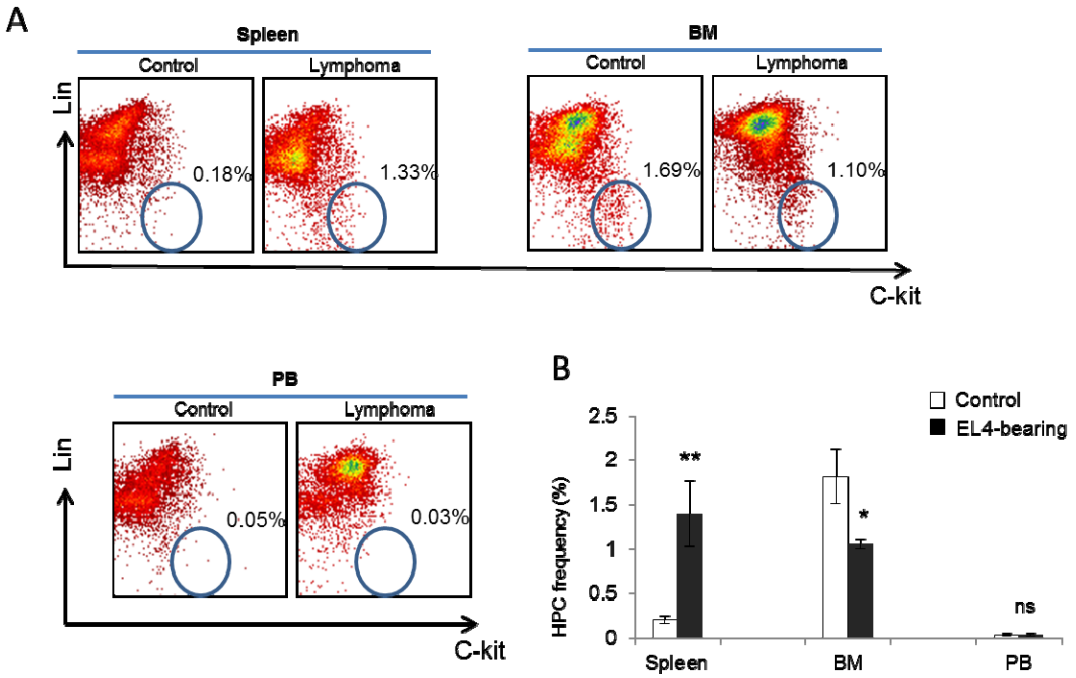

Figure 9. Lymphoma burden induces the expansion of hematopoietic progenitor/stem cells in spleens

(A). Representative flow cytometry scatter plot showing the frequency of C-kit^{+}Lin^{-} HPCs in spleen, BM and PB of EL4-bearing mice.

(B). Statistical summary of panel **A**: HPC frequency in spleen, BM and PB of EL4-bearing mice. n=4.

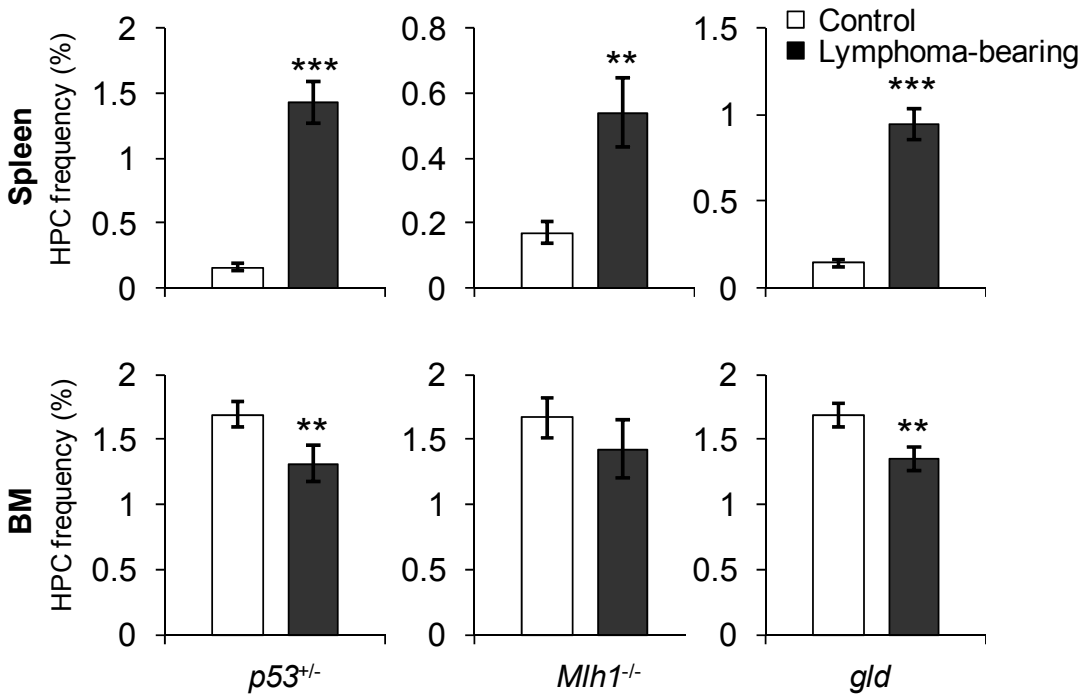

Figure 10. Statistical summary of HPC frequency in spleen and BM of different mutated lymphoma-bearing mice and their wild type control. n=4.

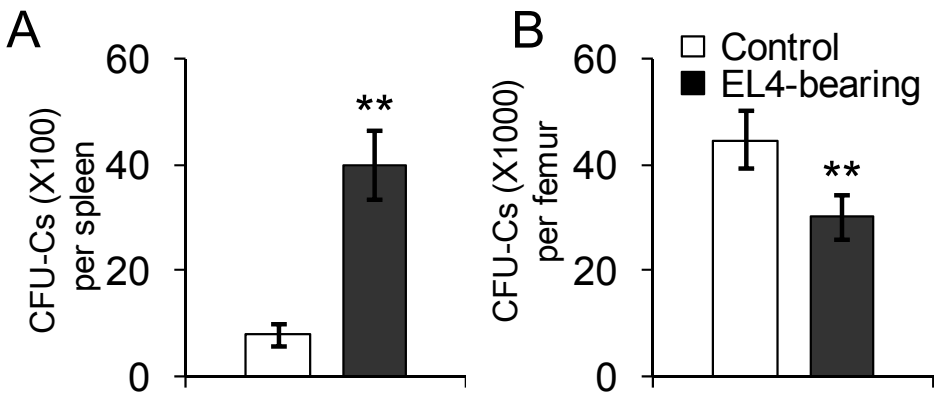

Figure 11. In vitro CFU-C assay of spleen and BM of tumor-bearing mice

(A).Colony-forming units of spleen of EL4-bearing mice and tumor-free control mice.

(B). Colony-forming units of BM of EL4-bearing mice and tumor-free control mice. The CFU-C assay was performed per the protocol from the manufacturer. The results were presented as the CFU-Cs per spleen or per femur. n=4.

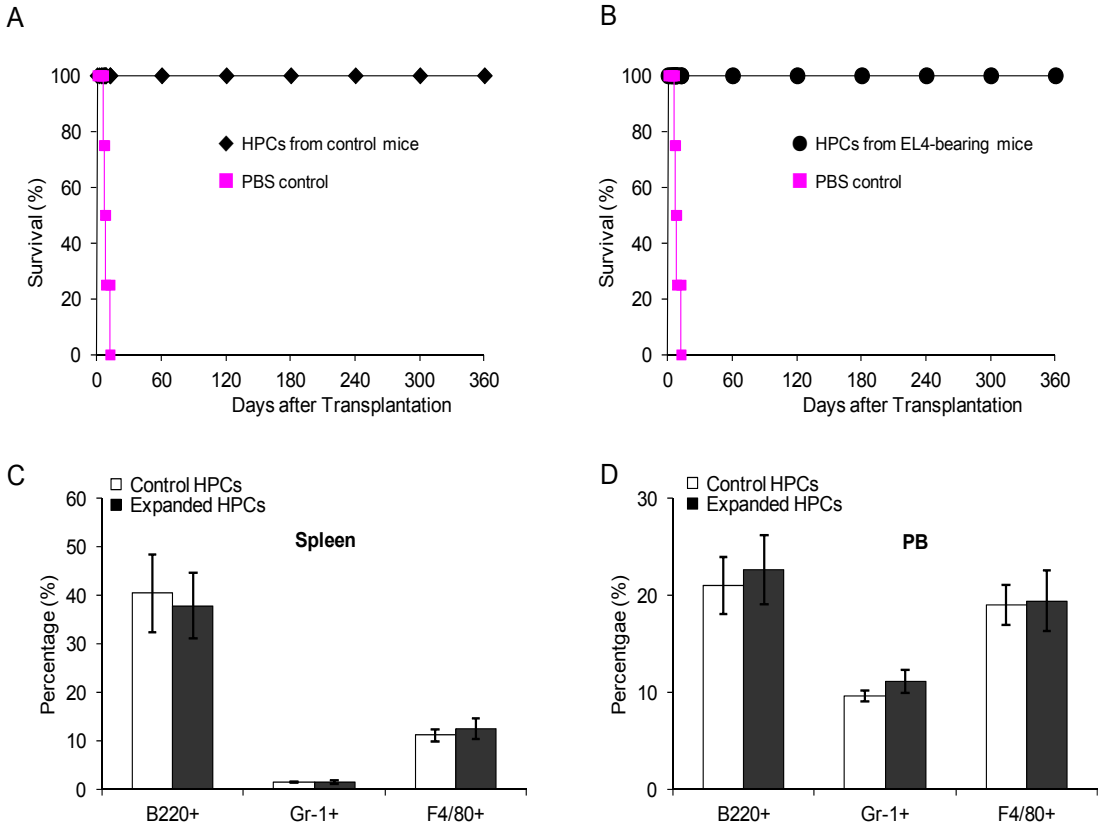

Figure 12. Splenic HPCs from tumor-free and tumor-bearing mice were equally capable of rescuing lethally-irradiated mice.

Splenic HPCs were isolated from tumor-free control mice and EL4-bearing mice using lineage cell depletion kit. Then $5X10^5$ of both cells were intravenously administered into lethally-irradiated C57BL/6 mice by tail-vein injection. PBS was as the control of HPC administration. The mouse survival was recorded during one year (A-B), following the analysis of different immune cell frequency in spleen (C) and PB (D) by flow cytometry. n=5.

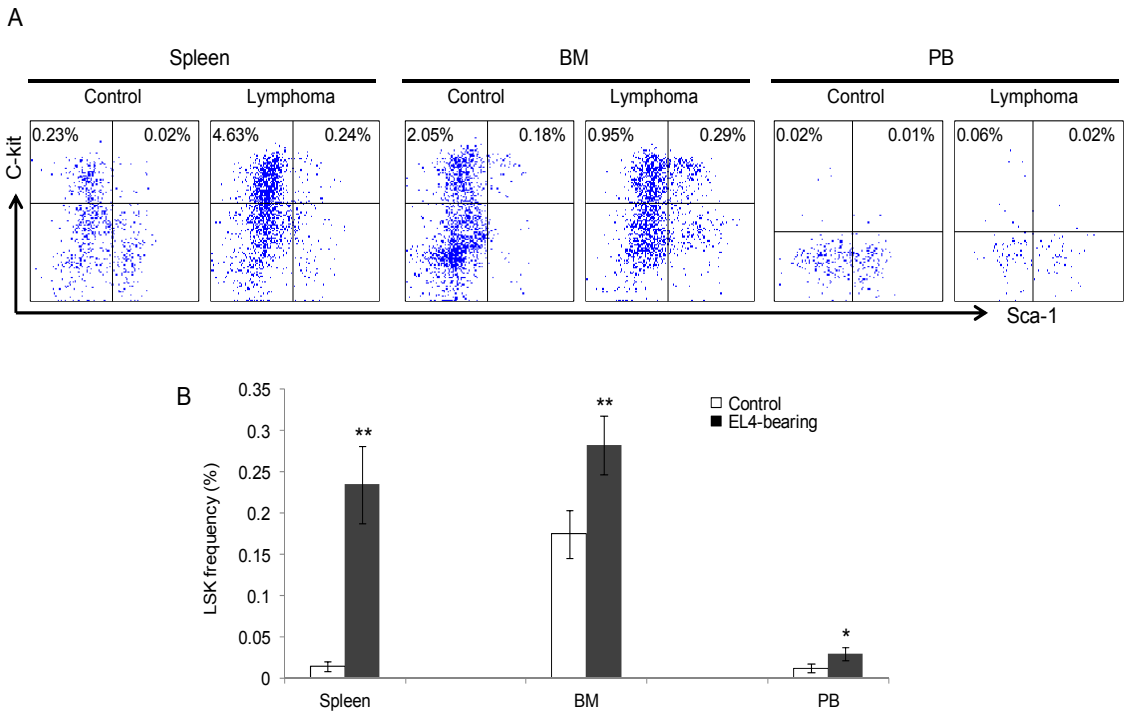

Figure 13. Lymphoma-bearing led to expansion of LSK (Lin$^-$Sca-1$^+$C-kit$^+$) cells in mouse spleen

(A). Representative flow cytometry scatter plots showing the frequency of Lin$^-$Sca-1$^+$C-kit$^+$ cells in spleen, BM and PB of EL4-bearing mice and tumor-free control mice.

(B). Statistical summary of LSK cell frequency in (A). n=4.

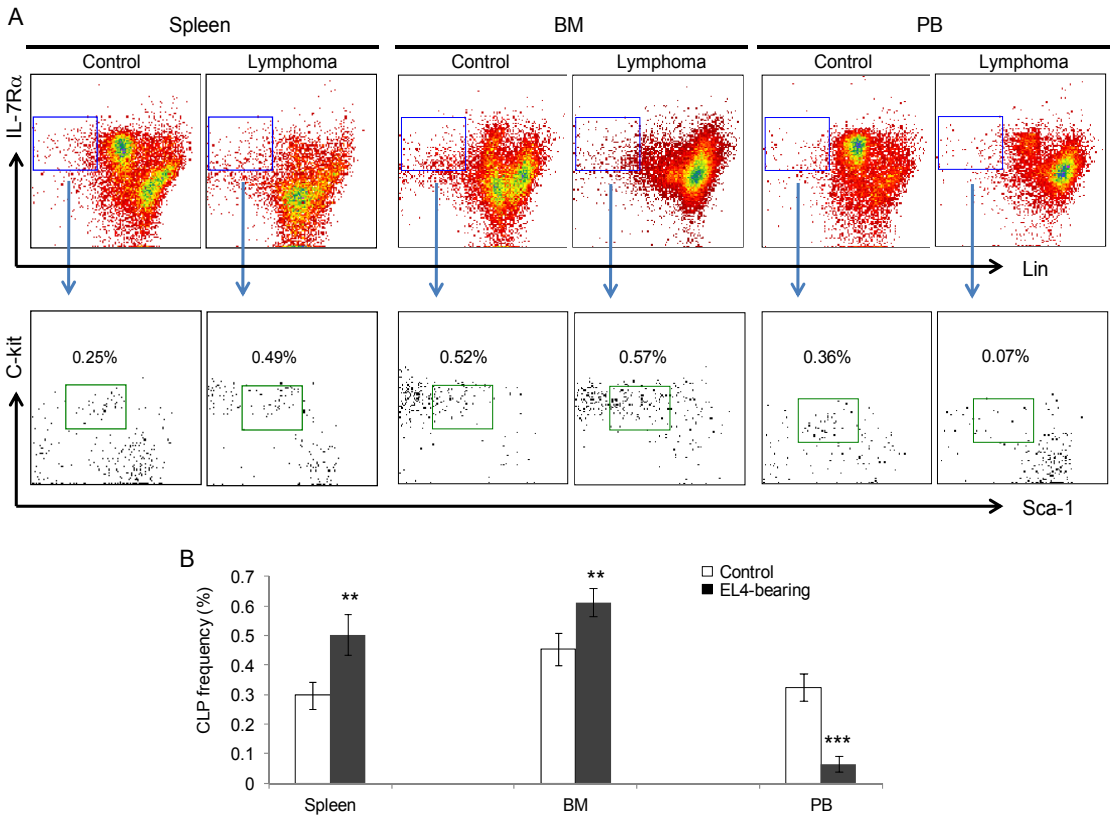

Figure 14. Lymphoma only induced a mild increase of common lymphoid progenitors in mouse spleen

(A). Representative flow cytometry scatter plots showing the frequency of common lymphoid progenitors (CLPs, $Lin^{-}IL\text{-}7R^{+}Sca1^{low}C\text{-}kit^{low}$) in spleen, BM and PB of EL4-bearing mice and tumor-free control mice.

(B). Statistical summary of CLP cell frequency in (A). n=4.

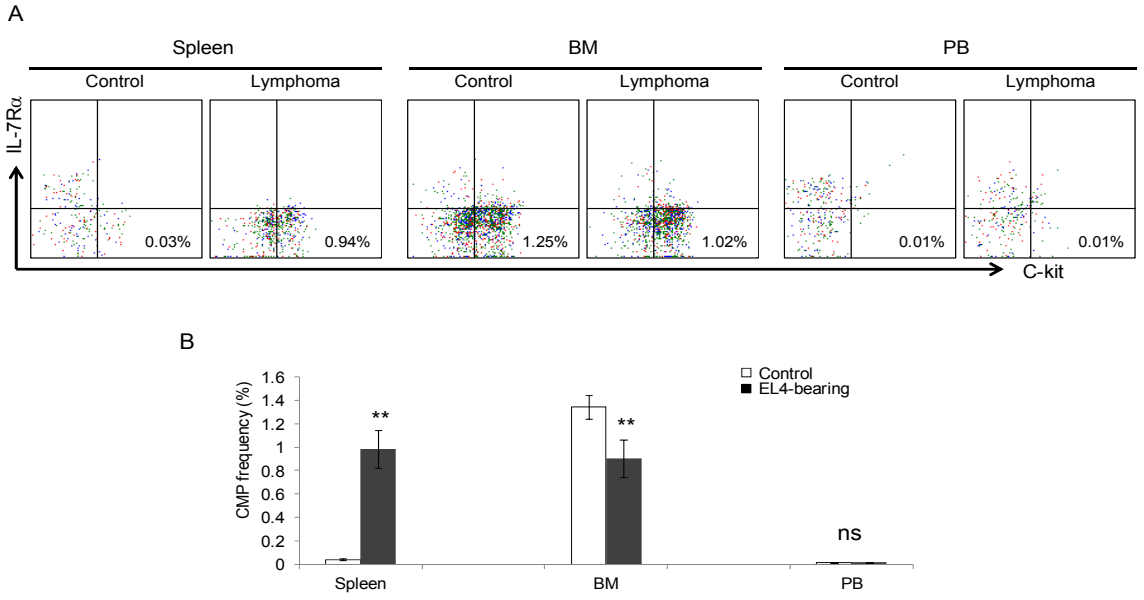

Figure 15. A dramatic increase of common myeloid progenitors in lymphoma-bearing mouse spleen

(A). Representative flow cytometry scatter plots showing the frequency of common myeloid progenitors (CMPs, $Lin^{-}Sca\text{-}1^{-}C\text{-}kit^{+}IL\text{-}7R^{-}$) in spleen, BM and PB of EL4-bearing mice and tumor-free control mice.

(B). Statistical summary of CMP cell frequency in (A). n=4.

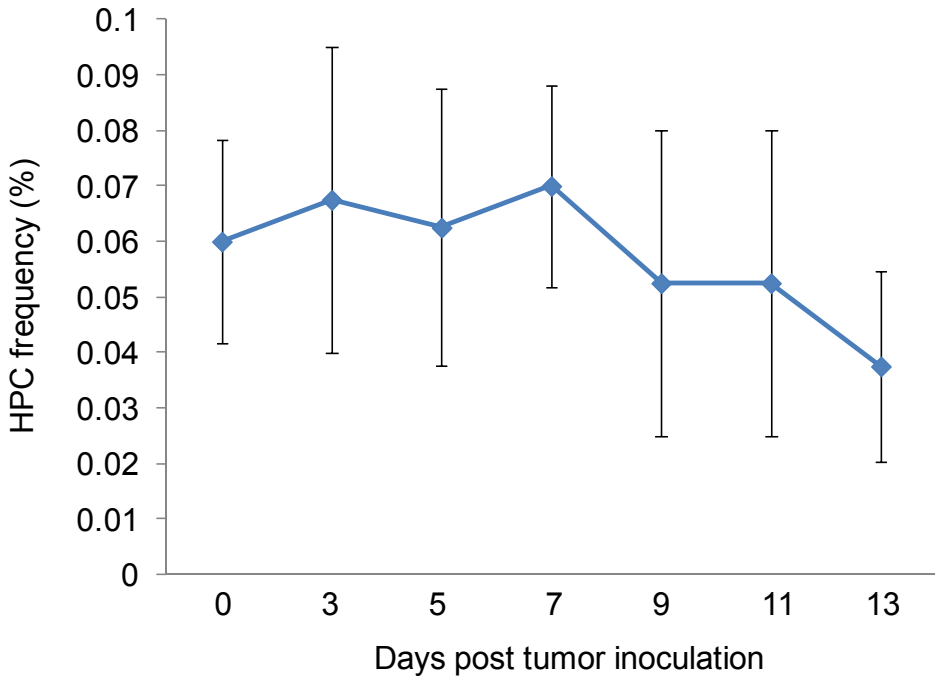

Figure 16. Frequency of HPCs in PB of tumor-bearing mice did not show a significant change.

The C-kit^{+}Lin^{-} HPCs in PB of EL4-bearing mice were measured by flow cytometry at the indicated time points post EL4 cell inoculation. Data shown are representative of two independent experiments.

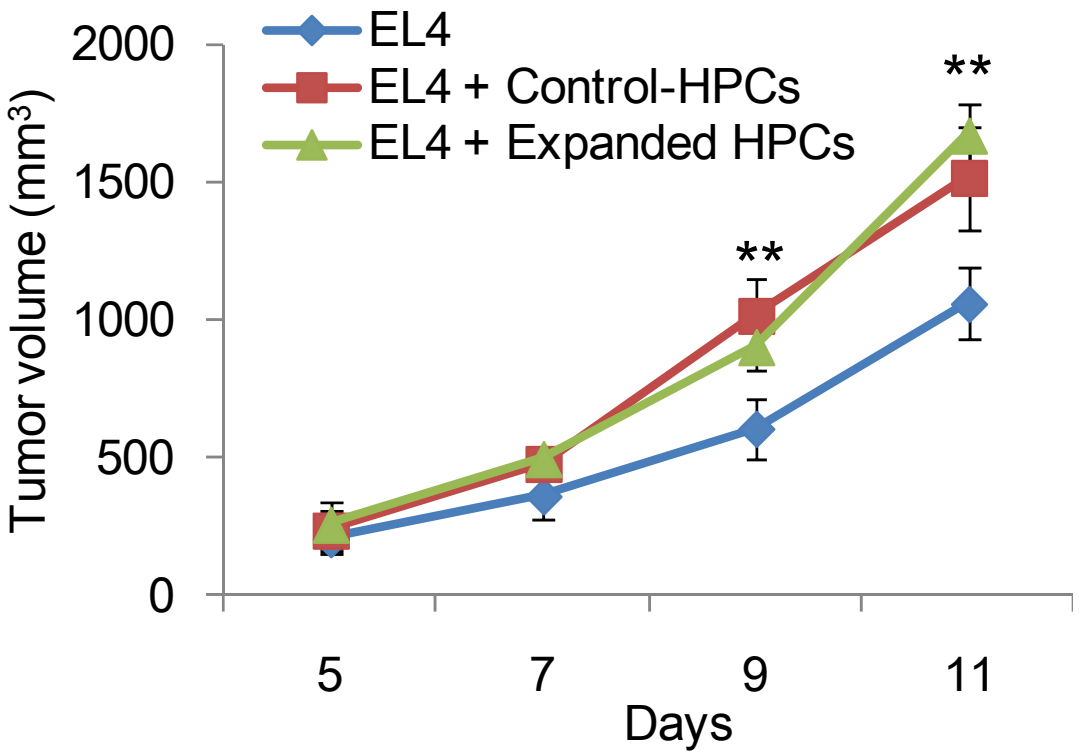

Figure 17. Infusion of HPCs enhanced lymphoma growth.

Splenic HPCs were isolated from either tumor-free control mice (control-HPCs) or from EL4-bearing mice (expanded HPCs) using mouse lineage depletion kit. $2X10^5$ of each HPCs were then intravenously infused into mice pre-administered with EL4 cells ($0.5X10^6$ cells, 2 days before, s.c.). At different time points, the lymphoma size was measured in each experimental group. n=4.

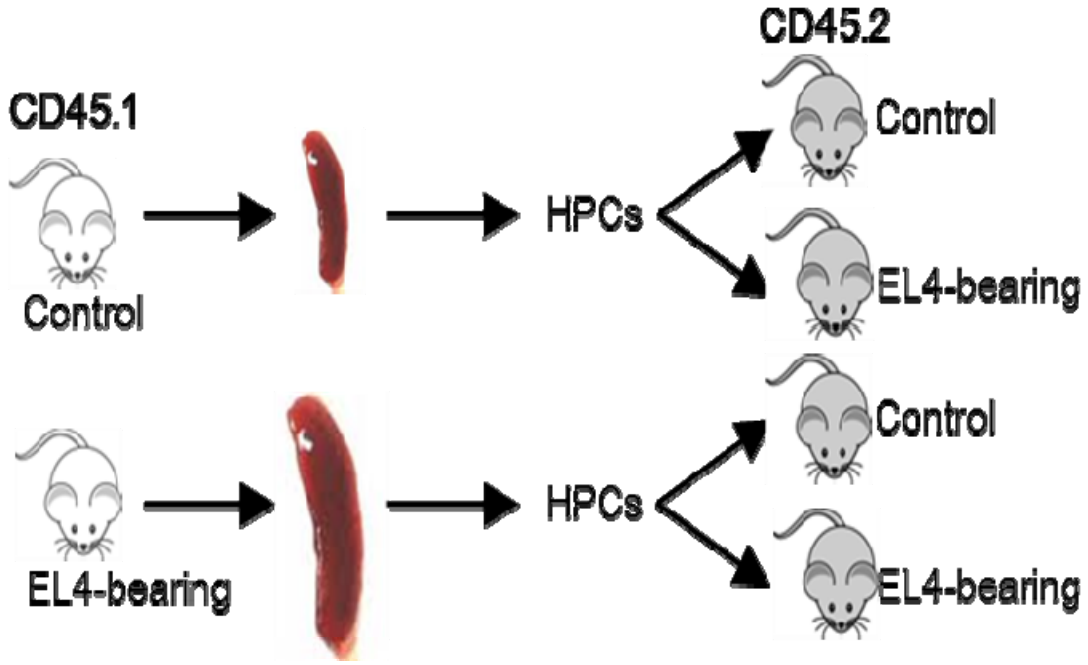

Figure 18. A scheme showing how the fate of donor HPCs was tracked. Briefly, splenic HPCs were isolated from tumor-free and EL4-bearing CD45.1 mice. Then these HPCs ($2X10^5$) were intravenously infused into either tumor-free control CD45.2 mice or EL4-pre-treated (2 days before, $0.5X10^6$ EL4 cells, s.c.) CD45.2 mice. Fourteen days later, the differentiation of the donor HPCs was examined.

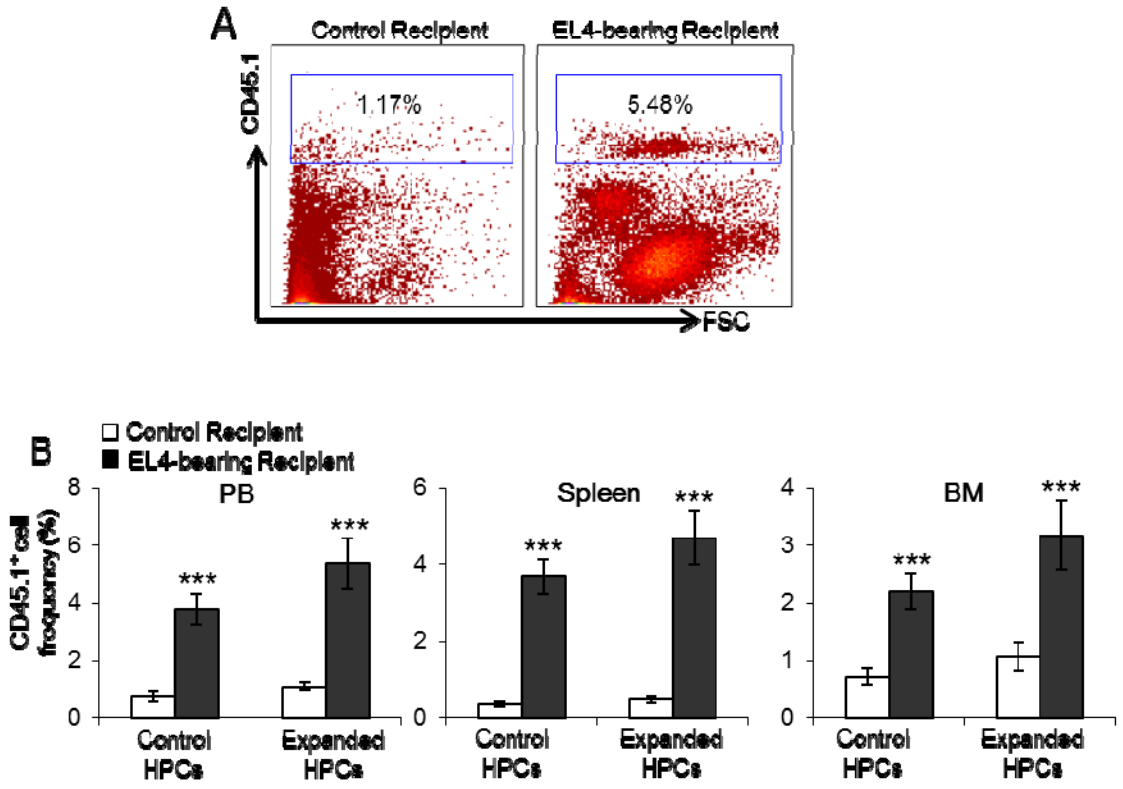

Figure 19. Tumor-bearing mice allow increased colonization by donor HPCs

As in **Figure 18**, 14 days later post HPC infusion, the donor CD45.1^{+} cell frequency in PB, spleen and BM of tumor-free control recipients and EL4-bearing recipients was analyzed by flow cytometry.

(A). A representative flow cytometry scatter plot shows the differentiation of expanded HPCs in PB.

(B). The statistical summary of **Figure 19A**. n=5.

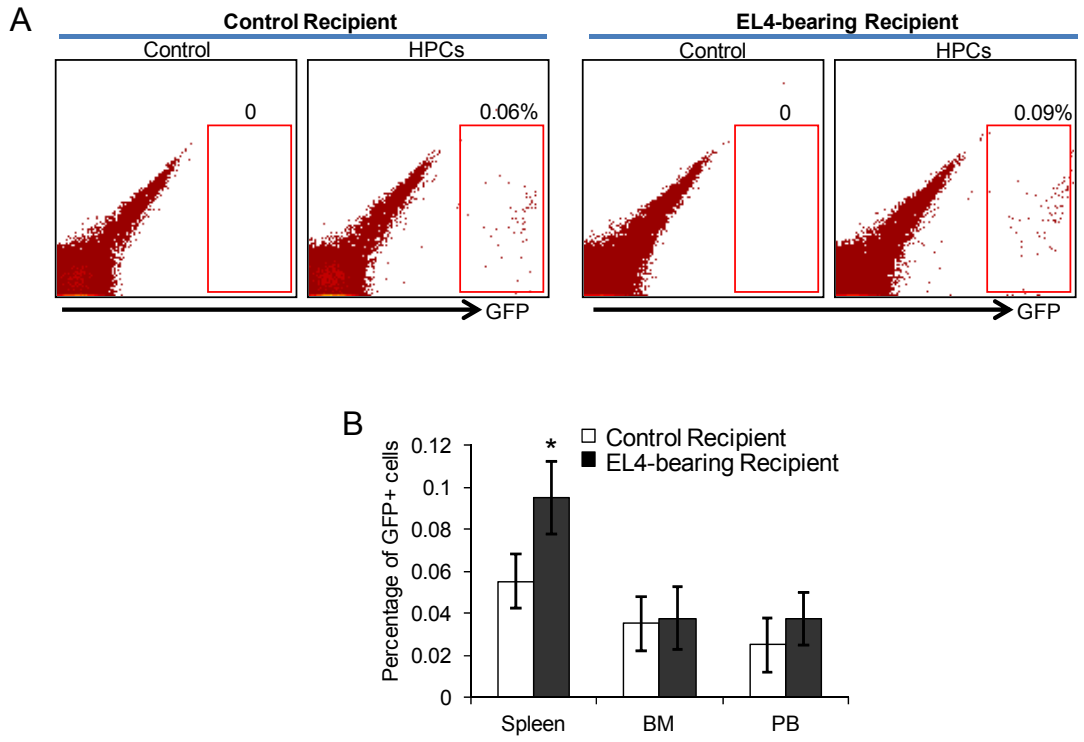

Figure 20. Donor HPCs had a higher capability of engraftment in spleens of tumor-bearing recipients compared to tumor-free control recipients.

As in **Figure 18**, the donor HPCs isolated from GFP-transgenic EL4-bearing mice were infused into either tumor-free control mice or EL4-pre-treated (4 days before, $0.5X10^6$ EL4 cells, s.c.) C57BL/6 mice. Two days post HPC infusion, the GFP^+ donor cells were measured by flow cytometry.

(A).Representative flow cytometry scatter plots.

(B).The statistical summary of the GFP^+ cell frequency in different tissues**.** n=4.

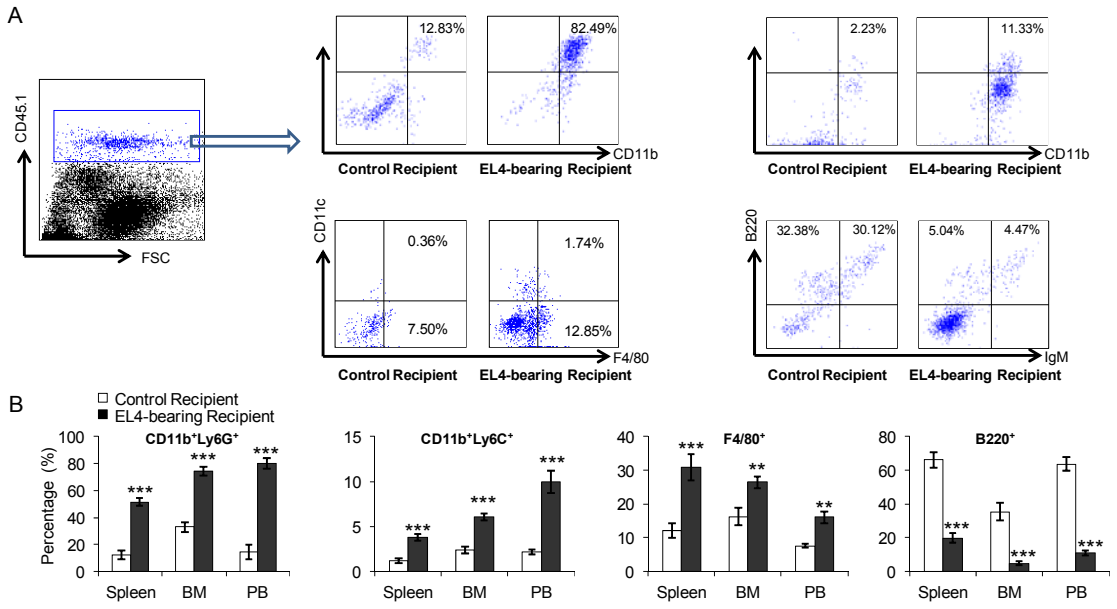

Figure 21. HPC differentiation was biased toward myeloid lineages in tumor-bearing mice

Differentiation of splenic HPCs. As in **Figure 18**, 14 days post donor HPC infusion, the differentiation of these donor cells was determined using different lineage cell markers including CD11b/Ly6G, CD11b/Ly6C, F4/80/CD11c and IgM/B220 by gating the CD45.1^{+} cells.

(A).Representative flow cytometry scatter plots.

(B).The statistical summary of panel (A) . n=5.

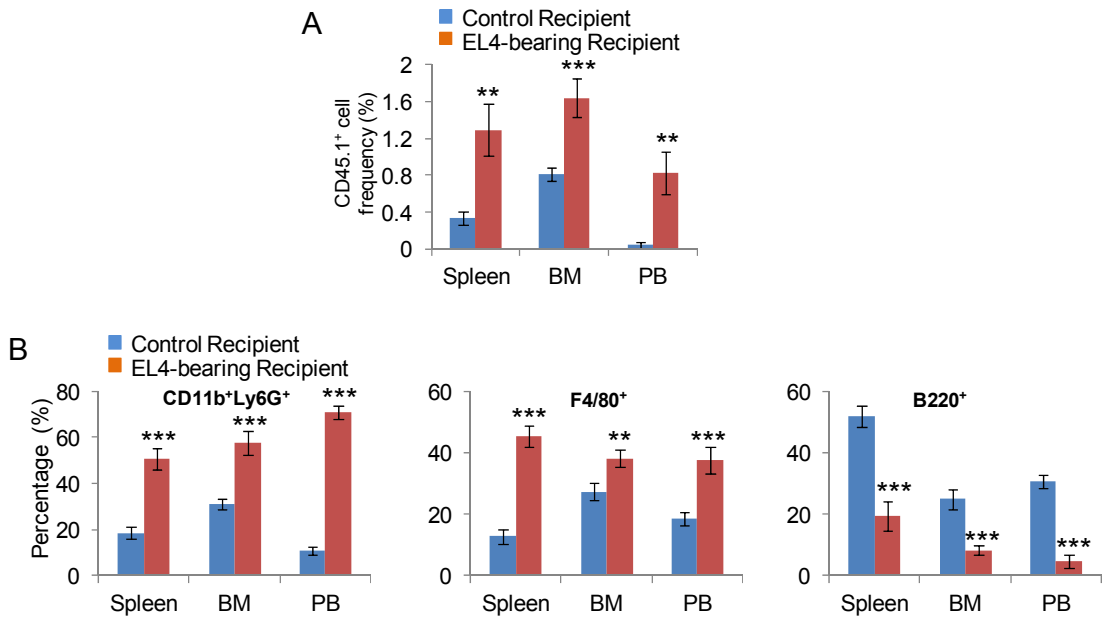

Figure 22. The differentiation of BM HPCs is also biased toward myeloid lineages in tumor-bearing mice

(A). Tumor-bearing recipients allowed increased colonization by BM-derived donor HPCs. The experiments were same as in **Figure 19B**, with splenic donor HPCs replaced with normal mice BM-derived HPCs. n=5.

(B). Differentiation of bone marrow HPCs. A similar experiment was conducted as in **Figure 21B**, with splenic donor HPCs replaced with normal mice BM-derived HPCs. n=5.

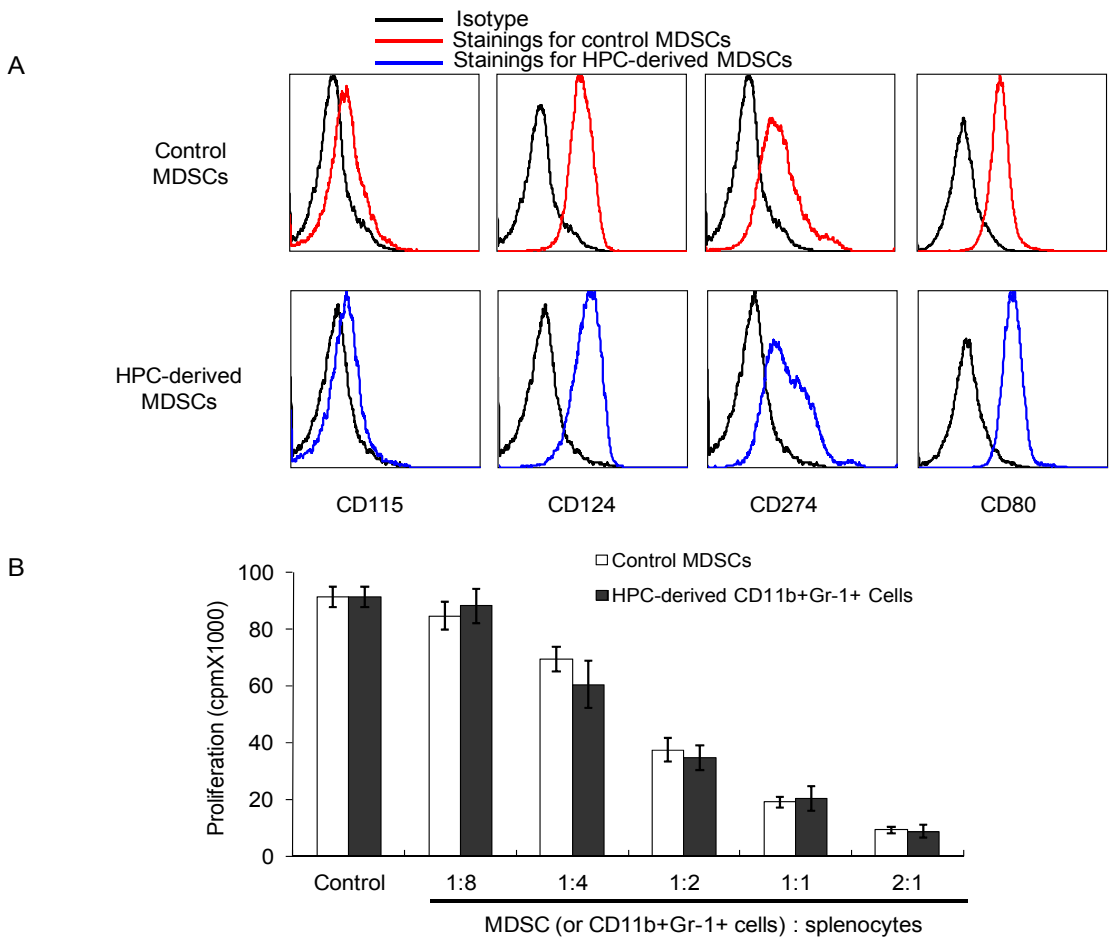

Figure 23. Tumor-expanded HPCs-derived CD11b$^+$Gr-1$^+$ cells are phenotypically and functionally similar to typical MDSCs

Donor HPCs were isolated from EL4-bearing CD45.1 mice and then 2X10^6 of these cells were intravenously infused into EL4-pre-administered (2 days before, 0.5X10^6 EL4 cells per mouse) CD45.2 mice. Fourteen days after HPCs infusion, the CD45.1$^+$CD11b$^+$Gr-1$^+$ cells in spleens were firstly isolated by a myeloid-derived suppressor cell isolation kit, and then sorted by cell sorter. The typical MDSCs were isolated using the same method. The phenotypes of these two sources of cells were determined by flow cytometry (A). To examine the

immunosuppressive effect of these two types of cells, both cells were co-cultured with splenocytes of OT-II mice at different ratios in the presence of OVA peptide 323-339. Two days later, the cell proliferation was assayed by ^{3}H-thymidine incorporation (B). Data are means ± SD of four wells from a representative of three experiments.

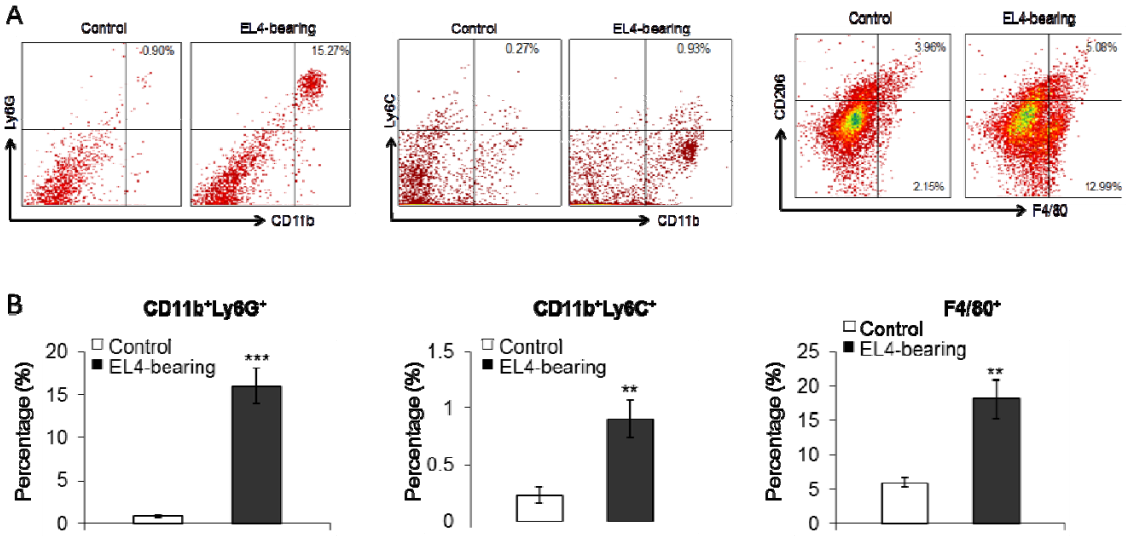

Figure 24. Increase of myeloid cells in spleens of tumor-bearing mice.

The indicated myeloid cell populations in spleens of EL4-bearing mice and tumor-free control mice were measured by flow cytometry. A representative flow cytometry pattern is shown in (A). The statistical analysis of the myeloid cell frequency is shown in (B). n=4.

All data shown are representative of at least three independent experiments.

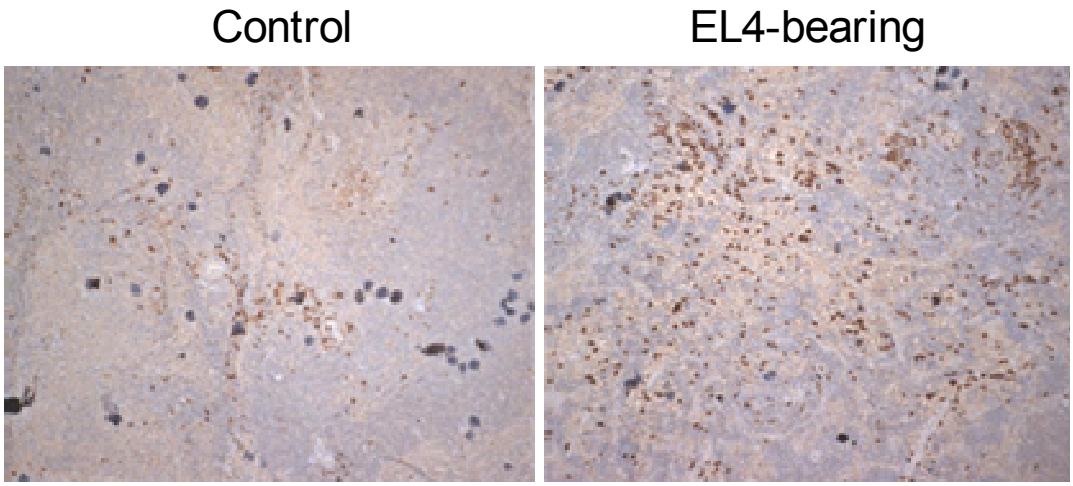

Figure 25. Increased abundance of macrophaes in tumor-bearing mice.

As shown by immunohistochemical staining of F4/80 in spleens of control mice and EL4-bearing mice.

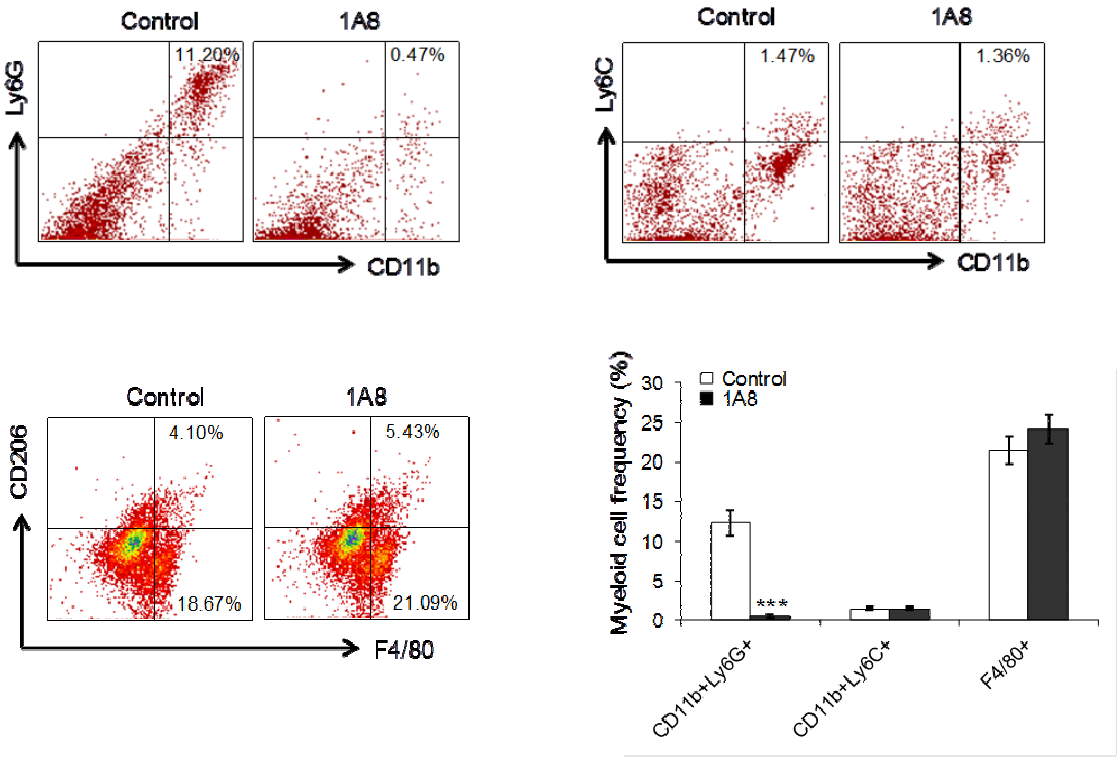

Figure 26. Anti-Gr-1 specifically depleted Ly6G$^+$ granulocytic MDSCs, but not Ly6C$^+$ monocytic MDSCs and macrophages

Depletion efficacy of anti-Gr-1 clone 1A8 on different myeloid cell population in spleens of tumor-bearing mice was analyzed by flow cytometry. The representative flow cytometry pattern and statistical analysis are shown. n=4.

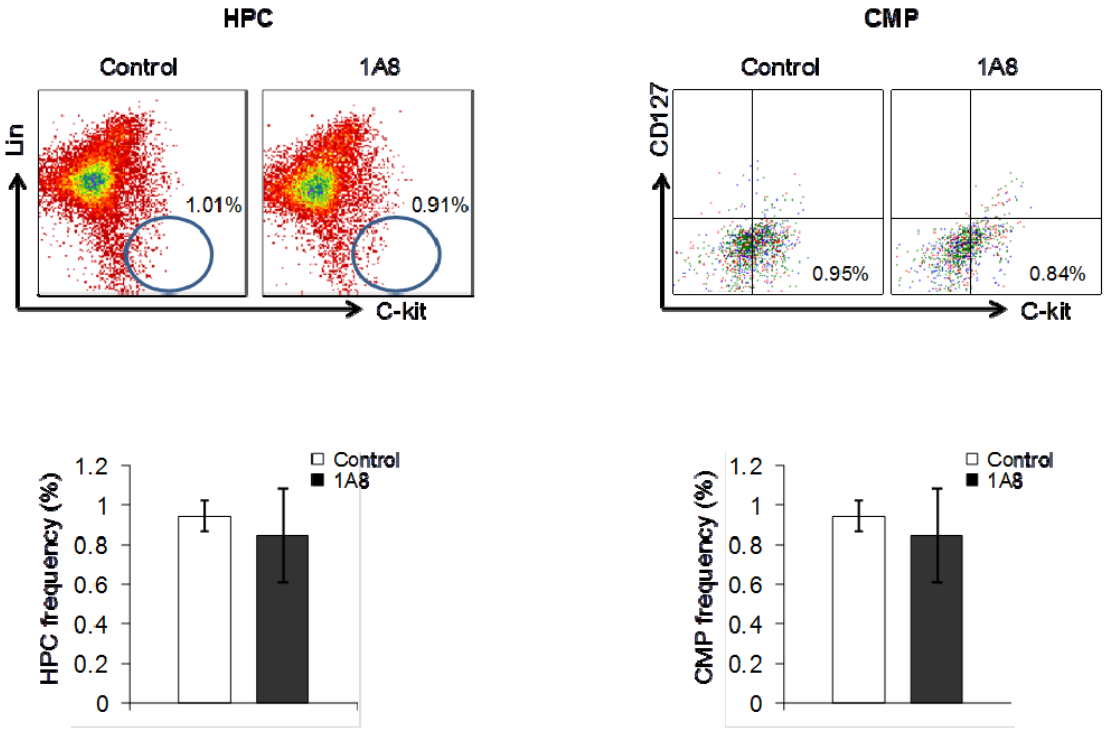

Figure 27. Ly6G$^+$ granulocytic MDSCs were not critical for tumor-induced spleen hematopoiesis

After a specific depletion of Ly6G$^+$ granulocytic MDSCs by anti-Gr-1 clone 1A8 in tumor-bearing mice, the frequency of splenic HPCs and CMPs was measured by flow cytometry. n=4. Data shown are representative of two independent experiments.

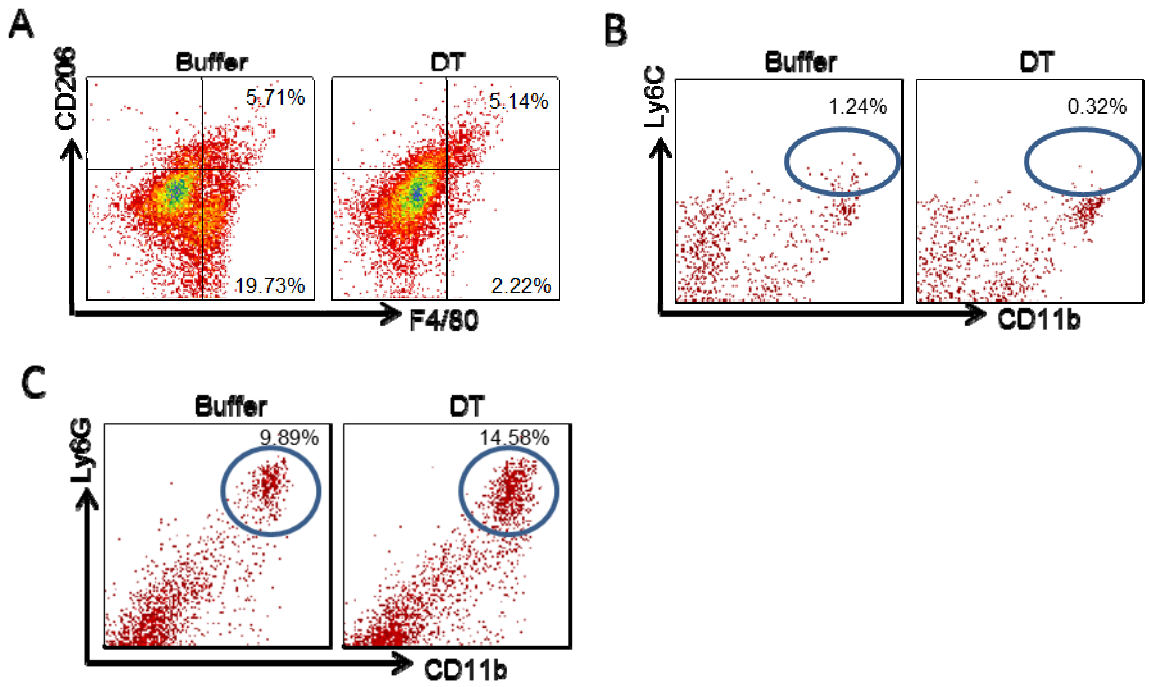

Figure 28. Effects of DT administration on different populations of myeloid cells in CD11b-DTR mice.

The frequencies of macrophages (F4/80^{+}) (A), monocytic MDSCs (CD11b^{+}Ly6C^{+}) (B) and granulocytic MDSCs (CD11b^{+}Ly6G^{+}) (C) in spleens of EL4-bearing CD11b-DTR mice treated with either buffer or DT, were examined by flow cytometry.

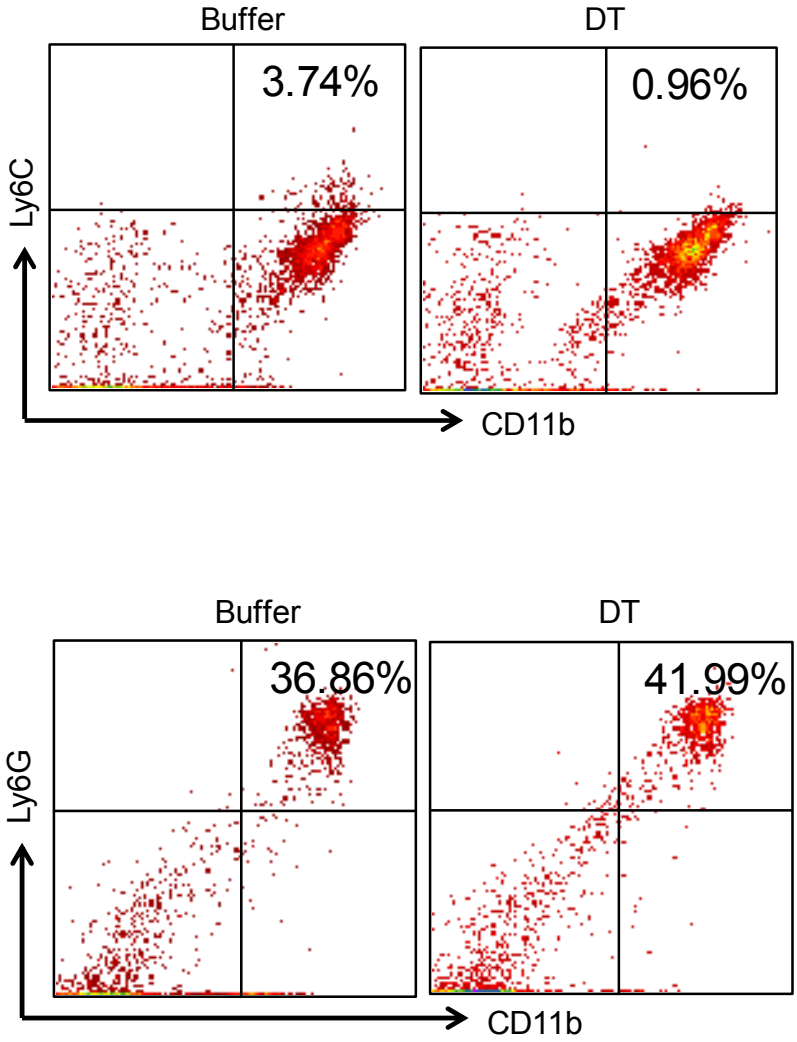

Figure 29. Influence of DT administration on different populations of myeloid cells.

The frequency of macrophages (F4/80$^+$), monocytic MDSCs (CD11b$^+$Ly6C$^+$) and granulocytic MDSCs (CD11b$^+$Ly6G$^+$) in PB of EL4-bearing CD11b-DTR mice treated with either buffer or DT, was examined by flow cytometry. n=4. Data shown are representative of at least three independent experiments.

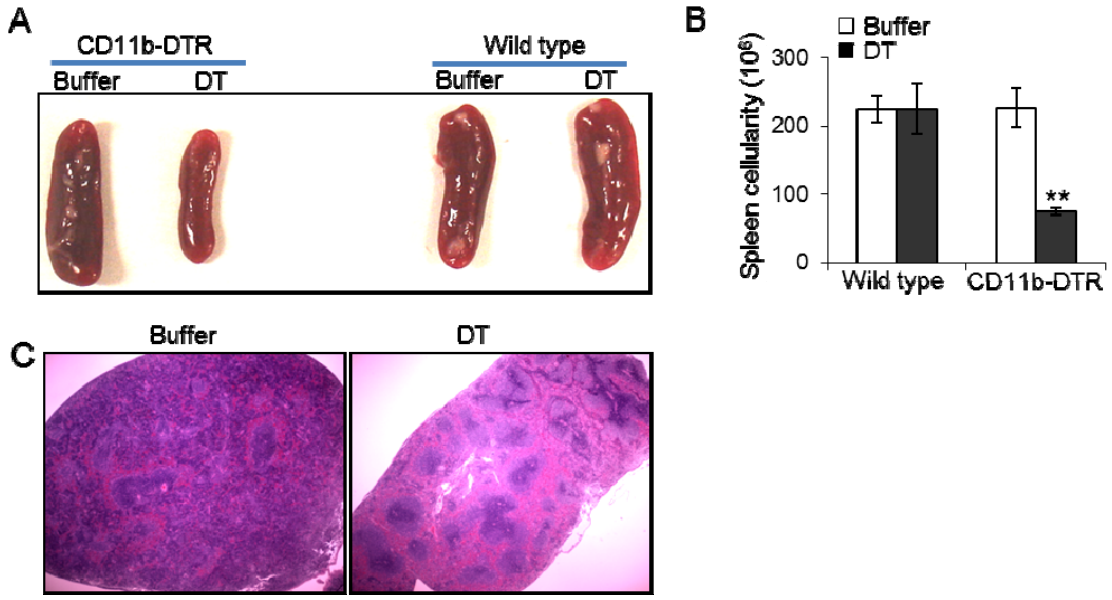

Figure 30. Depletion of macrophages/monocytes inhibited tumor-induced spleen hematopoiesis.

Impact of macrophage/monocyte ablation on spleen size (A), spleen cellularity (B), spleen histology (C), were shown in EL4-bearing CD11b-DTR mice, with wild type mice with and without treatment of DT as controls.

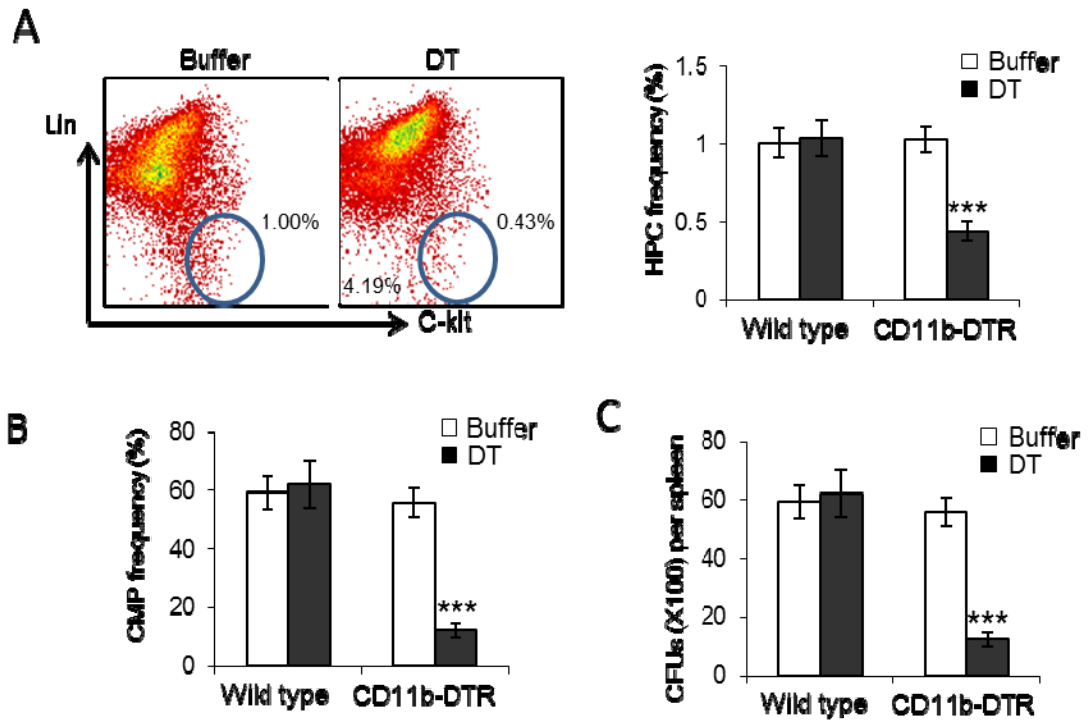

Figure 31 Depletion of macrophages/monocytes inhibited tumor-induced HPC expansion in spleen.

Impact of macrophage/monocyte ablation on splenic HPC frequency (A), CMP frequency (B) and CFU-Cs per spleen (C), were shown in EL4-bearing CD11b-DTR mice, with wild type mice with and without treatment of DT as controls. n=4.

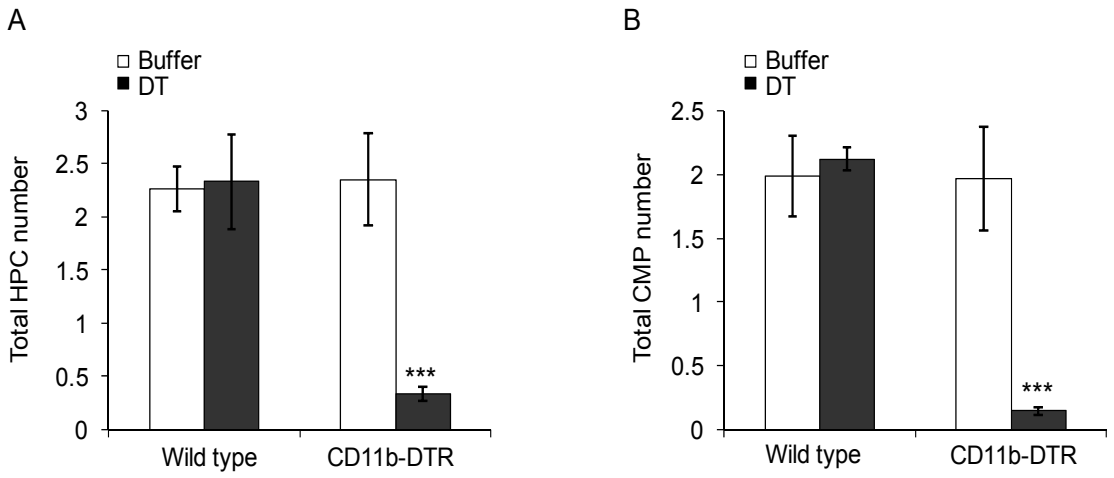

Figure 32. Depletion of macrophages/monocytes reduced the total number of HPCs and CMPs

The total number of splenic HPCs and CMPs in CD11b-DTR mice and wild type mice receiving either DT or buffer, was calculated as the total splenic cell number multiplying the frequency of HPCs or CMPs, which was determined by flow cytometry. n=4.

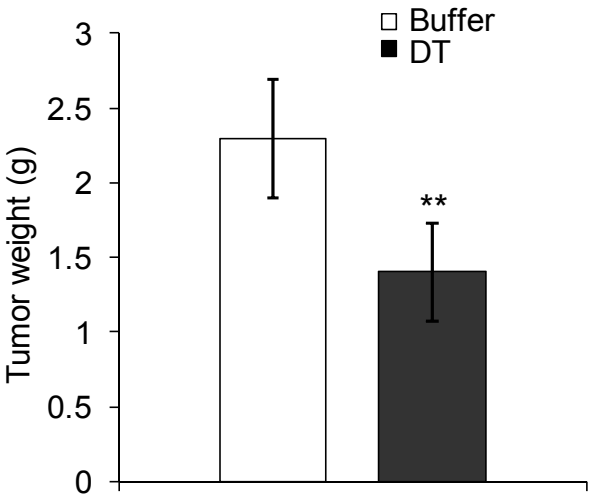

Figure 33. Macrophage/monocyte ablation led to reduced tumor weight

Post EL4 cell inoculation in CD11b-DTR mice receiving without and with DT, the tumor weights were measured on day 12. n=4.

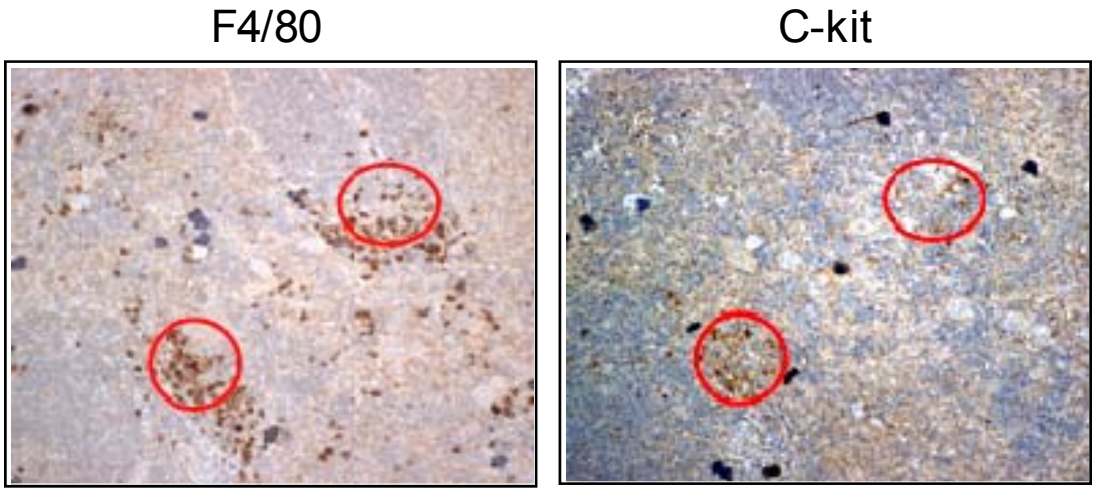

Figure 34. C-kit+ cells are clustered with F4/80^{+} cells in spleen of tumor-bearing mice.

The immunohistochemical staining of F4/80 and C-kit was performed on consecutive spleen sections from EL4-bearing mice. Magnification: 100X.

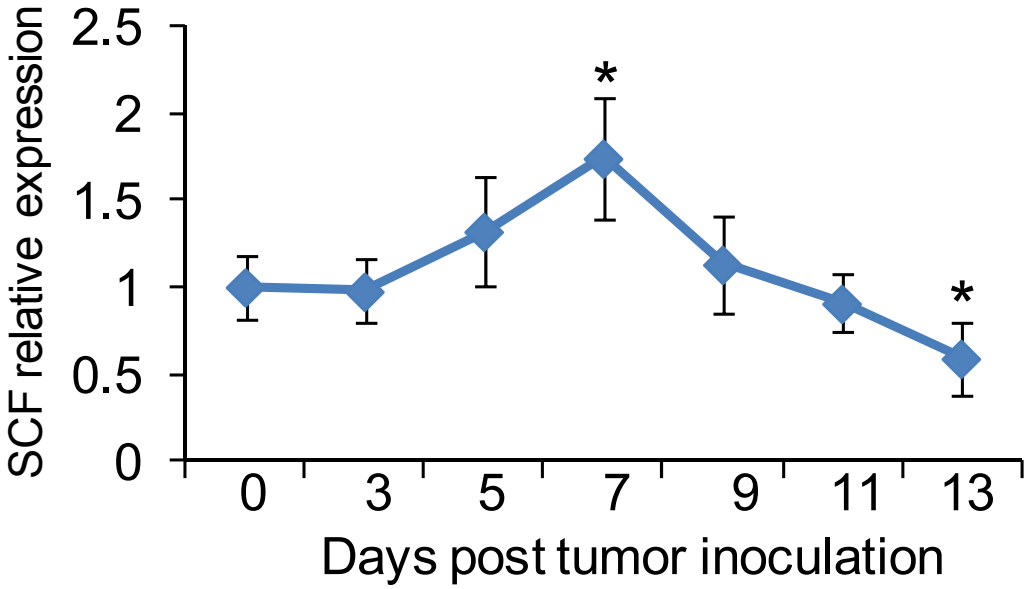

Figure 35. Transient up-regulation of SCF in spleens of tumor-bearing mice. The SCF expression in spleens of EL4-bearing mice was measured by real-time PCR at different time points post tumor cell inoculation. n=4.

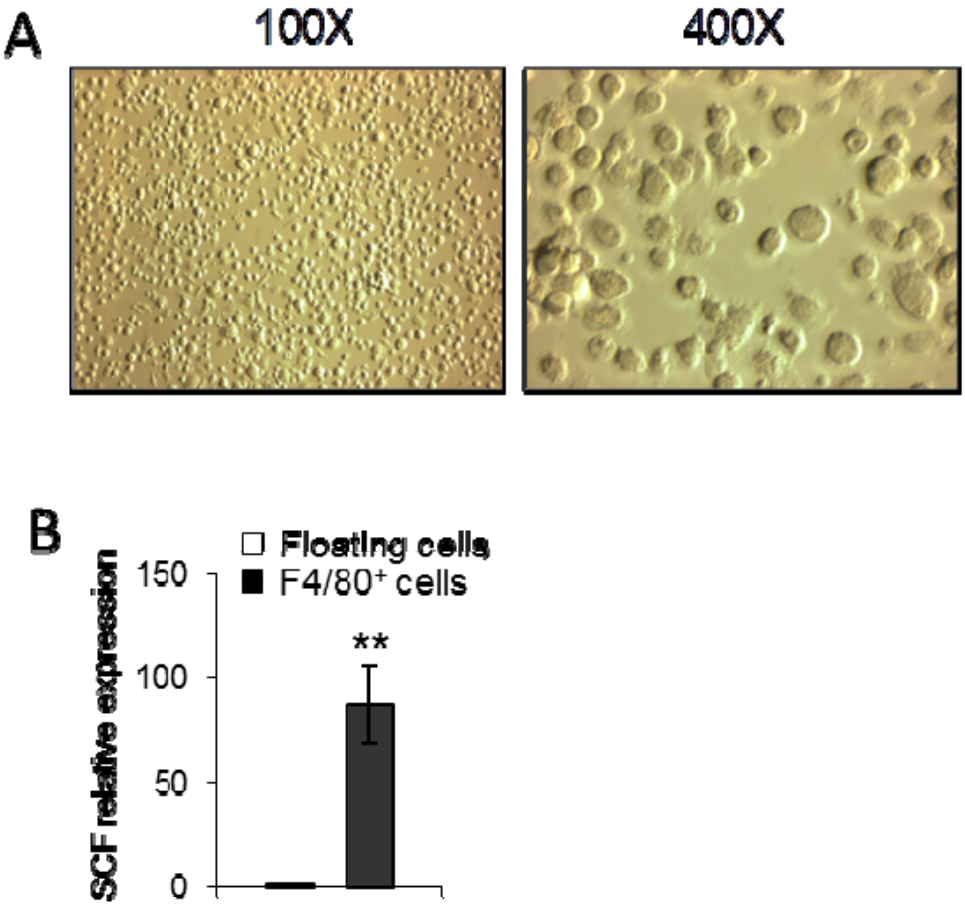

Figure 36. SCF was mainly expressed in CD11b$^+$F4/80$^+$ macrophage-like cells in vitro.

Spleen cells were isolated from EL4-bearing mice, and then plated in flasks at a density of 5X10^6 cells/ml. Two days later, the floating splenic cells and adherent CD11b$^+$F4/80$^+$ cells (morphology as shown in A) were collected respectively, for SCF expression assay by real-time PCR (B). n=4

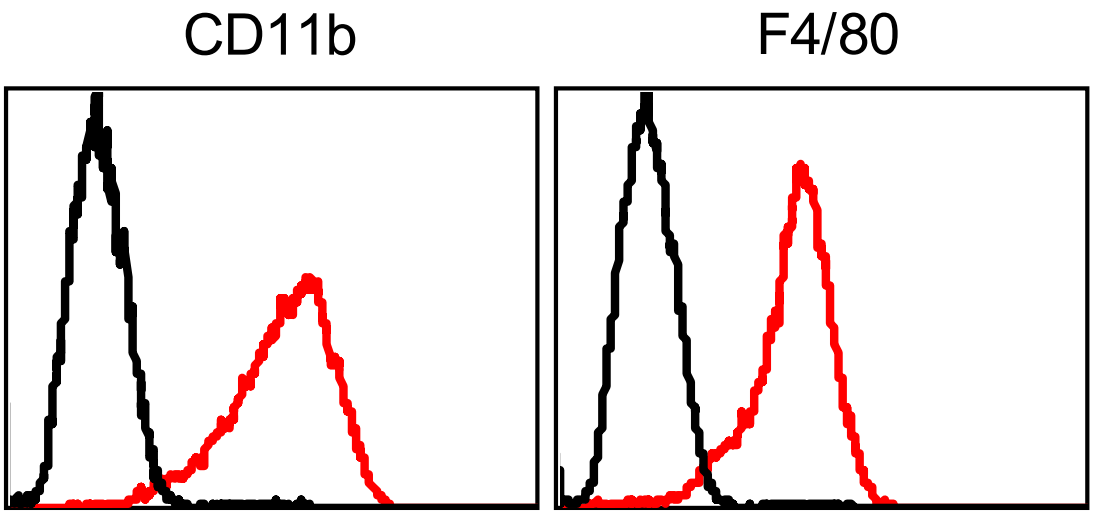

Figure 37. Adherent splenic cells were mostly CD11b$^+$ and F4/80$^+$ macrophage-like cells

Spleen cells were isolated from EL4-bearing mice, and then plated in flasks at a density of 5X10^6 cells/ml. Two days later, the adherent cells were collected for measurement of CD11b and F4/80 expression by flow cytometry.

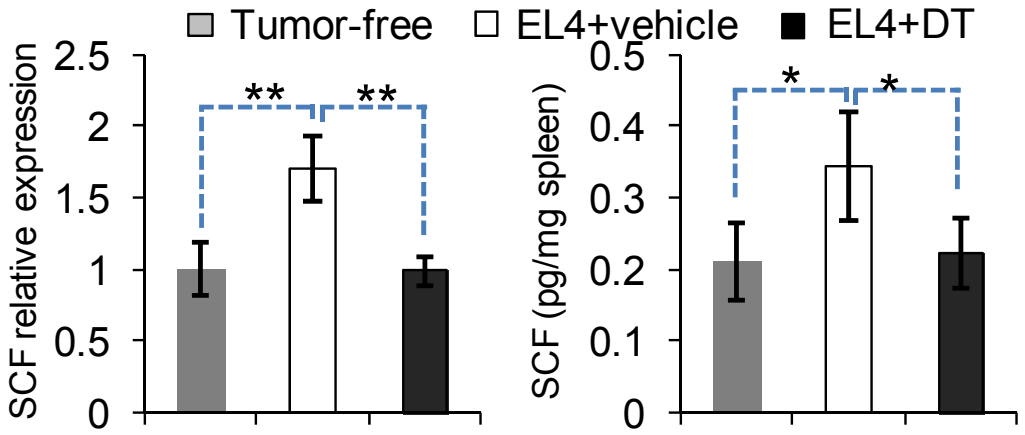

Figure 38. Depletion of macrophages/monocytes attenuated the SCF up-regulation in spleens of tumor-bearing mice in vivo.

SCF expression was measured by real-time PCR in spleens of tumor-free control CD11b-DTR mice, EL4-bearing CD11b-DTR mice treated without and with DT. The data were collected on day 8 post EL4 cell inoculation. n=4.

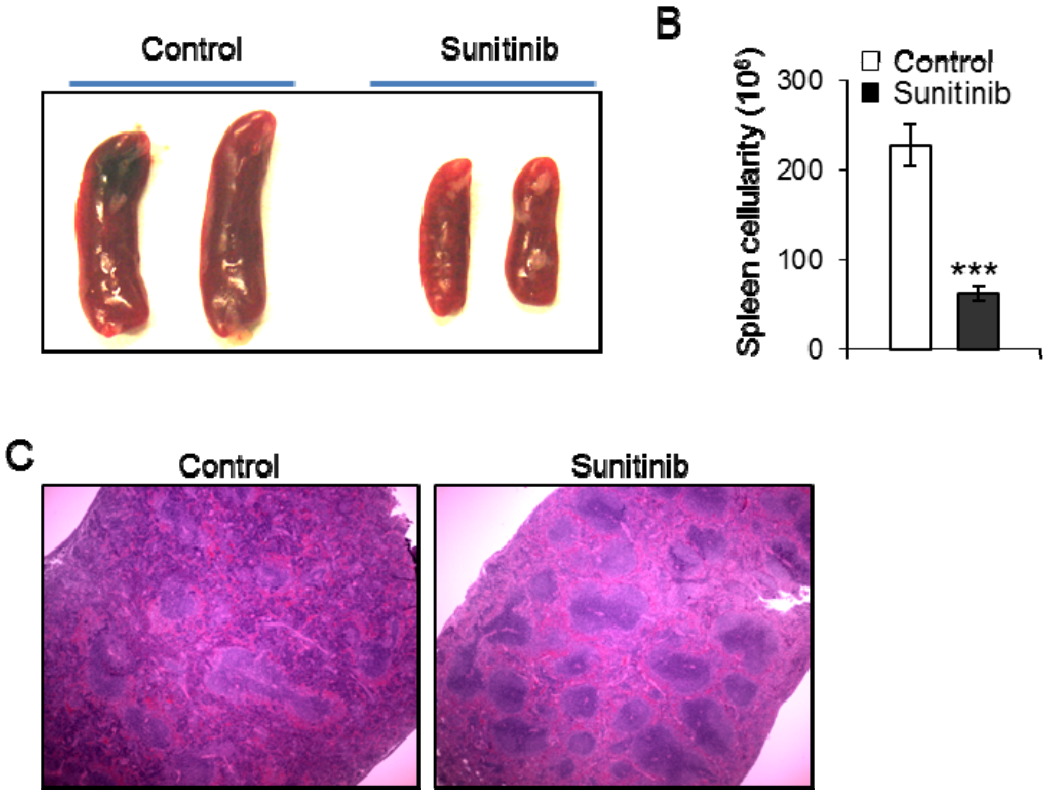

Figure 39. Sunitinib completely abolished tumor-induced spleen hematopoiesis.

The effect of administration of C-kit inhibitor sunitinib on spleen size (A), spleen cellularity (B), and spleen structure (C) were examined in EL4-bearing mice with and without treatment of sunitinib

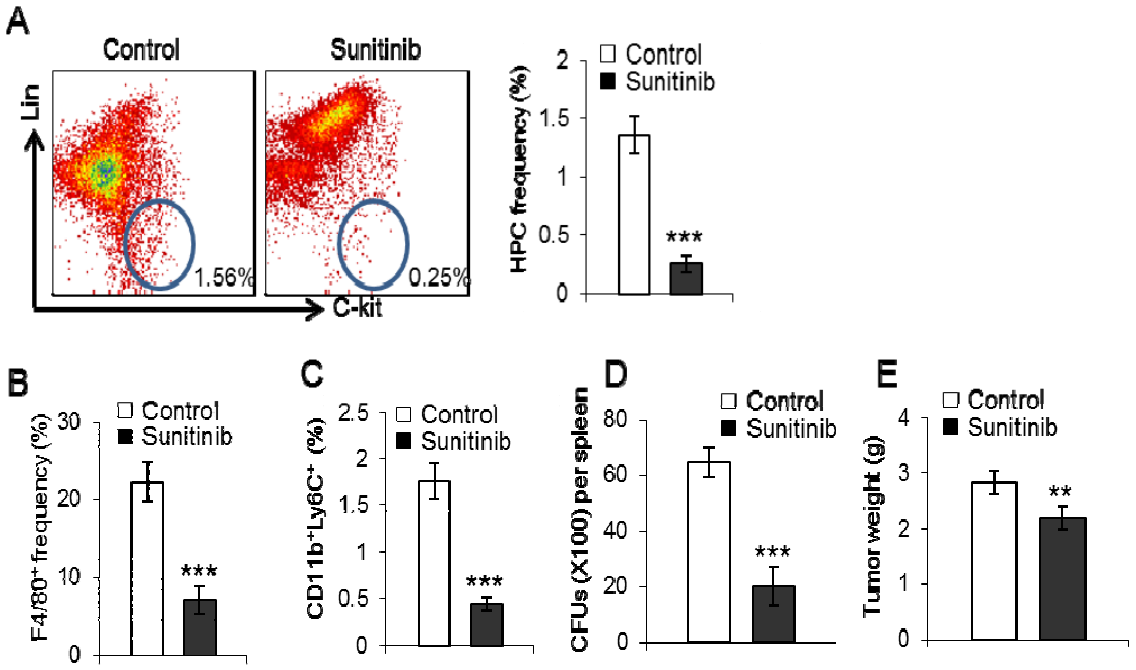

Figure 40. Sunitinib completely abolished tumor-induced splenic HPC expansion.

The effect of administration of C-kit inhibitor sunitinib on splenic HPC frequency (A), splenic macrophage (B) and monocytic MDSC (C) frequency, as well as CFU-Cs per spleen (D) and tumor weight (E), were examined in EL4-bearing mice with and without treatment of sunitinib. n=4.

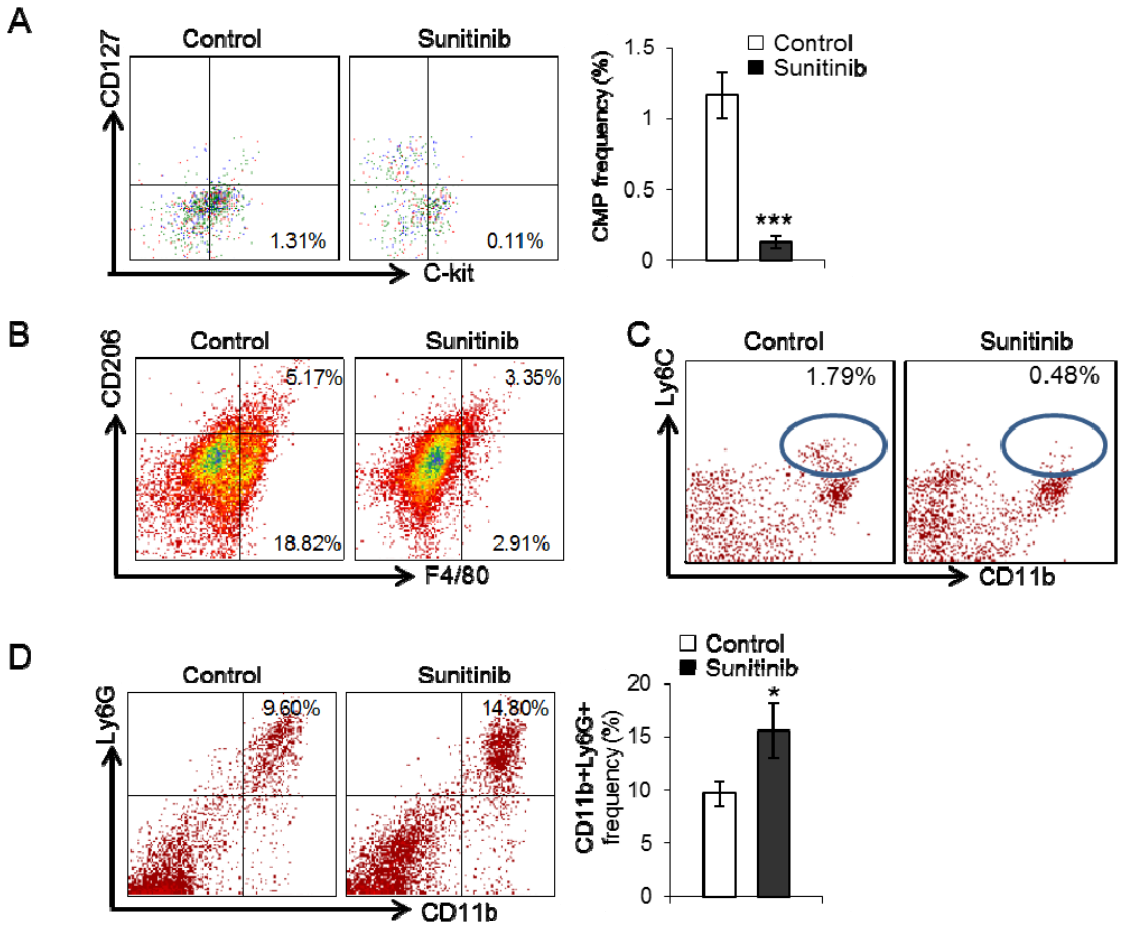

Figure 41. Sunitinib strikingly attenuated the splenic CMP expansion, reduced the frequency of macrophages/monocytes, but mildly increased the frequency of Ly6G^{+} granulocytic MDSCs

The effect of administration of C-kit inhibitor sunitinib on splenic CMP (A), F4/80^{+} macrophage (B), CD11b^{+}Ly6C^{+} monocytic MDSC (C) and CD11b^{+}Ly6G^{+} granulocytic MDSC (D) frequency, was examined in EL4-bearing mice with and without treatment of sunitinib. n=4. Data shown are representative of at least three independent experiments.

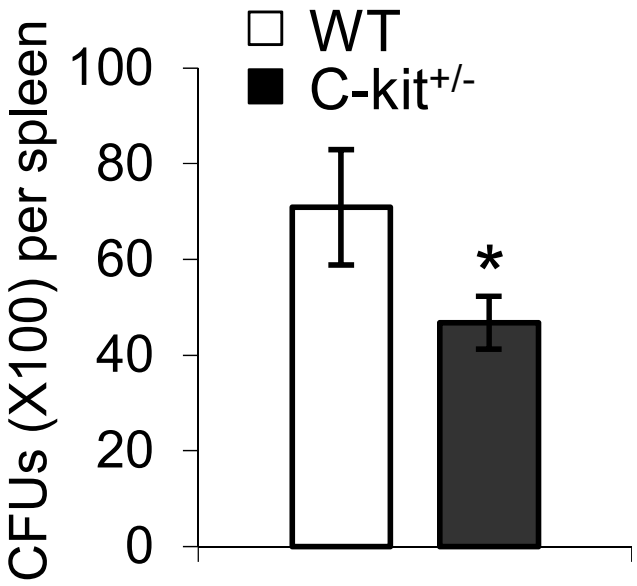

Figure 42. Tumor-induced spleen hematopoiesis was partially abolished in C-kit$^{+/-}$ mice.

Comparison of CFU-Cs per spleen in EL4-bearing C57BL/6 wild type and C-kit$^{+/-}$ mice. n=4.

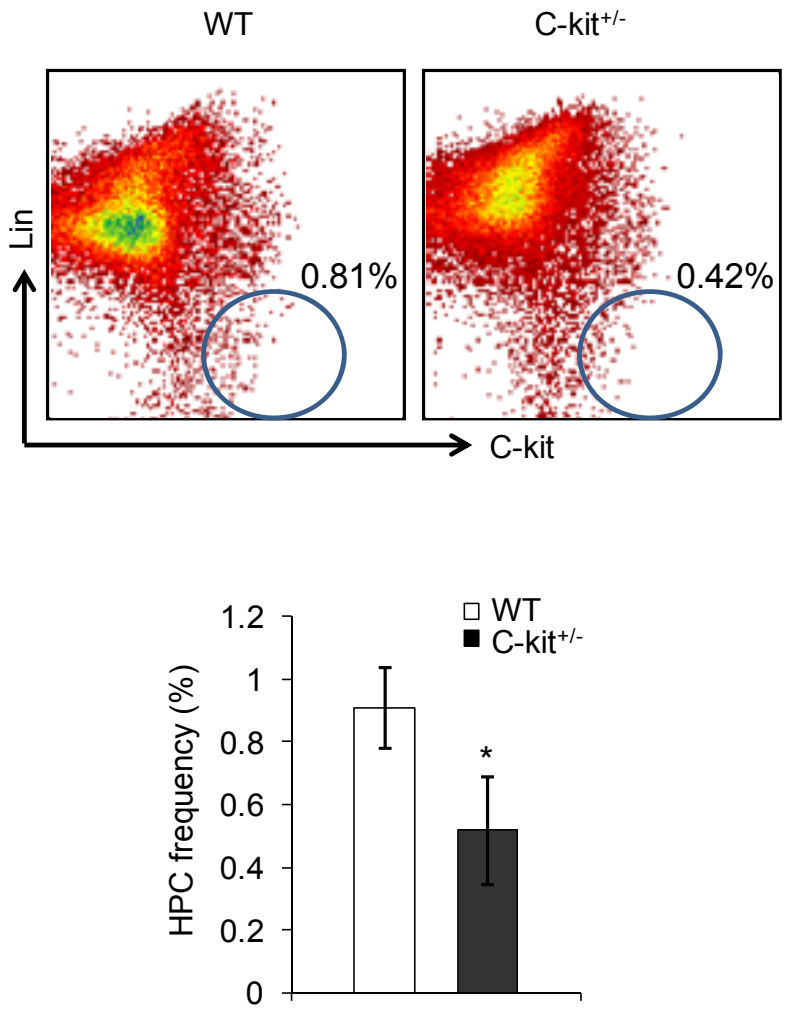

Figure 43. Reduced splenic HPC frequency in C-kit$^{+/-}$ mice

The HPC frequency in spleens of tumor-bearing C-kit$^{+/-}$ and wild type mice, was assayed by flow cytometry 12 days later post EL4 cell inoculation. n=4. Data shown are representative of at least three independent experiments.

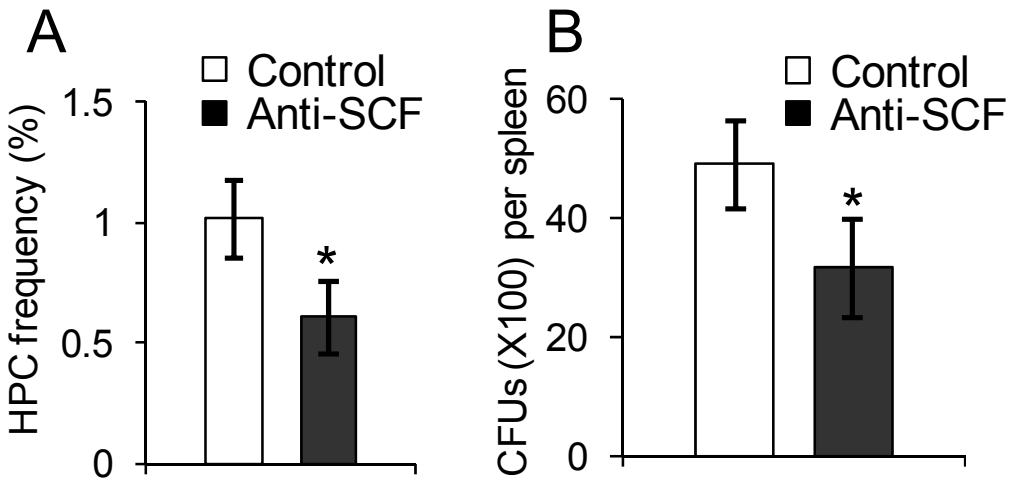

Figure 44. Neutralization of SCF significantly reduced spleen HPC expansion. The effect of anti-SCF administration on splenic HPC frequency (A) and CFU-Cs per spleen (B), were measured in EL4-bearing mice. n=4.

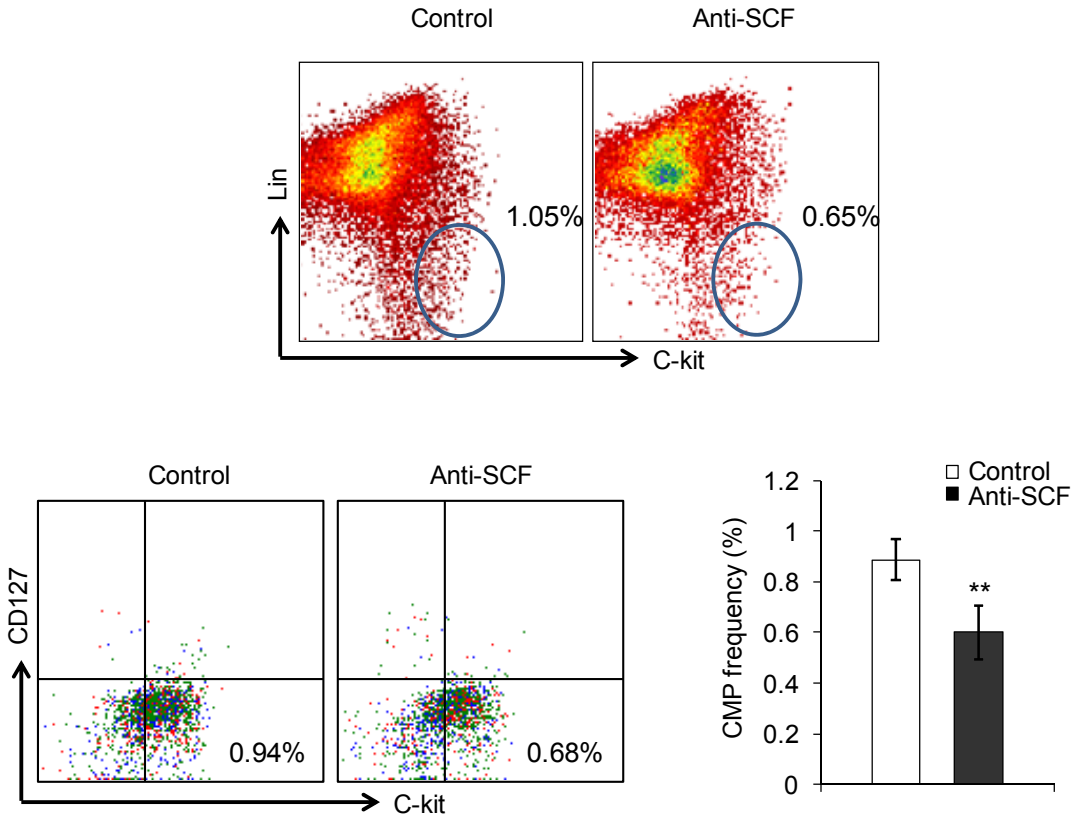

Figure 45. Neutralization of SCF partially inhibited the expansion of HPCs and CMPs in spleen

The effect of anti-SCF administration on splenic HPC and CMP frequency, was measured in EL4-bearing mice and tumor-free control mice using flow cytometry. n=4. Data shown are representative of three independent experiments.

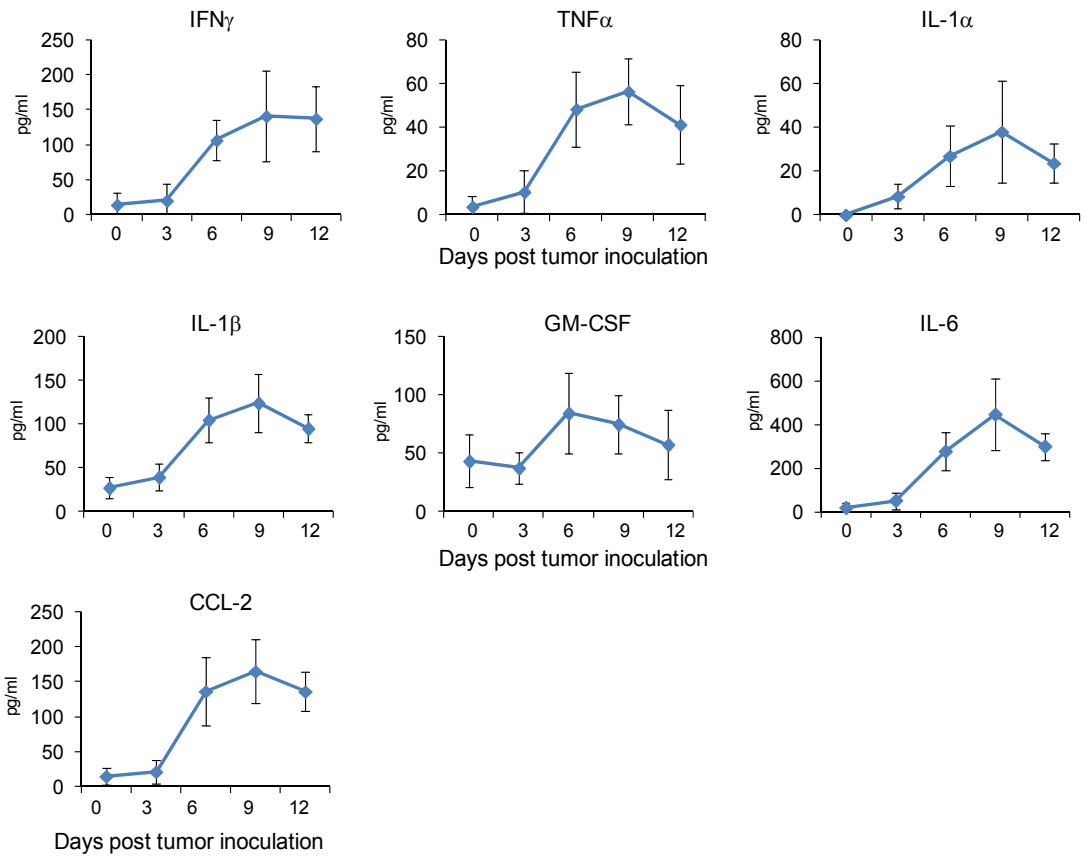

Figure 46. Inflammatory cytokine production in EL4-bearing mice.

At the indicated time points post-EL4 cell inoculation, the mice were sacrificed and the serum cytokines were measured by multiplexed bead array immunoassay. Representative of three independent experiments.

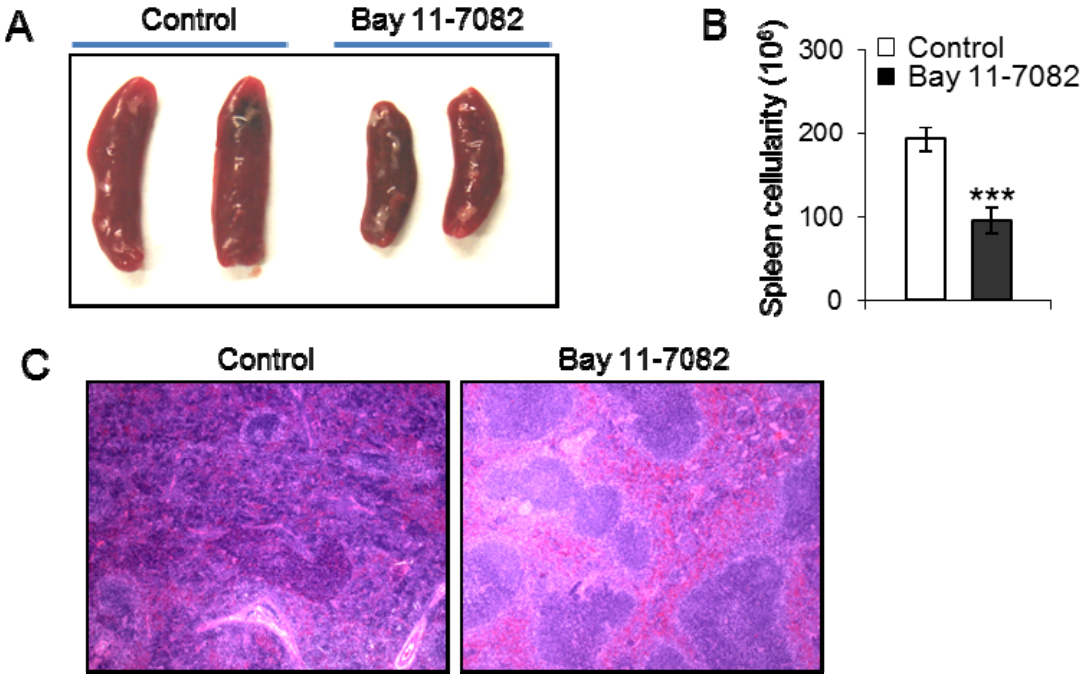

Figure 47. NFκB inhibitor largely abrogated tumor-induced spleen hematopoiesis.

The effect of administration of Bay 11-7082 on spleen size (A), spleen cellularity (B), spleen structure (C) were determined in EL4-bearing mice with and without treatment with Bay 11-7082.

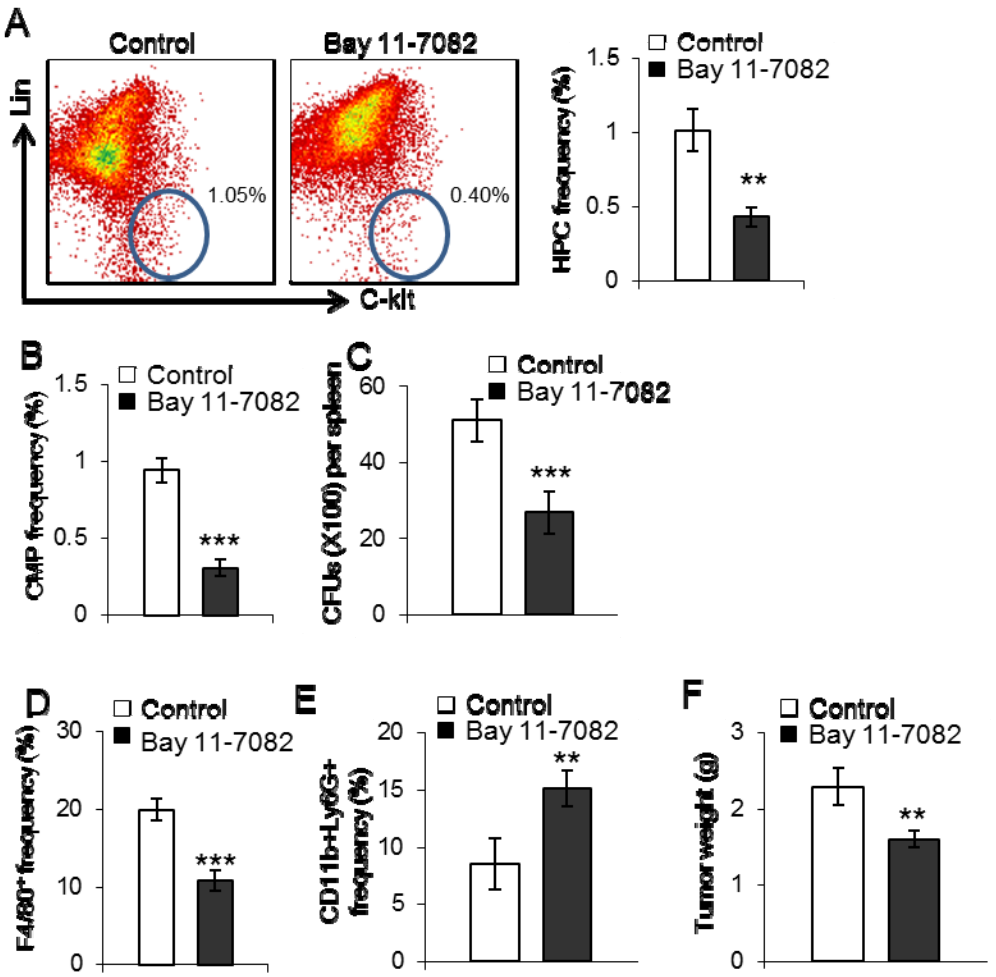

Figure 48. NFκB inhibitor largely abrogated tumor-induced splenic HPC expansion.

The effect of administration of Bay 11-7082 on splenic HPC frequency (A), CMP frequency (B), CFU-Cs per spleen (C), splenic macrophage (D) and granulocytic MDSC (E) frequency, and tumor weight (F), were determined in EL4-bearing mice with and without treatment with Bay 11-7082. n=4.

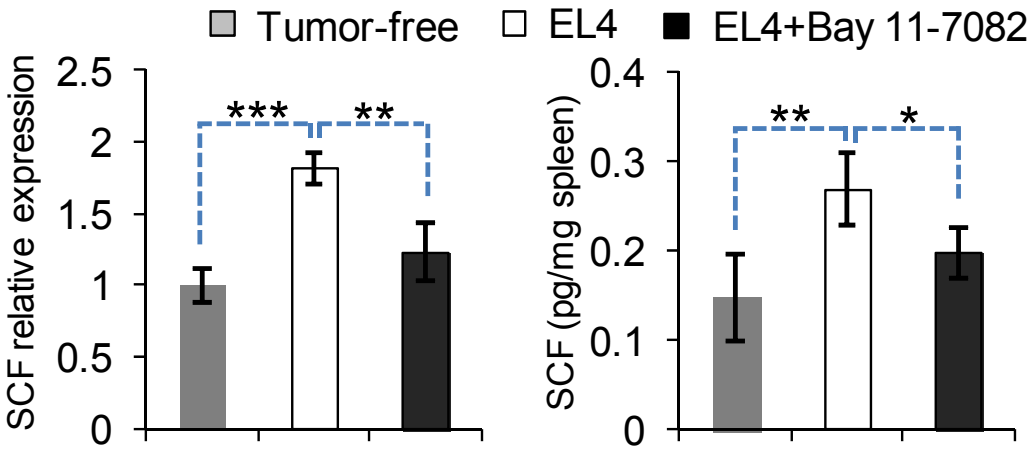

Figure 49. Bay 11-7082 blocked the SCF up-regulation in spleen of tumor-bearing mice in vivo.

SCF expression was measured by real-time PCR in spleens of tumor-free control mice, EL4-bearing mice, and EL4-bearing mice treated with Bay 11-7082. The data were collected on day 8 post EL4 cell inoculation. n=4.

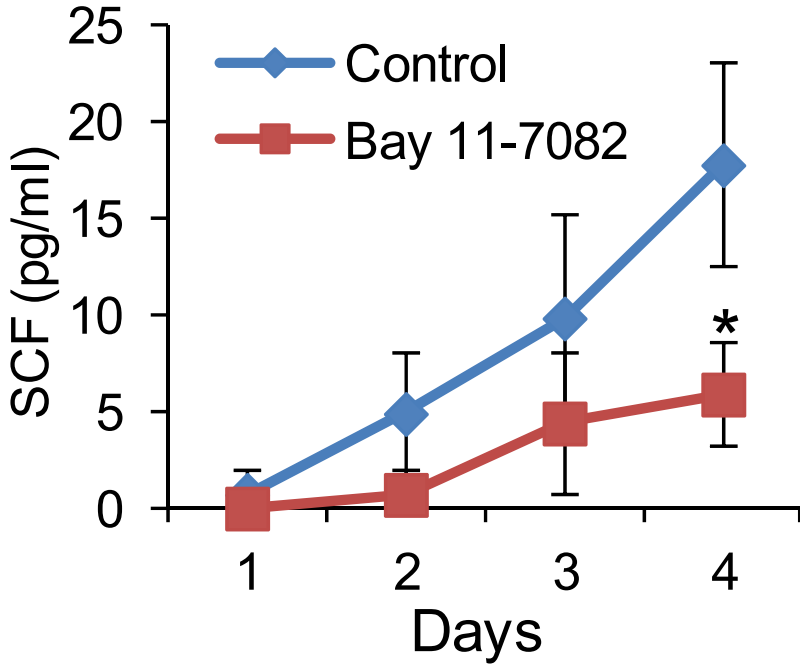

Figure 50. The splenic stromal cells isolated from tumor-bearing mice produced SCF in an NFκB-dependent manner

The splenic cells ($5X10^6$ cell/ml) obtained from EL4-bearing mice were cultured for 3 days in the presence and absence of Bay 11-7082 (5μM). The culture supernatants were then collected for SCF production assay by ELISA (M). n=4.

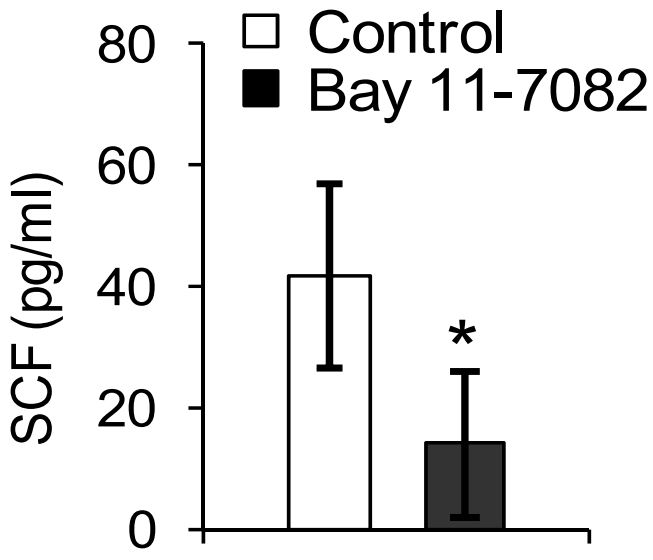

Figure 51. F4/80^{+} macrophage-like splenic stromal cells produced SCF in an NFκB-dependent manner in vitro.

The splenic cells were obtained from EL4-bearing mice. After in vitro culture for 24 hours, the adherent cells were further cultured for 3 days in the presence and absence of Bay 11-7082 (5μM). The culture supernatants were then collected for SCF production assay by ELISA. n=4.

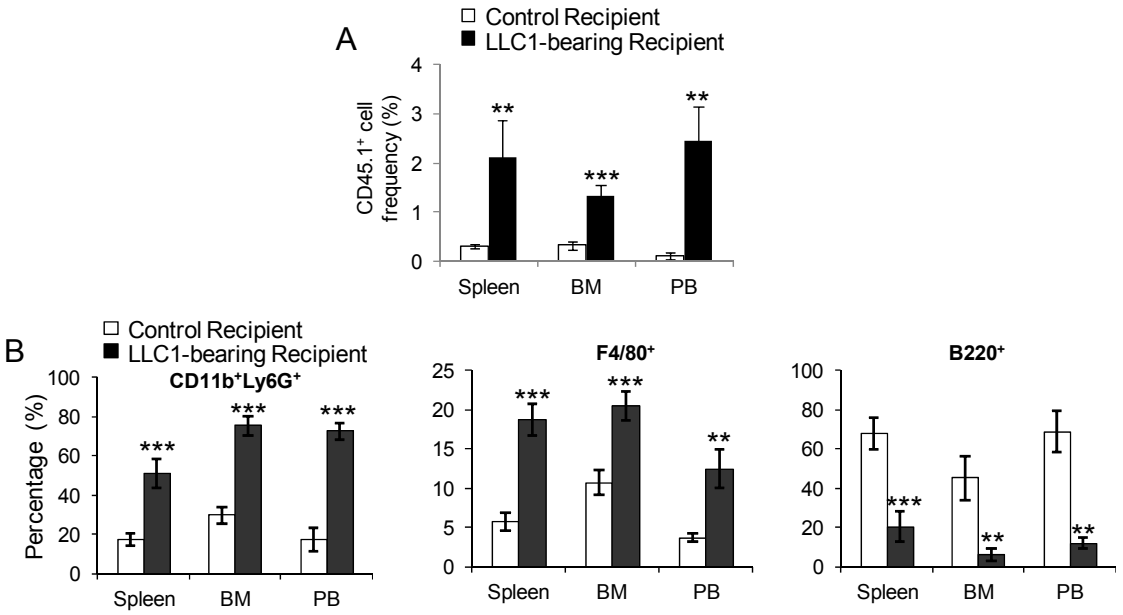

Figure 52. The HPC-myeloid cell expansion loop also applies to mouse carcinoma model

(A). Donor HPCs have increased colonizing capacity in tumor-bearing recipients in LLC1 lung carcinoma model. The experiment was conducted the same way as with **Figure 19B**.

(B). Donor HPCs primarily gave rise to myeloid cells in LLC1-bearing recipients. The experiment was performed the same as with that in lymphoma model (**Figure 21B**).

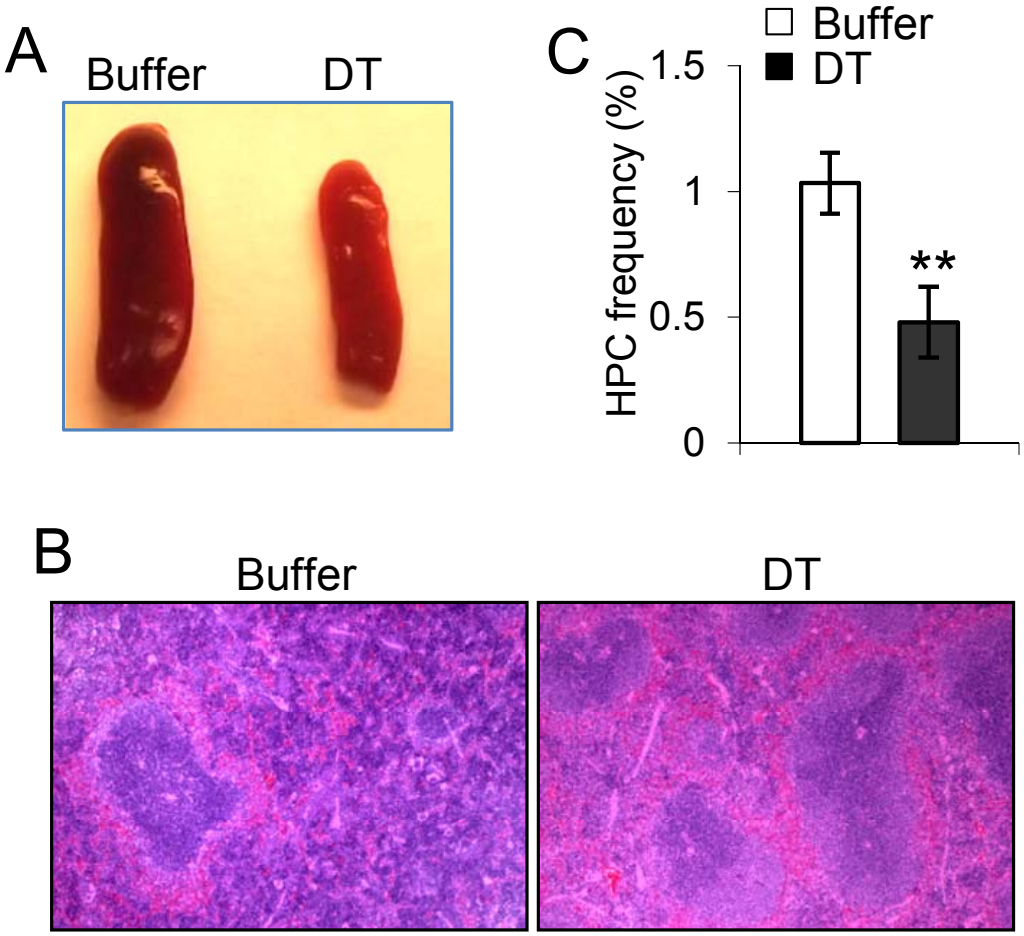

Figure 53. Depletion of macrophages/monocytes inhibited tumor-induced spleen hematopoiesis.

Depletion of macrophages/monocytes reduced spleen size (A), restored the spleen structure (B) and attenuated the elevation of splenic HPC frequency (C) in LLC1-bearing mice.

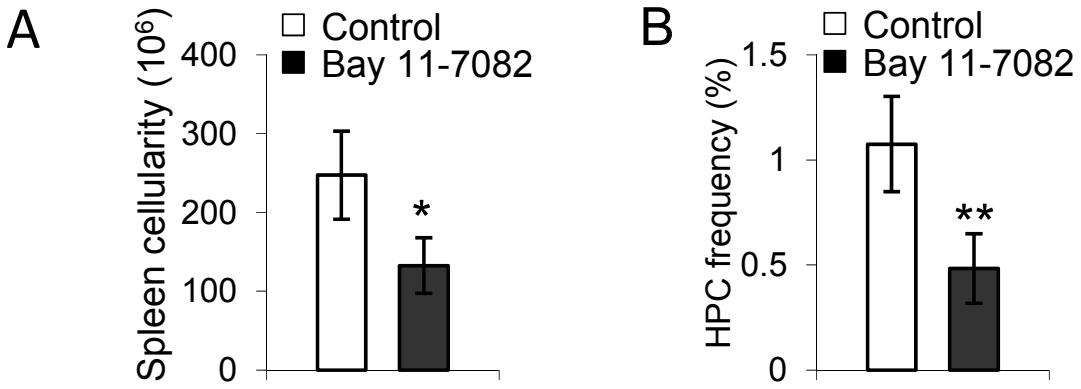

Figure54. NFκB inhibitor largely abrogated LLC1-induced splenic HPC expanison

Bay 11-7082 administration reduced spleen cellularity (A) and splenic HPC frequency(B) in LLC1-bearing mice.

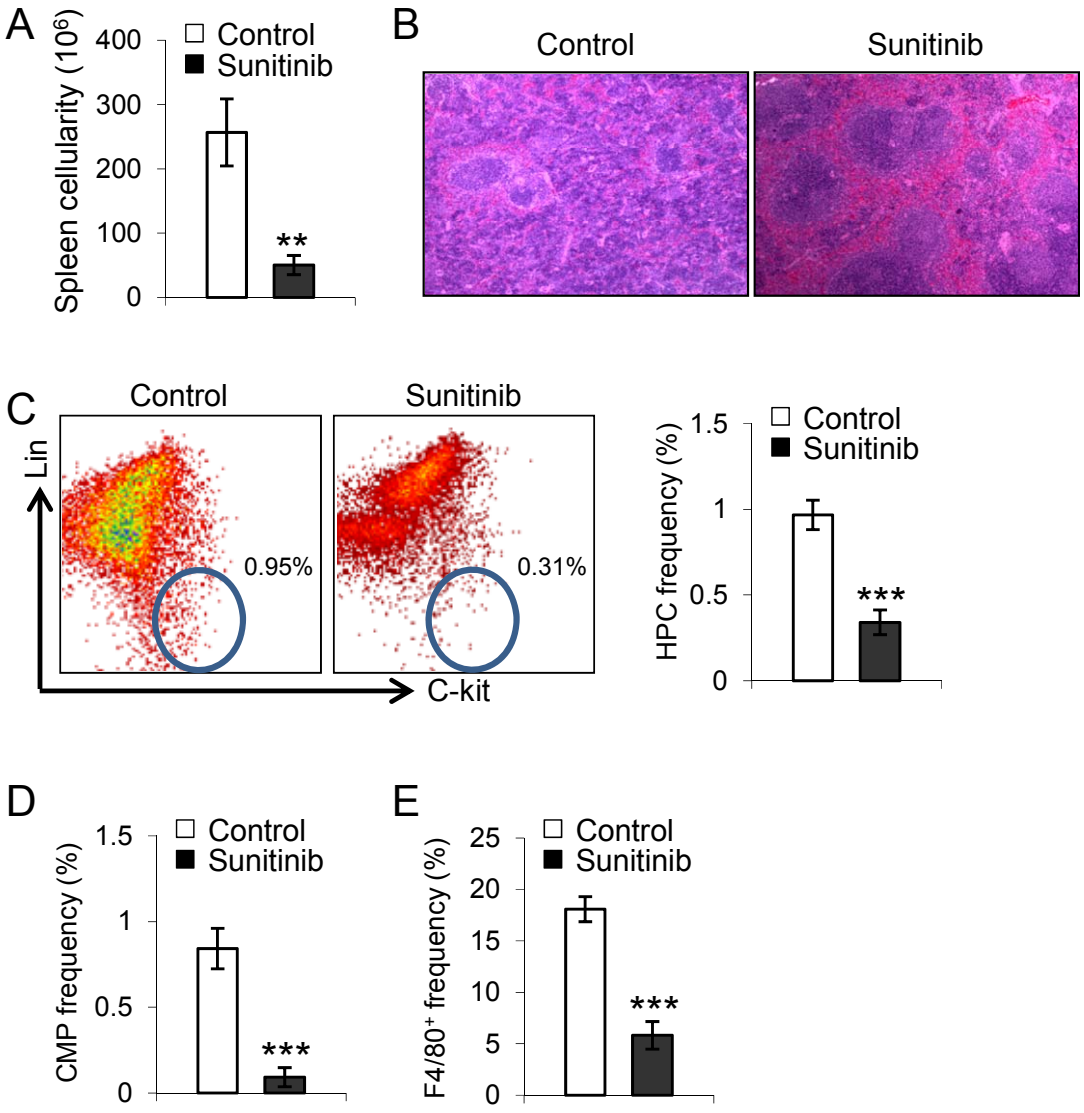

Figure 55. Sunitinib completely abolished LLC1-induced spleen hematopoiesis.

Treatment with sunitinib completely reversed spleen cellularity (A), restored spleen structure (B), reduced HPC (C), CMP (D) and macrophage (E) cell frequency in LLC1-bearing mice.

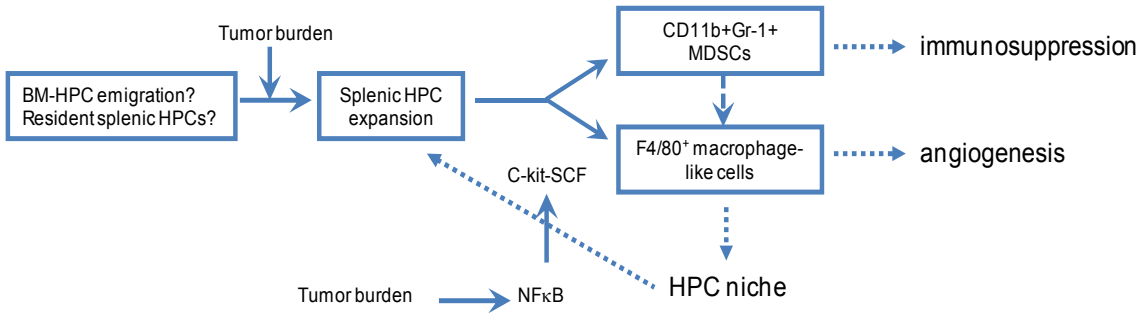

Figure 56. A possible mechanism of splenic HPC-myeloid self-amplifying loop.

Briefly, tumor burden leads to expansion of HPCs in spleen. The expanded HPCs are preferably differentiated into myeloid lineage cells in tumor-bearing hosts, including Ly6G^{+} granulocytic MDSCs and macrophages/monocytes. Macrophages/monocytes, but not granulocytic MDSCs, are critical in further supporting splenic HPC expansion possibly through production of SCF in an NFkB-dependent manner. Thus, splenic HPCs and myeloid cells form a loop to promote their amplification, a process likely to enhance tumor progression.

CHAPTER IV Discussion

Tumor burden disrupts hematopoiesis in bone marrow and leads to extramedullary hematopoiesis

In tumor-bearing mice the number of CFUs in bone marrow is generally reduced. Concomitantly, spleens become enlarged and harbor a greatly increased number of CFUs. The changes in the number of CFUs were correlated with changes in the frequency of HPCs in those two anatomic sites. These results suggest that the function of bone marrow as the major hematopoietic organ is diminished in tumor-bearing mice, and that spleens begin to function as a more important hematopoietic organ. This shift of hematopoiesis from bone marrow to spleen, possibly other extramedullary sites, is reminiscent of myeloproliferative disorders (Walkley et al., 2007b; Walkley et al., 2007a). They probably all involve disruption of HPC niches in bone marrow, enabling more HPCs to migrate to and colonize peripheral organs. It was demonstrated that CXCL12-CXCR4 axis is critical for the retention of HSPCs in bone marrow (Nie et al., 2008; Sugiyama et al., 2006). However, we were unable to detect an elevation of HPCs in peripheral blood (**Figure 16**), suggesting that the expansion of HPCs in spleen probably does not require a spike in the traffic of HPCs from bone marrow to spleen. Inside the spleen of tumor-bearing mice, the local HPCs, or the passing HPCs during steady circulation, may actively expand in situ, thanks to a self-amplifying loop of HPCs-myeloid cells. Some recent studies indeed show that spleens may serve as

important extramedullary hematopoietic organs and are the major sources of monocytes during tumorigenesis, myocardial infarction and atherosclerosis (Swirski et al., 2009; Robbins et al., 2012; Leuschner et al., 2012; Cortez-Retamozo et al., 2012) . Splenic hematopoiesis certainly plays a more important role under certain pathological conditions and, thus, merits greater attention in future studies.

It appears that the environment HPCs reside in, but not the tissue of origin of the HPC per se, determines the route of HPC differentiation. While HPCs isolated from the spleens of tumor-bearing mice primarily give rise to myeloid cells in tumor-bearing hosts, they behave like normal HPCs in tumor-free mice. This indicates that the differentiation routes of HPCs are highly influenced by their environmental cues, and that the spleen as a hematopoietic organ probably more favors myelopoiesis than BM.

Because HSCs in spleen tend to proliferate faster than those in bone marrow, this shift may better meet the demand for more myeloid cells to keep pace with tumor progression. Indeed, the myeloid-biased hematopoiesis will greatly benefit tumor progression, because MDSCs and TAMs are known to promote immunosuppression, angiogenesis and metastasis (Gabrilovich and Nagaraj, 2009; Murdoch et al., 2008; Qian and Pollard, 2010)(Cortez-Retamozo et al.,

2012). We previously showed that IFNγ induces increased hematopoiesis geared toward myeloid cells (Zhao et al., 2010). However, the myelopoiesis associated with tumor burden does not require IFNγ signaling, suggesting that myeolopoiesis in different conditions is not dictated by a single signaling pathway.

We showed that the contribution of donor HPC-derived cells to the pool of host hematopoietic cells was several fold higher in tumor-bearing hosts than in tumor-free hosts, indicating that tumor burden allows a higher rate of colonization by donor HPCs. What is responsible for the enhanced colonization of donor HPCs in the tumor-bearing mice? Colonization would first involve the engraftments of the donor HPCs in their new niches. It was previously shown that clearance of existing HSC niches would greatly increase the contribution of donor HSCs to the chimeric hematopoietic system (Czechowicz et al., 2007). Tumor burden, via the inflammatory environment it creates, might create more niches that accommodate HSPCs. Our results showed that the spleen of tumor-bearing mice allowed more engraftment of donor cells (**Figure 20**). Other possibilities that can be considered include preferential expansion of donor HPCs. It is also possible that HPCs are more likely to be retained in hematopoietic organs other than blood. More studies are needed to gain a more complete understanding of the mechanisms leading to the increased colonization of donor HPCs in tumor-bearing mice.

Monocytes/macrophages, but not MDSCs, contribute to splenic hematopoiesis

The HPCs in spleens were greatly expanded in tumor-bearing mice. Those HPCs are in turn preferentially differentiated into myeloid lineages. We demonstrated that HPCs and myeloid cells form a positive feedback loop in maintaining splenic myelopoiesis in tumor-bearing mice. We showed that monocytes/macrophages, the derivatives of HPCs, are crucial to the expansion of HPCs. In tumor-bearing mice in which monocytes and macrophages were efficiently depleted, splenic hematopiesis was greatly suppressed, as shown by the reduction in the frequency and total cell number of HPCs and CMPs. Remarkably, spleens were also restored to normal size and normal histology by macrophage depletion (**Figure 18**). Thus, the expansion of HPCs in spleen is dependent on monocytes/macrophages (**Figure 56**). While the detailed mechanism by which macrophages/monocytes promote HPC expansion remains to be elucidated, our immunohistochemistry analysis showed that C-kit positive cells, presumably HPCs, are clustered in the area with high density of macrophages. We also demonstrated that splenic macrophages are a source of SCF in vivo and in vitro. Importantly, the splenic hematopoiesis can be significantly suppressed by the inhibition of Kit signaling pathway. Therefore, by providing SCF, the ligand for C-kit, macrophages may serve as niches that can retain and accommodate HPCs and foster their proliferation. Because monocytes/macrophages critically rely on their

expression of SCF for their function in supporting the expansion of HPCs in spleen, these cells are analogous to the endothelial and perivascular cells that maintain HSCs in bone marrow. It was recently shown that when SCF was conditionally deleted in endothelial cells or leptin receptor-expressing perivascular stromal cells in bone marrow, HSCs were greatly depleted (Ding et al., 2012). Interestingly, deletion of SCF in hematopoietic cells or osteoblasts had no such effect. These results establish SCF and some of the SCF-expressing cells as the critical components in HSC niches. Further characterization of SCF-expressing macrophages in spleen of tumor-bearing mice may help to gain more insight into the formation of HSPC niches during EMH.

Several recent studies also showed a pivotal role of macrophages in maintaining endosteal HSC niche in bone marrow (Winkler et al., 2010; Chow et al., 2011; Christopher et al., 2011; Ehninger and Trumpp, 2011). Loss of support by macrophages results in HSC mobilization into blood. HSC mobilization caused by G-CSF is also mediated by loss of macrophages (Christopher et al., 2011). Further studies are needed to determine whether macrophages in bone marrow may play a similar role in promoting hematopoiesis as they do in spleen of tumor-bearing mice.

Inflammatory response-C-kit signaling drives HPC-myeloid cell loop

SCF and its receptor C-kit are essential for hematopoiesis in bone marrow (Broudy, 1997; Czechowicz et al., 2007; Ding et al., 2012). Not surprisingly, C-kit signaling is also involved in the tumor-induced splenic hematopoiesis. We demonstrated that splenic hematopoiesis could be significantly suppressed in three experimental conditions in which C-kit signaling was inhibited or reduced (**Figure 24**).When sunitinib, an inhibitor of C-kit tyrosine kinase, was applied to tumor-bearing mice, it could completely block the elevation of splenic hematopoiesis. In response to sunitinib, the spleens also regained their normal histology. Importantly, the percentage of monocytes/macrophages in spleen of tumor-bearing mice dropped to normal level, suggesting that the supply of new monocytes might be disrupted due to the inhibition of C-kit signaling. Because sunitinib may also reduce splenic hematopoiesis indirectly, by inhibiting tumor growth, we also characterized splenic hematopoiesis in tumor-bearing C-kit$^{+/-}$ mice. As expected, the number of CFUs per spleen in tumor-bearing C-kit$^{+/-}$ mice was significantly lower than in wild-type mice. Furthermore, blockade of SCF by neutralizing antibody could also significantly reduce the frequency of HPCs and the number of CFUs in the spleens of tumor-bearing mice, although it was not as effective as sunitinib. Aside from the potential effect of directly inhibiting tumor growth by sunitinib, the limitation in the amount of antibodies administrated may also be a factor. Nevertheless, C-kit signaling is clearly critical for HPC-myeloid

cell expansion loop in the spleen of tumor bearing mice.

Cancers are known to be associated with increased inflammatory response (Mantovani et al., 2008; Grivennikov et al., 2010). Inhibition of NFκB, the key regulator of tumor-induced inflammatory response, could completely keep splenic hematopoiesis in check in tumor-bearing mice. The suppressive effect of NFκB on splenic hematopoiesis was as effective as that of sunitinib. Interestingly, our in vitro and in vivo studies showed that the production of SCF by macrophages can be significantly inhibited by NFκB inhibitor. Therefore, the upregulation of C-kit signaling pathway that drives splenic hematopoiesis in tumor-bearing mice was due to the inflammatory microenvironment associated with tumor burden. In addition to its association with tumorigenesis, EMH is also involved in many other pathological conditions including myeloproliferative disorders. It would be interesting to test whether the HPC-myeloid cell expansion loop also operates in those conditions.

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PEER-REVIEWED PUBLICATIONS

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