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Dock2 participates in bone marrow lympho-hematopoiesis

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Abstract

Dock2 has been shown to be indispensable for chemotaxis of mature lymphocytes as a critical Rac activator. However, the functional expression of Dock2 in immature hematopoietic cells is unclear. In this study, we demonstrate that Dock2 is broadly expressed in bone marrow (BM) hematopoietic compartment, including hematopoietic stem/progenitor cell (HSC/HPC) fraction. Response of Dock2−/− HPCs to CXCL12 in chemotaxis and actin polymerization *in vitro* was impaired, although α4 integrin activation by CXCL12 was not altered. Myelosuppressive stress on HSCs *in vivo*, such as consecutive 5-FU administration and serial bone marrow transplantation, did not show hematopoietic defect in Dock2−/− mice. Long-term engraftment of transplanted Dock2−/− BM cells was severely impaired in competitive reconstitution. However, this was not intrinsic to HSCs but originated from the defective competition of Dock2−/− lymphoid precursors. These results suggest that Dock2 plays a significant role in BM lymphopoiesis, but is dispensable for HSC engraftment and self-renewal.

Keywords: Hematopoietic stem/progenitor cells; Dock2; CXCR4; CXCL12

Hematopoietic stem/progenitor cells (HSCs/HPCs) are mainly found in the bone marrow (BM) and are largely absent from the peripheral blood (PB) [1]. The dynamic travel from the BM to the PB is a characteristic feature of HSCs [2]. The critical retention axis for HSCs is believed to be chemokine CXCL12, which is produced in the BM microenvironment and its cognate receptor CXCR4, which is expressed on the HSCs, because AMD3100, a specific antagonist for CXCR4, induces HSC egress from the BM [3]. Clinically, granulocyte-colony stimulating factor (G-CSF) is most widely used to induce mobilization of HSCs from the BM to the circulation [2]. Recent studies have revealed that G-CSF induces marked reduction of CXCL12 in BM and bone [4–7]. CXCL12 plays a critical role in BM engraftment of HSCs following transplantation [8,9]. Among known chemokine receptors, CXCR4 is solely functional on HSCs [10]. Thus, identification of molecules, which work in the signaling cascade downstream of CXCR4, is necessary to understand the mechanism of HSC migration.

Cytoskeleton rearrangements are controlled by the members of the Rho guanosinetriphosphatases (GTPases) family [11,12]. The Rho family of small monomeric GTPases includes 22 genes coding for at least 25 proteins which can be divided into 6 families [13]. Among these, the Rac GTPase subfamily has been reported to play an important role in the integration of signals from cell surface receptors, such as CXCR4, c-kit, and β1 integrins leading to the control of cytoskeleton, and the proliferation and survival of HSCs [13].

The CDM family proteins, *Caenorhabditis elegans* CED-5, human Dock180 and *Drosophila melanogaster* Myoblast

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City, are implicated to mediate membrane extension by facilitating upstream function of Rac [14–19]. Dock2, a hematopoietic cell-specific CDM family protein, has been shown to be an essential Rac activator in mature lymphocytes [20]. Dock2-deficient (Dock2−/−) mice exhibit migration defects of T and B lymphocytes, but not of monocytes, in response to chemokines, resulting in several abnormalities, including T lymphocytopenia, atrophy of lymphoid follicles, and loss of marginal-zone B cells.

In this study, we found that Dock2 is broadly expressed in BM hematopoietic compartment including stem/progenitor cell fraction, and assessed its role in BM lympho-hematopoiesis.

Materials and methods

Mice. C57BL/6 mice were purchased from Charles River Japan (Tsukuba, Japan) and used as wild-type (WT) controls. Dock2−/− mice were generated by gene targeting [20] and backcrossed with C57BL/6 mice for more than eight generations before use. Dock2-GFP knock-in mice were generated as previously described [21]. C57BL6-CD45.1 congenic mice were purchased from Charles River Laboratories (Frederick Cancer Research Center, Frederick, MD). All animals used in this study were matched for sex and age in each experiment for comparison purpose (6- to 8-week-old). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Competitive reconstitution. BM nucleated cells (1×10^6 cells) from WT or Dock2−/− mice (CD45.2) were mixed with the same number of competitor CD45.1-BM cells (1×10^6 cells) and then transplanted into lethally irradiated (12 Gy, 2 split doses with 3 h-interval) CD45.1 recipients. Blood was harvested monthly and the origin (CD45.1 or CD45.2) of total leukocytes and each leukocyte subset were evaluated by flow cytometry. To assess the origin of BM hematopoietic reconstitution, mice were sacrificed at one, three, and six months after transplantation. Cell origin was similarly evaluated in B-lineage cells (IgM+B220+ and IgM-B220+), common lymphoid progenitors (CLPs: lineage-IL-7Rα+Sca-1^{dull}c-kit^{dull}), and HSCs (lineage-IL-7Rα-Sca-1+c-kit+) in the BM, and Pro-T cells (CD3-CD4-CD8-c-kit+CD25-) in the thymus.

Serial BM transplantation. Lethally irradiated (13 Gy, 2 split doses with 3 h-interval) WT mice transplanted with WT or Dock2−/− BM cells (4 million cells) were maintained for 1 month, and the BM cells (4 million) from these mice were harvested for secondary transplantation into lethally irradiated WT mice. Mice were observed in this manner of serial transplantation until 4 months (1 month after 4th transplantation).

Antibodies and flow cytometry, *transwell migration assay*, actin polymerization, static adhesion assay, RT-PCR, 5-fluorouracil (5-FU) exposure in vivo, and Statistical analysis. These methods can be found in online supplemental materials.

Results

We first examined Dock2 expression in HSCs/HPCs. RT-PCR revealed that Dock2 mRNA was detectable in mouse hematopoietic progenitor cell line FDCP-mix and lineage-Sca-1+c-kit+ (LSK) cells sorted from the BM of

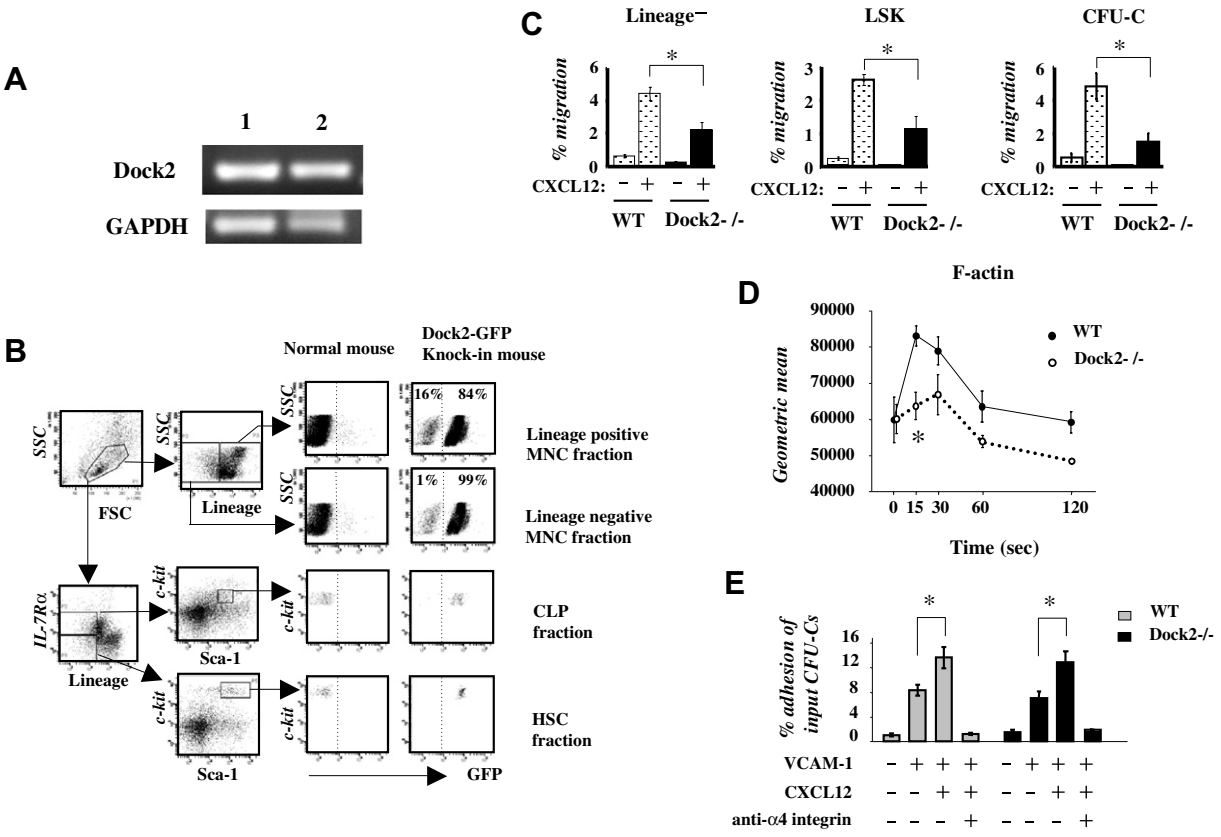

Fig. 1. Impaired CXCR4 function in Dock2−/− progenitors. (A) RT-PCR. 1: FDCP-mix, 2: lineage-Sca-1+c-kit+ (LSK) cells. (B) Flow cytometric analysis of bone marrow cells in Dock2-GFP knock-in mice. (C) Transwell migration toward CXCL12 of lineage- cells ($n = 6$), LSK cells ($n = 6$), and CFU-Cs ($n = 4$). (D) F-actin polymerization in lineage-c-kit+ cells evaluated by Alexa488-phalloidin binding following CXCL12 stimulation ($n = 3$). (E) Static adhesion of wild-type and Dock2−/− CFU-Cs to immobilized VCAM-1 ($n = 4$). $^*p < 0.05$.

wild-type C57BL/6 mice (Fig. 1A). We confirmed this at protein level by using Dock2-GFP knock-in mice. As shown in Fig. 1B, flow cytometric analysis revealed that vast majority of BM cells, including both lineage-positive and -negative mononuclear cell fractions, expressed Dock2. Common lymphoid progenitors (CLPs: lineage-IL-7Rα+Sca-1^{dull}c-kit^{dull}) and HSCs (lineage-IL-7Rα-Sca-1+c-kit+) also expressed Dock2 at high level (Fig. 1B).

We determined the response of Dock2−/− stem/progenitor cells to CXCL12 *in vitro*. Transwell migration of lineage-negative cells ($n = 6$, $p < 0.05$), colony-forming units in culture (CFU-Cs) ($n = 4$, $p < 0.05$) and LSK cells ($n = 6$, $p < 0.05$) from Dock2−/− mice was significantly reduced compared to WT control (Fig. 1C). It was likely due to impaired actin polymerization following CXCR4 signaling upon CXCL12 stimulation (Fig. 1D). We then evaluated α4 integrin expression and function. No difference in α4 integrin expression on the surface of LSK cells was observed between WT and Dock2−/− mice (data not shown). The adhesion of WT CFU-Cs to VCAM-1 was significantly enhanced in the presence of CXCL12, and similar enhancement was observed in Dock2−/− CFU-Cs (Fig. 1E). These data suggest that the major effects of CXCR4 signaling, such as migration and cytoskeleton reorganization, except α4 integrin activation, are highly regulated by Dock2 in HSCs/HPCs.

We next evaluated BM hematopoiesis in Dock2−/− mice. The results were consistent with previous report, wherein blood cell counts were comparable between WT and Dock2−/− mice (data not shown) [20]. As shown in supplemental Fig. S1A, steady-state BM of Dock2−/− mice contained normal number of nucleated cells. Although the number of mature lymphocytes (CD3+ T cells and B220+IgM+ B cells) was slightly reduced, the number of immature cells, such as B220+IgM− B cell precursors, CLPs, and HSCs was completely normal (supplemental Fig. S1B–F). We also tested B lymphoid long-term BM cultures (Whitlock and Witte type) [22] and found that both WT and Dock2−/− BM could establish cobblestone area and produced a comparative number of cells (data not shown). These data suggest that steady-state BM hematopoiesis is normal in the absence of Dock2.

To test the hematopoietic function of Dock2−/− mice under stress, chemotherapy-induced myelosuppression and serial BM transplantation were carried out. Treatment of mice with the antimetabolite, 5-FU, is selectively cytotoxic for cycling hematopoietic cells, induces BM hypoplasia, and can cause lethality. However, quiescent HSCs are thought to be resistant to the effect of 5-FU. When mice were treated weekly with 5-FU at a dose of 150 mg/kg, the survival frequency of Dock2−/− mice was significantly lower than the WT control group (Fig. 2A). However,

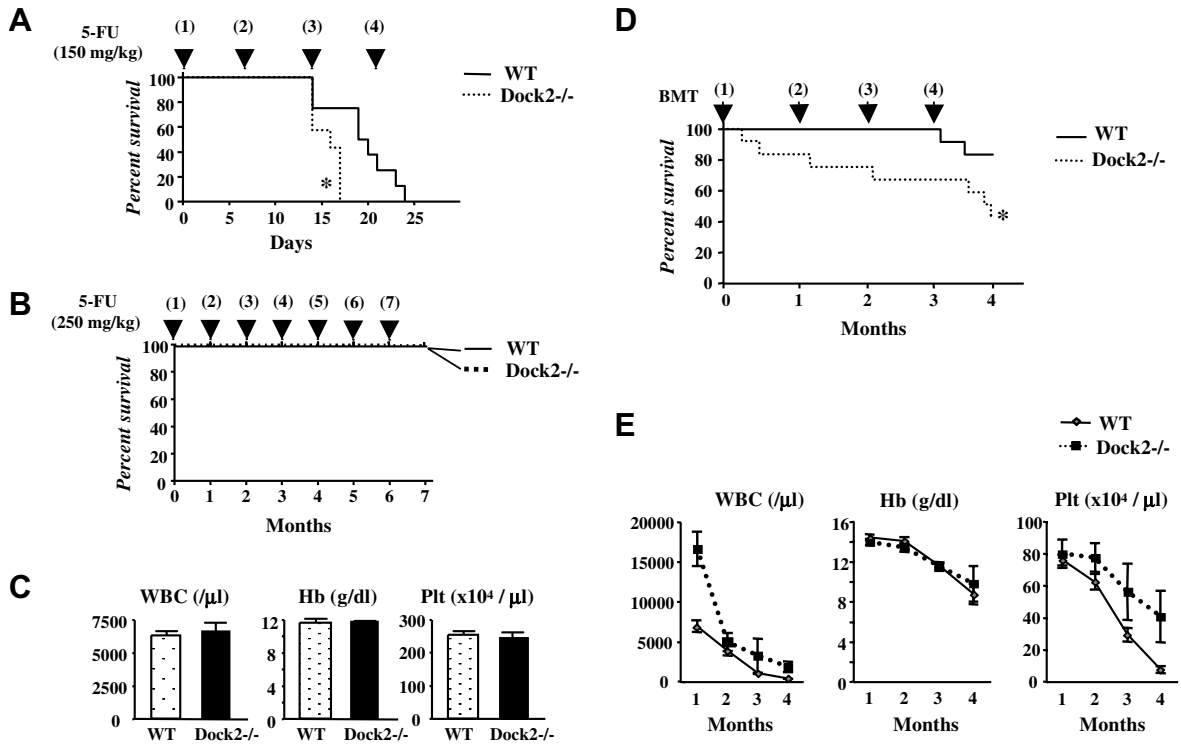

Fig. 2. 5-FU induced myelosuppression and serial bone marrow transplantation (BMT). (A,B) Wild-type or Dock2−/− mice were injected with 5-FU (A: 150 mg/kg, i.v., weekly, $n = 7$–8, B: 250 mg/kg, i.v., monthly, $n = 5$–7). $^{*}p < 0.05$. (C) Blood cell counts at 7 months in experiment (B). (D) Wild-type or Dock2−/− BM cells were transplanted into lethally irradiated wild-type mice. Bone marrow cells were harvested from these mice one month after transplantation for secondary transplantation into lethally irradiated wild-type mice. Survival frequency was assessed until 4 months (1 month after 4th transplantation) in this manner. $n = 12$, $^{*}p < 0.05$. (E) Blood cell counts of live mice at each evaluation point in this serial BMT experiments. WBC, white blood cell; Hb, hemoglobin; Plt, platelet.

when mice were treated monthly with 5-FU at a dose of 250 mg/kg, both WT and Dock2−/− mice survived for more than seven months (Fig. 2B). Blood cell counts were comparable between WT and Dock2−/− mice at seven months after the initial dose of 5-FU (7 doses) (Fig. 2C). We also examined stem-cell repopulating ability under stress of serial BM transplantation as a cell intrinsic model. Lethally irradiated WT mice transplanted with WT or Dock2−/− BM cells (4 million cells) were maintained for 1 month, and the BM cells (4 million) from each mouse were harvested for secondary transplantation into lethally irradiated WT mouse. Mice were observed in this manner of serial transplantation until 4 months (1 month after 4th transplantation). As shown in Fig. 2D, the survival frequency in mice transplanted with Dock2−/− BM was significantly lower than the WT control mice ($n = 12$, $p < 0.05$). However, the blood cell counts in survived mice were similar or even higher in Dock2−/− group compared to WT mice (Fig. 2E). These results suggest that the higher mortality of Dock2−/− mice under myelosuppressive stress was not intrinsic to hematopoietic defect, but probably due to infection or other reasons, and that hematopoietic stem cell engraftment and self-renewal are likely to be normal in the absence of Dock2.

To figure out the Dock2 function in BM lympho-hematopoiesis, we performed competitive repopulation assay. BM nucleated cells from WT or Dock2−/− mice (CD45.2) were mixed with competitor BM cells (CD45.1 congenic) and transplanted into lethally irradiated CD45.1 recipients (Fig. 3A). Blood was harvested monthly and the origin of leukocytes was assessed by flow cytometry. As shown in Fig. 3B, WT and Dock2−/− BM showed similar engraftment one month after transplantation, however, the contribution of Dock2−/− leukocytes severely decreased two to six months after transplantation. Detailed analysis of leukocyte subsets revealed that this reduction was not due to the stem cell defect but originated from selective reduction in lymphocytes, whereas the reconstitution of myeloid lineage cells, including neutrophils (Gr-1+F4/80−), and monocytes (Gr-1^{low}F4/80+) was similar to that of WT control (Fig. 3C). We also checked competitive repopulation of HSCs, CLPs, and B cell precursors in the BM one, three, and six months after transplantation in this competitive setting. As shown in Fig. 4A, the contribution of CD45.2 HSCs against competitor HSCs one month after transplantation was similar between WT and Dock2−/− mice, suggesting that the homing activity and early proliferation of Dock2−/− HSCs were normal. Although there was a mild trend toward reduced contribution of Dock2−/− HSCs (significant at three months), this difference disappeared six months after transplantation. CLPs showed similar trend (Fig. 4A). There was no difference between WT and Dock2−/− B lineage cells, including B220+IgM− B cell

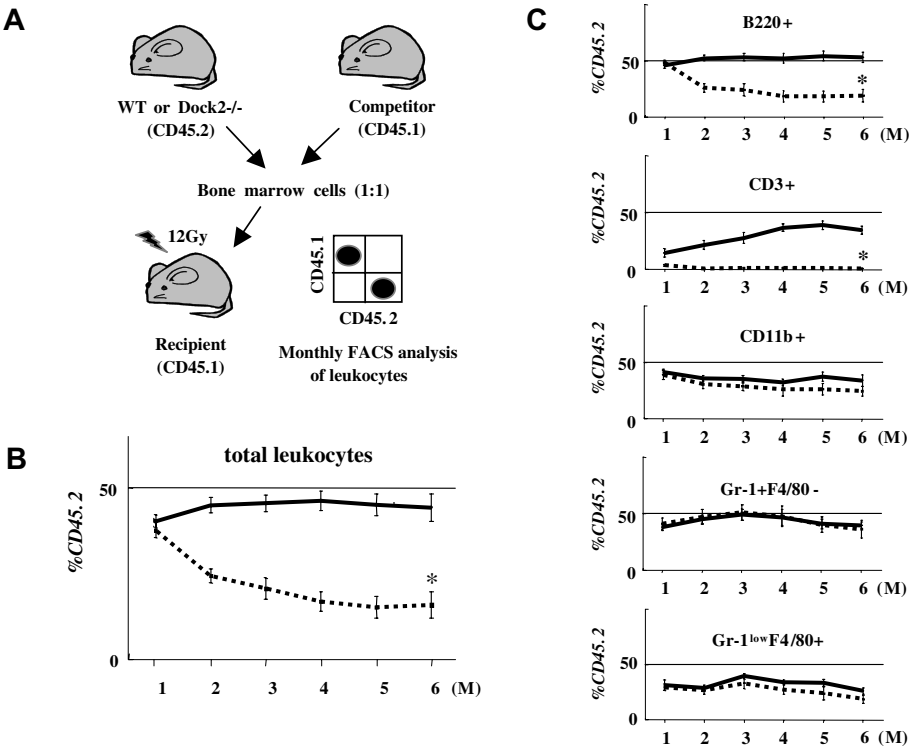

Fig. 3. Competitive reconstitution (peripheral blood). (A) Lethally irradiated wild-type CD45.1+ mice were injected with bone marrow (BM) mixture consisting of one million BM cells from wild-type or Dock2−/− (CD45.2+) mice and one million BM cells (competitor) from wild-type CD45.1+ mice. Recipient mice were bled monthly and the percentage of CD45.1/CD45.2 was assessed. (B,C) Chimerism of peripheral blood leukocytes: (B) in total leukocytes (C) in each cell fraction. $n = 10$, $^{*}p < 0.05$.

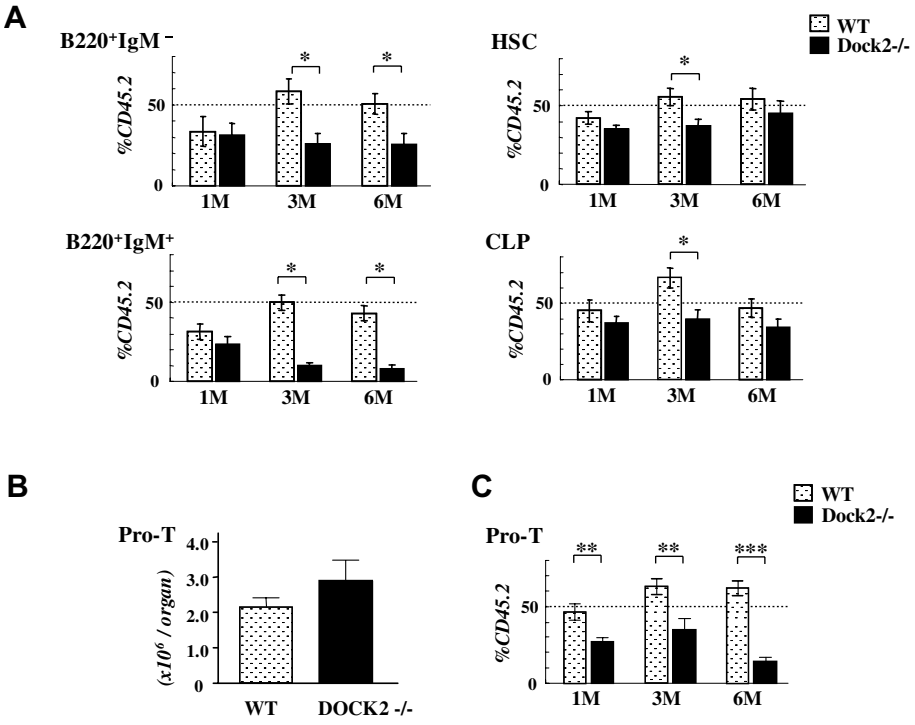

Fig. 4. Competitive reconstitution (bone marrow and thymus). (A) Chimerism of B lineage cells, common lymphoid progenitors (CLP), and hematopoietic stem cells (HSCs) in the bone marrow. (B) Number of pro-T (CD3-CD4-CD8-c-kit+CD25-) cells in the thymus of steady-state wild-type and Dock2−/− mice. (C) Chimerism of pro-T cells in the thymus in competitive reconstitution. M; months. $n = 6$–7 (1M), 8 (3M), 10 (6M) in (A) and (C), $n = 5$ in (B), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

precursors and B220+IgM+ mature B cells, one month after transplantation; however, Dock2−/− cells displayed a significantly inferior repopulation activity in this lineage three and six months after transplantation (Fig. 4A). The numbers of Pro-T (CD3-CD4-CD8-c-kit+CD25-) cells in steady-state thymus were comparable between WT and Dock2−/− mice (Fig. 4B); however, Dock2−/− pro-T cells showed significantly lower repopulation in the competitive setting (Fig. 4C). These results suggest that, although it is not evident in steady-state mice, Dock2−/− cells have defective repopulation activity at pro-T/B cell level.

Discussion

In our study, we demonstrate that Dock2 is broadly expressed in BM hematopoietic compartment including hematopoietic stem/progenitor cells, and the response of Dock2−/− progenitors to CXCL12 in chemotaxis and actin polymerization was impaired but not in CXCL12-induced α4 integrin activation. We also reveal that Dock2 regulates bone marrow lymphopoiesis, but the engraftment, survival and self-renewal of hematopoietic stem cells are likely to be normal in the absence of Dock2.

It has recently been reported that conditional deletion of CXCR4 in adult mice results in drastic reduction of BM precursor/mature B cells and CD34-LSK long-term repopulating stem cells but not CD34+LSK fraction, and the reconstitution by CXCR4-deleted BM was almost abolished including myeloid lineage cells in competitive setting [23]. The numbers of myeloid lineage cells in peripheral blood and lineage-IL-7Rα-Sca-1+c-kit+ cells in BM were comparable between wild-type and Dock2−/− mice at 6 months in our competitive reconstitution assay (Figs. 3 and 4), suggesting that the number and function of most primitive stem cells is normal in the absence of Dock2. The significant difference in HCSs/CLPs at 3 months in this competitive setting (Fig. 4A) suggests that Dock2 might selectively contribute to repopulation of short-term, but not long-term, repopulating stem cells [24]. In steady-state Dock2−/− mice, the numbers of lymphoid precursors (IgM-B220+ pre/pro-B cells in BM and CD3-CD4-CD8-c-kit+CD25- pro-T cells in thymus) were normal or even higher compared to wild-type mice (supplemental Fig. S1, Fig. 4, and [20]). However, competitive reconstitution of the immune system revealed that Dock2−/− lymphoid precursors have a significant defect (Fig. 4). Interestingly, Dock2−/− pre/pro-B cells in the BM were normal at one month after transplantation in competitive reconstitution assay (Fig. 4A), whereas Dock2−/− pro-T cells in the thymus were already decreased in the same mice (Fig. 4C). The proliferation potential in T cell precursors may be more affected by Dock2 deletion than in B cell precursors in the BM. Another possibility is that Dock2 in pro-T cells may play a significant role in egress from the BM and entry to thymus via blood circulation.

In serial transplantation, WBC and platelet counts in survived animals were higher in Dock2−/− mice after the first and the fourth transplantation, respectively

(Fig. 2E). Because Dock2−/− CD4+ T cells produce a large amount of IL-4 [25], it is possible that the altered cytokine network after transplantation might affect the hematopoietic recovery or release of mature cells from the BM to the circulation.

The α4 integrin is an essential adhesion molecule for recruitment of circulating lymphocytes into the lymphoid organs and peripheral sites of inflammation [26,27]. It is also known as a critical mediator for homing/engraftment of HSCs/HPCs following transplantation [28]. Chemokine CXCL12 stimulates α4 integrin adhesive activity in both mature T lymphocytes and immature HSCs/HPCs [8,29]. In contrast to a totally defective chemotaxis toward CXCL12 and actin polymerization [20], it has been reported that T cells from Dock2−/− mice display no alterations compared to wild-type mice in CXCL12-promoted adhesion mediated by α4 integrin [30]. This previous finding and our result suggest that α4 integrin activation in Dock2−/− hematopoietic cells may be compensated by other Rac-associated molecules, such as vav1 [31], Rap1 [32,33], and other CDM family protein. Despite the impaired chemotaxis *in vitro* (Fig. 1C), homing and engraftment of Dock2−/− HSCs/HPCs are not compromised *in vivo* (Figs. 3 and 4). It is possible that the major function of CXCR4 signaling in homing of HSCs/HPCs into BM is the activation of α4 integrin rather than chemotactic recruitment. Consistent with this, it has been reported that the important chemotactic receptor for stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor [34].

Our study revealed that Dock2 regulates CXCR4 signaling in immature hematopoietic cells. Further investigation on modulators of small GTPases may highlight a novel strategy to control the fate of stem cell.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.093.

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