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Inositol trisphosphate 3-kinase B (InsP3KB) as a physiological modulator of myelopoiesis

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Inositol trisphosphate 3-kinase B (InsP3KB) belongs to a family of kinases that convert inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) or IP3 to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4). Previous studies have shown that disruption of InsP3KB leads to impaired T cell and B cell development as well as hyperactivation of neutrophils. Here, we demonstrate that InsP3KB is also a physiological modulator of myelopoiesis. The InsP3KB gene is expressed in all hematopoietic stem/progenitor cell populations. In InsP3KB null mice, the bone marrow granulocyte monocyte progenitor (GMP) population was expanded, and GMP cells proliferated significantly faster. Consequently, neutrophil production in the bone marrow was enhanced, and the peripheral blood neutrophil count was also significantly elevated in these mice. These effects might be due to enhancement of PtdIns(3,4,5)P3/Akt signaling in the InsP3KB null cells. Phosphorylation of cell cycle-inhibitory protein p21cip1, one of the downstream targets of Akt, was augmented, which can lead to the suppression of the cell cycle-inhibitory effect of p21.

RESULTS

Peripheral Blood Neutrophil Count Is Profoundly Increased in InsP3KB Knockout Mice. InsP3KB was previously shown to be involved in T cell development and B cell selection and activation. Consistent with this, the absolute number of lymphocytes in peripheral blood is reduced significantly in InsP3KB knockout mice. On the contrary, peripheral blood neutrophil counts increased 2-fold from 0.63 ± 0.33 × 10⁶ per liter in wild-type mice to 1.21 ± 0.56 × 10⁶ per liter in InsP3KB knockout mice (Fig. 1 and Table 1). No significant difference was detected in the numbers of eosinophils, basophils, and monocytes, suggesting that the increased cell count is specific for neutrophils. A statistically significant decrease was also observed for red blood cell count and hemoglobin (HGB) in InsP3KB knockout mice; however, these changes were much smaller than detected reduction of lymphocyte count.

Neutrophil Apoptotic Death Is Enhanced in InsP3KB Knockout Mice. Neutrophils are terminally differentiated and usually have a very short life span. They die because of programmed cell death or apoptosis. The increased peripheral blood neutrophil count observed in InsP3KB knockout mice could be due to delayed neutrophil death. Thus, we used a well established in vitro system to explore neutrophil apoptotic death (Fig. 2). In this assay, the number of neutrophils undergoing death was quantified by using fluorescence-activated cell-sorting (FACS) analysis. We used


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to 12-week-old mice were used. Values are mean ± SD. * P < 0.01 versus wild-type mice by Student’s t test.

Annexin V, an anticoagulant protein that has high affinity and selectivity for phosphatidylserine (PS), to detect PS exteriorization and 7-AAD, a membrane-impermeable dye, to monitor cell membrane integrity (12). As reported (20), after 24 h of culturing, ~20% of wild-type neutrophils manifest clear morphological signs of apoptosis (Annexin V-positive and/or 7-AAD-positive). Disruption of InsP3KB resulted in a much enhanced apoptosis, with ~50% of cells undergoing apoptotic death in 24 h. This result suggests that increased peripheral blood neutrophil count is not due to delayed neutrophil death, because death of InsP3KB null neutrophils was in fact enhanced compared with the wild-type neutrophils.

Disruption of InsP3KB Promotes Myeloid Differentiation. Mature neutrophils are produced and released from marrow to circulate in peripheral blood, a process called neutrophil mobilization. Various cytokines, such as G-CSF and IL-8, can modulate the efficiency of mobilization. The increased peripheral blood neutrophil count in InsP3KB knockout mice may be a result of enhanced neutrophil mobilization. If this is the case, the number of neutrophils in bone marrow should be decreased in the knockout mice. However, such a decrease was not detected in InsP3KB knockout mice (Table 1), suggesting that alteration of neutrophil mobilization is not contributive to the increased peripheral blood neutrophil count in these mice.

The increased peripheral blood neutrophil count could be simply due to augmentation of neutrophil production in the bone marrow. To test this, we first measured the number of each hematopoietic progenitor cell type by using FACS analysis. We found that InsP3KB knockout mice had more bone marrow granulocyte monocyte progenitors (GMP) than control wild-type mice, as measured by the percentage of Lin−Sca-1+c-KIT−FcyRII/IIIcCD34+ cells in bone marrow. Because the common myeloid progenitor (CMP) population was unchanged, the increase of GMP counts suggested an enhancement of cell differentiation/proliferation of myeloid progenitor cells in InsP3KB knockout mice. Disruption of InsP3KB did not alter the amount of hematopoietic stem cells (Lin−Sca-1+c-KIT−), common lymphoid progenitors (CLP) population, and megakaryocyte erythroid progenitors (MEP) in bone marrow (Fig. 3 A and B and Table 2), suggesting that InsP3KB is a specific modulator of myelopoiesis.

The lineage analysis using flow cytometry suggested that the differentiation of multipotent hematopoietic stem cells toward GMP is somewhat enhanced by InsP3KB depletion, because more GMP cells were observed in the knockout mice. To further confirm this, we used a quantitative granulocyte monocyte colony-forming unit (CFU-GM) assay to functionally assess the number of committed myeloid progenitors in bone marrow (Fig. 3 C and D). As expected, InsP3KB knockout mouse-derived bone marrow contained more CFU-GM (99/10,000 bone marrow cells) than control wild-type mouse-derived bone marrow (52/10,000 bone marrow cells).

Disruption of InsP3KB Leads to Increased Proliferation of Myeloid Progenitor Cells. In the colony-forming unit assay described above, we noticed that elevation of CFU-GM colony number was accompanied by a dramatic increase in the number of cells per colony, particularly for the first 3 days in culture. For example, after 48 h, 12% of InsP3KB knockout bone marrow-derived colonies contained >32 divided cells, compared with only 7% for wild-type bone marrow-derived colonies (Fig. 4 A and B). A similar result was obtained when purified GMP cells were used (Fig. 4C). These results indicate an enhancement of proliferation of InsP3KB null myeloid progenitor cells. We further tested this
hypothesis using a flow-cytometric analysis of hematopoietic progenitors stained with intracellular fluorescent dye 5-(and -6)-carboxyfluorescein diacetate succinimidyl esters (CFSE) (Fig. 4 D–F). The CFSE label is stable, and only a modest reduction in fluorescent intensity was detected in 3 days in nondividing cells (data not shown). Thus, the serial halving of the fluorescence intensity of the CFSE-stained cells has been widely used to measure cell division and proliferation in various cell types. Cell labeling with CFSE is quite heterogeneous, with a broad fluorescent peak observed in flow-cytometry analysis (Fig. 4D). To improve the resolution of divisional clusters, we presorted cells based on a narrow gate of CFSE intensity (Fig. 4D). Consistent with what was observed in the colony assay, after 2 days of incubation in methylcellulose culture, 87% of InsP3KB null cells underwent at least one cell division, compared with only 70% for wild-type cells. In addition, it appeared that the time to finish a cell cycle was also significantly shortened in InsP3KB knockout cells. During the 48-hour incubation period, 21% of knockout progenitors accomplished four cell divisions with a doubling time of ≈12 h, whereas most wild-type progenitors can only finish fewer than three cell divisions with a doubling time of ≈18 h (Fig. 4E and F). Because cell death does not occur in the first 2 days of culturing, the decrease of CFSE intensity caused by cell death is minimal and should not affect data analysis. Collectively, our results demonstrated that disruption of InsP3KB can lead to a much higher proliferation rate in myeloid progenitor cells.

**Disruption of InsP3KB Results in Enhanced PtdIns(3,4,5)P3 Signaling in Hematopoietic Progenitors.** To further understand the mechanism by which InsP3KB regulates hematopoiesis, we first examined whether InsP3KB is expressed in hematopoietic progenitors (Fig. 5A). There are three InsP3K isoforms in mammalian cells. To determine which isoform(s) are expressed in hematopoietic progenitors, we conducted RT-PCR using each sorted hematopoietic cell type (Fig. 5A). To improve the resolution of divisional clusters, we presorted cells based on a narrow gate of CFSE intensity (Fig. 5A). Consistent with what was observed in the colony assay, after 2 days of incubation in methylcellulose culture, 87% of InsP3KB null cells underwent at least one cell division, compared with only 70% for wild-type cells. In addition, it appeared that the time to finish a cell cycle was also significantly shortened in InsP3KB knockout cells. During the 48-hour incubation period, 21% of knockout progenitors accomplished four cell divisions with a doubling time of ≈12 h, whereas most wild-type progenitors can only finish fewer than three cell divisions with a doubling time of ≈18 h (Fig. 5E and F). Because cell death does not occur in the first 2 days of culturing, the decrease of CFSE intensity caused by cell death is minimal and should not affect data analysis. Collectively, our results demonstrated that disruption of InsP3KB can lead to a much higher proliferation rate in myeloid progenitor cells.

**Table 2. Percentage of hematopoietic progenitor cell subsets in bone marrow**

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<th>LK</th>
<th>CMP</th>
<th>GMP</th>
<th>MEP</th>
<th>LSK</th>
<th>CLP</th>
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<td>Wild type</td>
<td>1.55 ± 0.70</td>
<td>0.21 ± 0.12</td>
<td>0.52 ± 0.22</td>
<td>0.70 ± 0.40</td>
<td>0.10 ± 0.07</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>InsP3KB−/−</td>
<td>1.80 ± 0.72</td>
<td>0.20 ± 0.11</td>
<td>0.75 ± 0.34</td>
<td>0.65 ± 0.17</td>
<td>0.12 ± 0.07</td>
<td>0.10 ± 0.05</td>
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</tbody>
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<tbody>
<tr>
<td>P</td>
<td>0.0916</td>
<td>0.7852</td>
<td>0.0089</td>
<td>0.6448</td>
<td>0.4302</td>
<td>0.0867</td>
</tr>
</tbody>
</table>
PtdIns(3,4,5)P3 signaling. Disruption of InsP3KB in neutrophils substantially enhances PtdIns(3,4,5)P3 signaling (9). Here, we investigated whether PtdIns(3,4,5)P3 signaling was also augmented in InsP3KB null progenitor cells. As described (9), we monitored PtdIns(3,4,5)P3 signaling by measuring phosphorylation of Akt. Similar with what was observed in neutrophils, Akt phosphorylation was significantly augmented in enriched InsP3KB null progenitor cells, whereas the total amount of Akt protein was unaltered (Fig. 5 B and C). One mechanism by which the PtdIns(3,4,5)P3/Akt pathway regulates cell cycle is via modulating the phosphorylation of cell cycle-inhibitory protein p21^{CIP1}, which is a target of Akt (13). Activation of p21^{CIP1} leads to G_{1} phase arrest and has been implicated in maintaining the quiescence of hematopoietic stem cells (14). Akt-dependent phosphorylation of p21^{CIP1} at Thr-145 diminishes the cell cycle-inhibitory effect of p21^{CIP1} and thus promotes cell cycle progression. Consistent with the enhanced Akt phosphorylation in InsP3KB null progenitor cells, the level of phosphorylated p21^{CIP1} was also increased in these cells, suggesting that the accelerated proliferation of myeloid progenitor cells may be a result of PtdIns(3,4,5)P3/Akt-mediated deactivation of p21^{CIP1} (Fig. 5 D and E).

Discussion
In the present study, we established a role for InsP3KB in myelopoiesis. Disruption of InsP3KB resulted in accelerated death of mature neutrophils but an elevated peripheral blood neutrophil count, indicating an augmentation of neutrophil production from bone marrow. FACS analysis of bone marrow cells revealed an elevation of GMP population in the knockout mice. Detailed cell cycle analysis showed a much shortened GMP cell cycle in InsP3KB null cells. Because this alteration was observed in an in vitro colony assay, the effect of InsP3KB on myelopoiesis is likely intrinsic to the progenitors rather than from the stromal environment. The accelerated proliferation of GMPs may be a result of enhanced PtdIns(3,4,5)P3/Akt signaling in the InsP3KB null cells. As a result, the phosphorylation of cell cycle-inhibitory protein p21^{CIP1}, a target of Akt, is enhanced, leading to a diminished cell cycle-inhibitory effect of p21^{CIP1}. Collectively, these results established InsP3KB as a negative regulator of myeloid differentiation and neutrophil production and is consistent with a recent report that demonstrated a crucial role of Akt in myelopoiesis (16).

It is worth pointing out that other mechanisms may also contribute to the observed hematopoiesis alteration in InsP3KB knockout mice. Ins(1,3,4,5)P4, the product of InsP3KB, can exert its function via binding to other cellular targets. Several proteins, including GAP1/Pibr (also known as Rasa3), a-catenin, and GAP1p, also specifically interact with Ins(1,3,4,5)P4 (17, 18), suggesting that the functions of these proteins might also be regulated by Ins(1,3,4,5)P4. Huang et al. (19) demonstrated that Ins(1,3,4,5)P4 can also bind to the PH domain of Tec family tyrosine kinase Itk, which plays an important role in TCR signaling. Interestingly, this binding changes the conformation of the Itk PH domain and subsequently promotes, instead of suppresses, the PH domain binding to PtdIns(3,4,5)P3 in T cells. Whether a similar mechanism also exists in hematopoietic progenitors was not investigated. InsP3K has also been reported to be a potential modulator of calcium mobilization, because it can decrease the level of Ins(1,4,5)P3, which mediates calcium release from internal stores, by converting it to Ins(1,3,4,5)P4. Miller et al. (7) recently reported that Ins(1,3,4,5)P4 regulates B cell selection and activation via modulating store-operated calcium channels. A much elevated calcium influx was detected in...
InsP3KB null B cells. Surprisingly, in InsP3KB null T cells, no substantial defects in Ins(1,4,5)P3 amounts or calcium mobilization was detected (5, 6). In neutrophils, disruption of InsP3KB does not affect the overall calcium signaling in the presence of extracellular calcium (9). However, more detailed investigation revealed a much decreased calcium release from intracellular stores and an enhanced calcium influx through store-operated calcium channels in InsP3KB null neutrophils stimulated with chemokines. The reduction of calcium release from intracellular stores appears to be a result of calcium depletion from the store (Y.J. and H.R.L., unpublished data). Whether disruption of InsP3KB can lead to abnormal calcium signaling in hematopoietic progenitor cells needs to be further investigated. Finally, although InsP3KB is known as an inositol phosphate kinase, we cannot completely rule out the possibility that some InsP3KB-related cellular functions might be mediated by kinase-independent mechanisms (e.g., as protein partner).

In current study, we showed that hematopoietic progenitor cell populations such as CMP, GMP, MEP, and CLP, express both InsP3KB and InsP3KC isoforms. Interestingly, InsP3KA, which was previously thought to be expressed solely in brain, was also detected in CMP and MEP populations. Because multiple InsP3K isoforms are expressed in hematopoietic progenitor cells, it will be intriguing to see whether a more dramatic phenotype in hematopoiesis can be observed when two or three InsP3K isoforms are deleted.

An unexpected result observed in this study is the accelerated apoptotic death of InsP3KB null neutrophils. We recently established PtdIns(3,4,5)P3/Akt deactivation as a causal mediator of neutrophil spontaneous death (12). Accordingly, because Akt activation was significantly enhanced in InsP3KB null neutrophils (9), a prolonged survival was expected in these neutrophils. The unexpected augmented death of InsP3KB null neutrophils is likely caused by other cellular defects in these cells. Ins(1,3,4,5)P4-mediated cell death was also observed in B cells (8) and primary pyramidal neurons (20). Marechal et al. (8) showed that Ins(1,3,4,5)P4 negatively regulates Ptdlns(4,5)P2-mediated Rasα3 plasma membrane translocation and activation in B cells. Rasα3 is a GAP (GTPase activating protein) acting on Ras. Depletion of intracellular Ins(1,3,4,5)P4 leads to enhanced plasma membrane translocation and activation of Rasα3 and thus reduces Ras/Erk activation, which, in turn, causes enhanced cell death and impaired development of InsP3KB null B cells. However, a similar mechanism was not detected in neutrophils. In fact, Rasα3 is barely expressed in neutrophils (data not shown).

Methods
Mice. InsP3KB-deficient mice were generated as described (5). Mice aged 8–14 weeks were used in this study. All procedures were approved and monitored by the Children’s Hospital Animal Care and Use Committee.

Complete Blood Count. Orbital peripheral blood (250 µl) was collected into K$_2$-EDTA-coated tubes. A complete blood count (CBC) and white blood cell count was analyzed by using ADVIA-120B Blood Analyzer (Bayer).

Flow-Cytometry Analysis and Cell Sorting. Bone marrow cells were flushed out from two femurs and two tibia into PBS supplemented with 2% FBS. Red blood cells were lysed by incubation with 600 µl of 1× ACK buffer (Invitrogen) at room temperature for 5 min. For sample analysis, five million bone marrow cells were incubated with an antibody mixture including antibodies for lineage markers (CD3ε-PE, CD4-PECy5, CD8-PECy5, CD19-PECy5, Gr1-PECy5, B220-PECy5), cKit-APC, Scal-biotin, CD34-FITC, and FcγRIII R-PE antibodies for 45 min on ice. After nonspecifically bound antibodies were removed by washing twice with 3 ml of PBS-2% FBS, IL7Rα-PE and Streptavidin-APC-Cy7 were added and then incubated on ice for another 30 min. Cells were washed and filtered before FACS analysis. All data were collected on a BD FACSAria II flow cytometer, and data analysis was performed by using FlowJo software. Fluorescence compensation was performed by using single fluorochrome-labeled wild-type mouse spleen cells. This was achieved by single-antibody staining with B220-PECy5, PE, FITC, APC, PE-Cy7, or APC-Cy7 antibodies. For purification of various progenitors, bone marrow cells from 1–3 wild-type mice were pooled together and stained as described above with the proportionally increased amount of antibody according to the cell number.

CFU-GM Assays. Bone marrow cells from wild type or InsP3KB deficient mice were seeded in semisolid Methocult GF M3534 medium containing rmSCF, rmFlt-3, and rhh-L6 (MethoCult GF M3534; Stem Cell Technology). Five thousand cells were used for each 35-mm nontissue culture dish. On day 3, the number of cells in each cell cluster (those containing more than two cells per cluster) were recorded and analyzed. Colonies that contained >30 cells were counted on day 7.

Cell Proliferation Analysis. Mouse low-density bone marrow cells were isolated as described (21). Purified cells were resuspended in PBS at a density of 10 million cells per milliliter. CFSE was then added to final concentration of 5 µM. After incubation for 10 min at 37°C, the cell population (~20%) were sorted by using a BD FACSaria flow cytometer (Fig. 4D). Sorted cells were recovered, mixed with Methocult GF M3534 medium, plated on 24-well nonadhesive culture dish (0.2 million cells per well), and incubated for 2 days. The cell division was analyzed by using a FlowJo software (proliferation analysis), which measures the serial halving of the fluorescence intensity of the CFSE stained cells.

RNA Isolation and RT-PCR. The total RNA was extracted by using Absolute MicroRNA kit (Stratagene). One-step RT-PCR was conducted by using a SuperScript One-Step RT-PCR system (Invitrogen). PCR amplification was performed by using primers specific for InsP3KA (5'-TGCACTGACTGTCTTACAAAC-3' and 5'-CCCTCCAGGGCTCCCTCGAG-3'), InsP3KB (5'-CGAGGTGATGCAGACGAGC-3' and 5'-CCTCCAGTGTGTTGCAGTCACA-3') or InsP3KC (5'-CTTCACTGCTGTCAGTATACACGAA-3' and 5'-CCCTCCAGGGCTCCCTCGAG-3'). GAPDH (5'-GGTGGTCTGATATGCGAGTAC-3' and 5'-GGTGGTCTGATATGCGAGTAC-3') gene was used as a control.

Western Blot Analysis. Low-density bone marrow cells (0.5 × 10^5 cells) were lysed with 100 µl of boiling protein loading buffer. After a brief sonication (5–10 sec,
10 µl of lysate was used for Western blot analysis. Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-Phospho-Akt (Ser-473) antibodies (Cell Signaling Technologies), respectively. Total p21 and phosphorylated p21 were detected with anti-p21 (C19) and anti-Phospho-p21 (Thr-145) antibodies (Santa Cruz Biotechnology), respectively.

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