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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 substantially 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.

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 × 10^6 per liter in wild-type mice to 1.21 ± 0.6 × 10^6 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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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− FeR11/II/III+CD34+ 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 deletion, 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 drastic 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 InsP3B null myeloid progenitor cells. We further tested this

![Fig. 1. The peripheral blood neutrophil count was increased in InsP3KB knockout mice. Peripheral blood cell counts (n = 24 for wild type and n = 15 for InsP3KB knockout) and bone marrow cell counts (n = 5) in wild-type and knockout mice were obtained by using an ADVIA-120B Blood Analyzer. Eight-to 12-week-old mice were used. Values are mean ± SD. *P < 0.01 versus wild-type mice by Student’s t test.](image)

![Fig. 2. InsP3KB deletion enhanced spontaneous neutrophil death. (A) Mouse bone marrow neutrophils were cultured in RPMI medium 1640 containing 10% FBS at a density of 2 × 10^6 cells per milliliter. Apoptotic cells were detected by Annexin V-FITC staining and 7-AAD staining. Ten thousand cells were collected at indicated time points and analyzed by using the Flowjo software. Region R1, viable cell; Region R2, early apoptotic cells; Region R3 and R4, late apoptotic cells and necrotic cells. (B) Time course of neutrophil spontaneous death. All values represent mean ± SD of four separate experiments.](image)

### Table 1. Peripheral blood and bone marrow cell numbers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>InsP3KB−/−</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow (×10^9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell</td>
<td>41.0 ± 7.1</td>
<td>36.4 ± 6.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>9.1 ± 3.5</td>
<td>7.80 ± 2.9</td>
<td>0.26</td>
</tr>
<tr>
<td>Peripheral blood (×10^3 per milliliter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total white blood cell</td>
<td>5.4 ± 1.9</td>
<td>4.1 ± 2.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.63 ± 0.33</td>
<td>1.21 ± 0.60</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>4.36 ± 1.56</td>
<td>1.76 ± 0.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Monocyte</td>
<td>0.07 ± 0.04</td>
<td>0.05 ± 0.03</td>
<td>0.146</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.26 ± 0.19</td>
<td>0.33 ± 0.26</td>
<td>0.44</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.04 ± 0.03</td>
<td>0.03 ± 0.03</td>
<td>0.35</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>9.5 ± 0.6</td>
<td>8.7 ± 0.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>14.5 ± 1.2</td>
<td>13.7 ± 1.2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

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 one 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 progenitor subpopulation. InsP3KB was detected in all cell populations examined, with a slightly lower expression in HSC and CLP populations. Interestingly, InsP3KA, which was previously thought to be expressed solely in brain (2–4), was also detected in the CMP and MEP populations, suggesting that it may play a role in megakaryocyte erythropoiesis differentiation. Because of the lack of enough material for Western blot analysis, we currently cannot monitor the protein level of each individual InsP3K isoform in hematopoietic progenitors.

**PtdIns(3,4,5)P3 signaling has been well documented as a cell cycle-promoting factor.** Recently, we demonstrated that InsP3KB and its product Ins(1,3,4,5)P4 negatively regulate...
Fig. 4. Disruption of InsP3KB accelerated cell division of myeloid progenitor cells. (A) The in vitro CFU-GM assay was conducted as described in Fig. 3. BMMCs were used in this assay. Shown are representative pictures of cell clusters/colonies at days 2 and 3. (B) The number of cell clusters containing the indicated number of cells was recorded and analyzed at day 3. (C) The in vitro CFU-GM assay was conducted by using purified GMP cells. Data shown are mean ± SD. (D and E) Flow-cytometric analysis of cell division of hematopoietic progenitors. The enriched progenitor cells were labeled with CFSE. To improve the resolution of divisional clusters, we resorted cells based on a narrow gate of CFSE intensity. Sorted CFSE-labeled cells were cultured at a density of 2 × 10^5 cells per milliliter in MethoCult GF M3534 for 48 h. Cell divisions were analyzed by serial halving of the fluorescence intensity of the CFSE-stained cells. Shown is a representative profile of three independent experiments. The % of Max is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells. They were calculated by using FlowJo software (based on 256 bins). (F) The percentage of cells undergoing the indicated number of cell divisions. Data shown are mean ± SD. **, P < 0.001 versus wild-type mice by Student's t test, *, P < 0.05.

PtIns(3,4,5)P3 signaling. Disruption of InsP3KB in neutrophils substantially enhances PtIns(3,4,5)P3 signaling (9). Here, we investigated whether PtIns(3,4,5)P3 signaling was also augmented in InsP3KB null progenitor cells. As described (9), we monitored PtIns(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 PtIns(3,4,5)P3/Akt pathway regulates cell cycle is via modulating the phosphorylation of cell cycle-inhibitory protein p21cip1, which is a target of Akt (13). Activation of p21cip1 leads to G1 phase arrest and has been implicated in maintaining the quiescence of hematopoietic stem cells (14). Akt-dependent phosphorylation of p21cip1 at Thr-145 diminishes the cell cycle-inhibitory effect of p21cip1 and thus promotes cell cycle progression. Consistent with the enhanced Akt phosphorylation in InsP3KB null progenitor cells, the level of phosphorylated p21cip1 was also increased in these cells, suggesting that the accelerated proliferation of myeloid progenitor cells may be a result of PtIns(3,4,5)P3/Akt-mediated deactivation of p21cip1 (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 PtIns(3,4,5)P3/Akt signaling in the InsP3KB null cells. As a result, the phosphorylation of cell cycle-inhibitory protein p21cip1, a target of Akt, is enhanced, leading to a diminished cell cycle-inhibitory effect of p21cip1. 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 GAP1IP4BP (also known as Rasa3), α-centaerin, 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 PtIns(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 and an enhanced calcium influx through store-operated calcium channels 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 PtdIns(4,5)P2-mediated Rasa3 plasma membrane translocation and activation in B cells. Rasa3 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 Rasa3 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, Rasa3 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 K2-EDTA-coated tubes. A complete blood count (CBC) and white blood cell count was analyzed by using ADVIA-1200 Blood Analyzer (Bayer).

Flow-Cytometry Analysis and Cell Sorting. Bone marrow cells were flushed out from two femurs and two tibias 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 (CD3e-PECy5, CD4-PECy5, CD8a-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-PECy7 and Streptavidin-APCCy7 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 FACSCanto 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, PECy7, or APCCy7 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, rmIL-3, and rmIL-6 (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, cells in the major peak (~20%) were sorted by using a BD FACSaria flow cytometer (Fig. 4D). Sorted cells were recovered, mixed with Methocult GF M3534 medium, plated onto 24-well nontissue 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′-TGCAGTACTGATTTACAAAC-3′ and 5′-CTCTCCAGGCCTCTTGATGA-3′), InsP3KB (5′-GGAGGTGACCGAGCAAGGCG-3′ and 5′-CTCCAGGATGTCTGCTGAC-3′) or InsP3KC (5′-GGACGCTGACACACAACTC-3′ and 5′-CACAAGAAGGAGGAACTGC-3′). GAPDH (5′-GGTCTGATGTCTGAGGA-3′ and 5′-GGACGATGGACAGTACTG-3′) gene was used as a control.

Western Blot Analysis. Low-density bone marrow cells (0.5 × 10^6 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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