Bone Marrow-derived Cells Require a Functional Glucose 6-Phosphate Transporter for Normal Myeloid Functions*

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Glycogen storage disease type Ib (GSD-Ib) is caused by a deficiency in the ubiquitously expressed glucose 6-phosphate transporter (Glc-6-PT). Glc-6-PT activity has been shown to be critical in the liver and kidney where a deficiency disrupts glucose homeostasis. GSD-Ib patients also have defects in the neutrophil respiratory burst, chemotaxis, and calcium flux. They also manifest neutropenia, but whether Glc-6-PT deficiency in the bone marrow underlies myeloid dysfunctions in GSD-Ib remains controversial. To address this, we transferred bone marrow from Glc-6-PT-deficient (Glc-6-PT−/−) mice to wild-type mice to generate chimeric mice (BM-Glc-6-PT−/−). As a control, we also transferred bone marrow between wild-type mice (BM-Glc-6-PT+/+). While BM-Glc-6-PT−/− mice have normal myeloid functions, BM-Glc-6-PT−/− mice manifest myeloid abnormalities characteristic of Glc-6-PT−/− mice. Both have impairments in their neutrophil respiratory burst, chemotaxis response, and calcium flux activities and exhibit neutropenia. In the bone marrow of BM-Glc-6-PT−/− and Glc-6-PT−/− mice, the numbers of myeloid progenitor cells are increased, while in the serum there is an increase in granulocyte colony-stimulating factor and chemokine KC levels. Moreover, in an experimental model of peritoneal inflammation, local production of KC and the related chemokine macrophage inflammatory protein-2 is decreased in both BM-Glc-6-PT−/− and Glc-6-PT−/− mice along with depressed peritoneal neutrophil accumulation. The neutrophil recruitment defect was less severe in BM-Glc-6-PT−/− mice than in Glc-6-PT−/− mice. These findings demonstrate that Glc-6-PT expression in bone marrow and neutrophils is required for normal myeloid functions and that non-marrow Glc-6-PT activity also influences some myeloid functions.

Glycogen homeostasis is maintained by the glucose-6-phosphatase-α (Glc-6-Pase-α) complex that consists of the Glc-6-Pase-α catalytic unit and the associated glucose 6-phosphate transporter (Glc-6-PT) (1, 2). Glc-6-PT translocates glucose 6-phosphate (Glc-6-P), the product of gluconeogenesis and glycogenolysis, from the cytoplasm to the lumen of the endoplasmic reticulum (ER) where the active site of Glc-6-Pase-α is located (3). Inside the ER, Glc-6-Pase-α catalyzes the conversion of Glc-6-P to glucose and phosphate (4). A deficiency in Glc-6-Pase-α causes glycogen storage disease type Ia (GSD-Ia, MIM232200), and a loss of Glc-6-PT causes GSD type Ib (GSD-Ib, MIM232220) (1, 2). While Glc-6-Pase-α is expressed primarily in the liver, kidney, and intestine (4, 5), Glc-6-PT is expressed ubiquitously (6). Both GSD-Ia and GSD-Ib patients manifest the characteristics of Glc-6-Pase-α deficiency; growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia (1, 2). However, GSD-Ib patients are unique in that they manifest symptoms of neutropenia and myeloid dysfunctions (7–9) that are not obviously related to metabolism in the gluconeogenic tissues, suggesting Glc-6-PT has other roles when expressed outside of the liver, kidney, and intestine.

The current treatment for GSD-Ib consists of a dietary therapy, to correct phenotypic Glc-6-PT deficiency, augmented with granulocyte colony-stimulating factor (G-CSF) therapy, to restore myeloid functions (1, 2, 9). While this combined therapy improves the metabolic abnormalities and myeloid functions of the patient, the underlying pathological process remains untreated, and long term liver and kidney complications as well as inflammatory bowl disease still develop in adult patients. Orthotopic liver transplantation has been advocated as a potential cure of GSD-Ib (10–13). While the transplanted GSD-Ib patients do show improved metabolic profiles, the consequence on the myeloid dysfunction remains controversial.

In two transplant patients, neutropenia was resolved, at least for the first 3 and 4 years after transplantation (12, 13), but in the other three patients, neutropenia persisted (10, 11). Since the primary function of the liver is to maintain glucose homeostasis, it is not immediately obvious how orthotopic liver transplantation could resolve myeloid dysfunctions. Since myeloid cells mature within the bone marrow in mammals (14), it is more likely that bone marrow transplantation would be able to

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‡ The abbreviations used are: Glc-6-Pase-α, glucose-6-phosphatase-α; Glc-6-PT, glucose 6-phosphate transporter; Glc-6-P, glucose 6-phosphate; ER, endoplasmic reticulum; G-CSF, granulocyte colony-stimulating factor; fMLP, f-Met-Leu-Phe; MIP-2, macrophage inflammatory protein-2; PBS, phosphate-buffered saline; RT, reverse transcription; HBS, Hanks’ balanced salt solution; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; BMT, bone marrow transplantation; CFU, colony forming unit(s); Hex-6-PDH, hexose-6-phosphate dehydrogenase.
resolve the myeloid dysfunctions. To this end we have performed myeloid transplantation experiments between wild-type and Glc-6-PT-deficient (Glc-6-PT<sup>−/−</sup>) mice to determine whether Glc-6-PT expression in the bone marrow is linked to myeloid functions.

We have generated Glc-6-PT<sup>−/−</sup> mice that manifest both the metabolic and myeloid dysfunctions of the human disorder (15). The Glc-6-PT-deficient mice exhibit neutropenia along with defects in neutrophil respiratory burst, chemotaxis, and calcium flux, in response to the bacterial peptide f-Met-Leu-Phe (fMLP). We have also shown that Glc-6-PT<sup>−/−</sup> neutrophils derived from these mice are defective in chemotaxis and in calcium flux, induced by the chemokines KC and macrophage inflammatory protein-2 (MIP-2) (15). Furthermore, local production of these chemokines, and the resultant neutrophil trafficking in vivo, are depressed in Glc-6-PT<sup>−/−</sup> ascites during an inflammatory response. The bone marrows of Glc-6-PT<sup>−/−</sup> mice have an elevated number of myeloid progenitor cells and a corresponding increase in serum G-CSF and KC levels (15). In this study, we show that wild-type mice receiving Glc-6-PT<sup>−/−</sup> bone marrow manifest the myeloid dysfunctions characteristic of GSD-Ib, which establishes that Glc-6-PT plays a functional role in bone marrow cells and is required for normal myeloid functions.

**EXPERIMENTAL PROCEDURES**

**Transplantation of Glc-6-PT<sup>+/+</sup> or Glc-6-PT<sup>−/−</sup> Mice with Glc-6-PT<sup>−/−</sup> Bone Marrows—**All animal studies were conducted under an animal protocol approved by the NICHD Animal Care and Use Committee. A glucose therapy was administered under an animal protocol approved by the NICHD Animal Care and Use Committee. A glucose therapy was administered under an animal protocol approved by the NICHD Animal Care and Use Committee. A glucose therapy was administered under an animal protocol approved by the NICHD Animal Care and Use Committee.

Glc-6-PT<sup>−/−</sup> mice were generated by Southern blot analysis using primers specific for the G6PT gene. Male and female Glc-6-PT<sup>−/−</sup> and wild-type littermate control (Glc-6-PT<sup>+/+</sup>) mice obtained from breeding pairs homozygous for the G6PT<sup>−/−</sup> allele were used in all experiments. Glc-6-PT<sup>−/−</sup> mice were generated by Southern blot analysis using primers specific for the G6PT gene. Male and female Glc-6-PT<sup>−/−</sup> and wild-type littermate control (Glc-6-PT<sup>+/+</sup>) mice obtained from breeding pairs homozygous for the G6PT<sup>−/−</sup> allele were used in all experiments.

**Hematological and Serum Analyses—**Blood samples were collected from the tail vein using EDTA-containing CAPRIJECT tubes (Terumo Medical Co., Elkton, MD) for the differential leukocyte counts. Manual 200-cell leukocyte differential counts of peripheral blood cells were performed on Hema 3 (Fisher Scientific)-stained smears. Bone marrow cells from femoral and tibial bones were harvested by flushing with 3 ml of Iscove’s modified Dulbecco’s medium containing 2% fetal bovine serum.

Serum glucose, total cholesterol, and uric acid were analyzed using kits obtained from Thermo Electron (Louisville, CO), triglycerides were from Sigma Diagnostics (St. Louis, MO), and lactate was from Trinity Biotech (St. Louis, MO).

**Isolation of Mouse Neutrophils and Cytokine Assays—**Mice were injected in the peritoneum with 3% thioglycollate (1 ml/25 g of body weight) 4 h prior to peritoneal lavage with 2 ml PBS. The lavage was centrifuged at 400 × g for 10 min to pellet cells and the supernatant used for cytokine analysis. The cytokines, G-CSF, KC, and MIP-2 were quantified using Quantikine ELISA kits (R&D Systems Inc., Minneapolis, MN).

**Respiratory Burst, Chemotaxis, and [Ca<sup>2+</sup>]<sup>i</sup> Measurements—**The respiratory burst of neutrophils was monitored by luminal amplified chemiluminescence using the LumiMax superoxide anion detection kit (Stratagene, La Jolla, CA) and Victor Light 1420 Luminoscence counter (PerkinElmer Life & Analytical Sciences, Inc, Wellesley, MA). The assay mixture (0.2 ml) contained 200 μM luminol, 250 μM enhancer, and 2 × 10<sup>6</sup> neutrophils in HBSS. Neutrophils were activated with 200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) in HBSS or HBSS as a control.

Chemotaxis was performed in 48-well chambers (Neuro-Probe, Gaithersburg, MD) using polyvinylpyrrolidone-free polycarbonate membranes with 3-μm pores as described previously (16). Lower chambers contained chemotactant dissolved in HEPES-buffered RPMI medium containing 1% bovine serum albumin (RPMI-BSA). Upper chambers contained 5 × 10<sup>6</sup> neutrophils in 50 μl of RPMI-BSA. After incubation at 37°C for 4 h, the membrane was removed, rinsed with PBS, fixed, and stained with Hema 3. Cells were counted in six randomly selected fields at 400-fold magnification and reported as the mean average.

**G6PT Is Required for Normal Myeloid Functions**

The transplant recipients were 3- or 5-week-old Glc-6-PT<sup>−/−</sup> mice which had received a lethal total body irradiation of 10 Gy (Gammacell 40 137Cs irradiation source; Nordion International, Kanata, Ontario, Canada). For 2 weeks following irradiation, the mice were housed in specific pathogen free conditions and provided with water treated with trimethoprim (0.16 mg/ml) and sulfadiazine (0.8 mg/ml) to prevent intestinal bacteria invading surrounding tissues of the immune compromised mice.

Donor bone marrow cells were harvested from 3- or 5-week-old Glc-6-PT<sup>−/−</sup> mice by flushing the femurs and tibias with Iscove’s modified Dulbecco’s medium containing 2% fetal bovine serum. Upper chambers contained 5 × 10<sup>6</sup> neutrophils in 50 μl of RPMI-BSA. After incubation at 37°C for 4 h, the membrane was removed, rinsed with PBS, fixed, and stained with Hema 3.
For intracellular calcium ion concentration measurements ($[\text{Ca}^{2+}]_i$), cells were loaded with FLIPER calcium 3 assay kit component A (Molecular Devices, Sunnyvale, CA) according to the following protocol. Cells were first washed in HEPES-HBSS buffer ($1 \times $ HBSS containing $10 \text{ mM HEPES}$) and resuspended at $10^7$ cells/ml of buffer. One hundred $\mu$l of cell suspension was pipetted into each well of a poly-l-lysine (Sigma) coated 96-well plate (Greiner, Longwood, FL), followed by $100 \mu$l of FLIPER calcium 3 assay kit component A dissolved in HEPES-HBSS. The plates were incubated at 37 °C for 30 min and then centrifuged at $1000 \times g$ for 5 min at room temperature. The plate was loaded into the reading chamber of a Flexstation fluorimeter (Molecular Devices) set at 37 °C. The appropriate ligands, fMLP (Sigma) and MIP-2 or KC (PeproTech Inc., Rocky Hill, NJ), were diluted in HEPES-HBSS containing 1% BSA and added to the plate robotically. Cells were excited at 485 nm and the fluorescence intensities detected at 515 nm.

**Hematopoietic Progenitor Cell Assays**—Progenitor cells were assayed in semisolid agar cultures by plating $2 \times 10^4$ bone marrow mononuclear cells in 1 ml of methylcellulose media (MethoCult M3231, Stem Cell Technologies, Vancouver, Canada) supplemented with the indicated cytokines. The number of colonies larger than 50 cells was counted on days 7–9. Recombinant murine cytokines (R&D Systems) used were: G-CSF (10 ng/ml), granulocyte macrophage-CSF (GM-CSF) (10 ng/ml), or macrophage-CSF (M-CSF) (2.5 ng/ml).

**Statistical Analysis**—Data are presented as the mean ± S.E. Statistical analysis using the unpaired t test was performed with The GraphPad Prism Program (GraphPad Software, San Diego, CA). Values were considered statistically significant at $p < 0.05$.

**RESULTS**

**Reconstitution of Glc-6-PT$^{+/+}$ Mice with Glc-6-PT$^{-/-}$ Bone Marrows**—We used bone marrow transplantation (BMT) to examine if the loss of Glc-6-PT expression in bone marrow is associated with the myeloid dysfunctions observed in GSD-Ib. Three- or five-week-old, irradiated Glc-6-PT$^{-/-}$ mice were reconstituted with bone marrows from Glc-6-PT$^{+/+}$ donors, to create BM-Glc-6-PT$^{-/-}$ mice. As controls, irradiated Glc-6-PT$^{+/+}$ mice were reconstituted with bone marrows from Glc-6-PT$^{+/+}$ donors, to create BM-Glc-6-PT$^{+/+}$ mice. Myeloid functions of the transplanted mice were assessed between 8 and 10 weeks after BMT. Approximately 80% of BM-Glc-6-PT$^{-/-}$ and BM-Glc-6-PT$^{+/+}$ mice survived BMT but none of the 76 irradiated Glc-6-PT$^{+/+}$ mice receiving no BMT survived, demonstrating that the irradiated Glc-6-PT$^{+/+}$ mice lacked the ability to reconstitute their own marrow and transplant recipients were being reconstituted from the donor cells.

Real-time PCR was used to measure the level of expression of the Glc-6-PT gene in RNA isolated from bone marrows of the BM-Glc-6-PT$^{-/-}$ and BM-Glc-6-PT$^{+/+}$ mice as well as the control Glc-6-PT$^{+/+}$ and Glc-6-PT$^{-/-}$ mice. Since the probe used in the assay (Assay ID Mm00484574_m1) flanks more than just the deleted sequences in the mouse Glc-6-PT mRNA, a low level of signal is detected in the Glc-6-PT$^{-/-}$ mice, as a result of a linear amplification of the non-deleted region (Fig. 1). Eight to 10 weeks after transplantation, the BM-Glc-6-PT$^{-/-}$ bone marrows express similar levels of Glc-6-PT.

**FIGURE 1.** Expression of the Glc-6-PT transcript in the bone marrows following BMT. The Glc-6-PT transcripts in bone marrows were quantified by real-time RT-PCR using a Glc-6-PT probe (Assay ID Mm00484574_m1) obtained from Applied Biosystems. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase RNA and then scaled relative to each other by dividing by the level of Glc-6-PT transcript in the Glc-6-PT$^{-/-}$ bone marrow.
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The BM-Glc-6-PT−/− mice manifest mild neutropenia and have only 65% of the wild-type neutrophil counts (Fig. 3A). As expected, neutrophil counts in the BM-Glc-6-PT−/− are similar to those of wild-type mice.

Intraperitoneal injection of thioglycollate leads to the recruitment of neutrophils into the peritoneal space and is an effective method of neutrophil isolation in mice. We therefore examined the effect of thioglycollate on the absolute cell counts in peritoneal exudates. Within the peritoneum, large numbers of neutrophils accumulate in Glc-6-PT−/− and Glc-6-PT−/− mice (Fig. 3A). The BM-Glc-6-PT−/− mice also manifest mild neutropenia and have only 65% of the wild-type neutrophil counts (Fig. 3A). As expected, neutrophil counts in the BM-Glc-6-PT−/− are similar to those of wild-type mice.

6-PT transcript as the Glc-6-PT−/− bone marrows and likewise the BM-Glc-6-PT−/− bone marrows express similar levels of Glc-6-PT transcript as the Glc-6-PT+/+ bone marrows (Fig. 1). Therefore the irradiated Glc-6-PT+/+ mice were successfully reconstituted with either Glc-6-PT−/− or Glc-6-PT−/− marrows, and little, if any, residual Glc-6-PT+/+ bone marrow remained in the BM-Glc-6-PT−/− mice.

The BM-Glc-6-PT−/− Mice Do Not Manifest Metabolic Abnormalities Characteristics of GSD-Ib—The BM-Glc-6-PT−/− control mice are severely neutropenic at age 1–3 weeks, and although their condition improves markedly at age 6 weeks (15), the neutrophil counts of 6–15-week-old Glc-6-PT−/− mice still only average 68% of the counts in the Glc-6-PT+/+ mice (Fig. 3A). The BM-Glc-6-PT−/− mice also manifest mild neutropenia and have only 65% of the wild-type neutrophil counts (Fig. 3A). As expected, neutrophil counts in the BM-Glc-6-PT−/− are similar to those of wild-type mice.

Intraperitoneal injection of thioglycollate leads to the recruitment of neutrophils into the peritoneal space and is an effective method of neutrophil isolation in mice (17). We therefore examined the effect of thioglycollate on the absolute cell counts in peritoneal exudates. Within the peritoneum, large numbers of neutrophils accumulate in Glc-6-PT−/− as well as in BM-Glc-6-PT−/− mice (Fig. 3B). In contrast, accumulation in the Glc-6-PT−/− peritoneum is decreased to 36% of controls (Fig. 3B). For BM-Glc-6-PT−/− mice, peritoneal neutrophil recruitment is reduced to 72.5% of that in the Glc-6-PT+/+ mice (Fig. 3B). This is a less severe reduction than seen with the Glc-6-PT−/− control mice, suggesting that neutrophil recruitment is only partially impaired by a deficiency of Glc-6-PT in the bone marrow and that Glc-6-PT expression in other tissues must contribute to neutrophil recruitment also.

Neutrophils in GSD-Ib patients (7–9) and Glc-6-PT−/− mice (15) exhibit reduced respiratory burst activities. In Glc-6-PT−/− mice, superoxide production in thioglycollate-recruited peritoneal neutrophils is markedly increased by exposure to PMA, and the activity remains elevated for more than 25 min (Fig. 3C). In BM-Glc-6-PT−/− neutrophils an activity similar to wild type is seen. However, for both the Glc-6-PT−/− and BM-Glc-6-PT−/− mice, PMA-stimulated superoxide production is reduced in neutrophils (Fig. 3C), implying Glc-6-PT expression in bone marrow is important for the respiratory burst.

Neutrophils from GSD-Ib patients are impaired in chemotaxis toward the bacterial peptide fMLP (7–9). Similarly, neutrophils from Glc-6-PT−/− mice are impaired in chemotaxis toward fMLP as well as the chemokines, KC, and MIP-2 (15).
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Thioglycollate-elicted peritoneal neutrophils from Glc-6-PT<sup>−/−</sup> mice exhibited a greater dose-dependent chemotactic response to fMLP, KC, and MIP-2 than neutrophils from Glc-6-PT<sup>+/−</sup> mice (Fig. 4A). The BM-Glc-6-PT<sup>−/−</sup> mice had a similarly impaired chemotaxis toward fMLP, KC, and MIP-2 (Fig. 4A). However, neutrophils from BM-Glc-6-PT<sup>+/+</sup> mice exhibited a dose-dependent chemotactic response indistinguishable from that of Glc-6-PT<sup>−/−</sup> mice.

In a similar fashion thioglycollate-elicted peritoneal neutrophils from both the Glc-6-PT<sup>−/−</sup> and BM-Glc-6-PT<sup>+/+</sup> mice showed a dose-dependent mobilization of Ca<sup>2+</sup> in response to fMLP, KC, and MIP-2 (Fig. 4B). Both the Glc-6-PT<sup>−/−</sup> and BM-Glc-6-PT<sup>+/−</sup> neutrophils were impaired in mobilization of Ca<sup>2+</sup> in response to fMLP, KC, and MIP-2 (Fig. 4B). Therefore, a deficiency of Glc-6-PT in the bone marrow also leads to problems regulating the intracellular Ca<sup>2+</sup> concentration in neutrophils.

**Increased Colony-forming Units in the Bone Marrow of BM-Glc-6-PT<sup>−/−</sup> Mice**—After birth, bone marrow is the primary origin and site of maturation and development of hematopoietic cells (14). In Glc-6-PT<sup>−/−</sup> mice, bone marrow aspirates combined from the femur and tibia contained a higher proportion of colony forming units (CFU) than Glc-6-PT<sup>+/+</sup> aspirates (15). Consistent with this, BM-Glc-6-PT<sup>−/−</sup> aspirates also contained a higher proportion of CFU than Glc-6-PT<sup>−/−</sup> aspirates. In vitro clonal stimulation of BM-Glc-6-PT<sup>−/−</sup> bone marrow aspirates with G-CSF, GM-CSF, or M-CSF revealed 1.9-fold more CFU-G, 1.4-fold more CFU-GM, and 1.3-fold more CFU-M than produced by either the wild-type littermates or BM-Glc-6-PT<sup>+/+</sup> mice (Fig. 5). This increase in CFU was comparable with the increase in CFU-G, CFU-GM, and CFU-M in the Glc-6-PT<sup>−/−</sup> mice (Fig. 5).

**Abnormal G-CSF and Chemokine Production in the BM-Glc-6-PT<sup>−/−</sup> Mice**—In the Glc-6-PT<sup>−/−</sup> mice, plasma G-CSF and KC levels were abnormally increased, suggesting an underlying problem with neutrophil production (15). Plasma G-CSF and KC values in Glc-6-PT<sup>−/−</sup> mice were 5.5- and 6.1-fold higher, respectively, than the Glc-6-PT<sup>+/+</sup> mice (Fig. 6A). Likewise, plasma G-CSF and KC values in BM-Glc-6-PT<sup>−/−</sup> mice are 6.4- and 3.5-fold higher, respectively, than the control animals (Fig. 6A). On the other hand, similar levels of G-CSF and KC were found in the plasma of control and BM-Glc-6-PT<sup>+/+</sup> mice.

Four hours after an intraperitoneal injection of thioglycollate, Glc-6-PT<sup>−/−</sup> mice exhibited a 14-fold increase in plasma G-CSF, a 29-fold increase in plasma KC, and a readily detectable level of MIP-2 (compare Fig. 6, A and B). A similar experiment with BM-Glc-6-PT<sup>+/+</sup> mice led to an 8-fold increase in plasma G-CSF, a 28-fold increase in plasma KC, and a readily detectable level of MIP-2, comparable with the wild-type mice. However, with BM-Glc-6-PT<sup>−/−</sup> mice where the steady state
plasma levels of the cytokines were already elevated, responses were more attenuated, with G-CSF increasing only 1.5-fold, and KC increasing 11-fold, a response similar to that of the Glc-6-PT<sup>−/−</sup> mice (Fig. 6A and B). The net effect of thioglycollate stimulation was that in both the BM-Glc-6-PT<sup>−/−</sup> and Glc-6-PT<sup>−/−</sup> mice, the plasma concentrations of MIP-2 were elevated almost 3-fold over levels in G6PT<sup>+/+</sup> mice, but the G-CSF and KC levels were equivalent for all mice (Fig. 6B).

Peritoneal levels of G-CSF, KC, and MIP-2 are generally below detection limits (18) unless stimulated by thioglycollate administration. In both BM-Glc-6-PT<sup>−/−</sup> and Glc-6-PT<sup>−/−</sup> mice, the induced expression of G-CSF, KC, and MIP-2 are attenuated relative to the levels induced in wild-type and BM-Glc-6-PT<sup>+/+</sup> mice (Fig. 6C). The BM-Glc-6-PT<sup>−/−</sup> mice deficient in Glc-6-PT expression in the bone marrow exhibited induced peritoneal levels of G-CSF, KC, and MIP-2 levels of 360 ± 110, 413 ± 107, and 56 ± 9 pg/ml, respectively, compared with the levels of 1123 ± 161, 1008 ± 137, and 373 ± 108 pg/ml, respectively, in Glc-6-PT<sup>+/+</sup> mice expressing Glc-6-PT in the bone marrow (Fig. 6C).

**DISCUSSION**

GSD-Ib is caused by a deficiency in Glc-6-PT. The most obvious, and well documented, role of Glc-6-PT is in the gluconeogenic tissues of the liver and kidney, where it transports Glc-6-P from the cytoplasm into the lumen of the ER for hydrolysis to glucose by Glc-6-Pase-α. However, there is evidence to suggest it may have different roles outside of the gluconeogenic organs. For instance, GSD-Ib patients manifest neutropenia along with myeloid dysfunctions in Ca<sup>2+</sup> mobilization, respiratory burst, and chemotaxis (7–9) not seen in GSD-Ia patients. Consistent with such alternative roles, Glc-6-PT expression is not restricted to the gluconeogenic tissues, like Glc-6-Pase-α, but is instead expressed ubiquitously (6). This suggests that bone marrow might be one of the non-gluconeogenic sites where Glc-6-PT expression is important. In this study, we have used a Glc-6-PT-deficient mouse strain that exhibits the same myeloid dysfunctions seen in human GSD-Ib patients (15) to examine this hypothesis.
G6PT Is Required for Normal Myeloid Functions

Before the primary role of Glc-6-PT in the bone marrow is not related to plasma glucose homeostasis but to myeloid functions.

We have previously shown that the Glc-6-PT\(^{-/-}\) mice exhibit neutropenia; reduced neutrophil respiratory burst; reduced neutrophil chemotaxis toward fMLP, KC, and MIP-2; reduced neutrophil mobilization of Ca\(^{2+}\) in response to fMLP and chemokines; and elevated levels of CFU-G, CFU-GM, and CFU-M (15). A near identical change in activities was observed in the BM-Glc-6-PT\(^{-/-}\) mice, but not in the BM-Glc-6-PT\(^{+/+}\) mice, suggesting that Glc-6-PT expression in the bone marrow is essential for normal myeloid functions and is independent of the expression of Glc-6-PT in surrounding tissues. Moreover, it demonstrates that neutrophils lacking Glc-6-PT have intrinsic defects in their respiratory burst, chemotaxis, Ca\(^{2+}\) mobilization, and chemokine production.

Some of the changes between the control Glc-6-PT\(^{-/-}\) mice and transplanted BM-Glc-6-PT\(^{-/-}\) mice were not identical, suggesting that some functions might be influenced by the Glc-6-PT\(^{+/+}\) background. For instance, thioglycollate-induced peritoneal neutrophil recruitment is only partially impaired in the BM-Glc-6-PT\(^{-/-}\) mice. The numbers of neutrophils accumulated in the BM-Glc-6-PT\(^{-/-}\) peritoneum (72.5% of wild-type) are twice as high as those of the G6PT\(^{-/-}\) peritoneum (36% of wild-type). Peritoneal recruitment of blood neutrophils in thioglycollate-elicted peritonitis is mediated primarily by the CXC chemokines, KC and MIP-2 (18). In plasma, while the steady state levels of G-CSF and KC are higher in both Glc-6-PT\(^{-/-}\) and BM-Glc-6-PT\(^{-/-}\) mice compared with Glc-6-PT\(^{+/+}\) and BM-Glc-6-PT\(^{+/+}\) mice, they all rise to similar levels upon stimulation. Within the peritoneum, however, the mice deficient in bone marrow Glc-6-PT express lower levels of G-CSF and KC upon thioglycollate stimulation. With the chemokine MIP-2, plasma levels are always higher in the mice lacking Glc-6-PT in the bone marrow than in the Glc-6-PT\(^{+/+}\) mice, but in the peritoneum, the trend is reversed. While the reduced peritoneal KC and MIP-2 levels in BM-Glc-6-PT\(^{-/-}\) and Glc-6-PT\(^{-/-}\) mice are consistent with the reduced neutrophil recruitment into the peritoneum, it cannot explain the 2-fold difference in recruitment between BM-Glc-6-PT\(^{-/-}\) and Glc-6-PT\(^{-/-}\) mice. This suggests that other factors that regulate neutrophil trafficking (19, 20), such as TNF-\(\alpha\) or the proinflammatory CC chemokines MCP-1, MIP-1\(\alpha\), and RANTES (regulated on activation normal T cell expressed and secreted), may be impaired and that their activities require the expression of Glc-6-PT in tissues outside of the bone marrow. Since fibroblasts, smooth muscle cells, and epithelial cells may play a role in regulating the synthesis of factors involved in neutrophil recruitment (21), Glc-6-PT may also play a key role in these tissues.

The GSD-Ib phenotype includes a loss of blood glucose homeostasis and impairment in myeloid functions. Both GSD-Ib patients and Glc-6-PT\(^{-/-}\) mice suffer from frequent hypoglycemic seizures and bacterial infections (7–9, 15). Even with glucose therapy it is difficult to sustain the life of Glc-6-PT\(^{-/-}\) mice much beyond weaning (21-day) and less than 15% of Glc-6-PT\(^{-/-}\) mice live past 4-weeks (15). Even when successfully maintained on glucose therapy, the weaned Glc-6-PT\(^{-/-}\) mice continue to suffer from frequent hypoglycemic seizures, and few live to 3 months of age. The Glc-6-PT\(^{-/-}\) mice are also growth-retarded, and their body weights are about 40–60% of the weights of their Glc-6-PT\(^{+/+}\) littermates (15). The short life span of the Glc-6-PT\(^{-/-}\) mice, compounded by their fragility, make it infeasible, at present, to conduct statistically significant studies of the effects of a wild-type bone marrow within a Glc-6-PT\(^{-/-}\) background. However, we were able to transplant wild-type bone marrow into three 5-week-old Glc-6-PT\(^{-/-}\) mice. All three survived transplantation, but all died a few weeks later. The serum glucose levels of two transplanted Glc-6-PT\(^{-/-}\) mice when dying were 23 and 24 mg/dl, respectively, suggesting that the most likely cause of death of the transplanted mice is hypoglycemia. The neutrophil counts of the two transplanted Glc-6-PT\(^{-/-}\) mice were 1594/\(\mu\)l and 1544/\(\mu\)l, respectively, compared with the mean value of 1800 ± 254/\(\mu\)l for wild-type mice and 1224 ± 150/\(\mu\)l for G6PT\(^{-/-}\) mice (Fig. 3A). It appears that transplanting of wild-type bone marrow to Glc-6-PT\(^{-/-}\) mice corrected neutropenia but not disturbed glucose homeostasis manifested by Glc-6-PT\(^{-/-}\) mice.

Despite our inability to conduct statistically significant studies, it is possible, however, to surmise what the outcome of this reverse transplant might be, based on the phenotype of GSD-Ia. Both GSD-Ia and GSD-Ib patients manifest virtually identical metabolic abnormalities but GSD-Ia patients do not display symptoms associated with myeloid dysfunctions (1, 2), raising the possibility that Glc-6-PT\(^{-/-}\) mice reconstituted with the Glc-6-PT\(^{+/+}\) bone marrows would be very similar to GSD-Ia mice. We would not expect to see any correction of the metabolic profiles controlled by the Glc-6-Pase-\(\alpha\)-Glc-6-PT complex in the gluconeogenic tissues, namely growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia associated with loss of Glc-6-P hydrolysis activity the liver, kidney, and intestine. However, based on the experiments we report here we would anticipate that these mice would have near normal myeloid functions, possibly with some abnormality in peritoneal neutrophil recruitment.

Our study shows that Glc-6-PT plays a critical role in normal bone marrow function. We (22) and others (23) have recently reported a second Glc-6-Pase activity, Glc-6-Pase-\(\beta\), which is ubiquitously expressed. Glc-6-Pase-\(\beta\) has the same active site structure (24) and kinetic properties as Glc-6-Pase-\(\alpha\) and can couple with Glc-6-PT, in the same way as Glc-6-Pase-\(\alpha\) (22), to hydrolyze Glc-6-P. Therefore, in the bone marrow, Glc-6-PT transports Glc-6-P from the cytoplasm into the lumen of the ER, where it serves as the substrate for at least two major metabolic pathways. The first is glucose production mediated by the ER-associated Glc-6-Pase-\(\beta\) that catalyzes the hydrolysis of Glc-6-P to glucose. The second is to fuel the endolysosomal peroxisome phosphate pathway to generate NADPH by the ER-associated hexose-6-phosphate dehydrogenase (Hex-6-PDH) (25). A defect in either, or both, of these pathways is likely to underlie the myeloid dysfunction.

Glucose is an energy substrate. One hypothesis is that normal myeloid function requires endogenous glucose production by the Glc-6-PT-dependent Glc-6-Pase-\(\beta\). In this case, a knock-out mutation of Glc-6-Pase-\(\beta\) should exhibit the myeloid dys-
functions of GSD-Ib but not the metabolic abnormalities. To this end, we have generated a Glc-6-Pase-β knock-out mouse line and characterizing its phenotype. Our preliminary results suggest that the Glc-6-Pase-β knock-out mice do manifest myeloid dysfunction mimicking the Glc-6-PT−/− mice. However, because Glc-6-PT and Glc-6-Pase-β are functionally coupled and co-dependent, the clear elucidation that lack of glucose production in the ER underlies part of the myeloid dysfunction awaits further experimentation. A second, non-exclusive hypothesis is that the endoluminal pentose-phosphate pathway is essential for myeloid function. One key role for NADPH in the ER is to confer oxoreductase activity to 11β-hydroxysteroid dehydrogenase-1 for the generation of cortisol/corticosterone (25, 26). If this pathway is important we would expect Hex-6-PDH knock-out mice to exhibit myeloid dysfunction. A recent report of Hex-6-PDH knock-out mice did not address myeloid dysfunction (27), but based on the observation that myeloid dysfunction in Glc-6-Pase-β mice is only seen upon appropriate challenge, this remains to be examined further.

In summary, we have demonstrated that the metabolic and myeloid dysfunctions seen in GSD-Ib can be separated. The metabolic dysfunctions are due to the loss of Glc-6-PT in the liver, kidney, and intestine, while the myeloid dysfunctions are due, to a large extent, to the loss of Glc-6-PT expression in the bone marrows and neutrophils. While orthotopic liver transplantation studies that targeting the expression of a normal Glc-6-PT gene to the liver can correct metabolic dysfunctions of GSD-Ib, this study suggests that it may be necessary to target the bone marrow cells if the myeloid dysfunctions are to be addressed also.

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