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Glycogen storage disease type Ib neutrophils exhibit impaired cell adhesion and migration

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Abstract

Glycogen storage disease type Ib (GSD-Ib), characterized by impaired glucose homeostasis, neutropenia, and neutrophil dysfunction, is an inherited autosomal recessive disorder caused by a deficiency in the glucose-6-phosphate transporter (G6PT). Neutrophils play an essential role in the defense against invading pathogens. The recruitment of neutrophils towards the inflammation sites in response to inflammatory stimuli is a tightly regulated process involving rolling, adhesion, and transmigration. In this study, we investigated the role of G6PT in neutrophil adhesion and migration using *in vivo* and *in vitro* models. We showed that the GSD-Ib (*G6pt*$^{-/-}$) mice manifested severe neutropenia in both blood and bone marrow, and treating *G6pt*$^{-/-}$ mice with granulocyte colony-stimulating factor (G-CSF) corrected neutropenia. However, upon thioglycolate challenge, neutrophils from both untreated and G-CSF-treated *G6pt*$^{-/-}$mice exhibited decreased ability to migrate to the peritoneal cavity. *In vitro* migration and cell adhesion of G6PT-deficient neutrophils were also significantly impaired. Defects in cell migration were not due to enhanced apoptosis or altered fMLP receptor expression. Remarkably, the expression of the β2 integrins CD11a and CD11b, which are critical for cell adhesion, was greatly decreased in G6PT-deficient neutrophils. This study suggests that deficiencies in G6PT cause impairment in neutrophil adhesion and migration via aberrant expression of β2 integrins, and our finding should facilitate the development of novel therapies for GSD-Ib.

Keywords

Glucose-6-phosphate transporter; CD11a; CD11b; Neutrophil adhesion; Migration

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Conflict of interest

None.

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1. Introduction

Neutrophils are the most abundant leukocytes in circulation and are essential for host defense, as they are responsible for eliminating invading pathogens through neutrophil recruitment, chemotaxis, phagocytosis, and destruction of the invading pathogens [1,2]. Recruitment of neutrophils from the blood stream to inflammatory sites is a critical event in the host defense against infection and in the repair of tissue damage. Generally, neutrophil recruitment is a tightly regulated process involving three distinct steps: i) selectin-mediated rolling, ii) firm adhesion via integrins, and iii) transmigration into tissue [3]. Generally, P-selectin glycoprotein ligand-1 and L-selectin are involved in neutrophil rolling upon contact with the endothelium [4,5], whereas lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (Mac-1) mediate neutrophil adhesion [1,3].

LFA-1 and Mac-1, which are predominantly expressed on neutrophils, are members of the β2 integrin family. They are heterodimers consisting of a distinct α subunit (CD11a or CD11b) and a β subunit (CD18) [6]. Leukocyte adhesion deficiency syndrome type I (LAD I) is caused by mutations in CD18, leading to an inherited defect of LFA-1, Mac-1, and p150,95 [7]. LAD I results in predisposition to life-threatening recurrent bacterial infections resulting from a severe defect in neutrophil and monocyte emigration to extravascular sites of inflammation [7]. A deficiency caused by inherited mutations of either CD11a or CD11b has not been reported, but $CD11b^{-/-}$ mice were found to be severely susceptible to bacterial infections [8,9].

Glycogen storage disease type Ib (GSD-Ib) is an autosomal recessive disorder, characterized by impaired glucose homeostasis, neutropenia, and neutrophil dysfunction [10,11]. GSD-Ib is caused by a deficiency in the glucose-6-phosphate transporter (G6PT) that transports cytoplasmic glucose-6-phosphate (G6P) into the lumen of the endoplasmic reticulum (ER), where a glucose-6-phosphatase (G6Pase) hydrolyzes G6P to glucose and inorganic phosphate [10]. G6PT is known to be functionally coupled not only with glucose-6-phosphatase-α (G6Pase- α), whose expression is restricted to the liver, kidney, and intestine, but also with G6Pase-β, which is ubiquitously expressed similar to G6PT. G6PT transports cytoplasmic glucose-6-phosphate (G6P) into the lumen of the endoplasmic reticulum (ER), where G6Pase hydrolyzes G6P to glucose and inorganic phosphate [10]. Previously, Chen et al. [12] generated a G6PT-deficient mouse model that mimics the phenotype of human GSD-Ib, manifesting neutropenia and neutrophil dysfunction. More recently, we demonstrated that GSD-Ib patients who were treated with granulocyte colony-stimulating factor (G-CSF) exhibited impaired neutrophil functions including chemotaxis, respiratory burst, and intracellular calcium mobilization, attributing, in part, to impaired neutrophil energy homeostasis [13].

In this study, we showed that *G6pt*$^{-/-}$ mice exhibited neutropenia in both the blood and the bone marrow, and that G-CSF therapy normalized the frequency and absolute neutrophil counts in the bloodstream. However, G-CSF therapy failed to correct impaired neutrophil recruitment into the peritoneal cavity during peritonitis in *G6pt*$^{-/-}$ mice. Compared to neutrophils from control mice, the G6PT-deficient neutrophils exhibited impaired adhesion to ICAM-1, fibrinogen, and TNFα-stimulated epithelial cells, resulting, in part, from

reduced expression of CD11a and CD11b in G6PT-deficient neutrophils. Together, these findings suggest that G6PT plays a critical role in neutrophil adhesion during recruitment into the extravascular sites of inflammation.

2. Materials and methods

2.1. Animals

All animal studies were conducted under an animal protocol approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee. To overcome the extremely low survival rate of the *G6pt*$^{-/-}$ mice, we infused neonatal *G6pt*$^{-/-}$ mice with a recombinant adeno-associated virus serotype 8 (rAAV8) vector carrying human G6PT (hG6PT) as previously described [14]. After gene therapy, the AAV8-hG6PT-infused mice were maintained until sacrifice at the age of 8–12 weeks.

2.2. Flow cytometry analysis

Peripheral blood cells were treated with Lysis/Fix buffer (BD Biosciences) to deplete erythrocytes and fix leukocytes. The fixed leukocytes were stained with fluorescein-isothiocyanate (FITC)-conjugated Gr-1 antibody and phycoerythrin (PE)-conjugated CD11b antibody (eBioscience). Bone marrow (BM) cells were isolated from the femurs and tibia of 8–12-week-old C57BL/6 control littermates and *G6pt*$^{-/-}$ infused with rAAV and stained with FITC-conjugated Gr-1 and PE-conjugated CD11b antibodies. All samples were acquired in a Guava EasyCyte Mini System (Millipore) and analyzed using FlowJo 7 software (TreeStar). Peripheral blood leukocytes and BM cells were counted using Guava ViaCount reagent (Millipore).

2.3. Induction of peritonitis and peritoneal neutrophil counts

Peritoneal neutrophils from *G6pt*$^{-/-}$ mice were prepared as described previously [12]. For G-CSF therapy, *G6pt*$^{-/-}$ and control littermates were subjected to daily subcutaneous injection of recombinant mouse G-CSF at 100 μg per kg body weight for 5 consecutive days. Then, peritonitis was induced by inoculation of 3% sterile thioglycolate broth (1 mL per 25 g body weight) for 4 h prior to peritoneal lavage with ice-cold PBS. The number and purity of recruited neutrophils were assessed by using the Guava ViaCount reagent and by staining with FITC-conjugated Gr-1- and PE-conjugated CD11b antibodies, respectively.

2.4. Isolation of murine bone marrow neutrophils

BM-derived neutrophils were isolated from the femurs and tibia of 8–12-week-old *G6pt*$^{-/-}$ and control littermates by using the anti-Ly-6G MicroBead kit (Miltenyi Biotec) as described by the manufacturer. Briefly, erythrocytes were depleted from isolated BM cells by lysis with Ack lysing buffer (Quality Biologicals) and up to 1×10^8 cells were resuspended in PBS supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Apoptotic cells were depleted using annexin V Microbeads (Miltenyi Biotec) as described previously [15]. Apoptotic cell-depleted leukocytes were mixed with 50 μL of Anti-Ly-6G-Biotin at 4 °C for 10 min. After adding 150 μL of the same buffer and 100 μL of Anti-Biotin MicroBeads, the mixture was incubated for 15 min at 4 °C, followed by washing

with 4 mL buffer. The labeled neutrophils were collected through a MACS column (Miltenyi Biotec). Purity and viability were assessed using Guava ViaCount reagent containing 7-aminoactinomyocin D. The morphology of isolated neutrophils was examined on Hema-3-stained (Fisher Scientific) cytospin slides.

2.5. In vitro migration and cell adhesion assays

Migration of BM-derived neutrophils toward fMLP (10^{-5} and 10^{-7} M) was measured as described previously [15].

To examine neutrophil adhesion to ICAM-1 and fibrinogen, 15-mm coverslips were precoated overnight at 4 °C with either purified mouse ICAM-1 (20 μg/mL, R&D) or fibrinogen (1 mg/mL, Sigma-Aldrich). BM-derived neutrophils (2×10^5) were activated by phorbol 12-myristate 12-acetate (PMA, Sigma-Aldrich) to a final concentration of 5 nM in RPMI 1640 medium supplemented with 1% BSA at 37 °C for 15 min and were transferred to a 24-well culture dish containing ligand-precoated coverslips. Neutrophil adhesion was performed by incubation at 37 °C for 30 min. The coverslips were washed three times with PBS and adhered cells were observed and counted under a Zeiss Axoskop2 microscope (Carl Zeiss Microimaging).

Cell adhesion to epithelial cells cultured in 24-well plates was tested after labeling PMA-activated neutrophils ($8 \times 10^5/cm^2$) with calcein AM (1μ; Invitrogen) at 37 °C for 15 min. Epithelial cells, MDCK and Caco2, were treated with TNFα (75 ng/mL, R&D Systems) at 37 °C for 6 h before neutrophil adhesion was performed. After washing the epithelial cell monolayers with RPMI 1640 supplemented with 1% BSA three times, calcein-labeled neutrophils were added, followed by incubation for 30 min at 37 °C. After washing the wells with PBS three times, 0.3 mL of PBS was added and adherent neutrophils were counted under a fluorescence microscope (EVOS FL Cell Imaging System; Thermo Fisher).

2.6. Quantitative real-time RT-PCR analysis

Total RNA was extracted from BM-derived neutrophils using the TRIzol Reagent (Invitrogen). mRNA expression was quantified by real-time reverse-transcription-polymerase chain reaction (RT-PCR) using an Applied Biosystems 7300 Real-Time PCR system. TaqMan probes used in this study were as follows: CD11b, Mm00434455_m1 and β-actin, Mm00607939_s1. Data were analyzed using SDS software (version 1.2; Applied Biosystems) and normalized to β-actin.

2.7. Western blot analysis

Neutrophils were lysed using RIPA lysis buffer supplemented with protease inhibitor cocktail (Thermo Fisher) for 15 min on ice with periodic vortexing. Following centrifugation at 13,000 rpm for 10 min at 4 °C, the protein lysate was quantified using BCA Protein Assay Kit (Thermo Fisher). Proteins were separated on 4–12% gradient gels and transferred on to polyvinylidene fluoride membranes. The membrane was probed with the following antibodies: mouse monoclonal CD11b (Abcam) and CD11a (Santa Cruz Biotechnology). Protein expression was quantified by densitometry with ImageJ software v1.48 (National Institutes of Health).

2.8. Statistical analysis

The unpaired *t*-test and analysis of variance were performed using Microsoft Excel 2015. Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. G6pt$^{-/-}$ mice manifest neutropenia in both blood and bone marrow

G6pt$^{-/-}$ mice under dietary glucose therapy continued to suffer from frequent hypoglycemic seizures with less than 15% living beyond 3 weeks [12]. To increase their survival, neonatal *G6pt*$^{-/-}$ mice were infused with rAAV8-hG6PT as previously described [14]. The rAAV8-hG6PT vector primarily targeted the liver and the expression of the introduced G6PT transgene in the bone marrow is transient [14]. Consequently the rAAV8-hG6PT-mediated correction of neutropenia in *G6pt*$^{-/-}$ mice is also transient [14]. We showed that the frequency of neutrophils in the peripheral blood and BM of *G6pt*$^{-/-}$ mice was significantly lower than that in control mice by 47.4 and 57.7%, respectively (Fig. 1A). Absolute neutrophil counts of *G6pt*$^{-/-}$ mice were significantly lower than those of control littermates, while white blood cell counts were higher in *G6pt*$^{-/-}$ mice by 24.6% (Fig. 1B).

3.2. G6PT deficiency impairs cell migration to the peritoneal cavity

Thioglycolate-induced peritonitis was used to examine the role of G6PT expression in neutrophil migration to the peritoneum. The total number of cells migrating to the peritoneal cavity was significantly reduced in *G6pt*$^{-/-}$ mice compared to that in control mice (Fig. 2B). Gr-1^{+} CD11b^{+} neutrophils were the main cell population (>85%) in the peritoneal cavity 4 h after thioglycolate-induced peritonitis in both *G6pt*$^{-/-}$ and control mice (data not shown).

Given that *G6pt*$^{-/-}$ mice are neutropenic both in the blood and in the BM (Fig. 1), we expected to observe decreased neutrophils during peritonitis. We therefore treated *G6pt*$^{-/-}$ mice with G-CSF for 5 days to increase absolute neutrophil counts in peripheral blood. As shown in Fig. 2A, the frequency of neutrophils in white blood cells was increased from 11.98 to 24.56 and 6.16 to 22.04 in control and *G6pt*$^{-/-}$ mice, respectively, revealing similar neutrophil populations in both mouse groups. However, peritoneal neutrophil counts were not significantly increased in G-CSF-treated *G6pt*$^{-/-}$ mice during peritonitis (Fig. 2B).

3.3. G6PT-deficient neutrophils exhibited reduced migration and adhesion

The reduced number of G6PT-deficient neutrophils recruited into the peritoneal cavity indicates a defect in the migratory activity of neutrophils. Thus, we examined the migration of BM-derived neutrophils from *G6pt*$^{-/-}$ and control mice in response to fMLP. As shown in Fig. 3A, a deficiency in G6PT significantly reduced neutrophil migration in response to 10^{-5} and 10^{-7} M of fMLP.

Cell migration is a multistep process that is closely linked to cell adhesion. Thus, we conducted cell adhesion assays using purified ICAM-1 and fibrinogen and epithelial cells. Compared to control neutrophils, there was a significant decrease in the number of G6PT-deficient neutrophils that adhered to either ICAM-1 or fibrinogen (Fig. 3B). Furthermore,

adhesion of G6PT-deficient neutrophils to TNFα-treated epithelial cells was significantly reduced (Fig. 3C).

3.4. G6PT-deficient neutrophils expressed reduced integrins CD11b and CD11a

β2 integrins are leukocyte-specific integrins that play an essential role in the firm adhesion and transmigration of neutrophils [6,16]. Thus, we analyzed the expression of CD11a and CD11b, which are the α chains of LFA-1 and Mac-1, respectively, in neutrophils from *G6pt*$^{-/-}$ and control mice. As shown in Fig. 4, the expression of CD11a and CD11b was significantly decreased in G6PT-deficient neutrophils along with a lower molecular mass CD11b (indicated by arrowhead) seen in G6PT-deficient neutrophils. The mRNA levels of CD11b were similar between *G6pt*$^{-/-}$ and control neutrophils (Fig. 4A).

4. Discussion

GSD-Ib is a rare inherited metabolic disorder caused by a deficiency in G6PT with an incidence of 1 in 500,000 [11]. GSD-Ib is characterized not only by hypoglycemia, growth retardation, hepatomegaly, hyperlipidemia, hyperuricemia, and lactic academia, but also by neutropenia and neutrophil dysfunction resulting in recurrent bacterial infections [11]. We have shown that neutropenia in GSD-Ib is caused by enhanced neutrophil ER stress and apoptosis [17,18]. More recently, we showed that impaired neutrophil energy homeostasis and activation of hypoxia-inducible factor-1α/peroxisome-proliferators-activated receptor-γ signaling underlie, in part, neutrophil dysfunction in GSD-Ib [13].

G-CSF is widely used to treat neutropenia patients of various origins, including GSD-Ib and G6Pase-β deficiency (or severe congenital neutropenia syndrome 4), to increase absolute neutrophil counts and alleviate the number and severity of bacterial infections [19–21]. In addition to recruiting neutrophils to inflammation sites, the functional capacity of neutrophils is critical for host defense [22]. The effects of G-CSF on neutrophil functions have been evaluated in numerous studies [23–28], but it remains unclear whether G-CSF can improve neutrophil functions. In this study, we showed that G-CSF treatment increases the absolute neutrophil number in the peripheral bloodstream of *G6pt*$^{-/-}$ mice, but neutrophil recruitment into the peritoneal cavity during peritonitis remains impaired in G-CSF-treated *G6pt*$^{-/-}$ mice, compared to control mice. These results suggest that impairment in neutrophil migration and adhesion seen in *G6pt*$^{-/-}$ mice cannot be rescued by G-CSF therapy.

The migration of leukocytes such as neutrophils and macrophages to inflammatory sites is a critical step in inflammation [3]. Previous studies reported that neutrophil accumulation during an inflammatory response by thioglycolate into the peritoneal space of *G6pt*$^{-/-}$ mice was significantly reduced to approximately 67% of that of their littermate controls [12]. This suggests that a recruitment defect in G6PT-deficient neutrophils, but the underlying mechanism remains unclear. In this study, we hypothesized that G6PT deficiency leads to impaired neutrophil migration, evident by the reduction in cell adhesion to the mesothelium or to ICAM-1 or fibrinogen. Integrins constitute the major family of cell surface receptors and play an essential role in mediating cell adhesion to extracellular matrix proteins [29]; integrin-mediated adhesion is known to be involved in various cellular functions, including embryonic development, apoptosis, and leukocyte homing and activation [30]. Integrins are

composed of two subunits, α and β, selected from among 16 α and 8 β subunits [30]. Heterodimerization of various α and β subunits generate more than 20 different integrins, including CD11b/CD18 (Mac-1) and CD11a/CD18 (LFA-1), which are the most abundant integrins in neutrophils [6]. In this study, we showed that levels of the α subunits, CD11b and CD11a, on neutrophils from *G6pt*$^{-/-}$ mice were significantly decreased compared to those of neutrophils from control mice. In addition, a significant level of an unglycosylated form (~130 kDa) of CD11b existed in G6PT-deficient neutrophils. Both CD11b and CD11a have been reported to be highly glycosylated, displaying sizes of 170 and 180 kDa, respectively [31]. Therefore, G6PT deficiency may cause deglycosylation at least in CD11b, but further studies are necessary to clarify this.

In conclusion, we showed that *G6pt*$^{-/-}$ mice manifested neutropenia in both the blood and in the bone marrow and that neutrophil recruitment into the peritoneal cavity during thioglycolate-mediated peritonitis was significantly reduced compared to that in control mice. G-CSF treatment of *G6pt*$^{-/-}$ mice which markedly improved neutropenia but failed to increase thioglycolate-induced neutrophil recruitment to the peritoneum, suggesting that impaired neutrophil migration cannot be rescued by G-CSF therapy. We showed that cell migration and adhesion were impaired in G6PT-deficient neutrophils, resulting, in part, from decreased expression of CD11b and CD11a. Taken together, our results demonstrate that the underlying cause of impaired neutrophil migration and adhesion in G6PT-deficient mice arises from aberrant expression of CD11b and CD11a.

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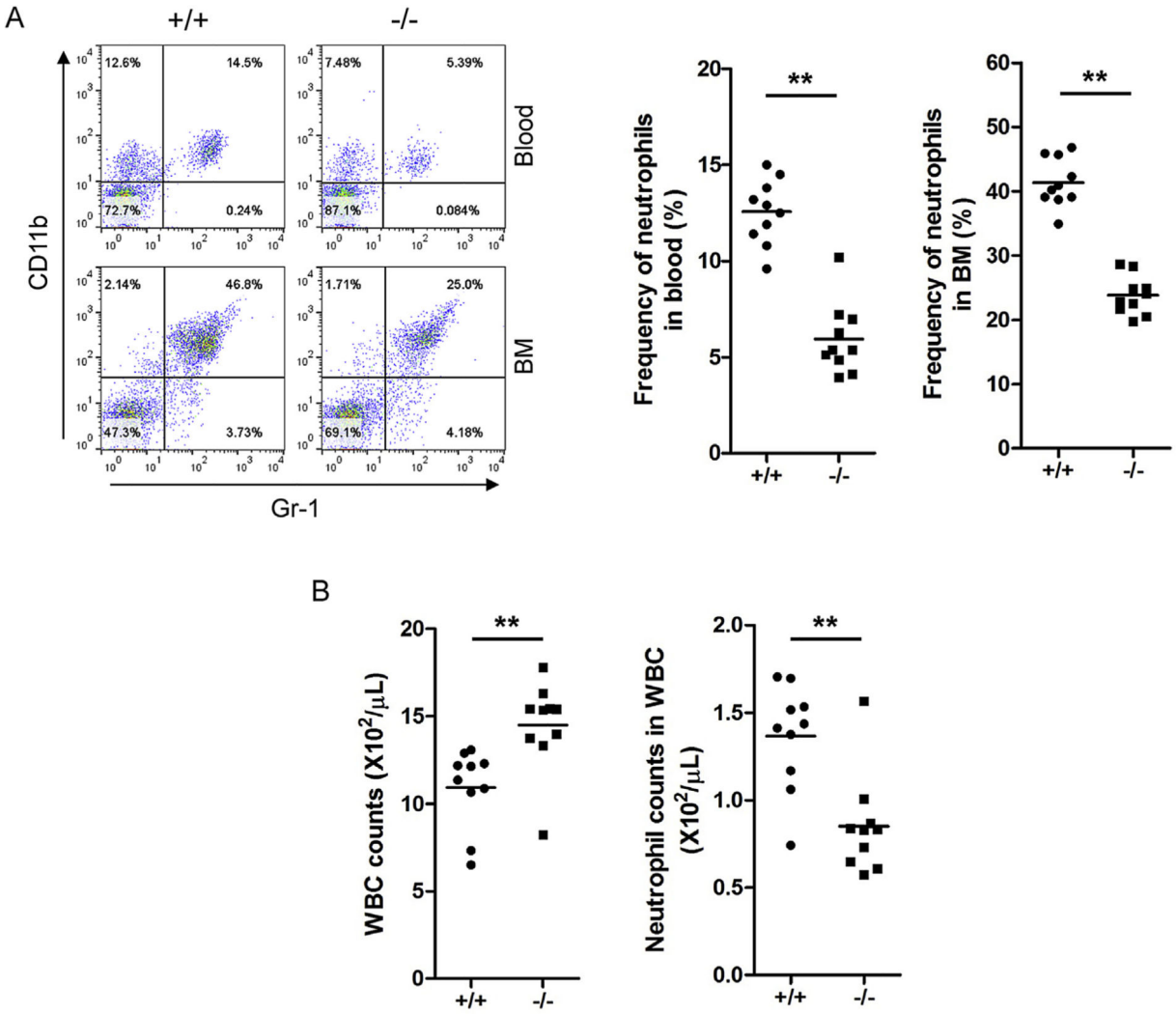

Fig. 1.

Neutrophil population in peripheral blood stream and bone marrow (BM) of *G6pt*$^{-/-}$ and control mice. (A) Representative flow cytometry data (left) and frequency of Gr-1^{+}CD11b^{+} cells in the blood and bone marrow (BM) of *G6pt*$^{-/-}$ and control mice (WT) (n = 10 per genotype). (B) Absolute number of white blood cells (WBC) and neutrophils in the blood of *G6pt*$^{-/-}$ (−/−) and control mice (+/+) (n = 10 per genotype). Each symbol represents an individual mouse, and horizontal lines represent the mean. Data represent the mean ± SEM (n = 10). **P* < 0.05, ***P* < 0.01.

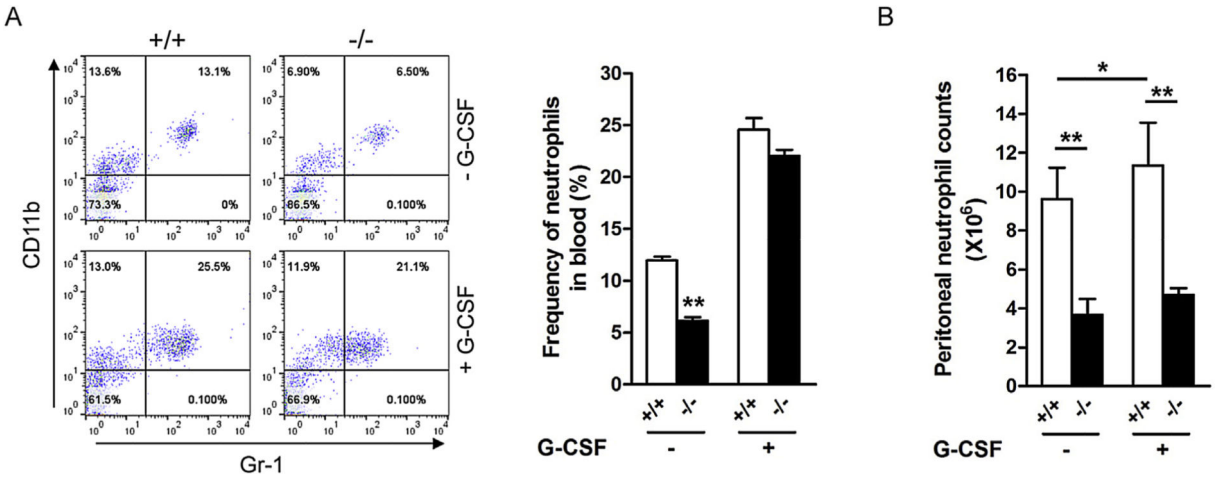

Fig. 2.

Neutrophil recruitment to the peritoneal cavity during peritonitis. (A) Representative flow cytometry data (left) and frequency of Gr-1^{+}CD11b^{+} cells in the blood of *G6pt*$^{-/-}$ (−/−) and control mice (+/+) (n = 5 per genotype) treated with G-CSF or PBS as a vehicle for 5 days. (B) Quantification of the total number of neutrophils migrated to the peritoneal cavity of *G6pt*$^{-/-}$ (−/−) and control mice (+/+) (n = 3 per genotype) 4 h after intraperitoneal injection of thioglycolate (3% w/v). Data represent the mean ± SEM (n = 10). **P* < 0.05, ***P* < 0.01.

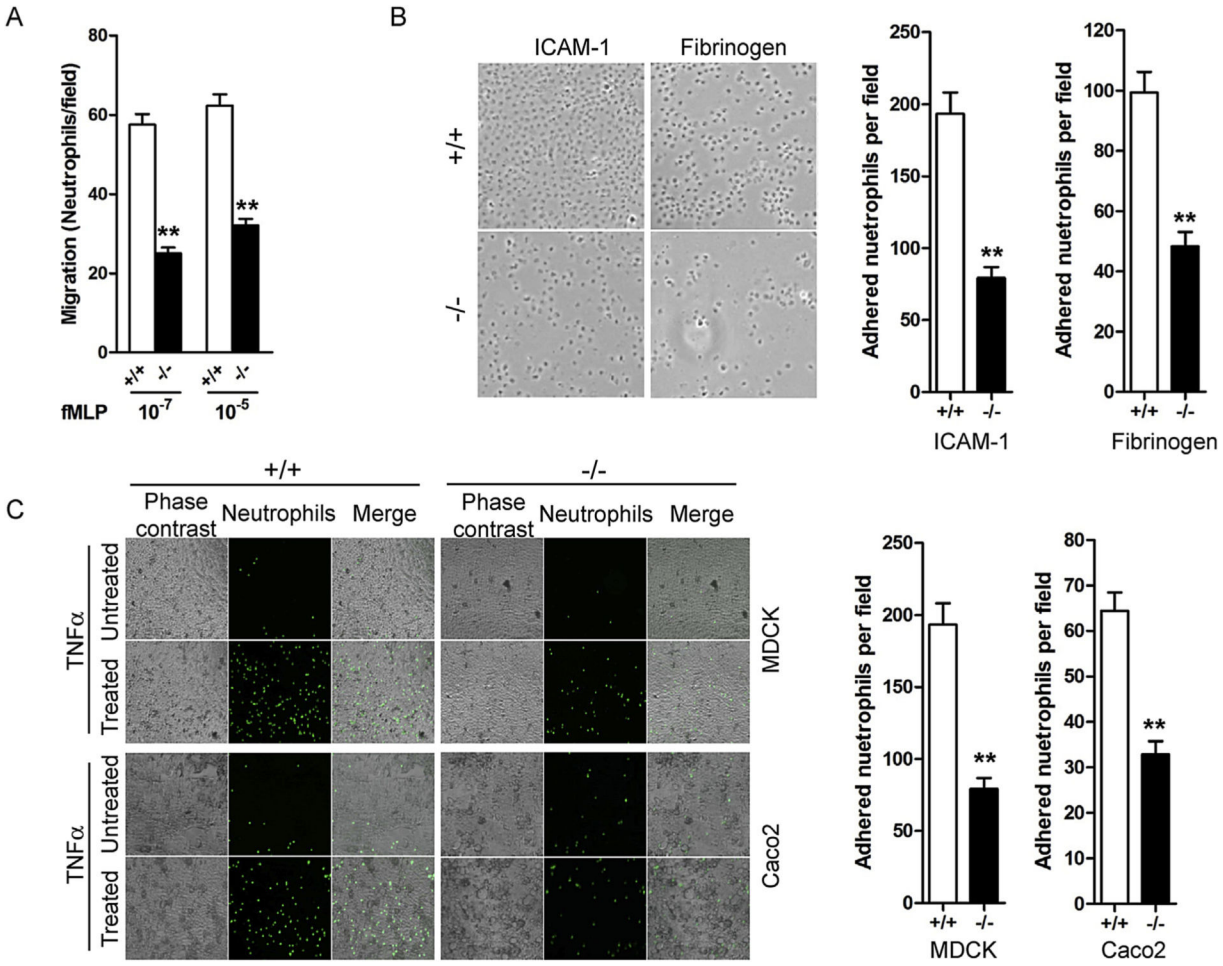

Fig. 3.

In vitro migration and adhesion of neutrophils from *G6pt*$^{-/-}$ and control mice. (A) Neutrophil migration in response to fMLP. (B) Adhesion of PMA-activated neutrophils to purified mouse ICAM-1 and fibrinogen. Data presented in the chart represent the average of three independent experiments with counting neutrophils in four random fields. (C) Adhesion of PMA-activated neutrophils to epithelial cells. Two epithelial cells, MDCK and Caco2, were treated with TNFα as described in Materials and Methods. Data presented in the charts represent the average of three independent experiments with counting neutrophils in four random fields. Data represent the mean ± SEM. *$P < 0.05$, **$P < 0.01$.

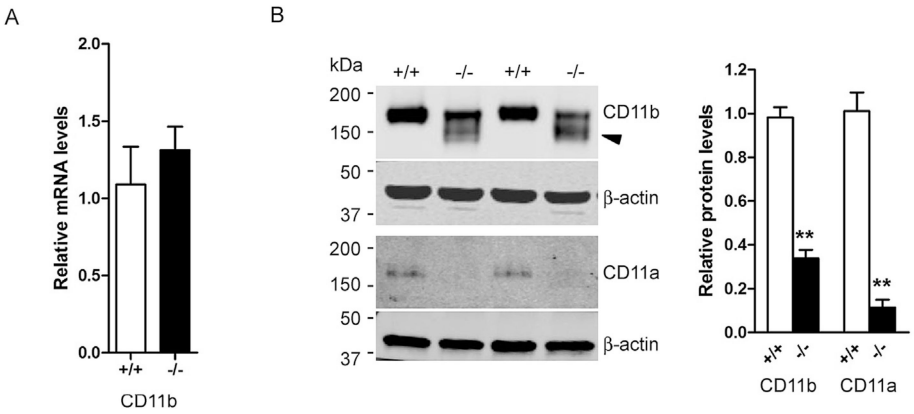

Fig. 4.

Analysis of the expression of CD11b and CD11a, on neutrophils from *G6pt*$^{-/-}$ and control mice. (A) Quantification of CD11b mRNA in G6PT-deficient (−/−) and wild-type neutrophils (+/+) by real-time RT-PCR. Expression is normalized to that of β-actin and measured relative to one wild-type neutrophil arbitrarily defined as 1. (B) Western blot analysis of protein extracts of neutrophils. Arrowhead indicates the deglycosylated form of CD11b. Relative CD11b and CD11a protein levels were quantified by densitometry of at least three independent western blots. Data represent the mean ± SEM. **P* < 0.05, ***P* < 0.01.