HHS Public Access

Author manuscript

*J Leukoc Biol*. Author manuscript; available in PMC 2018 August 17.

Published in final edited form as:

*J Leukoc Biol*. 2008 June ; 83(6): 1541–1550. doi:10.1189/jlb.1207838.

# Endotoxemia down-regulates bone marrow lymphopoiesis but stimulates myelopoiesis: the effect of G6PD deficiency

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## Abstract

Bone marrow (BM) dysfunction is an important component of immunomodulation. This study investigated alterations in cell content, apoptotic responses, and cell proliferation in BM, blood, and spleen in endotoxemic mice (LPS from *Escherichia coli*). As the decreased antioxidant status associated with glucose-6-phosphate dehydrogenase (G6PD) deficiency has been shown to modulate the innate immune response, we also tested whether a G6PD mutation (80% decrease in cellular enzyme activity) alters BM responses during endotoxemia. LPS decreased BM myeloid ($CD45^+CD11b^+$) and B lymphoid ($CD45^+CD19^+CD11b^-$) cell content compared with controls. In contrast, LPS increased $CD11b^+$ myeloid but decreased T and B cell counts in the circulation. Endotoxemia inhibited spontaneous, heat shock, and $H_2O_2$-induced apoptosis as well as proliferative activity in BM lymphoid cells. In contrast, BM myeloid cell apoptosis was not altered, and their proliferative activity was increased during endotoxemia. Following LPS, splenic myeloid cell content was increased, and T and B cell content was unchanged; furthermore, splenocytes showed increased apoptosis compared with controls. BM cell content, including lymphoid and myeloid cells, was greater in G6PD mutant than wild-type (WT) mice, and LPS decreased BM cell counts to a greater degree in mutant than WT mice. Endotoxemia caused widespread inhibition of BM cytokine and chemokine production; however, IL-6 production was increased compared with controls. LPS-induced IL-6 production was decreased in G6PD mutant animals compared with WT. This study indicates that endotoxin inversely affects BM myeloid and lymphoid cell production. LPS-induced down-regulation of B cell production contributes to the generalized lymphopenia and lymphocyte dysfunction observed following nonspecific immune challenges.

### Keywords

lipopolysaccharide; B cells; erythroid; apoptosis; proliferation; glucose-6-phosphate dehydrogenase; cytokines

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## INTRODUCTION

Sepsis or endotoxemia results in marked changes in the number of circulating polymorphonuclear phagocytes (PMN). Dependent on the dose and duration of the challenge, circulating PMN numbers tend to increase after endotoxin administration, whereas PMN blood counts are depleted following experimental polymicrobial sepsis [1, 2]. However, in contrast to the dynamic changes in circulating PMN numbers, it has been observed in endotoxemic and septic experimental rodent models that lymphocytes, including B and T cells, are markedly depleted following endotoxin as well as septic challenges [1–4]. Whereas lymphocyte apoptosis developing at the later stages of sepsis is a well-investigated area [4], the pathophysiological mechanism responsible for the acute and generalized lymphocytopenia manifesting early after infections has not been elucidated. Whereas it is well known that chronic infection results in anemia and decreased bone marrow (BM) activity [5, 6], and furthermore, severe injuries may modulate myelopoiesis [7, 8], the potential role of altered BM function and its relationship to the accompanying changes in blood and tissue cell composition during the acute phase of an innate immune response have not been thoroughly investigated. Thus, the studies tested the question of whether acute alterations in BM function contribute to the changes in peripheral myeloid and lymphoid cell dynamics during the early phase of endotoxemia.

The second aim of the investigations was to compare the endotoxin-induced BM responses as well as the accompanying changes at the periphery between glucose-6-phoshate dehydrogenase (G6PD) mutant and wild-type (WT) animals. G6PD is the rate-limiting enzyme of the hexose monophosphate shunt and a key component of the cellular antioxidant defense machinery [9–12]. The X-linked human G6PD deficiency is one of the most common genetic polymorphisms [11, 12]. G6PD polymorphism is associated with malaria protection, and the population frequency of the mutated alleles may reach 10–25% in malaria-endemic regions. The most common polymorphic forms are the Mediterranean with less than 10% residual erythrocyte G6PD activity and the African variants with 10–20% residual erythrocyte G6PD activity [11, 12]. In these human deficiencies, the G6PD activity decrease in nucleated white blood cells (WBC) is less pronounced with 20–30% residual activity in the Mediterranean forms, whereas near normal levels in the African variants.

Oxidative stress can modulate signaling pathways [13, 14] and could also contribute to organ dysfunction during the innate immune response [2, 3, 15, 16]. Furthermore, independent observations suggested increased incidence of sepsis and altered inflammatory course in G6PD deficiency [16, 17]. Therefore, we also tested the effect of a hypomorphic G6PD mutation on BM function and associated changes in the periphery. We used a G6PD mutant mouse strain that displays a 15–20% residual G6PD activity in erythrocytes, as well as in WBC. Thus, the residual cellular G6PD activity in this mutant mouse model represents a defect that is similar to that observed in the erythrocytes of the African type and in the WBC of the Mediterranean human variants. Cell composition changes, apoptosis, and cell proliferative activities were compared in different BM cell lineages together with the accompanying cell responses in blood and spleen.

## MATERIALS AND METHODS

### Reagents

Endotoxin-free, cell culture-grade buffers, media, and reagents were used in the experiments. FBS was purchased from Irvine Scientific (Santa Ana, CA, USA) and the protein assay kit from Pierce (Rockford, IL, USA). Flourochrome-conjugated antibodies, assay diluents, lysing, and permeabilizing flow cytometry solutions, Annexin-V, and FITC-BrdU kits were purchased from BD Biosiences (San Jose, CA, USA). For the in vitro cytokine studies, palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) and ultra-pure LPS (from *Escherichia coli*, 0111:B4) were purchased from InvivoGen (San Diego, CA, USA). All other reagents and chemicals of the highest grade available were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

### Animals and endotoxin treatment

Male G6PD-deficient mice (y/−) and their normal (WT, y/−) littermates (8–12 weeks old) derived from the same colony were used in the experiments as described in detail earlier [2, 18]. The inbred line from a mixed background [18] is similar to commonly used mouse strains in growth rate and size, and the G6PD mutant animals are phenotypically indistinguishable from WT. Original breeding pairs of G6PD mutant mice were purchased from the Medical Research Council (MRC) of England (Frozen Embryo and Sperm Archive Mammalian Genetics Unit, MRC, Chilton, UK). Animals were housed under 12 h light/dark cycles and fed standard rodent chow.

In the in vivo endotoxemic studies, we used sequential LPS (from *E. coli*, 026:B6, Sigma-Aldrich), with the initial i.p. injection of 10 mg/kg body weight (bw), followed by 25 mg/kg bw 3 h later [3]. This LPS protocol results in 40–70% mortality; however, only a negligible number of animals expire during the first 24 h of endotoxemia. Thus, survival bias does not skew the observations [3]. This LPS treatment caused increased mortality in G6PD mutant animals compared with WT as published previously [3]. The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals {Department of Health and Human Services, Publication No. [National Institutes of Health (NIH)] 85–23, Revised 1985, Office of Science and Health Reports, Data Reliability Review/NIH, Bethesda, MD, USA} and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School (Newark, NJ, USA).

### Genotyping

The G6PD gene in the mutant mouse shows a single base (A–T) difference from the published mouse G6PD sequences (BALB/c, C3H, C57BL/6) in the 5′ splice site consensus sequence at the 3′ end of exon 1, part of the untranslated region [18]. The mutation causes an 80% decrease in the cellular protein level of G6PD as a result of decreased mRNA stability or translation rate. The degree of G6PD activity decrease is similar in erythrocytes and nucleated WBC, as the structure and the stability of the mature protein are not affected by the mutation [18]. Animals were genotyped by using our allele-specific PCR method as we described in detail earlier [3]. Briefly, total genomic DNA was isolated from tail clippings using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). DNA was

subjected to amplification using primer sets that span the mutation site and uniformly amplify DNA from deficient and WT animals (control reaction, forward primer 5′-GGAAACTGGCTGTGCGCTAC, reverse primer 5′-TCAGCTCCGGCTCTCTTCTG). In parallel reactions, the same DNA was incubated in the presence of the same reverse primer together with the forward primer (5′-TGGCAGCGGCAACTAAACTCA), which reacts with the WT, or (5′-TGGCAGCGGCAACTAAACTCT), which reacts with the deficient G6PD allele, respectively. PCR reaction was carried out in the presence of 2 mM $MgCl_2$ with the following cycling: 94°C, 15 s; 68°C, 1 min; 28 cycles.

### Blood, splenocyte, and BM cell isolation and incubations

Blood was collected into heparinized tubes via cardiac puncture from fully anesthetized animals. Following the exsanguination, femurs were collected from the same animals. Femurs were cut at the diaphyses and BM cells flushed out by repeated injections of PBS containing 10% FBS through the bone channel. BM cells were sedimented and washed by centrifugation and suspended in a final volume to obtain 10 million/ml cells in the same PBS/FBS buffer. Next, the spleen was removed and placed into DMEM containing 10% FBS and penicillin streptomycin solution. Hypodermic needles were used to pull apart the splenic capsule, releasing spleen cells into suspension. The cell suspension together with the remaining splenic capsule was squeezed through a 70-μm nylon mesh cell strainer. Isolated BM cells or splenocytes were resuspended in DMEM containing 1% FBS for subsequent analyses or in vitro incubations.

Spontaneous, heat shock, and $H_2O_2$-induced apoptosis was measured using the Annexin-V apoptosis assay kit according to the manufacturer's protocol (BD Biosciences): Splenocytes and BM cells from control or endotoxemic animals were isolated and incubated at 37°C in a cell culture incubator for 4 h in the absence (spontaneous apoptosis) or in the presence of prevailing $H_2O_2$ concentrations. An additional set of cell aliquots was exposed to 43°C for 30 min (heat-shock) followed by a 3.5-h incubation at 37°C. At the end of the incubations, cells were collected, then washed, and resuspended in Annexin-V-binding buffer and incubated with Annexin-V and propidium iodide for 15 min at room temperature in the dark prior to flow cytometry analyses.

CFUs were determined as described earlier. Briefly, $10^6$ BM cells in 0.1 ml culture media were mixed with 0.4 ml methylcellulose (Methocult) and 0.5 ml Iscove's media containing 30% FBS, 2% BSA, 2.5 U/m rhGM-CSF, 0.2 mM β-mercaptoethanol and antiobotic/antimycotic solution. The cell suspension was placed in a culture dish and incubated in a humidified $CO_2$ incubator at 37°C. Media were refreshed at every 48 h, and after 2 weeks of incubation, the number of individual colonies was counted.

RBC deformability was analyzed with a laser-assisted ectacytometer (LORCA) as described previously [19]. Briefly, an aliquot containing ~30 million RBC was suspended in 1 ml 5% polyvinylpyrrolidone (m.w. 360,000; Sigma-Aldrich) in PBS at a final viscosity of 30 mPa at 37°C. Cell suspensions were exposed to increasing shear stress, and the elongation index (EI) was determined. $K_{EI}$ is calculated from the response curves and indicates the shear stress value in Pascal that is required to cause half-maximal erythrocyte elongation as described in details previously [19].

### Flow cytometry

The number of PMNs and lymphocyte subsets in blood and spleen was calculated by the number of total cell counts and the percent distribution of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ myeloid cells using antibodies against CD markers conjugated with FITC, allophycocyanin (APC), PerCP, or PE (BD Biosciences) in three- or four-color incubations. BM cell composition was determined by the cell distribution of $CD45^+CD19^+CD11b^-$ (B cells), $CD1b^+CD45^+CD19^-$ (myeloid cells), and $Ter119^+CD45^-CD11b^-$ (erythroid cells). As expected, CD3CD8− and CD3CD4-positive T cell content was negligible in BM from control as well as endotoxemic animals; thus, BM T cell content was not followed in the assays. Aliquots of 0.1 ml whole blood, splenocyte, or BM cell suspension were incubated with the respective markers for 15 min followed by incubation with BD FACS lysing solution (BD Biosciences) for 7 min at 37°C. Cells were washed twice with BD FACS wash buffer and then fixed with 1% methanol-free formaldehyde. FACS acquisitions were performed in a centralized flow cytometry facility. At least 30,000 events were collected for each analysis.

In vivo DNA synthesis rates by different BM cell lineages were assessed by using the FITC-BrdU flow kit (BD PharMingen, San Diego, CA, USA). BrdU (0.06 mg/kg bw in 0.4 ml vol) was injected i.p. into control or endotoxemic mice 2 h before harvesting the BM (i.e., at 22 h of endotoxemia). Following anesthesia and tissue collections, BM cell suspensions were prepared, and $10^6$ BM cells were incubated with fluorochrome-conjugated surface marker CD45, CD19, CD11b, and Ter119 antibodies in three-color incubations for 20 min at 4°C. Cells were washed with BD stain buffer then fixed and permeabilized by incubating with BD Cytofix/Cytoperm buffer for 30 min at 4°C. This was followed by incubation with BD Cytoperm-plus buffer for 10 min and subsequent incubation with the BD Cytofix/Cytoperm buffer for 5 min at 4°C. Cells were washed and treated with DNase at 37°C for 1 h. Finally, washed cell suspensions were incubated with the FITC-conjugated anti-BrdU antibody for 20 min at room temperature, washed with BD perm/wash buffer, and analyzed by flow cytometry.

### Cytokine antibody arrays and ELISAs

BM cell suspensions ($10^6$ cells/ml) were incubated in the absence or presence of 100 ng/ml LPS for 14 h at 37°C. Media were collected, and cytokine content was determined by using the Ray Bio Mouse Cytokine Antibody Array (Ray-Biotech, Inc., Norcross, GA, USA). Following the cell incubations, media samples were incubated with the membranes precoated with arrayed antibodies. Membranes were washed and processed according to the manufacturer's protocol, and the signals were detected by using a chemiluminescence imaging system at our centralized facility. Signals determined at individual spots were normalized to standards present on the same membrane. Normalized values detected on individual membranes obtained from endotoxemic and control animals or after the in vitro treatments were compared and expressed as relative change between samples in arbitrary units. Media content for selected cytokines was also determined using ELISAs (BD Biosciences) in a separate set of experiments.

### Statistical analysis

Statistical calculations were performed using JMP software (SAS Institute Inc., Cary, NC, USA). Results were analyzed using ANOVA, followed by *t*-test for pair-wise comparisons or Tukey-Kramer's test for multiple comparisons. Different study components were performed on six to eight different animals from each of the in vivo treatment groups, unless indicated otherwise. A statistically significant difference was concluded at $P < 0.05$.

## RESULTS

### Endotoxin-induced cell composition changes in the BM and periphery

In the first series of experiments, we determined the effect of endotoxemia on the WBC composition changes in blood, spleen, and BM. Endotoxemia decreased the number of circulating, total WBC counts in G6PD mutant animals, whereas the change in WBC count was not statistically significant in WT mice (Fig. 1A). Analysis of WBC subsets indicated an increase in circulating $CD11b^+$ cells (myeloid cells), whereas the number of B cells ($CD19^+$) as well as $CD4^+$ and $CD8^+$ T cells markedly decreased in endotoxemic animals compared with controls (Fig. 1, C–F). Circulating platelet numbers also decreased following LPS in WT and G6PD mutant animals compared with controls (Fig. 1B).

Figure 2 indicates cell composition changes in the spleens from the same animals. Total splenocyte yield was similar in WT and G6PD mutant mice, and LPS injection caused no statistically significant increase in total splenic cell content (Fig. 2A). Analysis of WBC subsets indicated that the numbers of splenic neutrophils and macrophages were markedly increased 24 h after LPS compared with controls (Fig. 2, B and C). In contrast, the number of splenic B cells and T cell subsets was not altered significantly by LPS treatment (Fig. 2, D–F).

Cell composition analyses of BM from the same animals are shown in Figure 3. Myeloid cells were strongly positive for CD45 and CD11b but were negative for CD19. B lymphoid cells stained positive for CD45 and CD19 but were weak/ negative for CD11b. As CD45 expression increases during B cell ontogeny [20, 21], and $CD19^+$ B cells clearly separated into two distinct subpopulations by the CD45-staining intensity, we analyzed mature (Fig. 3, flow panel R1 gate) and immature B cell contents (R2 gate) separately.

Total BM cell content was greater in G6PD mutant than WT animals under unchallenged, control conditions (Fig. 3A). LPS treatment markedly decreased BM cell content in WT and G6PD mutant animals (Fig. 3A). Under control conditions, G6PD mutant animals showed increased content of B lymphoid (Fig. 3, B and D) and myeloid (Fig. 3C) cells as compared with WT. Following LPS, myeloid content was depleted similarly in WT and G6PD mutant animals (Fig. 3C). Endotoxin depleted the mature B cell population only in G6PD mutant animals (Fig. 3B), whereas the less-mature B cell population was depleted similarly in WT and G6PD mutant mice (Fig. 3D). BM content of the remaining cells (R4), which contained a mixture of erythroid, other lymphoid, mesenchymal, and precursor cells, was also greater in G6PD than WT animals, and their BM content decreased following LPS treatment (Fig. 3E).

In a separate set of animals, we also tested the effect of endotoxin on circulating and BM erythroid cells. Twenty-four-hour endotoxemia did not alter the number of circulating erythrocytes in WT or G6PD-deficient animals (Fig. 4A). Erythrocyte dysfunction was assessed by determination of erythrocyte deformability. Whole blood was exposed to increasing shear stress, and from the response curve of erythrocyte elongation response, $K_{EI}$ was calculated. $K_{EI}$ represents the shear stress value (Pascal) required to cause half of the maximal erythrocyte elongation as we described earlier [19]. Figure 4B indicates that the LPS-induced increase in $K_{EI}$ was greater in G6PD mutant than WT animals, indicating the presence of more rigid erythrocytes in endotoxemic G6PD mutant animals (Fig. 4B). A BM erythroid line content identified by the marker set used ($Ter119^+$,$CD45^-$,$CD11b^-$) showed similar values in G6PD mutant and WT animals, and 24 h endotoxemia caused no significant decrease in BM erythroid cell content (Fig. 4, C and D).

### BM cell apoptosis and cell proliferation after endotoxemia

In the next series of experiments, we tested how the LPS-induced cell composition changes correlate with the apoptotic responses in BM and spleen (Figs. 5 and 6). Spontaneous apoptosis of BM lymphoid as well as myeloid lineages was similar (~7%) in control WT and G6PD mutant animals. Following endotoxemia, BM lymphoid cells showed decreased levels of spontaneous apoptosis compared with controls, whereas the number of spontaneously apoptotic myeloid cells was not affected significantly by endotoxin treatment (Fig. 5, A and B). Heat shock increased the number of apoptotic B cells and myeloid cells two- to threefold (Fig. 5, C and D; compare *y*-axes of Panels A and B). The heat shock-induced B cell apoptotic response remained decreased following endotoxemia as compared with controls, whereas the heat-shock response by myeloid cells was similar in control and endotoxemic animals. $H_2O_2$ administration increased B cell apoptosis in a concentration-dependent manner; however, the overall number of apoptotic B cells remained lower in endotoxemic than control animals (Fig. 5E). G6PD mutant B cells showed an increased tendency of the apoptotic response to $H_2O_2$ as compared with WT, reaching a statistically significant difference at 2 μM $H_2O_2$ treatment (Fig. 5E). BM myeloid cells showed no consistent increase in oxidant-induced apoptosis in WT or G6PD mutant animals (Fig. 5F).

In contrast to the observations in the BM, spontaneous splenocyte apoptosis was increased following endotoxemia (Fig. 6A). Heat shock proportionally increased splenocyte apoptosis in control and endotoxemic animals (Fig. 6B). Whereas splenocytes from control animals displayed resistance to $H_2O_2$-induced apoptosis, $H_2O_2$ treatment caused marked and concentration-dependent increases in the number of apoptotic splenocytes from endotoxemic animals (Fig. 6C). The response pattern was similar in WT and G6PD mutant mice.

As BM cell composition is also controlled by cellular proliferative activity, next, we tested the effect of endotoxemia on the in vivo DNA-synthesizing activity in BM cell lineages using an in vivo BrdU flow cytometry assay. Figure 7 indicates that in vivo BrdU incorporation by $CD45^+CD19^+$ BM lymphoid cells was decreased in endotoxemic animals compared with controls (Fig. 7A). In contrast, BrdU incorporation into myeloid cells ($CD45^+$,$CD11b^+$) was augmented following endotoxemia (Fig. 7B). The difference in BrdU incorporation rates by the cells of the erythroid lineage between endotoxemic and control

animals did not reach statistically significant levels (Fig. 7C). The endotoxin-induced changes in BrdU incorporation in all three lineages were similar in WT and G6PD mutant mice.

To further elucidate potential differences between WT and G6PD mutant BM myelopoietic activity, we also compared the colony formation activity (CFU) in long-term, in vitro BM cell cultures (2 weeks). Figure 7D indicates that under these chemically controlled, in vitro conditions, BM CFU activities were similar in endotoxemic and control animals. However, BM CFU activity was greater in G6PD mutant than WT animals obtained from endotoxemic or control mice, indicating an increased number of precursor cells in the G6PD mutant animals.

### Endotoxemia and BM cytokine production

Figure 8, A and B, summarizes observations about the cytokine responses from WT animals. The majority of the tested cytokines, chemokines, and inflammatory mediators showed decreased cytokine production from endotoxemic animals compared with controls (Fig. 8A). A few additional mediators also showed small but statistically nonsignificant decreases after endotoxemia (Fig. 8B). However, in striking contrast with this down-regulated general pattern, IL-6 production was increased following endotoxemia (Fig. 8B). We also determined IL-6 responses by the more quantitative ELISA assay using cells from a different set of experimental animals. Secondary in vitro stimulation by LPS or the TLR2 agonist Pam3CSK4 markedly and similarly stimulated BM IL-6 production from control WT and G6PD mutant animals (Fig. 8C, left panel). Endotoxemia increased IL-6 production, and the TLR2 agonist-induced IL-6 response was greater in endotoxemic than control animals. However, BM cells from endotoxemic G6PD mutant animals displayed attenuated TLR4- or TLR2-induced IL-6 production as compared with WT (Fig. 8C, right panel).

## DISCUSSION

This study demonstrates for the first time that BM myelo- and lymphopoiesis are inversely affected during the acute stage of endotoxemia. Whereas the biological importance of phagocyte mobilization early after nonspecific immune challenges is quite evident, the physiological role or the pathophysiological consequence of the accompanying acute lymphocytopenia involving B as well as T cells is less apparent. Our current observation indicating an endotoxin-induced depression in BM lymphopoiesis provides a possible underlying mechanism that is responsible for the depletion of circulating B cells early after nonspecific immune challenges. Decreased BM lymphopoiesis may be an important contributing factor to the generalized immunosuppression frequently observed in septic conditions.

Despite the fact that endotoxemia decreased myeloid and lymphoid cell content similarly in the BM, it is apparent that different mechanisms are responsible for these cell composition changes. Myeloid cell depletion is most likely the result of prompt release of myeloid cells from the marrow and their subsequent infiltration into immune-competent organs. This conclusion is supported by the observations that the decrease in BM myeloid content is accompanied by a simultaneous increase in the number of circulating neutrophils as well as

an augmented neutrophil infiltration into the spleen and presumably other immune-competent organs. Additionally, following endotoxin, the proliferative activity of BM myeloid cells (BrdU incorporation) is increased; meanwhile, they show largely unaltered, apoptotic responses. These observations together are consistent with an elevated myelopoiesis and immediate release of myeloid cells from the BM during the acute phase of endotoxemia.

In contrast to this LPS-induced response pattern of myeloid cells, BM resident B cells showed decreased de novo DNA synthesis accompanied by depleted B cell numbers in the circulation. The lack of increase in splenic B cell content makes it unlikely that 24 h endotoxemia caused elevated B cell migration into lymph and organs that would account for decreased numbers of circulating B cells. Thus, decreased BM lymphopoiesis is most likely an important component of the LPS-induced marked and fast-developing B cell depletion in the blood. The finding that LPS caused widespread BM cell depletion including cells negative for the markers used is consistent with a generalized inhibition of lymphopoiesis. However, further investigations using T cell progenitor markers will be needed to elucidate whether endotoxin also down-regulates the production of T lymphocytes.

Recent in vitro studies using isolated, naïve B cells and long-term (4–6 days) in vitro cell proliferation assays have demonstrated that B cell TLR activation is an important co-stimulus for BCR and Th cell-induced B cell proliferation [22, 23], as well as during the terminal phase of plasma cell differentiation [24]. Our observation, however, suggests that an acute in vivo TLR4 stimulus, in the absence of accompanying BCR activation and other costimulatory signals, is inhibitory on B cell proliferation and function. The more pronounced LPS-induced decrease in the number of low CD45-expressing B cells in WT BM suggests that the LPS sensitivity is different between the immature and mature B cell subpopulations.

It has been well documented in humans as well as animal models that sepsis results in lymphocyte apoptosis and generalized lymphopenia, which are believed to be major underlying causes of immune paralysis in septic patients [4, 25]. The observed, contrasting effects of endotoxemia on B cell apoptosis in the BM and spleen, with decreased apoptosis in BM versus increased apoptosis in the spleen, suggest a different sensitivity of differentiating and tissue-resident lymphocytes to apoptotic signals. These findings also suggest that the decreased BM lymphopoiesis and the elevated lymphocyte apoptosis at the periphery contribute to the generalized lymphocytopenia and associated lymphocyte dysfunction during endotoxemia. However, despite the fact that B cells remaining in the BM 24 h after endotoxin showed decreased apoptosis, this observation does not rule out the possibility that BM lymphocyte apoptosis occurred during preceding stages of endotoxemia, thereby also contributing to B cell depletion.

The widespread decrease in BM inflammatory mediator production together with the unchanged responsiveness of IL-4 and IL-10 following endotoxemia suggests complex alterations in the pro/anti-inflammatory balance, consistent with a depressed, functional status of the BM. However, in contrast to the general trend of down-regulated or unchanged mediator production, endotoxemia up-regulated BM IL-6 production, suggesting an

important function of IL-6 in the observed responses in agreement with independent observations [5, 6]. IL-6 has been shown to stimulate early hematopoiesis [26], B cell terminal differentiation, and malignant transformation [27–29] as well as to promote progenitor commitment toward the myeloid line [30, 31]. Therefore, an LPS-induced, elevated IL-6 production by the BM may represent a compensatory feedback response aimed at restoring depressed B cell production, or it may also promote myelopoiesis at the expense of lymphopoiesis.

Whereas the overall response pattern was similar in G6PD-deficient and WT animals, the increased BM cellularity in G6PD-deficient animals suggests that the decreased cellular antioxidant capacity associated with this mutation stimulates BM hematopoiesis [32]. The fact that myeloid colony formation in long-term, chemically controlled BM cultures also showed a greater activity in G6PD mutant than WT animals indicates an increased number of resident progenitor cells in G6PD-deficient animals. Previous studies indicated elevated blood IL-6 levels in endotoxemic G6PD mutant animals compared with WT, presumably as a result of augmented resident macrophage activation in the periphery [3, 33]. Blood concentration of IL-6 is markedly elevated following endotoxemia or sepsis, and increasing IL-6 levels have been shown to correlate with worsened outcome [34, 35]. The down-regulated IL-6 response in endotoxin-stimulated BM from G6PD mutant animals is consistent with an altered BM response under the G6PD-deficient conditions. It remains to be determined whether the down-regulated IL-6 production in G6PD mutant animals is associated with altered cell activation, or it is the reflection of different lineage composition between WT and mutant animals.

Although the LPS-induced circulating erythrocyte dysfunction, as reflected in decreased cell deformability, was greater in G6PD mutant than WT animals, endotoxemia had no marked effects on BM erythroid cell content or erythroid BrdU incorporation in these animals. This finding suggests that as a result of the longer lifespan of erythrocytes, an extended period of endotoxin exposure or a longer postendotoxin incubation time is required for a potential manifestation of anemia and the associated alterations in erythropoiesis.

Taken together, these investigations demonstrate that endotoxemia results in inverse effects on the BM's myelopoietic and lymphopoietic activities causing elevated myeloid proliferation but depressed B cell production. Parallel with these changes, BM cytokine production is down-regulated with the exception of IL-6, indicating a distinct, regulatory role of this cytokine in endotoxin-induced BM responses. Down-regulation of BM lymphopoiesis during the acute phase of an innate immune response may represent an important component of immune dysfunction contributing to the immune paralysis observed during prolonged septic conditions.

## Acknowledgments

This study was supported by NIH-National Institute of General Medical Sciences grant GM-69861.

## References


1. Remick DG, Newcomb DE, Bolgos GL, Call DR. Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. Shock. 2000; 13:110–116. [PubMed: 10670840]
2. Spolarics Z, Condon MR, Siddiqi M, Machiedo GW, Deitch EA. Red blood cell dysfunction in septic glucose-6-phosphate dehydrogenase-deficient mice. Am J Physiol Heart Circ Physiol. 2004; 286:H2118–H2126. [PubMed: 14751857]
3. Wilmanski J, Villanueva E, Deitch EA, Spolarics Z. Glucose-6-phosphate dehydrogenase deficiency and the inflammatory response to endotoxin and polymicrobial sepsis. Crit Care Med. 2007; 35:510–518. [PubMed: 17205013]
4. Hotchkiss RS, Coopersmith CM, Karl IE. Prevention of lymphocyte apoptosis—a potential treatment of sepsis? Clin Infect Dis. 2005; 41(Suppl 7):S465–S469. [PubMed: 16237649]
5. Fonseca RB, Mohr AM, Wang L, Sifri ZC, Rameshwar P, Livingston DH. The impact of a hypercatecholamine state on erythropoiesis following severe injury and the role of IL-6. J Trauma. 2005; 59:884–889. [PubMed: 16374277]
6. Livingston DH, Anjaria D, Wu J, Hauser CJ, Chang V, Deitch EA, Rameshwar P. Bone marrow failure following severe injury in humans. Ann Surg. 2003; 238:748–753. [PubMed: 14578739]
7. Santangelo S, Gamelli RL, Shankar R. Myeloid commitment shifts toward monocytopoiesis after thermal injury and sepsis. Ann Surg. 2001; 233:97–106. [PubMed: 11141231]
8. Cohen MJ, Carroll C, He LK, Muthu K, Gamelli RL, Jones SB, Shankar R. Severity of burn injury and sepsis determines the cytokine responses of bone marrow progenitor-derived macrophages. J Trauma. 2007; 62:858–867. [PubMed: 17426540]
9. Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J. 1995; 14:5209–5215. [PubMed: 7489710]
10. Kletzien RF. Glucose-6-phosphate dehydrogenase: a housekeeping enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J. 1994; 8:174–181. [PubMed: 8119488]
11. Beutler E. G6PD deficiency. Blood. 1994; 84:3613–3636. [PubMed: 7949118]
12. Luzzatto L, Mehta A. Glucose 6phosphate dehydrogenase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editorsThe Metabolic and Molecular Bases of Inherited Disease. New York, NY, USA: McGrawHill; 1995. 3367–3398.
13. Finkel T. Signal transduction by reactive oxygen species in non-phagocytic cells. J Leukoc Biol. 1999; 65:337–340. [PubMed: 10080536]
14. Jones DP. Redefining oxidative stress. Antioxid Redox Signal. 2006; 8:1865–1879. [PubMed: 16987039]
15. Efferth T, Fabry U, Glatte P, Osieka R. Increased induction of apoptosis in mononuclear cells of a glucose-6-phosphate dehydrogenase deficient patient. J Mol Med. 1995; 73:47–49. [PubMed: 7633942]
16. Spolarics Z, Siddiqi M, Siegel JH, Garcia ZC, Stein DS, Ong H, Livingston DH, Denny T, Deitch EA. Increased incidence of sepsis and altered monocyte functions in severely injured type A-glucose-6-phosphate dehydrogenase-deficient African American trauma patients. Crit Care Med. 2001; 29:728–736. [PubMed: 11373456]
17. Abu-Osba YK, Mallouh AA, Hann RW. Incidence and causes of sepsis in glucose-6-phosphate dehydrogenase-deficient newborn infants. J Pediatr. 1989; 114:748–752. [PubMed: 2715888]
18. Sanders S, Smith DP, Thomas GA, Williams ED. A glucose-6-phosphate dehydrogenase (G6PD) splice site consensus sequence mutation associated with G6PD enzyme deficiency. Mutat Res. 1997; 374:79–87. [PubMed: 9067418]
19. Condon MR, Kim JE, Deitch EA, Machiedo GW, Spolarics Z. Appearance of an erythrocyte population with decreased deformability and hemoglobin content following sepsis. Am J Physiol Heart Circ Physiol. 2003; 284:H2177–H2184. [PubMed: 12742829]

20. McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. Blood. 2001; 98:2498–2507. [PubMed: 11588048]
21. Carulli G, Cannizzo E, Zucca A, Buda G, Orciuolo E, Marini A, Petrini M. CD45 expression in low-grade B-cell non-Hodgkin's lymphomas. Leuk Res. 2008; 32:263–267. [PubMed: 17692374]
22. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur J Immunol. 2006; 36:810–816. [PubMed: 16541472]
23. Dye JR, Palvanov A, Guo B, Rothstein TL. B cell receptor cross-talk: exposure to lipopolysaccharide induces an alternate pathway for B cell receptor-induced ERK phosphorylation and NF-κ B activation. J Immunol. 2007; 179:229–235. [PubMed: 17579042]
24. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J Immunol. 2007; 178:7779–7786. [PubMed: 17548615]
25. Hotchkiss RS, Tinsley KW, Karl IE. Role of apoptotic cell death in sepsis. Scand J Infect Dis. 2003; 35:585–592. [PubMed: 14620139]
26. Kopf M, Ramsay A, Brombacher F, Baumann H, Freer G, Galanos C, Gutierrez-Ramos JC, Kohler G. Pleiotropic defects of IL-6-deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. Ann N Y Acad Sci. 1995; 762:308–318. [PubMed: 7545368]
27. Altmeyer A, Simmons RC, Krajewski S, Reed JC, Bornkamm GW, Chen-Kiang S. Reversal of EBV immortalization precedes apoptosis in IL-6-induced human B cell terminal differentiation. Immunity. 1997; 7:667–677. [PubMed: 9390690]
28. Morse L, Chen D, Franklin D, Xiong Y, Chen-Kiang S. Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor p18(INK4c) and IL-6. Immunity. 1997; 6:47–56. [PubMed: 9052836]
29. Cozen W, Gill PS, Ingles SA, Masood R, Martinez-Maza O, Cockburn MG, Gauderman WJ, Pike MC, Bernstein L, Nathwani BN, Salam MT, Danley KL, Wang W, Gage J, Gundell-Miller S, Mack TM. IL-6 levels and genotype are associated with risk of young adult Hodgkin lymphoma. Blood. 2004; 103:3216–3221. [PubMed: 15070705]
30. Bernad A, Kopf M, Kulbacki R, Weich N, Koehler G, Gutierrez-Ramos JC. Interleukin-6 is required in vivo for the regulation of stem cells and committed progenitors of the hematopoietic system. Immunity. 1994; 1:725–731. [PubMed: 7895162]
31. Zhu J, Garrett R, Jung Y, Zhang Y, Kim N, Wang J, Joe GJ, Hexner E, Choi Y, Taichman RS, Emerson SG. Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. Blood. 2007; 109:3706–3712. [PubMed: 17227831]
32. Tothova Z, Kollipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, McDowell EP, Lazo-Kallanian S, Williams IR, Sears C, Armstrong SA, Passegué E, DePinho RA, Gilliland DG. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell. 2007; 128:325–339. [PubMed: 17254970]
33. Wilmanski J, Siddiqi M, Deitch EA, Spolarics Z. Augmented IL-10 production and redox-dependent signaling pathways in glucose-6-phosphate dehydrogenase-deficient mouse peritoneal macrophages. J Leukoc Biol. 2005; 78:85–94. [PubMed: 15817708]
34. Ebong S, Call D, Nemzek J, Bolgos G, Newcomb D, Remick D. Immunopathologic alterations in murine models of sepsis of increasing severity. Infect Immun. 1999; 67:6603–6610. [PubMed: 10569781]
35. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. Shock. 2002; 17:463–467. [PubMed: 12069181]

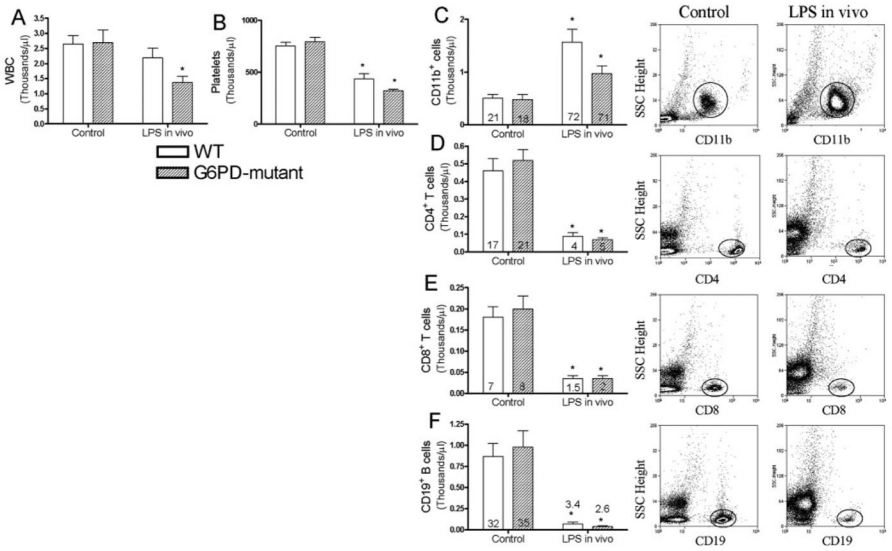


**Fig. 1.**

Endotoxemia increases the number of circulating neutrophils but depletes T and B lymphocytes. Twenty-four hours after LPS or saline injection (controls), blood was collected, and total WBC and platelet counts were determined (A and B). (C–F) Numbers of circulating $CD11b^+$ (mostly neutrophils) and different T cell ($CD4^+$ and $CD8^+$) and B cell ($CD19^+$) subsets as indicated. Numbers within the bars depict the mean values of percent cell distributions. Flow cytometry panels on the right show typical findings from a WT animal. *, Statistically significant difference as compared with control within the same genotype. Mean ± SEM; $n$ = 7–8 animals in each group. SSC, Side-scatter.

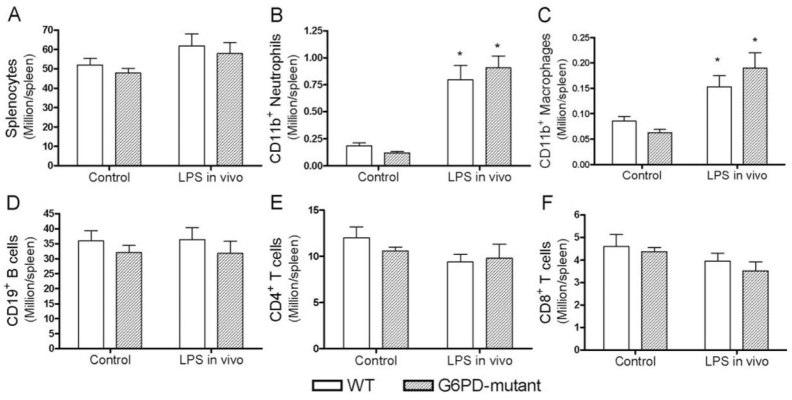


**Fig. 2.**

Endotoxemia increases neutrophil and macrophage infiltration into spleen. Twenty-four hours after LPS or saline injection, splenocyte suspensions were prepared, and cell counts were determined (A). Based on $CD11b^+$ staining and light-scatter properties, neutrophil and macrophage numbers as well as $CD4^+$ or $CD8^+$ T cells and $CD19^+$ B cell counts were calculated from total cell yields and the percent distribution of these cell populations (B–F). *, Statistically significant difference as compared with control within the same genotype. Mean ± SEM; $n$ = 7–8 animals in each group.

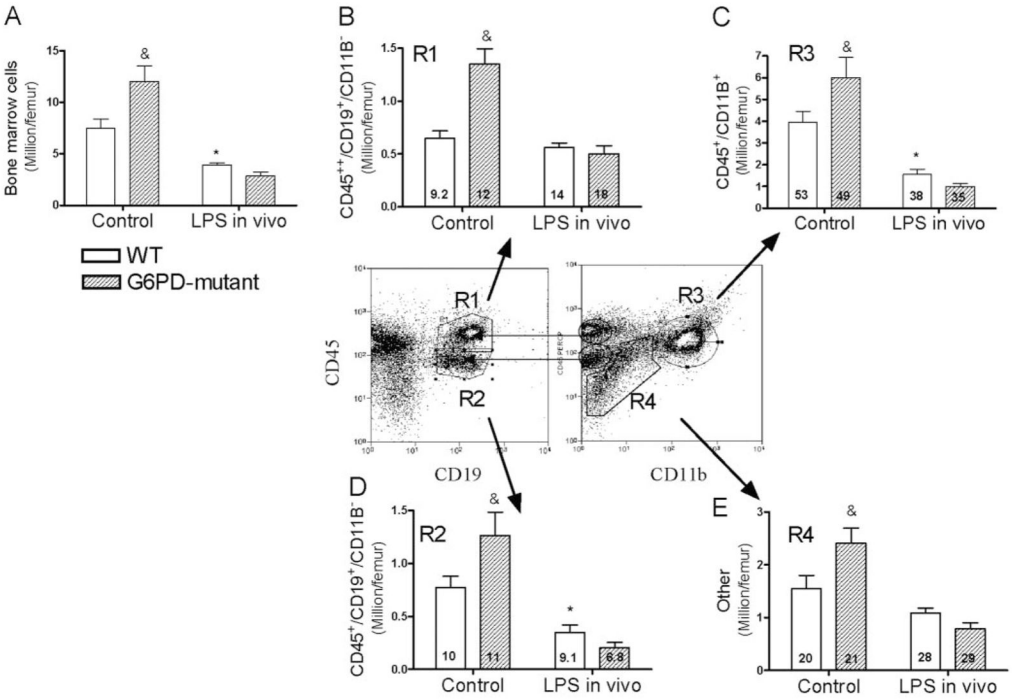


**Fig. 3.**

Endotoxemia down-regulates BM hematopoiesis. Twenty-four hours after LPS or saline injection, BM cell suspensions were prepared, and cell counts were determined (A). Following the incubations with a set of surface markers as described in Materials and Methods, BM content for different cellular subsets was determined by flow cytometry. B lymphoid cells were $CD45^{+}CD19^{+}/CD11b^{-}$(R1 and R2, B and D), whereas myeloid cells were $CD45^{+}CD11b^{+}CD19^{-}$ (R3, C). Cells with scattered staining for these markers are also shown (R4, E). *, Statistically significant difference as compared with control within the same genotype; &, compared with all other groups. Mean ± SEM; $n = 7–8$ animals in each group.

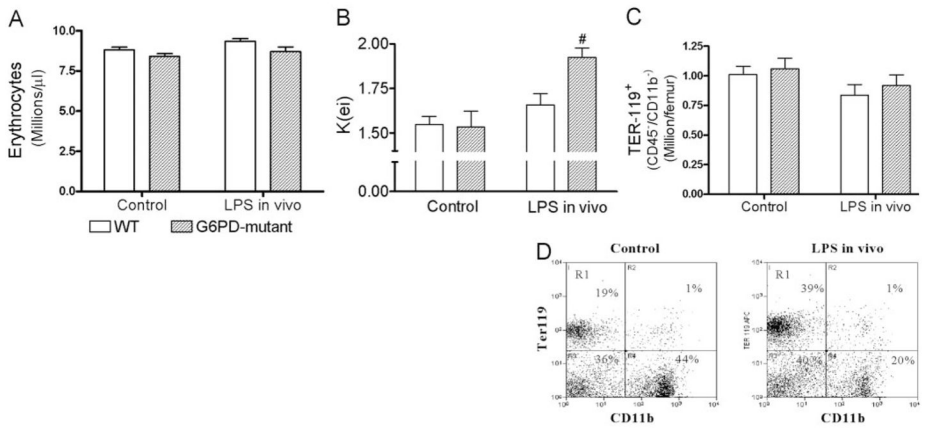


**Fig. 4.**

Erythroid cell responses following endotoxemia. Twenty-four hours after LPS or saline injection, circulating erythrocyte counts were determined (A). RBC deformability was measured by LORCA under prevailing shear stress, and $K_{EI}$ (shear stress causing half-maximal erythrocyte deformability) was calculated and compared (B). BM cell suspensions were also prepared, and erythroid content was determined using Ter199, CD45, and CD11b markers (C). Flow cytometry panels show a typical finding from a WT animal. #, Statistically significant difference as compared with control within the same genotype. Mean ± SEM; $n = 6$ animals in each group.

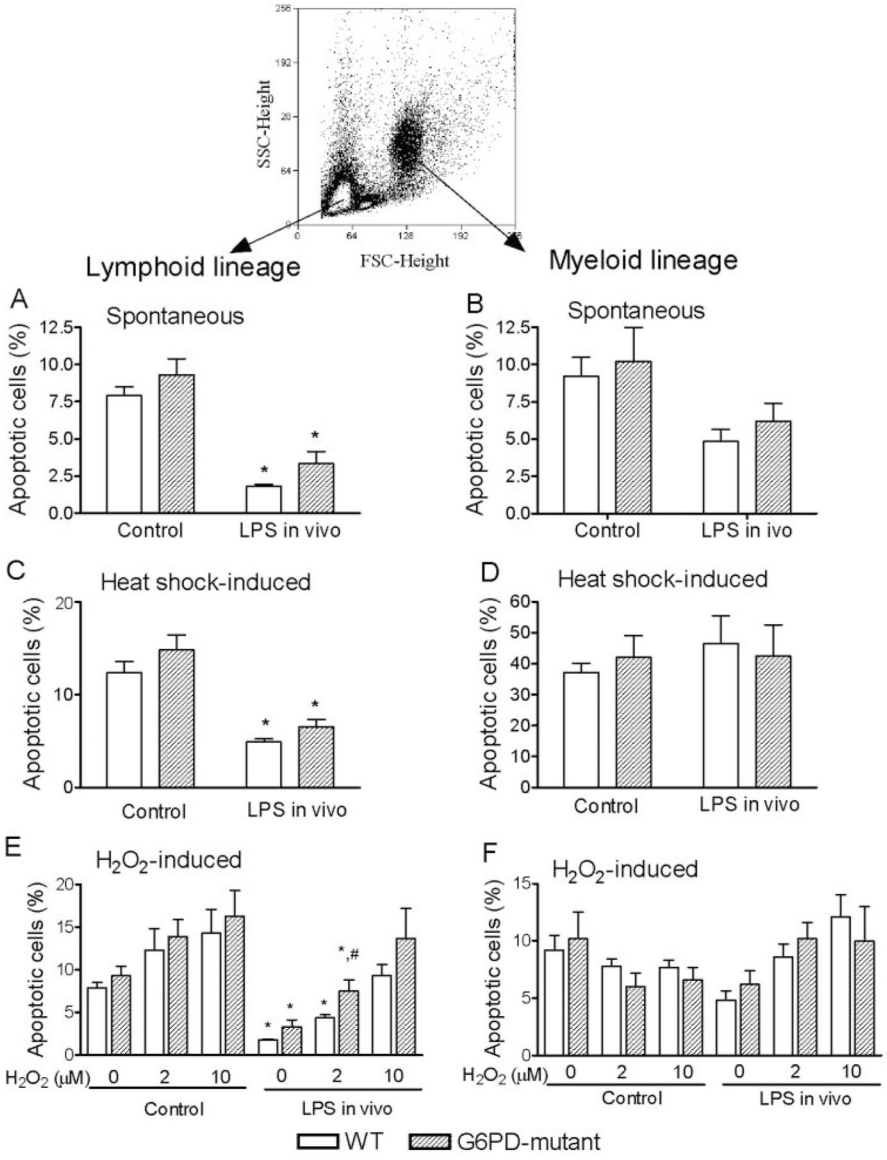


**Fig. 5.**

Residual lymphoid cells in endotoxin-depleted BM display resistance to apoptotic challenges. Twenty-four hours after LPS or saline injection, BM cell suspensions were prepared, and cells were incubated for 4 h at 37°C (A and B) or exposed to heat shock (C and D) or increasing concentrations of $H_2O_2$ (E and F) in parallel incubations. The number of apoptotic cells was determined by Annexin-V staining as described in Materials and Methods. The flow cytometry panel depicts a typical side/ forward scatter (FSC) from a WT animal, indicating two groups of small/less-complex and larger/more-complex populations. Based on back-gating from the experiments shown in Figure 3, these populations corresponded to the lymphoid ($CD45^+CD19^+$, panels on the left) and myeloid populations ($CD45^+CD11b^+$, panels on the right) and were analyzed accordingly. *, Statistically significant difference compared with control within the same genotype; #, compared with WT within the same treatment. Mean ± SEM; $n = 8$ in each group.

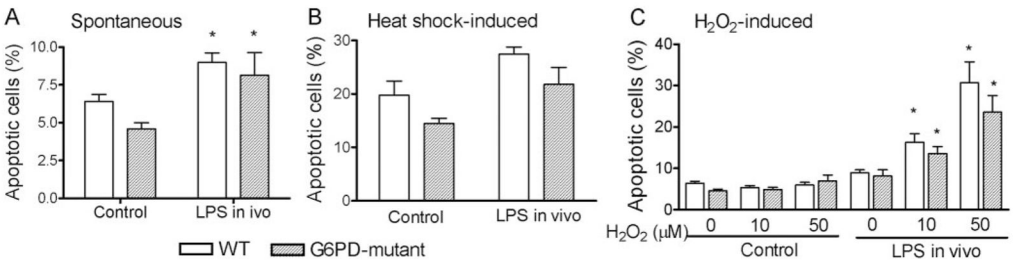


**Fig. 6.**

Increased splenocyte apoptosis following endotoxemia. Twenty-four hours after LPS or saline injection, splenocyte suspensions were prepared, and cells were incubated for 4 h at 37°C (A) or exposed to heat shock (B) or increasing concentrations of $H_2O_2$ (C) in parallel incubations. The number of apoptotic cells was determined by Annexin-V staining. *, Statistically significant difference compared with control within the same genotype. Mean ± SEM; $n = 8$ in each group.

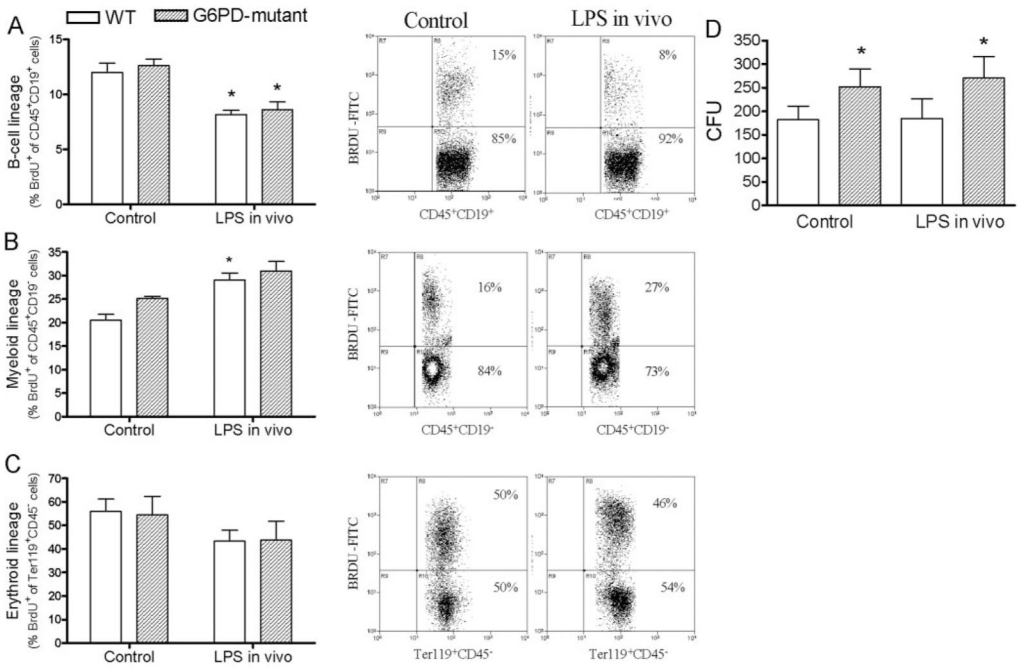


**Fig. 7.**

De novo DNA synthesis is inhibited in B cells but increased in myeloid cells in endotoxin-depleted BM. Twenty-two hours after LPS or saline injection, in vivo BrdU incorporation was determined, as described in Materials and Methods. BM cells were isolated, and BrdU staining in combination with the surface markers used was determined in B cells (A), myeloid cells (B), and erythroid cells (C). *, Statistically significant difference compared with control within the same genotype. (D) CFUs in BM from endotoxemic or control animals after culture in GM-CSF-containing media for 2 weeks. Mean ± SEM; $n = 6–8$ animals for the BrdU studies, or $n = 3$ animals for the CFU activity studies in each group.

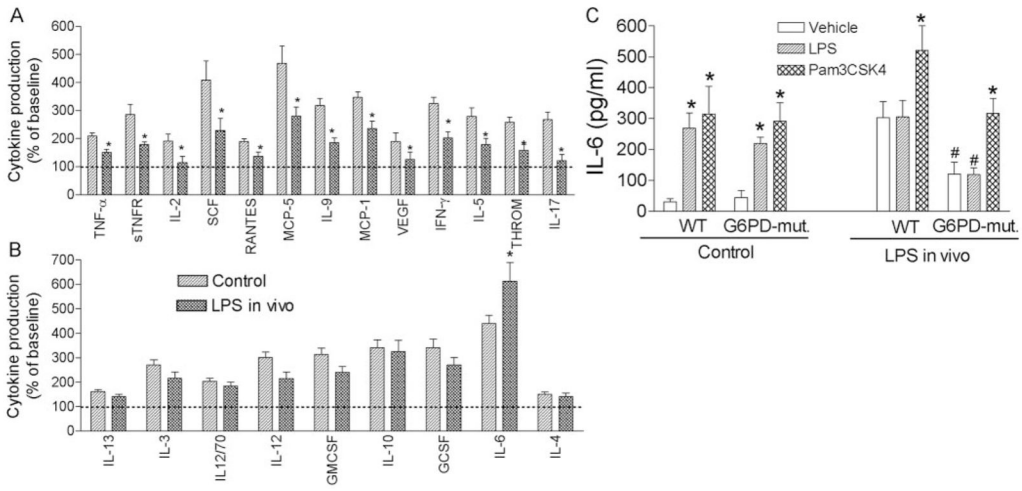


**Fig. 8.**

Endotoxemia down-regulates BM cytokine and chemo-kine production, except IL-6. (A and B) BM cells were prepared from control and endotoxemic WT animals, and suspension cultures of equal cell numbers were exposed to secondary LPS stimulus (100 ng/ml, 14 h). Media were collected and analyzed for cytokine content using a cytokine antibody array. Baseline (dotted line) represents values obtained in controls in the absence of in vitro LPS. (C) BM cells were prepared from an independent set of control and endotoxemic WT as well as G6PD mutant animals, and suspension cultures of equal cell numbers were exposed to LPS (100 ng/ml) or Pam3CSK4 (200 ng/ml) for 14 h. Cytokine content from conditioned media was measured by ELISA. *, Statistically significant difference compared with control within the same genotype; #, compared with endotoxemic WT in the corresponding in vitro treatment. Mean ± SEM; $n = 4$ in each group for cytokine arrays, or $n = 8$ in each group for the ELISA. sTNFR, Soluble TNF receptor; SCF, stem cell factor; VEGF, vascular endothelial growth factor; THROM, thrombopoietin.