Female X-Chromosome Mosaicism for NOX2 Deficiency Presents Unique Inflammatory Phenotype and Improves Outcome in Polymicrobial Sepsis

Rachna Chandra, Stephanie Federici, Zoltán H. Németh, Béla Horváth, Pál Pacher, György Haskó, Edwin A. Deitch and Zoltán Spolarics

*J Immunol* published online 18 April 2011
http://www.jimmunol.org/content/early/2011/04/18/jimmunol.1100205
Female X-Chromosome Mosaicism for NOX2 Deficiency Presents Unique Inflammatory Phenotype and Improves Outcome in Polymicrobial Sepsis

Rachna Chandra,* Stephanie Federici,* Zoltán H. Németh,† Béla Horváth,‡ Pál Pacher,§ György Haskó,* Edwin A. Deitch,* and Zoltán Spolarics*

Cellular X-chromosome mosaicism, which is unique to females, may be advantageous during pathophysiological challenges compared with the single X-chromosome machinery of males, and it may contribute to gender dimorphism in the inflammatory response. We tested the hypothesis of whether cellular mosaicism for the X-linked gp91phox (NOX2) deficiency, the catalytic component of the superoxide anion-generating NADPH oxidase complex, is advantageous during polymicrobial sepsis. Deficient, wild-type (WT), and heterozygous/mosaic mice were compared following polymicrobial sepsis initiated by cecal ligation and puncture. Compared with WT littermates, sepsis-induced mortality was improved in deficient mice, as well as in mosaic animals carrying both deficient and WT phagocyte subpopulations. In contrast, blood bacterial counts were greatest in deficient mice. Consistent with poor survival, WT mice also showed the most severe organ damage following sepsis. In mosaic animals, the deficient neutrophil subpopulations displayed increased organ recruitment and elevated CD11b membrane expression compared with WT neutrophil subpopulations within the same animal. The dynamics of sepsis-induced blood and organ cytokine content and WBC composition changes, including lymphocyte subsets in blood and bone marrow, showed differences among WT, deficient, and mosaic subjects, indicating that mosaic mice are not simply the average of the deficient and WT responses. Upon oxidative burst, interchange of oxidants between WT and deficient neutrophil subpopulations occurred in mosaic mice. This study suggests that mice mosaic for gp91phox expression have multiple advantages compared with WT and deficient mice during the septic course. The Journal of Immunology, 2011, 186: 000–000.

Females show better general health and longer life span than do males, and they present improved clinical course under a variety of conditions, including infections (1–9). The currently held notion is that sex hormones are responsible for gender dimorphic pathophysiological responses and the female advantage in overall health. However, gender dimorphism in pathological processes is also present in young prepubertal children, as well as in the elderly, which suggests that factors other than sex hormones are also involved (10–14). Recent work supports the possibility that the unique properties of the X chromosome, including female X-chromosome inactivation and cellular mosaicism for X-linked polymorphic genes, also contribute to gender-associated differences in physiology, pathophysiology, and disease progression (12, 15–18).

The sex chromosome differences between males and females represent altered gene content and entail gender specificity in X chromosome regulation and function. Males carry the Y chromosome passed on from the father and only one X chromosome inherited from the mother. In contrast, females carry two X chromosomes: one inherited from the mother (Xm, maternal), and one from the father (Xp, paternal). The potential double dose in the expression of X-linked proteins in females is compensated through random X-chromosome inactivation in individual cells, which is achieved by blocking gene expression in one of the parental X chromosomes. Cellular X-chromosome inactivation is complete at an early embryonic stage (19–21) and is maintained through the life span of each individual cell, resulting in cellular mosaicism for all X-linked mutations. Thus, cellular mosaicism for X-linked polymorphic gene expression is unique to females.

Importantly, although the Y chromosome contains only a few genes involved in sex determination during embryonic development, the X chromosome carries a large number of polymorphic genes encoding for proteins with central functions in immunology, signaling, redox processes, and metabolic systems (15, 22), and it expresses polymorphic small regulatory RNAs with potential genome-wide effects (23). Therefore, females carry two cell populations with distinct parental haplotypes, which is expected to manifest differences in regulatory and functional potentials and, thereby, broadening the regulatory and metabolic adaptability to changing environmental conditions.

The notion of the adaptability of mosaicism and its functional relevance is supported by the fact that elderly females frequently display cellular skewing that is characterized by increased representation of circulating blood cells expressing one of the parental X chromosomes. Sustained X chromosome skewing has also been observed in females heterozygous for severe X-linked deficiencies through selective pressure on progenitors (24–27). However, X-chromosome mosaicism may also manifest acute
skewing toward cell subpopulations that are advantageous in certain pathophysiological conditions through different degrees of gene expression, cell activation, proliferation, migration, or apoptosis (15). In support of this hypothesis, we presented a proof of principle study (28), using the X-linked gp91phox-deficient mouse strain, in which we showed that, during endotoxin shock, heterozygous mosaic animals convey a phenotype that is not simply the sum or average of deficient and wild-type (WT) responses. However, endotoxin shock is a fulminant inflammatory condition that is rarely observed in patients. Thus, it remains unknown whether female mosaicism provides a clinical advantage during polymicrobial sepsis, which is a major cause of death in the critically ill. Therefore, to test whether female mosaicism represents an advantage during the septic response, we used a gp91phox-deficient mouse strain, in combination with cecal ligation and puncture (CLP), which generates a clinical condition reminiscent of human peritonitis and systemic sepsis.

The X-linked gp91phox (NOX2)-deficient mouse strain was selected because gp91phox is the catalytic subunit of the NADPH oxidase complex in phagocytes, and this complex is an important component of antibacterial defense (29). Superoxide anion generated by gp91phox is dismutated into H₂O₂, which is used by myeloperoxidase (MPO) to produce HOCl for bacterial killing. Thus, a deficiency in gp91phox is expected to modulate the inflammatory response through altered bactericidal processes or altered phagocyte activation. Additionally, the deficiency is expected to alleviate oxidative stress-induced tissue injury, which is an important component of organ dysfunction during sepsis. Thus, studying this X-linked deficient mouse provides the opportunity to test whether the simultaneous presence of normal and deficient cell populations in mosaic females confers an advantage during the septic course over WT or deficient responses. To control for the confounding effects of male sex hormones, all experiments were performed in female animals. We found that mosaic mice present a clinically improved phenotype with dual advantages in survival and bacterial clearance in comparison with WT or homozygous gp91phox-deficient subjects.

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), and the protein assay kit was from Pierce (Rockford, IL). Fluorochrome-conjugated Abs, assay dyes, and lysing and permeabilizing flow cytometry solutions and kits were purchased from BD Biosciences and BD Pharmingen. All other reagents and chemicals of the highest grade available were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and CLP

Female gp91phox-deficient (−/−), heterozygous (+/−), and normal littermate (WT, +/+ ) mice matched by age (10–16 wk old, born within 2 wk) were used in the experiments. Initial breeders were purchased from The Jackson Laboratory (B6.129S6-Cybbtm1Din/J and WT control C57BL/6). This NOX2-deficient strain is well established on the C57BL/6 background. To control for potential environmental differences between animal facilities, mice were bred at our facility to produce all genotypes, including WT. Otherwise, healthy, gp91phox-deficient and WT animals are phenotypically indistinguishable, including growth rate and size. Occasionally, older deficient animals may present chronic granulomatous disease (CGD)-like symptoms (enlarged spleen, granuloma formation, and marked leukocytosis). The rate of CGD is ~4–6% of hemizygous males but is rarely observed in homozygous females and has not been found in heterozygous mice in our colony. The animals used in this study displayed no CGD-like symptoms. Animals were fed standard rodent chow. Polymicrobial septic peritonitis was induced using the CLP model, as described earlier (30). Briefly, animals were anesthetized by a s.c. injection of Nembutal (5 mg/100 g body weight). A midline abdominal incision was made, and the cecum was exposed, ligated, and punctured through opening holes at two sites with a 20-gauge hypodermic needle. Animals were resuscitated by s.c. injection of isotonic, pyrogen-free saline solution (0.025 ml/g body weight) immediately postoperatively and at 20 h post-CLP. When animals were followed for >24 h, they received daily saline resuscitations at the same dose. In a pilot experiment, we compared naive controls and sham-operated animals (opening the abdomen and moving the intestine but no ligation or puncture) and found no remarkable increase in inflammatory markers (31). Thus, we used nontreated naive animals as controls in selected experiments.

The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School.

Genotyping

The gp91phox gene in the mutant mouse has a PGKneo gene inserted in exon 3 (32). Total genomic DNA was isolated from tail clippings using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). DNA was subjected to PCR amplification using forward primers complementary to the PGKneo insert or WT sequences, respectively, and a common downstream primer. Forward primers, WT: 5′-AAGGAAAATCCTCCTCCTGCTGAAGA-3′ and deficient, 5′-GTTCTAATTCATCAGAGATTTACG-3′; common reverse primer: 5′-CGCACTGGAACCCCTGAGAAGG-3′. Gradient PCR reaction was carried out in the presence of 2 mM MgCl₂ with the following cycling: 94°C for 30 s (0.5°C decrease per cycle), and 72°C for 35 s; followed by 25 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 35 s; with final elongation of 72°C for 2 min. PCR amplicons were resolved on 3% agarose gels. The WT reaction produces a 240-bp product, and the deficient reaction produces a 195-bp product, whereas heterozygous samples present both amplicons.

Blood, splenocyte, and bone marrow cell isolation and incubations

Blood was collected into heparinized tubes via cardiac puncture from fully anesthetized animals. Following exsanguination, femurs were collected from the same animals. Femurs were cut at the diaphysis, and bone marrow (BM) cells were flushed into chilled PBS through the bone channel. BM cells were sedimented, 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 or splenocytes were resuspended in DMEM containing 1% FBS for subsequent analyses or in vitro incubations.

Flow cytometry

BM, blood, and spleen flow cytometry analyses and gating strategy were described in detail previously (33). Briefly, the number of polymorphonuclear neutrophils and lymphocyte subsets in blood and spleen was described in detail previously (33). Briefly, the number of polymorphonuclear neutrophils and lymphocyte subsets in blood and spleen was determined 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 Abs against CD markers conjugated with FITC, allophycocyanin, 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) and CD11b⁺CD45⁻CD19⁻ (myeloid cells). Aliquots of 0.1 ml whole blood, splenocyte, or BM cell suspension were incubated with the appropriate markers for 15 min following incubation with BD FACS lysing solution (BD Biosciences) for 7 min at 37°C. Cells were washed twice with BD FACS wash buffer and fixed with 1% methanol-free formaldehyde. FACS acquisitions were performed in a centralized flow cytometry facility, and ≥30,000 events were collected for each analysis.

Mosaic polymorphonuclear neutrophil subpopulations in blood and tissue samples (whole blood, BM, and peritoneal lavage) from heterozygous mice were identified with anti-mouse gp91phox mAb (BD Transduction Laboratories) using the BD “PhosphoFlow” protocol, with some modifications, and combined with secondary anti-IgG incubations.

Cell suspensions (0.1 ml) were incubated with BD PhosFlow Lys/fix Buffer (BD Pharmingen) at 37°C for 10 min. Cells were washed using PBS and centrifugal sedimentation (300 × g, 5 min), which was followed by permeabilization by BD PhosFlow Perm Buffer for 30 min. Cells were washed twice with BD Pharmingen stain buffer and then incubated with CD11b+PerCP and purified mouse anti-gp91phox mAb at room temperature for 30 min in the dark. Cells were then washed with stain buffer and
incubated with PE-conjugated rat anti-mouse IgG1 Ab (BD Pharmingen) for 20 min. Finally, cells were washed twice with stain buffer and analyzed by flow cytometry. Prior to initiating the studies with heterozygous mice, the gp91phox-staining method was validated by parallel incubations with isotype IgG and gp91phox Ab. Validation tests were also carried out by performing the staining protocols on cells from WT and deficient animals, as well as on mixed-cell suspensions, in parallel with mosaic samples. In addition to neutrophils, monocytes and macrophages are positive for CD11b. Pilot studies using macrophage markers and alternative gating showed a negligible presence of macrophages or monocytes within gates used for sampling neutrophils.

Blood differentials and cell counts in BM and spleen were determined using a computerized cell counter (Hematrue Veterinary Hematology Analyzer; Heska, Loveland, CO).

**FIGURE 1.** Mosaic neutrophil subpopulations in mice heterozygous for gp91phox deficiency. A, BM cell suspension or whole blood from mice heterozygous for gp91phox deficiency was processed according to the BD Phosflow Lyse/Perm-III Buffer Protocol. Samples were incubated with anti-CD11b-PerCP, as well as with anti-gp91phox or the corresponding isotype IgG, followed by incubation with PE-conjugated secondary Ab, as described in Materials and Methods. As shown, the well-defined CD11b<sup>+</sup> cell population (left panels) were separated into two cell populations based on the level of gp91phox expression (middle panels). The graphs (right panels) indicate that the ratio of Xp-expressing/Xm-expressing mosaic neutrophil subpopulations was ~1. B, BM (left panels) or WBC (right panels) was preincubated with DHR for 20 min, followed by gating with 1 μM PMA for 15 min. CD11b<sup>+</sup> myeloid cells were gated and analyzed for DHR fluorescence in deficient (top row), WT (middle row), and heterozygous mosaic (bottom row) animals. In WT animals, PMA resulted in a marked response, whereas there was no increase in DHR fluorescence in deficient samples. In mosaic animals, the presence of a two-cell population was evident, and the oxidative burst response of mosaic subpopulations corresponded well with the deficient and WT responses, respectively. Representative findings from several experiments with similar observations are shown.

**ELISAs, enzyme assays, and determination of blood bacterial counts**

ELISA kits for IL-10 (cat. #555252), IL-6 (cat. #555240), and MCP-1 (cat. #555260) were purchased from BD Biosciences (San Jose, CA), and the ELISA kit for MIP-2 (Dy452E) was obtained from R&D Systems (Minneapolis, MN). Plasma from freshly drawn heparinized blood was stored at −85°C until analysis. ELISAs were performed according to the manufacturer’s protocol. All of the compared samples from different genotypes were run simultaneously in duplicates on one plate. Values were determined from a calibration curve run parallel with the samples.

MPO activity was measured in frozen tissues homogenized in modified RIPA buffer containing 50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaVO<sub>4</sub>, and 1 mM NaF (pH 7.4). The homogenate was centrifuged at 13,400 × g for 10 min at 4°C. The supernatant was collected and stored at −80°C. MPO activity was measured as described earlier (34). MPO activity in tissue samples was determined using human leukocyte MPO as a standard (6908 U/ml from Sigma-Aldrich) and expressed as units of MPO/mg protein.

Plasma concentrations of lactate dehydrogenase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen, and creatine phosphokinase levels were analyzed using a clinical chemistry analyzer system (VetTest8008; IDEXX Laboratories).

Bacterial counts were determined from a 0.1-ml blood sample collected under sterile conditions and diluted serially in sterile physiological saline; 0.05 ml each dilution was aseptically plated on trypticase blood agar plates (BD Biosciences, San Jose, CA). The number of bacterial colonies was counted after a 24-h incubation at 37°C.

**Statistical analysis**

Statistical calculations were performed using JMP software (SAS Institute, Cary, NC). Results were analyzed using ANOVA, followed by the t test for pairwise comparisons or the Tukey–Kramer test for multiple comparisons. We used the log-rank test to assess survival differences among groups. Different study components were performed on six to eight animals from each of the in vivo treatment groups, unless indicated otherwise. A p value < 0.05 was considered significant, unless noted otherwise.

**FIGURE 2.** NOX2 mosaicism increases survival and improves bacterial clearance in polymicrobial sepsis. A, WT, gp91phox-deficient, and gp91phox mosaic animals were made septic by CLP. Animals received fluid resuscitation postoperatively and then repeatedly every 24 h and were observed for mortality. Mosaic and deficient animals showed improved survival compared with WT mice. *Statistically significant difference compared with WT, log-rank test. B, In a separate set of experiments, animals were subjected to CLP and resuscitation; bacterial counts in blood were determined 24 h later. Deficient animals showed the greatest bacterial counts following sepsis. *Statistically significant difference compared with mosaic animals. Mean ± SEM (n = 12–17 in each group).
**Results**

**Evidence of neutrophil mosaicism for gp91phox expression**

Fig. 1 presents the flow cytometry method used for tracking mosaic neutrophil subpopulations. BM or blood neutrophils were stained and gated by CD11b marker and forward scatter. CD11b staining identified a well-defined cell population (Fig. 1A, left panels). However, simultaneous staining for gp91phox expression resulted in two cell populations with low and high staining for gp91phox expression in heterozygous mosaic mice consistent with the presence of mosaic subsets (Fig. 1A, middle panels). On average, the ratio of the WT/deficient mosaic subpopulations was \( \sim 1:1 \) in blood, as well as in BM (Fig. 1A, right panels). Gp91phox staining intensities for the two mosaic subpopulations corresponded well with the respective staining intensities of deficient and WT samples (data not shown).

To provide additional evidence for the presence of mosaic subpopulations with functional differences in heterozygous animals, blood or BM neutrophils were also stained with dihydrorhodamine (DHR), which is an oxidant-sensitive fluorescent marker. Fig. 1B shows that upon initiating oxidative burst by the addition of PMA, homozygous deficient cells showed no response, whereas WT cells presented a marked increase in cellular oxidant content (Fig. 1B, top and middle panels). In contrast, heterozygous animals showed two cell populations: one without and one with a marked increase in cell oxidants, with staining intensities corresponding to deficient and WT samples (Fig. 1B, bottom panels).

**FIGURE 3.** Sepsis-induced cell-composition changes in blood and BM. The total numbers of major WBC subtypes and the percentage distribution of cells were determined from control or CLP-subjected mice. Bars represent absolute cell numbers calculated from cell counts and percentage distributions, whereas numbers within bars depict values as percentage of total cells. From total WBC yield in blood (A) and percent cell distributions, the total number of circulating neutrophils staining positive for CD11b (B), Th and cytotoxic T cells staining dual-positive for CD3/CD4 or CD3/CD8, respectively, (C, D), and B cells staining positive for CD19 (E) were determined. From BM cell yields (F) and percent cell distributions, the total number of myeloid cells staining double-positive for CD45 and CD11b (G), macrophages staining triple-positive for CD45, CD11b, and CD115 (H), and B cells staining double-positive for CD45 and CD19 (I) were determined. Mean ± SEM (\( n = 7–8 \) animals in each group). *Statistically significant difference between septic and control within the same genotype.
Improved survival and bacterial clearance in septic mosaic animals

WT, homozygous-deficient, and mosaic animals were subjected to CLP, followed by postoperative and daily fluid resuscitation; mortality was recorded for 8 d (Fig. 2A). WT animals showed the greatest mortality, with a 10% overall survival and 40-h median survival time (Fig. 2A). Mosaic and deficient animals showed statistically improved survival after CLP compared with WT: 40 and 50% overall survival, respectively (Fig. 2). The longest median survival time (100 h) was observed in mosaic subjects. Mean survival time of WT animals was 40 h, whereas deficient animals showed an intermediate value (65 h) between WT and mosaic mice (Fig. 2A).

An important component of resolving inflammation is clearing invading bacteria. Thus, in a separate set of experiments, we tested blood bacterial counts 24 h post-CLP (Fig. 2B). Deficient subjects showed the greatest numbers of circulating bacteria after CLP, whereas bacterial counts were similar in WT and mosaic animals (Fig. 2B).

Sepsis skews neutrophil ratios toward deficient sub-population in blood and spleen in mosaic subjects. Because splenocytes cannot be obtained for analyses before CLP, we also depicted WT/deficient neutrophil ratios in spleen and blood of CLP mice (Fig. 2B). In the BM, sepsis depleted B cell numbers in WT and deficient mice (Fig. 3E), in the BM, sepsis depleted B cell numbers in WT and deficient subject but caused no decrease in mosaic animals (Fig. 3I). Sepsis decreased BM macrophage numbers in WT mice, but it had no effect in deficient and mosaic subjects (Fig. 3H).

Skewed mosaic neutrophil content in blood and spleen following sepsis

The increase in blood neutrophil numbers in deficient subjects suggested that this might alter phagocyte tissue infiltration. Therefore, we tested whether the deficient subpopulations in mosaic animals responded differently from WT subpopulations following sepsis using blood and splenic neutrophil contents as markers. Because untreated mosaic animals show individual variability in the baseline WT/deficient neutrophil ratios (28), we sampled blood from the same animals 2 d prior to and 24 h after CLP (Fig. 4). The WT/deficient ratios in the spleen were also determined in these animals after CLP. This approach provided the possibility to test skewing within an individual subject, independent of the initial WT/deficient ratio of circulating neutrophils. Naive circulating neutrophils showed WT/deficient ratios between 0.4 and 1.2 in this set of mosaic animals (Fig. 4B, pre-CLP blood). However, this ratio decreased after CLP in each individual animal, indicating a relative increase in the number of circulating deficient neutrophils (pre-CLP blood versus post-CLP blood). Within the same animal, the WT/deficient ratio in blood and spleen was similar after sepsis (post-CLP blood versus post-CLP spleen). Because spleen analysis before and after CLP in the...
same animal is not feasible, we also depict WT/deficient ratios in spleen and blood from an independent control sample (Fig. 4B, unrelated subjects).

To gain additional insight into the status of neutrophil infiltration, we determined MPO activities in various organs, which are known sites of neutrophil accumulation during inflammation. Fig. 5 shows that following CLP, neutrophil accumulation was greater in lung and kidney from mosaic animals compared with WT or deficient subjects. Additionally, although liver MPO was increased similarly in mosaic and deficient subjects, splenic MPO was elevated in mosaic animals compared with deficient mice.

**Elevated CD11b expression in deficient mosaics subsets**

Membrane expression of CD11b is one of the markers of the adhesive potential in neutrophils. Thus, we compared the CD11b membrane-expression levels in mosaic subsets separated by gp91phox or DHR staining. PMA treatment caused a 100-fold increase in CD11b membrane expression in neutrophils compared with nonstimulated controls (data not shown). Under these activated conditions, CD11b content was statistically increased in cells from deficient mice compared with WT mice. CD11b expression was also increased in the deficient subpopulation compared with the WT subpopulation within mosaic animals (Fig. 6A, 6B). The elevated membrane expression by deficient neutrophil subpopulations compared with WT subpopulations was evident in blood, BM, and spleen from mosaic mice under naive and septic conditions (Fig. 6C–E).

**FIGURE 6.** Elevated neutrophil CD11b expression in the deficient subpopulations of mosaic animals. Myeloid cells from BM of WT and deficient animals (A) or mosaic mice (B) were stained for CD11b under PMA-stimulated conditions. Deficient neutrophils or deficient subsets from mosaics showed elevated CD11b expression compared with WT. In a separate set of experiments, neutrophil CD11b membrane expression was also determined in naive and septic animals from BM (C), spleen (D), and blood (E). Mean ± SEM (n = 6–7 animals in each group). *p < 0.05, compared with WT.

**Sepsis-induced cytokine response and organ dysfunction in mosaic animals**

The differences in survival and bacterial clearance among the tested gp91phox genotypes imply differences in the overall course of the inflammatory response. To confirm this, we first compared levels of IL-6 and IL-10, which are critically important in orchestrating the septic course, in blood and organs. Fig. 7 indicates that blood IL-6 at 6 h post-CLP was similar in WT, deficient, and mosaic subjects; however, at 24 h, it was lower in mosaic and deficient animals than in WT mice (Fig 7A). Blood IL-10 at 6 h after CLP was greater in mosaic animals than in WT or deficient subjects, whereas at 24 h it was similarly low in all mice (Fig. 7B). Lung IL-6 at 24 h post-CLP was increased in mosaic subjects compared with WT or deficient mice (Fig. 7C), whereas splenic IL-10 content was slightly decreased in mosaic animals compared with deficient animals. IL-6 and IL-10 levels in liver and kidney were not statistically different among genotypes (data not shown).

We also determined a set of blood organ dysfunction markers at 24 h post-CLP (Fig. 8). ALT, AST, bilirubin, and creatinine concentrations, which are indicators of liver and kidney dysfunction, were the most elevated in WT animals, consistent with poor WT survival. However, statistically significant differences (at p < 0.1) were only found between WT and unchallenged controls. The slight increases in ALT, AST, bilirubin, and creatinine in septic deficient or mosaic animals compared with controls did not reach statistical significance. Creatinine kinase and lactate dehydrogenase activity levels, which are indicative of muscle and metabolic dysfunction, were similar among genotypes.

**Intercellular communication between mosaic neutrophil subpopulations**

Cellular interplay through reactive oxidant species (ROS) between WT and deficient neutrophil subpopulations may represent a functional advantage in mosaic animals. For example, ROS released during a respiratory burst from the WT subpopulation could reach the NOX2-deficient subpopulation and be processed through the downstream enzymatic machinery, which could partially restore downstream enzymatic machinery, which could partially restore
redox-dependent cellular responses. To test whether oxidant interchange could occur between WT and deficient neutrophils, we measured PMA-induced oxidant content in WT and deficient cells, as well as in experimental 1:1 cell mixtures. In parallel, we compared “natural mixtures” of WT and deficient cells obtained from mosaic animals. Under nonstimulated conditions, DHR staining was similarly low in deficient, WT, and mosaic cells (Fig. 9A).

After PMA stimulation, when deficient or WT cells were incubated separately, cellular oxidant content was increased in WT, but not in deficient, samples (Fig. 9B versus Fig. 9A). However, mixing deficient and WT cells in a 1:1 ratio before PMA stimulation resulted in a slightly increased oxidant content in deficient cells (Fig. 9B). In mosaic samples, an increase in oxidant content within the deficient subpopulation was also observed, which was similar to that of deficient cells observed in the artificial cell mixtures (Fig. 9B versus Fig. 9C).

**Discussion**

It is widely believed that the female advantage over males with regard to health status and clinical outcomes from critically ill conditions is associated with differences in the sex hormone milieu. In general, studies have indicated beneficial effects of estrogens and harmful effects of testosterone and their derivatives (1–9). Likewise, it is well accepted that the gender dimorphic character of the innate immune response or clinical outcome from blood loss and severe injuries is also related to sex hormone effects. However, it is surprising that, despite the fact that the X chromosome displays major differences between males and females in X-linked gene variability, cellular content, and regulation, it has not been thoroughly investigated whether female X-chromosome mosaicism for parental haplotypes contributes to gender dimorphic physiology or the female advantage (12, 15–18). Moreover, although multiple studies suggested the potential role of the X chromosome in gender bias in autoimmune diseases (12, 35), it has not been investigated whether X-chromosome mosaicism could also affect the nonspecific innate immune response. Therefore, our current study represents the first attempt, to our knowledge, to demonstrate for the first time that the simultaneous presence of mosaic phagocyte subpopulations with or without the capacity to produce an oxidative burst is clinically advantageous during severe polymicrobial sepsis.

This clinical benefit in mosaics was manifested despite the fact that, conceptually, the presence of deficient cells could be advantageous through lessened oxidative stress or disadvantageous through compromised bacterial clearance. A priori, it could also be...
speculated that mosaicism simply reflects an intermediate condition between WT and deficient status due to the 1:1 ratio of WT and deficient phagocytes in mosaic mice. However, in contrast with these presumptions, our data indicated that gp91phox mosaicism presents its own phenotype, with uniquely modulated phagocyte responses presumably due to intercellular communications and feedback mechanisms between WT and deficient subpopulations and a related modulation of the systemic inflammatory response.

Consistent with these propositions, although bacterial killing was more efficient in mosaic and WT animals compared with gp91phox-deficient mice, sepsis-induced mortality was worst in WT mice, whereas deficient and mosaic mice showed similarly improved survival. These observations indicated that excessive neutrophil-mediated oxidative stress is likely more important in causing organ dysfunction and death than is a partially compromised bacterial clearance in this sepsis model. Most importantly, gp91phox mosaics seem to have a dual advantage: reduced oxidative stress with improved survival and the maintenance of efficient bacterial killing.

Additionally, we provided evidence that oxidant interchange between the WT and deficient neutrophil populations could take place, which may further contribute to the maintenance of efficient bacterial killing in mosaic animals. This is because superoxide anion or \( \text{H}_2\text{O}_2 \) released from the WT subpopulation can enter deficient subpopulations and subsequently be converted into \( \text{HOCl} \) through MPO, which is a critical step in bacterial killing and is expected to be functional in deficient cells. Thus, oxidant interchange between mosaic subpopulations may also alleviate oxidative stress, because at the site of neutrophil infiltration the deficient subpopulation may catabolize some of the ROS released from WT cells, thereby alleviating their noxious effects.

An additional important fact is that cellular redox status plays an important role in regulating signaling pathways, thereby influencing a variety of cell responses (36–39). An alleviated oxidative stress in mosaic subjects is expected to affect the activation of neutrophils per se, and this is also likely to impact redox-dependent responses by the endothelial and parenchymal cells in the target organs of neutrophil infiltration. Thus, it is not surprising that WT, deficient, and mosaic animals displayed differences in their overall systemic inflammatory response, as reflected in the different kinetics and concentration of blood and tissue cytokines. The associated differences in BM and blood WBC counts, including a different degree of lymphocyte depletion after sepsis, also suggest a uniquely influenced phenotype presented by mosaic animals, which is not simply the sum of the WT and deficient responses.

Variations in redox-dependent signaling and cytokine production are expected to alter neutrophil activation status. CD11b membrane expression reflects changes in neutrophil activation status, because CD11b is an important member of adhesion molecules, and its membrane content is markedly increased upon administration of inflammatory mediators, including phorbol esters mimicking protein kinase C activation. Interestingly, however, the lack of gp91phox alone in neutrophils from deficient mice or in the deficient neutrophil subpopulation from mosaic animals resulted in an elevated membrane expression of CD11b (Fig. 6). This increase in CD11b expression in deficient neutrophils was evident under resting conditions, as well as after phorbol ester stimulation, indicating an elevated cell-adhesion potential of deficient neutrophils. Consistent with this possibility, septic mosaic animals displayed increased splenic, lung, and kidney neutrophil infiltration compared with WT and deficient animals. This tissue infiltration in mosaics was likely to be skewed toward the deficient subpopulations, similar to that observed in the spleen. These observations, together with an unchanged WT/deficient ratio of the myeloid lineage in the BM (data not shown), suggested that sepsis-induced skewing of infiltrating neutrophils toward deficient populations is likely to be the result of increased cell-adhesion potential of deficient cells, rather than selection of progenitors in the BM. The fact that deficient cells from deficient mice showed a similar level of tissue recruitment as observed in WT cells suggested that a blunted degree of neutrophil activation in deficient animals may counterbalance their increased adhesion potential. Furthermore, the finding that neutrophil infiltration skewed toward the deficient subpopulation in mosaic animals suggested that the activation of deficient subpopulations could be partially restored by the presence of WT neutrophils. The fact that hepatic neutrophil infiltration was similarly increased in mosaic and deficient mice compared with WT mice suggested that the increased adhesion potential of deficient cells can be manifested in this organ, even in the absence of neutrophil redox activation. This may be related to the unique structural and cellular characteristics of the liver sinusoids and interstitium.

In summary of our major observations, we propose the following scheme: in WT mice, the presence of fully activated ROS-producing infiltrating neutrophils represents a disadvantage because they are major contributors to multiple organ dysfunction and failure. In contrast, bacterial clearance functions well in WT mice. The pattern is reversed in deficient subjects. Bacterial clearance is compromised; however, the lack of oxidative burst alleviates neutrophil-mediated stress in tissues and consequently improves mortality compared with WT animals. However, the simultaneous presence of deficient and WT subpopulations in mosaic animals presents multiple advantages. First, bacterial clearance could be maintained by the presence of the WT subpopulation, as well as by the possible oxidant interchange between mosaic subpopulations. Second, normal redox-dependent signaling and cell activation in the WT subpopulations provide the ability to repair some of the compromised functions in the deficient subpopulations through paracrine actions. Finally, tissue oxidative stress is also expected to be alleviated in mosaic animals because of the simple fact that half of the cells lack the ability to produce an oxidative burst, as well as because the deficient subpopulation may function as a sink of oxidants through their ROS-detoxifying machinery, especially at sites of neutrophil invasion where deficient and WT cells are in close proximity to each other.

Taken together, our observations using this model support the previously proposed hypothesis (15) that X-linked mutations or common polymorphisms could result in immune cell populations with different activation, functional, or regulatory potentials in mosaic females. The presence of mosaic subsets may provide a broadened functional repertoire in accommodating the dynamically changing physiology during inflammation. This is in contrast to males, whose X-linked phenotype is driven solely by the maternal haplotype and, therefore, are expected to manifest a more polarized biological response to a particular physiological condition.

Disclosures
The authors have no financial conflicts of interest.

References


