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Female X-chromosome mosaicism for gp91phox expression diversifies leukocyte responses during endotoxemia

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Abstract

Objective—To test the hypothesis, using an animal model, whether female X-chromosome mosaicism for inflammatory gene expression could contribute to the gender dimorphic response during the host response. X-chromosome-linked genetic polymorphisms present a unique biological condition because females display heterozygous cellular mosaicism, due to the fact that either the maternal or the paternal X chromosomes are inactivated in each individual cell in females. This is in contrast with the conditions in males who carry exclusively the maternal X chromosome.

Design—Prospective, randomized, laboratory investigation.

Settings—University research laboratory.

Subjects—Female mice deficient, heterozygous (mosaic) or WT for the X-linked gp91phox.

Interventions—We compared selected inflammatory markers among heterozygous (mosaics), WT and homozygous deficient animals in response to *in vivo* lipopolysaccharide (*Escherichia coli*, 20 mg/kg body weight). To test individual mosaic subpopulations of polymorphonuclear neutrophil responses, we also developed a flow cytometry assay that identifies the active parental X chromosomes in individual cells, using gp91phox expression as a marker.

Measurements and Main Results—Heterozygous mosaic mice presented white blood cell trafficking patterns similar to that observed in WT mice, despite the fact that the deficient subpopulation in mosaic animals displayed increased cell activation as reflected in elevated neutrophil CD11b expression and splenic infiltration. Mosaic animals also displayed splenic neutrophil infiltration, which was skewed toward the deficient subpopulation. Observations on splenic T-cell depletion and post lipopolysaccharide interleukin-10 responses indicated that the inflammatory response in mosaic animals does not simply display an average of the deficient and WT responses, but the mosaic subjects display a uniquely characteristic response.

Conclusions—The study supports the notion that female X chromosome mosaicism for polymorphic gene expression represents a unique condition, which may contribute to the gender dimorphic character of the inflammatory response. Mosaicism for X-linked polymorphisms may have clinical significance and needs consideration in genetic association or gender-related clinical studies.

Keywords

gender; X chromosome inactivation; gp91phox; inflammation; polymorphonuclear; lipopolysaccharide

It has been well documented that females, compared with males, show improved general health status and longevity as well as increased survival under a variety of disease processes, including infections (1–8). It is believed that sex hormones play an important role in causing gender-specific differences in the clinical courses after pathologic challenges. Recently, however, the unique properties of the X chromosome, including female X-chromosome inactivation and cellular mosaicism for X-linked polymorphic genes, have been suggested to also play important roles in the gender-associated differences in disease progressions (9–12).

Females carry two X chromosomes, one inherited from the mother (X_m, maternal) and one from the father (X_p, paternal), whereas males carry only one X chromosome that is passed on from the mother (X_m). The potential double dose of X-linked proteins in female cells could cause harmful effects in regulatory and metabolic processes; therefore, dosage compensation in females is achieved by X-chromosome inactivation at an early embryonic stage (13–15). Dosage compensation is the result of a random inactivation of one of the X chromosomes in an individual cell resulting in the block of gene expression from one of the parental X chromosomes that is maintained through the entire life span of each individual cell. Therefore, females are cellular mosaics for all their X-linked mutations. Because the X chromosome contains many genes encoding for proteins with central functions in metabolism, redox processes, and signaling systems (9), polymorphism of these genes would be expected to result in the presence of two cell populations with distinct regulatory and functional arsenals. Therefore, female X-chromosome cellular mosaicism is a unique condition, which differs from the heterozygosity of autosomal mutations where the polymorphic alleles manifest gene-dose effects or dominant/recessive traits uniformly in all cells.

We propose that, in response to physiologic and environmental challenges, female X-chromosome mosaicism may represent a broadened response arsenal and a more adaptive cellular system than the uniform cell populations in hemizygous males or rare homozygous females (9). To test this hypothesis, we employed a gp91phox-deficient mouse strain. The X-linked gp91phox (cybb) is the catalytic subunit of nicotinamide adenine dinucleotide phosphate oxidase complex, which is responsible for the production of superoxide anion in polymorpho- and mononuclear phagocytes. Using this mouse model, we tested whether mosaic animals display an average of the WT and deficient responses or they present a unique response pattern. Using our newly developed flow cytometry method for the detection of gp91phox expression, we also were able to test simultaneously respective responses of the two distinct mosaic subpopulations within an individual heterozygous subject. *In vivo* lipopolysaccharide (LPS, 24 hrs) was used as the challenge because it activates white blood cells and initiates a well-characterized nonspecific immune response. To control for the confounding effects of sex hormones, all experiments were performed on female animals. After comparing WT, homozygous, and mosaic responses, we found that mosaic animals present a distinct pattern, which shares WT-like and deficient-like response components at different degrees.

MATERIALS AND METHODS

Reagents

Endotoxin-free, cell culture grade buffers, media and reagents were used in the experiments. Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA), protein assay kit from Pierce (Rockford, IL). Fluorochrome conjugated antibodies, assay diluents, lysing and permeabilizing flow cytometry solutions and kits were purchased from BD Biosciences (San Jose, CA) and BD Pharmingen (San Diego, CA). All other reagents and chemicals of the highest grade available were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals and Endotoxin Treatments

Female gp91phox-deficient ($-/-$), heterozygous ($\pm$), and normal littermate (WT, $+/+$) mice matched by age (10–16 wks old, born within 2 weeks) were used in the experiments. All animals, including WT, were derived from our breeding colony. Otherwise healthy, gp91phox-deficient and WT animals are phenotypically indistinguishable, including growth rate and size. The mean body weight of the animals were WT: 22.4 g; heterozygous: 22.0 g; deficient: 23.2 g. Occasionally, older deficient animals may present chronic granulomatous disease-like symptoms (enlarged spleen, granuloma formation, and marked leukocytosis). The rate of granulomatous disease is about 4% to 6% of hemizygous males but is rarely observed in homozygous females and has not been found in heterozygous mosaics in our colony. The animals used in this current study displayed no granulomatous disease-like symptoms. Animals were fed with standard rodent chow. The estrus cycle was not tested in the mice. The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication, National Institutes of Health, No. 85-23, Revised 1985, Office of Science and Health Reports, Division of Receipt and Referral/National Institutes of Health, Bethesda, MD 20,205) and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School. Animals were injected intraperitoneally with lipopolysaccharide (LPS) (from *Escherichia coli*, 20 mg/kg body weight). This protocol resulted in negligible mortality 24 hrs after LPS in these animals; thereby, survival bias did not affect the observations. Controls received an equal amount of saline. At 24 hrs after LPS injection, the animals were anesthetized, and blood and tissues were collected and processed for analyses.

Genotyping

The gp91phox gene in the mutant mouse has a *PGKneo* gene inserted in exon 3 (16). Total genomic deoxyribonucleic acid was isolated from tail clippings, using the REDEExtract-N-Amp Tissue polymerase chain reaction kit (Sigma-Aldrich). Deoxyribonucleic acid was subjected to polymerase chain reaction amplification, using forward primers complementary to the *PGKneo* insert or WT sequences, respectively, and a common downstream primer. Forward primers WT: 5'-AAGAGAAACTCCTCTGCTGTGAA-3'; Deficient: 5'-GTTCTAATTCCATCAGAAGCTTATCG-3'; Common reverse primer: 5'-CGCACTGGAACCCCTGAGAAAGG-3'. Gradient polymerase chain reaction was carried out in the presence of 2 mM $MgCl_2$ with the following cycling: 94°C, 3 mins, 12 cycles of 94°C, 20 secs, 64°C, 30 secs, (0.5°C decrease per cycle) 72°C, 35 secs, followed by 25 cycles of 94°C, 20 secs, 58°C, 30 secs, 72°C, 35 secs with final elongation of 72°C for 2 mins. Polymerase chain reaction amplicons were resolved on 3% agarose gels. WT reaction produces a 240 bp, deficient a 195-bp product, whereas heterozygous samples present both amplicons.

Blood, Splenocyte, and Bone Marrow (BM) Cell Isolation and Incubations

A blood sample was collected into heparinized tubes via cardiac puncture from fully anesthetized animals. After the exsanguination, femurs were collected from the same animals. Femurs were cut at the diaphyses and BM cells were flushed out by repeated injections of phosphate-buffered saline 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 phosphate-buffered saline/FBS buffer. Next, the spleen was removed and placed into Dulbecco's modified Eagle's medium 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- μ m nylon mesh cell strainer. Isolated BM cells or splenocytes were resuspended in Dulbecco's modified Eagle's medium containing 1% FBS for subsequent analyses or *in vitro* incubations.

Flow Cytometry

BM, blood, and spleen flow cytometry analyses and gating strategy has been described previously (17). The number of polymorphonuclear neutrophilic leukocytes (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, 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), CD11b⁺CD45⁺CD19⁻ (myeloid cells). Aliquots of 0.1 mL whole blood, splenocyte or BM cell suspension were incubated with the respective markers for 15 mins followed by incubation with BD FACS lyzing solution (BD Biosciences) for 7 mins at 37°C. Cells were washed twice with BD FACS wash buffer and then fixed with 1% methanol free formalde-hyde. FACS acquisitions were performed in a centralized flow cytometry facility. At least 30,000 events were collected for each analysis.

Mosaic PMN subpopulations from tissue samples (whole blood, spleen, BM, and peritoneal lavage) of heterozygous mice were identified with anti-mouse gp91phox monoclonal antibody (BD Biosciences, San Jose, CA), using the BD "PhosphoFlow" protocol with some modifications and combined with secondary anti-IgG incubations. Cell suspensions (0.1 mL) were incubated with BD Phospho lyse/fix Buffer (BD Pharmingen) at 37°C for 10 mins. Cells were washed, using phosphate-buffered saline and centrifugal sedimentation (300 $\times$ g, 5 mins) which was followed by permeabilization by BD PhosphoFlow Perm Buffer for 30 mins. Cells were washed twice with BD Pharmingen stain buffer and then incubated with CD11b-PERCP and purified mouse anti-Gp91phox monoclonal antibody at room temperature for 30 mins in the dark. Cells were then washed with stain buffer and incubated with PE-conjugated rat anti-mouse IgG1 antibody [BD Pharmingen] for 20 mins. Finally, cells were washed twice with stain buffer and analyzed by flow cytometry. Before initiating the studies with heterozygous mice, the gp91phox staining method was validated first by parallel incubations with isotype IgG and gp91phox antibody. Validation tests were also carried out by performing the staining protocols on cells from WT and deficient animals, respectively, as well as on mixed cell suspensions in parallel with mosaic samples.

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

Enzyme-linked immunosorbent assay kits for interleukin (IL)-10 (555252), IL-6 (555240), and MCP-1 (555260) were purchased from BD Biosciences (San Jose, CA), Mip-2

(Dy452E) from R&D Systems (Minneapolis, MN). Plasma from freshly drawn heparinized blood was stored at -85°C until analysis. Enzyme-linked immunosorbent assays were performed according to the manufacturer's protocol. All 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.

Statistical Analysis

Statistical calculations were performed, using JMP software (SAS Institute Inc., Cary, NC). Results were analyzed, using analysis of variance followed by *t* test for pairwise 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. Significant difference was concluded at $p < .05$.

RESULTS

Identification of Mosaic Cells in Various Tissues

Flow cytometry was employed to identify mosaic subpopulations of PMNs in heterozygous animals, using an antibody against gp91phox combined with staining for the myeloid/neutrophil/macrophage marker CD11b. Figure 1 shows the applicability of the employed technique. CD11b staining identified a well-defined group of cells with similar fluorescent intensity in both WT and gp91phox-deficient animals (Fig. 1A, *top panels*). Gating on this CD11b positive cell population and testing for simultaneous staining for gp91phox revealed staining in deficient samples that overlapped with isotype IgG staining, whereas WT samples showed strong staining intensity for gp91phox (Fig. 1A, *histogram*).

This method was used in heterozygous animals to identify mosaic subpopulations of PMNs expressing different parental X chromosomes in various organs. Figure 1B presents a typical finding from a progeny of WT female and hemizygous deficient male. As shown, PMNs expressing the deficient (paternal) or WT (maternal) X chromosome is clearly distinguishable by the presence and absence of gp91phox expression in CD11b+ myeloid cells from blood, BM, spleen, and peritoneal fluid (Fig. 1B scatter plots). In this test, the value of WT/deficient mosaic cell ratio was near one, indicating approximately 50%–50% distribution of cells carrying the active X chromosome from respective parents in all organs tested (Fig. 1B, *histograms*).

Whereas after testing a larger sample of heterozygous animals the average value of mosaic WT/deficient-ratio was around one (50:50 paternal/maternal distribution), individual animals showed considerable variability in WT/deficient ratios. When an individual animal showed a skewed WT/deficient ratio, then this was uniformly manifested in all tissues independent of the direction of skewing as reflected in a strong linear correlation of WT/deficient ratios BM and blood (Fig. 1C, $p < .001$) and a similar tendency BM and spleen ratios (Fig. 1C, $p < .1$).

To further characterize and identify mosaic subpopulations in heterozygous mice, we also tested phorbol 12-myristate 13-acetate-induced oxidative burst in BM myeloid cells from mosaic animals (Fig. 1D). In WT mice, tested phorbol 12-myristate 13-acetate increased cell-associated dihydrorhodamine (DHR) fluorescence (indicative of cellular oxidant content) compared with vehicle controls, and the response was manifested in a single-peak histogram (Fig. 1D, *left histogram*). In contrast, mosaic animals displayed two cell populations with different level of oxidative bursts, one responding similarly to WT whereas the other showing attenuated DHR fluorescence (Fig. 1D, *right histogram, double arrows*).

Endotoxin-Induced Changes in Cell Trafficking

Because altered redox status is expected to modulate functional activation of PMNs, including cell attachment and transmigration, in the next series of experiments, we tested whether gp91phox deficiency or mosaicism alters the LPS-induced changes in tissue cell composition. A 24-hr LPS challenge was selected because at this window the LPS-induced white blood cell changes are well developed but mortality is negligible. Figure 2, A and B, indicates that LPS results in similar decrease in BM myeloid and B cell content in WT, deficient and mosaic animals. In the blood of unchallenged (control) animals, the number of circulating PMNs is about 60% greater in deficient animals than either WT or mosaic animals (Fig. 2C). Furthermore, after LPS, WT animals presented a 100% increase in the number of circulating PMNs, whereas deficient or mosaic animals showed no statistically significant increase (Fig. 2C). The number of circulating PMNs was similar in LPS-injected WT, deficient and mosaic animals. The LPS-induced marked depletion in circulating CD19+ B cells and CD4+ or CD8+ T cells were similar in WT, deficient and mosaic animals (Fig. 2, D–F).

Figure 3A indicates cell composition changes in the spleen. Under control conditions, deficient mice contain a greater number of resident splenic PMNs than heterozygous mice. The difference splenic PMN content from control WT and deficient mice did not reach a statistically significant level. LPS injection increases the number of splenic PMNs in WT, deficient and mosaic animals; however, in mosaic animals, PMN content was about half of that of deficient or WT mice after LPS (Fig. 3A). It was also noteworthy that, in response to LPS, deficient animals showed no remarkable changes in splenic B and T cell content, whereas both WT and mosaic animals presented depleted splenic B and T cell content (Fig. 3, B–D).

Skewed Responses of Mosaic Subpopulations

Figure 4 summarizes observations on CD11b membrane expression levels post LPS as measured by CD11b staining intensity, which is indicative of cell activation and cell attachment potential. Figure 4A indicates that, after LPS, PMNs that remained in circulating blood display decreased CD11b membrane expression in all genotypes. In contrast, the expression level of CD11b was increased in PMNs recruited into the spleen after LPS (Fig. 4B). Interestingly, in unchallenged controls, CD11b expression was greater in splenic PMNs from deficient than WT mice (mean fluorescence intensity difference, 73.2 ± 19 ; $p < .05$) (Fig. 4B).

We also determined whether the difference in CD11b membrane expression is also present in mosaic subpopulations within individual mosaic animals. Figure 4C indicates that CD11b expression is greater in the deficient subpopulation than the WT subpopulation (mean fluorescence intensity difference, 88 ± 9). This increased CD11b expression in deficient vs. WT PMN subpopulations in mosaic animals was independent of the WT/deficient PMN ratio.

These observations suggested that that the deficient PMN subpopulation within mosaic animals is at a greater degree of cell activation than the WT PMN subpopulation. Thus, we determined the LPS-induced changes in the WT/deficient ratios in different organs from heterozygous animals (Fig. 5). The mean WT/deficient ratio was near one in control animals in BM, blood, and spleen (Fig. 5, A–C). However, post LPS, WT/deficient ratio was decreased in BM and spleen and showed a borderline decrease in blood (Fig. 5, A–C). From the observed WT/deficient PMN ratios combined with the splenic PMN infiltration data presented in Figure 3, we calculated the proportions of newly infiltrating WT and deficient cells in response to LPS in mosaic animals. Figure 5D shows that LPS challenge of mosaic

animals resulted in approximately twice as many deficient as WT cells infiltrating into the spleen.

Finally, because altered myeloid cell function could modulate the overall inflammatory response, we compared the blood levels of a selected set of cytokines in these animals. We observed elevated IL-10 level at 24 hrs post LPS in mosaic animals compared with WT, whereas deficient blood contained an intermediate level WT and mosaic (Fig. 6). Blood levels of IL-6 were not different statistically 24 hrs post LPS, whereas MCP-1 and MIP-2 levels were below detection limits (data not shown).

DISCUSSION

This study reveals that, in subjects who are mosaics for myeloid cell gp91phox expression, the inflammatory response does not simply display an average of the deficient and “normal” (WT) responses, but the mosaic subjects display a uniquely characteristic response. Thus, at the proof of principle level, the study demonstrates for the first time that the inflammatory response could be uniquely different in female subjects who are heterozygous for X-linked polymorphisms compared with hemi- or homozygous individuals. Cellular mosaicism for X-linked polymorphic inflammatory gene expression could present an advanced functional repertoire by providing a broader range and scale of cell responses.

Using the presence or absence of gp91phox expression combined with flow cytometry for the identification and analyses of mosaic subpopulations in an individual subject revealed that some unchallenged animals display a variable degree of “spontaneous” skewing toward a particular subpopulation. This is reflected in the observed range of 0.4 –2.3 WT/deficient ratios in the number of circulating or tissue resident PMNs (corresponding with a bidirectional 30% to 70% paternal-maternal mosaic cell distribution range). This indicates that the presence/absence of functional gp91phox in BM stem cells does not represent a preconditioning status for directional skewing under normal conditions. These observations are in agreement with earlier studies indicating that only a few stem cells are responsible for driving myelopoiesis. Thus, selection from a small pool of stem cells simply by chance (18,19) may manifest skewing toward either deficient or WT populations after the amplification through myelopoiesis.

The fact that the mean value of WT/deficient ratio approached one in larger samples supports that myeloid stem cell activity is independent of gp91phox expression. However, post LPS, stem cell activity could be altered by the presence or absence of gp91phox, as previous studies suggested the role of NOX4 and NOX2 (gp91phox) in hematopoietic stem cell activity via redox regulation (20,21). Accordingly, our study revealed that after an inflammatory challenge, a “functional skewing” may occur in the periphery in mosaic animals independent of the initial (pre LPS) WT/deficient ratio in a particular animal. This is reflected most directly in the fact that the deficient subpopulations participated more in tissue infiltration than the WT subpopulations during endotoxemia in mosaics.

It has been shown that neutrophil infiltration-associated oxidative stress is an important component of tissue damage during endotoxemia (22,23). It has also been suggested that lack of gp91phox and consequent lack of oxidative burst in neutrophils alleviates tissue injury in a septic model, using male hemizygous gp91phox-deficient and WT animals (24). Other studies indicated an increased inflammatory response to LPS in nicotinamide adenine dinucleotide phosphate oxidase-deficient hemizygous animals (25). Our finding in mosaic subjects indicating that the more deficient than WT PMN subpopulation participated in tissue infiltration implies an alleviated oxidative stress *in toto* in the periphery of mosaic

animals, which may prove to be beneficial over WT during sepsis in agreement with earlier studies (24).

The finding that CD11b expression was greater in the deficient than WT PMN subpopulation in mosaics suggests that the basal activation status is elevated in gp91phox-deficient PMNs (25). However, the fact that this was not accompanied by elevated baseline splenic PMN content or attenuated PMN infiltration after LPS similar to that observed in deficient animals suggests a regulatory balancing role of WT subpopulations in mosaics probably through intercellular communications and feedback mechanisms. This notion is supported by several observations. First, deficient subjects had elevated splenic PMN content under control conditions. However, despite the fact that, on average, half of the cells were deficient in mosaics animals, the residual number of PMNs in mosaics was similar to that of WT animals; furthermore, post LPS, mosaic animals presented the least marked increase in splenic PMN infiltration. In addition, the observation that deficient animals displayed no statistically significant decreases in splenic B or T cell numbers whereas both WT and mosaic mice presented similar decreases in splenic lymphocyte content after LPS indicates that the presence of two cell populations in mosaics rescues these animals from the altered lymphocyte response observed in deficient mice. It is also a possibility that PMN apoptosis is modulated differently among these phenotypes, which could have contributed to the observed differences in cell composition changes (26,27).

LPS injection initiates a cascade of events, which is coordinated by complex cellular interplay and regulatory molecules, including cytokines (28). PMNs and macrophages are important cellular determinants in this cascade either by being sources of cytokines and other regulatory molecules or through stimulating tissue resident immune cell activation (28). Thus, a small change in early cell activation may alter the subsequently developing pattern of the systemic inflammatory response. Our observation indicating that heterozygous animals displayed the highest blood levels of IL-10 suggests that simultaneous presence of mosaic phagocyte subpopulations with or without the capacity of oxidative burst and respectively polarized activation status initiates/modulates the inflammatory cascade differently from that presented by animals carrying exclusively WT or deficient phagocytes.

The ultimate clinical importance of female X-chromosome mosaicism cannot be determined from this basic proof-of-principle investigative study. However, our observations provide justification for subsequent survival studies, as well as investigating organ and immune function in clinically more relevant sepsis models. Additional studies will be needed to test the potential overlaying modulating effects of sex hormones (29,30). Because the estrus cycle stage was not determined in these animals, we cannot tell if the changes observed would have been magnified or blunted at specific stages of the cycle. Nevertheless, our observations on the LPS-induced cytokine response together with the observed PMN and lymphocyte cell composition changes are consistent with the notion that gp91phox mosaicism represents a phenotype that is different from the simple mix of deficient and WT responses. Thus, clinically, it is likely that the immunomodulatory effects of mosaicism for X-linked polymorphic genes would contribute to gender-related differences in the host response.

CONCLUSION

Taken together, our study supports the notion that X-chromosome mosaicism for the expression of polymorphic genes presents a unique phenotypic condition in females. Because of the unusual population genetic aspects of X-linked mutations, hemizygous males are always more represented in the population than homozygous females (9,31,32). Thus, in addition to sex hormone modulation, cellular mosaicism for X-linked genetic

polymorphisms should also be considered as a contributing factor to the gender dimorphic character of the inflammatory response or other pathologic conditions.

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Figure 1.

Identification of mosaic cell subpopulations in heterozygous animals. *A*, Whole blood from gp91phox-deficient and WT animals was processed according to the BD phospho-lyse-fix-Perm-III buffer protocol. Samples were incubated with anti-CD11b-PERCP, as well as with either anti-gp91phox, or the corresponding isotype-IgG followed by incubation with PE-conjugated secondary antibody, as described in Materials and Methods. Staining intensity in gp91phox-deficient cells represents nonspecific background because it overlaps with the isotype-IgG staining. *B*, Myeloid cells from bone marrow (BM), blood, spleen, and peritoneal cavity from naive gp91phox heterozygous female mice were identified as described for part A. By the expression of gp91phox, two distinct cell populations carrying the active Xp or Xm can be readily identified in mosaic animals. *C*, Linear regression analysis of individual WT/deficient ratios BM and blood (circles) or BM and spleen (squares) from 11 animals. *D*, Heterozygous animals present two cell populations with different degree of oxidative burst after phorbol ester challenge. BM cells were preincubated with dihydrorodamine (DHR) for 20 mins followed by incubation in the presence or absence of 1µM of phorbol-myristate-acetate (PMA) for 20 mins. Subsequently, myeloid cells were gated and analyzed for DHR staining. A representative finding from a WT (left) and heterozygous mosaic animal (right) is shown. The moderate increase in PMA-induced fluorescence in the deficient mosaic subpopulation is most probably related to oxidant release from sources other than the nicotinamide adenine dinucleotide phosphate oxidase complex, which may include NOX isoenzymes or the mitochondria.

Figure 2.

Lipopolysaccharide (*LPS*)-induced changes in bone marrow (BM) and blood cell composition in WT, gp91phox-deficient and mosaic animals. Twenty-four hours after LPS or saline controls, BM cells and blood were collected and processed for flow cytometry analyses, as described in Materials and Methods. Graphs A and B depict BM myeloid cell content identified by CD45/CD11b double positive (A) and B-cell content identified by CD45/CD19 double positive staining (B). Graphs C–F depict the numbers of circulating CD11b⁺ (mostly neutrophils) and different T-cell (CD4⁺ and CD8⁺) and B-cell (CD19⁺) subsets as indicated. Cell numbers were calculated from percent distribution of cells and total white blood cell yield. Statistically significant difference ($p < .05$): *compared with control within the same genotype; #compared with WT or heterozygous controls. Mean $\pm$ SEM, $n = 7$ –8 animals in each group.

Figure 3.

Lipopolysaccharide (*LPS*)-induced changes in splenic cell composition in WT, gp91phoxdeficient and mosaic animals. From control or *LPS*-injected mice, splenocyte suspensions were prepared and cell counts were determined. Based on CD marker staining and light scatter properties, the counts of splenic polymorphonuclear neutrophils (A), CD4⁺ T-cells (B), CD8⁺ T-cells (C), and CD19⁺ B-cells (D) were calculated from total cell yields and the percent distribution of respective cell populations. Statistically significant difference ($p < .05$): *compared with control within the same genotype; #compared with deficient in the same treatment group. Mean $\pm$ SEM, $n = 7-8$ animals in each group.

Figure 4.

CD11b membrane expression is increased in the deficient polymorphonuclear neutrophilic leukocytes (PMN) subpopulations of mosaic animals and in spleen-infiltrated PMNs. CD11b staining (mean fluorescence intensity [MFI]) was measured in circulating (A) and splenic (B) neutrophils 24 hrs after LPS and compared among WT, deficient and mosaic animals. Graph C depicts CD11b expression levels in mosaic subpopulations identified by gp91phox expression, or lack of, as described for Figure 1. Statistically significant difference ($p < .05$): *compared with control within the same genotype; #compared with WT in the same treatment group; &compared with WT (paired comparison). Mean $\pm$ SEM, $n = 7-8$ animals in each group.

Figure 5. Gp91phox-deficient mosaic subpopulations dominate tissue cell composition changes after endotoxin. WT/deficient polymorphonuclear neutrophilic leukocytes (PMN) ratios were calculated, using the method described for Figure 1. *Graphs A–C* depict ratios in bone marrow (BM), blood, and spleen. *Graph D* presents the resident as well as the lipopolysaccharide (LPS)-induced newly infiltrated numbers of splenic PMNs, which were calculated from the WT/deficient (*def*) ratios combined with the cell infiltration values presented in Figure 3. *Statistically significant difference ($p < .05$) compared with control. Mean $\pm$ SEM, $n = 7$ –8 animals in each group.

Figure 6.

Heterozygous mosaic animals present robust interleukin (*IL*)-10 response post lipopolysaccharide (*LPS*). Blood IL-10 content was measured in plasma samples collected 24 hrs after LPS, using a commercial enzyme-linked immunosorbent assay kit. *Statistically significant difference ($p < .05$) compared with WT. Mean $\pm$ SEM, $n = 6-7$ animals in each group.